BIOCATALYSIS AND BIOMOLECULAR ENGINEERING

Edited by

CHING T. HOU AND JEI-FU SHAW

International Society of Biocatalysis and Biotechnology

National Chung-Hsing University

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To our wives, Mandy Hou and Yea-Shiow Shaw,
for their understanding and kind support during preparation of this book


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PREFACE

This book was assembled with the intent of bringing together current advances and in-depth reviews of biocatalysis and biomolecular engineering with emphasis on agricultural biotechnology. The book consists of selected papers presented at the fourth International Symposium on Biocatalysis and Biotechnology held at the Academia Sinica, Taipei, Taiwan November 19–21, 2008. At this symposium, 60 distinguished international scientists from the United States, Japan, Korea, Canada, Brazil, Belgium, Slovak Republic, France, and Taiwan, shared their valuable research results. Additionally, there were 20 selected posters, one session for American Oil Chemists Society Asian Section, and two workshops for Biotech Developments and over 600 attendees. A few chapters contained in this book were contributed by distinguished scientists who could not attend this meeting. This meeting was a great success and we greatly appreciate President Dr. Chi-Huey Wong of Academia Sinica for providing the venue for the meeting. The contributions of local organization committee members are highly appreciated: Andrew H.-J. Wang, and Ming-Che Shih of Academia Sinica, and Yung-Sheng Huang, Chang-Hsien Yang of the National Chung Hsing University.

Recent energy and food crises point out the important of bio-based products from renewable resources and agricultural biotechnology. It is inevitable to use modern tools of molecular engineering on plants, animals and microorganisms to solve these crises and improve the wellness of humankind. There is no comprehensive book on molecular engineering of agricultural biotechnology and bio-based products from renewable resources. The authors are internationally recognized experts from all sectors of academia, industry, and government research institutes. This is the most current book on molecular engineering of agricultural biotechnology and bio-based industrial products.

This book composes of 30 chapters divided into three sections. The first 10 chapters describe the world's newest research on improvement of agronomic and microbial traits. Included are: Insights into the Structure and Function of Acyl-CoA: Diacylglycerol Acyltransferase, Improving Enzyme Character by Molecular Breeding-Preparation of Chimeric Genes, Production and Accumulation of Unusual Fatty Acids in Plant Tissues, Preparation of Oleaginous Yeast by Genetic Modification and Its Potential Applications, Improving Value of Oil Palm Using Genetic Engineering, Potential in Using Arabidopsis Acyl-Coenzyme-A-Binding Proteins in Engineering Stress-Tolerant Plants, Modification of Lipid Composition by Genetic Engineering in Oleaginous Marine Microorganisms: Thraustochytrid, Integrated Approaches to Manage Tomato Yellow Leaf Curl Viruses, Carbohydrate Acquisition During Legume Seed Development, and Biotechnology Enhancement of Phytosterol Biosynthesis in Seed Oils. The second section includes 8 chapters devoted to Functional Foods and Biofuels: Dietary Phosphatydyl Inositol in Metabolic Syndrome, Biotechnological Enrichment of Cereals with Polyunsaturated Fatty Acids, Brown Seaweeds Lipids as Possible Source for Nutraceuticals and Functional Foods, Lipophillic Ginsenosides Derivative Production, Processes for Production of Biodiesel Fuel, Noncatalytic Alcoholysis Process for Production of Biodiesel Fuel—Its Potential in Japan and Southeast Asia, Use of Coniochaeta Ligniaria to Detoxify Fermentation Inhibitors Present in Cellulosic Sugar Streams, and Omics Applications to Biofuel Research. The third section with 12 chapters describes Renewable Bioproducts: Biotechnological Uses of Phospholipids, Application of Partition Chromatographic Theory on the Routine Analysis of Lipid Molecular Species, Dehydrogenase-Catalyzed Synthesis of Chiral Intermediates for Drugs, Engineering of Bacterial Cytochrome P450 Monooxygenase as Biocatalysts for Chemical Synthesis and Environmental Bioremediation, Glycosynthase from Inverting Hydrolases, Molecular Species of Diacylglycerols and Triacylglycerols Containing Dihydroxy Fatty Acids in Castor Oil, Biocatalytic Production of Lactobionic Acid, Recent Advances in Aldolase-Catalyzed Synthesis of Unnatural Sugars and Iminocyclitols, Production of Value-Added Products by Lactic Acid Bacteria, Enzyme Synthesis of Glycosides Using Alpha-Amylase Family Enzymes, Biological Synthesis of Gold and Silver Nanoparticles Using Plant Leaf Extracts and Antimicrobial Application, and Potential Approach of Microbial Conversion to Develop New Antifungal Products of Omega-3 Fatty Acids.

This book serves as reference for teachers, graduate students, and industrial scientists who conduct research in biosciences and biotechnology.

CHING T. HOU

Peoria, IL USA

JEI-FU SHAW

Taichung,Taiwan

CONTRIBUTORS

Hirofumi Adachi, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Japan

Zuzana Adamechova, Department of Biochemical Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinskeho 9, Bratislava, Slovak Republic

Tsunehiro Aki, Department of Molecular Biotechnology, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Japan

Nur Hanin Ayub, Malaysian Palm Oil Board, No. 6, Persiaran Institusi, Bandar Baru Bangi, Kajang, Selangor, Malaysia

Hassan Azaizeh, Institute of Applied Research Center (Affiliated with University of Haifa), The Galilee Society, Shefa-Amr, Israel

Vivek K. Bajpai, Department of Biotechnology, Daegu University, Kyoungsan, Kyoungbook, Republic of Korea

Pankaj K. Bhowmik, National Research Council—Plant Biology Institute Saskatoon, Saskatchewan, Canada

Kenneth M. Bischoff, Renewable Product Technology Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, 1815 N. University St., Peoria, IL, USA

Bahariah Bohari, Malaysian Palm Oil Board, No. 6, Persiaran Institusi, Bandar Baru Bangi, Kajang, Selangor, Malaysia

Milan Certik, Department of Biochemical Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovak Republic

Hsin-Liang Chen, Biodiversity Research Center, Academia Sinica, Taipei, Taiwan

Qin-Fang Chen, School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, China

Qilin Chen, National Research Council Canada—Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, Saskatchewan, Canada

Mee-Len Chye, School of Biological Sciences, The University of Hong Kong, Pokfulam, Hong Kong and State (China) Key Laboratory of Agrobiotechnology, Chinese University of Hong Kong, Shatin, Hong Kong

Fengjie Cui, Department of Food, Agricultural, and Biological Engineering, The Ohio State University/Ohio Agricultural Research and Development Center, Wooster, USA

Robert de la Pena, AVRDC—The World Vegetable Center, Shanhua, Tainan, Taiwan

Bruce S. Dien, Bioenergy Research Unit, NCAUR, USDA-ARS, Peoria, IL, 61604, USA

Ahmad Kushairi Din, Malaysian Palm Oil Board, No. 6, Persiaran Institusi, Bandar Baru Bangi, Kajang, Selangor, Malaysia

Wei Gao, School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, China

William A. Greenberg, Department of Chemistry, The Scripps Research Institute, 10550, N. Torrey Pines Rd., La Jolla, CA 92037, USA

Shoji Hagiwara, National Food Research Institute, NARO, Kan-nondai, Tsukuba, Ibaraki, Japan

J. J. Han, Doosan Glonet, B5F, Advanced Convergence Institute of Technology, 864-1 IUI-dong, Suwon, Gyeonggi, Korea

Ahmad Tarmizi Hashim, Malaysian Palm Oil Board, No. 6, Persiaran Institusi, Bandar Baru Bangi, Kajang, Selangor, Malaysia

T. Hatanaka, Kobe University, Kobe, Japan

Kiyoshi Hayashi, National Food Research Institute, 2-1-12 Kan-nondai, Tsukuba, Ibaraki, Japan

Xiaohua He, Western Regional Research Center, United States Department of Agriculture, Albany, California, USA

David Hildebrand, Agronomy Department, University of Kentucky, Lexington, Kentucky, USA

Tsugihiko Hirano, Renesas Northern Japan Semiconductor, Inc., 145-1 Nakajima Nanae-cho Kameda-gun, Hokkaido, Japan

Zhangyong Hong, Department of Chemistry, The Scripps Research Institute, 10550, N. Torrey Pines Rd., La Jolla, CA 92037, USA

Masashi Hosokawa, Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Japan

Ching T. Hou, Microbial Genomic and Bioprocessing Research Unit, National Centre for Agricultural Utilization Research, ARS, USDA, Peoria, IL, USA

Jiang-Ning Hu, Department of Food Science and Technology, Chungnam National University, 220 Yusung Gu, Gung-Dong, Daejeon, Republic of Korea

J. Hughes, AVRDC—The World Vegetable Center, Shanhua, Tainan, Taiwan

Zamzuri Ishak, Malaysian Palm Oil Board, No. 6, Persiaran Institusi, Bandar Baru Bangi, Kajang, Selangor, Malaysia

Hiroaki Iwasaka, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Japan

Ghulam Kadir Ahmad Parveez, Malaysian Palm Oil Board, No. 6, Persiaran Institusi, Bandar Baru Bangi, Kajang, Selangor, Malaysia

P. Kadirvel, AVRDC—The World Vegetable Center, Shanhua, Tainan 71499, Taiwan

Toshihide Kakizono, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Japan

Yasushi Kamisaka, Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology, Japan

Sun Chul Kang, Department of Biotechnology, College of Engineering, Daegu University, Gyungsan City, Gyungbook, Korea

Seiji Kawamoto, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Japan

Hiroko Kawasaki, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Japan

L. Kenyon, AVRDC—The World Vegetable Center, Shanhua, Tainan 71499, Taiwan

Beom Soo Kim, Department of Chemical Engineering, Chungbuk National University, 12 Gaeshindong, Heungdeokgu, Cheongju, Chungbuk, Korea

Hak-Ryul Kim, Department of Animal Science and Biotechnology, Kyoungpook National University, Daegu, Republic of Korea

Takaaki Kiryu, Osaka Municipal Technical Research Institute, Osaka, Japan

Taro Kiso, Osaka Municipal Technical Research Institute, Osaka, Japan

Motomitsu Kitaoka, Enzyme Laboratory, National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki, Japan

Takashi Kuriki, Biochemical Research Laboratory, Ezaki Glico Co., Ltd., 4-6-5 Utajima, Nishiyodogawa-ku, Osaka, Japan

K.T. Lee, Department of Food Science and Technology, Chungnam National University, 220 Gung-Dong Yusung-Gu, Daejon, South Korea

R. Li, Plant Science, University of Kentucky, Lexington, KY, USA

Qing-Shan Li, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakwa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan

Wen-Hsiung Li, Genomics Research Center, Academia Sinica, Taipei, Taiwan and Biodiversity Research Center, Academia Sinica, Taipei, Taiwan

Yebo Li, Department of Food, Agricultural, and Biological Engineering, The Ohio State University/Ohio Agricultural Research and Development Center, Wooster, OH, USA

Jiann-Tsyh Lin, Western Regional Research Center, ARS, USDA, Albany, CA, USA

Qin Liu, Agricultural Lipid Biotechnology Program, Department of Agricultural, Food and Nutritional Science, University of Alberta, 4-10 Agriculture/Forestry Centre, Edmonton, Alberta, Canada

Siqing Liu, Renewable Product Technology Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, 1815 N. University St., Peoria, IL61604, USA

Maria J. López, Departamento de Biología Aplicada, University of Almería, Almería, Spain

Thomas McKeon, Western Regional Research Center, United States Department of Agriculture, Albany, California, USA

Kazuo Miyashita, Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Mirato, Hakodate, Japan

Joaquín Moreno, Departamento de Biología Aplicada, University of Almería, Almería, Spain

Hiromi Murakami, Osaka Municipal Technical Research Institute, Osaka, Japan

Hiroshi Nabetani, Head of the Laboratory. Reaction and Separation Engineering Laboratory Food Engineering Division National Food Research Institute, National Agriculture and Food Research Organization, 2-1-12 Kan-nondai, Tsukuba, Ibaraki, Japan

Koji Nagao, Laboratory of Nutrition Biochemistry, Department of Applied Biochemistry and Food Science, Saga University, Saga, Japan

Mitsutoshi Nakajima, National Food Research Institute, NARO, Kan-nondai, Tsukuba, Ibaraki, Japan

Hirofumi Nakano, Osaka Municipal Technical Research Institute, 6-50, Morino-miya 1-chome, Joto-ku, Osaka, Japan

Maya Nanko, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Japan

Nancy N. Nichols, Bioenergy Research Unit, NCAUR, USDA-ARS, Peoria, IL 61604, USA

Mamoru Nishimoto, National Food Research Institute, 2-1-12, Kannondai Tsukuba Ibaraki, Japan

Takahisa Nishimura, Biochemical Research Laboratory, Ezaki Glico Co., Ltd., 4-6-5 Utajima, Nishiyodogawa-ku, Osaka, Japan

Hiromi Nishiura, Biochemical Research Laboratory, Ezaki Glico Co., Ltd., 4-6-5 Utajima, Nishiyodogawa-ku, Osaka, Japan

Koji Nomura, Biochemical Research Laboratory, Ezaki Glico Co., Ltd., 4-6-5 Utajima, Nishiyodogawa-ku, Osaka, Japan

Jun Ogawa, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto, Japan

Kazuhisa Ono, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Japan

Abrizah Othman, Malaysian Palm Oil Board, No. 6, Persiaran Institusi, Bandar Baru Bangi, Kajang, Selangor, Malaysia

Jocelyn A. Ozga, 4-10 Agriculture/Forestry Centre, Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada

Ramesh Patel, SLRP Associates, Biotechnology Consulting Firm, Bridgewater, NJ, USA

Umi Salamah Ramli, Malaysian Palm Oil Board, No. 6, Persiaran Institusi, Bandar Baru Bangi, Kajang, Selangor, Malaysia

S. Rao, Plant Science, University of Kentucky, Lexington, KY, USA

Dennis M. Reinecke, Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta

J.S. Rhee, Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1, Guseong-dong, Yuseong-gu, Daejeon, Korea

Ravigadevi Sambanthamurthi, Malaysian Palm Oil Board, No. 6, Persiaran Institusi, Bandar Baru Bangi, Kajang, Selangor, Malaysia

Rolf D. Schmid, Institut für Technische Biochemie, Universität Stuttgart, Allmandring 31, Stuttgart, Germany

Ming-Che Shih, Agricultural Biotechnology Center, Academia Sinica, Taipei, Taiwan

Yuji Shimada, Osaka Municipal Technical Research Institute, Osaka, Japan. 1-6-50 Morinomiya, Joto-ku, Osaka, Japan

Sakayu Shimizu, Divison of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakwa-oiwakecho, Sakyo-ku, Kyoto, Japan

Bungo Shirouchi, Laboratory of Nutrition Biochemistry, Department of Applied Biochemistry and Food Science, Saga University, Saga, Japan and Food Function and Labeling Program, National Institute of Health and Nutrition, 1-23-1 Toyama, Shinjuku-ku, Tokyo, Japan

Rodrigo M. P. Siloto, Agricultural Lipid Biotechnology Program, Department of Agricultural, Food and Nutritional Science, University of Alberta, 4-10 Agriculture/Forestry Centre, Edmonton, Alberta, Canada

J.K. Song, Chemical Biotechnology Research Center, Korea Research Institute of Chemical Technology, Yuseong-gu, Daejeon, Korea

Jae Yong Song, Department of Chemical Engineering, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea

Lucia Slavikova, Department of Biochemical Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinskeho 9, Bratislava, Slovak Republic

Kazuhisha Sugimoto, Biochemical Research Laboratory, Ezaki Glico Co., Ltd., Osaka, Japan

Masakazu Sugiyama, AminoScience Laboratories, Ajinomoto Co. Inc., 1-1 Suzukicho, Kawasakiku, Kawasakishi, Japan, 210-8681

Huang-Mo Sung, Department of Life Sciences, National Cheng Kung University, Tainan, Taiwan

Ahmed Tafesh, Institute of Applied Research Center (Affiliated with University of Haifa), The Galilee Society, Shefa-Amr, Israel

Koretaro Takahashi, Graduate School of Fisheries Science, Hokkaido University, 3-1-1 Minato, Hakodate, Japan

J.R. Thoguru, Plant Science, University of Kentucky, Lexington, KY, USA

Takayuki Tsukui, Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Japan

Vlada Urlacher, Institut für Technische Biochemie, Universität Stuttgart, Allmandring 31, Stuttgart, Germany

S. Venkatesan, AVRDC—The World Vegetable Center, P.O. Box 42, Shanhua, Tainan 71499, Taiwan

Mohd Basri Wahid, Malaysian Palm Oil Board, No. 6, Persiaran Institusi, Bandar Baru Bangi, Kajang, Selangor, Malaysia

Tzi-Yuan Wang, Genomics Research Center, Academia Sinica, Taipei, Taiwan

Yomi Watanabe, Osaka Municipal Technical Research Institute, 1-6-50 Morinomiya, Joto-ku, Osaka, Japan

Randall J. Weselake, Department of Agriculture, Food and Nutritional Sciences, University of Alberta, Edmonton, Alberta, Canada

M. Airanthi K. Widjaja-Adhi, Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Japan

Chi-Huey Wong, Department of Chemistry, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037, USA

Shi Xiao, School of Biological Sciences, The University of Hong Kong, Hong Kong, China

Teruyoshi Yanagita, Department of Applied Biological Sciences, Saga University, Saga, Japan

Suk Hoo Yoon, Korea Food Research Institute, San 46-1, Baekhyun-Dong, Bundang-Ku, Songnam-Si, Kyunggi-Do, Korea

Abdul Masani Mat Yunus, Malaysian Palm Oil Board, No. 6, Persiaran Institusi, Bandar Baru Bangi, Kajang, Selangor, Malaysia

Jitao Zou, Plant Biotechnology Institute, National Research Council Canada, 110 Gymnasium Place, Saskatoon, Canada

SECTION I

IMPROVEMENT OF AGRONOMIC AND MICROBIAL TRAITS

1

INSIGHTS INTO THE STRUCTURE AND FUNCTION OF ACYL-CoA: DIACYLGLYCEROL ACYLTRANSFERASE

RODRIGO M.P. SILOTO, QIN LIU, AND RANDALL J. WESELAKE

Agricultural Lipid Biotechnology Program, Department of Agricultural, Food and Nutritional Science, University of Alberta, 4-10 Agriculture/Forestry Centre, Edmonton, Alberta, Canada T6G 2P5

XIAOHUA HE AND THOMAS MCKEON

Western Regional Research Center, United States Department of Agriculture, Albany, California 94710, USA

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1.1 INTRODUCTION

Production of vegetable oils has been recognized as a rapidly developing field in plant biotechnology that goes beyond food-based applications. Many kinds of vegetable oils are used in soaps and cosmetics or converted to oleochemicals that are extensively used to replace petrochemicals in paints, plastics, fuels, and lubricants. The demand for biodegradable chemicals applied to industrial products has been increasing, and therefore a boost in the production of vegetable oils and fats is needed. Biotechnological approaches including traditional plant breeding and direct genome modification through genetic engineering are crucial tools to increase seed oil production without extending the area of crop cultivation, which has a direct impact on deforestation and competition with food production. Moreover, even a diminutive increase in seed oil content reflects in considerable profitability. Despite the unprecedented advances derived from molecular genetics and genomics research on the biochemical pathways of plant lipid metabolism in the last decade, the mechanisms regulating seed oil content are not fully understood. Many aspects of key enzymes are not yet determined even in model plants such as *Arabidopsis thaliana* (Hildebrand et al., 2008). For example, recent studies focusing on intracellular trafficking indicated that compartmentalization of enzyme activities within the endoplasmic reticulum (ER) membrane represents an additional mechanism adopted by plant cells to control oil production and may be essential for channeling of particular fatty acids into storage lipids (Dyer and Mullen, 2008).

Nevertheless, manipulation of genes involved in storage lipid biosynthesis has been used to increase accumulation of seed triacylglycerol (TAG), the main component of vegetable oils (Weselake, 2002). It was recently demonstrated that overexpression of plant and fungi genes encoding acyl-CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20), which catalyzes the final assembly of TAG, resulted in small but significant increases in seed oil content in canola and soybean tested under field conditions (Lardizabal et al., 2008; Weselake et al., 2008). Indeed, the level of DGAT activity in developing seeds seems to have a direct effect on the accumulation of TAG (Perry and Harwood, 1993; Cahoon et al., 2007). Surprisingly, little is known about the molecular mechanisms governing DGAT activity. The most basic information about structure and function of this enzyme is essential for rational designs to increase its performance in oilseeds and have a direct reflection in seed oil content. In view of the biotechnological importance of DGATs from plants and fungi, we summarize some of the structural and functional aspects of these enzymes with particular attention to membrane topology, functional polypeptide motifs, and subcellular localization. We use *in silico* approaches to compare the findings obtained with related enzymes in animals and prokaryotes.

1.2 DISCOVERY OF DGAT

The first proceedings reporting DGAT activity date from the 1950s (Weiss and Kennedy, 1956; Weiss et al., 1960), but the genes encoding DGATs were not isolated

until the late 1990s. The first *DGAT* cDNA was cloned by taking advantage of homology between an expressed sequence tag (EST) and an acyl-CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26), a related enzyme previously isolated by a complementation assay of mammalian cells devoid of cholesterol ester biosynthesis (Chang et al., 1993). The mouse (*Mus musculus*) *DGAT* gene isolated in 1998 encodes a protein, here referred to as MmDGAT1 that is 20% identical to mouse ACAT with the most conserved regions on the C-terminus portion of the enzyme (Cases et al., 1998). A plant *DGAT* gene was consequently isolated through the characterization of the locus *TAG1* in an *A. thaliana* EMS-induced mutant (AS11) with altered seed fatty acid composition and decreased DGAT activity (Katavic et al., 1995). The locus *TAG1* contains a 3.4-kb gene encoding a polypeptide showing 41% identity with MmDGAT1 (Zou et al., 1999). The polypeptide encoded by *TAG1* (AtDGAT1) exhibits DGAT activity when expressed in yeast and can complement DGAT function in AS11 (Jako et al., 2001). *DGAT* genes from fungi were identified through protein purification, an approach that was previously not successful with other DGATs, perhaps because of their membrane association. Polypeptides exhibiting DGAT activity were purified from lipid bodies of *Umbelopsis ramanniana*, formerly known as *Mortierella ramanniana* (Lardizabal et al., 2001). These *DGATs* shared little or apparently no homology with the previous *DGAT* genes, and therefore were classified as *DGAT2*. Curiously, genes homologous to *DGAT1* have not been found in fungi genomes, although it has been suggested that yeast *ACATs* (*ARE1* and *ARE2* in *Saccharomyces cerevisiae*) represent *DGAT1* orthologs in these organisms because they also display minor DGAT activity (Yen et al., 2008).

Several lines of evidence suggest that DGAT1 belongs to a class of enzymes with acyl-CoA transferase activity, which can utilize different acceptors in addition to diacylglycerols. For example, MmDGAT1 also possesses acyl-CoA:retinol acyltransferase (ARAT, EC 2.6.1.57) activity (Yen et al., 2005), while an *A. thaliana* acyl-CoA:fatty alcohol acyltransferase (wax ester synthase, WSD1) also displays DGAT activity *in vitro* (Li et al., 2008). In the case of DGAT2, a similar scenario is observed. In animals, *DGAT2* belongs to a gene family with seven members in humans (Cases et al., 2001). Three of these genes encode polypeptides with acyl-CoA monoacylglycerol acyltransferase (MGAT, EC 2.3.1.22) activity (Yen et al., 2002; Yen and Farese, 2003; Cheng et al., 2003). Two additional members display acyl-CoA:wax alcohol acyltransferase (AWAT, EC 2.3.1.75) activity, which is analogous to WSD1 (Turkish et al., 2005).

Orthologs of *DGAT1* and *DGAT2* have been identified through DNA homology in many other organisms and are widely distributed in eukaryotes. Currently, a relatively wide collection of *DGAT* genes is available which facilitates more detailed studies of enzyme structure and function through bioinformatic approaches. Many of these genes have been functionally characterized in recombinant systems as described in Table 1.1.

In prokaryotes, a bifunctional WS/DGAT was identified in *Acinetobacter calcoaceticus* (Kalscheuer and Steinbuchel, 2003). WS/DGAT has no sequence similarity to DGAT1, DGAT2, or any of the related acyltransferases from eukaryotes. Another nonhomologous DGAT, referred to as AhDGAT, was characterized in peanuts

TABLE 1.1 Eukaryotic DGATs Functionally Tested in Recombinant Organisms

cDNA	Original Organism	Host Used for Expression and Relevant Genetic Markers	Reference
HsDGAT1	*H. sapiens*	*S. cerevisiae* 12501 (*dga1*$^{-}\Delta$)	Inokoshi et al. (2009)
AtDGAT1	*A. thaliana*	*B. napus*	Weselake et al. (2008)
BnDGAT1	*B. napus*	*B. napus*	Weselake et al. (2008)
TmDGAT1	*T. majus*	*S. cerevisiae* H1246 (*are1*$^{-}\Delta$, *are2*$^{-}\Delta$, *dga1*$^{-}\Delta$, *lro1*$^{-}\Delta$), *A. thaliana* and *B. napus*	Xu et al. (2008)
ZmDGAT1	*Z. mays*	*Z. mays* and *S. cerevisiae* (*dga1*$^{-}\Delta$, *lro1*$^{-}\Delta$)	Zheng et al. (2008)
VgDGAT1	*V. galamensis*	*S. cerevisiae*	Yu et al. (2008)
VfDGAT1	*V. fordii*	*S. cerevisiae* SCY1998 (*dga1*$^{-}\Delta$, *lro1*$^{-}\Delta$)	Shockey et al. (2006)
AhDGAT	*A. hypogaea*	*E. coli*	Saha et al. (2006)
MmDGAT1	*M. musculus*	COS7-cells (*C. sabaeus*)	Yen et al. (2005)
EaDGAT1	*E. alatus*	*S. cerevisiae* H1266 (*are2*$^{-}\Delta$, *dga1*$^{-}\Delta$, *lro1*$^{-}\Delta$)	Milcamps et al. (2005)
AtDGAT1	*A. thaliana*	*S. cerevisiae* H1266 (*are2*$^{-}\Delta$, *dga1*$^{-}\Delta$, *lro1*$^{-}\Delta$)	Milcamps et al. (2005)
RcDGAT1	*R. communis*	*S. cerevisiae*	He et al. (2004)
TgDGAT1	*T. gondii*	*S. cerevisiae* SCY910 (*are1*$^{-}\Delta$, *are2*$^{-}\Delta$)	Quittnat et al. (2004)
HsDGAT1	*H. sapiens*	McA-RH7777 cells (*R. norvegicus*)	Liang et al. (2004)
BnDGAT1	*B. napus*	*P. pastoris*	Nykiforuk et al. (2002)
MmDGAT1	*M. musculus*	Sf9 insect cells (*S. frugiperda*)	Cases et al. (2001)
AtDGAT1	*A. thaliana*	*A. thaliana* AS11	Jako et al. (2001)
AtDGAT1	*A. thaliana*	*S. cerevisiae* SCY 062	Bouvier-Nave et al. (2000a)
CeDGAT1	*C. elegans*	*S. cerevisiae* SCY 062	Bouvier-Nave et al. (2000a)
NtDGAT1	*N. tabacum*	*S. cerevisiae* SCY 062	Bouvier-Nave et al. (2000a)
AtDGAT1	*A. thaliana*	*S. cerevisiae* SCY059 (*are1*$^{-}\Delta$, *are2*$^{-}\Delta$), *N. tabacum*	Bouvier-Nave et al. (2000b)
AtDGAT1	*A. thaliana*	Sf21 insect cells (*S. frugiperda*)	Hobbs et al. (1999)
AtDGAT1	*A. thaliana*	*S. cerevisiae* YMN5 (*Slc1*$^{-}\Delta$)	Zou et al. (1999)
MmDGAT1	*M. musculus*	H5 insect cells (*T. ni*)	Cases et al. (1998)
HsDGAT2	*H. sapiens*	*S. cerevisiae* 12501 (*dga1*$^{-}\Delta$)	Inokoshi et al. (2009)
RcDGAT2	*R. communis*	*A. thaliana* and *S. cerevisiae* (*dga1*$^{-}\Delta$)	Burgal et al. (2008)
UeDGAT2	*U. ramanniana*	*G. max*	Lardizabal et al. (2008)

TABLE 1.1 (*Continued*)

cDNA	Original Organism	Host Used for Expression and Relevant Genetic Markers	Reference
RcDGAT2	*R. communis*	*S. cerevisiae*	Kroon et al. (2006)
MmDGAT2	*M. musculus*	COS7-cells (*C. sabaeus*)	Stone et al. (2006)
VfDGAT2	*V. fordii*	*S. cerevisiae* SCY1998 (*dga1*$^{-}\Delta$, *lro1*$^{-}\Delta$)	Shockey et al. (2006)
HsDGAT2	*H. sapiens*	*S. cerevisiae* ScY2051 (*are2*$^{-}\Delta$, *dga1*$^{-}\Delta$, *lro1*$^{-}\Delta$)	Turkish et al. (2005)
MmDGAT2	*M. musculus*	Sf9 insect cells (*S. frugiperda*)	Cases et al. (2001)
HsDGAT2	*H. sapiens*	Sf9 insect cells (*S. frugiperda*)	Cases et al. (2001)
CeDGAT2	*C. elegans*	Sf9 insect cells (*S. frugiperda*)	Lardizabal et al. (2001)
ScDGAT2	*S. cerevisiae*	Sf9 insect cells (*S. frugiperda*)	Lardizabal et al. (2001)
UeDGAT2	*U. ramanniana*	Sf9 insect cells (*S. frugiperda*)	Lardizabal et al. (2001)

(Saha et al., 2006). Unlike other eukaryote enzymes, AhDGAT was purified from the soluble fraction of developing peanuts. Biosynthesis of TAG in the cytosol has been previously reported in a 10S multienzyme complex from the oleaginous yeast *Rhodotorula glutinis* (Gangar et al., 2001). Whether this soluble yeast DGAT and AhDGAT compose a novel class of DGATs is yet to be demonstrated.

1.3 MEMBRANE TOPOLOGICAL ORGANIZATION OF DGATs

The pattern in which a protein transverses the membrane bilayer is essential for elucidating the dynamics of the protein structure. DGAT1 and DGAT2 contain hydrophobic segments that are generally believed to constitute transmembrane domains (Fig. 1.1). DGAT1 displays more hydrophobic segments than DGAT2, which indicates a different topology and may relate to different physiological roles in TAG biosynthesis (Yen et al., 2008). Few experimental studies on DGAT topological organization in plants and yeast are available, and therefore we will mainly rely on *in silico* approaches to predict transmembrane segments and the orientation in the membrane bilayer.

A variety of web-based tools are available for predicting the topology of membrane proteins. Since only a few membrane proteins from bacteria are known to be beta-barrel shaped so far, the prediction algorithms are mostly developed for alpha-helical membrane proteins. Generally, five types of techniques have been used in these

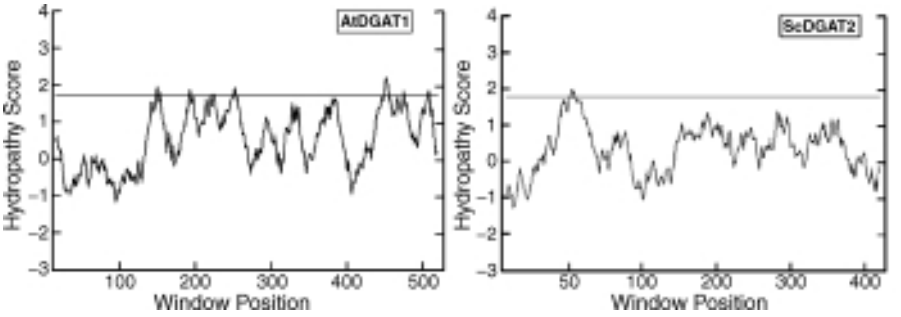

FIGURE 1.1 Kyte–Doolittle hydropathy plots of DGATs. Plots were generated by the method of Kyte and Doolittle (1982) using a window size of 19. Cutoff value (line) is 1.8 and peaks with score greater than 1.8 indicate possible transmembrane regions.

programs: hydrophobicity analysis combined with the positive inside rule (eg. TMpred and SOSUI), multiple sequence alignment (eg. ConPredII and TOPCONS), model-recognition approach (eg. MEMSTAT3, TMHMM, and HMMTOP), and support vector machine technique (eg. SVMtm) (Persson, 2006). An evaluation of the reliability of these methods indicated that a consensus prediction and model-based methods are best performing (Moller et al., 2001; Ikeda et al., 2002). The application of these algorithms for the prediction of TM domains in DGAT1 is described in Table 1.2 using AtDGAT1 and MmDGAT1 as models. For AtDGAT1, nine of the ten putative transmembrane domains are highly conserved among most of the prediction results except for the domains at 276–299 and 314–337 of AtDGAT1 and 251–276 and 285–312 of MmDGAT1 (highlighted TM5 and TM6 in Table 1.2). A model of nine-membrane-spanning topology agrees with our initial study on DGAT1 from *Brassica napus* (Foroud, 2005). In this work, protease mapping data showed that the region between 276 and 299 in BnDGAT1 (corresponding to TM5) is in the cytosol, in agreement with most of the prediction algorithms described in Table 1.2. Recent studies on DGAT1 from *Vernicia fordii* (tung tree) and *B. napus* indicated that the N-terminus faces the cytosolic side (Shockey et al., 2006; Weselake et al., 2006) as predicted by most algorithms in Table 1.2. The interaction of the N-terminus with lipid substrates in the cytoplasm may lead to a regulatory role of N-terminal region (Siloto et al., 2008) and there are several lines of evidence not only from *B. napus* DGAT1 but from mammalian DGAT1 and ACAT1 that favor this hypothesis (Cheng et al., 2001; Yu et al., 1999; Weselake et al., 2006). According to the work on VfDGAT1, the C-terminus of DGAT1 is also proved to orient toward cytosolic side, indicating an even number of membrane-spanning regions. This result disagrees with a nine-transmembrane topology model, and therefore further experimental testing will be required to examine the hypothesis of eight transmembrane domains.

Compared to DGAT1, DGAT2 is less hydrophobic, having a lower number of transmembrane domains and therefore a less intricate topology. The membrane topology of MmDGAT2 was experimentally determined revealing two transmembrane domains that are closely associated or a single hydrophobic domain embedded

TABLE 1.2 Prediction Results for Transmembrane Domains in DGAT1

	ConPredII	TOPCONS	MEMSAT3	HMMTOP	TMHMM	SVMtm	SOSUI	TMpred
AtDGAT1								
TM1	132–152	132–152	134–152	133–152	133–152	135–149	131–153	131–152
TM2	176–196	173–193	177–197	177–195	176–195	179–193	175–197	176–193
TM3	207–227	209–229	206–230	208–227	207–229	208–222	205–227	208–229
TM4	234–254	234–254	233–252	236–255	234–256	236–251	235–257	234–256
TM5	277–297	—	—	276–294	—	—	—	280–299
TM6	316–336	314–334	312–331	319–336	315–337	316–330	—	314–333
TM7	365–385	362–382	353–371	363–382	363–385	363–382	370–392	366–385
TM8	433–453	433–453	431–450	434–453	433–455	433–448	432–454	433–451
TM9	458–478	458–478	453–475	460–479	460–479	457–473	459–481	460–476
TM10	488–508	488–508	487–510	490–509	491–513	489–503	484–506	487–509
Orientation*	OUT	IN	IN	IN	IN	IN	N/A	OUT
MmDGAT1								
TM1	95–115	95–115	97–115	96–114	—	97–111	93–115	96–114
TM2	140–160	137–157	142–161	141–159	137–159	144–158	141–160	137–157
TM3	173–193	174–194	171–195	172–195	172–194	175–195	171–193	174–198
TM4	200–220	198–218	200–224	202–220	198–220	200–214	199–221	200–218
TM5	256–276	—	—	251–269	—	—	—	—
TM6	—	292–312	285–308	296–312	293–312	—		293–311
TM7	339–359	334–354	338–360	343–361	342–364	341–357	337–359	343–364
TM8	—	412–432	410–429	—	412–434	417–431	—	—
TM9	439–459	434–454	432–456	439–456	439–456	439–454	—	436–456
TM10	465–485	465–485	464–483	467–484	463–485	467–481	—	463–483
Orientation*	IN	IN	IN	IN	OUT	N/A	OUT	IN

Note: The polypeptides corresponding to AtDGAT1 and MmDGAT1 were submitted to a number of transmembrane prediction algorithms. The numbers correspond to the position of each transmembrane (TM) domain.

*Orientation of N-terminus. Cytosol: "IN". Lumen: "OUT". TMs highlighted in gray are not universally predicted. The websites used for each algorithm are: ConPred II, http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2; TOPCONS, http://topcons.net; MEMSAT3, http://bioinf.cs.ucl.ac.uk/psipred/psiform.html; HMMTOP, http://www.enzim.hu/hmmtop; TMHMM, http://www.cbs.dtu.dk/services/TMHMM; SVMtm, http://ccb.imb.uq.edu.au/svmtm; SOSUI, http://bp.nuap.nagoya-u.ac.jp/sosui; TMpred, http://www.ch.embnet.org/software/TMPRED_form.html.

in the membrane bilayer (Stone et al., 2006). The first transmembrane domain (TM1) of MmDGAT2 and ScDGAT2 was ubiquitously predicted, but the second (TM2) was identified by only a few algorithms (Table 1.3). Since the homology of DGAT2 from different organisms is lower than that of DGAT1, it is possible that ScDGAT2, which has a distinct hydropathy plot, could have a different topology compared with other fungi DGAT2s. This could be demonstrated by the prediction results of *Schizosaccharomyces pombe* SpDGAT2 (Table 1.3). Interestingly, the prediction of N-terminus orientation seems to be related to the length of the predicted N-terminal tail. DGAT2s with putative long tails are intended to face toward the cytosol, which agrees with work on VfDGAT2 and MmDGAT2 (Shockey et al., 2006; Stone et al., 2006). The same conclusion, however, cannot be made for DGAT2s with short tails.

1.4 ALIGNMENT OF DGAT1 POLYPEPTIDES

DGAT1 polypeptides are typically characterized by a hydrophilic N-terminus sequence followed by a number of hydrophobic stretches constituting potential transmembrane domains as previously discussed. The total number of predicted transmembrane domains in DGAT1 can vary according to the sequence and the algorithm used as shown above. When the sequences are aligned, however, many of these potential transmembrane domains are found in the same positions in most DGAT1 (Fig. 1.2). The first four transmembrane domains on the first half of the sequences and the last three transmembrane domains on the C-terminus are separated by short polar loops. Between these groups are two possible membrane-spanning regions that are separated by longer hydrophilic stretches. Here we will consider these nine potential transmembrane domains as landmarks to describe conserved motifs in DGAT1, acknowledging, however, that an experimental approach is required to verify these assumptions. We will also use the sequence of *A. thaliana* DGAT1 to describe the exact position of each motif.

An overview of the DGAT1 alignment from 30 different organisms indicates several conserved regions with about 7% of identical residues among plant and animal sequences. The hydrophilic N-terminus is composed of an average of 115 and 80 residues in plants and animals, respectively and is the least conserved region in DGAT1. An alignment of the N-terminal portion of DGAT1 from a broad range of organisms revealed a cluster of arginines in the first 30 residues (Fig. 1.3). The region comprising 20 positions preceding the first hydrophobic domain is also conserved and contains the motifs PAHRXXXESPLSSDAIFXQ and SLFSXXSGFXN, which are conserved in plants and animals, respectively. Other divergences discriminating DGAT1 from plants and animals include a serine at position 131 of AtDGAT1 conserved in plants and absent in animal DGAT1, and the motif WVXRQ in plants, corresponding to FL($^{L}/_{I}$)($^{R}/_{K}$)R in animals. These differences can be also observed in more ancient organisms such as *Toxoplasma gondii* and *Physcomitrella patens*. The long loop between the fourth and fifth transmembrane domains (between positions 260 and 278 of AtDGAT1) shows remarkable variability among all DGAT1s. Following this region lies the most conserved uninterrupted sequence of DGAT1

TABLE 1.3 Prediction Results for Transmembrane Domains in DGAT2

	ConPredII	TOPCONS	MEMSAT3	HMMTOP	TMHMM	SVMtm	SOSUI	TMpred
ScDGA2								
TM1	73–93	62–82	59–77	68–92	70–92	66–97	75–97	72–92
TM2	—	84–104	80–104	—	—	—	—	—
TM3	189–209	188–208	—	—	—	193–207	188–210	196–214
TM4	215–235	—	—	200–224	—	217–231	213–234	216–236
TM5	293–313	—	—	294–310	—	—	—	—
TM6	—	—	—	341–359	—	—	—	—
Orientation	IN	IN	IN	IN	IN	IN	N/A	IN
MmDGAT2								
TM1	70–90	71–91	61–85	68–92	73–95	73–93	66–88	76–96
TM2	—	93–113	88–112	—	—	95–109	93–115	—
TM3	—	—	160–178	—	—	—	—	—
TM4	—	—	—	—	—	—	—	224–248
Orientation	IN	IN	IN	IN	IN	OUT	N/A	IN
AtDGAT2								
TM1	19–39	20–40	18–36	19–42	15–37	19–43	16–38	27–50
TM2	44–64	43–63	39–60	47–64	39–61	45–60	44–65	—
TM3	109–129	—	103–121	112–129	—	112–126	—	112–130
TM4	134–154	—	—	—	—	138–153	136–153	134–154
Orientation	OUT	IN	IN	IN	OUT	OUT	N/A	OUT
SpDGAT2								
TM1	29–49	29–49	26–45	32–51	—	29–43	25–47	—
TM2	54–74	51–71	48–72	60–77	49–65	46–70	53–75	49–65
TM3	115–135	—	119–137	115–134	115–137	119–133	—	115–137
TM4	141–161	—	—	143–162	142–162	—	136–153	142–162
TM5	217–237	—	—	217–236	217–237	—	—	217–237
TM6	—	—	—	259–278	—	—	—	—
Orientation	OUT	IN	IN	IN	OUT	OUT	N/A	OUT

Note: The polypeptide corresponding to ScDGAT2, MmDGAT2, AtDGAT2, and SpDGAT2 were submitted to a number of transmembrane prediction algorithms. The numbers correspond to the position of each transmembrane (TM) domain.

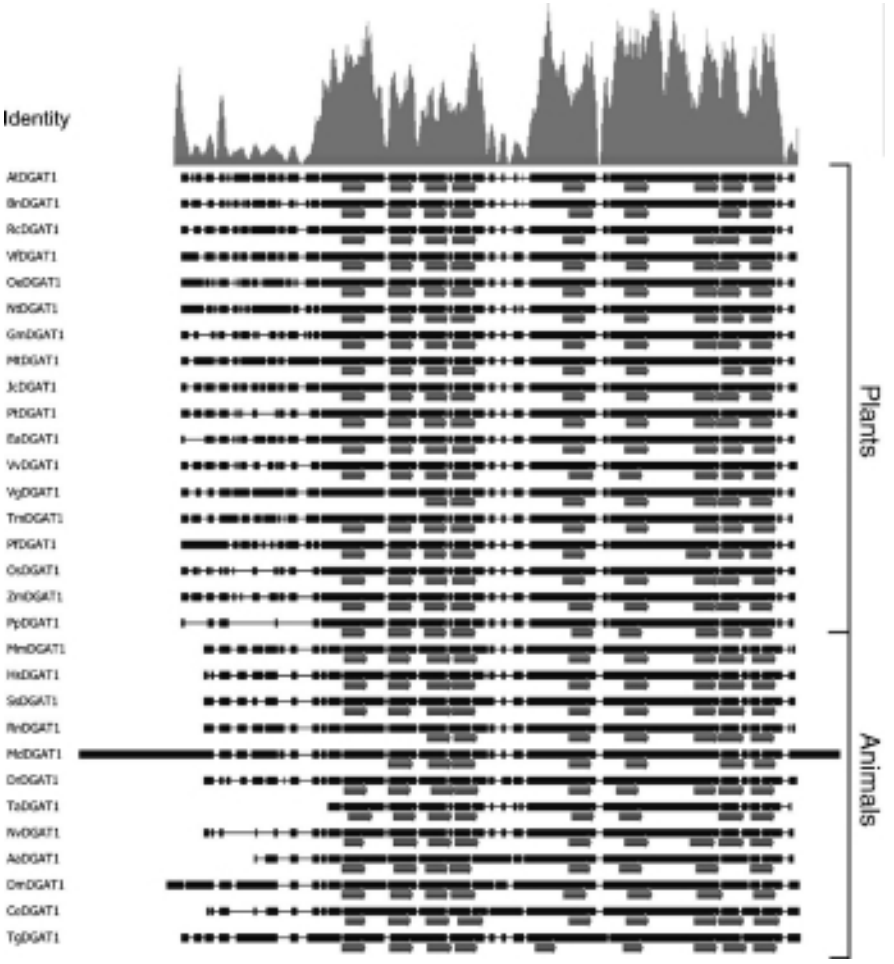

FIGURE 1.2 Alignment of transmembrane domains in DGAT1. The putative transmembrane domains of DGAT1 polypeptides from 12 animal and 18 plant organisms were predicted and the polypeptides were aligned. The identity of the alignment is graphed on the top using a window size of 6. The arrows denote the predicted transmembrane domains. The thick lines represent the sequence of each DGAT1, and the thin lines represent the gaps generated by the alignment. The picture was generated with Geneious Pro 4.6.0 and optimized manually. The transmembrane domains were predicted with transmembrane hidden Markov model (TMHMM). Accession numbers for the DGAT1 polypeptides are: AtDGAT1, NM_127503; AaDGAT1, XP_001658299; BnDGAT1, AAD45536; CeDGAT1, CAB07399; DmDGAT1, AAL78365; DrDGAT1, NP_956024; EaDGAT1, AAV31083; GmDGAT1, AAS78662; HsDGAT1, NP_036211; JcDGAT1, ABB84383; MdDGAT1, XP_001371565; MmDGAT1, NP_034176; MtDGAT1, ABN09107; NtDGAT1, AAF19345; NvDGAT1, XP_001639351; OeDGAT1, AAS01606; OsDGAT1, BAD53762; PfDGAT1, AAG23696; PpDGAT1, XP_001770929; PtDGAT1, XP_002330510; RcDGAT1, AAR11479; RnDGAT1, BAC43739; SsDGAT1, NP_999216; TaDGAT1, XP_002112025; TgDGAT1, AAP94209; TmDGAT1, AAM03340; VfDGAT1, ABC94471; VgDGAT1, ABV21945; VvDGAT1, CAN80418; ZmDGAT1, ABV91586.

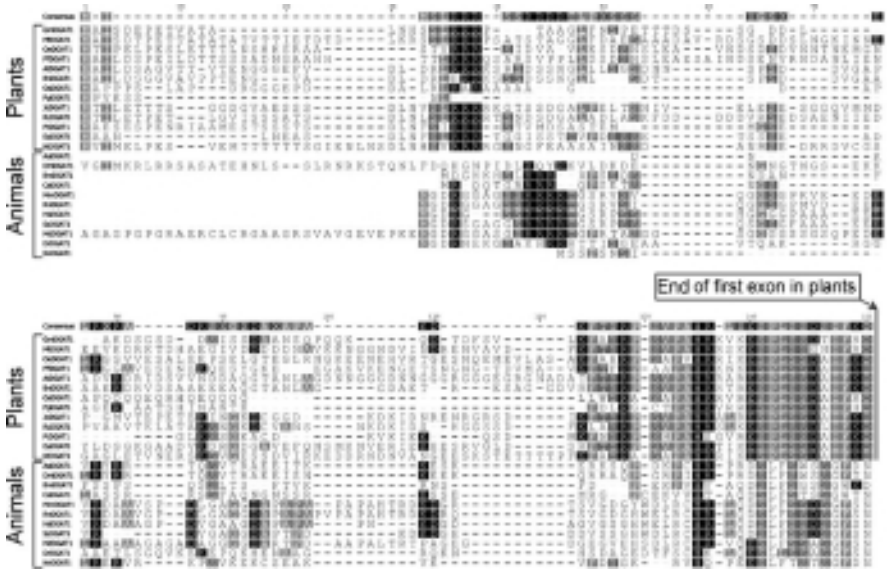

FIGURE 1.3 Alignment of the N-terminus polypeptide sequence of DGAT1 from plants and animals. Gray shades denote the polarity of blocks of conserved residues. The position corresponding to the end of the first exon in plants is indicated.

comprising the motifs PTLCYQXSYPR in plants and PTLCYEXXFPR in animals, preceding the fifth predicted transmembrane domain between positions 292 and 297 of AtDGAT1.

1.5 ALIGNMENT OF DGAT2 POLYPEPTIDES

DGAT2 polypeptides, in comparison to DGAT1, display fewer potential transmembrane domains and higher sequence divergence. An alignment of DGAT2 sequences from 20 organisms, covering plants and animals previously described in DGAT1, indicate approximately 5% of identical residues. Inclusion of 16 fungi sequences in this group decreases the identity to only 2.3%. A higher divergence might pose difficulties for identification of novel members of DGAT2 through sequence homology.

At least one transmembrane domain can be predicted for every DGAT2, but usually two transmembrane domains are conserved in the N-terminus portion and separated by a small loop (Fig. 1.4). This hydrophobic region is definitely very important because its removal results in lack of activity in ScDGAT2 (unpublished). An experimental approach indicated that the only membrane-spanning region in MmDGAT2 is composed of two transmembrane domains separated by a small loop that could be also interpreted as a single hydrophobic region embedded in the

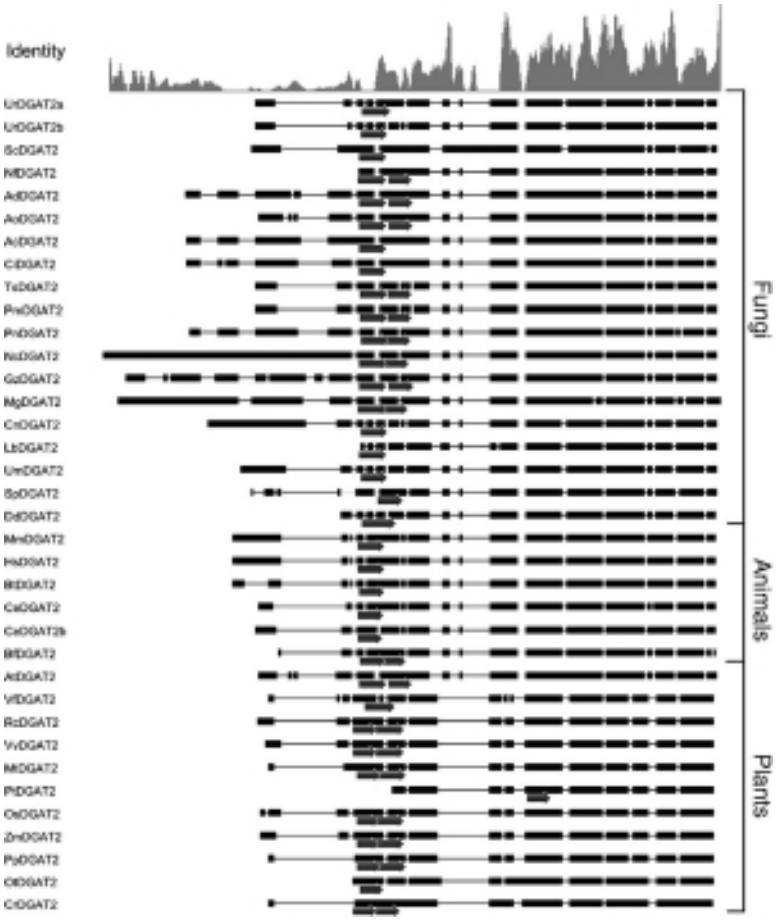

FIGURE 1.4 Alignment of transmembrane domains in DGAT2. The putative transmembrane domains of DGAT2 polypeptides from 18 fungi, 5 animals, and 11 plants were predicted and the polypeptides were aligned. The identity of the alignment is graphed on the top using a window size of 6. The arrows denote the predicted transmembrane domains. The thick lines represent the sequence of each DGAT2 polypeptide, and the thin lines represent the gaps generated by the alignment. The picture was generated as described for DGAT1. Accession numbers for the DGAT2 polypeptides are: AcDGAT2, XP_001540241; AdDGAT2, XP_001273210; AnDGAT2, CAK46407; AoDGAT2, XP_001822244; AtDGAT2, NP_566952; BfDGAT2, XP_002208225; BtDGAT2, CAD58968; CeDGAT2, CAB04533; CeDGAT2b, AAB04969; CiDGAT2, XP_001240299; CnDGAT2, EAL20089; CrDGAT2, XP_001693189; DdDGAT2, XP_635762; GzDGAT2, XP_381525; HsDGAT2, AAK84176; LbDGAT2, EDR14458; MgDGAT2, XP_368741; MmDGAT2, AAK84175; MtDGAT2, ACJ84867; NcDGAT2, CAE76475; NfDGAT2, XP_001261291; OsDGAT2, NP_001057530; OtDGAT2, CAL58088; PmDGAT2, XP_002146410; PnDGAT2, EAT89076; PpDGAT2, XP_001777726; PtDGAT2, XP_002317635; RcDGAT2, AAY16324; ScDGAT2, NP_014888; SpDGAT2, XP_001713160; TsDGAT2, EED21737; UmDGAT2, XP_760084; UrDGAT2a, AAK84179; UrDGAT2b, AAK84180; VfDGAT2, ABC94474; VvDGAT2, CAO68497; ZmDGAT2, ACG38122.

membrane (Stone et al., 2006). This region will be used as a landmark and the positions of conserved motifs also will be indicated in the UrDGAT2a polypeptide from *U. ramanniana*. The N-terminus portion preceding the transmembrane domains is quite variable in length and is usually smaller in animals and plants (with 38 and 30 residues in average, respectively) when compared with fungi (with 100 residues in average). The most conserved region in DGAT2 encompasses the motif RXGFX($^{K}/_{R}$) XAXXXGXX($^{L}/_{V}$)VPXXXFG($^{E}/_{Q}$) located approximately 150 residues after the second transmembrane domain (positions 259–281 of UrDGAT2a). Other conserved residues are the motif GGXXE (positions 204–208 in UrDGAT2a) and a phenylalanine, an arginine, and a proline in positions 164, 170, and 293 of UrDGAT2a, respectively. In addition, the motif YXXXXXHPHG is conserved in sequences from animals and fungi (positions 121–129 of UrDGAT2a) corresponding to YXXXXXEPH$^{S}/_{G}$ in plants. Preceding this motif is situated one of the most striking divergences in DGAT2 alignment, a hydrophilic segment of approximately 41 residues present in sequences from some fungi but absent in plants and animals. This region, corresponding to positions 144–185 of ScDGAT2, is also found as a much larger segment (158 residues) in *Yarrowia lipolytica* DGAT2. This hydrophilic segment, although nonessential, was demonstrated to modulate the enzyme activity of ScDGAT2 (unpublished results). Because this segment precedes a highly conserved motif, it is possible that it might represent a specialized function in DGAT2 from certain fungi.

1.6 STRUCTURE OF DGAT GENES

The architecture of genes encoding DGAT is largely available from whole-genome sequence databases or, as in the case of *V. fordii*, from sequencing of the respective genomic regions. In mammals, genes encoding DGAT1 share a similar architecture of 17 exons mostly grouped in the 3′ portion. A *DGAT1* representative from invertebrates (*Caenorhabditis elegans*), however, shows an unrelated distribution with only seven exons (Fig. 1.5A). In plants, DGAT1 genes from *A. thaliana*, *M. truncatula*, *Z. mays*, and *V. fordii* are composed of 16 exons, while DGAT1 from *O. sativa* contains 14 exons (Fig. 1.5B). The first exon of plant *DGAT1* genes comprises the largest coding sequence and encodes the hydrophilic N-terminus. Curiously, the last codon from the first exon of these genes encodes a glutamine in the same position of the alignment (motif IFX**Q**), denoting the end of the hydrophilic N-terminus and start of the first predicted membrane-spanning region (Fig. 1.3). The hydrophilic N-terminus is the most variable sequence of DGAT1 polypeptides, and therefore it is possible that segregation of this sequence in the first exon might have been used as an evolutionary mechanism to delimit variability in this region of the gene. This pattern was not observed in *DGAT1* sequences from animals. *DGAT2* genes show a structure that is dissimilar to that of *DGAT1*. Mammalian *DGAT2* genes share a common architecture with eight exons while the gene from *C. elegans* has only two exons (Fig. 1.5C). In plants *DGAT2* genes have eight exons in *A. thaliana* and ten exons in *V. fordii* and *O. sativa* (Fig. 1.5D).

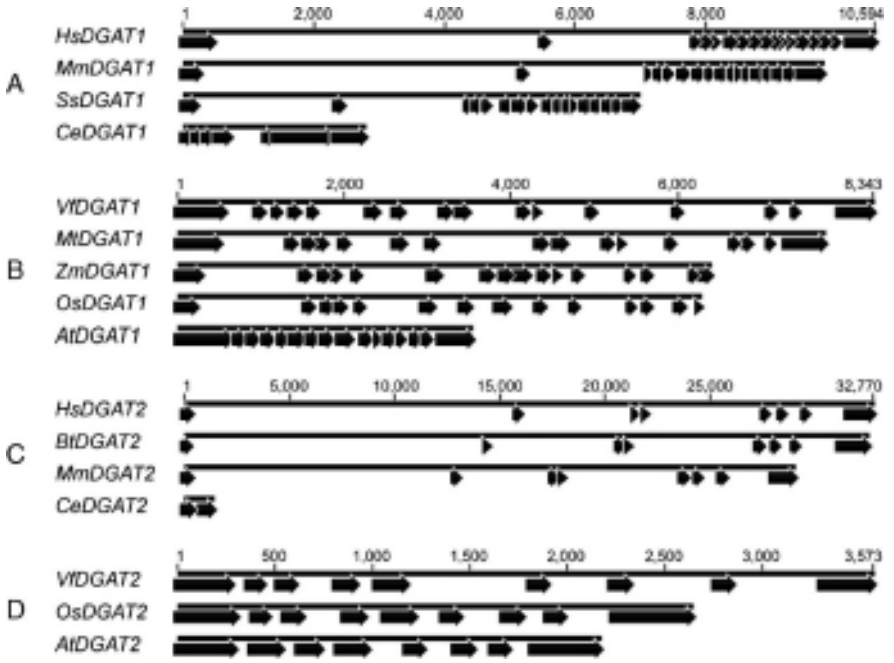

FIGURE 1.5 Architecture of DGAT genes. (A) *DGAT1* from animals, (B) *DGAT1* from plants, (C) *DGAT2* from animals, and (D) *DGAT2* from plants. The genomic sequences of each DGAT are represented by black bars. The arrows correspond to the regions comprising the coding region of the mRNA. The numbers correspond to the nucleotide positions. Accession numbers are: *HsDGAT1*, AC_000140.1; *MmDGAT1*, NC_000081.5; *SsDGAT1*, AY116586.1; *CeDGAT1*, NC_003283.9; *VfDGAT1*, DQ356679.1; *MtDGAT1*, AC174465.2; *ZmDGAT1*, AM433916.2; *OsDGAT1*, AP008212.1; *AtDGAT1*, NC_003071.4; *HsDGAT2*, NC_000011.8; *BtDGAT2*, NC_007313.3; *MmDGAT2*, NC_000073.5; *CeDGAT2*, Z81557.1; *VfDGAT2*, DQ356681.1; *OsDGAT2*, AP004757.3 and *AtDGAT2*, NC_003074.5.

1.7 FUNCTIONAL MOTIFS IN DGAT1

Most of the information available on the structure and function of DGATs is derived from comparisons of homologous enzymes. Alignments of polypeptide sequences encoding acyl-CoA-dependent acyltransferases from diverse organisms indicated a conserved histidine and an aspartic acid in the configuration **H**XXXX**D**. Substitution of the conserved histidine in the bifunctional enzyme 2-acyl-glycerophosphoethanolamine acyltransferase/acyl–acyl carrier protein synthase (Aas, EC 2.3.1.40 and 6.2.1.20, respectively) resulted in lack of acyltransferase activity (Heath and Rock, 1998). Substitution of the aspartic acid residue also resulted in significantly less activity. It was suggested that the histidine operates as a general base to abstract the proton from the hydroxyl group of the *sn*-1 glycerol-3-phosphate, facilitating nucleophilic attack on the thioester bond of acyl-CoA. The aspartic acid would work

in a charge relay system to increase the nucleophilicity of the hydroxyl group. This mechanism could be used by other acyltransferases, including DGAT. In fact a similar motif (**HH**XXX**DG**) is conserved in DGATs from prokaryotes (Daniel et al., 2004). In eukaryotic DGAT1, the motif **H**XXX**D** can be found closely after the fourth predicted transmembrane domain in DGAT1 from plants (positions 257–261 of AtDGAT1). Similarly, the motif **H**XXXX**D** is found in a region preceding the fifth predicted transmembrane domain of a few plants such as *A. thaliana*, *B. napus*, *R. communis*, and *V. fordii* (positions 342–347 of AtDGAT1) (Fig. 1.6). These motifs, however, are not conserved in animals and therefore might not compose the catalytic site of DGATs. Jako et al. (2001) identified the consensus sequence N(S/A/G)**R**(L/V)(I/F/A)(I/L)**E**N(L/V) in AtDGAT1 and proposed that the invariant arginine and glutamic acid on positions 149 and 153 could have functions analogous to those of histidine and aspartic acid residues, respectively. This region is highly conserved in all organisms including more ancient eukaryotes (*T. gondii* and *P. patens*) (Fig. 1.6). These residues

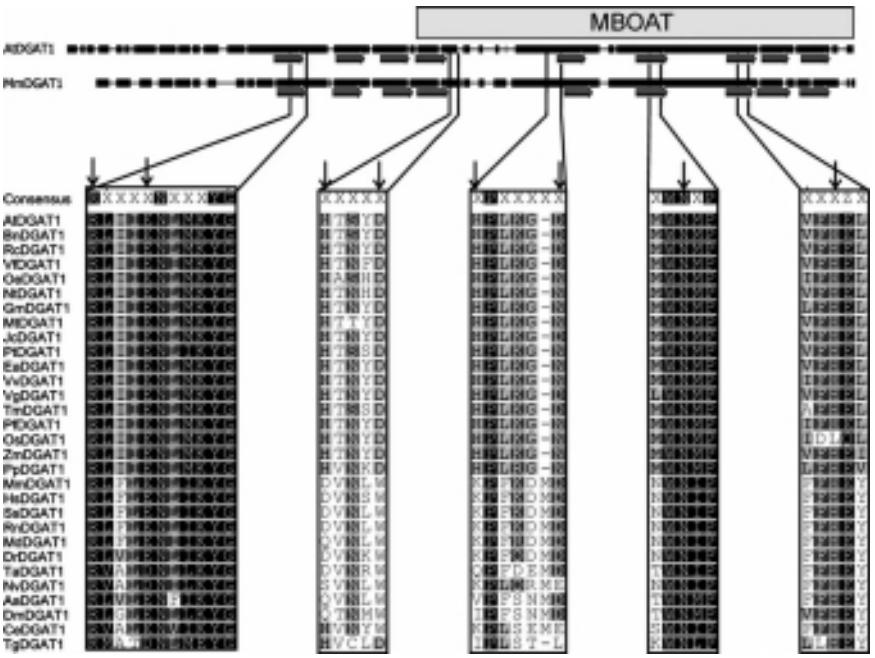

FIGURE 1.6 Alignment of putative active sites in DGAT1. A scheme MmDGAT1and AtDGAT1 is described on the top with the position of the MBOAT motif. The arrows in this scheme represent the predicted transmembrane domains. The thick lines represent the sequence of each DGAT1 polypeptide, and the thin lines represent the gaps generated though the alignment as previously shown. The vertical boxes contain the amino acid sequences for different DGATs indicated on the left. The arrows on these boxes indicate the position of conserved residues discussed in the text. Accession numbers for the DGAT polypeptides are the same as in Figure 1.2.

are present in the interface between a putative transmembrane domain and the adjacent hydrophilic loop, which would create an amphipathic environment for the substrates of DGAT. Moreover, DGAT1 is recognized as a member of a large protein family of membrane-bound O-acyltransferases known as MBOAT (NCBI domain ID pfam03062; Hofmann, 2000). Other members of the MBOAT family catalyze O-acylation reactions transferring acyl chains onto hydroxyl or thiol groups of lipids and proteins. For example, ACAT transfers an acyl chain from acyl-CoA to cholesterol, forming cholesteryl esters (Chang et al., 1993) while skinny hedgehog (ski) protein transfers a palmitoyl group onto cysteine residues of other proteins (Chamoun et al., 2001). The MBOAT family is characterized by a hydrophobic region (positions 234–509 of AtDGAT1) that contains a conserved asparagine (position 410 in AtDGAT1) and histidine (position 447 in AtDGAT1) (Fig. 1.6). It has been proposed that these residues could be involved in the catalytic activity. For example, this conserved histidine has been demonstrated to be a key residue for human ACAT1 activity (Guo et al., 2005). Whether any of these regions contribute to the catalytic site of DGAT1 is yet to be experimentally tested. Interestingly, *sn*-1 glycerol 3-phosphate acyltransferase (GPAT, EC 2.3.1.15) and lysophosphatic acid acyl transferase (LPAAT, EC 2.3.1.51), which are also membrane-bound O-acyltransferases catalyzing the first two acylation steps of TAG biosynthesis, are not classified as MBOAT members, suggesting that these enzymes might not share similar catalytic sites. It is also possible that these residues could act as supplementary catalytic sites being involved in other enzyme activities besides DGAT, such as ARAT and ACAT.

Other putative active sites in DGAT1 include the substrate binding sites. Sequences of DGAT1 from several plants indicate the presence of a putative diacylglycerol/phorbol ester binding motif that is apparently absent in ACATs (Zou et al., 1999; Nykiforuk et al., 2002; Xu et al., 2008). Phorbol esters such as phorbol-12-myristate-13-acetate (PMA) are commonly known to mimic diacylglycerols. The putative diacylglycerol/phorbol ester binding motif present in the positions 414 and 424 of AtDGAT1 forms the consensus **H**XXXX**RH**XXX**P** in DGAT1 from plants and animals. Xu et al. (2008) demonstrated that substitution of a phenylalanine by an arginine in position 439 of TmDGAT1 that is 16 positions after the predicted motif resulted in loss of DGAT activity. This could be a result of alterations in DAG interaction with DGAT. But, because this phenylalanine is positioned at a predicted transmembrane domain, substitution by a charged residue could also have structural implications. Acyl-CoA has been shown to interact with a recombinant N-terminal segment of BnDGAT1 and MmDGAT1 (Weselake et al., 2000, 2006; Siloto et al., 2008). The N-terminus sequence is highly variable, except for a region of 20 residues preceding the first hydrophobic domain, which shows remarkable conservation among plants and animals. Many of these variations, however, represent amino acid residues with similar properties, which could explain the acyl-CoA binding properties of DGAT1 from *B. napus* and *M. musculus*. Several lines of evidence suggest that acyl-CoA interaction with the hydrophilic N-terminus of DGAT1 regulates this enzyme allosterically. First, there is positive cooperativity exhibited for binding of 22:1-CoA in mouse and canola DGAT1 (Weselake et al., 2000; Siloto et al., 2008). Second, enzymes that are allosterically regulated

often form multimeric complexes to achieve cooperativity and the N-terminus of DGAT1 assists in the formation of dimers and tetramers as demonstrated for BnDGAT1 and HsDGAT1, respectively (Weselake et al., 2006; Cheng et al., 2001). For example, ACAT1 self-associates through the N-terminus, which also plays a regulatory role in this enzyme (Guo et al., 2001; Yu et al., 2002). Third, the acyl-CoA binding motif is not essential for enzyme activity because the removal of the N-terminus of RcDGAT1 results in a polypeptide with substantial enzyme activity, indicating that this is not the exclusive region to interact with acyl-CoA (unpublished data). Indeed, the fourth conserved block in GPATs and LPAATs, as described by Lewin et al. (1999), contains an invariant proline that has been proposed to participate in acyl-CoA binding. This proline was identified in plant DGAT1 polypeptides on the third predicted transmembrane domain corresponding to position 224 of AtDGAT1 and is in fact conserved in DGAT1 from all organisms. Substitution of this proline with an arginine in TmDGAT1 abolished DGAT activity, corroborating with the idea that this residue has a functional role (Xu et al., 2008). Another possible acyl-CoA binding site was proposed to be closely associated with the motif FYXDWWN in ACATs (Yen et al., 2008). This motif is present in DGAT1 and shows remarkable conservation with exception to CeDGAT1, where the second tryptophan is substituted by a phenylalanine. This motif is located on the loop preceding the third last putative transmembrane domain, relatively distant from the proline residue previously discussed, but near the asparagine residue conserved in MBOAT members. The paired tryptophans in this motif are a rare combination and have been previously demonstrated to participate in cholesterol binding. Guo et al. (2001) demonstrated, however, that substitution of the conserved tyrosine by alanine in yeast ACAT1 resulted in decreased affinity for acyl-CoA. Substitution of this same residue in TmDGAT1 (Y392A) resulted in decreased enzyme activity while a double mutation in tyrosine and tryptophan (Y392G/W395G) completely abolished enzyme activity (Xu et al., 2008).

Other putative functional domains predicted in DGAT1 include a leucine zipper and phosphorylation sites, although it is not yet clear whether these regions are important in the function, structure, or regulation of DGAT1. A putative leucine zipper motif was described in several DGAT1 from plants (Bouvier-Nave et al., 2000a; Nykiforuk et al., 2002). For example, in AtDGAT1 polypeptides five leucines (L222, L229, L236, L243, and L250) are consecutively spaced by six residues forming a classic leucine zipper (Hobbs et al., 1999). This leucine zipper, which might mediate interactions with other proteins, is present in a number of DGAT1 from plants but not from animals. Several studies indicated the presence of multiple potential phosphorylation sites in DGAT1 (Hobbs et al., 1999; Nykiforuk et al., 2002; He et al., 2004). Some of these sites are conserved in plant DGATs, such as the protein kinase C sites in the loop between the first and second transmembrane domains (positions 169–171 and 172–175 of AtDGAT1) and the casein kinase II sites (positions 254–257 and 403–406 of AtDGAT1). In addition, a tyrosine kinase site (positions 386–393 of AtDGAT1) is conserved in DGAT1 from plants and animals. This site overlaps with the FYXDWWN motif previously discussed as a putative acyl-CoA binding site. Although substitution of the conserved tyrosine by alanine in yeast

ACAT homologue resulted in lower affinity to acyl-CoA, phosphorylation could not be directly detected (Guo et al., 2001). Regulation of DGAT1 activity through phosphorylation is compelling not only because this is a common mechanism to control enzyme activity in eukaryotes but also because DGAT can scavenge DAG, an important molecule involved in phosphorylation signaling cascades (Carrasco and Merida, 2007). For example, the affinity of DAG to C1 domains of DAG kinase is modified by phosphorylation of residues situated close to this motif (Thuille et al., 2005). In addition, the fact that DGAT is expressed in vegetative tissues suggests that it can have additional roles beyond oil biosynthesis in seeds (Lu et al., 2003). Substitution of serine at position 168 in RcDGAT1 that corresponds to a protein kinase C site previously described resulted in a significant decrease in the enzyme activity (unpublished).

1.8 FUNCTIONAL MOTIFS IN DGAT2

The motifs previously described for DGAT1 cannot be found in DGAT2 sequences likely due to the little homology between DGAT1 and DGAT2. In fact, little is known about functional motifs of DGAT2. Stone et al. (2006) identified the conserved motif HPHG in positions 161–164 of MmDGAT2 as an important region for DGAT activity. Substitution of these residues, forming the sequences **A**PHG, H**G**HG, HP**A**G, and **AGA**G, resulted in a significant reduction of enzyme activity. More specifically, the histidine at position 163 of MmDGAT2 appeared to play a more important role for the enzyme function, which agrees with our mutagenesis work on ScDGAT2 (unpublished). This region is conserved in animal and fungi DGAT2, but in plants this motif is found as $EPH^{S}/_{G}$. Substitution of the glutamic acid by a histidine residue in plant DGAT2 did not result in an appreciable effect, but replacement of the motif HPHG in ScDGAT2 with residues EPHS found in plant DGAT2 resulted in loss of enzyme activity (unpublished). This indicates an important divergence on the structure/function of DGAT2 from fungi and plants. In addition, the motif $FLXLXXX^{n}$ (n indicates a nonpolar residue) was indicated as a putative neutral lipid binding domain in MmDGAT2 (Stone et al., 2006). Substitution of phenylalanine (position 80) and leucine (position 81) residues by alanine residues resulted in decreased DGAT activity. Substitution of the leucine in position 83 by an alanine resulted in lack of activity. This motif, present in the first predicted transmembrane domain of MmDGAT2 (positions 80–87), is conserved in vertebrate DGAT2 but not in plants or fungi orthologs. Substitution of the corresponding phenylalanine and leucine (positions 71 and 73) in ScDGAT2 results in a decrease of approximately 50% of the wild-type activity (unpublished). This same motif contains the putative membrane lipid attachment LGVAC found in prokaryotes through the interaction between the sulfhydryl group of a cysteine residue (position 87 of MmDGAT2) and DAG. Substitution of this cysteine by a serine in MmDGAT2 did not reduce DGAT activity, indicating that it does not function as a lipid attachment site. In fact, substitution of all cysteine residues in ScDGAT2 by alanine residues did not disrupt DGAT activity, indicating that this mechanism of DAG interaction is not present or at least essential in

this enzyme (unpublished data). In addition, substitutions on the conserved motif YFP located close to the transmembrane domains (positions 104–106 of UrDGAT2A) resulted in significant decreases in the enzyme activity.

1.9 SUBCELLULAR LOCALIZATION OF DGATs

To better elucidate the role of DGATs in cellular processes, their spatial location has been studied in different plants. In numerous earlier studies, DGAT location has been a subject of discrepancy whether it is associated with ER or oil bodies (Lung and Weselake, 2006). This debate could be the result of technique limitations because the general approach used in these studies was subcellular fractionation combined with enzyme assay in which cross-contamination can occur. For instance, in the study of germinating soybean cotyledon, the purified oil bodies also exhibited activities for ER markers (Settlage et al., 1995). This could be explained by association between oil bodies and ER (Cao and Huang, 1986; Settlage et al., 1995). Lacey and Hills (1996) applied different organelle markers to rule out the possible contamination in the assay and clearly demonstrated that *B. napus* DGAT is associated with ER. Similarly, Cao and Huang (1986) were able to localize maize DGAT in the rough ER (RER) by taking advantages of protein markers as well as the attachment of RER with polysomes in the presence of Mg^{2+} during fractionation. Actually the ER is regarded as the main site for TAG synthesis, and microsomal fractions from developing seeds of many plants as well as plant cultured cells have been extensively utilized for enzyme assays (Browse and Somerville, 1991; Weselake, 2005). Using more dependable techniques such as green fluorescent protein (GFP)-tagging and immunofluorescence, Shockey et al. (2006) have demonstrated that tung tree DGAT1 and DGAT2 are localized in the ER. Localization of both DGATs is dependent on a C-terminal ER retrieval motif. In VfDGAT1, the ER retrieval sequence YYHDL is part of the motif LLYYHDXMN conserved in all plant DGAT1. The ER retrieval domain in VfDGAT2 comprises the sequence LKLEI, where the two leucines are conserved in other DGAT2 sequences. Removal of the corresponding regions through C-terminus truncations in RcDGAT1 and ScDGAT2 resulted in decreased activity and decreased protein stability, respectively, indicating the importance of the C-terminus portion for both DGATs (unpublished). Interestingly, VfDGAT1 and VfDGAT2 do not colocalize in the ER, and therefore it is plausible that these polypeptides have distinct interactions with other proteins in the ER membrane. Mounting evidence based on studies with animals and plants indicate that DGAT1 and DGAT2, although catalyzing the same enzyme activity, have distinct physiological functions (Yen et al., 2008; Shokey et al., 2006; Burgal et al., 2008). In addition to the ER, DGAT activity was also found in chloroplasts of spinach leaves (Martin and Wilson, 1984) and more recently, Kaup et al. (2002), identified DGAT1 in the chloroplasts of senescing *Arabidopsis* leaves through immunoblotting. The mechanisms by which AtDGAT1 is transported to the chloroplast are yet to be determined.

In yeast, biochemical studies with *S. cerevisiae* indicated that DGAT activity is mainly in lipid droplets (Sorger and Daum, 2002). Indeed, DGAT2 in *U. rammaniana*

was purified from the lipid particle fractions (Lardizabal et al., 2001). In addition, two subcellular localization datasets generated by proteomic studies of *S. cerevisiae* indicated that ScDGAT2 localizes in ER and lipid droplets (Huh et al., 2003; Natter et al., 2005). Moreover, recombinant expression of ScDGAT2 in a yeast strain devoid of TAG biosynthesis indicated that ScDGAT2 localizes in the microsomal fraction as an integral membrane protein (unpublished). Due to the presence of conserved transmembrane domains, it is expected that yeast DGAT2 localizes in the ER. The mechanisms involved in its movement from the ER to lipid droplets, however, are not yet determined. *S. cerevisiae* lipid droplets contain a small fraction of proteins (Leber et al., 1994) when compared to the structurally related organelles in plants that are coated by oleosins (Tzen et al., 1993). The mechanism of oil body targeting in oleosins has been well studied and is assisted by a long hydrophobic domain composed of two chains that are separated by a motif with three conserved prolines (proline knot motif) that supposedly folds the domain, resulting in an unusual topological structure where the hydrophilic N- and C-termini face the cytoplasm (Tzen et al., 1992; Abell et al., 2004). Analysis of yeast DGAT2 revealed that the two potential transmembrane domains are separated by a very small loop. This region contains three prolines that are conserved in most sequences (Fig. 1.7). Such

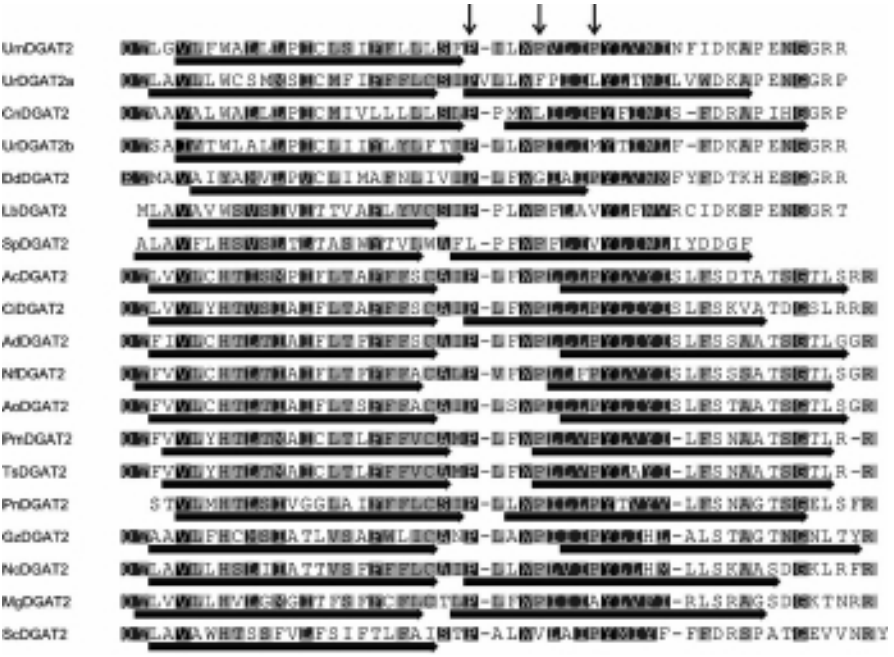

FIGURE 1.7 Alignment of the predicted transmembrane domain region from fungi DGAT2. Residues are highlighted in different shades of gray to black according to their similarity. The arrows denote the position of the predicted transmembrane domains. The positions of prolines conserved in many sequences are denoted by the arrows on the top. The alignment parameters and the accession numbers are the same as previously indicated in Figure 1.4.

similarities could explain the transfer of DGAT2 to lipid droplets, although this hypothesis needs to be experimentally verified. A study on murine DGAT2 has shown that the enzyme localizes on the ER and transfers to near the surface of lipid droplets when oleic acid is provided to drive TAG biosynthesis (Stone et al., 2009). Interestingly, determination of murine DGAT2 topological structure also showed both termini facing the cytosol and the possible presence of two adjacent transmembrane domains (Stone et al., 2006). DGAT2 from other organisms have a similar structure. VfDGAT2 has both N- and C-termini facing the cytosol with two predicted transmembrane domains separated by a small loop with a conserved proline (Shockey et al., 2006).

1.10 CONCLUSIONS AND FUTURE RESEARCH

Considerable progress has been achieved toward our understanding of DGATs and their involvement in the biosynthesis of TAG over the past decade. Many important aspects of the molecular mechanisms coordinating the catalytic activity, however, remain unclear. Most interestingly, DGAT1 and DGAT2 are unrelated polypeptides and yet catalyze the same reaction. Are the mechanisms involved in the acyltransferase catalytic function similar in DGAT1 and DGAT2? Are there any relationships between these enzymes that have not been identified with the current alignment algorithms? In an evolutionary perspective, did these enzymes evolve separately to catalyze the same reaction or do they have a common ancestor? These are some unanswered questions that require more fundamental research on DGATs. It would be valuable to have insights into the three-dimensional structure of DGATs because it would help to resolve some of these doubts.

Most of the information on putative structure–function relationships in DGATs has been deduced using bioinformatic approaches. The conclusions obtained with such approaches are valuable but still require experimental validation. Considerable progress has been made in shedding light on the topological organization of murine DGAT2 (Stone et al., 2006). It would be interesting to conduct similar experiments with a fungal DGAT2, particularly with ScDGAT2. This polypeptide contains unique characteristics as previously discussed, making it an interesting candidate for structural studies. Moreover, the correct topology of DGAT1 should be experimentally evaluated to determine whether the nine- or ten-transmembrane model is the correct one. In addition, to enhance our knowledge on DGAT catalytic activity, broader studies involving site-directed mutagenesis should be performed to identify functional regions. Currently, two studies have been conducted with a plant DGAT1 (*T. Majus*) and an animal DGAT2 (*M. musculus*) evaluating the influence of only a few residues (Xu et al., 2008; Stone et al., 2006). The polypeptide alignments presented in this chapter indicate the presence of multiple sites that could be involved in the catalytic activity of DGAT. This type of research could greatly advance if random mutagenesis techniques such as directed evolution or site-saturation mutagenesis could be applied to DGATs. One of the obstacles associated with such large-scale experiments is that standard methods to accurately measure DGAT activity require a

laborious assay with radiolabeled substrates (Coleman, 1992). Due to the association of DGATs with membranes, enzyme assays typically use microsomal fractions obtained through ultracentrifugation, which greatly decreases the throughput of the assay. Recently, we have demonstrated two assays to detect and measure DGAT activity in high-throughput scale (Siloto et al., 2009). Further development of such assays would definitively enhance our knowledge about the molecular mechanisms involved in DGAT activity. Furthermore, this is an attractive field of plant biotechnology for improving the performance of DGATs from plants and fungi, which have been already used to increase oil content in seeds (Weselake et al., 2008; Lardizabal et al., 2008).

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Abbreviations: EMS, ethyl methanesulfonate; TAG, triacylglycerol; DGAT, acyl-CoA:diacylglycerol acyltransferase; EST, expressed sequence tag; acyl-CoA: cholesterol acyltransferase, ACAT; ARAT, acyl-CoA:retinol acyltransferase; MGAT, acyl-CoA monoacylglycerol acyltransferase; TM, transmembrane; MBOAT, membrane-bound O-acyltransferases; PMA, phorbol-12-myristate-13-acetate; ER, endoplasmic reticulum; RER, rough ER; GFP, green fluorescent protein.

The two-character code preceding each DGAT gene and polypeptide indicates the organism of origin as follows:

Aa, *Aedes aegypti*; Ac, *Aspergillus clavatus*; Ah, *Arachis hypogaea*; An, *Aspergillus niger*; Ao, *Aspergillus oryzae*; At, *Arabidopsis thaliana*; Bf, *Branchiostoma floridae*; Bn, *Brassica napus*; Bt, *Bos taurus*; Ce, *Caenorhabditis elegans*; Ci, *Coccidioides immitis*; Cn, *Cryptococcus neoformans*; Cr, *Chlamydomonas reinhardtii*; Dm, *Drosophila melanogaster*; Dd, *Dictyostelium discoideum*; Dr, *Danio rerio*; Ea, *Euonymus alatus*; Gm, *Glycine max*; Gz, *Gibberella zeae*; Hs, *Homo sapiens*; Jc, *Jatropha curcas*; Lb, *Laccaria bicolor*; Md, *Monodelphis domestica*; Mm, *Mus musculus*; Mt, *Medicago truncatula*; Mg, *Magnaporthe grisea*; Nc, *Neurospora crassa*; Nf, *Neosartorya fischeri*; Nt, *Nicotiana tabacum*; Nv, *Nematostella vectensis*; Oe, *Olea europaea*; Os, *Oryza sativa*; Ot, *Ostreococcus tauri*; Pf, *Perilla frutescens*; Pm, *Penicillium marneffei*; Pn, *Phaeosphaeria nodorum*; Pp, *Physcomitrella patens*; Pt, *Populus trichocarpa*; Ps, *Picea sitchensi*; Rc, *Ricinus communis*; Rg, *Rhodotorula glutinis*; Rn, *Rattus norvegicus*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Ss, *Sus scrofa*; Ta, *Trichoplax adhaerens*; Tg, *Toxoplasma gondii*; Tm, *Tropaeolum majus*; Ts, *Talaromyces stipitatus*; Um, *Ustilago maydis*; Ur, *Umbelopsis ramanniana*; Vf, *Vernicia fordii*; Vg, *Vernonia galamensis*; Vv, *Vitis vinifera*; YL, *Yarrowia lipolytica*; Zm, *Zea mays*.

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2

IMPROVING ENZYME CHARACTER BY MOLECULAR BREEDING: PREPARATION OF CHIMERIC GENES

KIYOSHI HAYASHI, MOTOMITSU KITAOKA, AND MAMORU NISHIMOTO
National Food Research Institute, 2-1-12, Kannondai Tsukuba Ibaraki 300-8642, Japan

2.1 INTRODUCTION

Enzymes are useful tools for converting materials. Screening enzymes for the required character is still an important process, despite remarkable advances in biotechnology. Alteration of the enzyme character at the molecular level is a challenging and time consuming technique as the method of finding amino acid residues that improve the required enzyme character has not yet been established.

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The preparation of chimeric enzymes provides one method of modifying the character of the enzymes. The genes of the target enzymes are split and ligated into chimeric genes and then the ligated genes are expressed to obtain chimeric enzymes. We have successfully improved the character of enzymes including β-glucosidase (Kim et al., 2005; Goyal et al., 2001, 2002; Singh et al., 2002), xylanase (Shibuya et al., 2005; Kaneko et al., 2000), and aminopeptidase (Nirasawa and Hayashi, 2008), by chimeric enzyme preparation. The characters of the chimeric enzymes obtained are an admixture of the parental enzyme characters; thus, this technique can be considered as improving enzyme character by molecular breeding. Two examples of changing enzyme character through the construction of chimeric genes are described.

2.2 PREPARATION OF CHIMERIC β-GLUCOSIDASE TO IMPROVE THE ENZYME CHARACTER

2.2.1 Construction of Chimeric β-Glucosidases

We have cloned and expressed the β-glucosidase genes of *Agrobacterium tumefaciens* and *Thermotoga maritima*, and shown that the two enzymes have quite different characteristics (Watt et al., 1998; Goyal and Selvakumar, 2001). The two genes of the β-glucosidases have been used for preparation of chimeric enzymes to see how the enzyme character can be modified by constructing chimeric enzymes.

β-glucosidase was found to be composed of three regions: an N-terminal catalytic domain, a nonhomologous region, and a C-terminal domain of unknown function. The homology of the amino acid residues in the N-terminal and C-terminal domain are 45 and 37%, respectively (Fig. 2.1). Based on the sequence

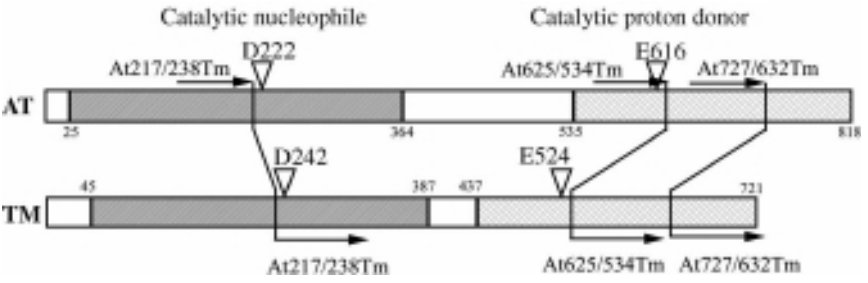

FIGURE 2.1 Homology in the amino acid sequences of the β-glucosidases from *A. tumefaciens* and *T. maritima*. AT and TM denote *A. tumefaciens* and *T. maritima*, respectively. Schematic representation of the sequence homology in the N-terminal (solid) and C-terminal (hatched) regions of the β-glucosidase genes is shown. Position of catalytic nucleophile/bases of D222 and D242, and catalytic proton donors of E616 and E524 are indicated in the figure (reproduced from Goyal et al., 2001).

FIGURE 2.2 Amino acid alignment of *A. tumefaciens* and *T. maritima* β-glucosidases in the C-terminal region. Identical and similar amino acid residues are designated by an asterisk (*) or dot (·), respectively (reproduced from Goyal et al., 2001).

alignment of the β-glucosidase, three different sites were selected as shuffling sites (Figs. 2.1 and 2.2). By comparing with the available X-ray crystal structure of the family 3 glycosyl hydrolases from barley (Varghese et al., 1999), the first shuffling site (At217-238Tm) was located five amino acids upstream of the catalytic site of D222 in the *A. tumefaciens* β-glucosidase. The second shuffling site (At625-534Tm) and the third shuffling site (At727-632Tm) were designed in the C-terminal domain. Of the three chimeric enzymes constructed, one chimera (At625/534Tm) was catalytically active, one chimera (At217/238Tm) was catalytically inactive, and a very low but unstable level of activity was observed for the third chimera (At727/632Tm).

In an earlier study, we showed that the folding information was distributed unevenly in the protein. The four different constructs truncated at the nonhomologous region resulted in active enzymes with slight modifications in character (Ying et al., 2004). The chimeric enzymes shuffled at the C-terminal domain of unknown function formed active enzymes (Kim et al., 2005; Goyal et al., 2001, 2002; Singh et al., 2002). However, the two chimeric enzymes shuffled at the N-terminal catalytic domain were only found in inclusion bodies, even though they were coexpressed with the GroEL/ES molecular chaperone (Hayashi et al., 2000b). Solubilization of the inclusion body in the buffer containing 8 M urea and subsequent refolding of these two proteins by slow dialysis was not successful in producing active enzymes (Hayashi et al. 2000a). Similar results were also obtained when the chimeric gene was shuffled at the N-terminal catalytic domain, At217/238Tm.

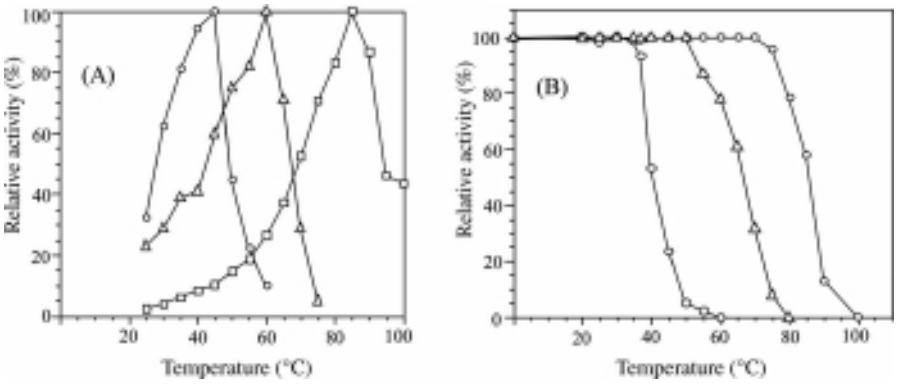

FIGURE 2.3 Temperature optimum (A) and heat stability (B) profiles of the chimeric and parental β-glucosidases. To estimate thermal stabilities of the β-glucosidases, each enzyme was preincubated for 30 min at various temperatures. The residual activities were then determined using standard assay conditions. *A. tumefaciens* (open triangle); At625/534Tm (open circle); *T. maritima* (open square) (reproduced from Goyal et al., 2001).

2.2.2 Thermal and pH Profiles of the Chimeric and Parental Enzymes

The effect of temperature on the activity of parental and chimeric enzymes is shown in Figure 2.3. The temperature optima for the *A. tumefaciens* and *T. maritima* enzymes were 65 and 85°C, respectively. The observed optimum temperature for the At625/534Tm chimeric enzyme was around 45°C, which is lower than that of the parental enzymes (Fig. 2.4A). This chimera does not show profiles intermediate to those of their parent enzymes unlike other chimeras (Kim et al., 2005; Goyal et al., 2001, 2002; Singh et al., 2002; Shibuya et al., 2005; Kaneko et al., 2000; Nirasawa and

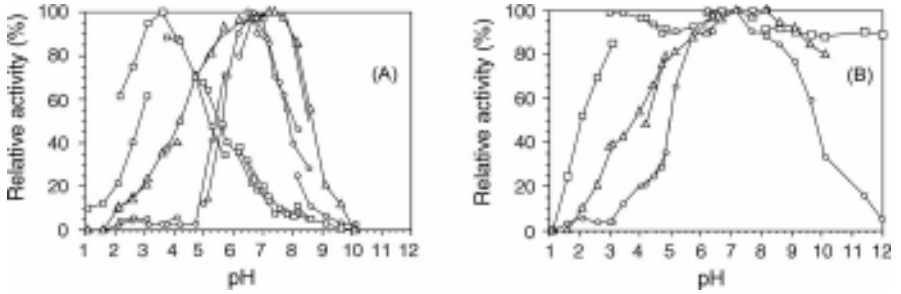

FIGURE 2.4 pH activity (A) and pH stability (B) profiles of the chimeric and parental β-glucosidases. The pH was adjusted with the following buffers: phosphate (pH 1.1–3.1), citrate (pH 2.16–4.12), acetate (pH 3.75–5.71), MES (pH 5.0–7.7), MOPS (pH 6.2–8.18), HEPES (6.48–8.56), and CHES (pH 8.15–10.12). For pH stability experiments, the enzymes were incubated at 30°C at different pHs for 30 min, then the residual activities were determined. *A. tumefaciens* (open triangle); At625/534Tm (open circle); *T. maritima* (open square) (reproduced from Goyal et al., 2001).

Hayashi, 2008). The temperature optimum for the chimeric enzyme does not correlate with the thermal stabilities of the parental enzymes. The chimeric enzyme At625/534Tm was stable up to 37°C, retaining about 90% of its maximum activity at 40°C, and was inactivated at 50°C (Fig. 2.4B). In contrast, the β-glucosidases of *A. tumefaciens* and *T. maritima* were stable up to temperatures of 55 and 75°C, respectively.

Marked differences in pH optima and pH stabilities were found in parental β-glucosidase. The pH optima for the *A. tumefaciens* and *T. maritima* enzymes were 7.2–7.4 and 3.2–3.5, respectively. The observed optimum pH for chimera At625/534Tm was around 6.2–6.5 (Fig. 2.4A), which is closer to that of the β-glucosidase of *A. tumefaciens*. Similarly, the pH stability of this chimeric enzyme was found to lie in the pH range from 5 to 9, whereas the *A. tumefaciens* and *T. maritima* enzymes displayed stability within the pH ranges of 4–11 and 3–12, respectively (Fig. 2.4B). The pH profile of the chimeric enzyme is closer to that of the *A. tumefaciens* β-glucosidase.

2.2.3 Substrate Specificities of the Chimeric and Parental Enzymes

By using various aryl glycosidases as substrates, the kinetic parameters of the β-glucosidases of *A. tumefaciens*, *T. maritime*, and the chimeric enzyme (At625/534Tm) were investigated. The observed K_m and k_{cat} values are summarized in Table 2.1. The specificity of the chimeric enzyme was different from that of the parent enzymes. The K_m values for the pNP-β-D-glucopyranoside of the parent enzymes, *A. tumefaciens* and *T. maritima*, were 0.012 and 0.0039 mM, respectively, while that for the chimeric enzyme At625/534Tm was 0.081 mM, which is slightly higher than

TABLE 2.1 Kinetic Parameters of the Parental and Chimeric Enzymes

Substrate	*A. tumefaciens*	*T. maritima*	At625/534Tm
pNP-β-D-glucopyranoside			
K_m (mM)	0.012	0.0039	0.081
k_{cat} (s^{-1})	95.4	6.4	3.3
k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	7950	1640	41
pNP-β-D-xylopyranoside			
K_m (mM)	0.005	2.64	0.95
k_{cat} (s^{-1})	28.9	18.4	0.16
k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	5780	6.96	0.17
pNP-β-D-fucopyranoside			
K_m (mM)	0.08	42.6	0.24
k_{cat} (s^{-1})	22.1	27.6	0.013
k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	280	0.648	0.054
pNP-α-L-arabinofuranoside			
K_m (mM)	0.24	18.9	0.66
k_{cat} (s^{-1})	119	9.0	0.025
k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	495	0.47	0.038

Note: Standard errors are within 10% of the given values.

those for the parent enzymes. In comparison, the k_{cat} value of the chimeric enzyme for pNP-β-D-glucopyranoside was 3.38 s^{-1}, which is lower than that observed for the β-glucosidases of *A. tumefaciens* (95.4 s^{-1}) and *T. maritima* (6.4 s^{-1}).

The above data indicates that the specificity of the chimeric enzyme is slightly closer to that of the *T. maritima* β-glucosidase because they showed more or less the same specificity toward the investigated substrates, although the chimeric enzyme contains only 10.3% amino acid residues of the *T. maritima* enzyme. The shuffled region of the C-terminal domain may play a significant role in the enzyme substrate specificity. The chimeric enzyme At625/534Tm differed from β-glucosidase of *A. tumefaciens* in specificity in that the chimeric enzyme was poor at hydrolyzing pNP-β-D-xylopyranoside. The chimeric β-glucosidase also hydrolyzed pNP-β-D-xylopyranoside, pNP-β-D-fucopyranoside, and pNP-β-L-arabinofuranoside, but the specificities were different from those of the parental enzymes.

2.3 PREPARATION OF CHIMERIC XYLANASE TO DETERMINE THE ENZYME ACTIVITY AT BASIC pH

Xylanases [3. 2. 1. 8] catalyze hydrolysis of the main polysaccharide chain of xylan, the main component of hemicellulose, in an endowise manner. Xylanases are classified into two families (families 10 and 11) according to the classification system used for glycoside hydrolases, which is based upon the primary structures of the enzymes (Henrissat and Bairoch, 1996). Xylanase A (XynA) produced by alkaliphilic *Bacillus halodurans* C-125 (the whole genome sequence was reported by Takami et al., 2000) is classified into family 10 of the glycoside hydrolases based on the amino acid sequence. XynA displays full hydrolytic activity toward *p*-nitrophenyl-β-D-xylobioside ($pNPX_2$) over the pH range from 5.3 to 8.8 (Nishimoto et al., 2002a, 2002b) identifying XynA as an alkaline xylanase. Alkaline xylanases are considered to be useful for pulp bleaching in the paper industry and the production of xylooligosaccharides from xylan (Blanco et al., 1995; Kulkarni and Rao 1996; Bissoon et al., 2002; La Grange et al., 2001). Therefore, it is important to understand how XynA is able to express activity in the basic region of pH.

Xylanase B (XynB) from *Clostridium stercorarium* F9 belongs to the glycosyl hydrolase family 10 and displays 51% identity to XynA by comparison of their amino acid sequences (Fukumura et al., 1995). The pH optimum of XynB is in the range of 5.3–6.5 (Honda et al., 2002), implying that the acidic range of the pH optimum is similar to that observed for XynA, whereas the basic range is significantly narrower than that of XynA. To determine the region(s) essential to maintaining XynA activity at basic pH levels, the construction of a number of chimeric enzymes of XynA with a standard xylanase was undertaken. XynB was selected as a shuffling partner with XynA in the construction of chimeric xylanases.

2.3.1 Construction of Chimeric Xylanases

The XynA from *B. halodurans* C-125 and the XynB from *C. stercorarium* F9, also a family 10 glycosyl hydrolase, share 51% amino acid identity. As shown in Figure 2.5,

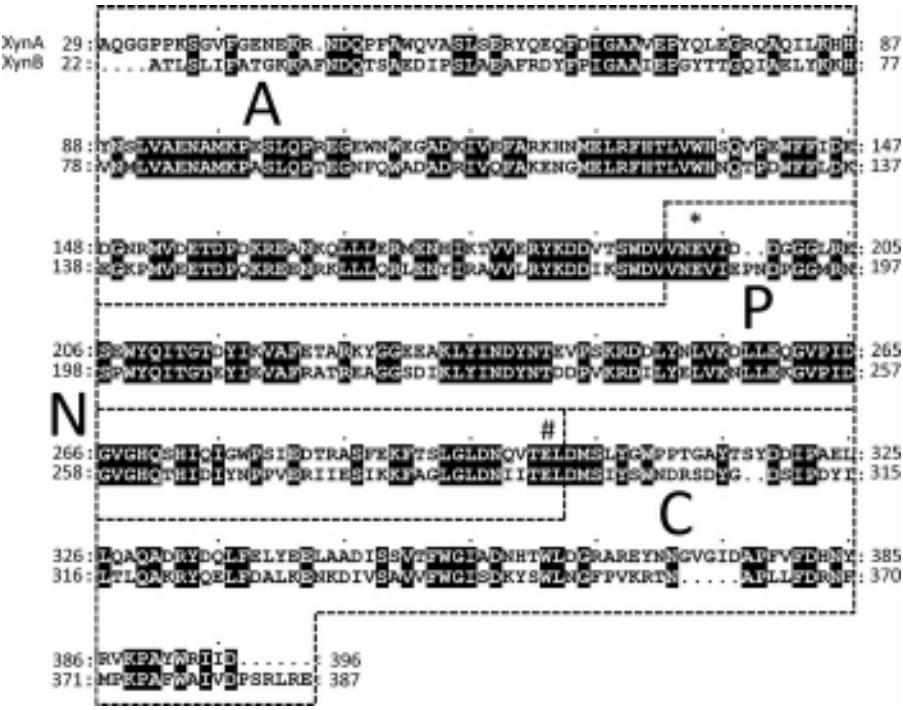

FIGURE 2.5 Multiple alignment of the parental mature xylanases from *Bacillus halodurans* C-125 (XynA) and *Clostridium stercorarium* F9 (XynB). The signal sequences of the XynA and XynB consist of 28 and 21 amino acid residues, respectively. The putative proton donor and catalytic nucleophile are shown as an asterisk and sharp symbol, respectively (reproduced from Nishimoto et al., 2002b).

XynA and XynB were divided into four parts at highly homologous regions: A and a (the amino terminal fragments; the upper and lower case letters denote the parts originating from XynA and XynB respectively), P and p (the parts containing the putative proton donor), N and n (the parts containing the putative catalytic nucleophile), and C and c (the carboxyl terminal fragments). The two catalytic residues of XynA and XynB, i.e., the proton donor and the catalytic nucleophile, were determined as Glu^{195} and Glu^{301}, and Glu^{184} and Glu^{292}, respectively (numbered from Met, the N-terminal residue of the open reading frame), identified by similarities in their amino acid sequences. The C-terminal borders of A, P, and N were designed to be Val^{192}, Asp^{265}, and Leu^{302}, respectively (XynA numbering). Six chimeric genes: APNc, APnc, Apnc, apnC, apNC, and aPNC, were constructed by substituting the relevant sections using an overlapping polymerase chain reaction (Zhong and Bajaj, 1993; Ahsan et al., 2001). The amplified chimeric gene fragments and pET28a vector (Novagen, Darmstadt, Germany) were ligated with *Nco*I and *Xho*I sites. Chimeric enzymes were purified with Ni-NTA agarose (QIAGEN, Hilden, Germany) chromatography from the transformed *Escherichia coli* cell lysate after induction with

isopropyl-1-thio-β-D-galactoside. Two of the six chimeric xylanases, namely APnc and Apnc, were produced and these enzymes showed activities comparable to those of the parental xylanases. However, no significant activity was found for any of the other four chimeric enzymes, i.e., APNc, apnC, apNC, and aPNC, in either the culture supernatants or the cell-free extracts. An SDS-PAGE analysis of the insoluble fractions obtained for these four chimeric enzymes revealed that the proteins were produced as inclusion bodies (data not shown).

2.3.2 Characterization of Chimeric Xylanases

Xylanase activity was determined from the amount of *p*-nitrophenol liberated using $pNPX_2$ (Kitaoka et. al., 1993) as the substrate. One unit of xylanase activity was defined as the amount of the enzyme liberating 1 μmol *p*-nitrophenol per minute. The specific activities and kinetic parameters of the purified xylanases were measured at pH 6.6. For the purified APnc and Apnc enzymes, specific activities of 51.3 and 47.8 U/mg were obtained, respectively. These values are comparable with those obtained for the parental enzymes, XynA and XynB (40.8 and 68.1 U/mg respectively). The major difference between XynA and XynB in terms of the kinetic parameters toward $pNPX_2$ is reflected in the K_m values (0.196 and 0.086 mM, respectively). The K_m values determined for the two active chimeric enzymes, APnc and Apnc (0.074 and 0.063 mM, respectively), were relatively close to that obtained for XynB. No difference was observed in the products from $pNPX_2$ in all the parental and chimeric enzymes. The thermal and pH stabilities of the parental and chimeric xylanases were assessed by determining the residual activity after incubation for 20 min at various temperatures or pH levels. APNC, APnc, Apnc, and apnc were stable at temperatures up to 50, 45, 45, and 60°C, respectively (Fig. 2.6A), and the

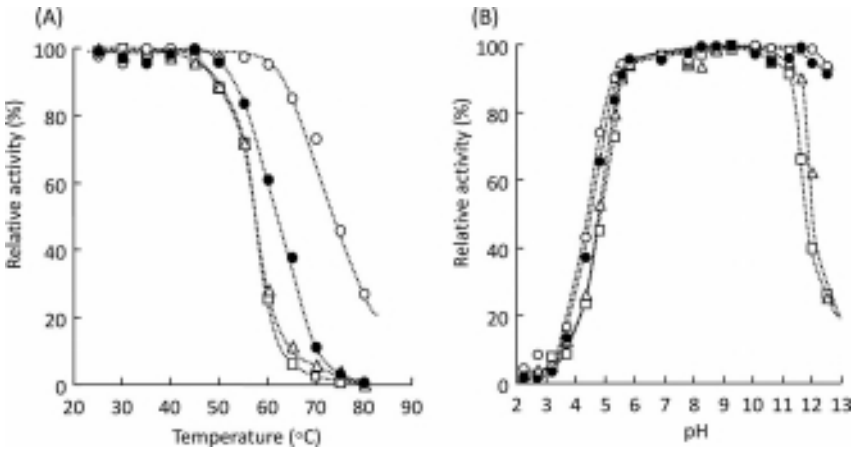

FIGURE 2.6 Thermal (A) and pH (B) stabilities of the chimeric and parental xylanases. XynA (closed circle); XynB (open circle); Apnc (open square); APnc (open triangle) (reproduced from Nishimoto et al., 2002).

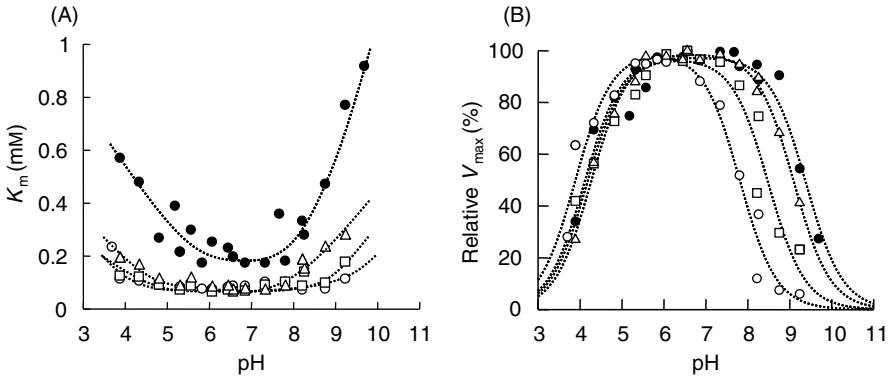

FIGURE 2.7 Comparison of the pH–activity profiles: pH versus K_m (A) and V_{max} (B) for the chimeric and parental xylanases. XynA (closed circle); XynB (open circle); Apnc (open square); APnc (open triangle) (reproduced from Nishimoto et al., 2002).

enzymes were stable over the pH ranges from 5.3 to 12.5, 5.6 to 11.6, 5.6 to 11.2, and 5.3 to 12.5, respectively (Fig. 2.6B). K_m and relative V_{max} values of the parental and chimeric xylanases were determined at various pH levels and these relationships are shown in Figure 2.7. It is interesting to note that the pH–K_m profiles obtained for the chimeric xylanases are similar to that obtained for XynB (Fig. 2.7A). The kinetic parameters and pK_a values were calculated by curve-fitting the experimental data to the Michaelis–Menten equation (2.1) and the bell-shaped curve (2.2) using the GraFit computer program (Leatherbarrow, 1990).

$$v = (k_{cat}[E]_0[S]_0)/(K_m + [S]_0) \tag{2.1}$$

$$V_{max}(\text{pH}) = V_{max}/((10^{(\text{pKa1}-\text{pH})} + 1)(10^{(\text{pH}-\text{pKa2})} + 1)), \quad (\text{p}K_{a1},\ \text{p}K_a \text{ for the nucleophile};\ \text{p}K_{a2},\ \text{p}K_a \text{ for the proton donor}) \tag{2.2}$$

The pK_{a1} and pK_{a2} values were calculated by regressing the experimental data with Eq. (2.2) and these values are given in Table 2.2. From these results, it can be seen that the pK_{a1} values obtained for both parental enzymes and the two chimeric xylanases were almost identical. In contrast, the pK_{a2} values obtained for APnc and Apnc were

TABLE 2.2 pK_a Values of the Parental and Chimeric Xylanases

	pK_{a1}	pK_{a2}
APNC (XynA)	4.1 ± 0.1	9.4 ± 0.1
APnc	4.3 ± 0.1	9.1 ± 0.1
Apnc	4.2 ± 0.2	8.5 ± 0.1
apnc (XynB)	3.9 ± 0.1	7.8 ± 0.1

9.1 and 8.5, respectively; values intermediate to those of the two parental enzymes, APNC (pK_{a2}, 9.4) and apnc (pK_{a2}, 7.8). The pK_{a2} value of XynB (apnc) increased from 7.8 to 8.5 by substitution of the N-terminal region with that from XynA (Apnc). This result indicates that the N-terminal fragment has some region that affects the pK_a of the proton donor. Moreover, the pK_{a2} value of Apnc increased to 9.1 by the substitution of the p section of Apnc to give the APnc chimeric enzyme, a value very close to that observed for XynA (9.4). The contribution of A and P to increasing the pK_{a2} were estimated to be similar because the exchange of A and P caused similar increases in pK_{a2}, 0.7 and 0.6, respectively. This result strongly indicates that the section containing the proton donor Glu^{195} also contains the main region needed to maintain a high pK_{a2} value. However, the pK_{a2} value obtained for APnc was still slightly less than that obtained for XynA, suggesting that the N and/or C parts may weakly affect the pK_{a2} value.

It is often reported that chimeric enzymes show properties intermediate to those of the parental enzymes (Kim et al., 2005; Goyal et al., 2001, 2002; Singh et al., 2002; Shibuya et al., 2005; Kaneko et al., 2000; Nirasawa and Hayashi, 2008). In this study, the specific activities and pK_{a2} values of APnc and Apnc were intermediate to those observed for the parental enzymes. However, the stabilities of the chimeric enzymes were less than those of the parental xylanases. This relative instability could be due to the loss of some interaction such as a hydrogen bond in the protein molecule, caused by the swapping of a domain.

2.4 FUTURE STUDIES

Because of the advances in biotechnology, the construction of chimeric genes, transformation of microorganisms, and expression of the chimeric genes are quite simple. However, there are limitations to obtaining the chimeric enzymes in a catalytically active form, i.e., the folding of the chimeric enzymes. Based on our experience, only one-third of the constructed chimeric genes are expressed as the catalytically active form. Coexpression with chaperone protein has been attempted and is found to be useful in some, but not in all, cases. Refolding of the denatured protein obtained as an inclusion body is not a successful technique. However, several new refolding methods have been developed. It would be interesting to apply these new methods to the constructed chimeric genes expressed as inclusion bodies. Accumulation of data on these chimeric enzymes will provide important information for elucidating the relationship between catalytic specificity and the three-dimensional structure of enzymes.

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3

PRODUCTION AND ACCUMULATION OF UNUSUAL FATTY ACIDS IN PLANT TISSUES

D. HILDEBRAND, J.R. THOGURU, S. RAO, AND R. LI
Plant Science, University of Kentucky, Lexington, KY 40546-0312, USA

T. HATANAKA
Kobe University, Kobe 657-8501, Japan

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3.1 INTRODUCTION

Plant membrane lipids contain only about six common fatty acids (Fig. 3.1) but storage triacylglycerol (TAG) can contain numerous fatty acids having different properties. Most 18:0-ACP formed in plant cells is desaturated to 18:1-ACP by a soluble desaturase (18:0-ACP-desaturase) in the chloroplast stroma (Browse and Somerville, 1991). Although Δ9-stearoyl (18:0)-ACP (acyl carrier protein) desaturases are mainly responsible for the synthesis of monounsaturated fatty acids in plants (Shanklin and Cahoon, 1998), several variant enzymes with different substrate specificities are also known (Cahoon et al., 1992; Cahoon and Ohlrogge, 1994; Schultz et al., 1996). Desaturases resembling cyanobacterial acyl lipid and mammalian and yeast Co-A desaturases (Fukuchi-Mizutani et al., 1998; Mekhedov et al., 2000) from *Arabidopsis* and other plants (Marillia et al., 2002) are known. Acyl-ACP-desaturase is structurally unrelated to either acyl-CoA desaturase or glycerolipid desaturase (Marillia et al., 2002). The main saturated fatty acid that is found and accumulates in plant lipids including TAG is palmitic acid, 16:0, and the main monounsaturated fatty acid in plants is oleic acid (*cis* Δ9-octadecenoic acid), Δ9–18:1 (Fig. 3.1). In 16:3 plants such as members of the cabbage family including *Arabidopsis* and canola Δ7–16:1 is an intermediate in the biosynthesis of 16:3 (Buchanan et al., 2000; Zhuang et al., 1996). Essentially all of the Δ7–16:1 (and

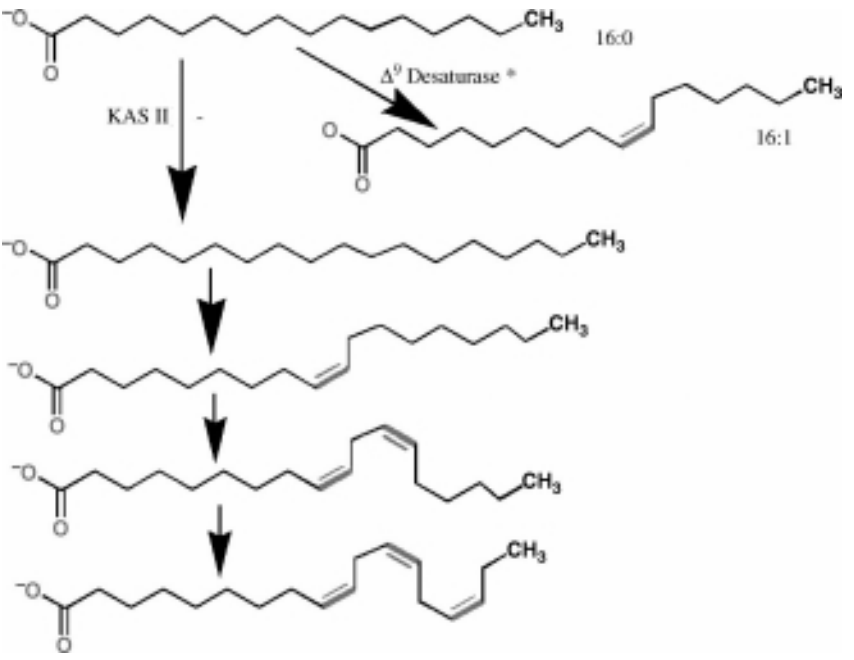

FIGURE 3.1 Biosynthesis of the five main plant fatty acids + diversion of palmitic acid (16:0) into palmitoleic acid (16:1) by a special Δ9 desaturase*. KASII = 3-ketoacyl-ACP synthase II.

Δ7,Δ10–16:2) is desaturated to Δ7,Δ10,Δ13–16:3 in 16:3 plants, which mainly accumulates in chloroplast galacto-lipids. Only a few plants make and accumulate palmitoleic acid, Δ9–16:1, including *Macfadyena unguis-cati* and *Macadamia* (Cahoon et al., 1998; Jones, 1939; Badami and Patil, 1980; Gummeson et al., 2000; Maguire et al., 2004).

In mammals and yeast monounsaturated fatty acids are mainly synthesized by ER Δ9-CoA desaturases. These desaturases can desaturate 16:0 to palmitic oleic acid, Δ9–16:1, in addition to desaturating 18:0 to 18:1 in plant and other tissues (Grayburn et al., 1992; Polashock et al., 1992; Wang et al. 1996; Moon et al., 2000). We recently cloned an oyster mushroom (*Pleurotus ostreatus*) Δ9 desaturase and showed it to be active with palmitic acid converting it to palmitoleic acid (Rao et al., 2010).

Another group of unusual fatty acids (UFA) of considerable interest are epoxy fatty acids such as vernolic acid (Va) (*cis*-12-epoxyoctadeca-*cis*-9-enoic acid). Va can accumulate up to 50–90% of total fatty acids in the seeds of *Vernonia galamensis*, *Euphorbia lagascae, Stokesia laevis, Crepis palaestina,* and *Bernardia pulchella* (Bafor et al., 1993; Pascual and Correal, 1992; Perdue, 1989; Thompson et al., 1994). These UFA have unique properties that make them valuable as renewable raw materials for the chemical industry, being used in making dyes, paints, coatings, adhesives, composites, plastics, and a variety of other products (Jaworski and Cahoon, 2003).

Recent efforts to express genes driving the synthesis of UFAs in commercial oil crops have generally met with only limited success, with much lower amounts of the desired fatty acid accumulating in the oils of transgenic plants (15–30%) compared with the native plant species (up to 90%) (Burgal et al., 2008; Cahoon et al., 2007; Jaworski and Cahoon, 2003; Singh et al., 2005; Thelen and Ohlrogge, 2002). It has been proposed that diacylglycerol acyltransferase (DGAT; EC 3.2.1.20) is one of the rate-limiting steps in plant storage lipid accumulation and plays an essential role in controlling both the quantitative and qualitative flux of fatty acids into storage TAGs (He et al., 2004; Jako et al., 2001; Lung and Weselake, 2006; Vogel and Browse, 1996; Yu et al., 2006). There are two distinct types of nonhomologous DGAT gene families designated as *DGAT1* and *DGAT2* encoding proteins with DGAT activity in plants (Lardizabal et al., 2001; Shockey et al., 2006) and animals (Cases et al., 2001). He et al. (2004) report that DGAT1 may play a dominant role in ricinoleic acid production in castor bean (*Ricinus communis*), while Kroon et al. (2006) present data indicating that DGAT2 is the major contributor to TAG formation in caster seed oil. Subsequently, Burgal et al. (2008) showed that castor DGAT2 can nearly double hydroxy fatty acid accumulation in *Arabidopsis* seeds (from $\sim$ 17 to $\sim$30%) by coexpression of this gene along with the castor hydroxylase compared with the hydroxylase gene alone. Moreover, Shocky et al. (2006) presented good evidence that DGAT2 may be involved in the selective accumulation of the unusual fatty acid, eleostearic acid, in tung (*Vernicia fordii*) oil.

Heterologous expression of single epoxygenase genes can result in the accumulation of epoxy fatty acids in seed oils, but amounts were considerably less than in the seeds of the source plants from which the genes were isolated. Transgenic expression of a cytochrome P450-type epoxygenase gene (*CYP726A1*) from *E. lagascae* led to the accumulation of Δ12-epoxy fatty acid in tobacco (*Nicotina tabacum*) callus or in

somatic soybean (*Glycine max*) embryos at 15 and 8% (w/w) of total fatty acids (Cahoon et al., 2002). Transgenic production of Va in *Arabidopsis* seeds with a single *C. palaestina* Δ12-epoxygenase (*Cpal2*) resulted in only 6.2% total epoxy fatty acids (Singh et al., 2001), much less than the 60% found in seeds of *C. palaestina*.

We previously isolated a cDNA clone (*SlEPX*) encoding a Δ12-epoxygenase from *S. laevis*, and seed-specific expression of this gene resulted in 2.4% of Va accumulation in transgenic *Arabidopsis* seeds (Hatanaka et al., 2004). DGATs from *V. galamensis* and *S. laevis* were found to have strong substrate preferences for Va bearing substrates including acyl-CoA and diacylglycerols (DAGs) with seed microsomes (Yu et al., 2006), but not *DGAT1* cDNA clones (*VgDGAT1*) expressed in yeast (Yu et al., 2008). Here, we report the cloning and characterization of a *V. galamensis DGAT2* cDNA (*VgDGAT2*). Coexpression of *SlEPX* and *VgDGAT1* or *VgDGAT2* cDNAs in petunia leaves and soybean somatic embryos leads to accumulation of Va with DGAT2 having greater proclivity for epoxy fatty acid accumulation. Va levels increased up to 14.7 and 25.8% in mature transgenic soybean seeds co-expressing *SlEPX* and *VgDGAT1* or *VgDGAT2*. This is the first report on the development of a commercial oilseed with high epoxy fatty acid levels by metabolic engineering.

3.2 RESULTS AND DISCUSSION

3.2.1 Cloning of a *P. ostreatus* Δ9 Desaturase Gene

NCBI BLAST searching with the sequence from the *P. ostreatus* genomic DNA clone and cDNA clone identified an N-terminal fatty acid desaturase domain and a C-terminal cytochrome b5 domain that is common to all known fungal Δ9 desaturases (Wongwathanarat et al., 1999). Primary sequence analysis of membrane desaturases from a wide range of organisms, including mammals, fungi, cyanobacteria, insects, and plants, reveals three conserved regions of histidine-box motifs: HXXXXH, HXXHH, and HXXHH (Man et al., 2006). Similar to the Δ9 desaturases from the above-mentioned organisms the *P. ostreatus* protein also contains eight histidines in three cluster motifs HRLWSH, HRSHH, and HNFHH. The *P. ostreatus* Δ9 desaturase amino acid sequence shows moderate to high homology to other known fungal desaturases. A distance tree from BLASTP results reveals that the hypothetical protein CG1G_11588 (*Coprinopsis*) has the highest homology to the *P. ostreatus* Δ9 desaturase with 58% identity and 75% similarity (NCBI accession # EAU81345).

3.2.2 Functional Analysis of the *P. ostreatus* Δ9 Desaturase Gene

GC analysis of the *P. ostreatus* fatty acids shows linoleic acid at 70% to be the major fatty acid followed by palmitic acid at ∼24% (Table 3.1). Similar lipid composition patterns have been reported for other *Pleurotus* sp. (Dimou et al., 2002). The lipid composition of *S. cerevisiae Ole*1 mutant rescued with the *P. ostreatus Ole*1 and *S. cerevisiae Ole*1 genes show palmitoleic acid to be most abundant (Table 3.1). Palmitoyl-CoA may be slightly preferred by the yeast *Ole*1 desaturase as a substrate

TABLE 3.1 Fatty Acid Composition of Wild-Type *P. ostreatus, S. cerevisiae*, and a Yeast Unsaturated Fatty Acid Auxotroph Rescued with *P. ostreatus* and *S. cerevisiae* Δ9 Desaturases

Fatty Acid	Content of Fatty Acids (%) in *P. ostreatus* Fruiting Bodies	Content of Fatty Acids (%) in *S. cerevisiae* (InVSc-1)	Content of Fatty Acids (%) in Yeast Auxotroph Rescued with Wild-Type *P. ostreatus ole*1	Content of Fatty Acids (%) in Yeast Auxotroph Rescued with Wild-Type *S. cerevisiae ole*1
16:0	23.6	31.9 ± 0.4	15.1 ± 0.3	16.1 ± 0.1
16:1Δ9	0.1	40.2 ± 0.6	47.4 ± 0.07	55.7 ± 0.2
18:0	1.4	12.8 ± 0.3	7.7 ± 0.1	5.1 ± 0.1
18:1Δ9	4.5	15.1 ± 0.6	29.9 ± 1.0	23.1 ± 0.1
18:2	69.6	—		
18:3	0.1	—	—	

Note: The yeast cells were cultured in YPD media (1% yeast extract, 2% Bacto-Peptone, 2% D-glucose) for 3 days at 28°C with shaking at 200 rpm. Values are means of three independent experiments ± standard errors.

compared with the *P. ostreatus* desaturase as indicated by the slight increase in the percent composition of palmitoleic acid in the total lipids of the rescued mutant. The oleic acid levels in the rescued mutant suggest that the *P. ostreatus* desaturase has higher activity with stearoyl-CoA than the yeast desaturase (Table 3.1).

These results when coupled with the *P. ostreatus* lipid composition (Table 3.1) indicate that there may be more than one Δ9 desaturase present in the oyster mushroom genome. The lipid composition of *P. ostreatus* indicated that 18:2 and 16:0 are the major fatty acids (Table 3.1). The levels of 16:1 compared to these two fatty acids are negligible. The Southern blot hybridization data also indicates that *P. ostreatus* has 2–3 Δ9 desaturases. Other Δ9 desaturase genes in *P. ostreatus* might contribute to the synthesis of the 18:1 precursor of 18:2. This is supported by the data available from the other known mushroom *L. edodes* Δ9 desaturase. Yeast *Ole1* mutant complementation with the *L. edodes* Δ9 desaturase showed much less 16:0 compared with 18:0 desaturation products. Similarly other known fungal Δ9 desaturases also show highest activities with 18:0 substrates (Wongwathanarat et al., 1999; Anamnart et al., 1997). It may be that Δ9 desaturase(s) with specificity for 18:0 substrates may be the major contributors to the *P. ostreatus* lipid composition. Alternatively, *P. ostreatus* may have much higher fatty acid elongation activity (e.g., 3-ketoacyl-ACP synthase II or KASII) from 16:0 to 18:0 (Fig. 3.1) than *S. cerevisiae.* When this *P. ostreatus* Δ9 desaturase is expressed in plant tissues, it increases 18:1 Δ9 levels more than 16:1 Δ9 (data not shown), which is also the case with the yeast Δ9 desaturase, suggesting higher relative elongation activity of 3-ketoacyl-ACP synthase II in *P. ostreatus* and plants compared with yeast.

Expression of the *P. ostreatus* Δ9 desaturase in petunia leaves shows increases in 18:1Δ9, 18:1Δ11, and 16:1Δ9, with the 16:1Δ9 levels below detection (<0.1%) in vector control transgenic leaves to several percent in most transgenics with levels as

TABLE 3.2 The Highest Expression Achieved in Desaturation of *P. ostreatus* Δ9 Desaturase Substrates

Fatty Acid	VC	1	2	3	4	5	6
14:0	1.0	1.1	0.5	0.8	0.5	0.1	0.1
16:0	14.1	21.9	16.1	25.6	17.6	20.5	21.2
16:1Δ7	0.8	0.9	0.9	0.3	1.4	0.0	0.0
16:1Δ9	**0.0**	**6.3**	**1.3**	**1.1**	**2.2**	**22.5**	**22.5**
18:0/16:3	2.5	7.2	3.1	5.1	3.6	10.1	10.5
18:1Δ9	0.5	2.8	1.6	1.7	1.9	5.5	5.7
18:1Δ11	**0.0**	**3.5**	**0.8**	**1.4**	**1.2**	**9.7**	**9.8**
18:2	9.0	8.2	10.5	12.1	11.5	7.6	7.4
18:3	70.9	46.4	64.0	49.0	57.9	23.9	22.7
22:0	1.2	1.6	1.1	3.0	2.2	0.0	0.0

Note: VC, vector control; 1–6, different petunia leaf transgenics.

high as ∼22% in some cases (Table 3.2). Oddly the increases in 18:1Δ9, 18:1Δ11, and 16:1Δ9 were not accompanied by decreases in 16:0 or 18:0, with the reductions mainly coming from 18:3.

3.2.3 Coexpression of *VgDGAT* with *SlEPX* Increases Vernolic Acid Levels in Agro-Infiltrated Petunia Leaves

RT-PCR amplification of target cDNA from petunia leaf tissue agro-infiltrated with the transgenes was used to further assess the utility of this system for the generation of the expected transcripts. Total RNA of the agro-infiltrated petunia leaf tissue was isolated using a standard isolation procedure and 5 μg used for first-strand cDNA synthesis with an oligo(dT) primer. An aliquot of the first-strand synthesis reaction was then used in combination with transgene-specific primers. Templateless control, RNA from uninfiltrated leaves, and vector control infiltrated leaves showed no amplification product of the transgene, while the complete experimental reaction yielded the same product of the transgene as in the positive control of plasmid DNA template. This indicates that the target transgenes expressed correctly in this system.

Based on the time course of the transgene expression, the agro-infiltrated petunia leaf tissues were sampled at 5 to 6 days post infiltration for total lipid extraction and subsequently for TLC and GC analysis. Va was not detected in the non-agro-infiltrated and empty-vector-control leaves, but was present in the petunia leaves expressing epoxygenase alone and combined expression with either *VgDGAT*. Compared to the expression of *SlEPX* alone, *VgDGAT1* coexpression increased Va level twofold, and *VgDGAT2* coexpression resulted in an enhancement of about five times more.

3.2.4 Coexpression of *VgDGAT* with *SlEPX* Increases the Accumulation of Vernolic Acid in Soybean Somatic Embryos

Va levels in transgenic somatic embryo lines were $5.0 \pm 0.6\%$, $9.1 \pm 0.5\%$, and $17.6 \pm 0.9\%$ (w/w) for *SlEPX* expression alone, and coexpression with *VgDGAT1* or

TABLE 3.3 Vernolic Acid and Total Oil Levels of Transgenic Soybean Somatic Embryos Transformed with the Vector Alone, VgDGAT1a, VgDGAT2, a *Stokesia* Epoxygenase (StEPX), *Stokesia* Epoxygenase + VgDGAT1a (VD1/StE), and *Stokesia* Epoxygenase + VgDGAT2 (VD2/StE) ± Standard Errors

Transgenic Line	Gene	Vernolic Acid (% of Total Oil)	Total Oil (mg/100 mg)
9322-1	Vector		20.1 ± 1.07 Δ
9322-2	Vector		17.8 ± 1.14
9322-6	Vector		18.6 ± 0.94
9242-1	VgDGAT1		23.1 ± 0.74
9242-2	VgDGAT1		24.9 ± 1.37 Δ
9242-3	VgDGAT1		23.2 ± 0.91
9247-4	VgDGAT1		19.9 ± 1.08
9247-5	VgDGAT1		20.3 ± 1.13
9247-6	VgDGAT1		19.1 ± 2.3
9387-3	VgDGAT2		22.3 ± 0.85
9387-5	VgDGAT2		18.2 ± 1.26
9995-1	VgDGAT2		23.2 ± 0.42 Δ
9995-2	VgDGAT2		22.5 ± 0.99
9995-4	VgDGAT2		20.5 ± 1.33
9996-1	StEXP	3.7 ± 0.91	14.5 ± 2.83
9996-3	StEXP	4.3 ± 0.72	16.6 ± 1.43
9996-5	StEXP	4.9 ± 0.36	15.1 ± 1.92
9996-6	StEXP	5.0 ± 0.62Δ	18.7 ± 1.06 Δ
9551-1	VD1 + StE	6.9 ± 0.74	20.6 ± 1.01
9551-3	VD1 + StE	6.8 ± 0.87	21.1 ± 2.02
9384-1	VD1 + StE	7.0 ± 0.57	22.9 ± 076 Δ
9384-6	VD1 + StE	7.1 ± 0.51Δ	20.3 ± 1.14
9381-2	VD2 + StE	13.2 ± 1.06	21.4 ± 0.79
9381-3	VD2 + StE	12.8 ± 1.26	19.9 ± 0.99
9381-4	VD2 + StE	11.6 ± 0.89	22.1 ± 1.08
9994-1	VD2 + StE	14.6 ± 0.93Δ	23.8 ± 0.89
9994-2	VD2 + StE	14.2 ± 1.04	23.9 ± 1.03 Δ
9994-5	VD2 + StE	10.9 ± 1.41	23.1 ± 1.14

VgDGAT2 (Table 3.3). However, no Va was found in vector control and either *VgDGAT*-expressing lines. Interestingly, the accumulation of Va was accompanied by a decrease in linoleic acid (C18:2) and α-linolenic acid (C18:3) levels and a slight increase in the oleic acid (C18:1) content in *SlEPX* lines compared with empty vector-transformed embryos. C18:2 and C18:3 were also reduced in *SlEPX/VgDGAT* transgenic lines with no changes for other fatty acids in the double transgenic lines. VgDGATs, particularly VgDGAT2, enhanced Va accumulation in soybean somatic embryos.

TABLE 3.4 Vernolic Acid and Total Oil Levels of Soybean T2 Seeds from Regenerated Transgenic Plants Transformed with *Stokesia* Epoxygenase + VgDGAT1a (VD1/StE)

Gene	Line Number	Vernolic Acid (%)	Total Oil (mg/100 mg dry WT)
VD1 + StE	9384-6-1-2	13.4	22.4
	9384-6-2-2	12.9	21.8
	9384-6-3-1	9.8	20.4
	9384-6-4-5	9.6	19.8
	9384-1-1-6	11.5	22.6
	9384-1-2-1	14.6	24.3
	9384-1-3-1	12.4	20.7
	9384-1-4-2	11.7	19.9
	9551-1-2-1	8.7	19.4
	9551-2-1-4	9.6	20.1
	9551-3-1-3	12.4	21.6
	9551-3-4-1	11.8	21.9

3.2.5 Higher Levels of Vernolic Acid in Mature Seeds of Regenerated Transgenic Soybeans Obtained by Coexpressing *SlEPX* with *VgDGATs*

The transgenic soybean somatic embryos were germinated and grown to maturity in a greenhouse. Seed chips of each progeny seed collected from the regenerated transgenic soybean plants were sampled for fatty acid analysis by GC and genotyping by PCR. Va was detected in the *SlEPX*-transgenic seeds and double transgenic seeds of *SlEPX* and each *VgDGAT*, but not in the seeds of null transgenic segregants, vector control lines, and only *VgDGAT1*- or *VgDGAT2*-transgenic lines (Tables 3.3 to 3.5). Va content in *SlEPX*-transgenic seeds ranged from 2.5 to 7.9% with an average of 5.5%. In *SlEPX/VgDGAT1* double transgenic seeds, the highest accumulation of Va

TABLE 3.5 Vernolic Acid and Total Oil Levels of Soybean T2 Seeds from Regenerated Transgenic Plants Transformed with *Stokesia* Epoxygenase + VgDGAT2 (VD2/StE)

Gene	Line Number	Vernolic Acid (%)	Total Oil (mg/100 mg)
VD2 + StE	9381-4-1-2	20.3	22.1
	9381-4-2-1	14.8	19.1
	9381-4-3-6	17.5	20.6
	9381-4-4-3	21.4	21.9
	9994-2-1-1	19.4	21.5
	9994-2-2-4	25.8	23.7
	9994-2-3-3	19.4	22.1
	9994-2-4-5	20.3	22.8
	9381-3-1-2	17.9	19.5
	9381-3-2-5	18.5	21.7
	9994-5-1-2	20.5	22.3
	9994-5-2-1	21.2	22.9

was 14.6% (9384-1-2-1line) with an average of 11.1%. The maximum level of Va was 25.8% (9994-2-2-4 line) with an average of 20.6%, which was found in the *SlEPX/VgDGAT2* double transgenic seeds. These data demonstrated that VgDGAT, especially VgDGAT2, can increase accumulation of Va in soybean seed oil.

In addition, the expression of transgenes caused some changes in fatty acid profiles in soybean seed oil compared with those in wild-type and vector control seeds. In *SlEPX*-containing seeds, C18:1 increased to some extent, whereas C18:2 was reduced considerably and C18:3 slightly decreased. Likely, in *SlEPX/VDGAT*-containing seeds, C18:2 and C18:3 decreased. Higher accumulation of Va was associated with lower C18:2. Again, data from mature transgenic soybean seeds revealed that VgDGATs, particularly VgDGAT2, are able to increase the accumulation of Va in soybean seed oil.

Seed-specific expression of a *S. laevis* cDNA (*SlEPX*) encoding a Δ12-epoxygenase resulted in 2.4% Va accumulation in transgenic *Arabidopsis* seeds (Hatanaka et al., 2004). In this study, seed-specific expression of *SlEPX* in both soybean somatic embryos and developing seeds also only yielded a small amount of Va production (Tables 3.3 to 3.5), much lower than that in *S. laevis* seeds. However, C18:1 levels were increased greatly in these *SlEPX* transgenic plants compared with the vector and untransformed controls (Tables 3.3 to 3.5). This phenomenon of limited accumulation of the target fatty acids and a marked increase in oleic acid (C18:1) at the expense of 18:2 and 18:3 fatty acids was also observed in other transgenic studies using divergent FAD2-like enzymes operating on different fatty acid substrates and performing a range of different modifications (Cahoon et al., 2006; Lee et al., 1998; Singh et al., 2001; Zhou et al., 2006).

Our investigation of expression profiles of genes involved in the final step of TAG synthesis indicates that DGATs are important in the synthesis and accumulation of epoxy and hydroxy fatty acids in seeds of high accumulators of those UFAs (Li et al., submitted). This was confirmed by our previous findings that microsomal DGATs from *V. galamensis* and *S. laevis* have strong substrate preferences for Va bearing substrates including acyl-CoA and DAGs (Yu et al., 2006). Based on these findings, we hypothesize that DGATs or other enzymes involved in TAG assembly coevolved for high accumulation of epoxy fatty acid in species such as *V. galamensis* and *S. laevis*.

Two *DGAT1* cDNA clones (*VgDGAT1a* and *VgDGAT1b*) were isolated from *V. galamensis* and their functions assayed in an *in vitro* yeast expression system (Yu et al., 2008). In the present study, we further cloned a *DGAT2* cDNA (*VgDGAT2*) from developing *V. galamensis* seeds. Sequence analysis showed VgDGAT2 shares high homology with other known DGAT2s and little homology with VgDGAT1 and other known DGAT1s. VgDGAT2 is at least 65% identical to other plant DGAT2 at the amino acid sequence level. Along with the most typical properties associated with the DGAT2 family, VgDGAT2 also contains two closely spaced membrane-spanning domains (Kroon et al., 2006; Shockey et al., 2006; Stone et al., 2006) and a C-terminal aromatic ER retrieval motif. Our present data show both *VgDGAT1* and *VgDGAT2* expression is associated with Va and total lipid accumulation during seed development of *V. galamensis,* indicating the potential of VgDGATs in increasing Va content in transgenic plant seeds.

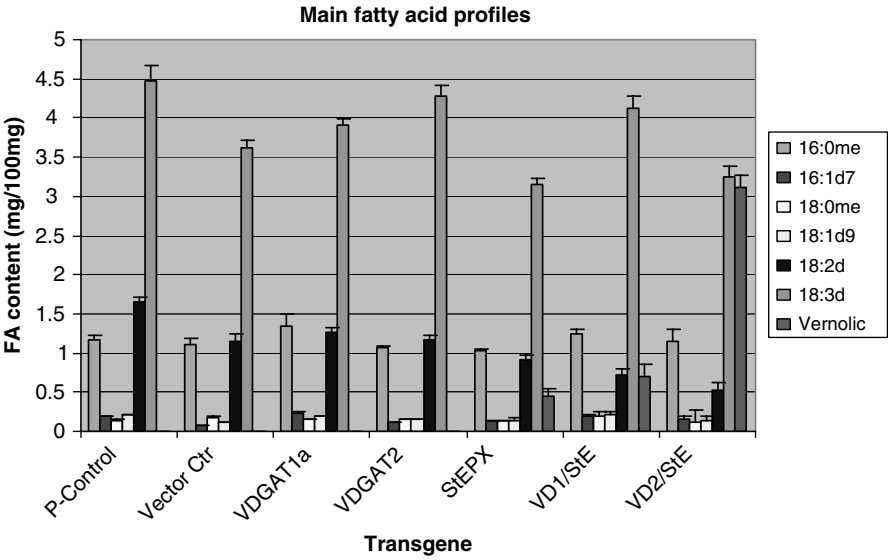

FIGURE 3.2 Fatty acid levels of petunia leaf controls, vector controls and leaves expressing VgDGAT1a, VgDGAT2, a *Stokesia* epoxygenase (StEPX), *Stokesia* epoxygenase + VgDGAT1a (VD1/StE), and *Stokesia* epoxygenase + VgDGAT2 (VD2/StE) ± standard errors.

To explore whether both VgDGATs function in increasing Va accumulation inferred from our expression analysis, the two cDNAs were coexpressed with *SlEPX* in petunia leaves. Compared with the small amount of Va in leaves expressing only *SlEPX*, coexpression with *VgDGAT1* did enhance Va accumulation, and *VgDGAT2* increased Va even more (Fig. 3.2). The effect of VgDGATs in increasing Va production was much stronger in soybean somatic embryos and developing soybean seeds (Tables 3.3 to 3.5). *VgDGAT1/SlEPX*-coexpressing soybean seeds accumulated Va up to 14.6%, and *VgDGAT2/SlEPX* coexpression enabled Va to increase to 25.8%. Clearly, our present data demonstrate that both VgDGATs facilitate the accumulation of Va, with VgDGAT2 having a greater impact. Burgal et al. (2008) report that only RcDGAT2 can increase hydroxy fatty acid levels in transgenic *Arabidopsis*, and RcDGAT1 does not. However, He et al. (2004) presented evidence that RcDGAT1 is important in oil biosynthesis in *R. communis*. The difference between VgDGAT1 and RcDGAT1 in UFA production in the transgenic plant seeds remains to be determined. Additionally, Burgal's data also showed transgenic expression of *Arabidopsis* DGAT2 (*AtDGAT2*) did not increase hydroxy fatty acid levels in transgenic *Arabidopsis* coexpressing fatty acid hydroxylase. This is consistent with our work on *Arabidopsis* DGAT1 and DGAT2 showing no specific function in UFA accumulation (Li et al., submitted). Taken together, our results indicate that both VgDGATs function in accumulation of Va, with VgDGAT2 as a more important contributor. These data support the hypothesis that some enzymes involved in TAG assembly have coevolved with epoxygenase for preferential incorporation of Va in storage oil. Future experimentation is needed to address the mechanism of VgDGAT2 significantly increasing

Va levels in the transgenic plant seeds. One possible important factor reported by Shockey et al. (2006) is the differential locations of DGAT2 and DGAT1 in the ER.

It has been reported that Va accumulation in transgenic *Arabidopsis* seeds often resulted in failure of germination and impaired growth and development (Singh et al., 2001). Similar phenomena are also reported for transgenic *Arabidopsis* seeds with higher level of hydroxy fatty acids (Burgal et al., 2008) and transgenic production of α-eleostearic acid in soybean somatic embryos and in wild-type *Arabidopsis* seeds by expression of a *Momordica charantia* conjugase (Cahoon et al., 2006, 2001). These observations suggest the possibility that high levels of UFA might damage metabolism and physiology of seeds. In our case, a number of progeny were collected from regenerated transgenic soybean plants. Several *SlEPX*/*VgDGAT* double transgenic seeds were found containing 9–16% Va. The next generation of these seeds was planted in the field in the 2008 growing season in Lexington, KY along with vector control and untransformed soybean lines. Germination, subsequent plant growth, development, and seed production were all normal. The seed weight and overall seed composition of *SlEPX*/*VgDGAT* transgenic lines showed no difference from the vector control and nontransgenic segregants (data not shown). These results indicate that the increased Va accumulation provided by VgDGAT1 and VgDGAT2 has no detrimental effect on the seed size or composition in comparison with parental or vector controls in a commercial oilseed.

3.3 CONCLUSIONS

The Δ9 desaturase gene cloned from *P. ostreatus* encodes an enzyme effective in desaturation of 16:0 to 16:1 in yeast, *S. cerevisiae Ole*1 cells.

Both VgDGAT1 and VgDGAT2 are important for the accumulation of Va, with VgDGAT2 contributing to the accumulation of this epoxy fatty acid to a greater level. Co-expressing *VgDGAT2* and *SlEPX* in a commercial oilseed oil crop can lead to accumulation as much as 25% of the industrial-valued Va in mature progeny seeds. This does not compromise plant growth or seed production in soybeans.

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4

PREPARATION OF OLEAGINOUS YEAST BY GENETIC MODIFICATION AND ITS POTENTIAL APPLICATIONS

YASUSHI KAMISAKA

Lipid Engineering Research Group, Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan

4.1 INTRODUCTION

Storage lipids are accumulated in cells in response to excess energy for cell growth. Recently, their production mechanisms have attracted particular attention to improve oil production in oleaginous plant seeds and microorganisms (Ratledge and Wynn, 2002; Hu et al., 2008; Dyer et al., 2008). As several important genes for storage lipid biosynthesis have been identified recently, the expression of these genes in plants and microbes has been actively investigated and it is seen that the expression of some genes caused an increase in oil content (Zou et al., 1997; Bouvier-Nave et al., 2000; Jako et al., 2001; Lardizabal et al., 2008). In addition to the storage lipid biosynthetic enzymes, regulatory mechanisms for the storage lipid biosynthesis may

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be important to increase oil content. These regulatory mechanisms may maintain storage lipids in coordination with intracellular energy levels, so that manipulation of these mechanisms may provide another strategy to increase oil content. Although some regulatory factors are elucidated especially in mammalian cells (Coleman and Lee, 2004), the applications of these regulatory factors to increase oil content have not been commonly reported.

The yeast *Saccharomyces cerevisiae* is a model organism for eukaryotes and its lipid metabolism has been extensively studied (Daum et al., 1998). Recently, *S. cerevisiae* attracted renewed interest as a model organism, since various global analyses have been actively conducted and their integrated approaches such as systems biology have been used to elucidate cellular phenomena in yeast (Hohmann, 2005; Mustacchi et al., 2006; Petranovic and Nielsen, 2008). We had thus started to study lipid accumulation mechanisms in *S. cerevisiae*, expecting that the global analyses would uncover new regulatory factors more readily. Since we had been also studying the lipid accumulation in an oleaginous fungus, *Mortierella ramanniana* var. *angulispora* (Kamisaka et al., 1999, 2004), the combination of the insights obtained in yeast and oleaginous fungus would allow us to understand and manipulate lipid accumulation mechanisms. This article presents our recent works to identify genes involved in lipid accumulation in yeast and prepare oleaginous yeast by genetic modification. Potential applications of the oleaginous yeast are also mentioned.

4.2 IDENTIFICATION OF GENES INVOLVED IN LIPID ACCUMULATION IN *S. cerevisiae*

Transposon insertion mutagenesis is one of the versatile tools in *S. cerevisiae* to identify genes responsible for particular phenotypes (Kumar et al., 2000). We used this technique to identify genes in lipid accumulation (Kamisaka et al., 2006), which had not been investigated by global analyses. We first enriched mutants as lighter density cells by Percoll density gradient centrifugation from transposon-mutagenized yeast cells, and then selected 18 transposon insertion mutants by screening using lipid body staining and triacylglycerol (TG) analysis. DNA sequencing of the transposon insertion sites in these mutants revealed five ORFs (Table 4.1). It is interesting to note that these ORFs are not directly involved in storage lipid metabolism, and thus we speculate that they are involved in the regulation of storage lipid metabolism. We prepared disruptants that lacked the entire regions of these ORFs using the PCR-based method (Nikawa and Kawabata, 1998) to confirm that the transposon insertion in these ORFs disrupted these gene functions. The lipid content of these disruptants was higher than that in the wild type (Table 4.2), indicating that these genes negatively regulated lipid accumulation. Nevertheless, the increase in the lipid content was not as high as expected, since some oleaginous fungi and yeast have a lipid content of 40–50% under optimal growth conditions (Ratledge, 1989). It was therefore likely that no single gene disruption could have profound impacts on lipid accumulation. We further prepared double disruptants of the five ORFs, but the effect of each gene disruption was not necessarily additive as expected.

TABLE 4.1 The ORFs Identified as Transposon Insertion Sites in Lipid Accumulation Mutants

Gene Name	Function
SNF2/YOR290c	RNA polymerase II transcription factor, ATPase
IRA2/YOL081w	Ras GTPase activator, negatively regulate cAMP synthesis
PRE9/YGR135w	Proteasome component, ubiquitin-dependent endopeptidase
PHO90/YJL198w	Phosphate transporter
SPT21/YMR179w	Regulator of transcription of Pol II promoter

Among the five ORFs we focused on *SNF2*, since the lipid content in the Δ*snf2* disruptant was higher and it was reported to function as a regulator of phospholipid synthesis repressed by inositol and choline (Kodaki et al., 1995). *SNF2* is well studied as a general transcription factor that forms part of the SWI/SNF chromatin-remodeling complex (Peterson and Tamkun, 1995). DNA microarray analysis of the Δ*snf2* disruptant was already reported, but any links of specific gene expression to storage lipid biosynthesis were not mentioned (Sudarsanam et al., 2000). Although extensive studies have focused on transcriptional regulation by Snf2p, little is known about the effect of *SNF2* expression on lipid accumulation. We found that the Δ*snf2* disruptant had larger amounts of TG but lower amounts of polar lipids than the wild type. In addition, the Δ*snf2* disruptant had larger amounts of oleic acid and lower amounts of palmitic acid and palmitoleic acid. Finally, the Δ*snf2* disruptant had a greater capability to incorporate exogenous fatty acids. These lipid characteristics of the Δ*snf2* disruptant suggest that *SNF2* affects the fatty acid synthesis and fatty acid

TABLE 4.2 Growth and Lipid Content of the Wild Type (YPH499) and the Gene Disruptants Cultured in the SD Medium

Strain	Dry Cell Weight (mg)	Total Fatty Acids (mg)	Lipid Content[a] (%)	
Wild type	40.0 ± 1.2	1.66 ± 0.16	4.15 ± 0.42	(1.00)
Δ*snf2*	36.4 ± 2.2	2.51 ± 0.17	6.93 ± 0.62	(1.67)
Δ*ira2*	24.5 ± 0.2	2.02 ± 0.06	8.20 ± 0.22	(1.98)
Δ*pre9*	33.4 ± 0.8	1.90 ± 0.20	5.69 ± 0.61	(1.37)
Δ*pho90*	40.5 ± 1.4	1.94 ± 0.04	4.79 ± 0.19	(1.15)
Δ*spt21*	39.8 ± 0.4	2.19 ± 0.05	5.50 ± 0.14	(1.33)
Δ*pre9/snf2*	38.8 ± 0.4	2.65 ± 0.22	6.83 ± 0.58	(1.65)
Δ*pre9/spt21*	31.8 ± 1.1	2.76 ± 0.18	8.68 ± 0.64	(2.09)
Δ*pho90/snf2*	39.8 ± 3.2	2.75 ± 0.28	6.91 ± 0.89	(1.67)
Δ*pho90/spt21*	44.3 ± 5.3	2.15 ± 0.14	4.86 ± 0.66	(1.17)

Note: Each strain was cultured in the SD medium at 30°C for 4 days (Kamisaka et al., 2006). Data are presented as means of triplicates ±SD per 50 ml culture. Values in parentheses represent the ratio to that in the wild type.

[a] Lipid content was expressed as total fatty acids (mg)/dry cell weight (mg) × 100.

transport through plasma membranes as well as lipid accumulation, although the exact mechanisms are not clear.

4.3 PREPARATION OF OLEAGINOUS YEAST *S. cerevisiae* BY GENETIC MODIFICATION

Although the Δ*snf2* disruptant had a higher lipid content than the wild type, it was not oleaginous enough to be a model of oleaginous yeast. The results suggested that other factors may be rate-limiting for further lipid accumulation, and we then investigated the overexpression of the TG biosynthetic enzymes because TG is a major storage lipid. Genes for diacylglycerol acyltransferase (DGAT) (*DGA1*) and phospholipid:diacylglycerol acyltransferase (*LRO1*) were cloned in yeast (Dahlqvist et al., 2000; Lardizabal et al., 2001), and it was demonstrated that the two enzymes were crucial for TG biosynthesis in yeast (Sorger and Daum, 2002; Sandager et al., 2002; Oelkers et al., 2002). We therefore conducted the overexpression of these genes in the Δ*snf2* disruptant to further increase the lipid content (Kamisaka et al., 2007).

During the course of the experiment, we found that transformation with the pL1177-2 vector containing a *LEU2* selection marker significantly increased cell growth and lipid accumulation in the wild type and Δ*snf2* disruptant without any insert genes. The effect was greater in the Δ*snf2* disruptant. We then investigated the effect of increasing concentrations of leucine in the medium on the cell growth and lipid accumulation instead of the transformation with pL1177-2. Concentrations of leucine higher than 250 μg/ml increased cell growth and lipid accumulation in both strains, and this increase was basically the same as that observed for the expression of *LEU2* by pL1177-2. These results indicated that leucine biosynthesis instead of the presence of leu2p was crucial for the increase in the cell growth and lipid accumulation. Based on the above observations, we prepared yeast transformants with pL1177-2 containing a *LEU2* selection marker and pL1091-5 containing a *URA3* selection marker.

By overexpression of *DGA1* or *LRO1* under the conditions optimized above, the lipid content of the wild type was increased 1.7- or 1.4-fold, consistent with previous observations (Bouvier-Nave et al., 2000; Dahlqvist et al., 2000). The lipid content of the Δ*snf2* disruptant, however, was changed differently by the overexpression of *DGA1* or *LRO1*. The overexpression of *DGA1* caused a 2.3-fold increase in the lipid content compared with the vector control, resulting in the total fatty acid content of 27.1%, whereas that of *LRO1* caused a 2.1-fold decrease. These results indicated that DGAT is rate-limiting for the TG biosynthesis and is somehow activated by the Δ*snf2* disruption. On the contrary, the effect of *LRO1* on lipid accumulation was reversed by the Δ*snf2* disruption. We then investigated the effect of overexpression of acyl-CoA synthase genes on lipid accumulation, since acyl-CoA synthase increased the level of one of the DGAT substrate, acyl-CoA. Among the four acyl-CoA synthase genes (*FAA1*, *FAA2*, *FAA3*, and *FAA4*), overexpression of *FAA3* together with *DGA1* slightly increased the lipid content in the wild type and maintained the lipid content in

the Δ*snf2* disruption. Although the additional overexpression of *FAA3* in the Δ*snf2* disruptant did not increase the lipid content, it increased the incorporation of exogenous fatty acids. The other three genes however decreased the lipid content in both strains, suggesting that these were involved in the degradation of fatty acids such as β-oxidation. Taken together, we prepared the oleaginous yeast by the Δ*snf2* disruption, the overexpression of *DGA1*, and the expression of *LEU2*. Cultivation of this oleaginous strain in the nitrogen-limited SD medium for 7 days resulted in the total lipid content of around 30% and larger lipid bodies with a diameter of around 2 μm (Fig. 4.1).

To confirm that the overexpression of *DGA1* induced an increase in DGAT activity, we measured the DGAT activity in the homogenate of each strain (Table 4.3). It was evident that the DGAT activity in the homogenate of the Δ*snf2* disruptant transformed with pL1091-5/DGA1 was significantly increased, which can explain the increased lipid accumulation in the strain. The DGAT activity, however, remained very low in

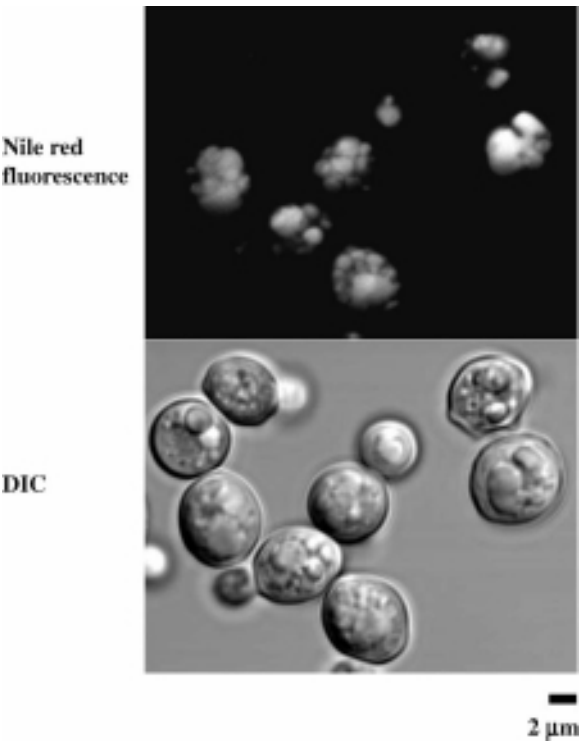

FIGURE 4.1 Staining of the Δ*snf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2 with Nile red. BY4741 Δ*snf2* disruptant transformed with pL1091-5/DGA1 and pL1177-2 cultured in the nitrogen-limited SD medium for 7 days was stained with Nile red (Kamisaka et al., 2007; Kamisaka, 2008). In each panel above is a fluorescent image and below is a differential interference contrast image. Bar = 2 μm.

TABLE 4.3 DGAT Activity in the Homogenate of BY4741 Yeast with the Overexpression of *DGA1*

Strain	DGAT Activity (pmol/min/mg protein)
WT (pL1091-5, pL1177-2)	1 ± 0
Δ*snf2* (pL1091-5, pL1177-2)	16 ± 1
WT (pL1091-5/DGA1, pL1177-2)	1 ± 1
Δ*snf2* (pL1091-5/DGA1, pL1177-2)	755 ± 134

Note: The homogenate was prepared from each strain that had been cultured at 30°C for 7 days in the nitrogen-limited SD medium (Kamisaka et al., 2007). The DGAT activity in the homogenate (3–10 μg protein) of each strain was assayed using [^{14}C]oleoyl-CoA as a substrate. Data are presented as means ± SD of triplicate values.

the homogenate of the wild type with the overexpression of *DGA1*. Furthermore, we expressed Dga1p with a 6x His tag to measure the amount of Dga1p. Immunoblotting with anti-6x His revealed that the homogenate of the wild type and Δ*snf2* disruptant transformed with pL1091-5/DGA1-6x His had similar amounts of Dga1p with a molecular mass of 50 kDa. The results indicated that the increase in DGAT activity in the Δ*snf2* disruptant with the overexpression of *DGA1* was not due to increased amount of Dga1p. Instead, Dga1p may be activated by the modification of Dga1p or cofactors interacting with Dga1p as suggested previously (Coleman and Lee, 2004; Lung and Weselake, 2006).

4.4 FUTURE PERSPECTIVES

The oleaginous yeast *S. cerevisiae* we prepared could be a model for oleaginous fungi and yeast. Although there have been extensive studies about lipid accumulation mechanisms in oleaginous fungi and yeast (Ratledge and Wynn, 2002), the proposed factors and mechanisms for lipid accumulation could not be easily evaluated due to the lack of genetic engineering information and tools in these oleaginous fungi and yeast. The oleaginous *S. cerevisiae* can be readily used to evaluate the effect of various factors on lipid accumulation by overexpression or disruption of the particular genes. In addition, various global analyses in *S. cerevisiae* may uncover new links of regulatory factors to lipid accumulation. *SNF2*, which was found as a negative regulator for the storage lipid biosynthesis, is one such regulatory factor, and further characterization of how *SNF2* affects DGAT activation and storage lipid biosynthesis would uncover other new factors involved in lipid accumulation.

The oleaginous *S. cerevisiae* can be used for designed oil production by metabolic engineering. Current targets are to use designed oil production for making nutritional oils such as polyunsaturated fatty acids and industrial oils such as fuels and lubricants (Veen and Lang, 2004; Graham et al., 2007; Cahoon et al., 2007). We have recently improved the production of stearidonic acid, a nutraceutical polyunsaturated fatty acid, by expressing rat Δ6-desaturase in the oleaginous *S. cerevisiae* (Kimura

et al., 2009). Further studies of this system may provide insights on how the target lipid products are accumulated using lipid accumulation processes, which may be applied to economically important oil producers such as oil crops and algae.

Finally, the oleaginous *S. cerevisiae* can be used for the screening of compounds modulating lipid accumulation. These compounds could be potential drugs for human diseases accompanying lipid accumulation, such as cardiovascular diseases and obesity. Since various global analyses in the oleaginous *S. cerevisiae* would make it possible to identify genes and proteins that reflect changes in lipid accumulation, the system using these indicator genes and proteins may provide rapid and high-throughput screening of compounds instead of the screening using lipid analysis.

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5

IMPROVING VALUE OF OIL PALM USING GENETIC ENGINEERING

GHULAM KADIR AHMAD PARVEEZ, ABRIZAH OTHMAN, UMI SALAMAH RAMLI, RAVIGADEVI SAMBANTHAMURTHI, ABDUL MASANI MAT YUNUS, AHMAD TARMIZI HASHIM, AHMAD KUSHAIRI DIN, AND MOHD BASRI WAHID

Malaysian Palm Oil Board, No. 6, Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor, Malaysia

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5.1 INTRODUCTION

Palm oil is the largest source of edible oil in the world is followed closely by soybean oil (Anon, 2007). Palm oil is the most price-competitive liquid cooking oil in many parts of the world and is also used in other food products such as shortenings, margarines, and spreads. Malaysia is the largest exporter of palm oil in the world. Its crude palm oil production is increasing annually; in 2008, it produced 17.73 million tonnes of palm oil, which is 12.1% or 1.91 million tonnes higher than the production in 2007 (Wahid, 2009). Previous studies have indicated that palm oil could lower serum cholesterol levels to the same degree as sunflower oil, which is rich in polyunsaturated fatty acids; moreover, studies also indicated that palm oil has antitumor properties due to the presence of high levels of vitamin E and tocotrienols (Qureshi et al., 1991; Kritchevsky et al., 1992; Tan, 1992).

The major problems faced by the oil palm industry in Malaysia are shortages of labor, scarcity of arable land, and occasional fluctuation in the price of the commodity. Recently, the price of palm oil has declined sharply (around 66% within a period of 8 months) from the highest level at RM 4179.00 (~US$1155) in early March 2008 to the lowest level at RM 1403.00 (~US$388) in late November 2008 (Wahid, 2009). One of the strategies to sustain the palm oil industry is through increased productivity. In order to remain competitive, the palm oil industry has to increase the productivity (yield per unit area) besides increasing the quality of oil and producing novel higher-value fatty acids. The process of obtaining transgenic plants must be much faster and more effective than can be achieved by conventional breeding (Parveez, 1998). The establishment of an effective and reliable oil palm tissue culture system and a method to transfer foreign genes into oil palm have made genetic engineering a promising tool for achieving the above-mentioned aims. Conventional improvement of oil palm, through plant breeding approaches, suffers from a number of limitations such as narrow gene pool (oil palm industry originated from only four palms introduced from Deli, Sumatera, Indonesia in 1911), a long generation time (normally takes about 7–10 years), and open pollination nature of the oil palm tree. From the first experience in producing transgenic oil palm in 1997, it could be roughly estimated that 4 to 5 years are required to produce valuable transgenic oil palm plantlets from the initial tissue culture stage (Parveez, 2000). This fact suggests that genetic engineering could reduce the time required to introduce a new trait into oil palm by around 80% compared with conventional breeding that requires a couple of rounds of crossing and back-crossings.

Initially, the genetic engineering of oil palm program, which was initiated in 1997, had aimed at increasing the monounsaturated oleic acid content by reducing the content of a major saturated fatty acid, palmitic acid (Cheah et al., 1995). However, many other useful compounds such as palmitoleic acid, ricinoleic acid, stearic acid, lycopene, and biodegradable plastics were targeted later to further increase the value of oil palm (Parveez et al., 1999; Parveez, 2005). It must be clearly noted here that currently, oil palm genetic engineering research is mainly carried out in the laboratory and the transgenic oil palms regenerated are planted in a fully contained biosafety screenhouse for evaluation. No transgenic oil palm has been planted openly for field

evaluation. Any of the transgenic oil palms produced, if proven suitable and safe after further evaluations, were subjected to regulatory approval for future commercialization as industrial feedstock or as niche edible products. The transgenic products are not targeted to enter the palm oil commodity market.

5.2 MATERIALS AND METHODS

5.2.1 Maintenance of Embryogenic Callus

Embryogenic callus was maintained on agar-solidified medium containing MS macro and micronutrients supplemented with 2.2 mg/l 2,4-D and 30 gm/l sucrose. The medium was adjusted to pH 5.7 with KOH prior to autoclaving. Embryogenic callus was cultured at 28°C, in the dark and subcultured every 30 days onto fresh medium (Parveez, 1998).

5.2.2 Bombardment of Embryogenic Calli

Five microliters of DNA solution (1 µg/µl), 50 µl of $CaCl_2$ (2.5 M), and 20 µl of spermidine (0.1 M, free base form) were added sequentially to the 50 µl particle suspension. The mixture was vortexed for 3 min and spun for 10 s at 10,000 rpm, and the supernatant was discarded. The pellet was washed with 250 µl of absolute ethanol. The final pellet was resuspended in 60 µl of absolute ethanol. Six microliters of the solution was loaded onto the center of the macrocarrier and air dried. Bombardments were carried out once at the following conditions: rupture disc pressure, 1100 Psi; rupture disc to macrocarrier distance, 6 mm; macrocarrier to stopping plate distance, 11 mm; stopping plate to target tissue distance, 75 mm; and vacuum pressure, 67.5 mmHg (Parveez, 1998).

5.2.3 Selection and Regeneration of Transformed Embryogenic Callus

Minimal inhibitory concentrations of selection agents for oil palm have been determined previously (Parveez et al., 1996). Embryogenic callus was exposed to medium containing Basta concentrations of 50 mg/l, one week after bombardment. Tissues were subcultured to fresh medium under selection at monthly intervals. Embryogenic cultures were maintained on media containing MS macro and micronutrients and Y_3 vitamins supplemented with 100 mg/l each of myoinositol, L-glutamine, L-arginine, and L-asparagine, 5 µM IBA, 0.7% agar, and 30 gm/l sucrose to form polyembryogenic cultures. The medium was adjusted to pH 5.7 with KOH prior to autoclaving. Embryogenic calli were incubated at 28°C in the presence of light and subcultured every 30 days onto fresh medium. Small plantlets were produced from polyembryogenic cultures on media containing MS macro and micronutrients and Y_3 vitamins supplemented with 100 mg/l each of myoinositol, L-glutamine, L-arginine, and L-asparagine, 0.1 µM NAA, 0.4 % agar, and 30 gm/l sucrose. The medium was adjusted to pH 5.7 with KOH prior to autoclaving.

Polyembryogenic calli were incubated at 28°C in light until sufficient shoots were produced. Roots were initiated from small plantlets on media containing MS macro and micronutrients and Y_3 vitamins supplemented with 300 mg/l L-glutamine, 100 mg/l myoinositol, 10 µM 2,4-D, 70 µM NAA, 0.15% activated charcoal, and 60 g/l sucrose. The medium was adjusted to pH 5.7 with KOH prior to autoclaving. Small plantlets were incubated at 28°C in light until roots were formed. The fully regenerated plantlets were later transferred into polybags and grown in a biosafety greenhouse.

5.2.4 Preparation of Total DNA from Embryoids and Small Plantlets

Resistant embryoids and leaflets were selected randomly and subjected to total DNA isolation according to the method of Ellis (1993). Tissues (10–50 mg) were placed in a 1.5 ml microfuge tube and immersed in liquid nitrogen. Frozen tissues were ground to fine powder in the presence of 400 µl of EB2 buffer (500 mM NaCl, 100 mM Tris-HCl {pH 8.0}, and 50 mM EDTA {pH 8.0}) and 20 µl of 20% SDS. Four hundred microliters of phenol mix (1:1 phenol:chloroform) was then added, thoroughly mixed, and centrifuged (12,000 rpm, 2 min, RT). The aqueous phase was transferred to a new tube and mixed with 800 µl of absolute ethanol. DNA was recovered by centrifugation (12,000 rpm, 5 min, RT). The pellet was washed with 70% ethanol and dissolved in 50 µl of TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0).

5.2.5 Polymerase Chain Reaction (PCR)

Amplification of selectable marker and useful genes were carried out using standard and touchdown PCR protocols (Sambrook et al., 1989). Fifty nanograms of oil palm DNA and 1 ng of transforming plasmid DNA were used in PCR reactions. In the standard procedure the following condition was used: 30 cycles at 92°C (50 s), 60°C (50 s), and 72°C (60 s). Amplified DNA fragments were examined by electrophoresis in 1.4% agarose gels in 0.5 × TBE (45 mM Tris-Borate; 1 mM EDTA, pH 8.0) buffer.

5.2.6 DNA Isolation and Southern Blot Hybridization

Total DNA extraction was carried out in plantlets according to modified CTAB method (Doyle and Doyle, 1990). Samples were chilled and ground in liquid nitrogen. Once the sample was sufficiently fine, 10 ml of CTAB extraction buffer (100 mM Tris-HCl, pH 8.0; 20 mM EDTA; 1.4 mM NaCl; 2% CTAB; 1% PVP, and 0.2% 2-mercaptoethanol) was added. The sample was then incubated at 65°C for 1 h. After 20 min at room temperature, 2 ml of chloroform:isoamyl alcohol (24:1) was added to the sample and centrifuged at 4°C, 13,000 rpm for 5 min. The aqueous phase was transferred into a new tube. The sample was extracted twice with 5 ml of chloroform: isoamyl alcohol (24:1). The DNA was precipitated by adding 6 ml of chilled isopropanol. After 20 min at room temperature, the sample was centrifuged at 13,000 rpm for 5 min. The supernatant was discarded and the pellet was dried at

room temperature. The pellet was washed using washing buffer (76% ethanol, 1 mM ammonium acetate), incubated at room temperature for 20 min before being centrifuged at 13,000 rpm for 5 min The pellet obtained was air dried and suspended in 500 µl of TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA).

For Southern blot, 20–30 µg of EcoR1-digested plant DNA was separated on 1.0% agarose gels. Blots were prepared by transfer to nylon membranes (Hybond-N, Amersham) using vacuum pump at 55 mbar pressure. Specific probes were randomly prime labeled with α P^{32} dCTP using Klenow fragment and hybridized onto the membranes. Hybridization was performed overnight at 65°C in hybridization buffer (20 × SSPE, 20% SDS, and 2 mg/µg herring sperm DNA) after being prehybridized in hybridization buffer plus 50 × Denhardt's solution at 65°C for 3 h. The membrane was also hybridized simultaneously with a probe derived from 1 kb Plus Ladder marker (Biodiagnostic) in order to detect the size of expected bands. Blots were washed twice at 65°C in 2 × SSC and 0.1% SDS for 15 min and once at 65°C in 1 × SSC and 0.1% SDS for 10 min. The membrane was exposed to X-ray film for 5 days at 80°C. The film was developed in the dark by soaking in the developer, tap water, and fixer (5% acid), and rinsed with water sequentially. The film was dried at room temperature.

Alternatively, gene fragments were labeled with DIG (Roche Molecular Biochemicals) according to the manufacturer's instructions. Labeled probes were then hybridized onto the membrane at 65°C. Membranes were washed twice with 2 × washing solution (5 min each) and twice with 0.5 × washing solution (15 min each at 65°C). Membranes were later blocked with a blocking reagent and incubated with anti-DIG to bind the antibody conjugates to the labeled DNA. The bounded antibody was detected by using a chemiluminescent assay (CSPD). The membrane was exposed to film at room temperature for 1–2 h.

5.2.7 High-Performance Liquid Chromatography Analysis for Biodegradable Plastics

Two grams of sample (embryoid/leaves) was oven dried at 80°C. Then, the dried sample was extracted under liquid nitrogen until it became powder. The powdered sample was transferred into a glass tube and 1 ml of concentrated H_2SO_4 was added before putting it in a 90°C water bath for 30 min [acid catalyzes depolymerization of PHB with elimination of water to yield crotonic acid (*trans* 2-butenoic acid)]. After 30 min, it was cooled on ice. Then, 4 ml of 0.014 N H_2SO_4 was added. After thoroughly mixing, the sample was filtered into a new glass tube through a 0.2 µm LC 13 PVDF. Then, 15 µl of filtered sample was transferred into suitable vials containing 135 µl of 0.014 N H_2SO_4 for HPLC analysis. Detection of crotonic acid was performed at 210 nm.

5.2.8 Total RNA Extraction

Total RNA extraction from transgenic samples was carried out using a method modified from Hosein (2001). Frozen leaf tissue was powdered in a mortar in liquid

nitrogen and then homogenized in 0.1 M Tris-HCl pH 8.0 in a GSA tube. The buffer was added in a ratio of 1 : 3 of tissue (weight) to buffer (volume). 1/10 volume of 5% (w/v) SDS was added to the homogenate and the solution was gently shaken on ice for 2 min to denature the protein. Equal volume of phenol (pH 8.0) was added to the mixture and the mixture was vortexed periodically for 5 min with frequent cooling on ice. The phases were separated by centrifugation at 4000 rpm (4°C) for 5 min and the aqueous layer was removed to a new centrifugation tube. The aqueous layer was re-extracted with an equal volume of buffered phenol until no residual protein interface was observed. Final extraction was done with an equal volume of buffered phenol:chloroform:isoamylalcohol (25:24:1, v/v/v). The aqueous layer was removed to a centrifuge tube and 8 M LiCl was added to achieve a final concentration of 2 M. The mixture was mixed by inversion and made to stand overnight at 4°C to precipitate the RNA. The RNA was pelleted by centrifugation at 10,000 (4°C) for 15 min. The supernatant was discarded and the pellet was resuspended in 1.5 ml of 2 M LiCl. The mixture was vortexed and spun again at 10,000 rpm for 10 min. The wash in 2 M LiCl was repeated. The pellet was dissolved in 1.5 ml of RNase-free water. 1/10 volume of 3 M sodium acetate pH 5.2 and 2.5 volume of 100% ethanol were added to the mixture. The mixture was mixed and stored overnight at −20°C to precipitate the RNA. The RNA was pelleted by centrifugation at 10,000 rpm (4°C) for 10 min. The supernatant was discarded and the pellet was rinsed in 2 ml of 70% cold ethanol and dried under vacuum. The RNA was dissolved in RNase-free water and stored at −80°C until used.

5.2.9 Real-Time Reverse Transcriptase (RT) PCR

Total RNA cleanup was done using the Qiagen RNase-free DNase Set according to the manufacturer protocol to remove DNA and other impurities. Concentration and purity were determined using the NanoDrop ND-1000 spectrophotometer. Then, 300 ηg/μl of cleaned total RNA was subjected to integrity analysis using Agilent 2100 Bioanalyzer (RNA 6000 Nano Assay Kit). Intact RNA was converted to cDNA by using High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR (TaqMan assay) was carried out with 10 μl 2 × TaqMan Universal PCR Master Mix, 1 μl 20 × Assay Mix (containing specific primers and probe), and 9 μl cDNA (diluted in RNase-free water). Gene fragment was used as a control for the specificity of primers and probes used in the amplification. PCR cycling parameters were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Real-time detection of fluorescence was performed on the ABI Prism 7000 sequence detection system.

5.2.10 Gas Chromatography Analysis

Plantlets or polyembryoids that are confirmed by PCR to carry the antisense palmitoyl-ACP thioesterase were subjected to gas chromatography to determine fatty acid composition. Methylation methods for fatty acid methyl esters (FAME) were performed. The detection of every compound was done based on the retention

time that was referred to F.A.M.E RM-6 Standards (C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, and C18:3) (Supelco).

Two grams of sample (embryoids) was extracted under liquid nitrogen until it became a powder. Then, 2 ml of toluene and 2 ml of 2.5% acidic methanol were added and vortexed. A condenser was attached and placed in a heating block, and then the sample was refluxed at 85°C for 2 h. After 2 h, the sample was transferred into a new vial and 3 ml of hexane and 5 ml of 5% NaCl were added. The sample was mixed and centrifuged for 5 min at 2500 rpm. The upper layer was transferred into a new vial and then 2 ml of hexane and 10 ml of 2% $KHCO_3$ were added to the sample. The sample was mixed and centrifuged again for 2 min at 2500 rpm. The upper layer was transferred again into a new vial and the hexane was evaporated in vacuum or under a nitrogen blanket. The sample was diluted to 1:5 v/v in hexane before injection into GC.

GC/FID analyses were performed on Agilent GC6890N (Agilent, USA) that is equipped with split/splitless injector at 150°C. One microliter of the solution of FAME 1:5 v/v in hexane was injected into GC. Agilent autosampler, AOL series (Agilent 7683 Series), and FID at 150°C were used (H_2 flow 40 ml/min, air flow 350 ml/min, and makeup He 45 ml/min). Data acquisition was performed by MSD and MSD data analysis software (Agilent, USA). A Supelco column, SP 2380 (0.25 mm × 30 m × 0.20 μm film), was used for the analysis. The temperature program is as follows: initial temperature 50°C for 2 min, final temperature 250°C, and ramp rate 4°C/min for 10 min. Helium was used as a carrier gas (1 ml/min). The FAC was calculated as normalized percentages from the peak areas.

5.3 RESULTS AND DISCUSSION

5.3.1 Production of Transgenic Oil Palm

Transgenic oil palm was first produced in 1997 using Biolistics-mediated transformation method after an extensive optimization of biological and physical parameters affecting DNA delivery, evaluating five different promoters and five selection agents (antibiotics and herbicide) for selecting transformed oil palm cells (Parveez, 1998). The transgenic oil palm status was verified using molecular and protein analyses (Parveez, 2000).

The transgenic plants were grown to maturity in polybags and kept in a fully contained biosafety screenhouse. The screenhouse is contained in a size 50 mesh and fixed with a double-layer door to trap and prevent insects and small animals from entering the screenhouse during opening and closing. Leaves, roots, and any other parts of the transgenic palms were removed from the palms and safely collected and autoclaved prior to appropriate disposal. The transgenic oil palms were proven fertile and demonstrated the inheritance of the transgenes (*bar* and *gusA*) into their progenies. Expression of the transgenes in the fruit and seed of mature transgenic oil palm was also demonstrated. There were no negative effects of the transgene's products in the transgenic palms observed. This finding is a good evidence for the safety of transgenic oil palm produced using Biolistics method.

5.3.2 High Oleate Transgenic Oil Palm

Enhancing the oleic acid content of palm oil is mainly required for use as a feedstock for oleochemical industries. The world's oleochemical market is expanding and it is expected that oleochemical production in Malaysia would follow the same increasing trend (Alan Brunskill, personal communication). A continuous supply of oleic acid as a feedstock for the above industry is essential. Therefore, efforts towards increasing the amount of oleic acid in oil palm have been given high priority. Before the efforts to increase oleic acid were initiated, the enzymatic mechanisms within the oil palm that cause its oil to produce around 44% saturated palmitic acid (C16:0) and 39% of monounsaturated oleic acid (C18:1) were elucidated. Based on the fatty acid biosynthesis pathway that is common to all plants (Fig. 5.1), extensive biochemical studies were conducted and revealed the following facts: (a) β-ketoacyl ACP synthase II (KAS II) enzyme activity was found to be rate-limiting in the oil palm fruits, which resulted in accumulation of palmitic acid and only about half of the palmitoyl-ACP substrate was converted into stearoyl-ACP and subsequently to oleoyl-ACP; (b) the palmitoyl-ACP thioesterase enzyme was very active and resulted in a high amount of palmitic acid accumulation; (c) palmitoyl-ACP thioesterase and oleoyl-ACP thioesterase are not related and their activities are not interdependent; (d) the stearoyl-ACP desaturase enzyme is very active, resulting in almost all the stearoyl-ACP intermediates desaturated to oleic acid, where increasing its activity is unlikely to increase oleic acid content; and finally (e) oleoyl-CoA desaturase is quite active in desaturating some of the oleoyl-CoA substrates into linoleioyl-CoA and eventually into linoleic acid, which requires to be down-regulated to reduce potential spillover into linoleic and linolineic acids (Sambanthamurthi et al., 2002). Using the same approach, efforts to increase oleic acid content in soybean were carried out. Soybean, which has 55% linoleic acid and 21.5% oleic acid, requires the pathway to be altered to increase oleic acid by preventing it from being desaturated to linoleic acid. Down-regulating the activity of oleoyl-CoA desaturase was carried out and resulted in an increase in oleic acid

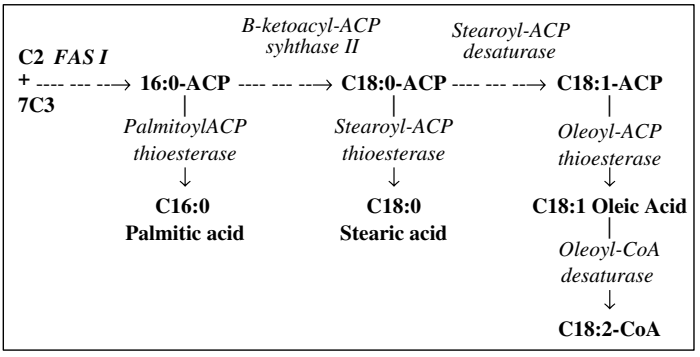

FIGURE. 5.1 Possible reactions involved in the modification of products of fatty acid synthetase.

content in soybean to 78.9% due to reduction in linoleic acid to 3% (Broglie et al., 1997). Using the same strategy, down-regulating the oleoyl-CoA desaturase resulted in a 15–78% increase in oleic acid content in cottonseed oil (Liu et al., 2000).

Based on biochemical studies, efforts to isolate the required genes to be manipulated and regulatory elements for targeting into mesocarp tissue were carried out. The genes for KASII (Ramli et al., 2006), palmitoyl-ACP thioesterase (Abrizah et al., 2000), stearoyl-ACP desaturase (Siti Nor Akmar et al., 1999), and oleoyl-CoA desaturase (Syahanim et al., 2004) were successfully isolated. Furthermore, as the targeted fatty acid changes were required to occur in the mesocarp of the oil palm fruit, the mesocarp-specific promoter was also successfully isolated (Siti Nor Akmar et al., 2001). Based on the biochemical studies, the following strategy was proposed to increase oleic acid in oil palm: (i) palmitoyl-ACP thioesterase gene activity is required to be down-regulated to reduce the synthesis of palmitic acid, (ii) KAS II activity needs to be up-regulated for efficiently using the higher pool of palmitoyl-ACP substrate and converting it into stearoyl-ACP, (iii) stearoyl-ACP desaturase activity will not be interrupted as it is actively desaturating maximum pool of stearoyl-ACP into olecyl-ACP and finally the activity of oleoyl-CoA desaturase needs to be down-regulated in order to minimize spillover of oleoyl-ACP into linoleoyl-CoA, which subsequently results in an increase in linoleic and linolineic acid content.

Based on the proposed strategy, four sets of transformation vectors were constructed, (i) antisense palmitoyl-ACP thioesterase gene driven by CaMV35S promoter (Abrizah et al., 2000), (ii) antisense palmitoyl-ACP thioesterase and sense KAS II genes driven by mesocarp-specific promoter, (iii) antisense palmitoyl-ACP thioesterase, sense stearoyl-ACP desaturase, and sense KAS II genes driven by mesocarp-specific promoter (Masani and Parveez, 2008); and (iv) antisense palmitoyl-ACP thioesterase, sense stearoyl-ACP desaturase, and antisense oleoyl-CoA desaturase genes driven by a mesocarp-specific promoter. The plasmids were bombarded into oil palm embryogenic cultures, followed by selection of transformants using herbicide Basta. The resistant colonies obtained were later proliferated into polyembryoids, followed by regeneration to produce transgenic plantlets. Currently, plantlets from a number of transformation events have been obtained and transferred onto soil in a fully contained biosafety screenhouse (Fig. 5.2). Initial molecular analysis using PCR revealed that most of the transgenic lines were positive for the selectable marker and palmitoyl-ACP thioesterase genes. The transgenic samples were also subjected to RT-PCR analysis. A number of samples were shown to have a reduced expression level of palmitoyl-ACP thioesterase gene compared with control. The positive samples were later subjected to fatty acid analysis, via gas chromatography, to examine whether there are any fatty acid changes taking place in the early stage of the plant development. This is possible as the first batch of transgenic cultures and plants were transformed with genes driven by constitutive promoter. The introduced gene was expected to increase the oleic acid content and decrease the palmitic acid content. Results of gas chromatography analysis indicated that only a small number of samples tested showed a reduction in palmitic acid and an increment in oleic acid composition as expected. Most of those samples showed reduction in palmitic acid and increase in oleic, linoleic, and linolineic acid contents. Some of the samples did not show any

FIGURE. 5.2 Transgenic oil palm plantlets in a biosafety nursery.

change. It is expected that once the palms are mature and produce fruits, more useful and reliable data could be obtained.

5.3.3 Production of Biodegradable Thermoplastics

Polyhydroxybutyrate (PHB), a type of biodegradable plastic or polyhydroxyalkanoate, is generally produced as a storage monomer by bacterial cells under minimal growth conditions (Senior and Dawes, 1973). PHB is synthesized in bacterial cells through three enzymatic reaction steps starting with the substrate acetyl-CoA being catalyzed by β-ketothiolase, followed by acetoacetyl-CoA reductase, and finally by PHB synthase acetyl-CoA (Anderson and Dawes, 1990) (Fig. 5.3). As acetyl-CoA is abundantly present in many organisms, such as bacteria, fungi, and plants, efforts to genetically engineer the organism for synthesizing polyhydroxyalkanoates prove beneficial. The polymer could be exploited for producing a wide range of environment-friendly industrial polymers. It was reported that production of PHA involving bacterial fermentation is more expensive than the petroleum-based production. Petroleum-based polymer production was estimated to be less than US$1.0/kg compared with around US$2.0 for bacterial fermentation-based production (van Beilen and Poirier, 2008; Philip et al., 2007). Due to the high cost of production using fermentation, production of biodegradable plastics in plants has been proposed as plants could produce millions of tonnes of oils and starch at a low price of around US$ 0.25–1.0/kg (Poirier et al., 1995).

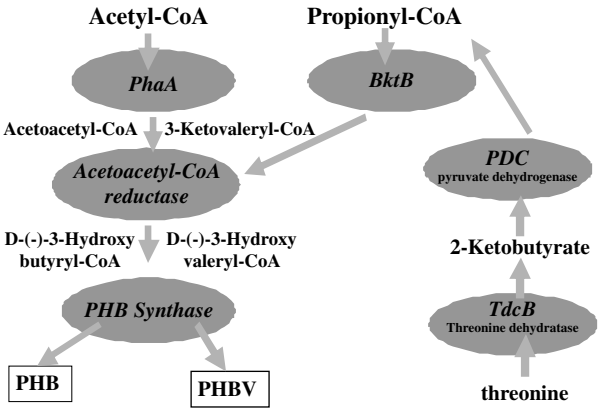

FIGURE. 5.3 Pathway for the biosynthesis of PHB and PHBV in oil palm.

Production of biodegradable plastics in plants was first demonstrated in cytoplasm of *Arabidopsis*; however, the yield was quite low, i.e., only 0.1% of its dry weight (Poirier et al., 1992). Later, when the polymer production was targeted in plastid, where the highest flux of acetyl-CoA is expected to be present, PHB synthesis was increased up to 14% of the dry weight (Nawrath et al., 1994). The PHB granules were all contained in the plastids. The granules were also similar to the ones produced in bacteria cells. When all the three genes were later transformed with a single plasmid, transgenic *Arabidopsis* plants successfully synthesized PHB up to 4% of their fresh weight (Bohmert et al., 2000). Besides PHB, synthesis of a more useful and beneficial copolymer, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) or PHBV, was also demonstrated in rapeseed and *Arabidopsis* (Slater et al., 1999). However, synthesizing monomer PHV requires propionyl-CoA as another substrate (Fig. 5.3). Therefore addition of another gene, either threonine deaminase (ilv) or threonine dehydratase (tdcB), is required to produce the substrate propionyl-CoA from threonine. Furthermore, another family member of β-ketothiolase enzyme (*bktB*) is needed to convert propionyl-CoA to β-ketovaleryl-CoA, which eventually is converted into hydroxyvalerate (PHV) by PHB-synthase. Using the bktB, phbB, phbC, and ilv genes targeted to plastids and driven by a constitutive promoter, Slater et al. (1999) were able to synthesize up to 1.6% of PHBV per dry weight in *Arabidopsis*. On the other hand, the use of a seed-specific promoter and targeting the genes to plastid resulted in up to 2.3% of PHBV synthesized per dry weight in rapeseed.

In this study, production of PHB and PHBV in oil palm was proposed by transforming PHB and PHBV genes into oil palm driven by constitutive promoter, mesocarp-specific promoter, or leaf-specific promoter (Parveez et al., 1999). All genes were targeted to plastid by including an oil palm acyl-carrier protein transit peptide (Rasid et al., 1999) by flanked by a RB7 matrix attachment region of tobacco (Matzke and Matzke, 1991) for stabilizing transgene expression by minimizing homology-dependent gene silencing (Matzke et al., 1989). A threonine dehydratase

(tdcB) (Guillouet et al., 1999) gene from *E. coli* was also used for synthesizing propionyl-CoA as a substrate for PHBV synthesis. Mesocarp is the most excellent target tissue as it is the oil-synthesizing tissue where acetyl-CoA substrate is abundant.

Construction of eight transformation vectors carrying PHB and PHBV genes driven by a combination of constitutive (actin, ubiquitin, and CaMV35S), mesocarp-specific, and leaf-specific promoters has been successfully carried out (Greg et al., 2001; Masani et al., 2008). The constructs were transformed biolistically into oil palm cultures and transformed cells were selected on a medium containing herbicide Basta. Resistant embryogenic calli were later obtained, normally after 5–6 months on selection medium. The resistant calli were proliferated and regenerated further until small plantlets were produced. The plantlets were transferred from the test tubes to soil in polybags and grown in a biosafety screenhouse. Molecular analyses, such as PCR, RT-PCR, and Southern blot, revealed that most of the transgenic plants were positive, demonstrating the integration and expression of the transferred genes (Parveez et al., 2008). The positive samples were later subjected to PHB or PHBV analysis, via HPLC, to determine the accumulation of PHB or PHBV in the plantlets. HPLC results demonstrated the presence of PHB in some of the plants tested. However, the yield is still low and requires more intervention. It is also expected that once the plants are mature, higher amount of biodegradable plastics could be detected in the fruits where the highest pool of acetyl-CoA is present.

5.3.4 High Stearate Transgenic Oil Palm

Oil palm contains an active Δ9 stearoyl-ACP desaturase that effectively desaturates stearoyl-ACP into oleoyl-ACP and finally into oleic acid (Fig. 5.1). Down-regulating the activity of Δ9 stearoyl-ACP desaturase should reduce the conversion of stearic acid to oleic acid. Therefore, by introducing an antisense copy of RNAi of the Δ9-stearoyl-ACP desaturase gene, stearic acid could accumulate in the oil palm. Knutzon et al. (1992) have altered rapeseed oil content through the antisense expression of a stearoyl-ACP desaturase gene to increase the amount of stearic oil from 1.8% up to 39.8%. This increase is due to concomitant reduction of oleic acid in the resulting transgenic plant. Similarly, down-regulation of Δ9-stearoyl-ACP desaturase gene also resulted in an increase of stearic acid content in cottonseed oil from 2% up to 40% (Liu et al., 2000). Down-regulating the activity of stearoyl-ACP desaturase enzyme in the mesocarp of oil palm is expected to result in the accumulation of a higher amount of stearic acid in the oil palm fruits. However, looking at the fatty acid biosynthesis pathway, the large pool of stearoyl-ACP may interfere with the equilibrium of the pathway and result in accumulation of palmitic acid as well. Therefore, manipulation of KAS II and palmitoyl-ACP thioesterase may also be important to be considered. Down-regulating palmitoyl-ACP thioesterase and stearoyl-ACP desaturase as well as overexpressing the KAS II gene may direct all the stearoyl-ACP pool towards stearic acid. All of the genes will be targeted into the mesocarp to ensure that fatty acid changes only take place in the mesocarp where the oil is synthesized. This new variety

of transgenic oil palm with high stearic acid content is expected to give rise to new applications such as cocoa butter substitute and personal care products such as lotions, shaving cream, and rubbing oils (Parveez et al., 1999).

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6

POTENTIAL IN USING *ARABIDOPSIS* ACYL-COENZYME-A-BINDING PROTEINS IN ENGINEERING STRESS-TOLERANT PLANTS

MEE-LEN CHYE, SHI XIAO, QIN-FANG CHEN, AND WEI GAO
School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, China

MEE-LEN CHYE
State (China) Key Laboratory of Agrobiotechnology, Chinese University of Hong Kong, Shatin, Hong Kong

6.1 PLANT LIPID-BINDING PROTEINS

Lipid-binding proteins that have been identified in plants include the lipid transfer proteins (LTPs), puroindolines, and the acyl-CoA-binding proteins (ACBPs)

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(Kader, 1996; Marion et al., 2007; Xiao and Chye, 2009). Seeds that act as a storage organ for lipids accumulate LTPs and puroindolines, both of which are small proteins of about 10 kDa (Marion et al., 2007). In contrast, ACBPs range from 10 to 73 kDa (Table 6.1) and the larger members contain additional domains such as kelch motifs and ankyrin repeats, which mediate protein–protein interactions (Xiao and Chye, 2009).

While puroindolines are confined to seeds of the Triticae and Avenae tribes (Marion et al., 2007), LTPs and ACBPs are ubiquitous proteins and have been reported in monocots and dicots (Kader, 1996; Chye et al., 2000; Guerrero et al., 2006; Marion et al., 2007; Xiao and Chye, 2009). In *A. thaliana*, at least 15 genes were reported to encode LTPs (Arondel et al., 2000) and subsequently >40 genes encoding putative LTPs have been identified (Blein et al., 2002). In contrast, only a mere six genes encode ACBPs in *Arabidopsis* (Table 6.1; Leung et al., 2004). The sequencing of whole plant genomes has recently revealed the presence of gene families encoding LTPs in many higher plants (Yeats and Rose, 2007). Proteins containing acyl-CoA-binding domains are not limited to plants, but extend to other eukaryotes including mammals, *Drosophila*, and yeast (Xiao and Chye, 2009).

These ACBPs are characterized by the presence of an acyl-CoA-binding domain that confers them the ability to bind long-chain acyl-CoA esters (Rasmussen et al., 1993; Chye, 1998; Chye et al., 2000; Leung et al., 2004, 2006; Knudsen et al., 2000; Burton et al., 2005). The 10-kDa rat ACBP was initially identified as a neuropeptide that inhibits the binding of diazepam to synaptic membranes (Guidotti

TABLE 6.1 Summary on the *Arabidopsis* ACBPs

Protein	Gene Name	GenBank Accession Number	M_r (kDa)	Subcellular Localization	References
ACBP1	At5g53470	NM_124726	37.5	PM and ER	Chye (1998); Chye et al. (1999); Li and Chye (2003)
ACBP2	At4g27780	NM_118916	38.5	PM and ER	Chye et al. (2000); Li and Chye (2003)
ACBP3	At4g24230	NM_118556	39.3	Extracellular	Leung et al. (2004, 2006)
ACBP4	At3g05420	NM_202498	73.1	Cytosol	Leung et al. (2004); Xiao et al. (2008b)
ACBP5	At5g27630	NM_122645	71.0	Cytosol	Leung et al. (2004); Xiao et al. (2008b)
ACBP6	At1g31812	NM_102916	10.4	Cytosol	Chen et al. (2008); Engeseth et al. (1996)

Note: PM, plasma membrane; ER, endoplasmic reticulum.

et al., 1983; Shoyab et al., 1986). The function of the 10-kDa ACBPs in mammals and yeast include acyl-CoA transport, maintenance of intracellular acyl-CoA pools, and protection of cytosolic acyl-CoAs from hydrolysis by cellular acyl-CoA hydrolases, as well as the protection of enzymes in lipid metabolism, such as acetyl-CoA carboxylase, acyl-CoA synthetase, and adenylate translocase, from inhibition by long-chain acyl-CoA esters (Faergeman and Knudsen, 1997). The 10-kDa ACBP has also been reported to be associated with long-chain acyl-CoAs in the regulation of lipid metabolism and gene expression, including that of the yeast gene *OLE1* that encodes Δ9-desaturase (Kragelund et al., 1999; McDonough et al., 1992).

In higher plants, the 10-kDa ACBPs were first identified from *Brassica napus* (Hills et al., 1994) and *Arabidopsis thaliana* (Engeseth et al., 1996). Since *de novo* fatty acid biosynthesis predominantly occurs in plastids, plastid-derived fatty acids need to be exported as palmitoyl-CoA (16:0-CoA) and oleoyl-CoA (18:1-CoA) esters to the endoplasmic reticulum (ER) for the biosynthesis of extraplastidial membrane lipids and triacylglycerols (Moreau et al., 1998; Ohlrogge and Browse, 1995). Since there appears to be an extensive exchange of acyl-CoAs (or their derivatives) between the plastids and the ER *via* the plant cytosol, the *Arabidopsis* 10-kDa ACBP was originally proposed to facilitate such transport and maintain a pool of long-chain acyl-CoA esters in the cytosol (Engeseth et al., 1996).

Larger proteins that contain an acyl-CoA-binding domain were first identified in *A. thaliana* (Leung et al., 2004; Xiao and Chye, 2009). Subsequently, they were also characterized in another plant species, *Agave americana* (Guerrero et al., 2006), as well as outside the plant kingdom, in *Cryptosporidium parvum* (Zeng et al., 2006) and *Caenorhabditis elegans* (Larsen et al., 2006). Phylogenetic analysis has shown that *Arabidopsis* ACBP1 and ACBP2, *A. americana* ACBP, and *C. parvum* ACBP1 group into one cluster (Fig. 6.1a). These ACBPs bear resemblance to each other by the presence of an acyl-CoA-binding domain as well as ankyrin repeats (Fig. 6.1b). BLASTP analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated that *A. americana* ACBP showed 60% overall amino acid identity to AtACBP1, with 85% amino acid conservation within the acyl-CoA-binding domain (Guerrero et al., 2006) and 75% amino acid conservation within the ankyrin repeats to AtACBP1 and AtACBP2. *C. parvum* ACBP1 bears 32–33% overall amino acid identity to the full-length sequences of AtACBP1 and AtACBP2, with 34–40% amino acid identity retained within the acyl-CoA-binding domain and 40% identity within the ankyrin repeats. *C. elegans* ACBP displays 37–40% amino acid identity to the acyl-CoA-binding domain of AtACBP1 and AtACBP2. Figure 6.1a further shows that the kelch-motif-containing *Arabidopsis* ACBP4 and ACBP5 are grouped in a different cluster.

The three-dimensional structures of LTPs in both lipid-bound and unbound forms have been solved from several plant species including *Arabidopsis*, rice, wheat, maize, barley, tobacco, and peach (Lerche et al., 1997; Lee et al., 1998; Tassin-Moindrot et al., 2000; Han et al., 2001; Da Silva et al., 2005; Pasquato et al., 2006; Lascombe et al., 2008). These investigations have revealed that LTPs bind lipids in a large hydrophobic cavity while puroindolines are characterized by a

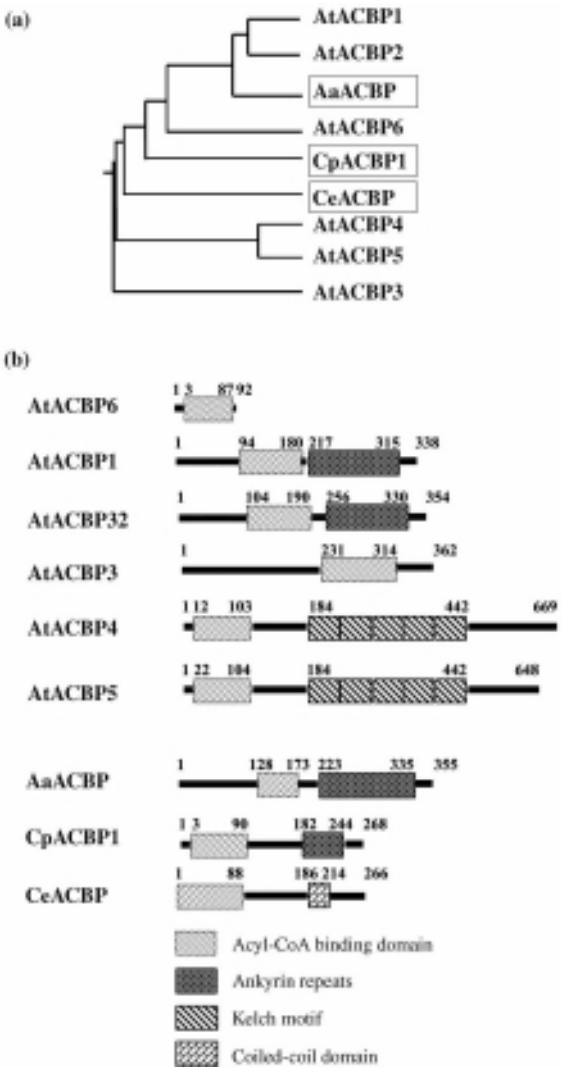

FIGURE. 6.1 Comparison of *Arabidopsis* ACBPs with large ACBPs from other organisms. (a) Phylogeny of large ACBPs and AtACBP6 using evolutionary trace server. At, *Arabidopsis thaliana*; Aa, *Agave americana*; Cp, *Cryptosporidium parvum*; Ce, *Caenorhabditis elegans*. The accession numbers and loci are as follows: AtACBP1 (At5g53470), AtACBP2 (At4g27780), AtACBP3 (At4g24230), AtACBP4 (At3g05420), AtACBP5 (At5g27630), AtACBP6 (At1g31812), AaACBP (AY650903), CpACBP1 (DQ406676), and CeACBP (NM_067130). Non-*Arabidopsis* ACBPs are boxed. (b) Comparison in structure of large ACBPs and the 10-kDa AtACBP6. The numbers indicate the amino acid positions.

unique tryptophan-rich domain that can interact with lipid aggregates (Marion et al., 2007). In the case of ACBPs, the structure of the bovine 10-kDa ACBP in complex with palmitoyl-CoA was first studied; it was observed to contain four α-helices in a bowl shape with an exposed acyl-CoA-binding site (Kragelund et al., 1993). The conserved positive charged residues of this ACBP were found to interact with the phosphate group of the ligand while the acyl chain was positioned between the hydrophobic surfaces of CoA and the protein. Among the 26 highly conserved residues in bovine ACBP, eight hydrophobic amino acids residing between the N- and C-terminal helices were shown to be essential in rate-limiting folding (Kragelund et al., 1999). Investigations on plant lipid-binding proteins have indicated that LTPs and ACBPs can bind acyl-CoA esters and phospholipids (Kader, 1996; Chen et al., 2008; Xiao and Chye, 2009). While only one ACBP is targeted extracellularly (Leung et al., 2006), many LTPs have been demonstrated to be apoplastic (Kader, 1996). Given this observation, ACBPs rather than LTPs are thus more likely involved in the intracellular trafficking of acyl-CoAs and phospholipids.

6.2 PROPOSED BIOLOGICAL ROLES OF PLANT LTPs

LTPs have been reported to be associated with plant development as well as plant defense (Kader, 1996). The mRNAs encoding LTPs are induced by cold stress, wounding, and microbial pathogens, suggesting putative functions that could be related to abiotic and biotic stresses (Molina et al., 1993; Pearce et al., 1998; Yubero-Serrano et al., 2003). LTPs and puroindolines display anti-microbial properties, suggesting their roles in defense against phytopathogens (Kader, 1996; Marion et al., 2007). Molina et al. (1993) demonstrated that LTPs from barley and maize leaves could inhibit the growth of pathogenic fungus (*Fusarium solani*) and bacteria (*Clavibacter michiganesis* subsp. *sepedonicus* and *Pseudomonas solanacearum*). Extracellularly targeted LTPs have also been suggested to provide protection on the cell surface against invaders (Kader, 1996). Furthermore, a putative apoplastic LTP has been proposed to bind a lipid derivative involved in long-distance signaling in systemic acquired resistance in *Arabidopsis* (Maldonado et al., 2002). Cryoprotectin, a specialized LTP homolog that was originally purified from acclimated cabbage leaves, is known to stabilize cell membranes during cold-induced stress despite its loss in lipid-transfer activity (Hincha, 2002).

Transgenic plants expressing LTPs and LTP-like proteins are conferred enhanced resistance to biotic stresses. LTPs have been proposed to bind elicitin receptor sites on the plant plasma membrane and interact with elicitins in defense signaling (Blein et al., 2002). Transgenic rice expressing an LTP from *Allium cepa* showed antimicrobial function and inhibited major rice pathogens such as *Magnaporthe grisea*, *Rhizoctonia solani*, and *Xanthomonas oryzae* (Patkar and Chattoo, 2006). A recombinant wheat LTP (Ltp 3F1) expressed in *Escherichia coli* displayed antifungal activities against *Alternaria*, *Bipolariis oryzae*, *Botrytis cinerea*, *Curvularia lunata*, *Cylindrocladium scoparium*, *R. solani*, and *Sarocladium oryzae*,

and transgenic tobacco lines overexpressing this LTP were better protected from fungal phytopathogens, *Alternaria*, *B. oryzae*, and *C. scoparium* (Kirubakaran et al., 2008).

6.3 *ARABIDOPSIS* ACBPs

In *Arabidopsis*, three (ACBP4, ACBP5, and ACBP6) of the six ACBPs are subcellularly localized in the cytosol (Table 6.1; Leung et al., 2004; Chen et al., 2008; Xiao et al., 2008b; Li et al., 2008). Furthermore, there are two membrane-associated ACBPs, ACBP1 and ACBP2, that are subcellularly localized to the ER and plasma membrane (Table 6.1; Chye et al., 1999; Li and Chye, 2003). The last member, ACBP3, is unique in its apoplastic localization (Table 6.1; Leung et al., 2006). Of these six ACBPs, only ACBP6 has corresponding well-characterized homologs in other eukaryotes (Engeseth et al., 1996; Faergeman and Knudsen, 1997).

Domains such as the ankyrin repeats are present in the ACBP1 and ACBP2 while kelch motifs are found in ACBP4 and ACBP5 (Fig. 6.1b; Leung et al., 2004; Li and Chye, 2004). These domains have been demonstrated to mediate protein–protein interactions in ACBP2 and ACBP4 by using yeast two-hybrid analysis, *in vitro* binding assays, and confocal microscopy of colocalized fluorescent-tagged proteins (Li and Chye, 2004; Li et al., 2008; Gao et al., 2009). Site-directed *in vitro* mutagenesis on His-tagged recombinant proteins has established the function of each acyl-CoA-binding domain in ACBP1 to ACBP5 in binding acyl-CoA esters (Chye et al., 2000; Leung et al., 2004, 2006). Results from *in vitro* binding assays also demonstrated that these recombinant ACBPs bind differentially to various acyl-CoA esters, suggesting their different functions within the plant cell. *In vitro* assays were also used to show that recombinant ACBP6 binds phosphotidylcholine (PC) (Chen et al., 2008).

The expression of several ACBPs in the six-membered ACBP family has been shown to be inducible upon exposure to abiotic and biotic stresses (Xiao and Chye, 2009). The expression of mRNAs encoding ACBP1 and ACBP2 is upregulated by Pb(II) treatment (Xiao et al., 2008a), *ACBP6* mRNA expression is elevated by cold treatment (Chen et al., 2008), and *ACBP4* mRNA expression is induced by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid, methyl jasmonate, and *B. cinerea* infection (Li et al., 2008). Their functions in relation to these various forms of stress have been investigated by using *Arabidopsis* ACBP-overexpression lines as well as by using gene-knockout mutants (Xiao et al., 2008a; Chen et al., 2008; Gao et al., 2009).

6.4 POTENTIAL OF *ARABIDOPSIS* ACBP1 IN PHYTOREMEDIATION

Pb(II) is a nonessential micronutrient and is harmful not only to plants but to animals, especially when it is concentrated and consumed through food chains (Clemens, 2001). So far, very few plant proteins have been identified to bind this toxic

environmental pollutant, and for those that do, they rarely confer both Pb(II) tolerance and Pb(II) accumulation in transgenic plants, the latter being a prerequisite for phytoremediation. Phytoremediation is a process by which plants are used to remove pollutants from the environment (Cunnnigham and Ow, 1996). The plants should be able to transfer the undesirable toxins from the contaminated soil/water via its roots for accumulation in "above-ground" tissues that can be subsequently harvested and safely disposed (Fig. 6.2). Since a previous study had identified the human form of the 10-kDa ACBP to be a molecular target of Pb(II) *in vivo* (Smith et al., 1998), experiments were conducted to address whether this Pb(II)-binding ability is present in plant ACBPs. Such conservation would enable the use of plant ACBPs in Pb(II) phytoremediation. Hence, *Arabidopsis* ACBP6 and the two membrane-associated ACBPs (ACBP1 and ACBP2) were tested and subsequently observed to bind Pb(II) *in vitro* (Xiao et al., 2008a).

Binding assays using *in vitro* translated ^{35}S-methionine labeled ACBP1, ACBP2, and ACBP6 and His-tagged recombinant ACBP1, ACBP2, and ACBP6 showed that ACBP1 and ACBP2 bind Pb(II) better than ACBP6 (Xiao et al., 2008a). Consistently, results from Northern blot analysis revealed that while the expression of *ACBP1* and *ACBP2* was induced following Pb(II) treatment, *ACBP6* expression was not. Further experiments using transgenic *Arabidopsis* ACBP1-overexpressors showed that they were more resistant to Pb(II)-induced stress than wild type and the *acbp1* knockout mutant exhibited increased sensitivity to Pb(II) in the growth medium (Xiao et al., 2008a). Growth of the mutant on Pb(II)-containing medium was restored upon its complementation using a cloned *ACBP1* cDNA (Xiao et al., 2008a). When the Pb(II) content of these plants was measured, the ACBP1-overexpressors were found to accumulate Pb(II) in shoots not roots, thus implying that ACBP1 can be used for phytoremediation (Xiao et al., 2008a). The ability of ACBP1 in accumulating Pb (II) in plant shoots makes it potentially useful in the removal of Pb(II) from contaminated soil. This strategy is cheap as plants photosynthesize, harness energy from the sun, and phytoremediate *in situ*.

6.5 POTENTIAL OF *ARABIDOPSIS* ACBP6 IN ENHANCING FREEZING TOLERANCE

Since the function of ACBP6 did not resemble its human homolog in Pb(II) binding, Chen et al. (2008) initiated investigations on the biological significance of the smallest member in the *Arabidopsis* ACBP family by using an *Arabidopsis acbp6* gene knockout mutant and ACBP6 overexpression lines. Mammalian 10-kDa ACBPs have been shown to bind and transfer acyl-CoA esters intracellularly as well as participate in gene regulation (Mikkelsen and Knudsen, 1987; Black et al., 2000; Petrescu et al., 2003). Evidence of its role in gene regulation arose from observations of its interaction with nuclear factor-4α a transcriptional activator in rat hepatocytes, which regulates genes in lipid and glucose metabolism (Elholm et al., 2000; Petrescu et al., 2003). ACBP6 homologs have been subcellularly localized in the cytosol and nuclei of cultured cells derived from monkey kidney

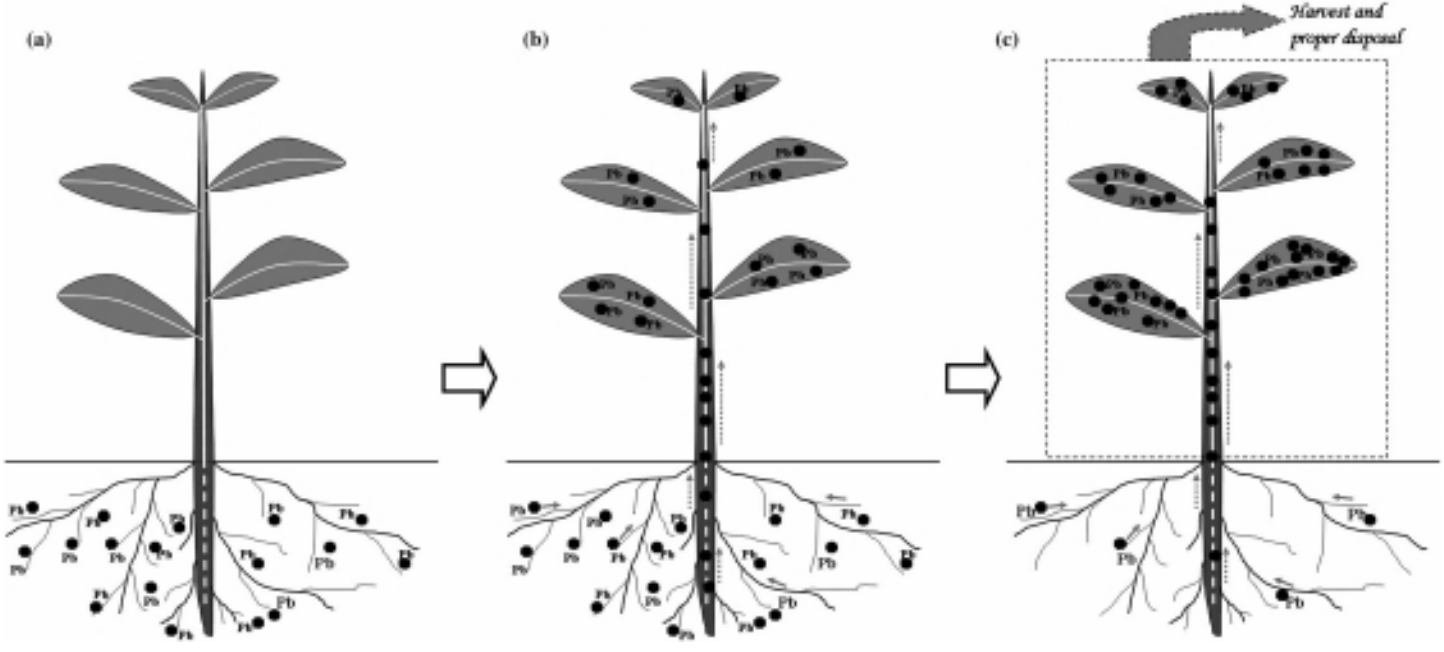

FIGURE. 6.2 Phytoremediation. Transgenic plant growing on soil contaminated with heavy metals, e.g. lead (Pb(II); dots in Fig. 6.2a). Roots take up the heavy metal from the soil (arrows in Fig. 6.2b) and translocate to shoots (broken lines in Fig. 6.2b). Deposition of the heavy metal in shoots for eventual harvest (Fig. 6.2c).

(Helledie et al., 2000) and human liver (Nitz et al., 2005). Chen et al. (2008) showed that ACBP6 is a cytosolic protein in *Arabidopsis* by using green fluorescent protein (GFP) as a protein tag followed by Western blot analysis and confocal microscopy.

Investigations were initiated to address if ACBP6 is associated with abiotic and biotic stresses based on previous results that its homologs are present in phloem exudates in cucumber, pumpkin, and rice (Walz et al., 2004; Suzui et al., 2006), implicating them in long-distance transport that may be stress related. Using Northern blot and Western blot analyses, *ACBP6* mRNA and protein were observed to be induced in *Arabidopsis* following cold treatment at 4°C. Investigations on the *acbp6* mutant, in comparison to wild type, indicated that the mutant was sensitive to freezing treatment at −8°C. In contrast, the overexpression of ACBP6 in transgenic *Arabidopsis* enhanced freezing (−8°C) tolerance, suggesting a role for ACBP6 in mediating freezing stress (Chen et al., 2008).

Further investigations revealed that ACBP6-overexpressors displayed increased expression of the mRNA encoding phospholipase Dδ (PLDδ). Lipid profiling analyses on cold-acclimated freezing-treated transgenic *Arabidopsis* plants overexpressing ACBP6 showed a decline in PC and elevation of PA, in comparison to wild type. Gain in freezing tolerance in ACBP6-overexpressors, with accompanying decreases in PC and accumulation of PA, is consistent with previous findings on PLDδ-overexpressing transgenic *Arabidopsis*. The participation of ACBP6 in the regulation of *PLD*δ expression would be reminiscent of the yeast 10-kDa ACBP that controls genes involved in stress responses. These findings on *Arabidopsis* ACBP6 indicate that it has potential applications in improving freezing tolerance in crop plants in agriculture, which would be advantageous to the expansion of the crop cultivation zones.

Other than those related to ACBP6, there are many independent pathways promoting freezing tolerance (Xin and Browse, 1998; Welti et al., 2002; Li et al., 2004), of which the most well understood is the one mediated by DREB transcriptional activators (Yamaguchi and Shinozaki, 2005, 2006). Chen et al. (2008) indicated that ACBP6-associated freezing tolerance is independent of the induction of cold-regulated cold-responsive gene expression. A better understanding of all these pathways is essential to extend research findings on freezing tolerance to crop improvement. Overcoming freezing tolerance is vital because like other abiotic and biotic factors, freezing limits crop productivity and causes significant losses in agriculture (Vasil, 2002).

6.6 POTENTIAL OF *ARABIDOPSIS* ACBP2 IN COMBATING OXIDATIVE STRESS

ACBP2 consists of several functional domains including an N-terminal membrane-targeting domain, an acyl-CoA-binding domain, and a C-terminal domain of ankyrin repeats (Chye et al., 2000). By using ACBP2-specific antibodies, ACBP2 was localized to the ER and plasma membrane using immunoelectron microscopy, confocal microscopy, and Western blot analysis of subcellular fractions (Chye et al., 1999,

2000; Li and Chye, 2003). When ACBP2–GFP and ACBP1–GFP fusions and their derivatives lacking N-terminal membrane-targeting domains were expressed transiently in onion epidermal cells, it was demonstrated that these N-terminal domains were functional in targeting the proteins to the ER and plasma membrane (Li and Chye, 2003). Single amino acid substitutions in the acyl-CoA-binding domain of ACBP2 were first shown to affect acyl-CoA binding activity, thereby confirming the significance of the acyl-CoA-binding domain in ACBPs (Chye et al., 2000). The C-terminal ankyrin repeats of ACBP2 were predicted to mediate protein–protein interactions and were subsequently confirmed to interact with AtEBP (Li and Chye, 2004) and a heavy-metal-binding farnesylated protein AtFP6 (Gao et al., 2009).

Such interactions of ACBP2 with AtFP6 prompted Gao et al. (2009) to further investigate the relationship between AtFP6, ACBP2, and heavy metals. *In vitro* translated AtFP6 and ACBP2 were generated and were both found to bind Pb(II), Cd(II), and Cu(II) (Gao et al., 2009). Subsequently, ethylenediaminetetraacetic acid was observed to inhibit the binding to various divalent metal ions (Gao et al., 2009). Furthermore, transgenic *Arabidopsis* ACBP2-overexpressors and AtFP6-overexpressors were better tolerant to Cd(II) in the growth media than the wild type (Gao et al., 2009). Lipid peroxidation and intracellular hydrogen peroxide (H_2O_2) accumulates following Cd(II) treatment because Cd(II) depletes antioxidant glutathione and inhibits the activities of antioxidative enzymes (Schützendübel and Polle, 2001). Subsequently, ACBP2-overexpressors were shown to display enhanced tolerance to H_2O_2 treatment, suggesting a possible role for ACBP2 in combating Cd(II)-induced oxidative stress. During peroxidized membrane lipid repair, peroxidized fatty acid residues are selectively removed and replaced by native fatty acids (Nigam and Schewe, 2000). Upon further binding assays, it was observed that recombinant ACBP2, as well as ACBP1, binds [^{14}C]linoleoyl-CoA and [^{14}C]linolenoyl-CoA *in vitro*, suggesting that they are likely involved in phospholipid membrane repair following lipid peroxidation resulting from heavy metal-induced stress (Gao et al., 2009). These findings on *Arabidopsis* ACBP2 indicate its potential use in combating metal-induced/oxidative stress in agriculture.

6.7 CONCLUSIONS AND PERSPECTIVES

Current investigations have revealed that *Arabidopsis* ACBP1, ACBP2, and ACBP6 are implicated in response to abiotic and biotic stresses. For those ACBPs that have been comparatively less well-characterized, there have been indications too that they are stress associated. For example, ACBP4 interacts with protein interactor AtEBP, an *Arabidopsis* ethylene-responsive element binding protein (Li and Chye, 2004), implying that ACBP4 may function in defense *via* ethylene and/or jasmonate signaling, since it is also subject to induction by methyl jasmonate (Li et al., 2008). Also, the upregulation of *ACBP4* mRNA upon *Botrytis* infection (Li et al., 2008) further implicates a role in defense against fungal infection. So far, lipid profile analysis on an *acbp4* knockout mutant and *acbp4*-complemented lines have shown

that while the mutant had decreased levels of membrane lipids including glactolipids and phospholipids, such decreases were restored to wild-type levels in the complemented lines (Xiao et al., 2008b). Further in-depth investigations on ACBP4 will be necessary to establish its function in response to biotic stress. Whether the remaining ACBPs, ACBP3 and ACBP5, are also associated with abiotic and biotic stresses will be an issue for future investigations.

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7

MODIFICATION OF LIPID COMPOSITION BY GENETIC ENGINEERING IN OLEAGINOUS MARINE MICROORGANISM, THRAUSTOCHYTRID

Tsunehiro Aki, Hiroaki Iwasaka, Hirofumi Adachi, Maya Nanko, Hiroko Kawasaki, Seiji Kawamoto, Toshihide Kakizono, and Kazuhisa Ono

Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan

7.1 INTRODUCTION

Continuous and sufficient intake of polyunsaturated fatty acids (PUFA) such as arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid (DHA) is effective for maintenance of human health, especially prevention of lifestyle-related illnesses, such as arteriosclerosis, apoplexy, and cardiopathy, and some types of allergic diseases (Calder, 2008; Colussi et al., 2007). On the other hand, xanthophylls, the lipid-soluble pigments such as astaxanthin and canthaxanthin, with high

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antioxidation activity, are also drawing special attention in the market of health commodities and medical supplies (Hussein et al., 2006). A stable supply of such functional lipids therefore expected. For more than a couple of decades, single-cell oils from some oleaginous microorganisms have been investigated as a practical source of the functional lipids, which can be an alternative to the natural source from marine and freshwater environment and genetically modified plants (Bhosale and Bernstein, 2005; Raghukumar, 2008).

Thraustochytrid is is a group of microheterotrophic eukaryotes belonging to the kingdom Chromista, and plays an important role as a primary decomposer in marine ecosystem (Honda et al., 1999). Its high growth ability, ease of cultivation and storage, and importance in the evolutional lineage can be advantages of an industrial producer as well as a model organism. We have isolated a number of thraustochytrid strains from coastal areas in Japan and Thailand and identified most of those belonging to the genera *Thraustochytrium*, *Schizochytrium*, or *Aurantiochytrium* (Huang et al., 2003; Yongmanitchai and Ward, 1989). Many strains in the latter two genera showed a high productivity of triacylglycerols rich in DHA and *n*-6 docosapentaenoic acid (DPA). Some of the isolates accumulated xanthophylls and also squalene, a biosynthetic intermediate of sterols, in their cells (Aki et al., 2003). Studies on the efficient production of valuable lipids and their utilization in aquaculture industry are ongoing (Yamasaki et al., 2006, 2007). Moreover, we have aimed to consolidate a genetic engineering system that enables us to elucidate the molecular mechanism of the biosynthesis of functional lipids and establish industrially useful mutants through molecular breeding. Here, we describe the modification of lipid composition in thraustochytrid using newly developed transformation system.

7.2 MATERIALS AND METHODS

Aurantiochytrium sp. CB15-5 was cultivated in GPY medium (3% glucose, 0.6% polypeptone, and 0.2% yeast extract) supplemented with 2% sea salts at 28°C for 2 days and used as a gene source and host cell for transformation. For isolation and auxotrophic test of transformant, GPY medium was supplemented with 0.5% sea salts, 50 mM sucrose, 100 μg/ml zeocin, and 0.1% tergitol in the presence or absence of 1 mM DHA. *Escherichia coli* DH5α carrying pUC18-based plasmid was cultivated in LB medium (1% trypton, 0.5% yeast extract, 1% NaCl, and 50 μg/ml ampicillin) and used for gene cloning.

Genomic DNA of *Aurantiochytrium* sp. CB15-5 was prepared as previously described (Huang et al., 2003). Oligo DNA primers for PCR were synthesized to isolate actin promoter region (5′-TCGAGCTCGGTACCCCTTCATACTC TCGCATTTCC-3′ and 5′-TTGGCCATTTTGCTAGTTGGGTGCTTGTTCTT-3′), actin terminator region (5′-AGAGTCGACATTGGAGTGATGGAATGCCC-3′ and 5′-CTTGCATGCTGTTGAAAGAGCTGAGGCCA-3′), bleomycin/phleomycin-resistant gene (5′-CTAGCAAAATGGCCAAGTTGACCAGTGCCGTT-3′ and 5′-CT CTAGAGGATCCCCTCAGTCCTGCTCCTCGGCCA-3′), and PUFA orf-C gene (5′-ATGGCGCTCCGTGTCAAGACGAACAAG-3′ and 5′-TTAGAGCGCGTTGG

TGGGCTCGTAGAC-3′, designed according to the nucleotide sequence of corresponding gene in *Schizochytrium* sp. ATCC 20888; Genbank #AF378329). Reaction mixtures (50 µl) consisted of 100 ng genomic DNA (for actin promoter and terminator and PUFA orf-C) or 20 ng pPhaT1 (for *Sh ble*), 50 pmol each oligo primers, 5 µl 10 × buffer, 0.2 mM dNTP, 1 mM $MgCl_2$, and 2.5 units KOD DNA polymerase (Toyobo). PCR reaction was performed according to thefollowing temperature cycles: 25 cycles of 98°C (15 s), 63°C (2 s), and 74°C (30 s) (for actin promoter); 25 cycles of 98°C (15 s), 68°C (30 s) (for actin terminator and *Sh ble*), or 94°C (5 min); followed by 30 cycles of 94°C (30 s), 55°C (30 s), and 72°C (5.5 min) (for PUFA orf-C). PCR products of actin promoter and *Sh ble* gene were inserted in this order at the *Sma*I site of plasmid pUC18 using in-fusion dry-down PCR cloning kit (BD Biosciences), by which the inserted genes were ligated without restriction site. After an additional insertion of actin terminator gene at 3′-adjacent site of *Sh ble* gene, the plasmid was digested with *Kpn*I and ligated with a DNA fragment (nucleotide numbers 1–1800) of PUFA orf-C gene. The resultant plasmid was linearized by digestion with *Xho*I (nucleotide position 913 of PUFA orf-C gene).

Aurantiochytrium sp. CB15-5 cultivated for 4 days in GPY medium was harvested by centrifugation, washed with BSS buffer (10 mM KCl, 10 mM NaCl, and 3 mM $CaCl_2$), and then washed twice and suspended with 50 mM sucrose. The cell suspension (80 µl) was mixed with 10 µg of the linearized plasmid in a 2-mm gapped cuvette, chilled on ice for 5 min, and pulsed at 500 V, 13 Ω, 50 µF in an electrocell manipulator (ECM600, BTX). The cells were suspended with GPY medium and incubated at 28°C for 1 h and spread on a GPY medium plate containing 100 µg/ml zeocin, 0.1% tergitol, and 1 mM DHA followed by incubation for 3 days.

Total lipid of cultivated cells was extracted with chloroform/methanol mixture (2:1, v/v) and methylesterified with 10% methanolic hydrochloride. Fatty acid methylesters were extracted with hexane and analyzed by gas chromatography (GC-17A, Shimadzu) equipped with a capillary column (TC-70, GL Science) and a flame ionization detector.

7.3 RESULTS AND DISCUSSION

In thraustochytrid, two distinct pathways are involved in the biosynthesis of PUFA (Qiu, 2003). One is a pathway similar to that found in many other eukaryotes, which consists of a series of fatty acid desaturation and elongation reactions and, in addition, a Δ4 desaturation reaction reported in the genus *Thraustochytrium* (Qiu et al., 2001). The other one deduced in the genus *Schizochytrium* utilizes a novel reaction that is similar to that of fatty acid and polyketide biosynthesis (Ratledge, 2004). Complementary DNAs coding for enzymes homologous to those found in eicosapentaenoic acid biosynthesis system in marine bacterium *Shewanella* have been isolated (Metz et al., 2001) and their heterologous expression resulted in the generation of DHA and DPA (Hauvermale et al., 2006). Considering the speculated functions of enzyme domains coded on the genes, PUFA might be synthesized by repetitive reactions

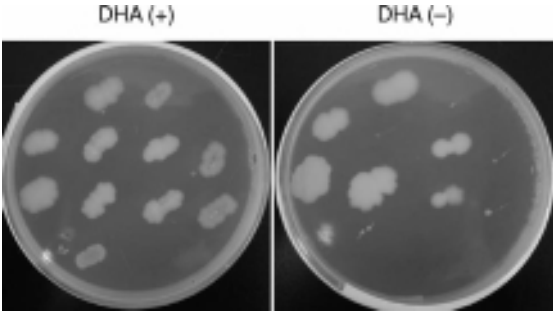

FIGURE 7.1 PUFA biosynthetic gene-disrupted mutants from *Aurantiochytrium* sp. CB15-5 show auxotrophic phenotype. Transformants carrying zeocin-resistant marker gene were inoculated on medium plate containing 100 μg/ml zeocin and 1 mM (left) or 0 mM (right) DHA and cultivated at 28°C for 2 days.

of condensation of substrate acyl chain with C_2 unit and reduction of hydroxyl group. Unlike fatty acid and polyketide biosynthesis, isomerization reactions should participate in forming methylene-interrupted *cis* double bonds that exist in PUFA molecules. Prior to the elucidation of the molecular mechanism underlying the novel enzymatic reaction, we investigated here the effect of disruption of the PUFA biosynthesis gene in *Aurantiochytrium*.

Gene disruption was carried out by the introduction of a plasmid carrying a zeocin-resistant gene marker and a part of the PUFA biosynthesis gene (orf-C) isolated from *Aurantiochytrium* sp. CB15-5 for homologous recombination. Some zeocin-resistant

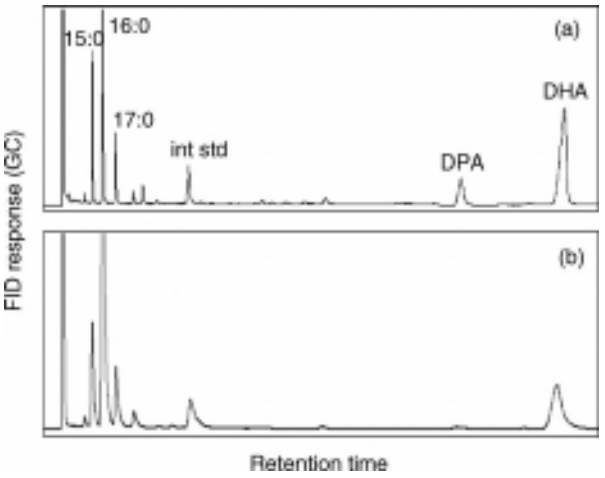

FIGURE 7.2 Fatty acid composition of parental strain (a) and PUFA biosynthetic gene-disrupted mutant (b). *Aurantiochytrium* cells were cultivated in liquid medium containing 1 mM DHA and their cellular lipids were extracted for fatty acid analysis by gas chromatography.

transformants were obtained and then their auxotrophic phenotype was checked for unsaturated fatty acids. As a result, five out of eleven transformants could not grow in the absence of DHA (Fig. 7.1), suggesting the loss of PUFA biosynthetic ability by the targeted gene disruption. In the presence of DHA in the growth medium, control strains contained DHA and DPA in their cellular lipids whereas DPA was not detected in the auxotrophic mutants (Fig. 7.2). Moreover, the mutants were rescued by the addition of other kinds of unsaturated fatty acids such as arachidonic acid and eicosapentaenoic acid (data not shown). These results suggest that the physiological role of PUFA in this organism is to provide the liquidity of lipid membranes for normal growth.

PUFAs synthesized at the early stage of cultivation in thraustochytrid are used as a component of membrane phospholipids and then accumulated in neutral lipids, mainly triacylglycerol, in the latter period (Morita et al., 2006). Since extragenously supplemented unsaturated fatty acids are incorporated in triacylglycerol, the gene-disrupted mutant lacking PUFA biosynthetic ability can be used to generate "structured lipids" carrying desired types of fatty acids. Furthermore, by using the targeted gene disruption technique reported here, the elucidation of unknown functions of PUFA and other biosynthesis systems will be feasible.

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8

INTEGRATED APPROACHES TO MANAGE TOMATO YELLOW LEAF CURL VIRUSES

R.C. DE LA PEÑA, P. KADIRVEL, S. VENKATESAN, L. KENYON, AND J. HUGHES

AVRDC—The World Vegetable Center, P.O. Box 42, Shanhua, Tainan 71499, Taiwan

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8.1 INTRODUCTION

At least 57 different species of viruses within the family Geminiviridae are reported to be capable of causing disease in tomato (*Solanum lycopersicum*), with the majority belonging to the genus *Begomovirus*. Tomato yellow leaf curl virus disease (TYLCVD) has so far been associated with at least 11 species and more than 25 strains of monopartite (old world) begomoviruses (Fauquet et al., 2008), the predominant species being tomato yellow leaf curl virus (TYLCV). The disease is widespread in the tropics and subtropics of Africa, India, and Southeast Asia, and yield losses can reach 100% if young plants are infected. Tomato leaf curl virus disease (ToLCVD) has symptoms that are subtly different from those of TYLCVD; it is distinguished from the latter by being caused by bipartite begomovirus species and by sequence differences in the DNA-A.

Symptoms of TYLCVD vary depending on the growth stage at the time of initial infection, environmental conditions, and the variety of tomato plant, and include severe stunting, marked reduction in leaf size, upward cupping, chlorosis of leaf margins, mottling, flower abscission, and significant yield reduction. The main whitefly vector of TYLCVD is *Bemisia tabaci*, which transmits the viruses in a persistent, circulative manner (Cohen and Nitzany, 1966). There are no reports of seed transmission in tomato, and mechanical transmission does not occur in nature (Moriones and Navas-Castillo, 2000).

Most begomoviruses possess a bipartite genome, composed of two circular single-stranded (ss) DNA molecules: DNA-A (2.6–2.8 kb) and DNA-B (2.6–2.8 kb). The begomoviruses associated with TYLCVD are unusual in that they have a monopartite genome, composed of a single genomic DNA-A, which includes genes essential for viral functions usually carried on DNA-B of the bipartite begomovirus species. The monopartite genome consists of six open reading frames (ORFs). The viral sense ORFs V1 and V2 overlap and encode the capsid protein (CP, 30 kDa) and movement protein (MP, 13 kDa), respectively. The other four ORFs are in the complementary sense and also overlap. C1 encodes the replication-associated protein (Rep, 40 kDa), C2 encodes the transcriptional activator protein (TrAP, 15 kDa), C3 encodes the replication enhancer protein (REn, 16 kDa), while C4 (12 kDa) is embedded as a different reading frame in C1 and may have functions concerned with virus movement and silencing suppression (Gronenborn, 2007). An approximately 300-nt intergenic region (IR) contains the origin of replication, which includes a potential stem-loop structure that is involved in the rolling-circle replication of viral ssDNA. The mode of replication of the virus genome in the plant cells and the relatively frequent occurrence of recombination between different begomovirus genomes makes the begomoviruses one of the most genetically diverse and rapidly evolving plant virus groups.

Methods used to manage TYLCVD depend on the ecology of the type of tomato cropping practice employed. In the open field, planting early in the season before the whitefly population builds up and making new plantings away from sources of infection such as old plantings can be effective. Other methods of reducing the sources of infection and infection pressure on the young tomato seedlings include enforcing

a mandatory vegetable (vector and virus host) crop-free period, destroying old tomato plants quickly after last harvest, practicing good host weed and volunteer tomato management, and early roguing out of infected plants (Polston and Lapidot, 2007). The use of yellow or reflective plastic mulches to disorient whitefly vectors has also been shown to slow the rate of increase in incidence of diseased plants in some locations. In some areas, the only viable means of growing TYLCVD-free tomatoes is through the use of physical barriers such as whitefly-proof screens to exclude whiteflies from the crop. The effectiveness of screenhouses or glasshouses in excluding whiteflies can be improved through the use of UV-absorbing plastic covering films, which interfere with whitefly navigation. Several different classes of pesticides have been used to control the whitefly vector of TYLCVD, although in some areas whiteflies have become resistant to some of these compounds. Currently, the neonicotinoids (e.g. imidacloprid and thiomethoxam) are the most widely used class of compounds, applied as a soil drench to field-grown tomatoes. Chemical control of whiteflies under protected conditions is much less common because of the potential adverse effects on insects introduced for pollination.

Various cultural and chemical approaches for the management of TYLCVD have not been found to be satisfactory (Tripathi and Varma, 2002). Genetic resistance offers the most practical and effective control of TYLCV. Sources of resistance to TYLCV and the whitefly vector are available in the wild and through genetic engineering. Tomato leaf curl virus resistance from the wild tomato species has been used successfully in breeding TYLCV-resistant cultivars. Similarly, whitefly resistance is present in wild species and genomic regions conferring resistance have been identified. The introduction of resistance genes from the wild to cultivated types reduces the disease incidence, but is a long-term process and often brings associated undesirable characters. The use of molecular markers has facilitated the transfer of genes and the ability to pyramid the TYLCV resistance factors. Although the control options are valuable individually, combination and complementation provides more effective control. Furthermore, the use of complementary approaches will offer more durable control of TYLCVD and prolong the effectiveness of a particular control measure.

8.2 HOST-PLANT RESISTANCE TO TYLCV

Developing tomatoes with natural resistance to TYLCV is the most cost-efficient method to control the disease. Effective sources of resistance to TYLCV originated from wild tomato relatives and are currently used in breeding programs to combat the disease. New resistance alleles are present in wild tomato germplasm collections. Crossing *S. lycopersicum* with other related species to introgress resistance genes is common, although some combinations would require techniques such as embryo rescue to obtain F_1 plants. Nevertheless, additional resistance genes are needed to provide a long-term solution to the changes in TYLCV populations.

Breeding programs increasingly rely on molecular markers to select genotypes carrying the resistance genes. Crosses are being made to develop genetic mapping

populations and introgress new genes from the wild tomato species and identify DNA markers for breeding. Developing effective, user-friendly markers is critical to marker-assisted selection (MAS) in improving breeding efficiency. DNA markers associated with TYLCV resistance genes have been developed. These molecular markers are used to select TYLCV-resistant genotypes in breeding programs and allow pyramiding of the TYLCV resistance genes.

8.2.1 Sources of Resistance to TYLCV

The cultivated tomato, *S. lycopersicum*, is extremely susceptible to TYLCV. However, high levels of resistance to TYLCV have been found in the wild species *Solanum pimpinellifolium*, *Solanum peruvianum*, *Solanum chilense*, *Solanum habrochaites*, and *Solanum cheesmaniae*. Several accessions of *S. peruvianum* and *S. pimpinellifolium* also showed good level of tolerance to TYLCV (Pilowsky and Cohen, 1990; Vidavsky et al., 1998; Pérez de Castro et al., 2008). The important sources of resistance to TYLCV found in different wild species are listed in Table 8.1. Among the different wild species showing resistance to TYLCV, *S. chilense*, *S. habrochaites*, and *S. peruvianum* are widely used in tomato breeding programs for developing TYLCV-resistant commercial cultivars (Picó et al., 2002; Lapidot and Friedmann, 2002; Ji et al., 2007a).

8.2.2 Genetics of Resistance to TYLCV

The genetics of resistance to TYLCV has been studied in a number of resistant accessions from breeding lines derived from *S. peruvianum*, *S. pimpinellifolium*, *S. chilense*, *S. habrochaites*, and *S. cheesmaniae* (Table 8.1). In most cases, the sources of resistance to TYLCV appear to be controlled by both major and minor genes. Five genes, namely *Ty-1*, *Ty-2*, *Ty-3*, *Ty-4*, and *Ty-5* have been reported so far. Zamir et al. (1994) reported a partially dominant gene, *Ty-1*, in *S. chilense*, LA1969. Banerjee and Kalloo (1987) reported that resistance to TYLCV in *S. habrochaites* f. *glabratum*, B6013, is controlled by two epistatic genes and further developed breeding lines with high levels of resistance to TYLCV (Kalloo and Banerjee, 1990). Later, Hanson et al. (2000, 2006) identified one of the genes in the breeding line H24 as *Ty-2*. Ji and Scott (2006) reported *Ty-3* in the breeding lines derived from *S. chilense*, LA2779. Ji et al. (2008) reported *Ty-4* in the breeding lines derived from *S. chilense*, LA1932. Recently, Anbinder et al. (2009) reported *Ty-5* in the tomato line *TY172* derived from *S. peruvianum*.

8.2.3 Molecular Mapping and Marker-Assisted Selection of TYLCV Resistance Genes

Zamir et al. (1994) first reported the mapping of *Ty-1* locus on chromosome 6 with the restriction fragment length polymorphic (RFLP) markers TG297 and TG97, with TG97 being the closest marker. The plants homozygous for *S. chilense* allele at TG297 and TG97 were highly resistant and produced no symptoms of the disease. PCR-based

TABLE 8.1 Sources of Resistance to TYLCV in Tomato

Species	Accessions	Breeding Lines Developed	Genetics	Reference
S. pimpinellifolium	LA121		Single major gene, incompletely dominant	Pilowsky and Cohen (1974)
	LA121, LA373		Quantitative, partially recessive	Hassan et al. (1984a) Geneif (1984)
	LA1478, LA1582		Single major gene, dominant	
	Hirsute-INRA, LA1478		Single major gene, dominant	Kasrawi (1989)
	Hirsute		Single major gene	Vidavsky et al. (1998)
	PI407543, PI407544		Single major gene, dominant	Hassan and Abdel-Ati (1999)
	UPV16991	L102	Single major gene, partially recessive	Pérez de Castro et al. (2008)
S. peruvianum	LA372, LA385, LA462, LA1274, LA1333, LA1373, INRA			Kasrawi et al. (1988)
	PI126935	M-60	Polygenic	Pilowsky and Cohen (1990)
	PI127830, PI127831			Muniyappa et al. (1991)
	PI-126944, PE-30			Picó et al. (1998)
	PI126926, PI126930, PI390681, LA441	TY172	Polygenic, partial dominance, *Ty-5*	Friedmann et al. (1998), Lapidot et al. (1997), Lapidot et al. (2000) Anbinder et al. (2009)
	EC104395		Polygenic	Vidavsky et al. (1998)

(*continued*)

TABLE 8.1 (*Continued*)

Species	Accessions	Breeding Lines Developed	Genetics	Reference
	PI126431, PI126929, PI126944, PI126945, PI390677, PI390682, LA378			Pilowsky and Cohen (2000)
	KC-315037, KC-315038, KC-315039, KC-315040, KC-315041			Azizi et al. (2008)
S. chilense	LA1969		Partially dominant gene, *Ty-1* and modifier genes	Zakay et al. (1991), Zamir et al. (1994), Vidavsky et al. (1998)
	PI126944, PI126945, PI127830, PI127831, PI128646			Pilowsky and Cohen (2000)
	LA1932, LA1938, LA1959, LA1960, LA1961, LA1963, LA1968, LA1969, LA1971			Picó et al. (1998), Picó et al. (1999)
	LA2779		*Ty-3*	Ji et al. (2006, 2007b)
	LA1932		*Ty-4*	Ji et al. (2008)
S. habrochaites	LA386, LA1252, LA1295, LA1352, LA1393, LA1624, LA1691			Hassan et al. (1982)
	LA386		More than one gene, dominant	Hassan et al. (1984b)

TABLE 8.1 (*Continued*)

Species	Accessions	Breeding Lines Developed	Genetics	Reference
	LA1777		Major dominant gene	Moustafa (1991)
	PI390658, PI390659, LA386, LA1777			Muniyappa et al. (1991)
	LA1777, LA386	lh902	Polygenic	Vidavsky and Czosnek (1998)
	LA1777		Minor genes	Momotaz et al. (2005)
	B6013	H-2, H-11, H-17, H-23, H24, H-36	Two epistatic genes, *Ty-2*	Banerjee and Kalloo (1987), Kalloo and Banerjee (1990), Hanson et al. (2000, 2006)
		Favi-9	One to two pairs of genes	Mazyad et al. (2007)
S. cheesmaniae	LA1401		Single major gene, recessive	Hassan et al. (1984b)
Not known		468-1-1-12	Single major gene, recessive	García-Cano et al. (2008)

markers for the TG97 locus have been developed at the Hebrew University of Jerusalem, Israel. In addition to *Ty-1*, several other resistance genes have been mapped on chromosome 6 within the proximity of the *Ty-1* region. For example, the *Mi-1* gene, derived from *S. peruvianum* conferring resistance to the root-knot nematode and aphid, is located within about 6 cM near the *Ty-1* locus (Zamir et al., 1994). A severe reduction of genetic recombination has been observed in this introgressed region containing *Mi-1* and *Ty-1* loci in *S. lycopersicum* plant materials. This allows the use of molecular markers linked to *Mi-1* as alternative markers for *Ty-1* (Milo, 2001) if alleles from *S. peruvianum* and *S. chilense* differed for these markers. If the alleles from these two species are the same for these markers, their use could lead to false positive results. Pérez de Castro et al. (2007) reported the

identification of a cleaved amplified polymorphic sequence (CAPS) marker, JB-1, tightly linked to *Ty-1*, which allows selection for *Ty-1* independent of *Mi-1*. The JB-1 marker was derived from a RFLP clone, CT21. It produced three different alleles upon restriction digestion of the PCR product with the *Taq*I enzyme. Allele 1 had a common band of approximately 400 bp, allele 2 consisted of a band slightly larger than 400 bp, and allele 3 had a band of 500 bp. Allele 2 and allele 3 were codominant and dominant over allele 1. Allele 3 detected lines with *Ty-1*, independent of the presence of *Mi-1*.

Hanson et al. (2000) mapped the *Ty-2* locus on chromosome 11 with the RFLP markers TG393 and TG36 using the breeding line H24, derived from *S. habrochaites* as the resistance source (Kalloo and Banerjee, 1990). Currently, several PCR-based markers for the *S. habrochaites* introgression have been developed. A CAPS marker, TG105A, shows robust amplification and restriction enzyme digestion with *Taq*I of the PCR product generates polymorphic bands for *S. habrochaites* and *S. lycopersicum*. Another PCR-based marker, T0302, also was developed for *Ty-2* that requires no restriction digestion. Linkage analysis showed that TG105A and T0302 are closely linked to each other and *Ty-2* is approximately 10 cM from these markers (Ji et al., 2007a).

Agrama and Scott (2006) conducted a quantitative trait loci (QTL) mapping analysis of resistance to TYLCV and tomato mottle virus (ToMoV) in *S. chilense* accessions, LA2779 and LA1932, using RAPD markers. The study revealed that three regions on chromosome 6 contribute to resistance to both TYLCV and ToMoV. The first region includes the *Ty-1* region, whereas the other two regions flank either side of the self-pruning (*sp*) and potato leaf (*c*) loci. RAPD markers linked to resistance in these regions have been identified using advanced breeding lines derived from the accessions LA2779 and LA1932 (Ji and Scott, 2005). Subsequently, Ji et al. (2007b) conducted a detailed mapping of begomovirus resistance and identified a large *S. chilense* introgression spanning markers from C2_At2g39690 to T0834 in LA2779-derived advanced breeding lines resistant to both TYLCV and ToMoV. A begomovirus resistance locus was mapped to the marker interval between cLEG-31-P16 and T1079 on the long arm of chromosome 6, and designated as *Ty-3*. In addition to the *Ty-3* locus, the large introgression also spans the *Ty-1* region near the *Mi* gene, suggesting the possible coexistence and linkage of resistance alleles at both *Ty-1* and *Ty-3* loci. In contrast, the LA1932-derived advanced breeding lines possess a much shorter introgression from cLEG-31-P16 to C2_At5g41480, which also carries a begomovirus resistance locus that is probably allelic at the *Ty-3* locus. Salus et al. (2007) designed PCR primers, FLUW-25F/R and P6-25F/R, from the BAC clone 56B23 (AY678298) that are effectively used for the selection of *Ty-3* locus.

Ji et al. (2008) found that the early breeding lines derived from LA1932 carry an additional introgression approximately 14 cM spanning markers from C2_At1g02140 to TG599 on the long arm of chromosome 3. The new TYLCV resistance locus was designated as *Ty-4*, and is flanked by markers C2_At4g17300 and C2_At5g60610.

Anbinder et al. (2009) mapped a major quantitative trait locus (QTL) and four additional minor QTLs contributing for TYLCV resistance in a breeding line TY172

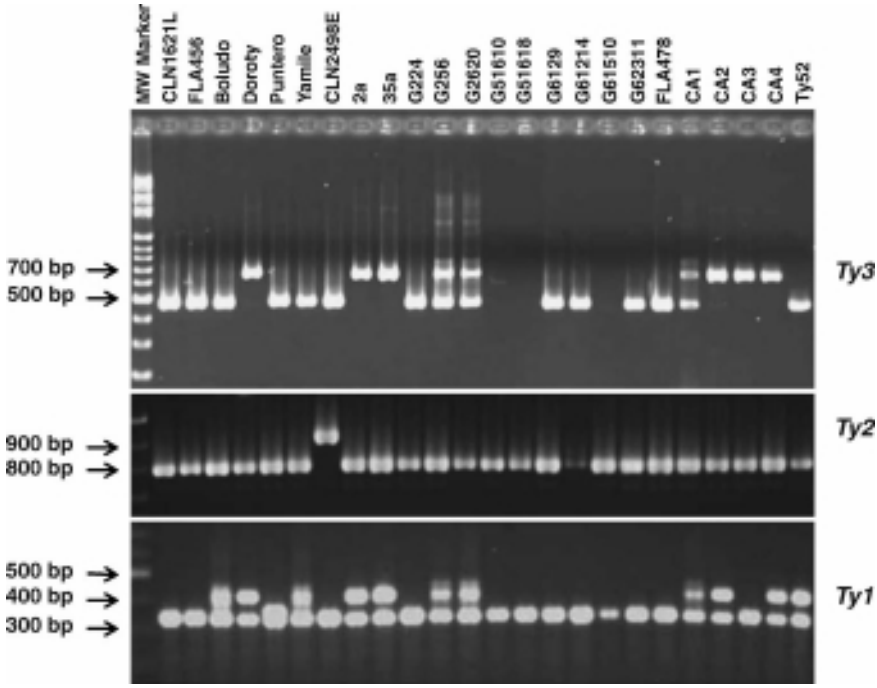

FIGURE 8.1 Marker-assisted selection of TYLCV resistance genes *Ty-1* (JB-1), *Ty-2* (T0302), and *Ty-3* (FLUW-25).

derived from *S. peruvianum*. The major QTL was termed *Ty-5*, mapped to chromosome 4 in the vicinity of *SINAC1* marker and accounted for 39.7-46.6% of the phenotypic variation. The minor QTLs, originated either from the resistant or susceptible parents, were mapped to chromosomes 1, 7, 9 and 11, and contributed 12% to the variation in symptom severity in addition to *Ty-5*. Identification and mapping of genes conferring resistance to TYLCV in *S. pimpinellifolium*, and *S. cheesmaniae* have been attempted, but none have been fine-mapped and no tightly linked markers are available to enable MAS (Chagué et al., 1997; Ji et al., 2007a).

Currently, PCR-based markers for *Ty-1*, *Ty-2*, and *Ty-3* are extensively used for MAS in public and private breeding programs. Figure 8.1 shows screening of JB-1 (*Ty-1*), T0302 (*Ty-2*), and FLUW-25 (*Ty-3*) markers across a set of tomato genotypes. The JB-1 marker produced two alleles, allele 1 (about 300 bp) and allele 2 (about 400 bp). Allele 1 was monomorphic but allele 2 indicated that the tomato lines Boludo, Doroty, Yamile, 2a, 35a, G256, G2620, CA1, CA2, CA4, and Ty52 carry the *Ty-1* locus. The marker T0302 produced a band of about 900 bp size associated with *Ty-2* locus and only CLN2498E carries the *Ty-2* gene among the genotypes tested. The marker FLUW-25 produced a band of about 700 bp size associated with the *Ty-3* locus. The genotypes Doroty, 2a, 35a, CA2, CA3, and CA4 are homozygous for *Ty-3*, whereas G256, G2620, and CA1 are heterozygous for *Ty-3*.

8.2.4 Pyramiding TYLCV Resistance Genes

Considering that resistance to TYLCV is controlled by multiple genes in its natural state, pyramiding of genes from different sources in a common background is an effective way to achieve higher and more durable resistance to TYLCV. Using the interspecific hybrids obtained from crosses between *S. pimpinellifolium* (LA121), *S. peruvianum* (CMV Sel. INRA), and *S. habrochaites* (H2), Kasrawi and Mansour (1994) showed that TYLCV resistance genes from different sources are complementary. Vidavsky et al. (2007) crossed TYLCV-resistant lines that originated from different wild tomato progenitors, *S. chilense*, *S. peruvianum*, *S. pimpinellifolium*, and *S. habrochaites*. All F_1 hybrids resulting from a cross between two resistant parents showed a relatively higher level of resistance, which in most cases was similar to that displayed by the more resistant parent. The combination of classical breeding together with molecular markers linked to the different sources of TYLCV resistance genes will facilitate pyramiding of these genes. Research is ongoing at AVRDC—The World Vegetable Center to stack TYLCV resistance genes, *Ty-1*, *Ty-2* and *Ty-3* to study gene complementation and develop breeding lines with multiple resistance genes using molecular markers (Peter Hanson, Personal communication).

8.3 RESISTANCE TO THE WHITEFLY VECTOR

The whitefly, *B. tabaci* (Hemiptera: Aleyrodidae), is polyphagous, adapted to a wide range of hosts, and distributed throughout the world (Oliveira et al., 2001). Whiteflies acquire the virus through phloem feeding; it is transmitted sexually from insect to insect, as well as to the progeny (Ghanim and Czosnek, 2000). *B. tabaci* and its B biotype, *B. argentifolii*, have contributed to the outbreak of TYLCV in many countries (Moriones and Navas-Castillo, 2000). Resistance to the insect vectors of plant viruses is likely to alter the development and feeding behavior of the vectors, thereby influencing virus acquisition and transmission. Hence, cultivars possessing resistance to both TYLCV and the whitefly could slow down the disease spread and crop damage.

8.3.1 Sources of Resistance to the Whitefly

Cultivated tomatoes and their wild relatives have been studied extensively for whitefly resistance. The highest levels of resistance to *B. tabaci* have been observed in the wild species *S. pennellii*, *S. habrochaites* f. *typicum*, and *S. habrochaites* f. *glabratum* (Gentile et al., 1968; De Ponti et al., 1975; Bas et al., 1992). Resistance to the whitefly in *S. peruvianum* (Channarayappa et al., 1992; Baldin et al., 2005) and *S. lycopersicum* var. *cerasiforme* (Sánchez-Peña et al., 2006) accessions also has been reported. None of the commercially available cultivars are highly resistant; however, considerable differences were observed in their levels of resistance against the whitefly (Curry and Pimentel, 1971; Heinz and Zalom, 1995; Srinivasan and Uthamasamy, 2005) (Table 8.2).

TABLE 8.2 Sources of Resistance to Whitefly, *Bemisia tabaci*, in Tomato

Genotypes	Species	Traits Observed	Reference
PI-127826	*S. habrochaites*	Number of whiteflies	Gentile et al. (1968)
LA716-64L, LA751-61L	*S. pennellii*		
Swift	*S. lycopersicum*	Number of whiteflies	Curry and Pimentel (1971)
LA716	*S. pennellii*	Trichome density, number of eggs	Heinz and Zalom (1995)
LA1353	*S. habrochaites*		
LA1340, LA1674, LA2560	*S. pennellii*	Adult settling (%), adult mortality (%), number of eggs	Muigai et al. (2002)
LA386, LA1353, LA1777, PI127826, PI127827	*S. habrochaites* f. *typicum*		
PI126449	*S. habrochaites* f. *glabratum*		
PI127826	*S. habrochaites*	Zingiberene content, number of whitefly	Freitas et al. (2002)
LA716	*S. pennellii*	Number of trichomes, number of eggs	Toscano et al. (2002)
PI127826, PI127827	*S. habrochaites*		
PI134417	*S. habrochaites* f. *glabratum*		
PI-127826	*S. habrochaites*	Period of development from egg to adults	Baldin et al. (2005)
PI-134417, PI134418	*S. habrochaites* f. *glabratum*		
LA716	*S. pennellii*		
LA444-1	*S. peruvianum*		
PI126931	*S. pimpinellifolium*		
LE231, LE1165	*S. lycopersicum*	Number of trichomes, number of eggs	Srinivasan and Uthamasamy (2005)
Wild populations	*S. lycopersicoum* var. *cerasiforme*	Trichome density, number of whiteflies	Sánchez-Peña et al. (2006)

8.3.2 Mechanism of Resistance to Whitefly

Painter (1951) classified the host-plant resistance mechanisms against insect pests into three categories: *Non-preference* or *antixenosis* (Kogan and Ortman, 1978), a quality that repels or disturbs insects, causing a reduction in colonization or oviposition; *antibiosis*, a quality that reduces insect survival, growth rate, or reproduction following the ingestion of host tissue; and *tolerance*, a capacity to produce a crop of high quality and yield despite insect infestation. Of these, antixenosis- and antibiosis-based resistance against whitefly has been well documented in tomato.

Trichomes present on the leaves and stems are the primary traits responsible for antixenosis- and/or antibiosis-based resistance to whitefly in tomato. The types and density of trichomes influence the preference of whiteflies for oviposition (Snyder and Carter, 1985). Seven types of trichomes occur in tomato, including glandular trichomes (types I, IV, VI, and VII) and nonglandular trichomes (types II, III, and V) (Luckwill, 1943). The wild genotypes of *S. habrochaites* and *S. habrochaites* f. *glabratum* possess type I, IV, VIa, and VIc glandular trichomes, but type VIc trichomes were associated with physical resistance to whitefly infestation and proliferation (Channarayappa et al., 1992). In *S. pennellii*, the type IV glandular trichomes are abundant (Liedl et al., 1995). The glandular trichomes contain several types of secondary compounds, which are either toxic or repellent to the insect pests. Williams et al. (1980) reported that insect resistance in *S. habrochaites* is caused, in part, by the presence of a methyl ketone, 2-tridecanone, found in the exudates of the leaf glandular trichomes. Freitas et al. (2002) reported that type IV and VI trichomes in *S. habrochaites* contribute to the increased zingiberene levels, which leads to higher levels of resistance to the whitefly. In *S. pennellii*, the type IV glandular trichomes produce exudates containing high levels of toxic acylsugars, which play a major role in the resistance against whitefly (Liedl et al., 1995). The commercial cultivars of tomato mostly exhibit nonglandular type III and V trichomes, which are not considered to be important in whitefly control mechanisms (Heinz and Zalom, 1995). Srinivasan and Uthamasamy (2005) found a positive relationship between leaf trichome density and oviposition preference by the whitefly. Higher oviposition occurred on leaves with dense trichomes. Higher pubescence might provide a more suitable microclimate for the oviposition. It is likely that whitefly rejects nonhairy leaved genotypes due to the lack of egg-laying sites. Hence, nonglabrous leaves may be an important criterion for whitefly resistance.

Nombela et al. (2000) reported that the *Mi* gene, introgressed from *S. peruvianum* and conferring resistance to nematodes and aphids, also may be involved in the partial resistance to whitefly shown by the commercial tomato cultivars carrying this gene. This suggests the possibility of alternative mechanisms that are distinct from trichome-mediated resistance to whitefly in tomato.

8.3.3 Genetics of Resistance to Whitefly

The genetics of the traits related to insect resistance in tomato appears to be complex and quantitative. For example, the accumulation of the desired levels of acylsugars for

protection from insects requires the presence of multiple genes. Mutschler et al. (1996) identified five QTLs on chromosomes 2, 3, 4, and 11, associated with acylsugar accumulation using an interspecific F_2 population developed from a cross between *S. lycopersicum* and *S. pennelli* (LA716). Subsequently, these QTLs were transferred into *S. lycopersicum* background through MAS. However, these five QTLs were not sufficient to accumulate the required levels of acylsugars to confer resistance in the progeny (Lawson et al., 1997). Additional QTLs involved in acylsugar production in LA716 were detected using an intraspecific population between LA716 and LA1912 (Blauth et al., 1998). The transfer of these QTLs into *S. lycopersicum* background is in progress (Mutschler and Lobato-Ortiz, 2006).

Similarly, Zamir et al. (1984) and Nienhuis et al. (1987) reported several genomic regions associated with 2-tridecanone content in *S. habrochaites* f. *glabratum*. Maliepaard et al. (1995) reported QTLs associated with trichome densities and resistance to greenhouse whitefly, *Trialeurodes vaporariorum*, using an interspecific population derived from the cross between moneymaker (*S. lycopersicum*) and CGN1.1561 (*S. habrochaites* f. *glabratum*). The QTLs for resistance to the whitefly and trichome densities were different, suggesting that the density of type IV trichomes was not involved in resistance to the whitefly. Freitas et al. (2002) reported that a single major locus controls high zingiberene content in PI-127826 (*S. habrochaites*), which is associated with type IV, VI, and VII trichome densities and resistance to whitefly.

Although high levels of whitefly resistance are present in wild tomato relatives, the trait is controlled by several genes; hence, the transfer of insect resistance into cultivated tomatoes has not been realized. Nevertheless, some level of insect resistance has been introgressed and could play an important role in complementing genes for resistance to TYLCV.

8.4 PATHOGEN-DERIVED RESISTANCE

The use of genetic engineering has successfully demonstrated the development of virus resistance through the production of transgenic plants. The concept of pathogen-derived resistance (PDR) has opened new avenues for the development of virus-resistant plants (Sanford and Johnston, 1985) including resistance to TYLCV. Transgenic tomatoes have been developed to suppress TYLCV using different segments of its own genome through gene-silencing strategies. The use of viral DNA in both sense and antisense orientations has been exploited with mixed results. The double-stranded RNA interference technology is highly efficient when compared to silencing through cosuppression and antisense. The RNA silencing is a host-dependent mechanism triggered against viruses leading to degradation of sequence-specific RNA (Hamilton and Baulcombe, 1999; Li et al., 2002). This type of phenomenon is known as post-transcriptional gene silencing (PTGS) in plants (Napoli et al., 1990), and RNA interference (RNAi) in *Caenorhabditis elegans* (Fire et al., 1998) and *Drosophila melanogaster* (Hammond et al., 2000). The PTGS is exploited against several RNA viruses with great success, since RNA viruses have

dsRNA in their replication cycle, in contrast to DNA viruses whose replication is through DNA intermediates.

8.4.1 Coat Protein-Mediated Resistance

The first transgenic tomato developed by Kunik et al. (1994) against TYLCV was using a coat protein gene of the same virus. An interspecific tomato hybrid, *S. lycopersicum* × *S. pennellii*, was transformed with the TYLCV CP gene (V1) and resistance was evaluated by subjecting plants to viruliferous whiteflies (20 whiteflies/plant) for 48 h. The response of transgenic tomatoes was either susceptible, delayed symptom expression, or recovery from viral infection without manifestation of complete resistance. All plants that recovered from TYLCV infection showed high-level expression of the CP gene. In contrast, transgenic tomato expressing the full length of the TYLCV CP gene showed complete to variable resistance (Raj et al., 2005). In this experiment, 8–10 viruliferous whiteflies/plant were used to evaluate the resistance. The variation in the resistance levels obtained in these experiments could be due to differences in whitefly (inoculum) pressure as well as the genome position and copy number of the transgene in the engineered plants.

8.4.2 Replicase-Mediated Resistance

In geminivirus, the virus encodes a 41-kDa multifunctional protein, which has a role in viral replication (Lazarowitz et al., 1992; Fontes et al., 1992, 1994) and represses its own expression (Sunter et al., 1993; Eagle et al., 1994). Noris et al. (1996) expressed the TYLCV N-terminal 250 amino acids of the Rep protein in both sense and antisense orientation under the control of CaMV 35S promoter in *Nicotiana benthamiana*. The resistance was evaluated by agroinoculation of TYLCV, but no complete resistance was observed. Transgenic progenies from the sense Rep gene performed better than antisense progenies. No symptoms were observed at 4 weeks post inoculation (WPI) but most of the plants showed symptoms at 15 WPI. The expression of the transgene was correlated with substantial reduction of viral DNA. Transgenic tobacco plants expressing the TYLCV C1 antisense RNA were obtained by Bendahmane and Gronenborn (1997) and the resistant lines were symptomless. The replication of the challenged TYLCV was completely suppressed after two generations of testing. Brunetti et al. (1997) used truncated C1 gene of the TYLCV (first 250 amino acids) to develop resistant transgenic tomatoes. Crosses were made between R_0 tomato carrying two loci (A and B; line 47) and accumulating high level of truncated Rep protein either with wild type or transgenic plant carrying antisense C1 gene. Altered/curling phenotype with high level of resistance to TYLCV was observed in the progenies obtained from crosses between the wild type and line 47. Resistant and susceptible phenotypes were observed from plants carrying both sense and antisense transgenes. Other studies have suggested that plants are unable to regenerate if they expresses a sense copy of the complete *Tomato yellow leaf curl Sardinia virus* (TYLCSV) Rep gene and the possible reason is the deleterious effects of the Rep protein on the cell (Bendahmane and Gronenborn, 1997). TYLCSV-resistant

transgenic plants expressing Rep-210 showed resistance to both homologous (TYLCSV) and heterologous (TYLCV-PT) forms of the virus (Lucioli et al., 2003). To reduce the toxicity of the Rep gene, the first 129 amino acids including the Rep-binding domain were used to develop transgenic tomatoes (Antignus et al., 2004). They observed that the transgenic tomatoes were completely resistant to the same virus with whitefly inoculation and variable resistance (immune to susceptible) was observed with agroinoculation. T-Rep gene provides resistance to only homologous virus TYLCV-Sar and not to TYLCV-Au and the related TYLCV-ES (Noris et al., 1996; Brunetti et al., 2001). Yang et al. (2004) expressed eight different constructs in transgenic tomatoes and observed that no TYLCV genomic DNA was detected by both hybridization and PCR amplification in the progenies transformed using constructs containing at least part of the Rep gene and the IR. No viable seeds were produced from plants transformed with the C4 gene, suggesting the toxic nature of the gene. The Rep protein was found unnecessary for resistance and in contrast to the study conducted by Brunetti et al. (1997, 2001). Praveen et al. (2005a, 2005b) developed transgenic tomatoes using the antisense orientation of the untranslatable ToLCV rep gene (1086 bp) with high level of resistance at the R_2 generation. Data from different studies have shown the variability of the effectiveness of transgenic plants transformed with similar silencing elements. The variability could be due to differences in construct used, copy number and genome location of the transgene, intactness of the insert, virus assay, and strain of the virus. Nevertheless, immunity could be achieved and highly resistant transgenic plants developed when sufficient numbers of transgenic events were generated.

8.4.3 Transgenic Plants Expressing Hairpin Vectors

Double-strand RNA molecules can be produced to generate small interfering RNAs (siRNAs), either by creating sense or antisense transgene, self-complementary inverted repeats, or through artificial microRNAs (amiRNA). It has been demonstrated that siRNA molecules can trigger PTGS against a geminivirus infection in the plant cell. Delivery of the synthetic siRNA molecules in tobacco BY2 protoplast inhibits the accumulation of the *African Cassava Mosaic Virus* (ACMV) when ACMV DNA A and DNA B are coinoculated with the synthetic siRNA designated to target the ACMV AC1 gene (Vanitharani et al., 2004). Transient and transgenic approaches are used to exploit the siRNA-mediated resistance against TYLCV.

Fuentes et al. (2006) developed transgenic tomatoes using intron-hairpin RNA constructs encoding 726 nucleotides of the 3′ end of the TYLCV C1 gene (Rep). Transgenic lines harboring a single copy of the construct showed immunity to TYLCV even after exposing 300 to many hundred whiteflies per plant for 60 days. Dot blot hybridization and ELISA revealed no TYLCV accumulation in the transgenic plants and showed that siRNA accumulation was correlated with resistance leading to PTGS. Ramesh et al. (2007) used the siRNA strategies to silence ToLCV AC1 and AC4 genes. To evaluate the resistance to ToLCV, virus-infected tomato plants were used as explants to develop transgenic tomatoes. Eighty to ninety-five percent of the transgenic plants harboring the antisense T-Rep, IR, and

Ihp genes were found to fully recover from ToLCV infection. It was reported that the hairpin RNA-mediated resistance mechanism is due to the prevention of RNAi suppression.

Abhary et al. (2006) constructed hairpin vectors using noncoding conserved regions of the TYLCV-Malaga virus, TYLCV-mild, and TYLCSV-Spain. A broad spectrum of resistance was obtained against different viruses that cause tomato yellow leaf curl disease, and no virus was detected by both PCR and dot blot hybridization. A positive correlation between resistance and the accumulation of TYLCV-specific siRNA was observed. Similarly, Zrachya et al. (2007b) demonstrated that the expression of hairpin vector containing 419 nucleotides of N-terminal TYLCV coat protein gene in both transgenic tomato and transient studies revealed that the resistance was mediated by siRNA and positively correlated with the accumulation of siRNA.

To study the siRNA-mediated silencing studies against ToLCV, Praveen et al. (2007) adopted two approaches. One approach was using an RNAi silencing target sequence from the TYLCV genome, designated via amiRNA, and the other approach was using transgene derived siRNAs. Both micro RNAs (miRNA) and siRNAs are regulated in plant development through transgene- or virus-induced silencing, respectively. The expression of amiRNA, intron spliced RNA, inverted repeat RNA, and short hairpin RNA using constructs developed from the IR, AV1, AC1, AC2, and AC4 showed 13–95% of the infected tomato plants recovered from infection. The best performance was observed in transgenic tomatoes expressing an amiRNA construct (95%).

8.4.4 Mechanism of Resistance

The mechanism of PDR is thought to be the inhibition of viral replication and movement within the plant system for both RNA and DNA viruses, until recently when the PTGS was discovered. TYLCSV DNA replication is inhibited by the binding of the Rep transgene to the viral DNA and blocking of the functional Rep synthesis by challenging the virus through transcriptional repression of the viral C1 gene through an interaction with its upstream recognition sequences (Noris et al., 1996; Antignus et al., 2004). The T-Rep protein acts as a dominant-negative mutant by competing with wild-type Rep for binding to the viral DNA (Brunetti et al., 1997). However, the resistance conferred by TYLCV T-Rep gene is of a protein-mediated type based on the correlation between the level of resistance and the degree of expression of the transgene in the plant tissue and is active at the single-cell level (Noris et al., 1996; Brunetti et al., 1997, 2001). Some plants initially resistant to TYLCSV became infected at a later stage (Brunetti et al., 1997; Noris et al., 1996). Late infections were associated with down-regulation of Rep-210 protein and its mRNA, and collectively suggested that the virus-mediated interference acts at a post-transcriptional level (Lucioli et al., 2003). The system of the Rep-210 amino acids is operated by two different types of mechanisms: (1) the homologous virus resistance is achieved by the ability of the Rep-210 to tightly inhibit the C1 gene transcription (RNA-mediated); (2) the heterologous virus resistance is due to the interacting

property of the Rep-210 oligomerization domain, but the contribution of oligomerization (protein-mediated) is secondary. The resistance can be overcome by the ability of the challenging virus to silence the homologous transgene and the Rep-130 (Lucioli et al., 2003). In contrast to protein-mediated resistance, the RNA-mediated resistance through PTGS blocks the movement of the challenging virus within the plant (Yang et al., 2004; Bendahmane and Gronenborn, 1997). Transient and transgenic expression of T-Rep gene of the TYLCSV revealed the efficient inhibition of the replication of the donor virus but not to the related strain (TYLCV Murcia strain). While the C4 protein does not have any role in virus resistance, the slow migration of the viral DNA was observed due to partial duplexes with incomplete minus strand.

After the discovery of the PTGS-mediated resistance, several studies were conducted to produce siRNA by the introduction of sense and antisense constructs to produce duplex RNA from various regions of the TYLCV. In the PTGS process, longer dsRNAs are cleaved by a host ribonuclease III-like enzyme, termed dicer, into siRNAs of 21–25 nucleotides in length. The resulting siRNA molecules associate with a multiprotein complex known as RNA-induced silencing complex (RSIC) and ultimately target homologous mRNA for degradation based on complementary base pairing (Baulcombe, 2004). Although PTGS lines accumulate low or undetectable amounts of Rep-210 protein and mRNA, and high amounts of siRNAs, the plants were susceptible under high whitefly pressure but resistant under low whitefly pressure. TYLCSV boosted the transgene silencing but it did not help with the recovery of the plant from viral infection (Noris et al., 2004). The dsRNA formed by the transgene antisense RNA with sense viral RNA (challenging virus) ultimately produced siRNA and led to the recovery of ToLCV infection or complete resistance (Yang et al., 2004; Praveen et al., 2005a). Positive correlation between resistance and accumulation of siRNA was observed (Praveen et al., 2005b; Fuentes et al., 2006; Abhary et al., 2006; Zrachya et al., 2007b). The RNA-directed DNA methylation resistance operated where transgenic virus-resistant lines did not accumulate detectable level of siRNA (Pooggin et al., 2003).

To counterattack the RNA silencing defense mechanism, most plant viruses have evolved RNA silencing suppressors (Voinnet et al., 1999). Unlike most virus RNA-mediated resistance that has been described, DNA viruses replicate through DNA intermediates and not through dsRNA, and therefore TYLCV is less susceptible to RNA silencing. The viral C2 and V2 protein of TYLCV-China and TYLCV-Is has been reported as a silencing suppressor, respectively (van Wezel et al., 2003; Dong et al., 2003; Zrachya et al., 2007a). The involvement of silencing suppressors helps to overcome the transgenic plant resistance to TYLCV (Noris et al., 2004).

8.5 INTEGRATED APPROACH TOWARDS STABLE RESISTANCE TO TYLCV

The dynamic changes in TYLCV and the development of pesticide resistance in whitefly populations have made the management of whitefly-transmitted TYLCV difficult in many areas. Successful management of this virus requires multiple

components. Resistant cultivars are the easiest to implement and the most cost-effective; they should be the cornerstone of an integrated TYLCV control program. However, effective breeding for resistance and deployment of resistance genes against TYLCV requires thorough knowledge of the virus. Screening programs have to consider the virus strains used in identifying resistant sources. In parallel, shifts in the virus populations have to be monitored to model the TYLCV management strategy.

Studies on known TYLCV resistance genes shows complementation between genes when they are stacked together; this method frequently is used in breeding programs to develop a horizontal form of resistance to TYLCV. Vidavsky (2007) reported that the most resistant hybrids were those that combined more than one source of resistance, and all sources of resistance appeared to be complementary. The highest level of resistance was achieved by combining the resistant sources derived from *S. peruvianum* and *S. habrochaites*. Complementation amongst *Ty-1*, *Ty-2*, and *Ty-3* resistance genes has been observed and is used in many tomato breeding programs to develop resistance across geographies and multiple strains of the virus.

Resistance to the whitefly vector is present in wild tomato relatives, albeit genetically complex, which makes introgression of the trait challenging. Limited attempts have been made to explore the host resistance to the whitefly while breeding for resistance to TYLCV in tomato. Channarayappa et al. (1992) first reported that some of the wild accessions carry resistance to both whitefly as well as TYLCV (Table 8.3). Momotaz et al. (2005) reported the presence of multiple genes controlling resistance to both whitefly and TYLCV in LA1777 (*S. habrochaites*). The combination of those genes might probably have caused a high level of TYLCV resistance in LA1777. Similarly, Kadirvel et al. (unpublished) observed the complementation of resistances to the whitefly and TYLCV in tomato hybrid genotypes (Table 8.3). LA1940 (*S. pennellii*) showed high level of resistance to both whitefly and TYLCV in the field and greenhouse conditions. When LA1940 was crossed with CLN1621L, a highly susceptible variety, the F_1 was susceptible to both whitefly and TYLCV. When the LA1940 was crossed with CLN2498E, a variety with moderate resistance to TYLCV carrying the *Ty-2* locus, the F_1 showed higher resistance to both whitefly and TYLCV when compared to CLN2498E (Fig. 8.2). Thus, the high level of resistance to TYLCV observed in the F_1 derived from the cross CLN2498E/LA940 could be due to the complementation of whitefly resistance from LA1940 and the *Ty-2* TYLCV resistance gene from CLN2498E. It was also observed that some of the TYLCV-resistant breeding lines such as CA4 and FLA456 were not preferred by the whitefly when compared to susceptible genotypes. Current research on genetics of resistance to insects in tomato provides an opportunity for combining whitefly resistance QTLs with TYLCV resistance genes, which could pave the way for increasing the durability of TYLCV resistance in the field (Mutschler and Lobato-Ortiz, 2006).

The use of genetic engineering has showed that immunity to TYLCV could be achieved. This is a standard that is very difficult and rarely achieved in conventional breeding. However, this type of resistance may impose considerable pressure for

TABLE 8.3 Reaction of Tomato Genotypes for Resistance to TYLCV and its Whitefly Vector, *Bemisia tabaci*

Genotypes	Species	Reaction to Whitefly	Reaction to TYLCV	Reference
PI390658, PI390659	*S. habrochaites*	Resistant	Resistant	Muniyappa et al. (1991)
PI127830	*S. peruvianum*	Resistant	Resistant	
LA386	*S. habrochaites*	Resistant	Resistant	Channarayappa et al. (1992)
LA1777, PI390513	*S. habrochaites*		Resistant	
LA407	*S. habrochaites* f. *glandulosum*		Mildly resistant	
Pusa ruby	*S. lycopersicum*	Susceptible	Susceptible	
LA1272	*S. pennellii*		Susceptible	
LA1407	*S. cheesmaniae*		Susceptible	
LA121	*S. pimpinellifolium*	Moderately resistant	Mildly resistant	
LA375, LA1335, PI19532	*S. pimpinellifolium*		Mildly resistant	
PI127830	*S. peruvianum*		Resistant	
PI126945	*S. peruvianum*		Mildly resistant	
LA385	*S. peruvianum*		Mildly resistant	
LA1954	*S. peruvianum*	Resistant	Moderately resistant	
LA451, LA364, LA366	*S. peruvianum*		Moderately resistant	
LA458	*S. chilense*		Susceptible	
LA1028, LA1306	*S. chimeleski*		Moderately resistant	
LA1326	*S. parviflorum*		Susceptible	
LA364	*S. parviflorum* f. *glandulosum*		Moderately resistant	
CLN1621L	*S. lycopersicum*	Highly susceptible	Highly susceptible	Kadirvel et al., unpublished
CLN2498E	*S. lycopersicum*	Susceptible	Moderately resistant	
CA4	*S. lycopersicum*	Moderately resistant	Resistant	
FLA456	*S. lycopersicum*	Moderately resistant	Resistant	
LA1940	*S. pennellii*	Highly resistant	Resistant	
CLN1621L × LA1940 (F1)	*S. lycopersicum* × *S. pennellii hybrid*	Susceptible	Susceptible	
CLN2498E × LA1940 (F1)	*S. lycopersicum* × *S. pennellii hybrid*	Moderately resistant	Resistant	

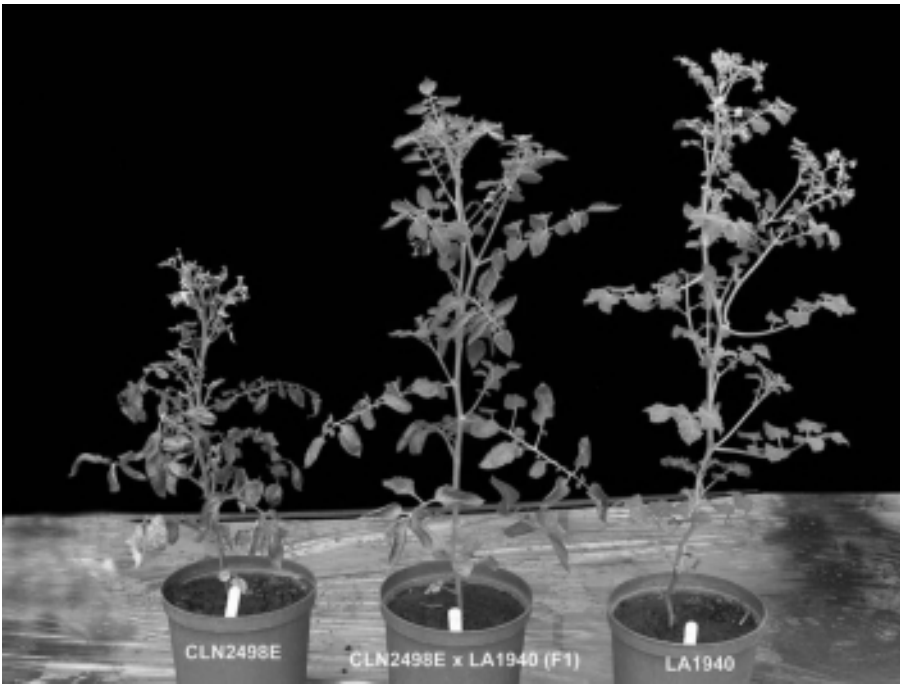

FIGURE 8.2 Reaction of *S. lycopersicum* (CLN2498E), *S. pennellii* (LA1940), and its F_1 hybrid (CLN2498E $\times$ LA1940) to TYLCV infection.

the virus to evolve and overcome the resistance. Although, there are transgenic approaches to achieve resistance against multiple virus strains, combining conventional and transgenic resistances has not been explored and may provide a more effective and stable resistance to TYLCV. Similarly, transgenic solutions to control the whitefly vector could complement the TYLCV resistance genes, or vice versa, in providing an effective and durable solution to TYLCV epidemics.

8.6 CONCLUSIONS

Tomato yellow leaf curl is a devastating disease and a major production constraint worldwide. Control measures are available; however, no single method provides an effectively stable solution to the TYLCV problem. Resistant varieties are currently the most effective way to reduce damage from TYLCV. Sources of resistance are available in the wild tomato species; resistance genes to TYLCV identified to date are introgressions from wild tomatoes. Breeding for resistance is confounded by the diversity of TYLCV and its whitefly vector such that individual resistance genes may be easily overcome by the virus. For example, *Ty-2* under moderate to heavy disease pressure does not provide resistance to TYLCV in Taiwan, compared to previous years in which it effectively controlled the virus. Pyramiding genes for resistance to

TYLCV increases the efficacy of control and broadens the effectiveness of resistance against multiple virus strains. Molecular markers linked to different sources of TYLCV resistance have been developed and have facilitated pyramiding of resistance genes against TYLCV. This approach has provided tomato breeding programs with a tool to effectively manage TYLCV. In addition, resistance to the whitefly vector is available, which on its own may not provide effective control of TYLCV, but may enhance the effectiveness and durability of the TYLCV resistance genes. The use of genetic engineering provides remarkable promise in both TYLCV and whitefly control. If public concern regarding genetically engineered vegetables is not addressed, this potential will not be realized in the near future. Other methods such as chemical control and manipulation of cropping practices could provide effective control of the whitefly vector and thereby reduce the risk of TYLCV infection. Some options available in combating TYLCV damage may not be highly effective on their own, but in combination, these complementary measures would provide highly effective, durable management of TYLCV epidemics.

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9

CARBOHYDRATE ACQUISITION DURING LEGUME SEED DEVELOPMENT

JOCELYN A. OZGA AND DENNIS M. REINECKE

Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada

PANKAJ K. BHOWMIK

National Research Council-Plant Biology Institute Saskatoon, Saskatchewan,Canada

9.1 INTRODUCTION: LEGUME SEED CROPS

Pea (*Pisum sativum* L.) is an important agricultural legume (pulse) seed crop for human and animal consumption. Pulses are rich, not only in protein and starch, but also in other nutrients such as fiber, vitamins, and minerals (Wang et al., 2008), which make pulses well suited to meet the demands of health-conscious consumers. Among a growing list of health-beneficial effects derived from pulse consumption (Leterme, 2002), the low glycemic index of pulses (Foster-Powell et al., 2002) can

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help control blood glucose and insulin levels and ameliorate some complications of diabetes (Rizkalla et al., 2002). Once referred to as "poor man's meat" because they are high in protein and inexpensive, pulses are valuable additions to a modern diet because of their good taste, convenience, ease of use, and nutritional role in managing and preventing diabetes (Leterme, 2002). By further understanding the mechanisms of nutrient transport and storage during pea seed development, targeted manipulation of specific aspects of these processes may lead to desirable changes in the seed nutrient profile including starch and protein content for human consumption and/or for industrial uses (Wang et al., 2003).

9.2 NUTRIENT PATHWAY FROM SEED COAT TO EMBRYO IN LEGUMES

In pea, sucrose is the principal photosynthate sugar that is transferred between cells and through vascular tissues to sink tissues (Smith and Denyer, 1992). The pea embryo, a strong terminal sink for sucrose, is not vascularly connected to the maternal seed coat tissue (Hardham, 1976). The seed coat sieve elements unload sucrose and other nutrients symplastically into the seed coat parenchyma cells; subsequently sucrose is unloaded into the endospermal apoplastic space between the seed coat and the developing embryo (Thorne, 1985; Van Dongen et al., 2003). As the embryo develops, the epidermal cells of the cotyledon play a key role in uptake of sugars and other nutrients from the endospermal space for growth and storage in the parenchyma cells of the cotyledons (Tegeder et al., 1999).

9.3 ROLE OF INVERTASES IN SUCROSE METABOLISM DURING LEGUME SEED DEVELOPMENT

Invertases (β-fructofuranosidase; EC 3.2.1.26) catalyze the irreversible hydrolysis of sucrose into its constituent monosaccharides fructose and glucose. Based on optimal pH requirements and subcellular localization, invertases have been classified into three isoforms (Sturm, 1999; Roitsch and González, 2004). Soluble-acid invertases are present in vacuoles, neutral or alkaline invertases occur in the cytoplasm, and insoluble-acid invertases are localized to the apoplast, usually ionically bound to the cell wall (cell wall invertases; CWI). CWI is active in growing zones and expanding sink tissues and facilitates assimilate unloading by increasing the concentration gradient of sucrose (Sturm, 1999; Roitsch and González, 2004). The role of CWIs in apoplastic cleavage of sucrose, and establishment and maintenance of sink metabolism has been studied and reported in different plant species, including faba bean (Weber et al., 1996), tomato (Godt and Roitsch, 1997), rice (Cho et al., 2005), and *Arabidopsis* (Sherson et al., 2003). A number of transgenic studies have also strengthened a correlation between altered CWI activity and the modification of sink strength in transgenic plants. Transgenic overexpression of CWI in potato (Sonnewald et al., 1997), CWI antisense repression in carrot (Tang et al., 1999),

and ectopic expression of a yeast-derived invertase targeted to the apoplast in the legume *Vicia narbonensis* L.(Neubohn et al., 2000) have confirmed that apoplastic invertases (CWI) play a crucial role in early differentiation and development of sink organs.

In the legume *Vicia faba*, CWI is expressed in the sugar unloading area of the seed coat, cleaving incoming sucrose within the apoplastic space separating the seed coat and embryonic tissues (Weber et al., 1995). During early seed development, seed-coat-associated CWI cleaves sucrose after it is unloaded from seed coat cells, thereby generating a high hexose environment surrounding the embryo. Subsequently, invertase activity declines in the seed coat, hexose content decreases (concomitant with an increase in sucrose content) in the surrounding embryo environment, and cotyledonary differentiation into a storage organ is initiated, including starch and storage protein accumulation. Generally, invertase activity predominates during early seed development, and sucrose synthase activity (a glycosyl transferase that converts sucrose into UDP-glucose and fructose) during seed storage and maturation (Sturm and Tang, 1999; Weber et al., 1995). Persistence of invertase activity at certain stages can delay embryo maturation (Weber et al., 1996; Silva and Ricardo, 1992). Somatic embryogenesis in carrot tissue cultures was characterized by an increase in alkaline invertase and a decrease in the acid invertase (Silva and Ricardo, 1992). Nonembryogenic cell lines, on the other hand, maintained very high acid-invertase activities. In *V. faba*, high hexose conditions were correlated with an extended mitotic activity in a large-seeded genotype when compared to a small-seeded genotype (Weber et al., 1996). In both the large- and small-seeded genotypes, when the hexose to sucrose ratio decreased, differentiation and storage activities were initiated in the cotyledons. Furthermore, sucrose feeding to mitotically active *V. faba* cotyledons *in vitro* induced differentiation (elongation of cells, enlargement of nuclei, appearance of starch grains and storage protein bodies) whereas a high ratio of hexoses to sucrose maintained mitotic activity in this tissue (Weber et al., 1996). It was concluded that the development of the embryo is coordinately regulated with that of the seed coat through metabolic signals that include the ratio of hexoses to sucrose.

9.4 PEA SEED COAT MORPHOLOGY AND SITE OF SUCROSE UNLOADING

The developing pea seed coat consists of distinct cellular layers (Van Dongen et al., 2003). The outermost cellular layer consists of epidermal cells that will elongate and differentiate into macrosclereids. A single layer of hypodermal cells is located immediately below the epidermis. The remainder of the seed coat tissue is made up of parenchyma cells that can be further classified into three distinct types: chlorenchyma, ground parenchyma, and branched parenchyma (Van Dongen et al., 2003).

The chlorenchyma cells contain a greater amount of chloroplasts compared to the other parenchyma cells in the seed coat. An important function of the chloroplasts in the seed coat appears to be the transient accumulation of starch during seed

development (Rochat and Boutin, 1992). The ground parenchyma cells are the most abundant of the parenchymous cell types in the seed coat. They appear similar to the chlorenchyma cells, but contain fewer chloroplasts. The innermost layer consists of the branched parenchyma (small irregularly shaped cells with extensive intercellular spaces). As the cotyledons of the embryo expand, the branched parenchyma and the innermost layers of the ground parenchyma of the seed coat are compressed and they deteriorate. The cell wall remnants of these tissues form a boundary layer between the functioning seed coat parenchyma cells and the cotyledons (Van Dongen et al., 2003).

The vascularly transported nutrients enter the seed through a single vascular bundle in the funiculus (connects the seed coat to the ovary wall) that continues into the seed coat as the chalazal vein and its two lateral branches (Hardham, 1976; Van Dongen et al., 2003). The chalazal vein consists of a central xylem strand surrounded by phloem elements and it extends approximately three-quarters around the seed circumference. The two shorter lateral branches contain only phloem tissue and run parallel to the radicle of the embryo (Hardham, 1976). Phloem-imported sucrose moves symplasmically from the seed coat vascular tissue (phloem of the chalazal vein and of the lateral branches) into the adjacent seed coat parenchyma cells before release into the apoplast (Fig. 9.1, seed coat) (Patrick and Offler, 1995; Patrick, 1997). Results from phloem-mobile fluorescent tracer experiments suggest that the site of phloem unloading in *P. sativum* is the ground parenchyma/chlorenchyma cells of the seed coat (Van Dongen et al., 2003). The branched parenchyma layer is probably not involved in sucrose unloading into the seed coat in

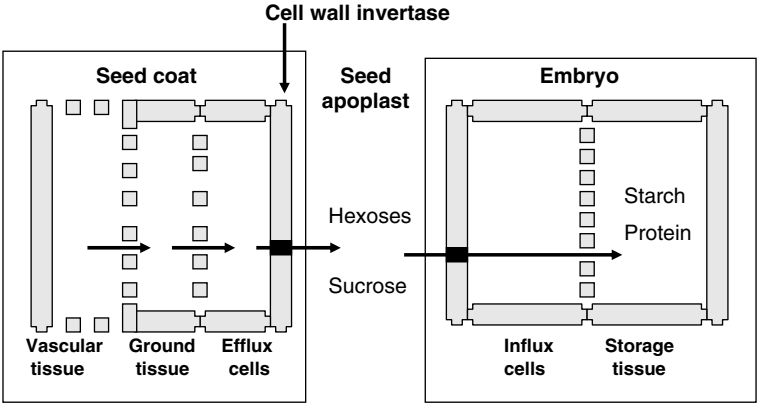

FIGURE 9.1 Symplasmic discontinuity between the seed coat (maternal) and embryo (filial) tissues in legume seeds necessitates solute efflux from the seed coat (represented by the black box in the innermost cell layer of the seed coat) and uptake by the embryo tissues (represented by the black box in the outermost cell layer of the cotyledons of the embryo). The activity of cell-wall invertases in the innermost layers of the seed coat parenchyma cells regulates the ratio of hexoses to sucrose in the vacuolated endospermal space (seed apoplast) of the seed.

the legume species *P. sativum* and *Phaseolus vulgaris* (Patrick et al., 1995; Van Dongen et al., 2003). Weber et al. (1997) have suggested that during the prestorage phase of seed development, CWI is specifically expressed in the outermost layer of *V. faba* seed coat parenchyma cells (likely homologous to the branched parenchyma of *Pisum*; Van Dongen et al., 2003). Furthermore, Weber et al. (1997) proposed that the developmentally regulated degradation of this layer (with loss of CWI activity) initiates the storage phase of the embryo through a switch from high to low ratios of hexoses to sucrose in the environment surrounding the embryo (vacuolated endospermic space; Fig. 9.1).

The importance of the seed coat in legume seed nutrient acquisition is highlighted in the studies with the pea seed mutant *E2748*. In this mutant line, the epidermal cells of the developing cotyledons do not take on transfer cell morphology (Borisjuk et al., 2002); instead they become enlarged, vacuolated, and tightly associated with adjacent seed tissues, indicating that these cells do not functional normally in sucrose uptake. Seed coat growth as well as composition, concentration, and dynamics of sugars within the endospermal vacuole were unchanged in line *E2748*, suggesting that seed coat growth is independent of that of the embryo and dependent on the maternal genotype. Furthermore, the seed coat appears to modulate both concentration and composition of sugars within the endospermal vacuole, regardless of normal embryo growth. This observation has significant implications for seed development, confirming the importance of the maternally derived seed coat tissue in regulating seed size (Hedley and Ambrose, 1980; Weber et al., 1996; Borisjuk et al., 2002).

9.5 EMBRYO ACQUISITION OF SUGARS

During the linear phase of storage acquisition in the pea embryo, the cotyledonary epidermal transfer cells mainly function in the uptake of sucrose. Intercellular movement of the symplasmic tracer 5-(6)-carboxyfluorescein suggests that symplasmic pathways interconnect the epidermal transfer cells to storage parenchyma cells (Tegeder et al., 1999). Sucrose/H + symporters located in the cotyledonary epidermal transfer cells are suggested to play a central role in acquisition of sucrose from the seed apoplast (Fig. 9.1, embryo) (Tegeder et al., 1999). Sucrose imported into the cotyledons of the embryo is subsequently converted to starch, a major seed reserve for subsequent seed germination. Sucrose is converted to starch during mid to late pea seed development by the coordinate regulation of starch synthesizing enzymes including ADP-glucose pyrophosphorylase, starch synthase, and starch-branching enzymes (Smith and Denyer, 1992; Smith et al., 1997).

9.6 MODIFICATION OF NUTRIENT PATHWAY DURING PEA SEED DEVELOPMENT

Agrobacterium tumefaciens-mediated transformation of pea has been demonstrated to be technically feasible (Schroeder et al., 1993; Grant et al., 1995; Bean et al., 1997),

which is an important prerequisite for metabolic engineering of sucrose metabolic pathways to alter partitioning of assimilates between the seed coat and the embryo and different storage products within the embryo. We have developed transgenic pea plants with a pea CWI gene (*PsInv-1*; Zhang et al., 1997) driven by a seed-coat-specific promoter. Preliminary data suggests that developing seeds of these transgenic lines have increased gene expression and activity of CWI, and decreased levels of sucrose concomitant with increased levels of glucose (Reinecke, Bhowmik, and Ozga, unpublished data). We will use these CWI-modified lines to determine the role of this enzyme in modulating sink potential and carbohydrate status in the seed. As the ratio of hexose to sucrose in the developing embryo environment is a key regulator of starch synthesis in legume seeds, it was hypothesized that modulation of the hexose/sucrose ratio after initiation of the starch accumulation phase may lead to production of different types of starch, variation in starch granule size, and the ratio of starch to protein in the mature seed, which are all important parameters for certain food and nonfood applications.

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10

BIOTECHNOLOGY ENHANCEMENT OF PHYTOSTEROL BIOSYNTHESIS IN SEED OILS

QILIN CHEN AND JITAO ZOU

National Research Council Canada-Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, Saskatchewan, Canada, S7N 0W9

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10.1 INTRODUCTION

Phytosterols have diverse and essential functions in plants. As integral components of the membrane lipid bilayer, they interact with glycerol lipids and sphingolipids to regulate membrane fluidity and permeability (Demel and De Kruyff, 1976; Schuler et al., 1991). Their physiological functions have been extensively reviewed by Clouse (2002) and Schaller (2003; 2004). In addition to their crucial role in plant development, phytosterols are considered important neutraceutical and pharmaceutical ingredients to human health. Phytosterols are structurally similar to cholesterol in their tetracyclic backbone skeletons but different from cholesterol in their side chains, and as food ingredients competitively inhibit intestinal absorption and also enhance fecal excretion of cholesterol (Moghadasian and Frohlich, 1999). It has been comprehensively reviewed that phytosterols are pharmaceutical bioproducts highly efficient in lowering LDL-cholesterol level while not affecting HDL-cholesterol and triglycerides levels, consequently reducing the risk of hypercholesterolemia (Katan et al., 2003; Calpe-Berdiel et al., 2009; Jones and AbuMweis, 2009). As an example, Sierksma et al. (1999) reported that administration of two tablespoon of spreads fortified with free sterol in mainly soybean oil or shea nut oil reduced total plasma cholesterol and LDL-C levels by 3.8 and 6%, respectively. Numerous companies have developed diversified products for the benefit of hypercholesterolemia subjects. Most of these products are mixtures of extracted phytosterols and/or phytostanols in the form of capsules, spreads, yoghurts, milk, margarine, cream cheese, pasta, and so on. The US Food and Drug Administration (FDA) issued a guideline in 2000 followed by a revision in 2008, which allows a health claim for foods that containing at least 0.65 g per serving of plant sterol esters (0.4 g of plant sterols), eaten twice a day with meals for a daily intake of at least 1.3 g of plant sterol esters (0.8 g of sterols) as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease. The American National Cholesterol Education Program Expert Panel (Adult Treatment Panel III) recommends phytosterol-enriched functional foods as part of an optimal dietetic prevention strategy in primary and secondary prevention of cardiovascular diseases (Expert panel on detection, evaluation, and treatment of high blood cholesterol in adults, 2001). The American Heart Association (AHA) sees phytosterols as a therapeutic optionfor individuals with elevated cholesterol levels (Lichtenstein et al., 2006). Other well-esteemed health-care professional societies, such as the Spanish Cardiology Society, the Association of Clinical and Public Health Nutritionists in Finland, and the National Heart Foundation in Australia, also recognize phytosterols as an important additional dietary option battling hypercholesterolemia. Such authoritative recommendations led to approval of phytosterols for use in foods in a number of countries. It was estimated that in 2005 3 billion US dollars were spent worldwide on various functional foods that have approved health claims for the management of elevated cholesterol levels. Phytosterols have also been suggested to possess other health-promoting effects including anticancer (Woyengo et al., 2009) and antioxidant activities. Phytosterols were reported to be toxic to breast cancer cells (Awad et al., 2000a, 2000b; Awad and Fink, 2000), colon cancer cells (Awad et al., 1998; Rao and Janezic, 1992), and prostate cancer

cells (Gregg and Fred, 2001). Δ5-avenasterol was demonstrated to have valuable antioxidant activity (White and Armstrong, 1986), likely due to the presence of an ethylidene group (Tian and White, 1994; Kochler, 2000; Silkeberg and Kochhar, 2000).

Commercial phytosterols are isolated from vegetable oils such as soybean oil, rapeseed or canola oil, sunflower oil, corn oil, and even tall oil, a by-product of the Kraft process of wood pulp manufacture. Due to poor solubility and bioavailability of free phytosterols, various doses of phytosterols have been reported for efficacy. Seed oils are among the best natural sources of phytosterols. Increasing phytosterol level in edible seed oil can lead to direct use of seed oil as food supplement, health-promoting agent, or therapeutic dietary option. Towards this end, a great deal of efforts in genetic engineering has been made to overproduce phytosterol in plant products, particularly in seed oil. This chapter will focus on the prospect of biotechnology in improving phytosterol content and composition in seed oil, with particular attention on the potential of an *Arabidopsis* sterol *O*-acyltransferase (SAT) gene, which was shown to be an efficient molecular tool for phytosterol ester overproduction in seed oils.

10.2 OCCURRENCE AND LEVELS OF PHYTOSTEROL IN SEED OILS

10.2.1 Phytosterol Structural Diversity

The major metabolic steps of phytosterol biosynthesis pathway leading to the production of diversified phytosterol molecules in plant tissues have been well defined, the details of which can be found in an excellent review by Benveniste (2004) and publications cited therein (Nakashima et al., 1995; Diener et al., 2000). To date, more than 250 different phytosterols have been identified in plants and marine organisms (Akihisa et al., 1991). These phytosterols can be conveniently divided into three classes, namely, 4-demethylsterols, 4-monomethylsterols, and 4,4-dimethylsterols, based on the number of carbons in the methyl groups on C-4. To pinpoint the origin of the diversity of methyl group, the biosynthesis of phytosterols can be divided into four sequential stages that are outlined as follow: (1) 2,3-oxidosqualene syntheses via mevalonate pathway, which is evolutionarily similar to sterol synthesis in yeast, plant, and mammalian cells; (2) 4,4-methylsterol formation, through which 2,3-oxidosqualene is first cyclized into the first plant-specific tetracyclic intermediate, cycloartenol, then converted into 24-methylenecycloartenol catalyzed by C-24-methytransferase. The conversion of 2,3-oxidosqualene is catalyzed by cycloartenol synthase, an equivalent of lanosterol synthase in yeast and mammals which cyclizes 2,3-oxidosqualene into lanosterol, leading to the end product of ergosterol in yeast and cholesterol in mammals. However, at least in *Arabidopsis*, it was recently revealed that phytosterols could be synthesized through the intermediate of lanosterol as well, although the contribution to sitosterol syntheses through lanosterol was only 1.5% relative to that via cycloartenol in wild-type *Arabidopsis* seedling (Ohyama et al., 2009). (3) 4-Methylsterol production, wherein

24-methylenecycloartenol is demethylated to generate cycloeucalenol, which is presumably the first 4-methylsterol. Cycloeucalenol is further sequentially converted into obtusifoliol, 4α-methylfecosterol, gramisterol (24-methylenelophenol), and citrostadienol via isomerization, demethylation, and reduction. (4) 4-Desmethylsterol production: End-product phytosterols, chiefly β-sitosterol, stigmasterol, and campesterol, belong to 4-desmethylsterols. β-sitosterol and stigmasterol are synthesized from citrostadienol, while campesterol is generated via gramisterol. In the course of the end-product formation, other 4-desmethylsterols, such as episterol and isofucosterol, are also generated as important intermediates.

In addition, phytosterols can be classified as Δ^5-phytosterols or Δ^7-phytosterols depending on the position of double bond in the rings. Δ^7-phytosterols are intermediates for three major Δ^5-phytosterols (β-sitosterol, campesterol, and stigmasterol). Phytosterols can be differentiated by side chains as well. The enzymatic properties and the number of sterol methyltransferase (SMTs) in individual organisms (Nes and Nichols, 2006) play a major role in determining side chain diversity.

10.2.2 Phytosterol Composition and Levels in Seed Oil

Most phytosterols in seed oils are 4-desmethylsterols. Among them, in most plant species β-sitosterol is the most abundant one, making up 38% in borage oil, 91% in avocado oil (Philips et al., 2002), 51% in rapeseed oil, and 95% in walnut oil (Verleyen et al., 2002). Certain phytosterols are only found in particular plant species or families. Borage, sesame, and evening primrose oils, for example, contain more Δ^5-avenasterol (isofucosterol) than others (Philips et al., 2002). A significant amount of brassicasterol is reported only for rapeseeds, which is about 7–13% of total phytosterols (Philips et al., 2002; Verleyen et al., 2002). In contrast to other seed oils with Δ^5-phytosterols (β-sitosterol, campesterol, and stigmasterol) as the major phytosterol components, in seed oil of Cucurbitaceae family plants Δ^7-sterols predominate. Spinasterols, including Δ^7-22,25-stigmastatrienol, Δ^7-stigmasterol, Δ^7-stigmastadienol, and Δ^7-avenasterol, reach as high as 95% of total sterol in pumpkin seed oil (Mandl et al., 1999). Other species of this family also showed similar composition (Akihisa et al., 1986, 1987; Garg and Nes, 1986; Taton and Rahier, 1996).

It is noteworthy that cholesterol, the dominant sterol molecule in animals, occurs in plants as well. In fact, many species of Solanaceae produce cholesterol at a significant level (Heftmann, 1984). In the pericarp tissue of mature green tomato fruit, cholesterol constituted some 6% of total sterol and 15–20% of sterol ester (Whitaker, 1988; Whitaker, 1991). The combined content of free sterol and sterol ester in 6 of 13 *Solanum* species reached >5% of seed oil (Zygadlo, 1994). It was postulated that the cholesterol content in plants depends on the activity of SMT. In mature *Arabidopsis* plants with disrupted SMT1, cholesterol became a major sterol species and made up 26% of total phytosterols in the whole plant at the expense of β-sitosterol. Cholesterol level in WT is at only 6% of total sterols (Diener et al., 2000).

Phytosterol content and their composition in seed oil have been determined in many plant varieties and cultivars. Canola oil has approximately twice the amount of total phytosterol (4590–8070 μg/g) as that of sunflower (2100–4540 μg/g) or

soybean (2340–4660 μg/g) (Vlahakis and Hazebroek, 2000). Although β-sitosterol, campesterol, and stigmasterol appear as major phytosterol species, their proportion nonetheless depends on plant species (Vlahakis and Hazebroek, 2000; Verleyen et al., 2002). Even for the same crop species, the content and composition vary with cultivars, varieties, and lines (Vlahakis and Hazebroek, 2000).

Phytosterol levels in seed oil also fluctuate with growth and environmental factors. The effect of temperature on accumulation of phytosterols in vegetative (Willemot, 1980; Uemura and Yoshida, 1984; Palta et al., 1993) and reproductive (Bartley, 1996; Whitaker, 1993, 1994; Huang and Grunwald, 1986) tissues has been reported for a number of plant species. Total phytosterols in seed oils of soybean plants grown under 35/25°C, averaged at 6580 μg/g, increased to 96% and 64%, respectively, as compared with 20/10°C and 27/17°C. Temperature elevation leads to changes in phytosterol composition with more campesterol and decreased β-sitosterol and stigmasterol (Vlahakis and Hazebroek, 2000). The change of ratio between campesterol and β-sitosterol most likely involves the behavior of some enzymes, including but likely not limited to SMT (Schaeffer et al., 2001). When exposed to mild water deficit, the molar ratio of stigmasterol/β-sitosterol was increased in *maize* (Grunwald, 1974; Navari-Izzo et al., 1989) and sunflower (Navari-Izzo et al., 1990), and maize. It was suggested that the ratio of different phytosterols has an impact on membrane permeability (Grunwald, 1974; Douglas and Walker, 1983), thereby influencing adaptation to environmental temperature.

Most industrial seed oil refining involves multiple extraction procedures. As a result, a portion of phytosterols is inadvertently removed from oils (Mounts et al., 1996). Depending on the type of oil and refining conditions, a loss of phytosterols can occur at a rate of 10–70% (Kochhar, 1983). Phytosterol decreased progressively with increasing temperature or time (Jawad et al., 1984). Furthermore, degumming and bleaching eliminate phytosterol by another 15% in canola oil (Prior et al., 1991), and 36%, 18%, and 24%, respectively, for fully refined corn, soybean, and rapeseed oil when compared with their crude oils (Ferrari et al., 1996). Moreover, refining seems to remove phytosterols discriminately (Jawad et al., 1984), with campesterol being most susceptible to significant changes in the ratio of free phytosterols and phytosterol esters (Ferrari et al., 1996; Verleyen et al., 2002). Generally, refining reduced total phytosterol and free phytosterol content, while relative content of phytosterol ester increased (Phillips et al., 2002). Due to higher solubility of phytosterol esters in seed oil, phytosterol esters of corn, soybean, and rapeseeds oil were reduced in refining process by only 3–16% while the total phytosterols were decreased by 18–36% (Ferrari et al., 1996). Current refining regime also causes significant oxidation, isomerization, and other intermolecular transformations of phytosterols including dehydroxylation, hydrolysis, and dehydrogenation (Piironen et al., 2000; Jawad et al., 1984).

10.2.3 Phytosterol Conjugates in Seed Oil

In addition to free sterol form, phytosterols are also found in the form of three conjugates, phytosterol glycosides (SGs), acylated phytosterol glycosides (ASGs), and acylated phytosterol esters (SEs) including fatty acid ester and hydroxycinnamic

acid ester. SEs are present in plants as storage form or for transportation purpose. They are synthesized via acylation of 3β-OH in phytosterol. SEs can be found in lipid bodies in the cytoplasm and are present at a substantial level in seeds. In seed oil, phytosterol esters make up a significant percentage of total phytosterols. According to Verleyen et al. (2002), SE in rapeseed and corn represented 56–60% of total sterols, while other tested vegetable oils such as soybean and sunflower contained relatively lower SE portions (25–40%). β-sitosterol dominates the acylated phytosterols in all seed oil samples, but its percent weight varies between 52% and 95% due to different seed sources. The content of SEs change with seed oil sources. Corn, soybean, sunflower, and canola produce higher level of SEs as compared with other seed oils (Verleyen et al., 2002). In canola, for example, phytosterols constitute about 0.5% of seed oil (Sabir et al., 2003).

Phytosterols are glucosylated with a hexose (usually glucose) in C-4 to form SGs. The hexose moiety can be further esterified with a fatty acid, most commonly in C-6, thus forming ASGs. SGs and ASGs appear to be present only in plants. Interestingly, β-sitosterol-β-D-glucoside in the plasma membrane is the primer molecule for cellulose synthesis in plants (Peng et al., 2002). SGs and ASGs have been reported from plant tissues such as potato tuber (Galliard et al., 1975), apple pulp (Takakuwa et al., 2005), oranges (Nagy and Nordby, 1971), eggplant (Zimowski and Wojciechowski, 1996), tomatoes (Whitaker, 1991), bell pepper (Whitaker and Lusby, 1989; Yamauchi et al., 2001), and a number of herbal or medicinal plants (Shaiq et al., 2002; Yao et al., 2007; Rios et al., 1989). For seed oil, SG and ASG were detected in palm (Homberg and Bielefeld, 1982; Murui and Siew, 1997), soybean (Hoed et al., 2008; Jantzen and Gohdes, 1934), and flax seed oil (Philips et al., 2005). The contents of the two conjugates varied sometimes drastically with plant species, and even in different reports for the same species.

10.3 PHYTOSTEROL ENHANCEMENT THROUGH GENE ENGINEERING

The *de novo* biosynthesis of phytosterols involves more than 20 enzymes, a metabolic process likely regulated at multiple steps. Carbon flux into the isoprenoid pathway and then to cycloartenol, and the flux from cycloartenol to Δ^5-end-product phytosterols have received the most attention (Harker et al., 2003a). A great deal of efforts has been devoted to biochemical characterization of a few essential enzymes, particularly 3-hydroxyl-3-methylglutary Co-enzyme A reductase (Suzuki et al., 2004; Narita and Gruissem, 1989; Nieto et al., 2009), squalene synthase (SQS) (Fulton et al., 1995; Uchida et al., 2009), and SMTs (Wang et al., 2008; Nes, 2003). This was followed by genetic engineering works in efforts to enhance phytosterol content in plant products.

10.3.1 3-Hydroxyl-3-Methylglutary Co-enzyme A Reductase

3-Hydroxyl-3-methylglutary Co-enzyme A reductase (HMGR) catalyzes the formation of mevalonic acid, a key intermediate for triterpene synthesis. This has been

widely accepted to be the rate-limiting enzyme in sterol biosynthesis in spite of some debates (Chappell et al., 1995; Nes and Venkatramesh, 1999). Harker et al. (2003a) reported co-ordination of HMGR, SMT, and an unknown sterol acyltransferase in phytosterol accumulation in developing seeds. In *Arabidopsis*, HMGR1 and HMGR2, with divergent N-terminal domains but highly conserved transmembrane domains and C-terminal catalytic domains, have been well characterized. *HMGR1* is widely expressed in various tissues and developmental stages (Enjuto et al., 1994), but *HMGR2* is exclusively expressed in meristematic and floral tissues (Enjuto et al., 1995). Disruption of *HMGR1* and *HMGR2* in *Arabidopsis* caused triterpenoids level 65 and 25% lower than that in wild type plants, respectively (Ohyama et al., 2007). Loss of function in HMGR1 decreased sterol levels in seedling and inflorescence by some 53 and 25%, respectively (Suzuki et al., 2004). Activity of HMGR was regulated at different levels, from transcriptional to post-translational (Nieto et al., 2009). Over-expression of a full-length *HMGR* did not increase phytosterol accumulation (Re et al., 1995; Hey et al., 2006). Evidence has been presented that plant HMGRs could be regulated through reversible phosphorylation by SNF1-related protein kinase-1 (Halford et al., 2003) and degradation triggered by accumulated end phytosterol product (Korth et al., 2000). The proteolytic degradation is mediated by the N-terminal membrane-spanning region. Deletion of N-terminal membrane-spanning region abolished the sterol-mediated turnover of the enzyme (Jingaei et al., 1987), which consequently allowed accumulation of phytosterol (Harker et al., 2003b; Hey et al., 2006). Therefore, a truncated HMGR has been employed to increase phytosterol level. Tomato transformed with an *Arabidopsis HMGR1* under the control of 35S constitutive promoter elevated phytosterol by up to 2.3-fold in ripe fruits of T_0 generation plants (Enfissi et al., 2005). Similar to this, transgenic tobacco lines expressing 35S promoter controlled *HMGR1* from *Hevea brasiliensis* (H.B.K.) Müll. Arg. increased total phytosterols in leaves by up to six fold through production of phytosterol esters (Schaller et al., 1995). Engineered tobacco seeds constitutively or seed-specifically expressing an N-terminal truncated *Hevea brasiliensis* HMGR increased phytosterol by 2.4- or 3.2-fold, respectively (Harker et al., 2003b).

10.3.2 Squalene Synthase

Squalene synthase (SQS) mediates the head-to-head condensation of two molecules of farnesyl pyrophosphate (FPP) to form squalene, the first and committed step from the central isoprenoid pathway towards sterol and triterpenoid biosynthesis. SQS may regulate sterol synthesis by directing FPP into either sterol or nonsterol isoprenoids depending on the changing cellular requirement. Inhibition of SQS activity by squalestatin, an irreversible inactivator of plant SQS, accompanied dramatic reduction in phytosterol content (Wentzinger et al., 2002). SQS was found to play an important regulatory role in the synthesis of phytosterol and triterpene in *Panax ginseng* (Lee at el., 2004). Overexpression of SQS of *P. ginseng* origin in plants enhanced production of phytosterols in adventitious roots. β-sitosterol and stigmasterol, the major sterol molecules in ginseng, were 1.6–3 times higher than that in WT control. Surprisingly the content of squalene was decreased, and no

cycloartenol increase was detected in the study (Lee et al., 2004). Similar results were reported elsewhere (Seo et al., 2005).

10.3.3 3-Hydroxysteroid Oxidase

3-Hydroxysteroid oxidase catalyzes oxidation of the carbon 3-hydroxyl group of 3-hydroxysteroids to produce ketosteroids (Corbin et al., 1994). The enzyme is produced by a phylogenetically diverse group of microorganisms and usually occurs as a secreted protein. 3-hydroxysteroid oxidases reported useful in overproduction of phytosterol and phytostanol include those naturally occurring in some microorganisms, such as *Streptomyces* spp., *Brevibacterium*, spp., *Pseudomonas* spp., and *Mycobacterium* spp. (Smith and Brooks, 1976). However, no 3-hydroxysteroid oxidase of plant origin has been cloned so far. It was suggested that when engineered with 3-hydroxysteroid oxidase without the chloroplast target peptide, cotton plants grew with severe defects in vegetative and seed development (Venkatramesh et al., 2003). Transgenic tobacco, *Nicotiana tabacum* expressing an *Actinomyces* 3-hydroxysteroid oxidase-encoding gene resulted in a 5.5-fold increase in total phytosteroids. 3-Ketosteroids and phytostanols were produced from nondetectable levels to amounts accounting for 50% and 27% of total phytosteroids, respectively. As compared with WT control, the ratio among phytosterol species was strikingly skewed. Sitosterol and campesterol, making up 36% of total sterol in WT control, almost disappeared in transgenic plants (Heyer et al., 2004). Expression of the *Streptomyces* A19249 3-hydroxysteroid oxidase in *Brassica napus* in leucoplast converted 18–22% of β-sitosterol, 17–24% of campesterol, and 26–43% of brassicasterol into their corresponding phytostanols in the best-performing plants. Similarly in *Glycine max*, approximately 40–80% of phytosterols (sitosterol, campesterol, and stigmasterol) were converted into phytostanols. But total phytosterol and phytostanol content was not significantly affected in either crop (Venkatramesh et al., 2003). Therefore, the effect brought about by the expression of a 3-hydroxysteroid oxidase on phytosteroid is likely determined by factors such as specificity of the enzymes, transformed plant species, and tissues (leaf, stem, seeds, etc.). No data has clearly shown that the expression of 3-hydroxysteroid oxidase is able to enhance total phytosterol and phytostanol in the seeds of transgenic plants.

10.3.4 Sterol *O*-acyltransferases SATs

10.3.4.1 Rationale for Identification of SATs

Advantage of Phytosterol Esters over Free Sterols as a Food Ingredient As early as in 1951, β-sitosterol was found effective in decreasing serum cholesterol in chickens and was therefore suggested as a therapeutic agent for hypercholesterolemia (Peterson, 1951). Eli Lily Company introduced the first phytosterol product, "Cytellin" as a drug for hypercholesterolemia treatment (Pollak and Kritchevsky, 1981). Both soya oil (rich in campesterol and stigmasterol) and tall oil (from pine trees, 93% β-sitosterol) were utilized as phytosterol sources. But its low water solubility and the resulting low bioavailability required a daily intake of up to

18 g of β-sitosterol to achieve a reduction in serum cholesterol levels (Farquhar and Sokolow, 1958), resulting in the product becoming unmarketable. The hydrophobic and lipophobic properties only allow phytosterols to be formulated as tablet or capsule, while making them difficult and inefficient as food ingredient. To circumvent this problem, phytosterol crystal size was reduced by means of grinding or suspension to enhance its bioavailability (Dawson and Rudel, 1999), and dietary mixed micelles were intensively investigated to realize decent phytosterol solubilization (Meĺnikov et al., 2004). Despite these efforts, the inconvenience of dosing and problems with palatability limited the use of phytosterols as cholesterol-lowering agents. An interesting finding was that phytosterol esters were 10 times more soluble in oil than free sterols (Jandacek et al., 1977). Consequently, acylation has been the key to phytosterol solubilization into various foods, especially fat-based food for lowering intestinal cholesterol absorption (Mattson et al., 1977). In addition, the fatty acyl moiety hardly compromises the power of phytosterols; a mere 2.8 g sitostanol/day given as sitostanol ester in margarine significantly reduced LDL cholesterol by 16% (Miettinen et al., 1995). To obtain the benefit from the higher solubility of phytosterol esters, much effort has been placed on the methodologies for esterifying phytosterols. Esterification of phytosterol could be carried out via two routes, direct esterification using free fatty acids or trans-esterification using fatty acid methylesters (Chung et al., 2000). King et al. (2001) reported production of sterol ester through lipase-catalyzed reactions in supercritical carbon dioxide. By using the established parameters and procedure, the yield of cholesterol/sitostanol ester ranged from 90% to 99%. However, these *in vitro* esterification reactions were performed at an inevitably high cost. Employing plant systems as bioreactors via gene engineering thus came to light in the post-genomic era.

Potential of SATs to Increase Phytosterol in Seed Oil Phytosterol esters are the forms for phytosterol storage. They are ubiquitous in all plants (Dyas and Goad, 1993), localized in cytoplasm (Brandt and Benveniste, 1972) or as lipid bodies in the elaioplasts of the tapetum in developing *B. napus* anthers (Hernandez-pinzon et al., 1999; Wu et al., 1999). Phytosterol acyltransferase activity was found to increase during rapeseed and tobacco seed development (Harker et al., 2003a), correlating with accumulation of phytosterol esters that make up about 60% of total sterols in rapeseed oil (Verleyen et al., 2002). In addition, overproduction of phytosterols by means of genetic engineering was reported mainly through the accumulation of phytosterol esters (Schaller et al., 1995; Gondet et al., 1992). Therefore, SATs should be considered as key enzymes for genetic engineering to boost phytosterol esters in seed oil.

10.3.4.2 Two Sterol O-Acyltransferase Families: ACATs and LCATs To our knowledge, two classes of SATs, namely, acyl-CoA:cholesterol *O*-acyltransferase (ACAT) and lecithin:cholesterol acyltransferase (LCAT), have been reported. ACATs catalyze the transfer of acyl group from fatty acyl-CoAs to 3-OH of a free sterol, while LCATs esterify 3-OH of free sterol using phosphatidlycholine as

fatty acyl donor. A 4-kb cDNA encoding an ACAT1 was first isolated from human macrophage in 1993 (Chang et al., 1993), which led to the identification of the ACAT family enzymes. Homologs of human ACAT1 in yeast (Yang et al., 1996; Yu et al., 1996), mice (Uelmen et al., 1995; Green et al., 1996), hamsters (Cao et al., 1996), rabbits (Pape et al., 1995), nonhuman primates (Anderson et al., 1998), and rats (Matsuda et al., 1998) were successfully identified. The cloning of two SAT-encoding genes in yeast and the observation that ACAT activity in different tissues displayed different susceptibility to inhibition by chemical inhibitors (Kinnunen et al., 1988; Maduskuie et al., 1995) suggested the multiplicity of sterol acyltransferases contributing to sterol esterification process (Meiner et al., 1996). This was then corroborated by the isolation of ACAT2 genes in humans (Oelkers et al., 1998), nonhuman primates (Anderson et al., 1998), and mouse (Cases et al., 1998a). ACAT1 and ACAT2 were proposed to be descendants of ancestral genes in yeast (Yang et al., 1996) and perform different cellular functions (Meiner et al., 1996). Surprisingly, ACAT homolog (At2g19450) in *Arabidopsis* was identified as an acyl-CoA:diacylglycerol acyltransferase (DGAT1) with no significant sterol acyltransferase activity (Zou et al., 1999). However, due to its high identity to ACATs, the DGAT1 was still recognized as a member of the ACAT family (Cases et al., 1998b).

LCAT is a glycoprotein that catalyzes the transfer of acyl groups from the sn-2 fatty acid of phosphatidylcholine (PC) to esterify free cholesterol, which is believed to play a pivotal role in reverse cholesterol transport by facilitating formation of cholesterol esters in high-density lipoprotein cholesterol for subsequent hepatobiliary excretion (Miller et al., 1995). The LCAT was first identified in humans (McLean et al., 1986). In *Arabidopsis* there are six LCAT homologs. Two of them, At1g04010 and At5g13640, have been experimentally verified as phospholipid sterol acyltransferase (PSAT) (Bana et al., 2005) and PC diacylglycerol acyltransferase (PDAT) (Ståhl et al., 2004), respectively.

Arabidopsis AtPSAT AtPSAT had a strong preference for phosphotidyl-ethanolamine (PE) over PC as acyl group donor. *In vitro* assay with acetone-treated microsomal preparation showed that AtPSAT prefers end phytosterol products, β-sitosterol and campesterol, than phytosterol intermediates such as cycloartenol, 24-methylene cycloartenol, and obtusifoliol. However, when equal amounts of β-sitosterol and an intermediate were present in *in vitro* reaction, AtPSAT preferentially acylated the intermediate rather than the end product. Phytosterol esters were reduced in T-DNA insertion mutant plants grown in liquid medium; however, phytosterol content in AtPSAT overexpression lines were not reported (Bana et al., 2005), although overexpressing AtPSAT under the control of NAPIN promoter was documented to be capable of increasing seed oil (Lassner and Eenennaam, 2007). Instead, another *Arabidopsis* LCAT member, LCAT4 (At3g03310), when overexpressed specifically in seeds, increased phytosterol level by up to 50% (Lassner and Eenennaam, 2007).

Arabidopsis AtSAT1 Another SAT, AtSAT1, was identified out of 15 *Arabidopsis* membrane-bound *O*-acyltransferase (MBOAT) protein family members based on the

rationale that all isolated ACATs belong to the MBOAT family through yeast complementation in combination with chromatographic approach (Chen et al., 2007). Amino acid sequence alignment analysis indicated a low percent identity (7.8–12%) of AtSAT1 with other ACATs. AtSAT1 protein is a shorter polypeptide with 345 amino acids, only approximately two thirds of other ACATs' length, largely due to truncation at the N-terminus. The RxWNxxVxxxLxxxVY motif crucial for fatty acyl-CoA binding (Guo et al., 2001), and a His residue (His245 in AtSAT1) constituting part of active site (Hofmann, 2000), are present in all ACATs; but, two structurally conserved motifs, FY xDWWN and HSF regions, proposed to be crucial for ACAT activity and stability (Cao et al., 1996; Oelkers et al., 1998) are absent in AtSAT1.

Heterogeneous expression of *AtSAT1* enabled 100-fold accumulation of sterol ester in *are1are2* double mutant yeast strain at the expense of free sterols. Qualitative and quantitative analysis revealed that a biosynthetic intermediate, lanosterol, rather than the yeast sterol end product ergosterol, was accumulated as the esterified sterol species. Similarly, overexpression of *ATSAT1* in *Arabidopsis* under the control of a seed-specific NAPIN promoter increased phytosterol ester production by more than twofold and the total sterol content by 64% in some transgenic lines. In wild-type plant seeds, β-sitosterol and campesterol are the major phytosterol moieties and constitute 80.4% and 17.5% of total SE, respectively; cycloartenol and 24-methylene cycloartenol esters are present at a barely detectable level. Strikingly, in the transgenic lines overexpressing *AtSAT1*, cycloartenol esters were the most prominent sterol species, representing up to an average of 64.1% of total SE, while the level of 24-methylene cycloartenol esters was increased to 5.6% as well. As a result, esterified β-sitosterol and campesterol were reduced to averages of 25.2 and 5.2%, respectively.

These results clearly show that *AtSAT1* highly preferred lanosterol in yeast and cycloartenol in *Arabidopsis*. Both of the two sterol intermediates are important biosynthetic intermediates generated via 2,3-oxidosqualene cyclization, suggesting the presence of other SAT(s) responsible for esterification of phytosterol end products. Zweytick et al. (2000) reported *in vivo* sterol substrate specificity, although their results slightly differed, but showed that ARE1 mainly acylated the intermediate lanosterol and, to a less extent, zymosterol; while ARE2 esterifies the end product ergosterol. The substrate preference of SATs for different sterols was also reported for human ACAT1, ACAT2, and LCAT1 (Ryan et al., 2003).

10.3.5 Combination of Multiple Genes to Amplify the Power of Single Gene Effect

As already demonstrated, overexpressing key genes in the sterol synthesis pathway can lead to phytosterol overproduction. These enzymes are positioned at different sites on the phytosterol biosynthesis pathway likely controlling intermediate flux and consequently formulating phytosterol composition. Coexpression of more than one key gene, constitutively or tissue/organelle-specifically, in a plant of interest could be performed to maximize the potential of gene engineering. These genes are usually installed separately into different expression cassettes so that their expression could

be controlled under preferred promoters. This strategy has been successfully utilized in the development of insect and herbicide tolerance (Urwin et al., 1998; Tang et al., 1999; Zhao et al., 2003). Attempts were made to increase phytosterol level in soybean oil in combinations of HMGR and 3-hydroxysteroid oxidase, and HMGR and SMT (Venkatramesh et al., 2000). As compared with single HMGR transformation, addition of another gene, either 3-hydroxysteroid oxidase or SMT, led to no further increase in phytosterol content. But, the power of combining multiple genes in phytosterol overproduction should not be disregarded merely due to failure from likely inappropriate gene combinations. Considering that phytosterol metabolism involves tens of enzymes and unknown regulatory network, fundamental research must be carried out to ensure success in gene-stacking strategy.

10.4 REMARKS AND PERSPECTIVES

The efficacy of phytosterol as a food ingredient in reducing cholesterol level in humans is indisputable based on medical research covering more than half a century. Improving phytosterol content in vegetable oil offers the prospect of reducing risks associated with obesity and cardiovascular diseases, thereby mitigating the tremendous social and economic costs that modern society is saddled with. Research in recent years has made impressive progress in identifying molecular components involved in phytosterol biosynthesis. The imperative of utilizing these molecular tools for a biotechnology approach is well recognized. Continued efforts are however needed in order to reach the goal of biofortification of phytosterols in food products.

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SECTION II

FUNCTIONAL FOODS AND BIOFUELS

11

DIETARY PHOSPHATIDYLINOSITOL IN METABOLIC SYNDROME

BUNGO SHIROUCHI,[1,2] KOJI NAGAO,[1] AND TERUYOSHI YANAGITA[1]

[1]*Laboratory of Nutrition Biochemistry, Department of Applied Biochemistry and Food Science, Saga University, Saga 840-8502, Japan*

[2]*Food Function and Labeling Program, National Institute of Health and Nutrition, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8636, Japan*

11.1 INTRODUCTION

Lifestyle-related diseases, such as obesity, hyperlipidemia, atherosclerosis, type 2 diabetes, and hypertension, are widespread and increasingly prevalent in industrialized countries. Accompanied by the rapid increase in the number of elderly people, this

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becomes a medical and a socioeconomic issue. A clustering of metabolic disorders in an individual, defined as metabolic syndrome, is known to increase cardiovascular morbidity and mortality (Grundy et al., 2005). Although the pathogenesis of metabolic syndrome is complicated and precise details of the underlying mechanisms are not known, it has been suggested that the quality of dietary fats may be an important modulator in terms of the risks associated with this syndrome (Yanagita and Nagao, 2008; Nagao and Yanagita, 2008).

Although triglycerides (TGs) make up the majority of dietary fat, phospholipids (PLs) compose about 3–8% of the daily intake of total dietary fats (Åkesson, 1982; Yanagita, 1984). Growing evidence indicates that dietary PLs, especially phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), have beneficial effects compared with dietary TGs. For example, dietary PC, PE, and PS have been reported to have lipid-lowering effects and improve brain function (McDaniel et al., 2003; Buang et al., 2005; Shirouchi et al., 2007; Cohn et al., 2008). Despite the fact that the use of PC and PS for nutritional and therapeutical purposes has increased in recent years, reports examining the physiological functions of dietary phosphatidylinositol (PI) are scarce. PI is a minor component of dietary PLs and is found in legumes and seeds such as soybeans, peanuts, rapeseeds, and sunflower seeds (Weihrauch and Son, 1983). It has been known that PI in biological membranes plays key roles in mediating cellular responses to external stimuli (Holub, 1986; Colodny and Hoffman, 1998). In the present study, we investigated the physiological function of dietary PI in metabolic syndrome model Zucker (*fa/fa*) rats. Zucker (*fa/fa*) rats have hyperphagia because they have a missense mutation on the leptin receptor gene, and develop a syndrome with multiple metabolic and hormonal disorders that shares many features with the human metabolic syndrome (Phillips et al., 1996).

11.2 EFFECT OF DIETARY PI ON THE DEVELOPMENT OF NON-ALCOHOLIC FATTY LIVER DISEASE IN METABOLIC SYNDROME MODEL RATS

Non-alcoholic fatty liver disease (NAFLD) is often associated with features of the metabolic syndrome and is emerging as the most common liver disease worldwide (Harrison and Diehl, 2002; Youssef and McCullough, 2002; Fan et al., 2007). NAFLD is the preferred term to describe the spectrum of liver damage, ranging from hepatic steatosis to steatohepatitis, liver fibrosis, and cirrhosis. Most liver-related morbidity and mortality is associated with the development of cirrhosis. Cirrhosis is most likely to occur in individuals who have progressed from hepatic steatosis to steatohepatitis. Although the processes through which steatohepatitis evolves from hepatic steatosis are not fully understood, it is necessary to develop effective therapies for the treatment of NAFLD and to discover nutrients that will reduce the risk of NAFLD.

Male Zucker (*fa/fa*) rats were assigned to two groups, with dietary fats composed of 7% soybean oil (TG group), and a mixture of 5% soybean oil + 2% soybean PI (PI group). The fatty acid compositions of experimental diets are given in Table 11.1.

TABLE 11.1 Fatty Acid Composition of Experimental Diets

	TG Diet[a]	PI Diet[b]
Fatty acid composition, wt (%)		
14:0	0.4	0.4
16:0	10.0	11.4
16:1	0.3	0.2
18:0	1.3	1.7
18:1	18.1	16.3
18:2 n-6	61.6	61.4
18:3 n-3	7.0	7.2
Others	1.3	1.4
Total	100	100

[a] Contained 7% soybean oil as dietary fat.
[b] Contained 5% soybean oil + 2% soybean PI as dietary fat.

Figure 11.1 shows hepatic TG levels, activities of aspartate aminotransferase (AST) in serum, and hepatic carnitine palmitoyltransferase (CPT) activities of Zucker (*fa/fa*) rats after 4 weeks of consuming the diets. Although there was no significant difference in the final body weight or food intake between the groups (data not shown), the PI diet significantly decreased liver weights (by 21%, data not shown) and hepatic TG levels (by 77%, Fig. 11.1) of Zucker (*fa/fa*) rats. Consistent with the alleviation of hepatomegaly and hepatic steatosis by the PI diet, the activities of hepatic injury markers such as AST (by 37%, Fig. 11.1) and ALT (by 47%, data not shown) were markedly decreased in the serum of PI-fed rats than in the TG-fed rats. Although we did not perform a hepatic histological evaluation, these data suggest that dietary PI protects Zucker (*fa/fa*) rats from the development of NAFLD.

To further examine the effect of dietary PI on the liver, hepatic enzymes related to TG metabolism were analyzed. Activities of phosphatidate phosphohydrolase and fatty acid synthase, which are key enzymes in the regulation of TG and fatty acid *de novo* synthesis, did not differ between the groups (data not shown). However, the

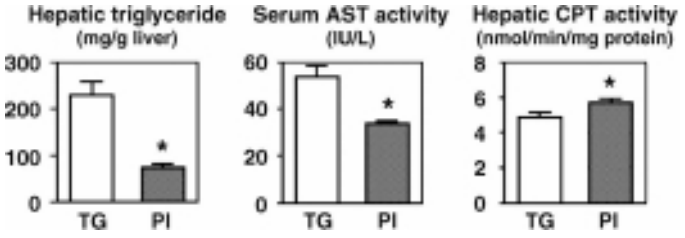

FIGURE 11.1 Effect of dietary PI on hepatic triglyceride levels, activities of aspartate aminotransferase (AST) in serum, and hepatic carnitine palmitoyltransferase (CPT) activities in Zucker (*fa/fa*) rats. Rats were fed diets containing either 7% soybean oil (TG group) or 5% soybean oil + 2% soybean PI (PI group) for 4 weeks. Values are expressed as means ± standard error of six rats. Asterisk shows significant difference at $P < 0.05$.

activity of CPT, a key enzyme of fatty acid β-oxidation, was significantly greater in the PI group as compared with the TG group (Fig. 11.1). Although the mechanisms responsible for the development of NAFLD are unclear, it has been suggested that hepatic steatosis results from accelerated mobilization from expanded visceral fat stores and their deposition in the liver as well as decreased hepatic fatty acid β-oxidation (Harrison and Diehl, 2002; Youssef and McCullough, 2002). Thus, we suggest that the prevention of NAFLD by dietary PI is partially attributable to the enhancement of CPT activity in the liver.

11.3 EFFECT OF DIETARY PI ON SERUM ADIPONECTIN LEVELS AND HEPATIC INFLAMMATORY MOLECULE mRNA LEVELS IN METABOLIC SYNDROME MODEL RATS

Insulin resistance is the essential first pathological step in the development of NAFLD (Marceau et al., 1999; Marchesini et al., 2003). In fact, hepatic steatosis is now proposed as a feature of the insulin resistance syndrome along with type 2 diabetes, visceral obesity, hyperlipidemia, and hypertension (Marceau et al., 1999; Marchesini et al., 2003). After 4 weeks of feeding period, Zucker (*fa/fa*) rats given the TG diet had severe hyperinsulinemia. As shown in Fig. 11.2, serum insulin level tended to decrease (by 35%) in the PI group as compared with the TG group. Recently, it has been recognized that adipose tissue not only stores excess energy in the form of fat but also secretes physiologically active substances called adipocytokines (Matsuzawa et al., 1999). Among those, adiponectin is one of the most abundant secretory proteins from adipose tissue in rodents and humans (Matsuzawa, 2005). Because several reports indicate that adiponectin can lead to enhanced insulin action *in vitro* and *in vivo*, it is strongly suggested that adiponectin plays a protective role against insulin resistance (Matsuzawa, 2006). In the present study, serum adiponectin level was markedly increased (by 43%) in the PI group as compared with the TG group

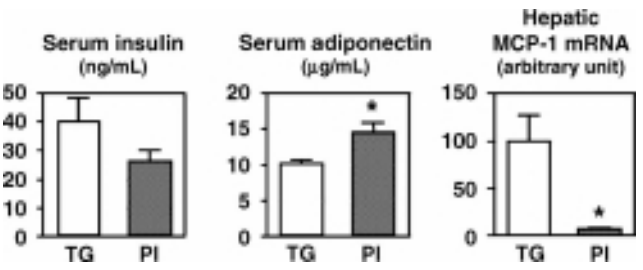

FIGURE 11.2 Effect of dietary PI on serum insulin levels, serum adiponectin levels, and hepatic monocyte chemoattractant protein-1 (MCP-1) mRNA levels in Zucker (*fa/fa*) rats. Rats were fed diets containing either 7% soybean oil (TG group) or 5% soybean oil + 2% soybean PI (PI group) for 4 weeks. Values are expressed as means ± standard error of six rats. Asterisk shows significant difference at $P < 0.05$.

(Fig. 11.2). These results suggest that dietary PI improves insulin resistance through increased serum adiponectin level.

The pathogenesis of steatohepatitis, the more advanced form of NAFLD, has yet to be clearly defined, but the recent major theory is the "two-hit" hypothesis (Day and James, 1998). The first "hit" is the TG accumulation within the liver. It was proposed that lipid-laden hepatocytes are more susceptible to a second "hit", i.e., injury by oxidative stress and inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α. In addition, lipid peroxidation products trigger cytokine production within the liver, and this accelerates TNF-α-mediated liver injury. In fact, an overexpression of TNF-α mRNA is found in the liver of nonalcoholic steatohepatitis patients (Crespo et al., 2001). Moreover, it has been recognized that monocyte chemoattractant protein-1 (MCP-1), a member of the CC chemokine family, induces inflammatory responses by recruiting inflammatory cells and is up-regulated by inflammatory stimuli such as TNF-α (Baggiolini, 1998). Interestingly, recent findings also indicate that transgenic mice expressing MCP-1 exhibit insulin resistance and hepatic steatosis, whereas a disappearance of MCP-1 in knockout mice and an acute inhibition of MCP-1 by expression of a dominant-negative mutant in mice resulted in improvement of insulin resistance and hepatic steatosis (Kanda *et al*., 2006). In the present study, mRNA expression of TNF-α tended to decrease (by 58%, data not shown) and MCP-1 mRNA expression was drastically decreased (by 93%, Fig. 11.2) in the liver of PI-fed rats compared with TG-fed rats. Because adiponectin has protective effects against both inflammation (Czaja, 2004) and fibrosis (Kamada et al., 2003), we consider that the increase in serum adiponectin by dietary PI may contribute to the prevention of development and progression of NAFLD in Zucker (*fa/fa*) rats.

11.4 EFFECT OF DIETARY PI ON CHOLESTEROL LEVELS IN METABOLIC SYNDROME MODEL RATS

As shown in Fig. 11.3, alleviation of hepatomegaly and hepatic steatosis by the PI diet were partly associated with a reduction in the cholesterol accumulation in the liver (by

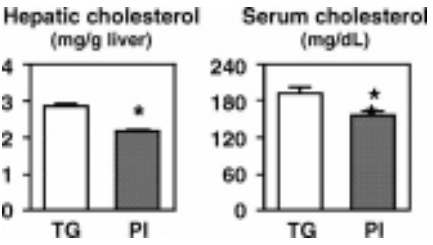

FIGURE 11.3 Effect of dietary PI on hepatic cholesterol levels and serum cholesterol levels in Zucker (*fa/fa*) rats. Rats were fed diets containing either 7% soybean oil (TG group) or 5% soybean oil + 2% soybean PI (PI group) for 4 weeks. Values are expressed as means ± standard error of six rats. Asterisk shows significant difference at $P < 0.05$.

40%, Fig. 11.3). As a consequence, serum cholesterol level was also decreased in PI-fed rats compared with TG-fed rats (by 19%, Fig. 11.3).

11.5 EFFECT OF DIETARY PI ON HEPATIC mRNA LEVELS AND FECAL BILE ACID LEVELS IN METABOLIC SYNDROME MODEL RATS

Acyl-CoA:cholesterol acyltransferase1 (ACAT1), a rate-limiting enzyme of cholesterol esterification; cholesterol 7α-hydroxylase (CYP7A1), a rate-limiting enzyme of bile acid synthesis; 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, a rate-limiting enzyme of cholesterol synthesis; and low-density-lipoprotein (LDL)-receptor relate cholesterol homeostasis. The gene expression of cholesterol biosynthetic enzymes and receptor, such as HMG-CoA reductase and LDL-receptor, are regulated by sterol regulatory element binding protein-2 (SREBP-2), a transcriptional factor. Given the results that the PI diet did not affect the mRNA levels of HMG-CoA reductase, LDL-receptor, and SREBP-2 compared with the TG diet (data not shown), the cholesterol-lowering effect of dietary PI was not attributable to the reduction of cholesterol biosynthesis. On the other hand, ACAT1 mRNA level tended to decrease by the PI diet (by 63%, data not shown), whereas CYP7A1 mRNA level tended to increase by the PI diet (by 2.3-fold, Fig. 11.4). Additionally, the PI diet increased the levels of fecal bile acids compared with the TG diet (to 2.3 fold, Fig. 11.4). Previous studies showed that dietary PC and PE revealed hypocholesterolemic effects through the increased fecal excretion of neutral steroid, not bile acids, compared with the TG diet (Imaizumi et al., 1983). Thus, we suppose that the hypocholesterolemic effect of PI diet was attributable to suppressed lipoprotein synthesis and enhanced bile acid synthesis in the liver, and was a unique mechanism that differs from that revealed by other PLs.

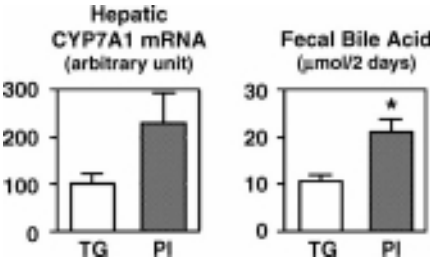

FIGURE 11.4 Effect of dietary PI on hepatic cholesterol 7α-hydroxylase (CYP7A1) mRNA levels and fecal bile acid levels in Zucker (*fa/fa*) rats. Rats were fed diets containing either 7% soybean oil (TG group) or 5% soybean oil + 2% soybean PI (PI group) for 4 weeks. Values are expressed as means ± standard error of six rats. Asterisk shows significant difference at $P < 0.05$.

11.6 CONCLUSIONS

The present study shows that dietary PI prevents the development of nonalcoholic fatty liver disease and mild hypercholesterolemia in the metabolic syndrome model rats (Shirouchi et al., 2008, 2009).

Although composition of dietary fatty acids can affect lipid and glucose metabolism, there was no significant difference of fatty acid composition between TG diet and PI diet in this study (Table 11.1). Therefore, we suppose that the beneficial effects demonstrated in this study were attributable to the physiological functions of PI itself or its constituent base inositol. Given that dietary PC and PE have lipid-lowering effects, comparison concerning physiological effects on the development and prevention of metabolic syndrome among PLs, such as PC, PE, and PI, would be of great interest for future study.

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12

BIOTECHNOLOGICAL ENRICHMENT OF CEREALS WITH POLYUNSATURATED FATTY ACIDS

MILAN CERTIK, ZUZANA ADAMECHOVA, AND LUCIA SLAVIKOVA

Department of Biochemical Technology, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinskeho 9, 812 37 Bratislava, Slovak Republic

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12.1 INTRODUCTION

The increasing demand for high-value lipids has focused commercial attention on the provision of suitable biosynthetic framework for their production. One of the main targets is the natural construction of functional food containing healthy and dietary important polyunsaturated fatty acids (PUFAs), such as γ-linolenic acid (18:3 n-6; GLA), dihomo-γ-linolenic acid (20:3 n-6; DGLA), arachidonic acid (20:4 n-6; AA), eicosapentaenoic acid (20:5 n-3; EPA), docosapentaenoic acid (22:5 n-3 or n-6; DPA), and docosahexaenoic acid (22:6 n-3; DHA). However, inadequacy of natural sources of PUFAs resulted in "hunting" for appropriate technology that could be able to either enrich common food and feedstuffs with PUFAs or directly produce PUFAs in high enough quantities.

The extensive research and development of PUFA manufacturing carried out over the past several years has been aimed at biotechnological techniques. Successes in genetic transformation of some plants leading to making modified "green varieties" with the capacity to synthesize PUFAs (Murphy, 2002; Napier, 2002) or developing fermentation methods with oleaginous microorganisms forming tailor-made PUFAs in high yields (Certik and Shimizu, 1999a; Dyal and Narine, 2005; Ward and Singh, 2005) has opened new possibilities for natural production of these valued metabolites. Because cereals represent a major food and feed intake and they did not contain essential PUFAs, the question arises as to how to enhance the amount of PUFAs in cereal-based diet. Thus, this chapter deals with biotechnological enrichment of cerealss with PUFAs particularly by fungal solid-state fermentations.

12.2 IMPORTANCE AND SOURCES OF POLYUNSATURATED FATTY ACIDS

PUFAs, with their unique structural and functional characteristics, are distinguished by two main functions (Horrobin, 1995). The first function relates to their roles in regulating the architecture, dynamics, phase transition, and permeability of membranes, and modulating the behavior of membrane-bound proteins such as receptors, ATPases, transport proteins, and ion channels. In addition, PUFAs control the expression of certain genes (3) and thus affect some processes including fatty acid biosynthesis and cholesterol transport in the body. Much more interest is focused on the second role of PUFAs as precursors of a wide variety of metabolites (such as prostaglandins, leukotrienes, and hydroxy-fatty acids) regulating critical biological functions. The various roles played by PUFAs make it apparent that they are required in every organ in the body in order for the organs to function normally. Therefore, it is not surprising that insufficient dietary consumption of PUFAs leads to abnormalities in the skin (atopic eczema), cardiovascular, endocrine, nervous, inflammatory, immune, respiratory, and reproductive systems, diabetes, premenstrual syndrome, etc. Because mammals lack the ability to synthesize PUFAs, these must be supplied in the diet. Moreover, PUFAs also have a number of uses in various fields and the biomedical and nutraceutical applications of commercial PUFA-rich preparations have been reviewed by Gill and Valivety (1997).

The main oil sources relatively rich in C_{18} PUFAs are the seeds of some plants with dominant synthesis of linoleic acid (18:2 n-6) and α-linolenic acid (18:3 n-3). Occurrence of significant levels of essential GLA in plants is rare and the most commercially important sources of this fatty acid are seeds of evening primrose (*Oenothera biennis*) with 8–10% GLA, borage seeds (*Borago officinalis*) containing 24–25% GLA, and black currant seeds (*Ribes nigrum*), which contain 16–17% GLA. In contrast, PUFAs above C_{18} cannot be synthesized by higher plants in any significant amounts owing to a lack of the requisite enzymes. On the other hand, the beneficial affect of fish oil has been ascribed to its C_{20} and C_{22} n-3 fatty acid content, notably, EPA, DPA n-3, and DHA. However, fish oils possess objectionable tastes and odors, and their satisfactory utilization requires the removal of cholesterol and small amounts of potentially toxic impurities (e.g., pollutants). There are also additional drawbacks to the use of fish oils as a PUFA source, such as variations in oil quality and the presence of fatty acids with antagonistic properties (e.g. arachidonic acid). Total content of n-3 fatty acids depends considerably on the season and geographic location of harvest sites as well as on the species of fish and the type and availability of its primary food source, namely marine microorganisms. Furthermore, it has been predicted that if n-3 PUFAs come to be widely used as prophylactic drugs, the total present annual production of marine fish oil would be insufficient to meet the worldwide demand.

As alternatives to agricultural and animal oil products, oleaginous microorganisms have also been intensively studied. Certain fungi, marine bacteria, heterotrophic and phototrophic microalgae, and mosses contain various PUFAs, and may thus represent suitable sources of them (Certik and Shimizu, 1999b; Ward and Singh, 2005). The diversity of microbial species can facilitate the selection of strains producing oil with predominant fatty acids. Particularly active in the synthesis of PUFAs are species of fungi belonging to Zygomycetes (Certik and Shimizu, 1999a). Moreover, these oleaginous fungi producing PUFA could be economically valuable because most of their PUFAs occur in the triacylglycerol fraction of their lipids.

12.3 BIOTECHNOLOGICAL STRATEGY FOR CEREALS ENRICHED WITH PUFAs

Cereals represent a major food supply for humanity and are associated with virtually every day of the history of civilization. Eight cereal grains (wheat, maize, rice, barley, sorghum, oats, rye, and millet) provide 56% of the food energy and 50% of the protein consumed on the earth (Cordain, 1999). Because humanity has become dependent on cereal grains for the majority of its food supply, it is necessary to understand the nutritional implications of cereal grain consumption on human health.

Although cereal grains are rich in proteins and carbohydrates, many of them are deficient in several essential nutrients. From the point of view of lipids, cereal grains are quite low in fats averaging 3.6% of fat in their total caloric content. While linoleic acid is the major fatty acid of n-6 family found in grains, α-linolenic acid (n-3 family) is detected only in small quantities in cereals. Calculated ratio of n-6 (18:2)/n-3 (18:3) fatty acids varied from 11–12 (buckwheat, barley, wheat) to 96 (amaranth)

(Certik et al., 2006). It should be emphasized that α-linolenic acid is a precursor of essential fatty acids of n-3 family. Its balanced intake is important for the normal development of brain and reduces the risk of coronary heart diseases as well. Therefore, a diet based primarily on cereal grains not only encourages improper dietary balance between n-6 and n-3 fatty acids but also is deficient in other essential PUFAs of both n-6 (GLA, DGLA, AA, DPA n-6) and n-3 families (EPA, DPA n-3, and DHA). It may lead to increased incidence of various diseases resulting from insufficient PUFA intake as mentioned above.

There are several strategies to enhance the content of PUFAs in a cereal diet (Fig. 12.1). One possibility is to simply add these compounds to food or feed. Unfortunately, inadequacy of natural plant or microbial sources rich in PUFAs does not allow them to be used in large quantities as food/feed supplements. In addition, supplementation of diet by synthetically prepared PUFAs has been strictly restricted. The other approach may involve application of gene engineering techniques to prepare new cereal varieties with desired fatty acid profile. Nevertheless, these methods are limited because of the difficulties with gene transformation in various cereals. Moreover, transgenic plants containing PUFA are not approved for application in the food/feed industry in many countries. Thus, an attractive perspective to enhance the content of PUFAs in cereals might be based on fermentation methods. Success in the microbial PUFAs production has led to a flourishing interest in developing fungal fermentation processes and enabled several processes to attain commercial production levels (Certik and Shimizu, 1999a). Of them, application of lower oleaginous fungi to utilize and transform cereals during solid-state fermentations creates new prospects for cereal-based products enriched with PUFAs (Slavikova and Certik, 2005; Certik et al., 2008a).

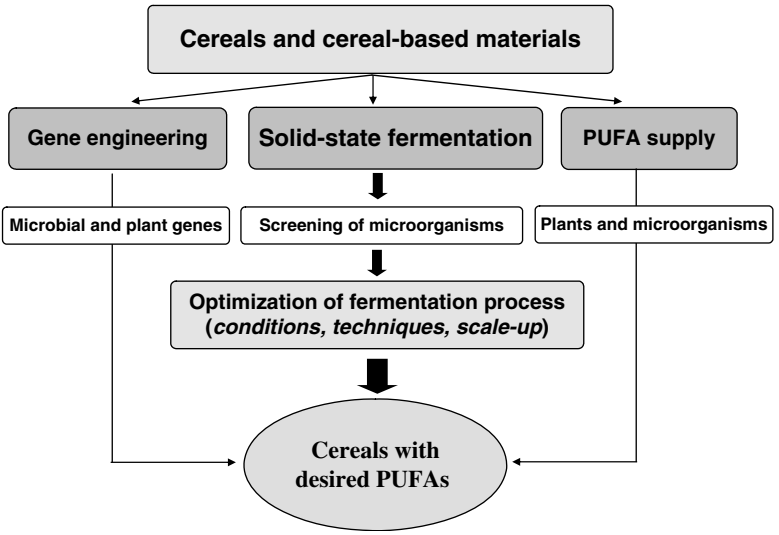

FIGURE 12.1 Strategy for cereals enrichments with PUFAs.

12.4 SOLID-STATE FERMENTATIONS

Solid-state fermentation is a process in which microorganisms grow on a moist solid substrate in the absence of free water (Pandey, 2003). Moist solid substrates, which are polymeric in nature and insoluble in water, act as a source of carbon, nitrogen, minerals, water, and other nutrients and moreover provide suitable anchorage for the microorganisms. SSF simulates fermentation reactions occurring in the nature and allows microbial utilization of raw agromaterials or by-products of the agrofood industries. SSF is therefore a powerful tool for effective utilization of agromaterials (e.g. cereals) and their valorization to various types of value-added bioproducts with desired properties (Certik et al., 2002, 2006) and might provide another opportunity to fill marketing claims in food, feed, pharmaceutical, veterinary, and environmental fields.

Biotechnological processes must be highly effective and competitive compared to the other commonly used techniques in order to attain commercial feasibility. The extensive research and development of PUFA production by SSF is basically aimed at improving the economic competitiveness compared to plant- and animal-derived oils. Emphasis is laid on increasing the product value, using inexpensive substrates, screening for more efficient strains, and reducing the processing steps that can lead to bioproducts with high PUFA content. Because SSF can be carried out on a variety of agricultural materials and residues that have limited nutritional values (including cereal wastes), it is necessary to optimize the potential of microorganisms for the transformation of these substrates into desired metabolites (Certik et al, 2008a, 2008b).

However, many scale-up problems of SSF processes employed in PUFA production have to be solved before a laboratory-scale process can be transferred to the commercial level. There are fewer studies on the factors influencing the microbial growth and lipid formation in large-scale SSF than those with submerged fermentation. The two most important factors are mass transfer (including both transfer of oxygen to the growing microorganisms and transfer of nutrients and enzymes within the substrate solid mass) and removal of reaction temperature generated during fermentation. In general, development of a desirable SSF process for fungal PUFAs involves many steps that are as follows: (i) isolation, screening, and selection of an appropriate microorganism, (ii) optimization of physicochemical and nutritional parameters as well as standardization of process unit operation on a laboratory scale, (iii) scale-up studies and, if necessary, the design and establishment of the pilot plant, (iv) generation of engineering data, design, and layout of the commercial plant, (v) construction of the plant and solving any difficulties in plant operation during the commissioning step, and finally (vi) regular operation of the plant for the production of microbial metabolite(s). Scale-up is therefore the crucial link in the transfer of laboratory-scale processes to commercial production scale and brings several problems such as variations in the biomass formed, large-scale inoculum development, medium sterilization, aeration, agitation, maintenance of constant heat and water balance, pH control, contamination control, heterogeneity, downstream processing, and water and solid waste handling (Lonsane et al., 1992).

Nevertheless, PUFA production by SSF could be a useful method for a newly developing market, as the risk to producers and the investment cost may be expected to be less dramatic.

12.4.1 Microorganisms for Cereal Utilization

A number of lower filamentous fungi belonging to Mucorales have been tested for their effectivity to produce PUFAs during their growth on various substrates. Filamentous molds are considered as ideal microorganisms for SSF processes because: (a) the surface of cereal substrates is sufficiently covered by the fungal mycelium during cultivation, (b) microscopic observations revealed that fungal hyphae penetrate into the cereal particles resulting in efficient utilization of substrates, (c) they produce necessary enzymes for hydrolysis of sources bounded in biopolymers, and (d) they grow at reduced water activity that prevents bacterial contamination. Slovak group has pioneered SSF process, in which microorganisms belonging to *Mucoraceae* easily and efficiently utilized cereals containing starch, proteins, and low amount of lipids, and accumulated lipid with dietetic valuable PUFAs (Slugen et al., 1994). Because these fungi simultaneously decrease antinutrient compounds in the substrates (e.g., phytic acid) and partially hydrolyze substrate biopolymers, prefermented mass with a high content of PUFAs may be used as inexpensive food and feed supplement. Screening of those microorganisms has led to selection of *Thamnidium elegans*, *Cunninghamella echinulata*, *Cunninghamella elegans*, *Mucor mucedo*, and *Mortierella isabellina* as producers of GLA (Certik et al., 2006; Certik, 2008) and *Mortierella alpina* as a producer of DGLA, AA, and EPA (Certik, 2008; Certik et al., 2008a). Thus, fungal PUFAs were accumulated in the newly formed cereal bioproduct and their amount depended on the substrates, microorganisms, and cultivation conditions used (Table 12.1).

12.4.1.1 Microbial PUFA Biosynthesis Because microorganisms have often been considered for the production of PUFA-rich oils, the prospect for efficient microbial PUFA production requires knowledge of the mechanisms by which their biosynthesis and accumulation is commonly accomplished within the cell. PUFA biosynthesis in aerobic organisms is associated with membrane-bound enzymes, and the desaturation system of the aerobic pathway is composed of three proteins: NAD(P)H-cytochrome b_5 reductase, cytochrome b_5, and the terminal cyanide-sensitive desaturase. There are three types of desaturases: (i) acyl-CoA desaturases, (ii) acyl-ACP desaturases, and (iii) acyl-lipid desaturases. Generally, stearoyl-CoA or stearoyl-ACP is the substrate for the first double bond introduction to form oleoyl-CoA or oleoyl-ACP, respectively. Subsequent desaturation takes place in the endoplasmic reticulum where fatty acids bound to phospholipids (especially phosphatidylcholine) are desaturated rather than thiol CoA esters. However, besides this dominant desaturation pathway, direct conversion of fatty acyl-CoA to the corresponding PUFA also occurs in the endoplasmic reticulum (Certik and Shimizu, 1999a).

TABLE 12.1 Production of γ-Linolenic Acid (GLA), Dihomo-γ-Linolenic Acid (DGLA), Arachidonic Acid (AA) and Eicosapentaenoic Acid (EPA) by Solid-State Fermentations of Selected Fungi Utilizing Various Cereal Substrates

Strain	Substrate	PUFA	Yield (g/kg BP)
T. elegans	Spelt flakes/SMG	GLA	7.2
	Oat flakes/SMG	GLA	5.9
	Wheat bran/SMG	GLA	5.0
	Wheat bran/SMG/sunflower oil	GLA	10.0
	Wheat bran/SMG/plant extract	GLA	20.0
	Crushed corn	GLA	10.0
	Rye bran/SMG	GLA	4.2
	Buckwheat/SMG	GLA	4.7
	Millet/SMG	GLA	6.5
	Amaranth/SMG	GLA	4.7
C. echinulata	Barley	GLA	6.1
C. elegans	Barley	GLA	7.0
M. isabellina	Barley	GLA	18.0
M. alpina	Rice	AA	21.4
	Wheat sprout/SMG	AA	36.1
	Wheat bran/SMG	AA	42.3
	Rye bran/SMG	AA	21.9
	Peeled barley	AA	16.2
	Oat flakes	AA	31.2
M. aplina	Cresed sesame seeds	DGLA	21.3
M. alpina	Peeled barley/linseed oil/SMG	EPA/AA	23.4/36.3

Note: Ratio of substrate/SMG was 1:3 (w/w). (SMG - spent malt grains)

The first double bond in the aerobic pathway dominant in eukaryotic cells and some bacteria is invariably introduced into the Δ^9 position of saturated fatty acids; thus, palmitoleic (16:1 *cis* 9) and oleic (18:1 *cis* 9) are the most common monoenes in microorganisms. Oleic acid is then, in general, desaturated by Δ^{12} desaturase to yield linoleic acid, which may be further converted by Δ^{15} desaturase to α-linolenic acid. Thus, these three fatty acids are the basic precursors of the n-9, n-6, and n-3 fatty acid cascades (Fig. 12.2). The next steps for PUFA production are desaturation of appropriate fatty acid precursors by Δ^6 desaturase followed by successive chain elongations and subsequent desaturation(s) to yield the respective C_{20} and C_{22} PUFAs. The n-9 family of PUFA is synthesized from oleic acid and sequential participation of Δ^6 desaturase, elongase, and Δ^5 desaturase to finally produce MA. The n-6 family of fatty acids is usually formed from linoleic acid via desaturation (Δ^6, Δ^5, Δ^4) and elongation steps from linoleic acid through GLA, AA, and adrenic acid (22:4 *cis* 7,10,13,16) to DPA n-6. Finally, two routes operate for n-3 PUFAs synthesis in microorganisms (Certik et al., 1998). In the first route, which is temperature independent, ALA is metabolized via the n-3 pathway to EPA, DPA-ω3, and DHA. The second temperature-dependent route involves the conversion of

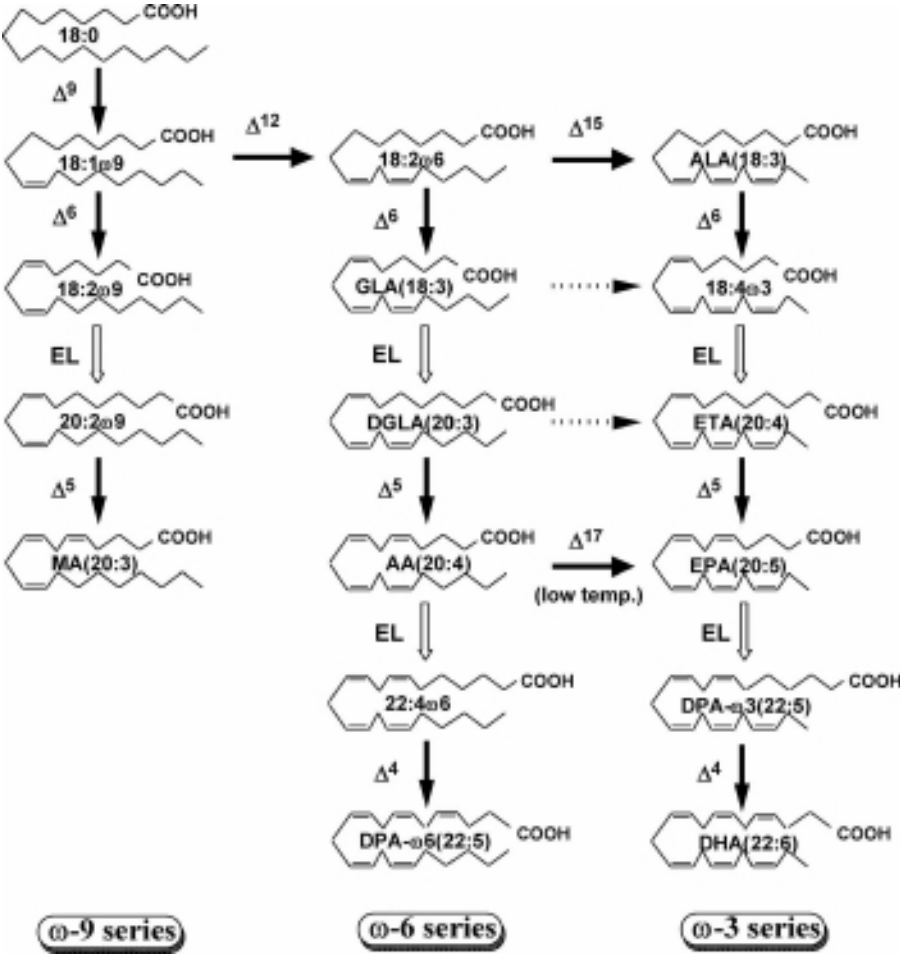

FIGURE 12.2 Biosynthetic pathways of polyunsaturated fatty acids in microorganisms. EL, elongase; Δ^4, Δ^5, Δ^6, Δ^9, Δ^{12}, Δ^{15}, and Δ^{17}, desaturases.

n-6 fatty acids into corresponding n-3 PUFAs catalyzed by two possible enzymes, Δ^{15} and Δ^{17} desaturases (ω^3 desaturases).

12.4.2 Cereal Substrates

A plenty of cereal materials have been tested with the emphasis on achieving bioproducts with high PUFA content (Certik et al., 2006, 2008a). Cereals are well-balanced sources of assimilable carbon with adequate levels of organic nitrogen and other nutrients necessary for fungal proliferation. Microorganisms utilized substrates with varying effectivity and enriched them with oil containing PUFAs. The lipid amount in prefermented products was generally doubled and, depending on

the substrate, ranged from 2% to 12%. GLA or AA concentration in lipids extracted from prefermented mass was permanently low when cereals alone were applied as substrates. On the other hand, the fungal growth on cereals mixed with internal support (e.g., spent malt grains, SMG) resulted in maximal quantities of GLA or AA in isolated lipids. A constantly high yield of GLA or AA in prefermented mass was reached when a mixture of wheat bran/SMG (3:1) served as a substrate for the SSF process (Table 12.1).

12.4.3 Effect of Cultivation Conditions on Production of PUFA-Enriched Cereals

12.4.3.1 Suitable Inoculum and Cultivation Techniques Selection of a suitable inoculum is one of the key factors for each fermentation process. The most used techniques are inoculation with spore suspension or vegetative mycelium. In the case of SSF inoculation with pre-fermented solid material containing microbial culture and residual substrate can also be employed. Because some fatty acids served as a precursor of individual PUFAs, the effect of linoleic acid addition to inoculum on GLA production was also studied. The best GLA yield was obtained by vegetative mycelium without linoleic acid supplement (Slavikova et al., 2005).

Cultivation in plastic bags equipped with cotton plugs offers a good fermentation environment for laboratory-scale experiments. Spreading the substrate in the bags to obtain a thick layer supports appropriate heat removal, oxygen transfer, and the possibility of gentle homogenization. Nevertheless, the handling of bags is quite difficult, especially on a large scale, because plastic bags are susceptible to rupture during substrate sterilization. Erlenmeyer flasks and Petri dishes (covered with glass or plastic foil) represent further possible systems for SSF. The high accumulation of GLA in total fatty acids (14%) was observed in Erlenmeyer flasks. It was probably caused by adequate air supply, as oxygen availability is the limiting factor in the GLA biosynthesis. Although the best GLA yield was observed in the Petri dishes covered with plastic foil, this system is not very suitable for practical use (Slavikova et al., 2005).

12.4.3.2 Internal Support Solid-state fermentations are often carried out with the help of internal support or matrix. Utilization of cereals by fungi was also rapidly improved when SMG were employed as an internal support. The ratio of substrate/SMG is important for satisfied fungal growth and depends on both substrate and microorganism. The substrate/SMG ratio of 3:1 was found to be optimal for the fungal biotransformation of cereals to GLA bioproducts (Certik et al., 2006). Cereals without SMG, in most cases, led to agglomeration of substrate particles and created more compact mass, which in turn interfered with microbial respiration and affected substrate utilization negatively. On the other hand, unbalanced substrate/SMG ratio provided probably limited surface for microbial attack and thus poorer availability of assimilable compounds from substrates. A positive effect of SMG on GLA production is also demonstrated in Figure 12.3, where *T. elegans* grown in the presence of SMG converted linoleic acid from substrates to GLA with higher

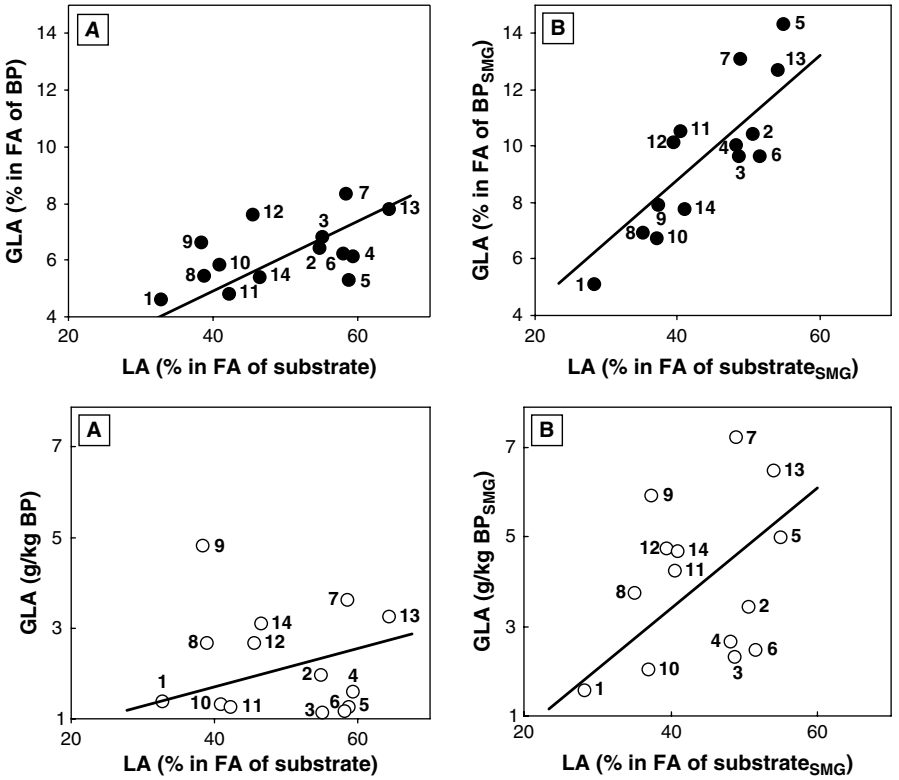

FIGURE 12.3 Effect of the addition of spent malt grains (SMG) on bioconversion of linoleic acid (LA) from substrates to GLA in bioproducts after solid-state fermentation of used cereals by *T. elegans* at 24°C for 4 days. Used cereals: 1, rice; 2, barley; 3, peeled barley; 4, wheat; 5, wheat bran; 6, spelt-wheat; 7, spelt- wheat flakes; 8, oat; 9, oat flakes; 10, rye; 11, rye bran; 12, buckwheat; 13, millet; 14, amaranth. (A: cultivation without SMG; B: cultivation with SMG; filled symbols—relationship between LA content in fatty acids of substrate and GLA content in fatty acids of the bioproduct; open symbols—relationship between LA content in fatty acids of the substrate and GLA yield in the bioproduct).

efficiency (Certik et al., 2006). It is known that both fungal growth and linoleic acid transformation to GLA by Δ^6-desaturase require high oxygen availability. Porous substrates with internal support provided better respiration and aeration efficiency due to an increased interparticle space. This resulted in improved bioconversion of linoleic acid from substrates to GLA. Moreover, SMG may also remove the heat generated during fermentation and finally promote GLA accumulation in the bioproduct.

12.4.3.3 Moisture and Water Activity Appropriate moisture of the substrate is required for satisfactory fungal growth during SSF. In solid-state systems lacking free

water, a certain amount of water still remains necessary for fungal survival. Water is needed for intracellular transport of mass, for physical protection against turgor forces, for evaporative cooling of substrate layers, and, probably the most important, as a solvent for nutrients. There is pronounced distinction among species with respect to the minimum water activity required for their growth. Therefore, restricted water availability can prevent the outgrowth of undesired microorganisms in nonsterile fermentations, especially in combination with extreme pH, thus reducing the need for sterilization. On the other hand, inadequate moisture content leads to suboptimal growth, low degree of substrate swelling and, thereby, declined accessibility of nutrient sources to fungal enzymes. Conversely, high moisture reduces substrate porosity, impairs oxygen diffusion, increases the risk of bacterial contaminations, and restricts gas exchange. Preliminary trials with peeled barley, wheat bran, and a mixture of wheat bran and SMG at 3:1 suggested that dry substrate/water ratio of 1:1.5 was favorable for both lipid and GLA production by filamentous fungi (Slavikova et al., 2002a). This ratio might be associated with better fungal assimilation of starch from cereals because starch gelatinization occurs in well-moistened substrates. The optimal condition for GLA formation was found at a moisture content of 60%. Water content of over 75% suppressed both lipogenesis and GLA production. It was postulated that water in high concentration may fill the interparticle space in the substrate layer affecting the transfer of gases and oxygen transport, which is required for PUFA generation by desaturation system (Certik et al., 2006). The water activity of fermented substrate also significantly influenced fungal growth (formation of air mycelium) and PUFA production. Basically, water activity (a_w values) more than 0.9 provided high PUFA yield.

12.4.3.4 Nutritional Regulation The growth of fungi on cereal substrates resulted in a constant lipid yield with stable fatty acid profile. The basic physiology of microbial lipid overproduction is that the organism is cultivated on a medium consisting of carbon source excess and a limited quantity of other important nutrients such as nitrogen (Certik, 2000). Further improvement of PUFA formation could be achieved by physiological regulation of the SSF process employing the following steps: (a) gradual elevation of carbon/nitrogen ratio with addition of appropriate carbon source; (b) optimization of oxygen availability and mineral supplementation; and (c) transformation of exogenously added oils consisting of precursors of PUFAs.

The optimum C/N ratio for lipid accumulation depends on the type and actual concentration of both carbon and nitrogen sources, and is different for individual microorganism. Oleaginous microorganisms usually do not express their potential to accumulate lipids to any great extent in media with C/N ratios less than 20:1 (Certik, 2000). Cereal materials such as rice bran, wheat bran, oat flakes, peeled or pearled barley provide a suitable source of nutrients for fungal growth and lipid production, with high carbon content in starch, and adequate levels of organic nitrogen resulting in C/N ratio ranging commonly from 20 to 60. However, when glucose or whey was added to cereal substrates, lipid accumulation and GLA content was improved by 80% and 60%, respectively (Slavikova et al., 2002b).

Supplementation of cereals by selected ions (e.g. Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}) was also tested. It was found that lipid biosynthesis was significantly stimulated by Mg^{2+} and Zn^{2+} ions whereas the presence of Ca^{2+} reduced lipid accumulation in prefermented cereals. Fungal production of GLA also increased by 50% when Mg^{2+} ions were employed.

The ability of filamentous fungi to utilize exogenous fatty acids opens new possibilities to prepare PUFAs in high yield. There is a stock of relatively cheap vegetable oils containing individual fatty acid precursors. SSF was applied for fungal utilization of cereals enriched with several plant oils with the aim to enhance the overall content of PUFAs in prefermented mass. Fermentation of cereals with addition of sunflower oil, which consisted of 60–70% of linoleic acid, led to a 100% increase in GLA yield (Slavikova and Certik, 2005).

12.4.3.5 Modification of PUFA Yield by Plant Extracts The biosynthesis and profile of fatty acids is controlled by enzymes involved in lipogenesis, so the activation or inhibition of these metabolic steps is also a useful tool for improving carbon flux to individual PUFAs. For example, linoleic acid is directly converted to GLA by fungal Δ^6 desaturase; regulation of this metabolic step might be crucial for further improvement of GLA amount in cereals. From this point of view, application of various plant extracts that could possess bioactive compounds regulating fatty acid biosynthetic machinery seems to be a promising way to prepare bioproducts with a high yield of preferred PUFA. Because there are a variety of polar or nonpolar compounds in plants, two types of solvents (ethanol and acetone, respectively) were used to prepare plant extracts. Application of ethanol extracts from ginger or sweet flag improved the GLA yield by 30% or 25%, respectively (Certik et al., 2003). On the other hand, biosynthesis of GLA was reduced by 70% when acetone extract from tansy was added to the substrate.

12.4.4 Cereal Bioproducts Enriched with PUFAs

T. elegans effectively utilized various types of cereals and enriched them with oil containing GLA. Depending on the substrate and the optimization of cultivation conditions, several cereal products enriched with GLA were achieved: substrates supplemented with sunflower oil resulted in bioproducts containing up to 25% lipids with total GLA yield of 10 g/kg of bioproduct; prefermentation of substrates supplemented with plant extract yielded up to 20 g GLA/kg of bioproduct. In addition, *M. isabelina* sufficiently enriched barley with 18 g GLA/kg (Table 12.1).

Solid-state fermentations were also employed to improve the market for fungal AA-rich bioproducts. Screening of many fungi has resulted in selection of *M. alpina* that has also been used for preparation of microbial oil with high AA content by submerged fermentations (Certik and Shimizu, 1999a). The growth of this fungus on agroindustrial materials during SSF was much slower compared with *T. elegans* and prefermentation was completed after 10–14 days. Nevertheless, the search for optimal substrate revealed that *M. alpina* satisfactorily converted the mixture of wheat bran/SMG (3:1, w/w) leading to a bioproduct with 4.2% AA (Table 12.1).

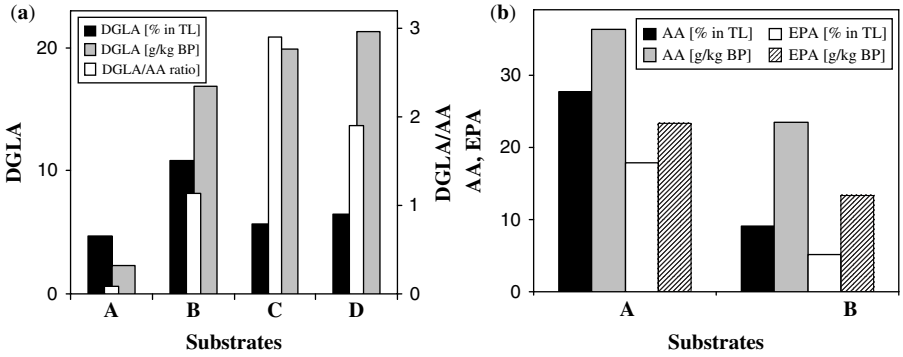

FIGURE 12.4 Dihomo-γ-linolenic acid (DGLA), arachidonic acid (AA), and eicosapentaenoic acid (EPA) content in total lipids (TL) and bioproduct (BP) after solid-state fermentation of *Mortierella alpina* grown on various substrates (substrates do not contain DGLA, AA, or EPA). (a) A, peeled barley; B, peeled barley/sesame seeds (1:1, w/w); C, crushed sesame seeds (13 days SSF); D, crushed sesame seeds (18 days SSF). (b) A, peeled barley/linseed oil/spent malt grains (0.5:1:3, w/w); B, wheat flour/linseed oil/yeast extract/spent malt grains (0.5:2:0.1:3, w/w).

Basic cultivation of *M. alpina* usually leads to a standard fatty acid profile with predominant concentration of AA and only low level of DGLA. Since bioconversion of DGLA to AA is catalyzed by Δ^5 desaturase, inhibition of this metabolic step is accompanied by a rapid increase of DGLA/AA ratio. This strategy was applied during SSF for the preparation of bioproduct enriched with DGLA by *M. alpina* (Certik and Shimizu, 1999a). Addition of sesame seeds (they contain sesamin analogues, which are known to be effective inhibitors of Δ^5-desaturase) to peeled barley rapidly enhanced DGLA levels in the bioproduct and DGLA/AA ratio as well (Fig. 12.4a). However, when *M. alpina* utilized only crushed sesame seeds, the DGLA/AA ratio in the bioproduct after 13 days of cultivation reached a value of 2.9 that corresponded to almost 20 g DGLA/kg of bioproduct. Subsequent cultivation for further 5 days led to the final 21.3 g DGLA/kg of bioproduct (Certik et al., 2008b).

The SSF process has been developed to prepare EPA-rich bioproducts using fungi that can rapidly utilize, incorporate, and modify exogenously added oils. Linseed oil consists of about 57% α-linolenic acid, which is a precursor of PUFAs belonging to n-3 fatty acid family. Because *M. alpina* possesses appropriate enzymatic apparatus for possible transformation of α-linolenic acid to EPA (Certik et al., 1998), linseed oil was added to the substrate with the aim to shift formation of n-6 fatty acids (AA) to ω-3 fatty acids (EPA). This effort has led to prefermentation of the optimized mixture of peeled barley/linseed oil/SMG (0.5:1:3, w/w) by *M. alpina*, which simultaneously yielded 23.4 g EPA/kg of bioproduct (17.8% EPA in oil) and 36.3 g AA/kg of bioproduct (27.6% AA in oil) (Fig. 12.4b). Thus, such a strategy allows the preparation of oils with desirable n-6/n-3 PUFA ratio, finally leading to more beneficial human applications (Certik et al., 2008b).

12.4.5 Application of Cereal-PUFA Bioproducts

Wheat, due to its wide application in cereal goods, is one of the potential substrates for prefermentation with PUFA-forming fungi. Wheat-based PUFA-enriched bioproduct was employed for making cereal goods (1, 2, 5, and 10% of the PUFA-bioproduct was added to flour wheat) such as rolls, bakery products with higher oil level, and pasta. Elevated concentration of the bioproduct increased water absorption of the dough, which resulted in changed rheological properties of the dough such as reduction of dough rise, prolonged dough development time, and higher softening degree of dough. The brown color and crackness of the final cereal goods intensified with an enhanced amount of the PUFA bioproduct. Sensorial analysis also revealed that increasing the amount of the bioproduct affected typical fungi flavor (originated from filamentous fungi applied in SSF) in the cereal goods positively (Certik et al., 2005; Dodok et al., 2005).

12.5 GENETIC TRANSFORMATION OF PLANTS

Recombinant DNA technology has significantly augmented the conventional crop improvement. Genetic transformation offers direct access to a vast pool of useful genes not previously accessible to plant breeders. Current genetic engineering techniques allow coordinated approaches to the introduction of novel genes/traits into the selected plants. Dramatic progress has been made over the past two decades in manipulating genes from diverse sources and inserting them into microorganisms and crop plants.

There is considerable interest in the possibility of using plant genetic engineering to produce foodstuffs that have been modified to improve the levels of endogenous nutrients and enhance their nutritional composition or alternatively to introduce non-native compounds (e.g., unusual lipids or fatty acids) into plants (Tucker, 2003; Bhalla, 2006). Transgenic plants are therefore considered as "green factories" for the synthesis of useful compounds in a sustainable and cheap manner (Alonso and Maroto, 2000; Napier et al., 2006). There are also significant economic and ecological drivers for developing transgenic plants as novel sources of such nutrients; many of these compounds are currently obtained from nonsustainable or subeconomic sources.

Genetic engineering can be used to change the metabolic pathways to increase the production and amounts of various useful metabolites in plants (e.g., PUFAs). Altering the composition of fatty acids is being increasingly targeted for value addition. It is now possible to change the fatty acid profile by introducing genes responsible for the biosynthesis of PUFAs. On the other hand, more unsaturated fatty acids (e.g., linoleic or linolenic acid) content may be decreased in order to elevate the content of monounsaturated fatty acids (e.g., oleic acid), which allow processing without the traditional use of hydrogenation, and thus avoiding the undesirable trans-fatty acids (Sharma et al., 2002).

12.5.1 Biochemical Pathways for PUFA Production in Plants

Transgenic plants engineered to produce major PUFAs, such as GLA, DGLA, AA, EPA, and DHA, could be one way of overcoming the foreshadowed shortage of these nutritionally important fatty acids. During the last few years, many of the genes that are responsible for the biosynthesis of PUFAs have been cloned from various organisms, including fungi, algae, mosses, plants, nematodes, and mammals (Chen et al., 2006; Warude et al., 2006). However, successful changing of PUFA metabolic pathway would require the introduction of genes controlling each of the biosynthetic steps in the pathway for the synthesis of PUFAs from their adequate C18-precursors.

12.5.1.1 Conventional PUFA Pathway The first step in PUFA pathway is the introduction of a double bond at the Δ^6 position by the action of the Δ^6-desaturase. This enzyme is able to operate on both linoleic acid, to produce GLA, and α-linolenic acid, to produce stearadonic acid (18:4 n-3, SDA), thereby initiating the pathways for n-6 and n-3 PUFA synthesis, respectively. In recent years, Δ^6-desaturase genes have been cloned from a range of plants (e.g., *Anemone leveillei*, borage, *Echium* sp., *Marchantia polymorpha*, and *Primula* sp.), fungi (e.g., *M. alpina*, *Mucor rouxii*, and *Pythium irregulare*), diatoms (e.g., *Phaeodactylum tricornutum*), and mosses (e.g., *Ceratodon purpureus* and *Physcomitrella patens*) (for references see Zhou et al., 2006; Warude et al., 2006). To continue the transgenic production of other PUFAs, Abbadi et al. (2004) expressed the genes for the conventional Δ^6-pathway (Δ^6-desaturase, elongase, Δ^5-desaturase) in linseed under the control of seed-specific promoters. They observed very high levels (>25% total fatty acids) of Δ^6-desaturated fatty acids (GLA, SDA) but only low levels of the subsequent elongation products, with AA and EPA accumulating to 0.9 and 0.8% of total seed fatty acids. Thus, it was concluded that there was a "bottleneck" preventing the elongation step. The very inefficient elongation of GLA and SDA was almost certainly due to the absence of these fatty acids in the acyl-CoA pool, which in turn was likely to be due to poor acyl exchange with phospholipids.

12.5.1.2 Reverse-Engineering PUFA Pathway On the other hand, successful accumulation of C20 PUFAs in transgenic plants was first reported by Qi et al. (2004), where both EPA and AA were synthesized in transgenic *Arabidopsis* plants, accumulating to 3.0 and 6.6%, respectively. Although the achieved levels of these PUFAs were somewhat lower than the optimal target range, the fact that these fatty acids were synthesized and accumulated with no deleterious effects to the host transgenic plant demonstrated an important "proof-of-concept." However, several important points must be emphasized. Firstly, the accumulation of higher PUFAs was carried out by "reverse-engineering" of the PUFA pathway, which was not the conventional Δ^6-desaturase/elongase route but the alternative Δ^9-elongase/Δ^8-desaturase system (Napier et al., 2004). In that respect, the relative success in this approach was derived from the ability of the (initiating) Δ^9-elongase to utilize

endogenous linoleic and α-linolenic acid substrates present in the acyl-CoA pool. This in turn would provide C20 substrates for the subsequent desaturases and be less dependent on the "switching" of substrates from glycerolipids and acyl-CoA pool (Napier et al. 2004). Secondly, the final transgenic lines accumulating AA and EPA were the result of three sequential genetic transformations, in which the D9-elongase, Δ^8-desaturase, and Δ^5-desaturase were introduced as separate transgenes and integration events. Finally, in addition to the accumulation of AA and EPA, some nonmethylene interrupted C20 fatty acids (sciadonic acid 20:3 Δ5,11,12, n-6 and juniperonic acid 20:4 Δ5,11,14,17, n-3) were also detected (Napier et al., 2006).

Although there have not been any reports dealing with successful transformation of "PUFA genes" to cereals, studies mentioned above represent important biotechnological breakthroughs in a major step forward in the production of very long PUFAs in transgenic plants. They also provide some new insights into the biochemical pathways under manipulation and provide useful new tools for the dissection of the underlying enzymatic reactions. Thus, these biotechnological techniques offer a great promise to prepare tailor-made plants enriched with valued oil compounds that are used in medicine, pharmaceutical, food, and feed industry.

12.6 CONCLUSIONS AND PERSPECTIVES

The development of biotechnological enrichment of cereals with PUFAs will undoubtedly depend on their commercial potential and demand. Current achievements with naturally prepared PUFA-cereal bioproducts by fungal solid-state fermentation confirm the potential of these fermentation techniques. PUFA-cereal bioproducts should in general be considered safe for the production of food or feed ingredients, so they may be used as an inexpensive food and feed supplement. Therefore PUFA-based cereals may fill marketing claims in food, feed, pharmaceutical, and veterinary fields. Thus, the association of oleaginous lower filamentous fungi with solid-state fermentations has created new perspectives for economic competitiveness and a market of cereal-based bioproducts containing PUFAs.

ACKNOWLEDGMENT

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13

LIPOPHILIC GINSENOSIDE DERIVATIVES PRODUCTION

JIANG-NING HU AND KI-TEAK LEE

Department of Food Science and Technology, Chungnam National University, 220 Yusung Gu, Gung-Dong, Daejeon, 305-764, Republic of Korea

13.1 INTRODUCTION

Ginseng, the root and rhizome of *Panax ginseng* C.A. Meyer (Araliaceae), has been widely used in Asian regions for more than 2000 years. The genus name of *P. ginseng* "*Panax*", which derived from "panacea", was given by the Russian botanist, meaning cure all and longevity. The species name "ginseng" comes from the Chinese word "rensheng" which means "human" as ginseng root resembles the human body (Nocerino et al., 2000). Besides *P. ginseng*, some other species of the genus *Panax* are also called ginseng; these include *Panax quinquefolius* L. in the northeastern parts of the United States and Canada; Japanese Chikusetsu Ginseng (*Panax japonicus*

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C.A. Meyer) as an alternative to ginseng; *Panax notoginseng* (*P. notoginseng* Burk.) in Yun-nan Province in China; *Panax pseudoginseng* in Nepal and eastern Himalayas; and *Panax vietnamensis* in Vietnam (Yun, 2001). Ginseng is a very famous phytomedicine used all over the world and has served as an important component of many Chinese prescriptions for thousands of years due to its well-known health benefits both as a tonic and as a sedative agent as described in the oldest Chinese Materia Medica, the *Shen Nong Ben Cao Jing*. Ginseng still occupies a permanent and prominent position in the herbal list. In this review, we attempt to systematically summarize the information on ginseng, including chemical composition, traditional usage, modern pharmacological activities, metabolism pathway in the human body, and bioconversion to obtain lipophilic ginsenoside derivatives *in vitro*.

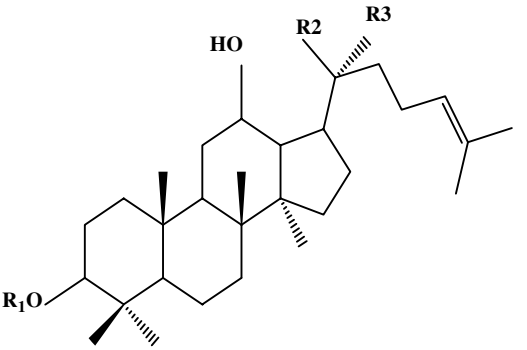
13.2 CHEMICAL COMPOSITION AND TRADITIONAL USAGE OF *Panax ginseng*

The main components of *P. ginseng* include ginseng saponins, polysaccharides, amino acids (glutamine and arginine, in particular), flavonoids, essential oils, and so on. The principal active ingredients have been investigated to be polysaccharides and ginseng saponins, which exhibit multiple pharmacological actions both *in vitro* and *in vivo* (Steve Helms, 2004).

Acidic polysaccharides (10,000–150,000 MW) have recently been observed to have immunomodulating (Park et al., 2001), radioprotective (Kim et al., 2007), and antitumor effects (Kim et al., 1998). These polysaccharides contain various sugar moieties, uronic acid, and less than 5% protein by weight. They are readily soluble in water.

Ginseng saponins, generally called ginsenosides, are unique to *Panax* species, and believed to be responsible for most of the actions of ginseng (Attele et al., 1999). Additionally, ginsenosides operate through many mechanisms of action, and it was suggested that each ginsenoside may have its own specific tissue-dependent effect (Murphy and Lee, 2002). They consist of a gonane steroid nucleus with 17 carbon atoms arranged in four rings. Based on their structural differences, all ginsenosides are of the dammarane type and are further separated into the panaxadiol (e.g. Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, and Rs1) (Fig. 13.1) and panaxatriol type (e.g. Re, Rg1, Rf, Rg2, and Rh1) (Fig. 13.2) with the exception of ginsenoside Ro, which is an oleanane-type triterpenoid (Fig. 13.3) (Shoji, 2001).

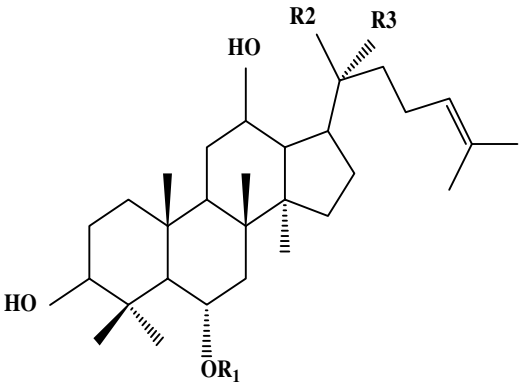
As noted in the oldest Chinese Materia Medica, *Shen Nong Ben Cao Jing*, ginseng was described as having many pharmacological functions such as restorative, tonic, nootropic, anti-aging, longevity imparting, and so on (Xiang et al., 2008). According to traditional Chinese medicine theories, traditional Chinese medicine emphasizes individual treatment, and mainly uses herbs to improve the pathological conditions to adjust the balance of the human body. In Asian countries, ginseng products are usually used as a general tonic and adaptogen to help the body to resist the adverse effects of a wide range of physical, chemical, and biological factors and to restore homeostasis



	R_1	R_2	R_3
1. Ginsenoside -Ra_1	- glc (2-1) glc	-glc (6-1) arap (4-1) xyl	-Me
2. Ginsenoside -Ra_2	- glc (2-1) glc	-glc (6-1) arap (2-1) xyl	-Me
3. Ginsenoside -Ra_3	-glc (2-1) glc	-glc (6-1) arap (3-1) xyl	-Me
4. Ginsenoside -Rb_1	- glc (2-1) glc	- glc (6-1) glc	-Me
5. Ginsenoside -Rb_2	- glc (2-1) glc	- glc (6-1) arap	-Me
6. Ginsenoside -Rb_3	- glc (2-1) glc	-glc (6-1) xyl	-Me
7. Ginsenoside -Rc	-glc (2-1) glc	- glc (6-1) araf	-Me
8. Ginsenoside -Rd	-glc (2-1) glc	- glc	-Me
9. Ginsenoside -Rg_3	-glc (2-1) glc	-H	-Me
10. Ginsenoside -F_2	- glc	-glc	-Me
11. Ginsenoside -Rh_2	-glc	-H	-Me
12. Ginsenoside -Compound K	-H	-glc	-Me
13. Ginsenoside -Protopanaxadiol	-H	-H	-Me

FIGURE 13.1 The chemical structure of protopanaxadiol-type ginsenosides from *Panax ginseng*.

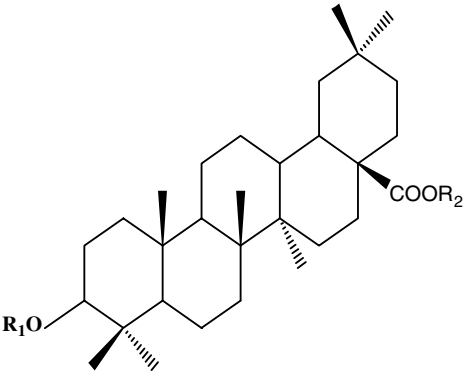
(Brekhman and Dardymov, 1969). These tonic and adaptogenic effects of ginseng are believed to enhance the physical performance and general vitality in healthy individuals, to increase the body's ability to fight stress in stressful circumstances, and to support resistance to diseases by strengthening normal body function, as well as to reduce the detrimental effects of the aging processes (Tyler, 1993; O'Hara



	R_1	R_2	R_3
1. Ginsenoside -Re	-Rha (2-1) glc	-O-glc	-Me
2. Ginsenoside -Rf	-glc (2-1) glc	-OH	-Me
3. Ginsenoside –Rg_1	-glc	-O-glc	-Me
4. 20 (S)-Ginsenoside –Rg_2	-Rha (2-1) glc	-OH	-Me
5. 20 (R)-Ginsenoside –Rg_2	-Rha (2-1) glc	-Me	-H
6. Ginsenoside –Rh_1	-glc	-OH	-Me
7. Ginsenoside –protopanaxadiol	-H	-OH	-Me
8. 20-gluco-Ginsenoside -Rf	-glc – glc	-O-glc	-Me
9. Ginsenoside Rg_4	-Glc(2-1)rha	-OH	-Me
10. Ginsenoside F_1	-H	-O-glc	-Me
11. Ginsenoside F_3	-H	-O-glc (6-1) Arap	-Me
12. Ginsenoside F_5	-H	-O-Glc (6-1) Araf	-Me

FIGURE 13.2 The chemical structure of protopanaxatriol-type ginsenosides from *Panax ginseng*.

et al., 1998). In spite of being used for several centuries with effectiveness in Asia, many mechanisms of ginseng activity are still unclear. The traditional Chinese medicine theories about ginseng are difficult to understand. Since the beginning of the 20th century, people have attempted to use modern chemistry and pharmacology to study ginseng. At the end of the 1960s in particular, modern study of ginseng has made significant progress. Recently, the pharmacological activities of the chemical



	R1	R2
Ginsenoside-Ro	-β-D-Glucuronic acid (2-1)-glc	-glc

FIGURE 13.3 The chemical structure of oleanane-type pentacyclic triterpene ginsengoside from *Panax ginseng*.

constituents and the therapeutic uses of ginseng have generally been defined (Xiang et al., 2008). The pharmacological activities of ginseng focus on central nervous system, cardiovascular system, anticancer, immune system, and so on. The main pharmacological activities of ginseng and its major components (ginsenosides) were systematically summarized by Radad et al. (2004) and Xiang et al. (2008), respectively.

13.3 METABOLISM OF GINSENOSIDES IN HUMAN BODY

Although many researches have reported that ginsenosides have significant functions and pharmacological effects on human health, the metabolic pathway of these ginsenosides in human body is still not clear. Ginsenoside is not the real active component in the human body. It acts as a prodrug that is metabolized to the active form by intestinal bacterial deglycosylation (Hasegawa, 2004; Kobashi et al., 1992; Kobashi and Akao, 1997).

13.3.1 Deglycosylation of Ginsenosides by Intestinal Bacteria

According to the theories of traditional Chinese medicine, medicinal herbs are normally administered orally. Hence, these herbs and their ingredients must contact gastric juice and digestive and bacterial enzymes in the intestines. There is

no exception to ginseng. Ginsenosides are usually considered as major active compounds in the human body. However, more reports have shown that other than the exception of slight oxygenation ginsenoside is hardly decomposed by gastric juice. The total bioavailability of intact ginsenosides from the intestines is extremely low (Rb1, 0.1–4.4%; Rb2, 3.7%; and Rg1, 1.9–18.4%) (Kobashi and Akao, 1997; Takino, 1994; Xu et al., 2003). When ginseng extract was orally administered to humans, ginsenoside F1, compound K (M1), ginsenoside Rh1, and protopanaxatriol ginsenoside (M4) were detected in the urine and blood (Tawab et al., 2003). These results suggested that protopanaxadiol-type ginsenosides may be metabolized, mainly to compound K (M1), and protopanaxatriol-type ginsenosides to ginsenoside Rh1, ginsenoside F1, and protopanaxatriol (M4). Yang et al. reported that ginsenoside Rd was administrated intravenously to the volunteers (Yang et al., 2007). Results showed that most of the ginsenoside Rd remained unchanged and was excreted through rat urine; only a little amount of Rd was subject to change during metabolic processes in humans. The metabolic pathways of ginsenoside Rd differed depending upon the different administration routes. Among the metabolic compounds, ginsenoside Rb1 dominated the profile obtained from intravenous administration, while ginsenoside Rg3 dominated the profile obtained from oral administration. Conclusively, the main metabolic pathways are supposed to be as follows: protopanaxatriol type, Rb1 → Rd → F2 or ginsenoside X → ginsenoside LXXV → M1 (ginsenoside compound K); Rb2 → M6 → M2 → M1, and Rc → M7 → M3 → M1 (M1 is gradually hydrolyzed to M12); protopanaxatriol-type, Re → Rg1 → M11 (F1) or M8 (Rh1) → M4 (Hasegawa, 2004).

13.3.2 Esterification of Intestinal Bacterial Metabolite with Fatty Acid

Ginsenosides are deglycosylated by intestinal bacteria to the active form M1 after oral administration. M1 was selectively accumulated in the liver, and most M1 was excreted as bile. Approximately 24 mol% of dosed M1 was esterified with fatty acids in the liver without structural variation. Esterified M1 (EM1) was not excreted in the small intestine and provided stronger activities than M1. Structural analysis indicated that EM1 was a family of fatty acid M1 monoesters, including stearate, oleate, or palmitate. The fragment pattern of EM1 suggests that fatty acid is linked to M1 at C-3 of the aglycone moiety or C′-6 of the glucose moiety (Hasegawa et al., 2000).

M4, as the main bacterial metabolite of protopanaxatriol-type ginsenosides after oral administration, was completely absorbed from the small intestine into the mesenteric lymphatics followed by the rapid esterification of M4 with fatty acids, which occurred at C-3 of the aglycone moiety, and its accumulation in the tissues including the liver and lungs. Esterified M4 (EM4) spread to other organs in the body followed by its excretion as bile. The esterified M4 did not directly affect the tumor *in vitro*, whereas it stimulated splenic NK cells to become cytotoxic to tumor cells (Hasegawa et al., 2002). Some pharmacological activities of these ginsenoside metabolites have been summarized by Hasegawa et al. (2004).

13.4 PRODUCTION OF LIPOPHILIC GINSENOSIDE DERIVATIVES USING ENZYME

13.4.1 Degradation of Ginsenoside by Microorganisms

In order to get more amounts of lipophilic ginsenoside derivatives (such as M1 and M4) *in vitro*, recently, researchers have focused on the transformation of major ginsenosides into their metabolites using methods such as acid treatment (Yang et al., 2001), alkali treatment (Yang et al., 2003), heating (Kim et al., 2000), and enzymatic conversion (Yang et al., 2001) *in vitro*. Among these methods, enzymatic hydrolytic treatment is considered ideal due to the specific hydrolysis of the glucose moiety from ginsenoside skeleton. According to the clue of pathway of ginsenoside metabolism in human body, many researches tried to transform ginsenosides to their metabolite using intestinal bacteria and other bacteria (Chi and Ji, 2005; Ko et al., 2003; Park et al., 2001). Our research group has also successfully identified one fungus (*Aspergillus niger*) from soil to transform ginsenosides to compound K (Hu et al., 2008). In this study, the conditions of β-glucosidase production from *A. niger* were optimized using the response surface methodology, and we found that wheat bran, KH_2PO_4, and stirring speed had the maximum effects on β-glucosidase production. Under the optimized conditions of wheat bran concentration of 34.51 g/L, KH_2PO_4 concentration of 1.78 g/L, and stirring speed of 161.60 rpm/min, *A. niger* produced the highest β-glucosidase activity of 4650.14 U/mL.

The optimized conditions were subsequently applied to transform ginsenoside Rb1 into compound K. The metabolite of ginsenoside Rb1 transformed by *A. niger* in our study was completely compound K within 8 h. The putative conversion pathway of Rb1 examined by HPLC chromatograms was Rb1, Rd, F2, and compound K (Fig. 13.4). Other reports showed a similar pathway for protopanaxatriol to different extents using different food microorganisms. Yousef et al. reported that the ginseng root pathogen *Pythium irregulare* had the ability to biotransform a mixture of 20(*S*)-protopanaxadiol ginsenosides by an extracellular glycosidase into ginsenoside F2 through the hydrolysis of the terminal monosaccharide units from disaccharides present at C-3 and/or C-20 of ginsenosides Rb1, Rc, Rb2, and G-X□ (Yousef and Bernards, 2006). Another example showed that β-glucosidase from China white jade snail hydrolyzed the 20-C, β-(1 → 6)-glucoside of ginsenoside Rb1 to produce ginsenoside Rd, but did not hydrolyze further to F2 or compound K (Luan et al., 2006). Several ginsenosides such as Rb2 and Rc have been transformed into ginsenoside compound K by food microbial enzymes within 24 h (Chi et al., 2005).

13.4.2 Synthesis of Ginsenoside Esters using Commercial Enzymes

In order to increase the lipophilic solubility of ginsenosides, which would increase their tract and cellular absorption, esterification provides a route to obtain more nonpolar ginsenoside derivatives. Until now, several research groups have already reported the feasibility of the lipase-catalyzed acylation of various polyhydroxylated compounds, such as flavonoids, sugars, and glucosides as well as ginsenosides in both

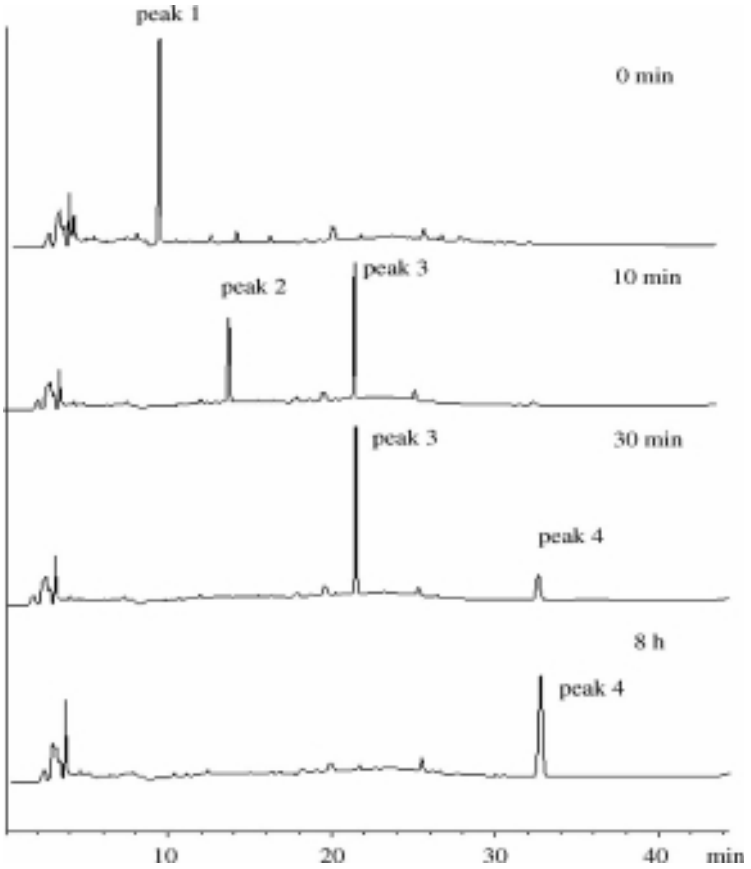


FIGURE 13.4 HPLC chromatograms of transformation of Rb_1 into compound K during different periods by *A. niger*. Peak 1, ginsenoside Rb_1; peak 2, ginsenoside Rd; Peak 3, ginsenoside F_2; Peak 4, compound K.

toxic and less toxic organic media (Gao et al., 2000; Pedersen et al., 2002; Danieli et al., 1997; Ardhaoui et al., 2004; Zhang et al., 2007). Recently, our research group also attempted to study the synthesis of ginsenoside Rb1 esters using *Candida antarctica* lipase B (Novozyme 435) (Hu et al., 2008). We investigated the lipase-catalyzed synthesis of ginsenoside Rb1 esters with different acyl donors (vinyl acetate, vinyl butyrate, and vinyl decanoate) in different solvents (*tert*-butanol, *tert*-amyl alcohol, and a mixture of *tert*-butanol and pyridine). The enzymatic reactions were carried out in a temperature-controlled water shaker at 185 rpm. GRb1 (2 mg/mL), 4 Å molecular sieves (50 mg/mL), and an acyl donor were added in a suitable solvent such as *tert*-amyl alcohol. The reactions were performed by adding Novozyme 435 enzyme and shaking the reaction mixture at a temperature ranging from 25 to 65°C; samples were withdrawn at different times ranging from 12 to 60 h and then filtered through a

syringe filter (0.45 μm) prior to HPLC-MS/MS analysis. The effects of three acyl donors with different carbon chains (vinyl acetate, vinyl butyrate, and vinyl decanoate) and three reaction solvents (*tert*-amyl alcohol, *t*-BuOH, and a mixture solvent of 1:1 of *t*-BuOH and pyridine) on the GRb1 conversion rate showed quite different results. The reaction with vinyl butyrate showed the highest conversion while the lowest conversion rate was observed in the reaction with vinyl decanoate (Fig. 13.5A).

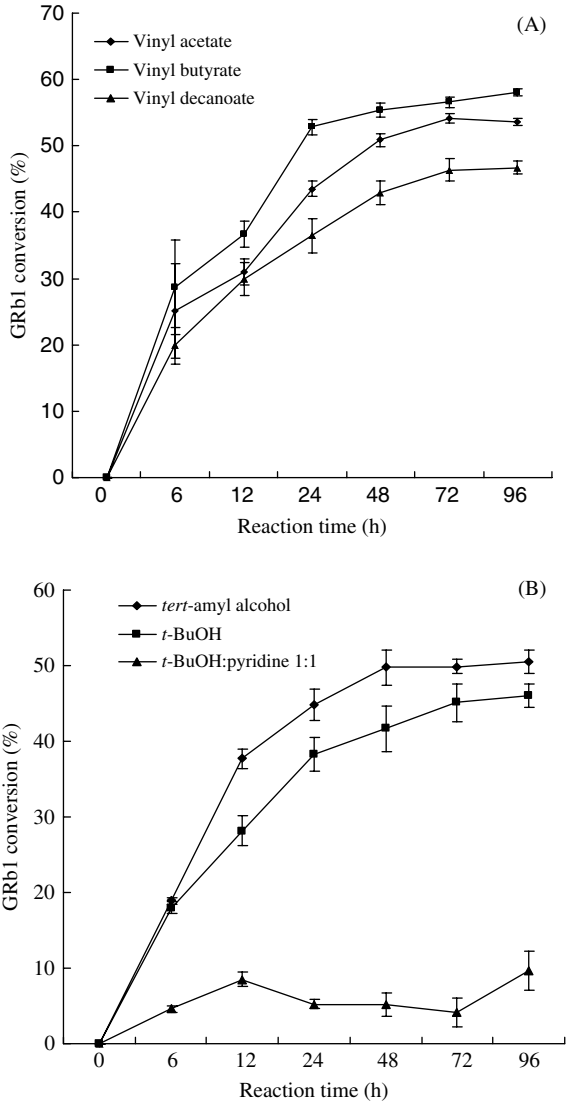


FIGURE 13.5 Effect of different acyl donors (A) and different organic solvent systems (B) on GRb1 conversion in *tert*-amyl alcohol by Novozyme 435 at 45°C.

It seems that acyl donors with short carbon chains were more effective than those with long carbon chains. A similar observation was made by Pedersen et al. (2002) in which the formation of sucrose esters was higher with butanoic acid than with decanoic acid.

In the literature, *tert*-amyl alcohol, *t*-BuOH, acetone, acetonitrile, and the mixture solvent of *t*-BuOH and pyridine were usually used in the enzymatic acylation of natural glycosides due to the high capacity of dissolving natural glycosides (Danieli et al., 1997; Teng et al., 2004; Kontogianni et al., 2003; Gebhardt et al., 2002). As observed, the organic medium indeed influenced the conversion of GRb1 ester. The esterification reaction in *tert*-amyl alcohol showed the highest conversion rate while the reaction in the mixture solvent of *t*-BuOH and pyridine showed very low conversion rate (Fig. 13.5B). The final conversions in *tert*-amyl alcohol and *t*-BuOH after 96 h were 50.54% and 46.02%, respectively. However, in the mixture solvent of *t*-BuOH and pyridine, the conversion was only 9.68% after 96 h.

The esterification reactions were monitored by HPLC-MS/MS. HPLC chromatograms suggested that one species was produced in the reaction with vinyl decanoate while more than one species were generated in the reaction with vinyl acetate and vinyl butyrate (Fig. 13.6). The deprotonated molecules $[M\text{-}H]^-$ of peak 1, 2, 3, and 4 at *m/z* 1107, 1149, 1149, and 1191 suggested that two GRb1 monoacetate and one

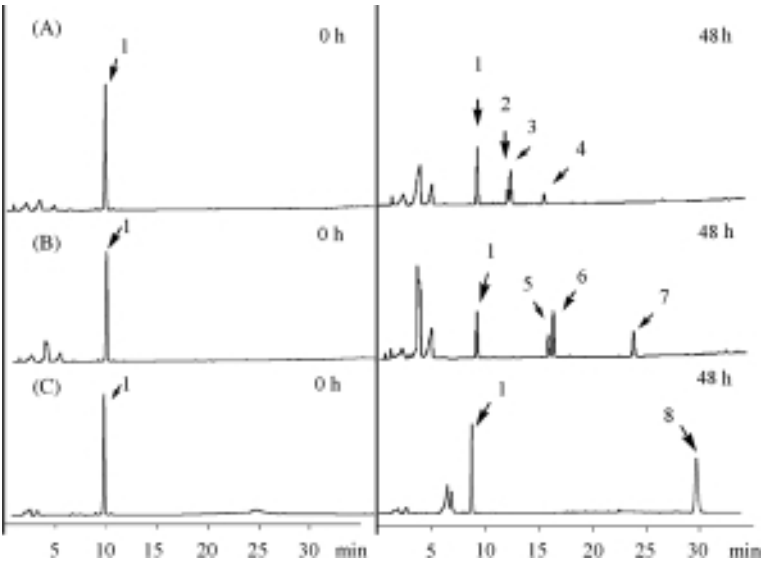


FIGURE 13.6 HPLC chromatograms of the lipase-catalyzed esterification of GRb1 with different acyl donors at 0 or 48 h. (A) Reaction with vinyl acetate; (B) reaction with vinyl butyrate; (C) reaction with vinyl decanoate. Peak: 1, GRb1; 2, 3, and 4, GRb1-acetate species; 5, 6, and 7, GRb1-butyrate species; 8, GRb1-decanoate species.

GRb1 diacetate were formed in the reaction with vinyl acetate; peak 5, 6, and 7 at *m/z* 1177, 1177, and 1247 suggested that two GRb1 monobutyrate and one dibutyrate were generated in the reaction with vinyl butyrate; peak 8 at *m/z* 1261 suggested that one GRb1 monodecanoate was produced in the reaction with vinyl decanoate (Fig. 13.7). The result was in good agreement with that by Gebhardt et al. who reported that two monoacetylginsenoside Rb1 and one di-*O*-acetylginsenoside Rb1 were obtained from the process of acylation of GRb1 by Novozyme 435 (Gebhardt et al., 2002). To allow the increase in GRb1 lipophilicity, the esterification condition of

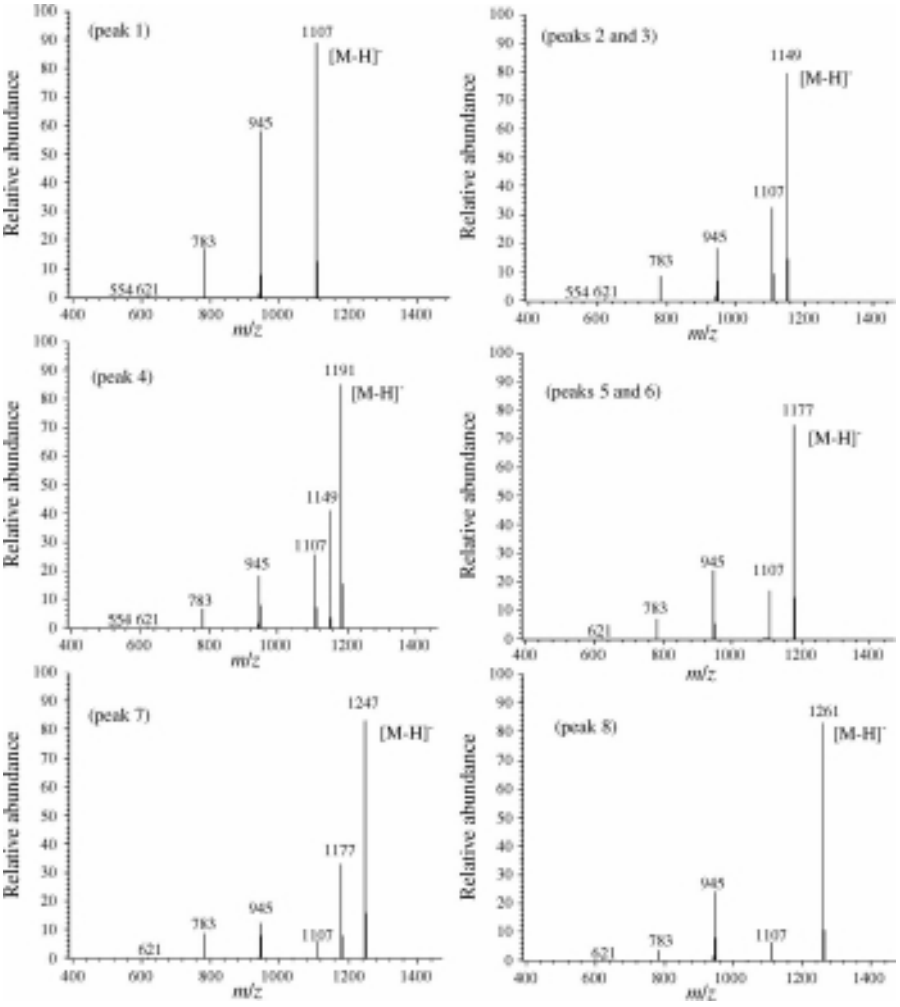


FIGURE 13.7 Mass spectra of GRb1 and new species in the esterification of GRb1 with acyl donors.

GRb1 with vinyl decanoate in *tert*-amyl alcohol was further optimized using response surface methodology (RSM). The maximum GRb1 conversion to GRb1 decanoate ester was predicted to be 61.51% under the following conditions: 40.2 h, 52.95°C, substrate mole ratio 275.57, and enzyme amount 39.81 mg/mL.

In addition, Teng et al. have synthesized many ginsenosides including ginsenoside Rd, Rg3, 20(r)-Rg3, Rh2, Re, Rh1, Rg2, gypenoside VII, and pseudoginsenoside F11 with vinyl acetate, catalyzed by Novozyme 435 in organic solvents to afford different monoacetyl ginsenosides (Teng et al., 2004). Ginsenoside Rd was also acylated with vinyl decanoate or vinyl cinnamate to generate Rd decanoate esters and Rd cinnamate esters, respectively. The acylation of glucosylated ginsenoside Rd, Rg3, 20 (r)-Rg3, Rh2, gypenoside VII, and Rh1 occurred at the primary 6-OH function group of the terminal glucose moiety of the C(3) or C(20) of the dammarane-type aglycone. In contrast, ginsenosides Re, pseudoginsenoside F11, and Rg2, containing mixed sugar moiety, resulted in acylation of both the rhamnose and the glucose moieties. In the case of ginsenoside Re and pseudoginsenoside F11, acylation at the secondary 4-OH function group of the terminal rhamnose moiety, attached at C(3) of the aglycone, is preferred. Among these ginsenosides, the "upper" gentiobiose moiety linked at C-20 proved to be more active than the "lower" sophorose unit linked at C-3 in ginsenoside structures for biocatalyzed acylation and galactosylation (Gebhardt et al., 2002).

Until now, there are few researches reported that natural glycosides and their esters have significant different functions. It has been reported that acylation of cholestane saponins could increase the antitumor potency (Mimaki et al., 1997). As for ginsenosides, there are no reports showing the functions between ginsenosides and their esters. However, compared with ginsenosides, ginsenoside esters have more lipophilic polarity. We expect that ginsenoside esters could increase their tract and cellular absorption in intestines. More pharmacological researches should be further conducted to confirm that.

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14

BROWN SEAWEED LIPIDS AS POSSIBLE SOURCE FOR NUTRACEUTICALS AND FUNCTIONAL FOODS

M. Airanthi K. Widjaja-Adhi, Takayuki Tsukui, Masashi Hosokawa, and Kazuo Miysahita

Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, 041-8611, Japan

14.1 INTRODUCTION

Seaweeds, the largest and most complex marine algae, are photosynthetic like plants that form the basic biomass in the intertidal zone. As seaweeds lack many distinct organs as found in terrestrial plants, whole parts are available as biomass resource. There are about 6000 species of seaweeds divided into three main classes: green (chlorophytes), red (rhodophytes), and brown (phaeophytes). These three groups are not thought to have a common multicellular ancestor. Seaweeds form a part of the

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staple diet in Japan, Korea, and China apart from being used as delicacies in some parts of the Western world. They are therefore branded as sea vegetables (Wong and Cheung, 2000). Seaweeds have been harvested for centuries by many countries, especially in the Asian continent, for several uses. The total world production of commercial aquatic plants, including seaweeds, in 2006 was 16,218,885 tons to which aquacultured seaweeds contributed almost 77.6% (FAO, 2009). Brown seaweeds are the major class contributing about 59% of the total culture production in 2006 followed by red (40%) and green (<1%) seaweeds.

Seaweeds are principally used as sources of phycocolloids, thickening and gelling agents for various industrial applications including uses in foods, though they still continue to be widely consumed as food in Asian countries. They are nutritionally valuable, in both fresh as well as dried forms, as ingredients in a wide variety of prepared foods (Wong and Cheung, 2000). The quality of protein and lipid in seaweed is better compared with other vegetables mainly due to their high content of essential amino acids and the relatively high levels of both omega-3 and omega-6 highly unsaturated fatty acids (HUFA).

The unique and phenomenal biodiversity of the marine environment makes a large pool of novel and bioactive molecules. Marine micro/macroalgae are important potential sources for these bioactives. With increasing commercial demand intensive research has been done on the production of omega-3 HUFA. From this viewpoint, marine and freshwater microalgae have been considered as good sources for these omega-3 HUFA. Some species of these algae contain large amounts of high-quality functional omega-3 HUFA, eicosapentaenoic acid (20 : 5n-3, EPA), and docosahexaenoic acid (22 : 6n-3, DHA). Fish oil is a major source for the commercial production of omega-3 PUFA (Guschina and Harwood, 2006). It contains both EPA and DHA, while several microalgae show preferential production of EPA and DHA. Thus, in order to obtain purified EPA or DHA microalgae is a better source than fish oil.

Seaweeds are also important potential sources for omega-3 HUFA together with sources of omega-6 HUFA such as arachidonic acid (20 : 4n-6). Of late, seaweeds have been explored for some of the lipid-related bioactives and nutraceuticals present in them. It has been recently reported that the seaweeds contain some interesting phytochemicals such as carotenoids, phytosterol, polyphenols, and functional omega-3 and omega-6 HUFA. Carotenoids, phytosterols and polyphenols in general, especially those of terrestrial origin, have been thoroughly reviewed with respect to their occurrence, biological functions, and possible health benefits; however, there has been relatively little information on those of marine origin. The present chapter is aimed at throwing new light on the seaweed lipid characteristics from the view point of its utilization as a nutraceutical and functional food material.

14.2 SEAWEEDS LIPIDS AS A RICH SOURCE OF FUNCTIONAL HUFA

Marine lipids generally contain a wider range of fatty acids than terrestrial plants and animals (Berg and Barnathan, 2005). Omega-3 HUFAs such as EPA and DHA are

typical of marine lipids whereas omega-6 HUFA, mainly linoleic acid, are predominant in common vegetable oils. The importance of EPA and DHA in human health promotion has been confirmed through research. Although many papers have been published on the health beneficial effects of EPA and DHA, there is still an increased level of interest in nutritional and health related issues associated with EPA and DHA.

Another important HUFA, arachidonic acid (20 : 4n-6), is obtained from the diet or synthesized from its parent essential fatty acid, linoleic acid (18 : 2n-6), by desaturation and chain elongation. EPA and DHA are also derived from α-linolenic acid (18 : 3n-3) by alternating with the same desaturase and elonganase as in the biosynthesis of arachidonic acid. The synthesis of functional HUFA from linoleic acid or α-linolenic acid is limited, giving rise to potential shortage at times when need exceeds synthesis rate, such as during the fetal and early neonatal periods. The present diet in most of the countries is low in omega-3 HUFA and rich in omega-6 HUFA. This causes imbalance of omega-3 HUFA/omega-6 HUFA ratio in tissue and blood lipids, implicating in many diseases.

The brain has high arachidonic acid and DHA contents. Newborn human brain weighs about 400 g and increases to 1100 g at the age of 2 years. Both arachidonic acid and DHA are supplied from transplacental transfer in pregnancy and from mother's milk after birth. Infant formula also contain both omega-6 and omega-3 long-chain HUFA whose sources are egg yolk lipids and marine oils, respectively. Brown seaweed lipids are a rich source of two series of long-chain HUFA, omega-6 and omega-3 HUFA (Bhaskar et al., 2008). These HUFA, with proven nutritional significance, have attracted considerable interest as both nutraceuticals and pharmaceuticals (Senanayake and Fichtali, 2006).

We have reported the compositions of major fatty acids of total lipids (TL) from six brown seaweeds (Terasaki et al., 2009) (Table 14.1). Major HUFA from these algae were linoleic acid (18 : 2n-6; 3.8–11.2%), α-linolenic acid (18 : 3n-3; 4.3–10.1%), octadecatetraenoic acid (18 : 4n-3; 5.6–11.8%), arachidonic acid (20 : 4n-6; 6.1–15.2%), and EPA (20 : 5n-3; 9.7–13.2%). Palmitoleic acid (16 : 1n-7; 2.0–6.6%) was the major monoenoic fatty acid apart from oleic acid (18 : 1n-9; 5.6–14.8%), which is in contrast to higher plants (Harwood and Jones 1989). Arachidonic acid is a precursor of eicosanoids with pharmacological significance, while 18 : 4n-3 is a precursor for EPA and DHA. The importance of EPA and DHA in human health promotion has been confirmed through research. Nutritional standards and nutritionists recommend a ratio of 1 : 1.5 to 1 : 2 between omega-3 and omega-6 HUFA (Hamazaki and Okuyama, 2003; Gebauer et al., 2004). From the results, it is very clear that the seaweeds analyzed in this study have a nutritionally desirable ratio of omega-3 to omega-6 HUFA.

14.3 BROWN SEAWEEDS ARE A RICH SOURCE OF POLYPHENOLS

Phlorotannins are the largest group of polyphenols in brown seaweeds (Kang et al., 2004; Nakai et al., 2006; Shibata et al., 2008; Zou et al., 2008). Based on

TABLE 14.1 Main Fatty Acid Composition of Brown Seaweed Lipids

Fatty Acid	*S. horneri* ($n=25$)	*S. thunbergii* ($n=5$)	*S. fusiforme* ($n=17$)	*C. hakodatensis* ($n=17$)	*M. intestinale* ($n=10$)	*A. japonicus* ($n=8$)
14 : 0	3.8 ± 0.6	3.4 ± 0.7	3.3 ± 0.6	3.8 ± 0.6	5.7 ± 1.3	6.1 ± 0.4
16 : 0	26.2 ± 5.2	20.3 ± 4.3	21.5 ± 5.5	21.5 ± 6.3	18.4 ± 5.6	19.6 ± 2.2
16 : 1n-7	4.1 ± 1.0	2.8 ± 2.1	6.4 ± 2.5	3.9 ± 1.4	2.0 ± 2.1	6.6 ± 2.3
18 : 1n-9	8.2 ± 1.5	11.1 ± 3.0	5.6 ± 2.0	5.6 ± 1.6	14.4 ± 4.7	14.8 ± 5.8
18 : 2n-6	5.9 ± 1.1	7.9 ± 1.1	4.3 ± 0.6	3.8 ± 0.4	11.2 ± 1.9	4.6 ± 0.9
18 : 3n-3	5.3 ± 0.8	7.0 ± 1.5	10.1 ± 2.5	8.6 ± 1.7	5.3 ± 1.1	4.3 ± 1.0
18 : 4n-3	5.6 ± 3.4	11.8 ± 6.4	6.5 ± 4.1	7.9 ± 3.9	8.9 ± 5.1	5.8 ± 2.2
20 : 4n-6	14.6 ± 1.6	15.2 ± 1.7	11.6 ± 1.6	14.8 ± 1.7	8.5 ± 1.6	6.1 ± 0.7
20 : 5n-3	9.7 ± 3.0	11.2 ± 1.5	11.5 ± 3.9	12.0 ± 3.9	12.2 ± 3.5	13.2 ± 2.4
18 : 4n-3 + 20 : 5n-3	15.3	23.0	18.0	19.9	21.1	19.0

Note: Adapted from Terasaki et al. (2009).

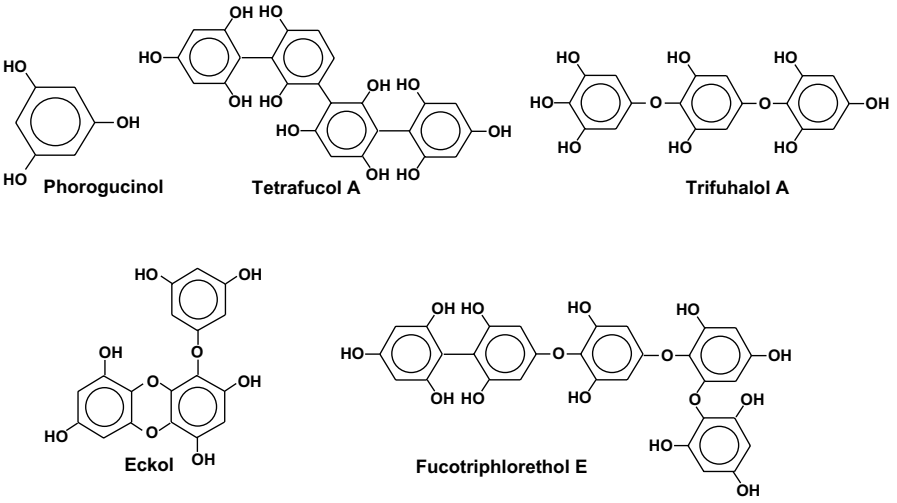

FIGURE 14.1 Chemical structures of phlorotannins in brown seaweeds.

the type of structural linkages between the phloroglucinol subunits (Fig. 14.1), phlorotannins can be systematically classified into four major subclasses: fucols, fuhalols, eckol, and fucophlorethols (Fig. 14.1) (Jung et al., 2006; Parys et al., 2007). As shown in Figure 14.1, fucols are phlorotannin polymers in which the phloroglucinol units are connected only by C–C (aryl–aryl) bonds. Fuhalols are connected exclusively via ether bonds. Eckols are characterized by the occurrence of at least one three-ring moiety with a dibenzo-1,4-dioxin element substituted by a phenoxyl group at the C-4 position. Fucophlorethols contain both biaryl and aryl–ether linkages.

Nakai et al. (2006) screened the antioxidant activities of 50% ethanol extracts of 25 common Japanese seaweeds. The highest radical scavenging effect was obtained for *Sargassum ringgoldianum*. The chemical structure of the main active fraction contained bifuhalol oligomers. The partially purified phlorotannin-rich fraction exhibited significant scavenging potencies on superoxide anion radicals, which were around five times higher than that of catechin. Shibata et al. (2008) identified several phlorotannins including eckol, phlorofucofuroeckol A, dieckol, and 8,8′-bieckol from the Japanese Laminariaceous brown seaweeds (*Ecklonia cava, Eisenia bicyclis*, and *Ecklonia kurome*). All the phloroglucinol oligomers inhibited phospholipid peroxidation in the liposome system and were potent DPPH radical scavengers. With the exception of eckol, all the phlorotannins exhibited extraordinary superoxide anion radical scavenging abilities, which was around 2–10 times more effective than ascorbic acid and α-tocopherol. Other series of phenolic compounds such as bromophenols (Duan et al., 2007; Li et al., 2007, 2008) or catechins (Yoshie et al., 2000) have been identified from red and brown or red and green algae respectively.

Algal polyphenols, especially phlorotannins derived from brown algae, can function as herbivore deterrents, digestive inhibitors, and antibacterial agents; thus, they are putative defensive agents against the natural enemies of these algae (Glombitza et al., 1997; Lüder and Clayton, 2004; Targett and Arnold, 1998). Nagayama et al. (2002) found the antibacterial or bactericidal activity of algal phlorotannins against food-borne pathogenic bacteria as being more effective than that of catechins. Antibacterial and antioxidant activities of algal polyphenols will be useful to preserve food products against deterioration.

Studies have revealed that algal polyphenols possess many biological activities, including anti-inflammatory (Shin et al., 2006), hepatoprotective (Kim et al., 2005; Zhao et al., 2004), antitumor (Kim et al., 2006; Yuan and Walsh, 2006), antihypertensive (Jung et al., 2006), and HIV-1 reverse transcriptase (Ahn et al., 2004) activities as well as antidiabetic activity on the basis of inhibition of α-glucosidase (Iwai, 2008). Their multiple physiological activities, thus, may offer many advantages for potential applications in the nutraceutical, pharmaceutical, and cosmetic industries. However, the evaluation of the effects exerted by algal polyphenols causes several problems when moving from simple experimental systems to the complexity of human body. The major problem is their bioavailability and the difficulties in unraveling the complex mechanisms of absorption and metabolism. To date, most of the studies on the biological activities of algal polyphenols have been done *in vitro* using cultivated cell lines. A basic research will be needed to define the absorption rate of algal polyphenols, identification of their metabolites, and the real molecular events that underline the biological effects of algal polyphenols.

14.4 CAROTENOIDS IN BROWN SEAWEEDS

In marine environments, carotenoids are widely present in both algae and animals. Palermo et al. (1991) reported the presence of β-carotene, zeaxanthin, fucoxanthin (Fx), and fucoxanthinol in the red algae. In brown algae Fx is the dominant carotenoid (Dembitsky and Maoka, 2007). The number of naturally occurring carotenoids reported continues to rise and has now reached more than 700. Fx is the most abundant carotenoid contributing more than 10% of the estimated total production of carotenoids in nature (Matsuno, 2001). Marine photosynthetic organisms such as micro-/macroalgae synthesize β-carotene *de novo* from isoprenyl diphosphate *via* phytoene and lycopene, and then alter it to produce other derivatives. Marine animals do not synthesize carotenoids *de novo* and those found in the animal bodies are either the result of the direct accumulation of carotenoids from food or are partly modified through metabolic reactions.

Representative seaweed carotenoid, Fx, has been reported to be an effective antioxidant. Electron spin resonance (ESR) analysis showed the quenching ability of Fx against both organic radicals DPPH and 12-doxyl-steraic acid (12DS) (Nishino, 1998). Yan et al. (1999) demonstrated the strong DPPH radical scavenging activity of Fx. Fx has a unique structure including an unusual allenic bond and 5,6-monoepoxide in its molecule. This structure affects its antioxidant activity.

The ability of carotenoid to quench singlet oxygen increases with increasing number of conjugated double bonds (Conn et al., 1991), whereas the antioxidant activity of carotenoids increases with the presence of functional group in terminal rings as seen in astaxanthin and Fx (Miller et al., 1996). Murakami et al. (2000) screened 19 natural carotenoids for their structure–function relationship with respect to radical scavenging activity using human promyelocytic HL-60 cells. They found that the presence of an allenic bond as seen in Fx is a factor for the inhibition of superoxide and NO generation, while the presence of 4-oxo-β-end group in the structure of astaxanthin and canthaxanthin enhances the NO generation.

Fx has higher biological activity than carotenoids from terrestrial origin (Guerin et al., 2003; Hosokawa et al., 2009; Miyashita, 2008; Miyashita and Hosokawa, 2009). It has been recognized that oxidative damage plays a central role in the development of degenerative diseases, including inflammatory diseases, cardiovascular diseases, and cancer. Anticancer and anti-inflammatory effects are also found in Fx (Hosokawa et al., 2009). On the other hand, Fx can modulate gene expressions related to the improvement in human health. In this case, their nutritional activities depend on their specific chemical structures. The antiobesity effect of edible seaweed carotenoid, Fx, is very interesting, as its molecular mechanism has been made clear and its activity depends on the protein and gene expressions of UCP1 in WAT, although UCP1 is usually only found in BAT (Maeda et al., 2005). Fx has also been reported to have antidiabetic effects and causes the enhancement of DHA synthesis in the mouse liver (Maeda et al., 2007; Tsukui et al., 2007).

With increasing interest in the physiological effects of brown seaweed lipids containing several bioactives such as Fx, omega-3 and omega-6 long-chain HUFA, and polyphenols, more detailed data on the lipid content and its profile has been expected. Brown seaweed also contains fucosterol (Fs) as the dominant sterol. Dietary intake of plant sterols (as part of a normal diet, or as a supplement) is known to reduce blood cholesterol levels. The same effect has been also found in Fs (Ikeda et al., 1988). Furthermore, Fs has been known to show antioxidant (Lee et al., 2003) and antidiabetic (Lee et al., 2004) activity.

We have reported the quantitative data on TL, Fx, and Fs in all 15 brown seaweeds (Terasaki et al., 2009) (Table 14.2). *Sargassum horneri*, commonly referred to as Akamoku, had the highest Fx (3.7 ± 1.6 mg/g dry weight) and Fs (13.4 ± 4.4 mg/g dry weight) contents, followed by Uganomoku (*Cystoseira hakodatensis*; Fx: 2.4 ± 0.9; Fs: 8.9 ± 2.0). A relatively higher quantity of Fx was also observed in *Melanosiphon intestinalis* (1.9 ± 0.9), *Sargassum thunbergii* (1.8 ± 1.0), *Saragassum confusum* (1.6 ± 0.8), and *Analipus japonicus* (1.4 ± 1.0). In general, species from Sargassaceae were rich in Fx and Fs as compared with species from other groups. Higher contents of Fx and Fs in *S. horneri* indicate the potential of this seaweed species as a source of Fx. *S. horneri* also showed a TL content of 62.6 ± 18.7 mg/g dry weight. In our preliminary examination, the young *S. horneri* (length; 1–10 cm) monocultured under strictly controlled conditions had Fx and Fs contents of 14.6 and 32.3 mg/g dry weight), respectively, indicating the superior value of this seaweed as a source of Fx and Fs.

The results of this study also indicate the seasonal variations in Fx and Fs contents of six seaweeds (*S. horneri*, *C. hakodatensis*, *S. thunbergii*, *S. fusiforme*, *A. japonicas*,

TABLE 14.2 Content of Total Lipid, Fucoxanthin, and Fucosterol from Some Edible Brown Seaweed in Japan

Family	Species	Japanese Name	Total Lipid (mg/g Dry Weight)	Fucoxanthin (mg/g Dry Weight)	Fucosterol (mg/g Dry Weight)
Sargassaceae	*Sargassum horneri*	Akamoku	62.6 ± 18.7	8.6 ± 3.8	13.4 ± 4.4
	Sargassum thunbergii	Umitoranoo	31.8 ± 13.1	4.3 ± 2.3	5.6 ± 2.0
	Sargassum fusiforme	Hijiki	27.5 ± 11.9	2.7 ± 1.5	5.9 ± 2.8
	Cystoseira hakodatensis	Uganomoku	42.9 ± 6.2	5.6 ± 2.1	8.9 ± 2.0
	Sargassum confusum	Fujisujimoku	47.4 ± 12.8	3.7 ± 1.9	5.5 ± 1.7
	Sargassum patens	Yatumatamoku	58.9 ± 25.1	4.6 ± 3.1	7.8 ± 0.1
Fucaceae	*Silvetia babingtonii*	Ezoishige	39.1 ± 11.8	1.7 ± 0.4	5.8 ± 0.7
	Fucus distichus	Hibamata	31.1 ± 12.1	2.2 ± 0.6	4.7 ± 0.7
Laminariaceae	*Kjellmaniella crassifolia*	Gagome	15.7 ± 6.4	1.6 ± 1.0	2.0 ± 1.0
	Alaria crassifolia	Chigaiso	27.1 ± 1.1	2.7 ± 0.9	2.4 ± 0.6
Alariaceae	*Undaria pinnatifida*	Wakame	37.5 ± 8.4	2.8 ± 1.3	3.9 ± 1.7
Asperococcaceae	*Melanosiphon intestinale*	Kitaiwahige	34.9 ± 17.1	4.5 ± 2.8	3.8 ± 2.0
Ralfsiaceae	*Analipus japonicus*	Matsumo	42.6 ± 16.0	3.3 ± 2.3	6.3 ± 2.9
Scytosiphonaceae	*Scytosiphon lomentaria*	Kayanomori	16.5 ± 2.2	1.1 ± 0.2	1.4 ± 0.4

Note: All the brown seaweeds ($n = 4$–25) were collected from the coast of Hakodate, Japan. Total lipids (TL) were extracted from the frozen sample with methanol (1 : 10 w/v). (Adapted from Terasaki et al., 2009).

and *M. intestinalis*). A positive relation between Fx and Fs was observed, with higher Fs content coinciding with higher Fx content in all the species analyzed. In addition, the highest Fx and Fs contents were observed between January and March (i.e., the period between winter and spring). During this period, duration of sunshine, PAR, and seawater temperature were the lowest for the areas (Miyashita and Takagi, 1987) from which samples were collected. Low light during the winter period leads to increased production of Fx in brown algae through the xanthophyll-cycle pathway that involves formation of Fx from zeaxanthin.

14.5 FUTURE DIRECTION

Brown seaweed lipids can be extracted from the raw materials with organic solvents such as ethanol. The lipids obtained, however, have difficulty in the application and handling for the product formulation because of their high viscosity and low oxidative stability of Fx, onega-3, and omega-6 HUFA. On the other hand, these active compounds are much stable in the matrix with other biomolecules such as seaweed powder including proteins, polysaccharides, and fibers, although the reason has not yet been made clear (Prabhasankar et al., 2009). The seaweed powder can be obtained after drying raw material. Brown seaweed belonging to *Sargassum* species would be a good source for nutraceutical and functional foods, as it is rich in highly bioactive compounds such as Fx, Fs, omega-3 HUFA (EPA and 18 : 4n-3), and omega-6 HUFA (arachidonic acid) (Fig. 14.2). However, raw powder contains a considerable amount of minerals that are not suitable for food material.

Lipid concentrated seaweed powder was obtained by the enzymatic treatment. As shown in Figure 14.3, lipid fraction and Fx was concentrated by the alginate lyase treatment with the removal of water-soluble degradated polysaccharides, minerals, and other products. This seaweed powder may be used in nutraceutical products. Alginate, laminaran, fucoindans, and galactofucans are representative polysaccharides contained in brown seaweeds. The composition of each polysaccharide differs from brown seaweed species. Although there are many types of enzymes available for

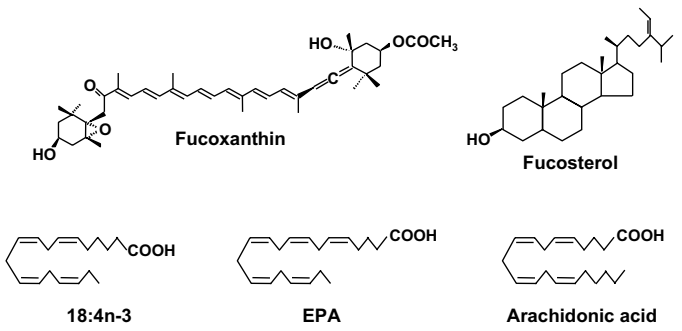

FIGURE 14.2 Chemical structures of bioactive compounds in brown seaweed lipids.

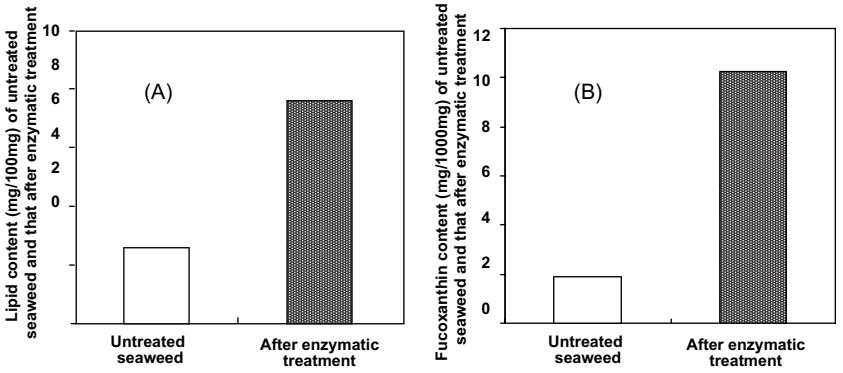

FIGURE 14.3 Lipid content (A) and fucoxanthin content (B) of seaweed (*Undaria pinnatifida*) before and after enzymatic treatment. Dried *Undaria pinnatifida* (0.5 mg) was treated with alginate lyase S (1 mg; Nagase Co., Tokyo) in 100 ml of water at 37°C for 1 h. The reaction products were washed with water, and then lipids were extracted with chroloform/methanol (2 : 1, v/v).

polysaccharides and protein degradation, those for seaweeds are limited. Effort is needed to establish feasible recovery methods to enhance the utility of these important marine resources.

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15

PROCESSES FOR PRODUCTION OF BIODIESEL FUEL

YOMI WATANABE AND YUJI SHIMADA

Osaka Municipal Technical Research Institute, 1-6-50 Morinomiya, Joto-ku, Osaka 536-8553, Japan

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15.1 INTRODUCTION

Carbon dioxide emitted from fossil resources is becoming a big concern for global warming. On the other hand, carbon dioxide originating from biological resources is fixed by photosynthesis, so the total carbon balance in the global environment is maintained. Therefore, the replacement of fossil resources with biological resources suppresses an increase in the amount of carbon dioxide in the environment, and can contribute to the prevention of global warming. From this viewpoint, a great deal of attention is currently focused on the production of biofuels from biological resources. In this chapter, we introduce two kinds of biofuels [bioethanol and biodiesel fuel (BDF)], and also discuss recent studies for the ecofriendly production of BDF, which include a biochemical process using an immobilized lipase.

15.2 BIOFUELS

Bioethanol and BDF are the two main biofuels. In many cases, bioethanol and BDF are used as fuels for vehicles after mixing with gasoline and light oil, respectively.

15.2.1 Bioethanol

Bioethanol is produced from rice, barley, corn, sugarcane, sugar beet, etc. and is widely used as a beverage, an industrial chemical, and a fuel. The demand of the world in 2007 was about 13 billion gallons, and about 90% was produced in the United States and Brazil. About 70% of the total bioethanol produced is used as a fuel. Bioethanol in particular is blended with gasoline at a concentration of 10%, and is used as a fuel (E10) for vehicles. Recently, the United States aimed to reduce the present consumption of gasoline by 20%, and started to increase the bioethanol production targeting 35 billion gallons by 2017. The implementation of this plan brought about a remarkable rise in grain prices, resulting in a jump in food prices. Hence, there was an urgent need to develop a new technology for the production of bioethanol from leaf/stem and woody waste, which are not edible.

The main process for bioethanol production is fermentation by microorganisms, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Zymomonas mobilis*. *S. cerevisiae* can produce ethanol (EtOH) at a high concentration, and *S. pombe* is tolerant to high osmotic pressure (Wilkie et al., 2000). *Z. mobilis* produces ethanol in a high yield from sugar, but by-produces sorbitol, which suppresses further fermentation (Lee and Huang, 2000). These strains produce EtOH from glucose, fructose, and sucrose, but not from starchy and cellulosic biomass because of lack of their ability to convert it to fermentable carbohydrates. Two processes (saccharification and fermentation) are, therefore, necessary for the production of EtOH from starchy and cellulosic biomass, and a biological or chemical process is adopted for saccharification. Much attention is currently focused on the creation of a new microorganism that undergoes both saccharification and fermentation.

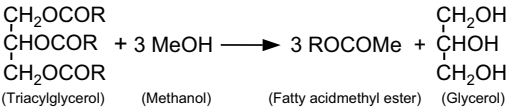

FIGURE 15.1 Conversion of TAG to FAME.

15.2.2 BDF

BDF is a fatty acid (FA) alkyl ester produced by transesterification of triacylglycerol (TAG) with alcohol (Fig. 15.1). Rapeseed, soybean, and palm oils are used as TAGs, and methanol (MeOH) is usually used as an alcohol because of its low price. Hence, BDF is generally a fatty acid methyl ester (FAME).

TAG can be used only as a fuel for diesel engines working with heavy oil because its molecular weight is large (ca. 900) and the viscosity and flash point are high. Meanwhile, the molecular weight of BDF is small (ca. 300) and its viscosity and flash point are low. Accordingly, BDF can be used as a light oil substitute for diesel engines, which are used widely. To stabilize the quality of BDF, it is blended with light oil at a concentration of 20%, and the resulting oil (B20) is used as a fuel for diesel engines.

BDF is used as a petroleum diesel fuel substitute for the following reasons. (i) The exhaust gas from BDF contains low concentration of unburned hydrocarbon, CO, small particle matter, and SO_x, compared with that from petroleum diesel fuel. (ii) The total amount of CO_2 in the environment does not increase through the use of BDF as a fuel because the CO_2 generated by combustion of BDF is converted to vegetable oil by photosynthesis. (iii) BDF is biodegradable. In addition, (iv) production of BDF from waste edible oil is useful for reduction of waste materials.

FAMEs have been used originally as raw materials for production of fatty alcohols and detergents. Recently, an agricultural policy for cultivation of oil seeds and the preferential taxation with the use of BDF were also provided, leading to a significant increase in the production of BDF from rapeseed oil in EU: the demand for BDF in 2006 was 5 million tons (Fig. 15.2). BDF is also produced from surplus soybean oil in the United States. The demand in 2006 reached 1 million tons, and was estimated to double in 2007.

15.3 VARIOUS PROCESSES FOR BDF PRODUCTION

Homogeneous reactions with alkaline catalysts have been adopted traditionally for conversion of vegetable oils (TAGs) to FAMEs. In this chemical process it is easy to control the reaction. The reaction time is short, and the cost of production is also cheap. The process, however, cannot be applied to the production from various kinds of raw materials. To overcome the drawback, several ecofriendly processes are proposed (Table 15.1).

When a raw material (vegetable oils, TAGs) includes free fatty acids (FFAs) and water, the homogeneous reaction with alkaline catalysts decreases the degree of conversion significantly. The problem can be solved by using acidic catalysts,

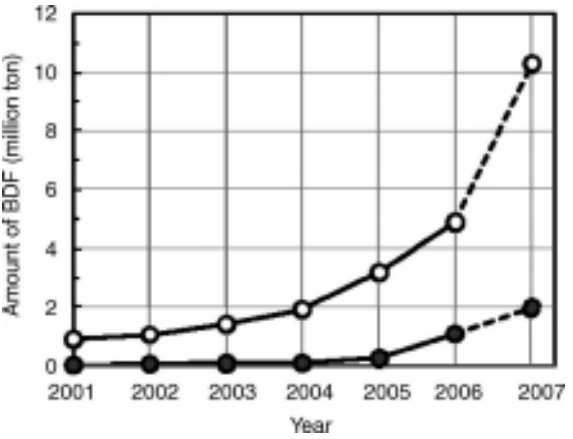

FIGURE 15.2 Production of BDF in EU and the United States. The amount of 2007 is estimated (The National Biodiesel Board, the European Biodiesel Board).

although larger amounts of MeOH are required. Glycerol by-produced in homogeneous catalysis includes alkali or acid. Much expense is, therefore, required for the refinement of glycerol. In addition, alkaline and acidic catalysts contaminating the product (BDF) are removed easily by washing with water, but the resulting water must be treated as waste.

These problems can be solved by adopting reactions with solid catalysts. In these reactions, however, durability of the solid catalysts and elution of a part of catalyst to the reaction solution are pointed out as problems. Furthermore, it is strongly desired to develop a new solid catalyst that converts even raw materials contaminated with FFAs and/or water to BDF efficiently.

To solve drawbacks in reactions with catalysts, other processes are proposed: supercritical MeOH process and superheated MeOH vapor bubble process. The adoption of these processes required not only much expense for the construction of plants, but also much energy for the reactions.

TABLE 15.1 Various Processes for the Production of BDF

Process	Reaction	Catalyst
Chemical	Homogeneous catalysis	Alkaline catalyst (NaOH, KOH)
		Acidic catalyst (H_2SO_4)
	Solid catalysis	Metal oxide
		Ionic exchange resin
	No catalysis	Supercritical MeOH
		Superheated MeOH vapor
Biochemical	Whole-cell catalysis	Immobilized *Rhizopus oryzae*
	Enzyme catalysis	Soluble lipase
		Immobilized lipase

The biochemical process is also proposed as an energy-saving and ecofriendly reaction. The adoption of the process facilitates the refinement of the product (BDF) and by-product (glycerol). The first is a process that uses whole-cell catalyst. The cost for its preparation is expected to be cheap, but the durability is short. Furthermore, the degree of conversion is not so high. The second is a process that uses a lipase. The process achieves a high degree of conversion, but the cost of lipase is high. To solve the problem, the adoption of immobilized lipase is proposed.

The processes described in this section are introduced in the following sections.

15.4 PROCESSES WITH CHEMICAL CATALYSTS

15.4.1 Homogeneous Catalysis

BDF is produced presently from vegetable oils by a homogeneous reaction with alkaline catalysts, such as NaOH and KOH. The process mainly consists of (i) removal of FFAs and water from a raw material oil, (ii) methanolysis of TAGs with an alkaline catalyst, (iii) separation of reaction mixture into BDF and glycerol layers by standing, (iv) removal of alkali, soap, and glycerol from BDF layer by washing with water, and (v) dehydration of washed BDF layer. The methanolysis of the process (ii) is conducted usually by agitation at 70°C for one to several hours. An equimolar quantity of MeOH for total fatty acids (FAs) in TAGs is theoretically necessary for complete conversion, but more than 2 mol MeOH is used to achieve a high yield of BDF. The remaining MeOH in the reaction is recovered by distillation and is recycled. The repetition of steps (ii) and (iii) is known to be effective in increasing the content of BDF.

In this process, contamination of water in the raw material oil induces hydrolysis of TAGs and by-produces soaps, resulting in a decrease of the degree of conversion. Contamination of FFAs in the raw material also by-produces soaps, leading to a drastic decrease in the recovery. The pretreatment (removal of FFAs and water) is, therefore, necessary to achieve a high yield. When FFAs in the oils are present only in a small amount, BDF can be produced without preremoval of FFAs because by-produced soaps are removed by washing BDF layer with water. Meanwhile, when the oil includes more than several percent of FFAs, efficiency of removal of FFAs by neutralization with NaOH becomes low. In this case, a homogeneous reaction with acidic catalyst, e.g. H_2SO_4, is effective because the reaction catalyzes not only methanolysis of TAGs, but also methyl esterification of FFAs.

A reaction with alkaline/acidic catalyst requires washing with water for removal of alkali/acid and by-products. The resulting alkaline/acidic water generated in this process must be treated as a waste. In addition, the by-product (glycerol) includes alkaline/acidic catalyst, as well as FAMEs, FFAs, and soaps. Because the purification cost of the alkaline/acidic glycerol is too expensive, the glycerol is presently treated as an industrial waste.

15.4.2 Solid Catalysis

The use of solid catalysts is expected to produce BDF from TAGs without the following processes: the water washing of product (BDF) and the treatment of alkaline wastes (glycerol and water). It has been reported so far that carbonate and acetate of alkali (alkaline earth) metals and phosphate of metals are effective for methanolysis of TAGs (Suppes et al., 2001; Leclercq et al., 2001). The reactions, however, required high reaction temperature (>200°C) and high pressure. To solve the problem, Kouzu et al. (2006, 2008) developed a new catalyst, calcium oxide, that was prepared by burning without contact with CO_2. A reaction with reflux using the solid catalyst achieved 99% conversion. But waste edible oil usually includes small amounts of FFAs, water, and unknown polar compounds. These contaminants eluted the catalyst in the product, resulting in a significant decrease in the ability and durability of the catalyst. Hence, a pretreatment for conversion of FFAs in waste edible oil to FAMEs was attempted by using cation-exchange resin. But the recovery of the solid catalyst was limited to 80%, and the process still required the water washing of the product and the treatment of wastes (glycerol and water).

The use of ion exchange resin solved the problem of elution of metal in the product (BDF). In general, ion exchange resin has low resistance to heating and its methanolysis activity is low. Shibasaki-Kitakawa et al. (2007) found that an anion exchange resin (PA306s; Mitsubishi Chemical Co., Japan) showed strong activity for methanolysis of TAGs. When a reaction was conducted at 50°C for 5 h in a mixture of TAGs, 10 mol MeOH for total FAs (3.3 mol for TAGs), and 20–40 wt% of the ion exchange resin, 80% of TAGs were converted to FAMEs and unreacted FAs were adsorbed on the ion-exchange resin as FFAs. The eluate after removing the ion-exchange resin was 99% FAMEs. The activity of the resin decreased gradually by repeated use. The activity was regenerated by washing with citric acid and then with NaOH water solution, followed by equilibrating with EtOH. To convert waste edible oil containing FFAs to BDF, a continuous process was proposed. This process consisted of (i) methyl esterification of FFAs in the waste oil using a cation-exchange resin, (ii) methanolysis of TAG using an anion exchange resin, and (iii) regeneration of the two ion exchange resins (Shibasaki-Kitakawa and Yonemoto, 2006). This process, however, still includes the following problems: the treatments of the oil substances released from the regeneration step of ion-exchange resins and the solutions used in the step.

15.5 PROCESSES WITHOUT CATALYSTS

15.5.1 Supercritical MeOH Method

The reaction with supercritical MeOH does not require organic solvents and catalysts. Hence, the steps for their removal after the reaction can be omitted. Solubility of MeOH in TAGs is very low under ordinary conditions, but is very high under supercritical conditions because of the formation of a hydrophobic environment. The resulting homogeneous reaction mixture underwent efficiently methanolysis

of TAGs. Kusdiana and Saka (2001) succeeded in the production of 95–99% FAMEs by supercritical fluid reaction at 350°C and 20 MPa for 4 min in a mixture of TAGs and 40 mol MeOH for total FAs. Under these conditions, water did not disturb methyl esterification of FFAs or methanolysis of TAGs (Kusdiana and Saka, 2004a). Hence, this method does not require pretreatment for removing FFAs and water in a raw material oil.

BDF can be produced under milder conditions than those in the reaction described above. This involves a two-step reaction consisting of hydrolysis of TAGs and methyl esterification of FFAs (Kusdiana and Saka, 2004b; Minami and Saka, 2006). A mixture of an equal amount of oil and water was kept for 1 h in the subcritical state at 270°C and 7 MPa, so that TAGs were hydrolyzed almost completely. To the oil layer (FFAs) recovered by standing was added 6 mol MeOH for total FAs. Keeping the mixture for 30 min at 270°C and 20 MPa, FAMEs were produced with a content of 94%. When BDF was produced by the two-step reaction, glycerol produced in hydrolysis was removed into the water layer. Therefore, the amount of glycerol in the BDF became smaller than that in the BDF produced by the one-step reaction described above.

15.5.2 Superheated MeOH Vapor Bubble Method

A reaction was proposed and conducted without a catalyst at near ambient pressure (0.1 MPa) (Ishikawa et al., 2005). A reactor containing a raw material oil was heated at 280°C, and MeOH was introduced from the bottom at a flow rate. Consequently, MeOH was converted to gas and reacted with TAGs. Unreacted MeOH went out from the top of reactor and was recovered by cooling. This method converted rapeseed oil to 97% FAMEs. The area of gas–liquid interface affected the degree of conversion. It can be pointed out that the design of the plant for an increase in the area of gas–liquid interface becomes an important key.

15.6 BIOCHEMICAL PROCESSES FOR BDF PRODUCTION

There are processes using enzymes as catalysts. Enzymes catalyze reactions under mild conditions so that creatures can maintain their lives, unlike chemical catalysts. The products of the reactions are also biodegradable. Hence, enzyme-catalyzed reactions are safe and ecofriendly in general.

Lipases are classified as enzymes that catalyze hydrolysis of TAGs. They also catalyze esterification and transesterification by setting suitable conditions. BDF can be produced theoretically *via* lipase-catalyzed reactions. Enzymes including lipases, however, are generally unstable in short-chain alcohols, leading to their inactivation. A new reaction system must be devised to avoid the inactivation by short-chain alcohols. Several reaction systems have been proposed so far: production of BDF from (i) TAGs, (ii) FFAs, and (iii) a mixture of TAGs and FFAs (named acid oil) using (i) soluble lipases, (ii) immobilized lipases, and (iii) microbial cells carrying lipase activity as catalysts.

15.6.1 Process with Soluble Lipases

Many industrial lipases are supplied in a liquid state. The products are prepared by removal of microbial cells from culture broth, followed by concentration of the filtrate. In other case, the lipases are supplied as powdered enzymes after drying the concentrate. The powdered lipase is used generally after dissolving with water.

Mittelbach (1990) reported first that lipases from *Rhizomucor miehei*, *Pseudomonas fluorescens*, and *Candida* sp. catalyze alcoholysis of sunflower oil with short-chain alcohols in ether. It is, however, desirable not to use any organic solvents for the industrial production. *Burkholderia cepacia* lipase is reported to have strong activity even in an organic solvent-free system (Kaieda et al., 2001): a mixture of soybean oil, 1–2% water, and the lipase was agitated, and 1/3 mol MeOH for total FAs was added three times to the mixture (see Section 6.2.1). After 90 h of reaction, almost all TAGs were converted to FAMEs. The reaction systems with *Candida rugosa* lipase (Watanabe et al., 2004) and *Rhizopus oryzae* lipase (Kaieda et al., 1999) were also reported. Because these lipases were less stable for MeOH than *B. cepacia* lipase, dilution of MeOH by adding 10–20% water was necessary to avoid the inactivation. However, hydrolysis proceeded simultaneously and 10–20% FFAs remained at the end of reaction. The reaction could be controlled easily when using soluble lipase, but the difficulties in recovering and reusing the lipase might result in high production cost.

15.6.2 Processes with Immobilized Lipase

The use of immobilized lipase is effective in reducing the production cost because the lipase can be recovered easily and reused. Nelson et al. (1996) reported first that immobilized *Candida antarctica* lipase (Novozym 435, Novozymes, Bagsvaerd, Denmark) catalyzed methanolysis of TAGs. But the reaction was conducted in *n*-hexane, and the immobilized lipase was not reused because of rapid inactivation. The inactivation was avoided by a method as described later (see Section 6.2.1). Accordingly, immobilized *C. antarctica* lipase is used widely as one of the effective catalysts for the production of BDF.

The components of the refined vegetable oil, which is used as a raw material for the production of BDF, are TAGs. Meanwhile, crude vegetable oils, waste edible oils, and oils by-produced in the steps of vegetable oil refining are a mixture of TAGs and FFAs, although their ratio is different. Furthermore, FFAs are released as wastes or by-products from production processes of oil/fat-related compounds. These composites (TAGs, FFAs, and a mixture of TAGs and FFAs) are candidates for raw materials candidates for the in production of BDF. It was reported recently that immobilized *C. antarctica* lipase was effective in the continuous production of BDF from the three composites. The processes are introduced in the following sections.

15.6.2.1 Production from TAGs Enzymes are generally unstable in short-chain alcohols such as MeOH and EtOH. On the other hand, one of the raw materials in BDF

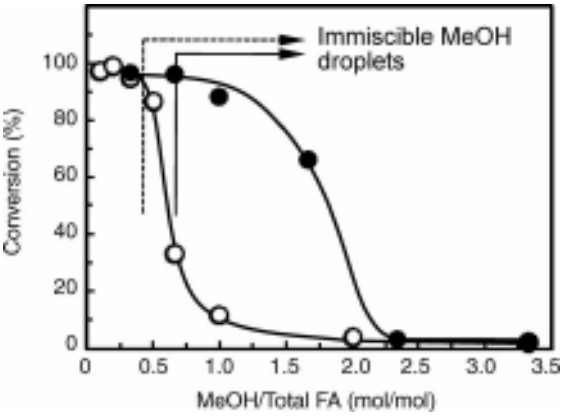

FIGURE 15.3 Irreversible inactivation of immobilized *C. antarctica* lipase by contact with immiscible MeOH phase. One reaction mixture (○) was composed of vegetable oil and MeOH, and the other mixture (●) was composed of acylglycerols (AGs)/33% FAMEs mixture and MeOH. The reaction mixture was shaken with 4 wt% immobilized lipase at 30°C for 24 h. The amount of MeOH was expressed as the molar ratio to the amount of total FAs in the system. The conversion was expressed as the molar ratio to the amount of total FAs in the system. The conversion was expressed as the amount of MeOH consumed for the ester conversion of AGs (when the amount of MeOH is less than that of FAs in AGs), and as the molar ratio of FAMEs to total FAs in the system (when the amount of MeOH is more than that of FAs in AGs). Arrows indicate the region in which a part of MeOH exists as immiscible droplets.

production is MeOH. It is necessary for efficient production of BDF to overcome instability of lipase for MeOH.

More than an equimolar amount of MeOH for total FAs (stoichiometric amount) is necessary to achieve complete conversion of TAGs to FAMEs. The solubility of MeOH in vegetable oil (TAGs) is low, so that a part of MeOH in the oil forms immiscible droplets when mixing TAGs and a stoichiometric amount of MeOH. When *C. antarctica* lipase came in contact with MeOH phase (MeOH concentration, 100%), the enzyme was easily inactivated. On the other hand, when TAGs were mixed with a soluble amount of MeOH in TAGs (<1/3 of stoichiometric amount), the MeOH did not inactivate the lipase (Fig. 15.3) (Shimada et al., 1999).

This finding led to the proposal of a new reaction system. The reaction was started in a mixture of TAGs and 1/3 of stoichiometric amount of MeOH. After consuming MeOH almost completely, 1/3 mol of MeOH was added to the reaction mixture two times. The three-step reaction is shown schematically in Figure 15.4, and a typical time course is shown in Figure 15.5 (Shimada et al., 1999).

When MeOH was completely consumed in the reaction of TAGs and 1/3 mol MeOH, the reaction mixture consisted of 33% FAMEs and 67% acylglycerols (mainly TAGs). The solubility of MeOH in the mixture was higher than that in vegetable oil, and even 2/3 mol MeOH was dissolved completely. The lipase was not at all inactivated in the reaction mixture in which MeOH was completely dissolved

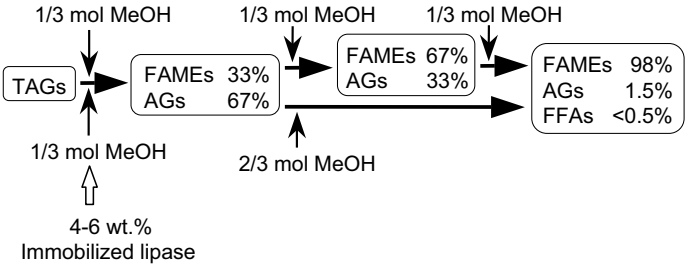

FIGURE 15.4 Production of BDF from TAGs by stepwise addition of MeOH.

(Fig. 15.3) (Watanabe et al., 2000). A two-step reaction system was also proposed based on the result. The reaction was started in a mixture of oil and 1/3 mol MeOH, and 2/3 mol MeOH was added to the mixture after consuming the first 1/3 mol of MeOH completely. The two-step reaction is shown schematically in Figure 15.4, and a typical time course is shown in Figure 15.5 (Watanabe et al., 2000).

Three- and two-step methanolysis converted 98% TAGs to FAMEs. The two-step reaction was advantageous because of the reduction of reaction time (Fig. 15.5). Immobilized lipase used in the two- and three-step reactions was very stable under the conditions determined, and could be used for more than 50 times (100 days) without any significant loss of activity (Watanabe et al., 2000).

Waste edible oil could be also converted to BDF by the stepwise methanolysis (Watanabe et al., 2001). Although the oil included 0.2% water and 2.5% FFAs, these

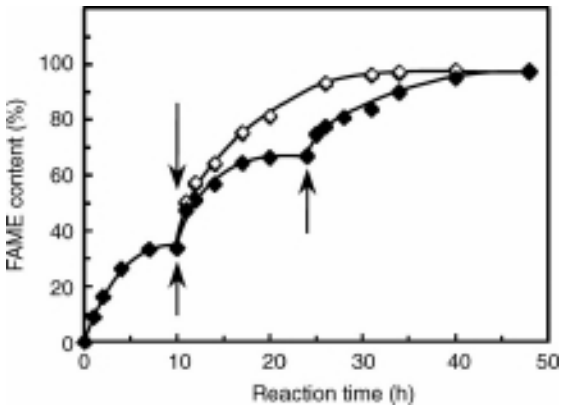

FIGURE 15.5 Time course of two- and three-step methanolysis of vegetable oil using immobilized *C. antarctica* lipase. The three-step reaction was carried out as follows (◆): A mixture of vegetable oil, 1/3 mol MeOH for total FAs, and 4 wt% immobilized *C. antarctica* lipase was shaken at 30°C. The second and third 1/3 mol MeOH were added at 10 and 24 h, respectively. The two-step reaction is as follows (◇): The first-step reaction was conducted under the same conditions as those of the three-step reaction, and 2/3 mol MeOH was added to the reaction mixture at 10 h.

contaminants did not disturb three-step methanolysis and a high conversion of TAGs to BDF was achieved.

Here we have described that the stepwise methanolysis kept the reaction mixture homogeneously and overcame the inactivation of lipase by MeOH. The lipase was also found to be stable in a homogenous reaction mixture containing *t*-butanol (Li et al., 2006; Wang et al., 2006). TAGs underwent methanolysis with 1.3 mol MeOH for total FAs in TAGs using the same volume of *t*-butanol to TAGs, 3 wt% immobilized *Thermomyces lanuginosa* lipase, and 1 wt% immobilized *C. antarctica* lipase. The reaction system converted TAGs to FAMEs in 95% yield and the immobilized lipase was reused for 200 cycles (Li et al., 2006). The system is also applicable to acid oil (Wang et al., 2006).

Whole-cell carrying lipase activity also could be used as a catalyst for the production of BDF from TAGs. The whole-cell catalyst was prepared by growing *R. oryzae* mycelia on the surface of polyurethane foam: named biomass support particle (BSP) (Ban et al., 2001). The reaction was conducted by adding 1/3 mol MeOH three times to the mixture containing 15% water using BSPs, resulting in 90% conversion. When the reactions were conducted in *t*-BuOH, the whole-cell catalyst was reused several times although the yield of FAMEs decreased to <65% (Li et al., 2008). The whole-cell catalyst system is interesting as a method for producing immobilized catalyst cheaply, because microbial cells can be used directly as catalysts. However, further efforts are required to improve the degree of conversion and stability.

15.6.2.2 Production from FFAs The oil and fat industry brings FFAs as by-products or wastes. Immobilized *C. antarctica* lipase efficiently catalyzes methyl esterification of FFAs. The lipase also recognizes polyunsaturated fatty acids (PUFAs) strongly. An oil rich in PUFA is produced industrially by the hydrolysis of fish oils with a lipase that acts on PUFA weakly (Shimada et al., 2000), and large amounts of FFAs were released from this process. The waste FFAs (Watanabe et al., 2002) and purified PUFAs (Shimada et al., 2001) were efficiently converted to their methyl and ethyl esters, respectively. Because MeOH and FFAs are miscible with each other, *C. antarctica* lipases generally do not get inactivated in a mixture of FFAs and more than an equimolar amount of MeOH. A reaction system for methyl esterification of FFAs is shown schematically in Figure 15.6.

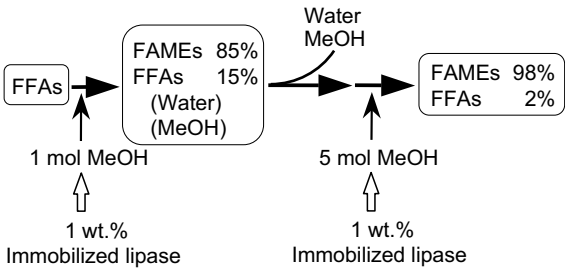

FIGURE 15.6 Production of BDF from waste FFAs by two-step methyl esterification.

The degree of esterification of FFAs reached 80–90% when using an equimolar amount of MeOH, but did not exceed 95% esterification even though lager amounts of MeOH were used. The result showed that water generated by esterification disturbs the achievement of a high degree of esterification. Hence, the first esterification was conducted with an equimol of MeOH, and 85% FFAs were converted to FAMEs. After the removal of generated water and remaining MeOH from the reaction mixture, the second esterification was conducted with 5 mol MeOH for unreacted FFAs. The two-step reaction accomplished more than 98% esterification. The immobilized *C. antarctica* lipase could be reused more than 50 times without any significant loss of activity.

Methanolysis described in Section 6.2.1 needed 4–6 wt% of immobilized lipase and 30 h of reaction period to reach almost steady state. On the other hand, methyl esterification of FFAs reached steady state at 10 h even when using 1 wt% immobilized lipase. These findings showed that the lipase catalyzes methyl esterification more strongly than methanolysis, although a quantitative evaluation is difficult because the substrates are different.

15.6.2.3 Production from Waste Acid Oil

Process Comprising Methyl Esterification and Methanolysis The third material for the production of BDF is a mixture of TAGs and FFAs. When a mixture of an equal weight of refined vegetable oil (TAGs) and FFAs was allowed to react with an equimol of MeOH for total FAs using immobilized *C. antarctica* lipase, >90% of FFAs were converted to FAMEs but >95% of TAGs were unreactive. This phenomenon can be explained as follows (Fig. 15.7). Immobilized *C. antarctica* lipase catalyzes methyl esterification more rapidly than methanolysis. The esterification generated water, which strongly inhibited methanolysis of TAGs. Accordingly, most of the TAGs remained in the reaction mixture (Watanabe et al., 2005).

Based on this hypothesis, it was attempted to convert a mixture of TAGs/FFAs to FAMEs by a two-step process with immobilized *C. antarctica* lipase. The first-step reaction was conducted in a mixture TAGs/FFAs and an equimol of MeOH for total FAs. Consequently, >95% FFAs were converted to FAMEs, but most of the TAGs

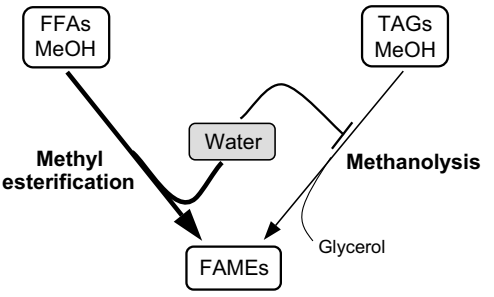

FIGURE 15.7 Reactions in a mixture of FFAs, TAGs, and MeOH with immobilized *C. antarctica* lipase.

were unreactive. MeOH and water generated as the first-reaction product were removed by evaporation, and the resulting mixture (TAGs and FAMEs) was then treated with an equimol of MeOH for unreacted FAs. The two-step process converted >98% FAs to FAMEs (Watanabe et al., 2005).

Acid oil was released from a neutralization step in vegetable oil refining. The process comprising methyl esterification of FFAs and methanolysis of TAGs was applied to the conversion of a waste acid oil [77.9% FFAs, 10.8% acylglycerols (AGs), 11.3% other lipophilic compounds] to BDF (Fig. 15.8A). The first-step methyl esterification was conducted in a mixture of the acid oil and an equimol of MeOH for total FAs using 1 wt% immobilized *C. antarctica* lipase. Contrary to the expectation, the lipase inactivated readily in the reaction mixture. The inactivation was avoided by the addition of large amounts of MeOH (5 mol for total FAs). The result is assumed to be due to dilution of unknown inhibitors with MeOH. After the removal of MeOH and water in the first-step reaction mixture, an equimol of MeOH

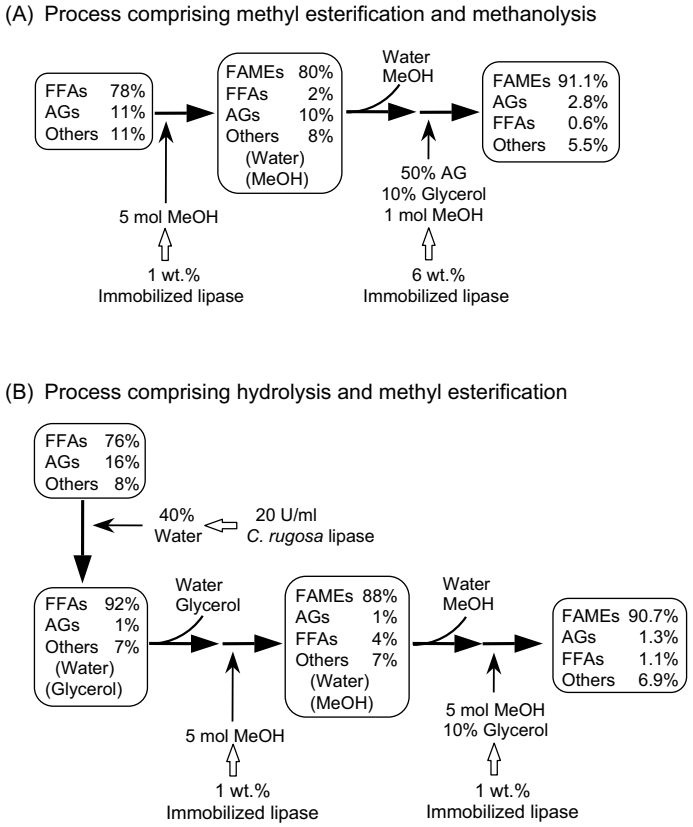

FIGURE 15.8 Production of BDF from acid oil obtained from a neutralization step in vegetable oil refining. (A) Process composing methyl esterification of FFAs and methanolysis of TAGs. (B) Process composing hydrolysis of TAGs and methyl esterification of FFAs.

for unreacted FAs was added to the resulting mixture and remaining AGs were attempted to be converted to FAMEs. The immobilized lipase was inactivated readily by the unknown inhibitors in the waste acid oil. This inactivation was avoided almost completely by the addition of vegetable oil at the final concentration of 50%, and, furthermore, by the addition of glycerol to give a concentration of 10%. The two-step reaction converted the waste acid oil to a mixture including 91.1% FAMEs, 0.6% FFAs, and 2.8% AGs. In the two-step process, immobilized *C. antarctica* lipase was reused >50 times without significant loss of activity (Watanabe et al., 2007a).

Process Comprising Hydrolysis and Methyl Esterification The process described in the previous section (Fig. 15.8A) required a large amount of lipase: 1 and 6 wt% immobilized lipase for methyl esterification and methanolysis, respectively (7 wt% in total). As described previously (Fig. 15.7), immobilized *C. antarctica* lipase catalyzes methyl esterification more strongly than methanolysis. Hence, a new process was proposed, which comprises hydrolysis of a waste acid oil and methyl esterification of the resulting FFAs (Fig. 15.8B) (Watanabe et al., 2007b).

A waste acid oil (76.3% FFAs, 15.8% AGs, and 7.9% other lipophilic compounds) was first hydrolyzed in 40% water. Unknown inhibitors in the acid oil did not disturb the hydrolysis, and the acid oil was converted to FFAs. When the resulting oil layer was treated with an equimol of MeOH for total FAs using 1 wt% immobilized *C. antarctica* lipase, the lipase was inactivated readily. The inactivation was avoided by the addition of 5 mol (34%) MeOH. Generated water and unreacted MeOH were removed from the reaction mixture by evaporation, and the second esterification was conducted. The immobilized lipase was not inactivated significantly in the second esterification. Accordingly, the reaction was conducted with 5 mol (2.2%) of MeOH for residual FFAs. In addition, the lipase activity was maintained with a high degree of esterification by the addition of 10% glycerol, even though the lipase was used repeatedly. In the second esterification, a small amount of water generated by the reaction moves to the glycerol layer. Consequently, the water does not participate in the esterification, leading to maintenance of a high degree of esterification. The two-step reaction could be repeated more than 100 times without any significant loss of activity. The adoption of this process successfully reduced the amount of immobilized lipase from 7 to 2 wt% in total (Watanabe et al., 2007b).

15.7 CONCLUSIONS

Fossil resources must be replaced with biological resources, which are recyclable, to construct a sustainable society. This consideration increases the demand of BDF in the EU every year. When we consider energy in future, the use of FAMEs as a fuel is too luxurious. Natural energies, such as solar and wind energies, can be used as a fuel by converting them to electricity. At present, FAMEs are used as a biofuel, but should be used in the future as raw materials of chemicals rather than as a fuel. Even if such a time comes, the study is continued to develop a new process that is energy saving and does not release any wastes. But the raw materials may be replaced from

edible oil to waste edible or nonedible oils. In addition, not only FAMEs, but also glycerol must be used as materials for production of various industrial chemicals. We should accordingly develop a technology for the conversion of nonedible oil to FAMEs and glycerol. We hope this review will contribute in constructing a sustainable society.

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16

NONCATALYTIC ALCOHOLYSIS PROCESS FOR PRODUCTION OF BIODIESEL FUEL: ITS POTENTIAL IN JAPAN AND SOUTHEAST ASIA

HIROSHI NABETANI, SHOJI HAGIWARA, AND MITSUTOSHI NAKAJIMA
National Food Research Institute, NARO, Kan-nondai, Tsukuba, Ibaraki, 305-8642, Japan

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16.1 PROMISING MATERIALS FOR THE PRODUCTION OF BIODIESEL FUEL

Biodiesel fuel is a replacement for diesel as a fuel produced from biomass resources. It is usually defined as a fatty acid methyl ester (FAME) derived from vegetable oil or animal fat.

In European countries, such as Germany and France, biodiesel is commercially produced mainly from rapeseed oil, whereas in the United States, soybean oil is more frequently used. The amount of biodiesel fuel produced in Europe in 2004 was more than 2,000,000 tons. This amount is expected to rise to about 4,000,000 tons in 2007. This quantity implies that about half of the harvested rapeseed in Europe is converted into FAME and used as biodiesel fuel.

In many other countries such as Japan and countries in Southeast Asia, lipids that cannot be used as a food source could be more suitable materials for the production of biodiesel because its production from edible oils could result in an increase in the price of edible oils, thereby increasing the cost of some foodstuffs. Therefore, used edible oil, lipids contained in waste effluent from the oil milling process, by-products from oil refining process, and crude oils from industrial crops such as jatropha could be more promising materials for use in these countries.

In Japan, it is not feasible to use virgin vegetable oil as a raw material for producing biodiesel because Japan has to import a large quantity of the edible oil it uses from foreign countries. However, it could be possible to utilize used edible oil. In Japan, about 4000 tons of FAME is produced from used edible oil and utilized as a biodiesel fuel.

Waste effluent from oil-milling processes and by-products from oil-refining processes contain substantial amounts of lipids. For example, free fatty acids are obtained from the refining process as by-products. The free fatty acids account for 5–10% of crude oil. These materials are present already and need not be collected. Therefore, if these materials are converted into FAME at a low cost, they could provide a useful source of biodiesel fuel.

Jatropha (*Jatropha curcas* L.) is an oil crop, which can grow in marginal land such as in the eastern part of Indonesia where the amount of rainfall is less than 400 mm/year (Fig. 16.1). The oil content of jatropha seeds is 30–40%. Oil productivity of the jatropha plant is considered higher than that of the soybean or rapeseed. The first harvest can be obtained within eight months of planting. Then, about 20 kg of seed can be harvested twice a year from one plant. The plant life is about 50 years. However, jatropha oil contains toxins and is unsuitable for human consumption. Therefore, utilization of jatropha oil in the production of biodiesel does not affect the supply and price of edible oil. However, if crude jatropha oil can be converted into FAME at a low cost, it will be a promising material for biodiesel fuel. Agriculture could therefore be expanded into marginal land by introducing jatropha plants, which will benefit the residing communities.

As mentioned above, the materials available in Japan and Southeast Asia for the production of biodiesel fuel have common characteristics; they contain a considerable amount of impurities and are high in free fatty acids.

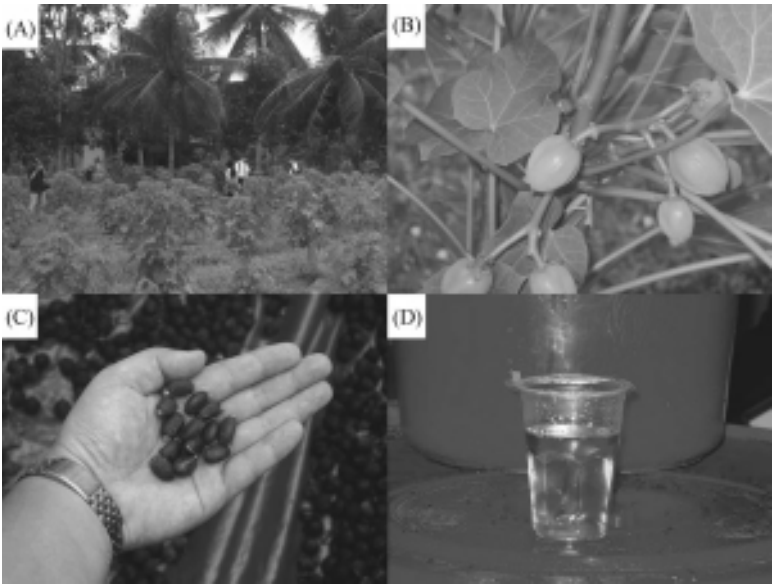


FIGURE 16.1 Jatropha plants 6 months after planting (A), fruits (B), seeds (C), and crude oil obtained by screw press (D).

16.2 PROBLEMS WITH THE CONVENTIONAL ALKALINE CATALYZED ALCOHOLYSIS REACTION PROCESS FOR THE PRODUCTION OF BIODIESEL FUEL

In the alcoholysis reaction for the production of biodiesel fuel from vegetable oil, triglyceride, which is the main component of vegetable oil, is reacted with methanol to form FAME and glycerol (Fig. 16.2). FAME is used as a biodiesel fuel. In the

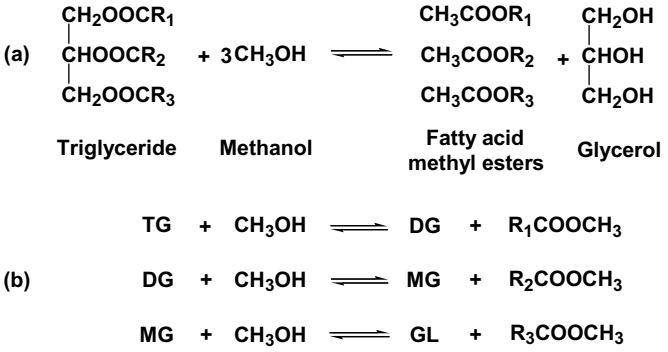


FIGURE 16.2 Alcoholysis of triglyceride with methanol. (a) overall reaction; (b) three consecutive and reversible reactions (TG, triglyceride; DG, diglyceride; MG, monoglyceride; GL, glycerol).

conventional process, alkaline catalysts such as NaOH and KOH are used to promote the reaction.

In the FAME production process using the alkaline catalyst method, the catalyst has to be removed from the products after the reaction, failing which, glycerol, which is a by-product of the process, cannot be utilized in other industries. However, the process of removing the alkaline catalyst increases the total cost of biodiesel fuel production.

In the case of the alkaline catalyzed alcoholysis reaction process, free fatty acids contained in raw materials have to be removed prior to the reaction in order to maintain the activity of the catalyst. However, the removal of free fatty acids reduces the yield of the process.

16.3 ADVANTAGES OF A NONCATALYTIC ALCOHOLYSIS REACTION PROCESS OVER THE CONVENTIONAL ALKALINE-CATALYZED PROCESS

In a system with a noncatalytic alcoholysis reaction, the purification process to remove the catalyst after the reaction is not required. Therefore, configuration of the total system can be simplified and the by-product, glycerol, can be directly utilized in other industries. In this way, the total cost of biodiesel fuel production will be reduced.

In a system with a noncatalytic alcoholysis reaction, triglycerides as well as free fatty acids could be converted into FAME. Therefore, the neutralization process for the removal of free fatty acids is not required prior to the reaction process. This eventually improves the yield of the total system.

Therefore, some groups in Japan, including the authors' group, are working on the development of a noncatalytic alcoholysis reaction process for the production of biodiesel fuel.

16.4 RESEARCH ON THE NONCATALYTIC ALCOHOLYSIS REACTION FOR BIODIESEL FUEL PRODUCTION IN JAPAN

16.4.1 Supercritical Methanol Method

Saka and Kusdiana (2001) at the University of Kyoto investigated the methyl-esterification reaction in supercritical methanol without using any catalyst. The experiment was performed in a batchwise reaction vessel preheated to 623 and 673 K and at a pressure of 45–65 MPa. They demonstrated that at a preheating temperature of 623 K, 240 s of supercritical treatment with methanol was sufficient to convert rapeseed oil into methyl esters, and although the prepared methyl esters were the same as that produced from the most commonly used method using an alkaline catalyst, the yield of methyl esters obtained at 623 K was higher than that obtained at 673 K. It was found that the supercritical methanol process requires a shorter reaction time and uses a simpler purification procedure. In addition, by using the supercritical

methanol method, methyl esters were produced not only from triglycerides but also from free fatty acids.

Then, they tried to combine the methyl-esterification reaction in supercritical methanol with the hydrolysis reaction in subcritical water (Kusdiana and Saka, 2004). In this process, triglycerides are first hydrolyzed into free fatty acids. Then the free fatty acids are converted into methyl ester in supercritical methanol. They reported that the monoglyceride content in the product was reduced and the quality of the final product was improved by using the two-step process.

16.4.2 Simultaneous Reaction of Transesterification and Cracking (STING) Method

Recently, Iijima et al. (2005) at the National Agricultural Research Center in Tsukuba, Japan proposed a new method called simultaneous reactions of transesterification and cracking (STING). In this method, transesterification and cracking proceed simultaneously under supercritical condition and triglycerides, diglycerides, monoglycerides, methyl esters, which consist of medium-chain fatty acids, higher alcohols, lower alcohols, and other hydrocarbons are formed. These components constitute one phase and are used as a biodiesel fuel. Therefore, no by-products are formed in this process.

No glycerol is formed as a by-product using the STING method. Therefore, the yield of the process is improved.

The product has lower viscosity and a lower pour point as compared to FAME formed by the conventional alkaline catalyzed method. Therefore, the quality of the product is higher.

16.4.3 Superheated Methanol Vapor Bubble Method

In order to further decrease the cost required for the production of biodiesel fuel, the authors proposed another method called the superheated methanol vapor bubble method.

Figure 16.3 shows the schematic flow diagram of a reactor for the superheated methanol vapor bubble method (Yamazaki et al., 2007; Joelianingsih et al., 2008). Superheated methanol vapor is continuously bubbled into the oil in the reactor vessel and reacted with triglycerides to form FAME and glycerol. The FAME and glycerol formed flows out of the reactor together with unreacted methanol vapor and is collected using a condenser. Condensed FAME is separated from glycerol by sedimentation and used as a biodiesel fuel. Unreacted methanol vapor is sent back to the reactor vessel and reused. No catalyst is used in this method.

A reaction using the superheated methanol vapor bubble method can be conducted at atmospheric pressure. Therefore, both initial and running costs required for the process can be reduced. The general applicability of this method could be greater than that of the supercritical methanol method or STING method.

Figure 16.4 shows the effects of material composition on the outflow rate of FAME in the superheated methanol vapor bubble method. Free fatty acid can be converted into FAME using this method. The outflow rate is several times higher than that with

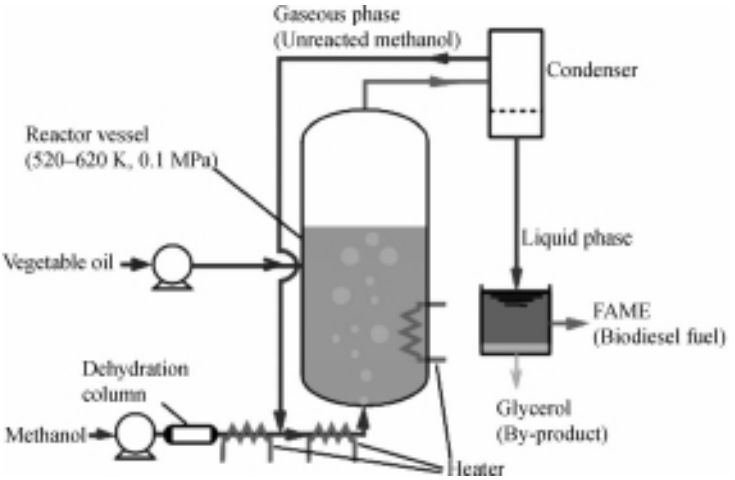
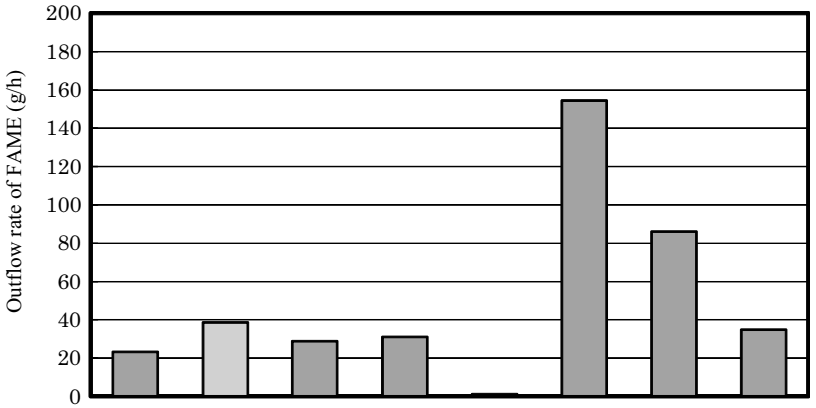


FIGURE 16.3 Schematic flow diagram of a reactor based on the superheated methanol vapor bubble method (FAME: fatty acid methyl ester).



Sample		Fresh oil (FO)	Used oil	DG	FO:DG =50:50	MG	Oleic acid (OA)	FO:OA =50:50	FO:OA =98:2
Composition	TG	98.7	90.5	13.5	56.0	-	-	49.3	96.4
	DG	1.0	5.0	82.5	41.8	-	-	0.5	1.0
	MG	0.4	4.0	4.0	2.2	100.0	-	0.2	0.4
	FFA	-	0.5	-	-	-	100.0	50.0	2.2
Outflow rate of FAME (g/h)		23.1	38.5	28.8	31.0	1.2	154.4	85.9	34.8

FIGURE 16.4 Effects of impurities contained in waste edible oils on the outflow rate of fatty acid methyl ester (FAME) from a reactor based on the superheated methanol vapor bubble method (TG, triglyceride; DG, diglyceride; MG, monoglyceride; FFA, free fatty acid).

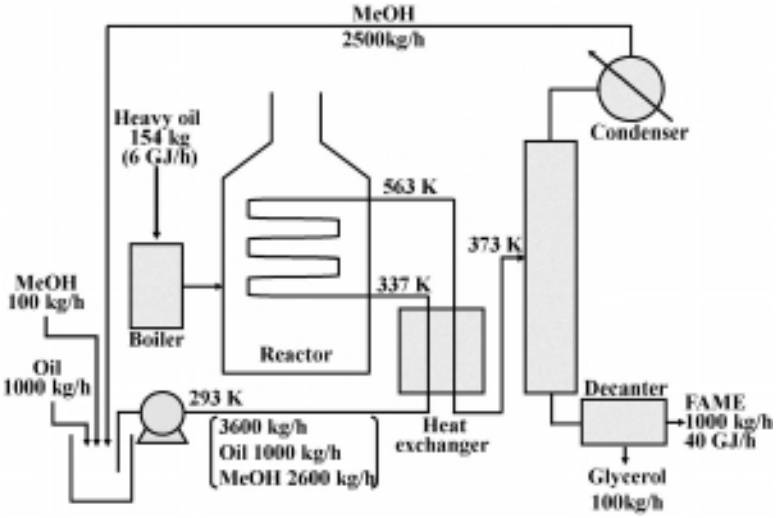


FIGURE 16.5 Flowchart of energy and materials in a practical scaled noncatalytic reactor based on superheated methanol vapor bubble method.

triglycerides (Joelianingsih et al., 2007). This result implies that this method is suitable for materials available outside Europe and the United States, which are rich in free fatty acids.

Based on the data obtained using a bench scale reactor, an industrial scale reactor, which could produce 1000 kg of methyl esters in an hour, was designed, and material balance and economic efficiency of the reactor were estimated. Figure 16.5 shows a flow diagram of the design process (Ishikawa et al., 2005). In this case, triglycerides were used as the raw material. In order to produce 1000 kg of methyl esters, 154 kg of heavy oil needs to be burned as a heat source for the reactor.

The cost of producing 1 L of methyl esters from used edible oil using this reactor was estimated to be about 40 yen (0.33 cents), which was much lower than the cost required for the conventional alkaline catalyst method.

In 2006, the authors' group constructed a pilot scale reactor, which produced about 40 L of FAME in a day using the superheated methanol vapor bubble method.

In 2007, the authors' group designed a practical scale reactor, which produced about 400 L of FAME in a day. This reactor has been constructed recently (Fig. 16.6). However, the efficiency of the reactor remains to be demonstrated.

16.5 CONCLUSIONS

The current state of research on the production of biodiesel fuel conducted in Japan is introduced in this paper.

The authors hope that the results of this research will reduce the cost involved in producing biodiesel fuel and contribute to the prevention of global warming,

FIGURE 16.6 Demonstration plant based on the superheated methanol vapor bubble method (design productivity: 400 L/d).

reduction in consumption of fossil fuels, and the utilization of rural areas in Japan as well as in many other countries.

ACKNOWLEDGMENT

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17

USE OF *Coniochaeta ligniaria* TO DETOXIFY FERMENTATION INHIBITORS PRESENT IN CELLULOSIC SUGAR STREAMS[1]

NANCY N. NICHOLS AND BRUCE S. DIEN
Bioenergy Research Unit, NCAUR, USDA-ARS, Peoria, IL 61604, USA

MARIA J. LÓPEZ AND JOAQUÍN MORENO
Departamento de Biología Aplicada, University of Almería, Almería, Spain

17.1 INTRODUCTION

Lignocellulosic biomass, such as agricultural residues and energy crops, is an abundant potential feedstock that could be converted to fuels and chemicals so as to avoid competition with food crops. Conversion of biomass to products (Fig. 17.1)

[1]Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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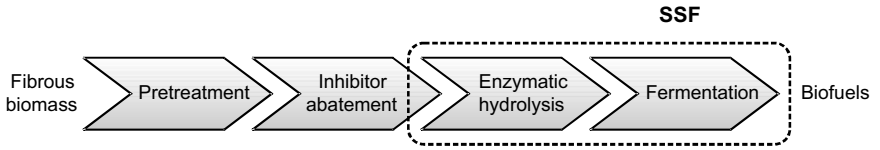

FIGURE 17.1 Conversion of lignocellulosic biomass to ethanol or other products. Enzymatic saccharification and microbial fermentation may be combined in a single process known as simultaneous saccharification and fermentation (SSF). Inhibitor abatement is carried out after pretreatment.

involves physical and/or chemical pretreatment, followed by enzymatic saccharification and microbial fermentation (Mosier et al., 2005; Hahn-Hägerdal et al., 2006; Lin and Tanaka, 2006; Yang and Wyman, 2008). Pretreatment renders cellulose accessible for enzymatic digestion and, in some cases, releases fermentable sugars from hemicellulose (Mosier et al., 2005; Yang and Wyman, 2008). Due to the fibrous nature of lignocellulose, pretreatment conditions often are severe enough to cause the formation of side-products that are inhibitory to fermentation. These inhibitory products arise from degraded sugar monomers and partial solubilization of lignin and include furan aldehydes, phenolic compounds, and aromatic and aliphatic aldehydes and acids (reviewed in Palmqvist and Hahn-Hägerdal, 2000b and Klinke et al., 2004). Furan aldehydes arise from dehydration of sugars that are liberated during dilute acid pretreatment; furfural forms from pentoses, and 5-hydroxymethylfurfural (HMF) from hexoses. Acetic acid is released from acylated plant hemicellulose and, in some cases, from lignin. Formic and levulinic acids are formed by the degradation of sugars and HMF during acid hydrolysis of lignocellulose. Aromatic acids, aldehydes, and alcohols arise from the hydrolysis or oxidation of lignin; the particular compounds formed depend on lignin composition and pretreatment method.

As a result of these inhibitors, lignocellulosic hydrolysates present a hostile environment for fermenting microbes. Toxicity correlates generally with hydrophobicity of compounds (Zaldivar et al., 1999; Klinke et al., 2004). For furans and aromatic compounds, aldehydes are the most toxic to microbes, followed by acids and alcohols (Zaldivar et al., 1999, 2000; Zaldivar and Ingram, 1999). The net effect on the culture is to inhibit microbial growth, ethanol productivity, and sometimes ethanol yield. The effect on the microbial cell is to disrupt membrane integrity, macromolecular synthesis, and/or glycolytic and fermentative enzymes (Zaldivar et al., 1999; Palmqvist and Hahn-Hägerdal, 2000b; Gorsich et al., 2006; Hristozova et al., 2006, 2008). Furfural and HMF are especially problematic because they are both potent inhibitors and their effects are synergistic with other compounds (Zaldivar et al., 1999; Palmqvist et al., 1999).

Inhibitors present a direct challenge to processing of biomass because depending upon their concentrations, they will reduce product yields, delay fermentations, or even cause them to fail (Palmqvist and Hahn-Hägerdal, 2000a; Klinke et al., 2004). Therefore, inhibitory compounds must be mitigated prior to fermentation (Fig. 17.1).

Methods for inhibitor removal include dilution, adsorption, extraction, and precipitation (Palmqvist and Hahn-Hägerdal, 2000a; Mussatto and Roberto, 2004; Zautsen et al., 2009). Many of these methods have significant disadvantages such as excess waste generation and/or cost. We have recently developed a novel method for removing inhibitors that uses biological abatement. Bioabatement uses a microbe to metabolize the inhibitory by-products arising from the pretreatment of lignocellulose. Its advantages include the ability to treat liquid–solid mixtures, no requirement for recharging unlike resins, minimal need for chemical inputs, and limited generation of waste streams. Use of this methodology might also be amenable to process water recycling schemes. This chapter reviews the discovery and characterization of a bioabatement microbe, *Coniochaeta ligniaria* NRRL30616, and use of the strain to detoxify lignocellulosic biomass hydrolysates.

17.2 DISCOVERY OF MICROORGANISMS FOR ABATEMENT OF FERMENTATION INHIBITORS

A microbial enrichment strategy was used to isolate suitable microbes for the bioabatement of lignocellulose hydrolysates (López et al., 2004). Furfural-contaminated soil was used as the environmental source of microbes (Nichols et al., 2005, 2008). The inoculum was initially serially enriched for microbes that are able to grow in a mineral medium containing furfural, HMF, and ferulic acid as the sources of carbon and energy. These carbon sources are representative of fermentation inhibitors found in hydrolysates, and so the microbes enriched in this step were able to metabolize at least one of the carbon sources and tolerate the combination of inhibitory compounds during growth. The subsequent transfer of enrichment cultures into pH-neutralized corn stover acid (CSH), prepared by dilute-acid pretreatment was followed by selecting microbes that withstand inhibitors present in an actual hydrolysate.

The microbes isolated from this two-step enrichment consisted of bacteria, including *Acinetobacter*, *Arthrobacter*, *Flavobacterium*, *Methylobacterium*, and *Pseudomonas* strains, and one fungal strain. The one fungal isolate, *C. ligniaria* NRRL30616, was selected for further study because of its superior growth on the inhibitory chemicals in a mineral medium and in pH-neutralized corn stover dilute-acid hydrolysate. *Coniochaeta* are Ascomycete fungi found in soil, plants, and decaying wood (Weber et al., 2002). *C. ligniaria* NRRL30616 is capable of eliminating furfural and HMF from CSH (Fig. 17.2) (López et al., 2004).

17.3 *C. ligniaria* METABOLISM OF INHIBITORS

C. ligniaria, like many soil microbes, has evolved the ability to metabolize a wide array of substrates. In addition to furfural and HMF, strain NRRL30616 grows on other inhibitory chemicals as sole sources of carbon and energy, including acetate, ferulic acid, 4-hydroxybenzaldehyde, syringaldehyde, vanillin, and vanillic

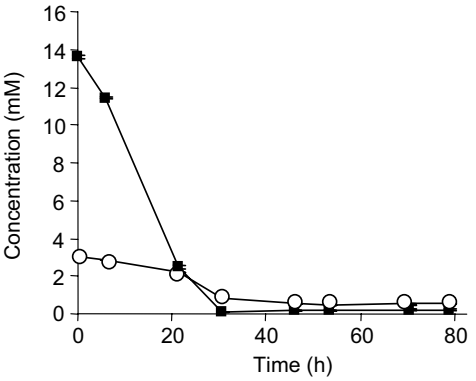

FIGURE 17.2 Removal of furfural (■) and HMF (○) from corn stover hydrolysate by *C. ligniaria* NRRL30616 (adapted from López et al., 2004).

acid (Nichols et al., 2005). All of these compounds are present in dilute-acid treated lignocellulose, such as corn stover hydrolysate. *C. ligniaria* was further evaluated for growth on an actual corn stover prepared by dilute-acid pretreatment. As shown in Figures 17.3 and 17.4, the chemical profile of corn stover hydrolysate was altered after

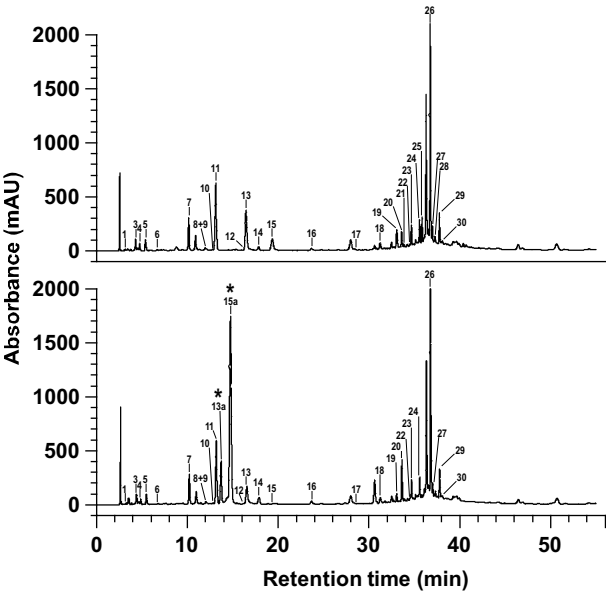

FIGURE 17.3 HPLC–UV chromatograms of corn stover dilute acid hydrolysate before (top) and after (bottom) 48 h incubation with *C. ligniaria* NRRL30616. Asterisks above peaks 13a and 15a indicate HMF alcohol and furfuryl alcohol, respectively; these two peaks result from metabolism of HMF and furfural by NRRL30616 and were not present in uninoculated hydrolysate. The identity of other peaks is discussed in Nichols et al. (2008).

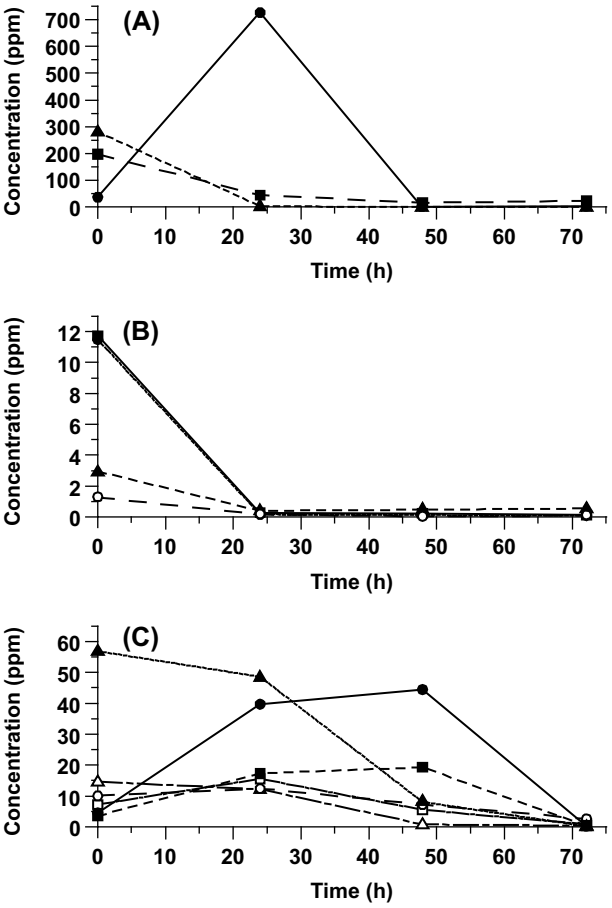

FIGURE 17.4 Fate of inhibitory compounds in corn stover hydrolysate during 72 h incubation with NRRL30616. (A) Furfural (□), 2-furoic acid (●), and HMF (○). (B) 4-Hydroxybenzaldehyde (●), 3,4-dihydroxybenzaldehyde (○), vanillin (□), and syringaldehyde (△). (C) Ferulic acid (○), vanillic acid (●), 4-hydroxycoumaric acid (□), salicylic acid (■), and 3,4-dihydroxybenzoic acid (▲). Reproduced from Nichols et al. (2008).

abatement with *C. ligniaria* NRRL30616. Figure 17.3 qualitatively shows the compounds present in hydrolysate before and after bioabatement, as detected by HPLC-UV. Figure 17.4 shows the concentration of selected compounds measured during 72 h of incubation. Inhibitory compounds in several classes (furan aldehydes, aromatic aldehydes, and aromatic acids) were metabolized. The concentration of some compounds increased transiently, suggesting their potential role as metabolic intermediates during the removal of the various inhibitors (Fig. 17.4). Transient accumulation of aromatic acids, furan derivatives (furfuryl alcohol, HMF alcohol, and

furoic acid), and some citric acid cycle intermediates was observed during growth in corn stover hydrolysate (Nichols et al., 2008).

Because many microbes efficiently metabolize sugars, it seemed possible that *C. ligniaria* NRRL30616 might consume the fermentable sugars present in the hydrolysate in preference to the inhibitory compounds. To test for this, the strain was grown in media containing mixtures of furfural and either glucose (Nichols et al., 2005) or xylose. In fact, furfural was consumed prior to both glucose and xylose (Fig. 17.5). Furfural was consumed from the mixtures more rapidly than either sugar,

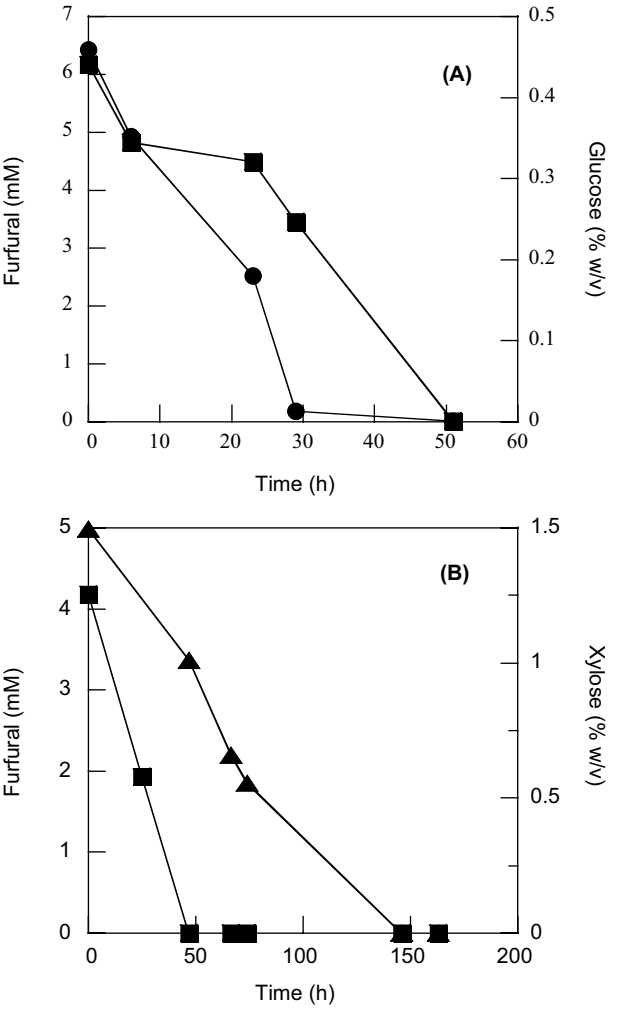

FIGURE 17.5 Detoxification of furfural by *C. ligniaria* NRRL30616 in the presence of sugars. (A) Furfural (circles) is metabolized prior to glucose (squares). (B) Furfural (squares) is metabolized prior to xylose (triangles). Data for glucose metabolism was previously reported by Nichols et al. (2005).

despite the fact that NRRL30616 grows faster on glucose or xylose (2.7–3.1 h doubling time) than on furfural (7.0–10.0 h doubling time when grown on furfural as a sole carbon and energy source). Similar results were observed in corn stover hydrolysate, where furfural and HMF degradation begins immediately, but sugars are consumed only after extended incubation (Nichols et al., 2005, 2008). Preferential metabolism of furfural prior to glucose or xylose by NRRL30616 is likely a stress response to detoxify furfural, rather than a true substrate preference. This phenomenon has practical consequences for bioabatement; consumption of sugars by the abatement microbe is undesirable because sugar monomers should be left available for conversion to the product by the fermenting microorganism.

It is likely that isolate NRRL30616 acquired enhanced ability to withstand furfural-related stress prior to its enrichment and isolation in the laboratory. NRRL30616 was isolated from furfural-contaminated soil, and was therefore subjected in nature to selection for the ability to metabolize, or at least withstand, the toxic effects of furfural. In fact, NRRL30616 was compared to several related fungal strains, and although some closely related strains could grow on furfural, HMF, and other inhibitory compounds, NRRL30616 had superior ability to detoxify corn stover hydrolysate (Nichols et al., 2005). In addition, fungal soil isolates such as *C. ligniaria* may also be expected to have the ability to degrade lignocellulosic polymers. In a screen for lignocellulose-degrading enzymes, NRRL30616 and related strains were shown to produce cellulase, xylanase, and two lignin peroxidases (manganese peroxidase and lignin peroxidase), but not laccase, in solid- and semisolid-state cultures (López et al., 2007). The degradative activities of *C. ligniaria* may be useful for processing lignocellulosic biomass.

17.4 BIOABATEMENT OF PRETREATED HYDROLYSATES

It was expected that removal of inhibitors from hydrolysates by NRRL30616 would enhance subsequent fermentation to ethanol. To prove the effectiveness of bioabatement, NRRL30616 was used to condition corn stover hydrolysate prior to ethanol fermentation. After corn stover was pretreated with dilute acid, the resulting hydrolysate was neutralized and inoculated for bioabatement with NRRL30616. Then the hydrolysate was fermented to determine whether the altered chemical profile resulted in improved ethanol fermentation. Solids were removed before the growth of the bioabatement strain and then recombined with the liquid hydrolysate for simultaneous saccharification with cellulase and β-glucosidase and fermentation by *Saccharomyces* sp. LNH-ST, a yeast strain engineered to ferment xylose (Ho et al., 1999). Fermentations were also conducted using *E. coli* FBR5, a bacterial strain engineered to selectively produce ethanol rather than the usual mixture of lactate, acetate, and ethanol (Dien et al., 2000).

As shown in Figure 17.6 for *Saccharomyces* sp. LNH-ST, bioabatement with NRRL30616 resulted in improved fermentations. In the example shown (Fig. 17.6A), treated hydrolysate yielded 1.7% ethanol, while no ethanol was produced from unabated hydrolysate. Instead, glucose liberated by saccharification of cellulose

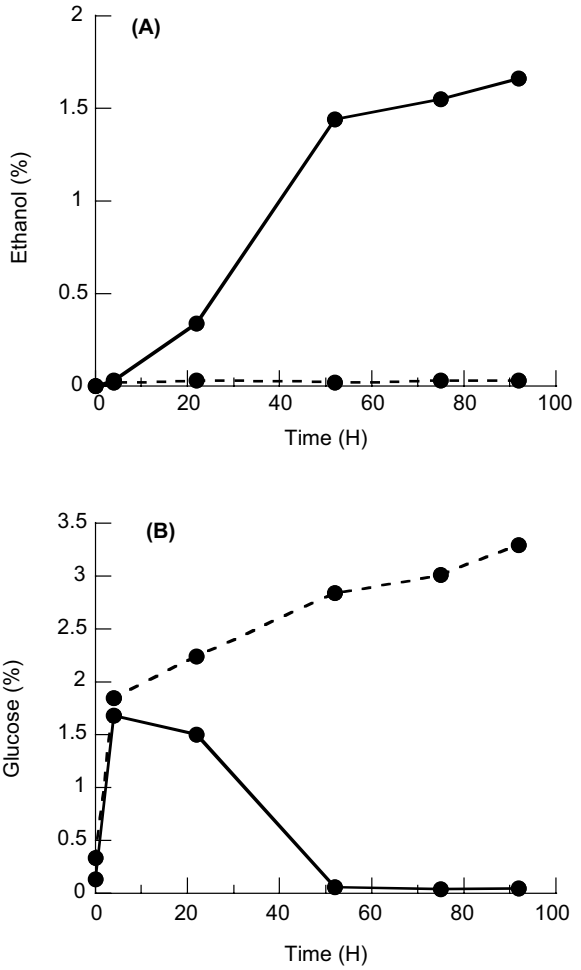

FIGURE 17.6 Effect of bioabatement on SSF of corn stover hydrolysate using *Saccharomyces* sp. LNH-ST. Solid lines represent fermentation of hydrolysate after bioabatement (24 h growth) using *C. ligniaria* NRRL30616. Dashed lines show results for the negative control (hydrolysate that was not abated with *C. ligniaria* prior to SSF). Concentration of (A) ethanol and (B) glucose during fermentation. Reproduced from Nichols et al. (2005).

accumulated in the hydrolysate (Fig. 17.6B). Fermentation of pentoses by recombinant ethanol-producing microbes was also shown to occur more efficiently in hydrolysates that were conditioned with NRRL30616 than in untreated hydrolysates (Nichols et al., 2008).

Strain NRRL30616 was also evaluated for bioabatement of hydrolysates prepared with switchgrass. Switchgrass presents a possible unique challenge for dilute acid hydrolysis and microbial fermentation, because fructose, present during early stages of growth, is highly susceptible to conversion to HMF upon exposure to acid and heat

(Dien et al., 2006). Reduction in furfural and HMF concentrations by *C. ligniaria* was observed in switchgrass hydrolysate. As observed for corn stover, preconditioning with *C. ligniaria* yielded switchgrass hydrolysates with improved fermentability and no lag phase (not shown). Because commercial fermentations are operated under nonaseptic conditions; a long lag phase would not be feasible because it would foster microbial contamination.

17.5 CONCLUSIONS

The biological abatement of biomass hydrolysates taps the ability of specially selected microbes to metabolize detrimental substances present in lignocellulose-derived sugars. The use of microbial cells for detoxification relies on the presence and operation of a suite of pathways for metabolism of compounds including furans, aromatics, and organic acids. In abatement of fermentation inhibitors, the selected microbe may be expected to efficiently undertake detoxification of the compounds that are perceived to be most problematic. The need for an appropriately selected microbe to tolerate and grow in the inhibitory milieu of biomass hydrolysates was the impetus for isolation of *C. ligniaria* NRRL30616.

Other microbes have also been identified for potential use in bioabatement of hydrolysates. The growth of *Trichoderma reesei* in willow hemicellulose hydrolysate decreased the ultraviolet absorbance of hydrolysates, presumably by degrading phenolic compounds, and improved the fermentability of the remaining cellulose fraction (Palmqvist et al., 1997). Enzymatic detoxification, using fungal laccase and lignin peroxidase, also resulted in improved fermentability (Jönsson et al., 1998). Likewise, a *Saccharomyces cerevisiae* mutant, unable to utilize glucose or mannose for growth, was used to deplete acetic acid from hardwood hydrolysate and yielded a sugar stream that allowed enhanced growth of xylose-fermenting yeasts (Schneider, 1996). Detoxification of corn stover and corn starch pyrolysis liquors by the white rot fungus *Phanerochaete chrysosporium* and by bacterial strains *Pseudomonas putida* and *Streptomyces setonii* was demonstrated by Khiyami et al. (2005a, 2005b). A Gram-negative bacterium, *Ureibacillus thermosphaericus*, was also shown to detoxify and improve fermentability of waste wood hydrolysate (Okuda et al., 2008). In a related work, a *C. ligniaria* isolate and four bacterial strains were isolated based on their ability to remove phytotoxic compounds from torrefied grass used as potting soil and seed germination medium (Trifonova *et al.*, 2008). These examples show that appropriately selected microbes, including *C. ligniaria*, may be well suited for targeted removal of fermentation inhibitors by metabolism.

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18

OMICS APPLICATIONS TO BIOFUEL RESEARCH

TZI-YUAN WANG,[1] HSIN-LIANG CHEN,[2] AND WEN-HSIUNG LI[1,2]
[1]*Genomics Research Center, Academia Sinica, Taipei, Taiwan*
[2]*Department of Life Sciences, National Cheng Kung University, Tainan, Taiwan*

HUANG-MO SUNG
Biodiversity Research Center, Academia Sinica, Taipei, Taiwan

MING-CHE SHIH
Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan

18.1 INTRODUCTION

The shortage of the oil supply calls for the exploration of biomass ethanol. The U.S. Departments of Energy and Agriculture have jointly mapped a plan to replace 30% of the national consumption of liquid transportation fuels with bioethanol by 2030

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(www.genomicsgtl.energy.gov/centers). The switch will create a huge impact on the energy and chemical industries. Associated with this switch, many chemical feedstocks currently derived from petrochemical sources may instead be obtained from biological and agricultural sources through fermentations and bioconversions. Lignocellulosic biomass (i.e. nongrain plant materials) is currently being promoted as an alternative feedstock source for the production of ethanol. The major component of plant cell wall (lignocellulose materials) is cellulose, along with hemicellulose and lignin. Cellulose is a polymer of glucose and makes up about 45% of the dry weight of wood.

Lignocellulose is difficult to hydrolyze because it is (i) associated with hemicellulose, (ii) surrounded by a lignin seal that has a limited covalent association with hemicellulose, and (iii) much of it has a crystalline structure with a potential formation of six hydrogen bonds, four intramolecular and two intermolecular, giving it a highly ordered, tightly packed structure. Pretreatments aim at increasing the surface area of cellulose by (i) removing the lignin seal, (ii) solubilizing hemicellulose, (iii) disrupting crystallinity, and/or (iv) increasing pore volume. The value of a cellulase system that attacks crystalline cellulose lies in the observation that many of the pretreatments that increase surface area also increase crystallinity. These include dilute sulfuric acid, alkali, and ethylenediamine. The rate-limiting step in the conversion of cellulose to fuels is its hydrolysis, especially the initial attack on the highly ordered, insoluble structure of crystalline cellulose, since the products of this attack are readily solubilized and converted to sugars. A great deal of effort has gone into the development of methods for conversion of cellulose to sugars. Most of this work has emphasized the biochemistry, genetics, and process development of fungi (especially *Trichoderma reesei*) coupled to the further conversion of the produced sugars to ethanol by yeast (*Saccharomyces cerevisiae*) (Demain et al., 2005; Garcia-Campayo and Wood, 1993; Gomez de Segura et al., 1998; Gomez de Segura and Fevre, 1993; Hebraud and Fevre, 1990a, 1990b; Li and Calza, 1991; Ljungdahl, 2008; Mountfort and Asher, 1989; Pearce and Bauchop, 1985; Percival Zhang et al., 2006; Wood et al., 1995). Recent advances in genomics, transcriptomics, and proteomics make it possible to hunt for cellulases more efficiently. This chapter introduces the biological concept of biomass conversion and how omics approaches can be applied to explore microbial genomes and elucidate the functions of cellulases and related genes.

18.2 NEXT GENERATION: RENEWABLE ENERGY BIOMASS PROGRAM

Corn and sugarcane are two main feedstocks for current generation bioethanol, while soybean is the main source for biodiesel production. Ethanol is an important element of transportation fuel production. In 2001, about 12% of gasoline sold in the United States contained 8–10% ethanol as a fuel additive to boost octane and reduce carbon monoxide and other toxic air emissions. Due to current high demand and tight supply of fossil fuel, it is essential to develop bioethanol as a renewable energy source. Currently, in the United States ethanol is derived mostly from corn kernel starch,

which is catabolized to glucose by α-amylase and amyloglucosidase prior to fermentation by yeast. The usage of ethanol as fuel is advantageous because it reduces dependence on foreign oil, has a positive impact on the environment, and mitigates atmospheric fossil carbon emissions. However, even if all harvested corn were used for ethanol production, it would amount to less than 10% of current U.S. transportation fuel consumption (Hill et al., 2006). In addition, recent usage of corn for ethanol production has driven food price upward dramatically. Recognizing this shortfall, nongrain plant materials are being promoted as an alternative feedstock source for ethanol production.

The major bulk of plant material is cell wall, which consists of cellulose (40–50%), hemicellulose (20–30%), and lignin (20–30%), depending on the plant species. The fundamental cellulosic ethanol process needs a chemical-based pretreatment to break the raw materials and microbased, mainly yeast, fermentation to convert xylose (5C sugar) and glucose (6C sugar) into ethanol (Fig. 18.1). The key step is to breakdown cellulose into glucose and hemicellulose into xylose. Cellulose $(C_6H_{10}O_5)_n$ is a polymer of glucose in beta-1,4-linkage. It is the major component of plant cell wall and is the most abundant form of living terrestrial biomass. Hemicellulose has a random, amorphous structure with little strength and is present in almost all plant cell walls along with cellulose. Hemicellulose is easily hydrolyzed by dilute acid or base as well as by enzyme treatment. Sugar monomers in hemicellulose can include xylose, mannose, galactose, rhamnose, and arabinose.

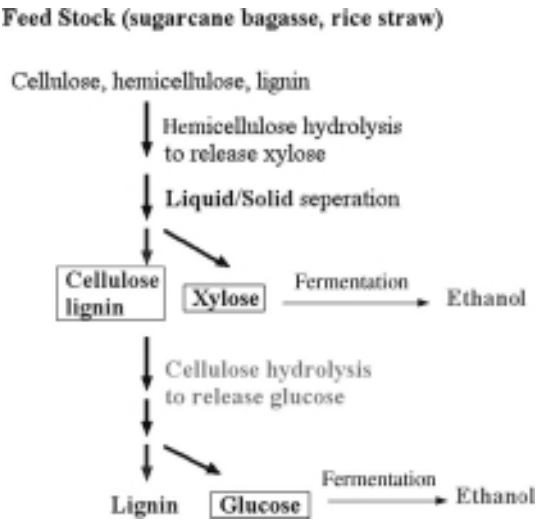

FIGURE 18.1 Cellulosic ethanol process. The pretreatment process converts the feedstocks into liquid (xylose) and solid (cellulose and lignin) as raw materials for further cellulase degradation and yeast fermentation.

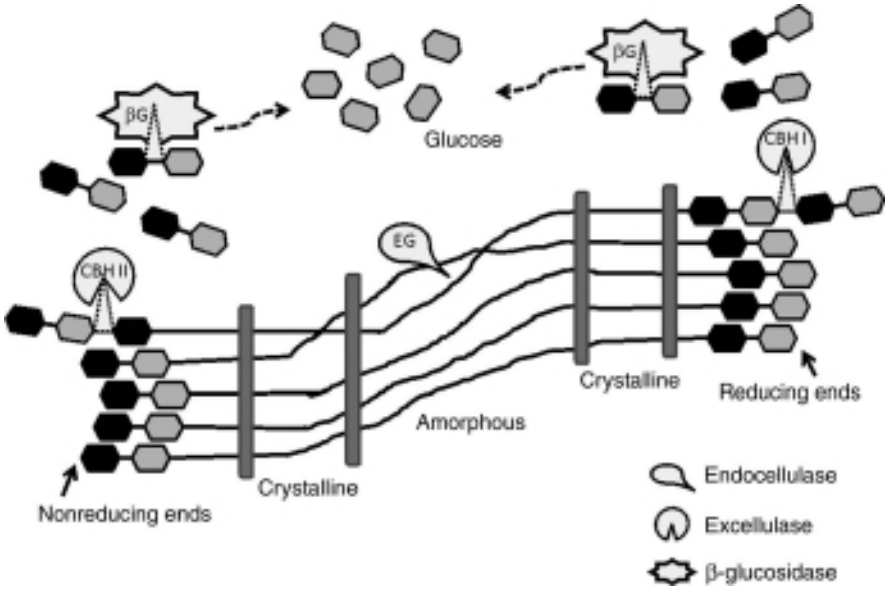

FIGURE 18.2 A combination of three enzymes is required to degrade cellulose. Endocellulase can hydrolyze internal β-1,4-linkage of cellulose into short oligosaccharides; two major types of exocellulase (CBH I, CBH II) then hydrolyze the oligosaccharides into cellobiose from the ends. The β-glucosidase can further hydrolyze cellobiose into two molecules of glucose.

There are three major types of enzymes found in cellulase systems that can degrade crystalline cellulose (Fig. 18.2): (1) endoglucanases (endo-β-1,4-glucanases, EG; EC 3.2.1.4); (2) exoglucanases (exo-β-1,4-glucanases, EXG), including cellodextrinases (1,4-β-D-glucan glucanohydrolases; EC 3.2.1.74) and cellobiohydrolases (CBH) (1,4-β-D-glucan cellobiohydrolases; EC 3.2.1.91); and (3) β-glucosidases (β-D-glucoside glucohydrolases, BGLU; EC 3.2.1.21) (for reviews see Lynd et al., 2002, 2005). Endoglucanases break internal bonds to disrupt the crystalline structure of cellulose, exposing individual cellulose polysaccharide chains. Endoglucanase could hydrolyze carboxymethylcellulose (CMC), $H_3PO_4^-$, or alkali-swollen (amorphous) cellulose really well, but was not able to attack crystalline cellulose such as cotton fiber or Avicel to a significant extent. Hydrolysis of amorphous cellulose yields a mixture of glucose, cellobiose, and other soluble cellooligosaccharides; glucose and cellobiose are the principal products of the reaction. Second, exoglucanases cleave 2–4 units from the ends of the chains produced by endocellulase, resulting in tetrasaccharides or disaccharide (cellobiose). Two main types of exocellulases (CBH): the first type, CBH I, works processively from the reducing end, and the other type, CBH II, works processively from the nonreducing end of cellulose. Exoglucanase is able to digest crystalline cellulase such as Avicel but is unable to digest CMC and cellobiose, which makes Avicel a good substrate for measuring

cellobiohydrolase activity. The other is β-glucosidase that hydrolyzes cellobiose into monosaccharides, but is unable to digest cellulose. Most β-glucosidases can cleave cellobiose into two units of glucose. However, some β-glucosidases can hydrolyze aryl-β-glucosides but not cellobiose.

18.3 MAIN FEEDSTOCKS FOR NEXT-GENERATION BIOFUELS

Grasses such as *Miscanthus* and switchgrass are being proposed as good feedstocks for bioethanol production. However, due to limited farmland, we propose to develop two agricultural residuals, rice straw and sugarcane bagasse, as well as Napier grass, *Pennisetum alopecuroides*, as feedstocks for current-generation biofuels in Taiwan. Farmers used to burn tons of the rice straw and bagasse, which emitted great amounts of carbon dioxide (CO_2) into the air and might contribute to causing global warming. Converting these raw materials into cellulosic ethanol would reduce carbon dioxide release and recycle them as an alternative energy source in cars, machines, and even industries. Napier grass has the advantage of fast growth, biomass production, disease resistance, adaptability, minimal management, and easy propagation. More than 60% of its cell wall is cellulose and hemicellulose. These energy crops can be further improved by breeding of new cultivars with lower lignin contents (Torney et al., 2007). In addition, genetic engineering can also be used to express endoglucanase and other lignocellulolytic enzymes in these crops as bioreactors or for autohydrolysis.

Two main obstacles in cellulose breakdown are as follows: (1) lignins prevent access of cellulose to enzyme attack and (2) cellulose in crystalline form cannot be degraded efficiently by cellulases. So, there is a great need to find powerful cellulases to break down cellulose into glucose. Microbes from a variety of locations, such as decaying woods and plant materials or from guts of grass-feeding animals, termites, or grasshoppers, possess cellulases to digest cell wall components. These microbes seem to be good sources for cellulases, but how can we look for good cellulases? We need to identify the cellulases, clone and express the enzymes, and quantify their activity with the same evaluating platform.

18.4 IDENTIFICATION OF CELLULASE GENES BY GENOMIC APPROACHES

A way to identify microbial cellulase genes is through purification of cellulase proteins and cloning of cDNAs based on protein sequences. However, this approach is tedious and inefficient. In addition, microbial cellulases are often produced in small amounts and cannot be attained in sufficient quantities for subsequent works. The microbial genome sequencing project administered by the Joint Genome Institute (JGI), the U.S. Department of Energy (www.jgi.doe.gov/), has sequenced more than 500 microbial genomes and established a searchable database for sequence retrieving. Many of these microbes are lignocellulytic and their genomes are therefore potential sources for efficient cellulase genes. In addition, there are a number of new-generation

DNA sequencers with high-throughput sequencing capability. These next-generation sequencers speed up the gene identification and discover more specific enzymes and regulatory genes within weeks or months (Droege and Hill, 2008; Hanriot et al., 2008; Hashimoto et al., 2009). With these rich genomic resources, it is possible now to hunt for cellulase genes at the genomic scale.

Phanerochaete chrysosporium, for example, is a white-rot fungus that can efficiently degrade lignin and gain access to cellulose and hemicellulose of plant cell walls. The genomic sequence is completed (Martinez et al., 2004; Sato et al., 2009; Vanden Wymelenberg et al., 2006). Its genome contains genes for cellulases, xylanases, and lignin-degrading enzymes, which can be explored for biomass conversion and industrial usage. We searched the *P. chryosporium* genome and collected putative cellulases genes as candidates for cloning (Table 18.1). After domain structure analyses, we chose EXG, EG, and BGLU genes, which contain both catalytic glycosyl hydrolase (GH) domain and cellulose binding motif (CBM) in their coding sequences, for our heterologous protein expression studies. The presence of CBM increases substrate-binding affinities. Recent studies showed that functional cellulases indeed could be found in these candidates (Sato et al., 2009; Vanden Wymelenberg et al., 2006).

For most genomic sequences in the JGI database, we found that their annotations are mostly incomplete. Therefore, in order to identify cellulase genes from these genomes, one needs to perform a BLAST search with EXG, EG, or BGLU sequences from related species. After cloning of putative cellulase genes, sequencing analyses need to be performed to validate the open reading frames of target genes, since we have found errors in some of the sequences retrieved from the genomic database.

18.5 HOW CAN TRANSCRIPTOMIC STUDY HELP IDENTIFY CELLULASE GENES IN A MICROBE?

Microarray can be used to establish molecular profiling of key microbes to provide a global view of cellular activity and build regulatory and metabolic maps to guide future genetic engineering. We have used *Clostridium thermocellum* to establish a platform for transcriptomic studies in biofuel research. *C. thermocellum* is a rare organism that is both cellulolytic and ethanogenic, which is a natural cellulose-degrading bacterium that can utilize cellulose as the sole carbohydrate and energy source. It possesses a cellulase system, cellulosome, that is highly active on crystalline cellulose. *C. thermocellum*, therefore, is a promising organism for the industrial process, consolidated bioprocessing (CBP), which directly converts cellulosic materials to ethanol by fermentative microorganism (Raman et al., 2009).

We found that *Clostridium* can grow, though very slowly, on different agricultural materials, such as powders of rice straw, sugarcane bagasse, or Napier grass, even without any pretreatment. In addition, we found that *C. thermocellum* can grow on microcrystalline cellulose, i.e., cellobiose, but not glucose, as the sole carbon source. To design microarray slides for our study, we downloaded the genomic sequence of *C. thermocellum* from JGI at http://genome.jgi-psf.org (Brown et al., 2007). After

TABLE 18.1 Annotated Cellulase Genes in *P. chrysosporium*

Gene	GH Family	Predicted Function	Comments
Pc.3.82.1	GH6	Cellobiohydrolase II	Carries a CBM1
pc.116.24.1	GH7	Cellobiohydrolase	Carries a CBM1
ug.139.1.1	GH7	Cellobiohydrolase	Carries a CBM1
pc.139.19.1	GH7	Cellobiohydrolase	cbh1-1 (5): cel7
pc.139.26.1	GH7	Cellobiohydrolase	Carries a CBM1
pc.33.51.1	GH7	Cellobiohydrolase	Carries a CBM1
pc.95.47.1	GH7	Cellobiohydrolase	Carries a CBM1
pc.47.15.1	GH61	Putative endoglucanase	Carries a CBM1
pc.48.13.1	GH61	Putative endoglucanase	Carries a CBM1
pc.60.56.1	GH61	Putative endoglucanase	Carries a CBM1
pc.92.53.1	GH61	Putative endoglucanase	Carries a CBM1
pc.48.27.1	GH61	Putative endoglucanase	Carries a CBM1
Gx.9.81.1	GH61	Putative endoglucanase	
gx.92.11.1	GH61	Putative endoglucanase	
Pc.9.32.1	GH61	Putative endoglucanase	
gx.92.22.1	GH61	Putative endoglucanase	
gw.174.14.1	GH61	Putative endoglucanase	
Pc.9.34.1	GH61	Putative endoglucanase	
Pc.9.36.1	GH61	Putative endoglucanase	
pc.38.73.1	GH61	Putative endoglucanase	
pc.61.18.1	GH61	Putative endoglucanase	
pc.10.35.1	GH61	Putative endoglucanase	
pc.88.21.1	GH61	Putative endoglucanase	
pc.92.56.1	GH61	Putative endoglucanase	
pc.132.24.1	GH9	Putative endoglucanase	
gx.11.93.1	GH3	Putative beta-glucosidase	Carries a CBM1
gx.11.93.1	GH3	Beta-glucosidase	
pc.114.39.1	GH3	Putative beta-glucosidase	
pc.2.181.1	GH3	Putative beta-glucosidase	
pc.47.98.1	GH3	Putative beta-glucosidase	
gx.63.15.1	GH3	Putative beta-glucosidase	
Pc.80.2.1	GH3	Putative beta-glucosidase	
pc.83.26.1	GH3	Putative beta-glucosidase	
Gx.66.3.1	GH3	Putative beta-glucosidase	
Pc.80.1.1	GH3	Putative beta-glucosidase	
gx.140.9.1	GH3	Putative beta-glucosidase	
gx.231.5.1	GH3	Putative beta-glucosidase	

retrieving sequences of putative EXG, EG, BGLU, and carbohydrate metabolic genes, a total of 180 genes were selected. We used Picky algorithm (download from http://www.complex.iastate.edu/download/Picky/) to design 55–60 mer oilgonucleotide corresponding to a unique sequence in each gene, then spotted these probes onto slides.

We have used these arrays to conduct gene expression profiling studies, using RNAs from *C. thermocellum* grown under different carbon sources. When

C. thermocellum was grown on sugarcane bagasse, rice straw, or Napier grass, a few glucoside hydrolase genes were down-regulated, but many more hydrolase genes and cellulosome enzyme encoded genes were up-regulated. The enzyme α-L-arabinofuranosidase encoded genes were also up-regulated, indicating that *C. thermocellum* can also induce enzymes to hydrolyze cross-linking glycans in the cell wall. Moreover, we found that *C. thermocellum* cellulase genes display substrate specificity. For example, some glucoside hydrolase genes were only induced in cells grown on rice straw and purified microcrystalline cellulose media but not on the other two substrates. The up-regulated genes and the substrate-specific genes thus identified are potential candidate cellulase genes for the bioethanol production system. We are currently doing a genome comparison of these genes to identify the key structure for degradation of different cellulose substrates. The details are under investigation.

In summary, we found that both *C. thermocellum* and *P. chrysosporium* can grow on powders of rice straw, sugarcane bagasse, or Napier grass, even without any pretreatment. Microarray analyses indicate that GH genes and cellulosome enzyme encoded genes were differentially regulated in *C. thermocellum* grown under different substrates. However, a serious disadvantage of *C. thermocellum* and its relatives is that its genetics is little understood and there is no transformation tool. Transcriptomic study will help us make intelligent choices in selecting candidate cellulase genes for further studies. More recently, new-generation sequencers also provide alternative tools for the transcriptomic studies. In the future, these new tools can accelerate new enzyme research, especially in the field of microbiology.

18.6 HOW CAN PROTEOMIC STUDY HELP IDENTIFY CELLULASE GENES IN A MICROBE?

Microbial genomes often contain substantial numbers of GH genes, many of them induced by different carbon sources. Sometimes, a transcriptome can only tell the expression level of mRNA, not the expression level of functional proteins. Our previous case study demonstrates that different cellulases might be expressed in different substrates. Therefore, proteomic studies can offer complementary data for transcriptomics in the hunt for desired cellulases.

The white-rot basidiomycete *P. chrysosporium* employs extracellular enzymes to completely degrade the major polymers of wood: cellulose, hemicellulose, and lignin, which is a good example for omics approach. A secretome dataset of *P. chrysosporium* was generated using the most recent assembly (www.jgi.doe.gov/whiterotwww.jgi.doe.gov/whiterot). A total of 874 potentially secreted proteins were predicted in a previous study (Vanden Wymelenberg et al., 2006). Eighteen unique peptide sequences were assigned to eight specific GH genes in their study, most of which have been implicated in degradation of hemicelluloses. Both genomic and proteomic studies can give more insight into metabolism of *P. chrysosporium*, which can directly and empirically indicate the functional GH genes for further biofuel applications.

Proteomic approaches also have been used to study the heavy metal stress response (Ozcan et al., 2007). A 2-D reference map in p*I* range 3–10 was constructed for the soluble protein fraction of *P. chrysosporium* grown with or without heavy metals. Functional annotation could be made for 517 spots out of 720 that were subjected to MALDI–TOF–MS analysis, according to the specific accession numbers from the *P. chrysosporium* genomic database. Including three new proteins, Nine key proteins were demonstrated as the most strongly induced. The proteome or secretome approaches provided an efficient way to identify the regulatory and/or key proteins differentially expressed under those conditions, such as substrate digestion, stress response, etc.

18.7 CONVERSION OF CELLULOSE TO ETHANOL

The main goal of bioethanol research is to develop a cost-effective bioprocess for conversion of lignocellulose to ethanol. To reach the final goal, an effective saccharification–fermentation biomass process for ethanol production is needed. Among the many kinds of bioprocesses such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and CBP, CBP is the most preferred process because it has potential for high efficiency and low cost (Lynd et al., 2002, 2005). In CBP, cellulase production, hydrolysis, and fermentation occur in one single process, whereas in SHF, SSF, and SSCF, there is a dedicated cellulase production step that is separated from hydrolysis and fermentation (Lynd et al., 2002, 2005). The key requirement for CBP is a microbe or a group of compatible microbes that can carry out cellulase production, hydrolysis, and fermentation, in a single process. Such microbes are currently unavailable or inefficient.

Based on the rich genomic resources, advances in bioinformatics, transcriptomics, and proteomics, we have designed a strategy to engineer fungal and bacterial strains for efficient conversion of cellulose to ethanol. In this strategy, microbes that produce ethanol with high titers, such as *Saccharomyces cerevisiae*, are used as starting points to engineer recombinant organisms expressing heterologous cellulase genes that enable these organisms to produce ethanol with pretreated lignocellulose as the sole carbon source. As illustrated in Figure 18.3, we will use genomic and proteomic approaches outlined in prior sections to identify efficient cellulase genes from various microbial genomes. We will first construct recombinant *S. cerevisiae* strains that express EG, EXG (exoglucanase), or/and BGLU from different microbial species. Different combinations of recombinant yeast strains will be co-cultured and tested for their ability to utilize cellulose as the main carbon source. We will then perform growth experiments to select progeny that can grow efficiently using cellulose as the sole carbon source. We will subsequently determine the molecular basis of the growth advantage of the selected yeast strain. Lastly, we will generate recombinant yeast strains that express all three cellulase genes, EXG, EG, and BGLU, in a single yeast cell. We will compare ethanol production efficiencies between the yeast strain that contains all three cellulase genes and the co-culture of three strains that express an individual cellulase gene.

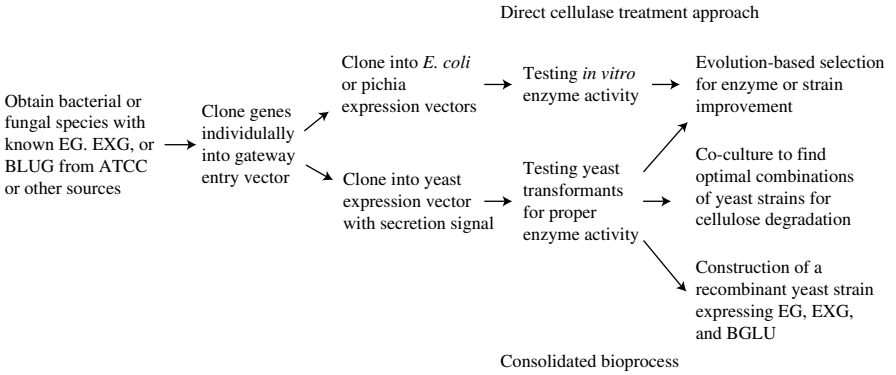

FIGURE 18.3 Scheme for metabolic engineering of fungal and bacterial strains for biomass conversion.

18.8 CONCLUDING REMARKS

Currently, the key barriers to economical production of bioethanol from lignocellulosic material are the high cost of cellulase enzymes and difficulty in finding combinations of cellulases or microbial mixtures that can work synergistically and efficiently toward various feedstocks. The rate-limiting step in the conversion of cellulose to fuels is its hydrolysis, especially the initial attack on the highly ordered insoluble structure of crystalline cellulose. Recent advances in genomics, transcriptomics, and proteomics offer an opportunity to identify efficient cellulases from microbial genomes.

Among the many kinds of bioprocesses, CBP is often considered as the most preferred process because of its high efficiency and low cost (Lynd et al., 2002, 2005). In CBP, cellulase production, hydrolysis, and fermentation occur in one single process. The key requirement for CBP is a microbe or a group of compatible microbes that can carry out cellulase production, hydrolysis, and fermentation in a single process. Such microbes are currently unavailable or inefficient. We have outlined a strategy to generate recombinant fungal and bacterial CBP strains that can efficiently utilize cellulose to produce ethanol. By taking advantage of available omic tools, we believe this strategy is likely to succeed in generating CBP strains.

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SECTION III

RENEWABLE BIOPRODUCTS

19

BIOTECHNOLOGICAL USES OF PHOSPHOLIPIDS

JEONG JUN HAN

Doosan Glonet, B5F, Advanced Convergence Institute of Technology, 864-1 IUI-dong, Suwon, Gyeonggi, Republic of Korea

JAE KWANG SONG

Chemical Biotechnology Research Center, Korea Research Institute of Chemical Technology, P.O. Box 107, Yuseong-gu, Daejeon 305-600, Republic of Korea

JOON SHICK RHEE

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1, Guseong-dong, Yuseong-gu, Daejeon 305-701, Republic of Korea

SUK HOO YOON

Korea Food Research Institute, Baekhyun-Dong, Seongnam-Si, Kyunggi-Do, Republic of Korea

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19.1 INTRODUCTION

There are a great variety of phospholipid structures found in nature and derivatized for enhanced functionality. The term "phospholipid" designates any lipid-containing phosphoric acid as a mono- or di-ester, and "glycerophospholipid" refers to any derivative of glycerophosphoric acid that contains at least one *O*-acyl group attached to the glycerol residue (Fig. 19.1) (IUPAC-IUB, 1978; Silvius, 1993). Ether phospholipids contain either an *O*-alkyl or an *O*-(1-alkenyl) group found in, for example, plasmalogen (1-*O*-alk-1′-enyl-2-acyl glycerophospholipids) (Brites et al., 2004). In addition, sphingosine-containing phospholipids (sphingophospholipids) are further classified into two major classes: phosphocholine derivatives of ceramides, commonly known as sphingomyelin, and glycosylated derivatives of inositol phosphoceramides, commonly known as phytoglycolipids (Olsen and Jantzen, 2001). In this paper, we divided phospholipids into glycerophospholipids such as phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI), lysophospholipid, and sphingophospholipid for the sake of convenience; the lysophospholipid referred to any glycerophospholipid that was lacking one of the *O*-acyl groups.

Phospholipids generally contain the hydrophilic "head" consisting of a negatively charged phosphate and a polar moiety and the hydrophobic "tail" generally consisting of a fatty acid moiety. This zwitterionic property renders them surface-active; thus, they are mainly being researched for an emulsifier for a variety of industrial products such as foods, cosmetics, drugs, nutritional supplements, and chemicals. The phospholipids such as PC and PE have the tendency to form molecular bilayers and

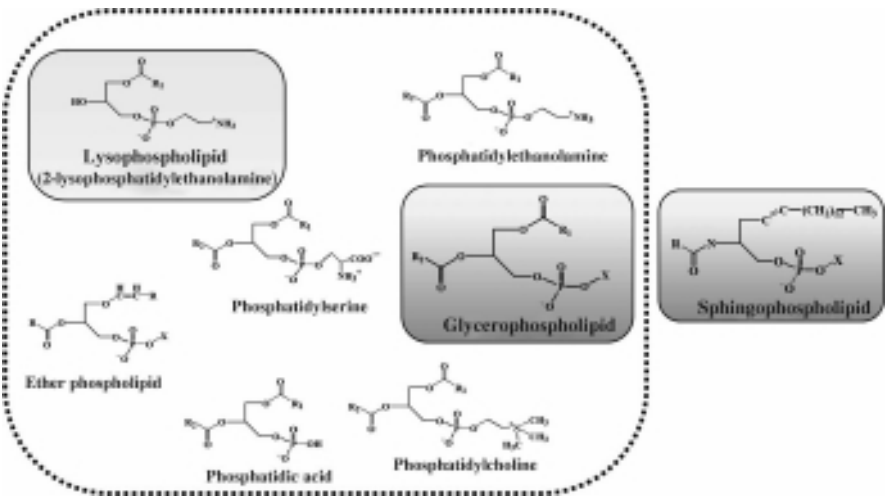

FIGURE 19.1 Schematic representation of phospholipids such as the glycerophospholipid and the sphingophospholipid. R, R1, and R2, hydrocarbon tails of FA; X, a polar alcohol moiety such as choline, serine, ethanolamine, myo-inositol, glycerol, or phosphatidylglycerol.

minimize their surface by forming vesicles. In particular, such liposomes have been tested with regard to their suitability as carriers for drugs or other physiologically active materials. Phospholipids are also biologically and physiologically active compounds and thus useful as an active ingredient of foods, drugs, cosmetics, and agrochemicals. The major natural resources for industrial preparation of a variety of useful phospholipids are vegetable oils, seed oils, and egg yolks (Gober et al., 1993). The phospholipids separated from these resources, mostly PC, PE, PS, and PI, can be used directly in industrial products and further as starting materials for modification to other valuable phospholipids. A large number of reports and scientific papers have been published dealing with the physical, chemical, and enzymatic modification of phospholipids to synthesize biotechnologically important ones; some reviews already gave an overview of the production of phospholipids and the characteristics of biocatalytic enzymes such as phospholipases and lipases (D'Arrigo and Servi, 1997; Servi, 1999; Guo et al., 2005; Ulbrich-Hofmann et al., 2005).

Phospholipids have many favorable functions and can be used as valuable ingredients for food, cosmetics, agriculture, and pharmaceuticals. Traditionally, phospholipids have been used mainly in foods and animal feeds. As many biological activities and functions of phospholipids have been elucidated, they are becoming very important and crucial components in medicinal, biotechnological, and cosmetic applications. In this paper, we will provide a concise review of the functionality and the practical and potential use of phospholipids for food, cosmetics, agricultural application, and as pharmaceuticals.

19.2 PHOSPHOLIPIDS FOR FOOD AND NUTRACEUTICALS

Commercially available phospholipids are commonly derived from crude soybean oils, sunflower oils, and egg yolk. A typically industrial processing of soybean lecithin to different end-products by physical or chemical methods is depicted in Figure 19.2. To obtain commercial lecithin from the crude soybean oil, the general procedure usually includes the following processes: hydration of phosphatides, lecithin sludge separation, and sludge drying. For powder-type lecithin, acetone treatment is usually included. The resultant soybean lecithin has a typical composition of PC, 20%; PE, 15%; PI, 20%; PA, 15%; and other components. More high-purity phospholipids as well as individual species of phospholipids have been produced from commercial sources using practicable techniques, such as solvent fractionation and chromatographic technology (Guo et al., 2005).

PC is a major constituent of most biological membranes, and the dietary PC is a source of choline, an essential nutrient for humans and animals. Choline deficiency caused a variety of abnormalities in animals, such as infertility, growth retardation, bone abnormalities, and increased sensitivity to carcinogenesis (Ziesel, 1990). PC also plays a vital role in keeping the liver and nervous systems healthy (Kidd, 1996; Chung et al., 1995). Once PC broke down in the body, the free choline was supplied to the brain where the neurotransmitter acetylcholine was manufactured (Blusztajn et al., 1987). Levels of acetylcholine diminished with age, and such decline was

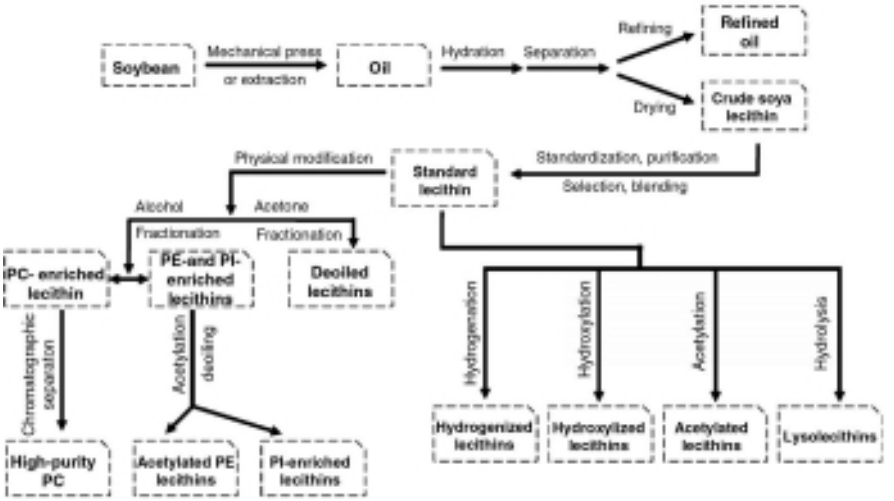

FIGURE 19.2 Schematic diagram of industrial processing of soybean lecithins (Guo et al., 2005).

known to be associated with age-related memory impairment. Choline is also involved in human reproduction and fetal and infant development; the formulas not made with cow's milk must include choline in all FDA-approved infant formulas (FDA, 1991). Because the most effective way to get choline levels elevated in the body is by taking PC (Hirch et al., 1978), many commercial diet supplements containing PC are being sold in the market as a dietary choline source. In those cases, PC as a purified extract of lecithin is commonly recommended for treating a variety of liver, nerve, memory, and other conditions, including multiple sclerosis and memory loss.

The relative abundance of PS in the brain reflects involvement in nerve cell functions, including nerve transmitter release and synaptic activity; some clinical studies suggested that PS could support brain functions to decline with age (Crook et al., 1991, 1992; Cenacchi et al., 1993). PS in nerve cell membranes seemed to help activate and regulate proteins related to nerve cell functions such as generation, storage, transmission, and reception of nerve impulse. PS was supposed to actually benefit memory, learning, concentration, and mood (Palmieri et al., 1987). Research has shown that the consumption of 800 mg per day of phosphatidylserine for 10 days significantly lowered the level of cortisol in the blood after exercise (Monteleone et al., 1992). Further research looked at the effects of daily phosphatidylserine supplementation (600–800 mg) on the subjects undergoing intensive or moderate weight training (Fahey and Pearl, 1998; Starks et al., 2008). The researchers found that phosphatidylserine significantly decreased postexercise cortisol levels, reduced muscle soreness, and reduced the levels of depression normally associated with overtraining. Therefore, PS also has a great potential as a dietary supplement. Although most of the clinical data of PS were obtained using bovine brain source, the PS prepared from soy or egg lecithin showed the same effects (Suzuki et al., 2001).

The PS can be synthesized by PLD-catalyzed transphosphatidylation reaction of lecithin and serine (Iwasaki et al., 2003) and is being sold as a dietary supplement and pharmaceutical formulations (Della et al., 1992).

Long-chain-polyunsaturated fatty acids (LC-PUFAs) such as arachidonic acid and docosahexaenoic acid are critical components of cellular membranes and are especially enriched in the developing retina and gray matter of the brain (Clandinin et al., 1980; Bazan et al., 1986). They were correlated with improved visual acuity and cognitive development (Birch et al., 1998). Lipid components of infant formula are usually obtained from vegetable oils, which contain fatty acids up to 18 carbon atoms in length. Therefore, supplementation of LC-PUFAs has been needed for infant formula. Other lipid sources such as fish oil, oil from unicellular microorganisms, and egg oil fraction can be sources of LC-PUFA. From some clinical trials with premature infants, it was found that LC-PUFA provided as a component of phospholipids was more efficiently absorbed than those provided in the triacylglycerol form (Wijendran et al., 2002). Therefore, phospholipids containing arachidonic acid or docosahexaenoic acid may be desirable sources for dietary infant formula. It was reported that intracerebroventricular administration of DHA-conjugated PC induced a significant increase in paradoxical sleep time and total sleep time and this effect might be caused by fatty acid residues rather than choline residue (Hashimoto et al., 1993). The conjugated DHA from fish egg has been commercialized as a food supplement for deep sleep and is being sold in Japan.

Lysophospholipids show a high solubility in water, thereby rendering them enhanced emulsification properties in oil/water emulsions and an ability to form emulsions that are more stable to variations of pH and temperature. Furthermore, the ability of the lysophospholipids to form an emulsion is less influenced by the presence of ions, such as magnesium or calcium ions. These properties of the lysophospholipids are particularly suitable for use in many industrial applications such as food technology, cosmetics, and pharmaceutical industries. Modified phospholipids such as PS and lysophospholipids can be produced by phospholipase-catalyzed reaction. Lots of papers and patents for the synthesis of modified phospholipids have been reported, and the general reaction scheme for the production of modified phospholipids is well illustrated in Figure 19.3 (Guo et al., 2005).

Sphingomyelin is a sphingophospholipid and consists of a ceramide unit linked at position 1 to phosphorylcholine; it is found as a major component of all animal tissues but not of plants or microorganisms. It resembles PC in its physical properties. Sphingomyelin is a precursor for a number of sphingolipid metabolites that have important functions in cellular signaling (Merrill and Konrad, 2002). They function in cell-to-cell communication, signal transduction, and immunorecognition. Sphingomyelin is converted to ceramide by sphingomyelinase. Ceramide is hydrolyzed to sphingosine by the removal of the amide-linked fatty acid. Such sphingolipids are also constituents of most foods, but the amounts are relatively small, and there is no evidence that dietary sphingolipids are required for growth or survival. However, both sphingolipids and their digestion products, i.e., ceramides and sphingosines, are highly bioactive compounds that have profound effects on cell regulation. They have a structure similar to that of a glycerol-based phospholipid, having a polar head group as

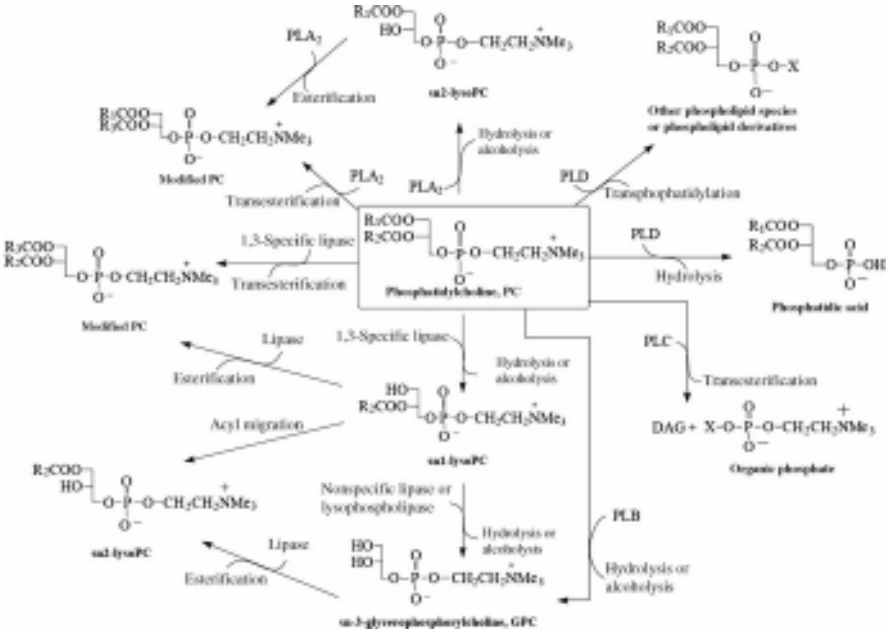

FIGURE 19.3 Enzyme-catalyzed transformation of phospholipids (Guo et al., 2005).

well as two hydrophobic hydrocarbon chains (one is the sphingosine and the other is a fatty acid chain) (Huwiler et al., 2000).

There are some reports of the health effects of sphingolipids, i.e., prevention and treatment of cancer, lowering cholesterol level, prevention of type I diabetes, and antimicrobial activity (Merril, 1997). It was shown that a diet of sphingomyelin reduced the chance of malignant cancer in rats (Schmelz et al., 1996). Matsuyama et al. (2000) reported that the nutrition formulation containing sphingomyelin had a promoting effect on the maturation of gastrointestinal tract. Therefore, sphingolipids as a food ingredient may offer possibilities of lowering the risk of gastrointestinal cancer, lowering cholesterol levels, lowering the risk of thrombosis, and a reduced risk of bacterial or viral infections. In addition, they may help to prevent type I diabetes. Dietary supplements including ceramide or sphingomyelin extracted from natural sources such as milk, rice bran, and rice germ are being sold as a diet supplement in the market. Especially, ceramides from plant sources are glycosylated sphingolipid and major sugar is glucose. These plant origin ceramides show epidermal proliferation, skin moisturizing effect, whitening, and anti-wrinkle effect, and are being sold worldwide as beauty ingredients in food (Marsh et al., 1995; Tsuji et al., 2006).

19.3 PHOSPHOLIPIDS FOR COSMETICS

Phospholipids serve the following effects and functions in cosmetics: emulsifier, wetting agent, foam stabilizer, antioxidant, conditioning and softening agent,

moisturizer, penetrating agent, emollient, and pigment dispersant. Therefore, they have been used for cosmetic formulation such as skin cream, lotions, foundations, cleansing creams, sunscreens, shampoos, hair conditioners, shaving cream, face powder, lipstick etc. For example, the ethanol-soluble extract of soy lecithin was recommended as a spray for conditioning hair due to its property of eliminating electrostatic charge and holding curls at high humidity (Claffey et al., 1982). Highly purified phospholipids could be used for the preparation of liposomes. Liposomes can encapsulate water-soluble ingredients in their inner water space, and oil-soluble ingredients in their phospholipid membranes. Because of their similarity to phospholipid domains of cell membranes and an ability to carry substances, liposomes can be used to protect active ingredients and provide time-release properties in medical or cosmetic treatment. The phospholipids being used for liposomes are mainly PC, PE, phosphatidylglycerol (PG), and PS. They are usually synthetic phospholipids having dioleoyl, distearoyl, dipalmitoyl groups. But the hydrogenated soy PC is also widely used as a stable emulsifier of cosmetic base. Blume et al. (1999) reported the application of liposomes in encapsulation of active materials such as vitamin, plant extracts, and nutrients for skin. In that system, ascorbic acid encapsulated in liposomes achieved four times the penetration of ascorbic acid in an emulsion. Liposomes may also be used to transport lipophilic active materials such as vitamins A, E, and C to excised skin (Padamwar and Pokharkar, 2006). Similar liposomes were also effective for the transport of alpha-hydroxy acids with a resultant increase in skin moisture, improved smoothness, and a reduction in wrinkles. Recently, the skin beautifying effects of PS such as anti-wrinkle and anti-inflammatory were also reported (Cho et al., 2008). The anti-inflammation effect of PS is at least partly dependent on the activation of peroxisome proliferator-activated receptors, a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes related to cellular differentiation, development, and metabolism of higher organisms (Ramos et al., 2007).

It was reported that phosphatidic acid (PA) has intensive growth-promotional effects on hair epithelial cells and epidermal keratinocytes (Takahashi et al., 2003). PA was also shown to have hair-growing activity to induce the anagen phase of the hair cycle in the *in vivo* murine model. In contrast, lysophosphatidic acid (LPA) showed the lower growth-promoting effects on hair epithelial cells relative to PA and the minimal or no growth-promoting activity on epidermal keratinocytes.

LPA is present in many cell membranes of organisms, and is recognized as an important bioactive lipid mediator with diverse biological activities. It shows a variety of cellular responses, including mitogenic and antimitogenic effects on the cell cycle, induction of cancer cell invasion, regulation of formation of actin stress fibers and focal adhesion assembly, cell motility, and mobilization of intracellular calcium (Murakami-Murofushi et al., 2002). Among its biological activities, LPA has some effects that may be applicable in the cosmetic field. LPA shows an effect of regenerating the damaged skin tissues and new blood vessels (Mosher and Checovich, 1996; Price et al., 1999). LPA exerts a synergistic effect in repairing the damaged skin tissues and scars in combination with sphingolipid long-chain base (Xu et al., 2007). LPA is a skin activator with glycosaminoglycan-production-accelerating

effects, and thus is useful for cosmetic preparations that prevent skin aging (Doi et al., 1995). It also has the effect of inhibiting hyperproliferation of epithelial cells resulting from psoriasis or the like (Piazza and Mazur, 1996). LPA also inhibits apoptosis and maintains or restores cell functions (Bathurst et al., 1998). In addition, LPA shows an activity that regulates skin wrinkles in mammalian skin and may be applied to the anti-aging of skin (Piazza and Mazur, 1993). LPA also shows a strong hair-growing activity and thus can be used as an active ingredient of cosmeceuticals for hair growth (Kamimura et al., 2002).

Sphingolipids are one of the major components of lipid barrier and comprise more than 40% of the total skin lipids. Ceramides contain fatty acids linked by an amide bond to the amine group of a long-chain sphingoid base. They can be produced during the hydrolysis of sphingomyelin by sphingomyelinase or phospholipase C. In general, they are present at low levels only in tissues, but they are also important as intermediates in the biosynthesis of the complex sphingolipids. Sphingomyelin and ceramide have important functions in cellular signaling processes such as the regulation of apoptosis and cell differentiation (Cutler and Mattson, 2001). Ceramides play an important role in maintaining skin barrier homeostasis as key structural components of stratum corneum (Man et al., 1993). Other important physiological functions such as skin-soothing and wound-healing efficacy have also been proposed. Ceramides also have a moisturizing effect (Chamlin et al., 2002). Skin barriers take the form of keratinized dead cells piled up like bricks, and the regions between the cells are filled with lipids composed of mainly ceramides, cholesterol, and fatty acids with an appropriate ratio, thereby forming a lipid lamellar barrier that prevents loss of moisture. About a third of moisture in the skin is bound to ceramides; that is, ceramides are present as a water-bound form. Thus, ceramides are a decisive factor responsible for maintaining moisture in the skin and they are being used in cosmetics as moisturizing agents.

19.4 PHOSPHOLIPIDS FOR AGRICULTURAL APPLICATION

Soybean phospholipid with organic or inorganic acid, alcohol, and water in a certain proportion has an effect of disinfection, bacteriostasis, preservation, and maintaining freshness when it is sprayed on the surface of foods, fruits, vegetables, and flowers. The dispersion solution of phospholipid is reported to be effective in the prevention of loss of freshness of vegetables, fruits, and mushroom after harvest (Misato and Honma, 1978). Phospholipid dispersion solution is also effective in delaying the falling of blossoms of cut flowers (Shikanuma and Shibuya, 1987).

PG is a ubiquitous lipid that is the main component of some bacterial membranes, and is also found in membranes of plants and animals where it appears to perform specific functions. In plants, PG is found in all cellular membranes, but appears to be especially important in the thylakoid membrane where it can comprise 10% of the total lipids with a high proportion (up to 70%) in the outer monolayer, and it seems to play an important role in photosynthesis (Sato et al., 2000; Hagio et al., 2000). Therefore, it is anticipated that PG might be applicable as a physiologically active agent for plant growth.

Treating a plant with a formulation containing lysophospholipid may enhance the health of plant or seed and protect it from stress-related injury. It may also enhance or accelerate the recovery of an injured plant and enhance the germination of seeds and seedling vigor (Farag and Palta, 2003). LPE and lysophosphatidylinositol (LPI) were especially found to be effective agents in enhancing the ripening and storage characteristics of fruit, whether applied pre- or post-harvest. They enhance ethylene production in fruit and decrease respiration so as to maintain the fruit's firmness for a long time. Similar effects occur in leaves and other green plant tissue. Other lysophospholipids and ethanolamine-containing phospholipids appear to have similar effects (Palta and Ryu, 2002).

Inositol sphingophospholipids from *Phytophthora capsici* have a protective effect on pepper plants against pathogenesis by *P. capsici* (Lhomme et al., 1990). It was reported that ceramide aminoethylphosphonate from *Phytophthora infestans* elicited the accumulation of phytoalexins, a response frequently associated with the activation of disease resistance mechanisms in plant tissue.

19.5 PHOSPHOLIPIDS FOR PHARMACEUTICALS

Drugs administered orally can be absorbed through the oral mucosa or the lining of the stomach and intestine. However, the rate of absorption depends on the ability of the drug to pass through the lipid barrier of epithelial membranes. Several drugs are poorly absorbed in the digestive tract, and must be administered by injection. Many therapeutic compounds are also rapidly inactivated in the liver. However, bioactivity or bioavailability of such agents may be increased by conjugation of many biological active agents to phospholipid via a phosphodiester bond. Conjugation of one or more phospholipid moieties to lipophilic compounds can make them more hydrophilic, more soluble in aqueous media without abrogating their ability to traverse biological membranes, and then the conjugated compound may be rapidly absorbed in the intestine and thus avoid first-pass inactivation in the liver (Chasalow, 1998).

Kumar and Hostetler (1991) reported the application of phospholipid prodrugs of aspirin, other salicylates, and nonsteroidal anti-inflammatory drugs in the therapy of chronic inflammatory disorders. The drugs were linked to either or both of the glycerol hydroxyls of a phospholipid or to available hydroxyls of phospholipid head groups by ester bonds. The drugs may also be linked to available amines of phospholipid head groups by amide bonds. The phospholipid moiety can be removed later by enzymes such as phospholipase C or D, sphingomyelinase, and nonspecific esterases, and then the original compound can be liberated with its biological activity intact. Such phospholipid prodrugs may reduce the gastrointestinal irritation and toxicity of drugs when administered at high doses and may impart desired characteristics, such as increased bioavailability, increased site specificity, or new physiological activity. The drug–phospholipid conjugates also enabled the drug to be transported to a specific site, or to be released within a specific organ (Senderikhin et al., 2001). The applicable agents were antibiotics like cephalosporin, fusidic acid,

and vitamins, dihydroepiandrosterone, estradiol, testosterone, aspirin, and any other pharmaceutically active agents having an alcohol group. Sridhar and Hostetler (1996) introduced the method of converting a drug, or other pharmaceutical compounds that are not absorbed through the intestine because of their chemical structure, to orally available form. They reported that the lipid derivative comprising a 1-acyl- or alkyl-phospholipid covalently linked to a functional group of the taxol-related compounds either directly through a phosphate ester or through a linker molecule enhanced the oral bioavailability and/or tissue levels of the administered pharmaceutical compound.

Derivatization of amine group of phospholipids can give desirable functions. The pharmaceutical formulations containing *N*-acylphosphatidylethanolamine may be effective for their anti-inflammatory action (Cestaro and Pistolesi, 2003). The hydrolyzed product, *n*-acylethanolamines, also has pharmacological properties, e.g., canabinoid receptor agonist (Hanus et al., 1993), anti-inflammatory and anti-anaphylactic activity (Facci et al., 1995), and anorexic effect (de Fonseca et al., 2001).

The cell membrane plays a role in physiological homeostasis and allows only selected molecules to penetrate. Therefore, the barrier should be broken down temporarily when the delivery of impermeable pharmaceutical agents is desired. Liposomes can be used as drug carriers of hydrophobic or hydrophilic drugs entrapped in their hydrophobic or hydrophilic compartments, respectively. Liposomes have been used to deliver chemotherapeutic agents, immunomodulators, and antifungal agents in animals (Lopez-Berestein et al., 1984) and humans (Lopez-Berestein et al., 1985). Some studies showed that the liposomal delivery reduced certain types of drug-related toxicities such as doxorubicin cardiotoxicity (Forssen and Tokes 1981; Herman et al., 1983) and *cis*-diaminedichloroplatinum nephrotoxicity, and might increase antitumor activity as a result of a slow-release mechanism (Mayhew et al., 1978; Patel and Baldeschwieler, 1984).

Among the applications of liposome in pharmaceutical and medical fields, we additionally describe the application of liposome to gene delivery. Gene therapy using liposome can deliver particular genes in specific cells. Antisense oligonucleotides and ribozymes can be delivered to suppress the expression of unwanted genes. The liposomal gene delivery is the most widely studied system because it has advantages of no immunogenicity, low cost, and safety. However, the level of delivery and gene expression was generally low as compared with the viral delivery system.

Recently, advances have been made to improve transfection efficiency. Several approaches have been described to improve intracellular delivery by vesicular systems (Templeton and Lasic, 1999). By imparting a charge to the lipid vesicles, encapsulation efficiency can be increased. Positively charged liposomes can complex negatively charged DNA plasmids into small and colloidally stable particles, which significantly increases transfection efficiency (Wrobel and Collins, 1995). In fact, phospholipids themselves are not sufficient to impart cationic property to liposomes. Thus, other lipids having cationic properties such as dioeoyl trimethyl ammonium propane and dioctadecyl dimethyl ammonium bromide were introduced and have been studied. Murphy et al. (2001) introduced a technology to efficiently combine compacted DNA with phospholipids, e.g., PE or PC and hydrophobic peptides,

to produce homogenous complexes that are completely resistant to nuclease. Stable complexes of cationic liposomes with plasmid DNA could be prepared by (1) including a small amount of poly(ethylene glycol)–phospholipid conjugate or (2) condensing the DNA with polyamines prior to the formation of liposome–plasmid complexes. These preparations were stable for months at 4°C and gave reproducible high transfection activity for *in vivo* gene delivery after intravenous injection in mice (Hong et al., 1997). Gao and Huang (1996) reported the application of several high-molecular-weight cationic polymers, such as poly(L-lysine) and protamine. It could enhance the transfection efficiency of several types of cationic liposomes 2–28-fold in a number of cell lines *in vitro*. However, problems such as lack of a general strategy for tissue targeting, drug concentrations that are sometimes lower than desired, difficulty of large-scale production, and unpredictable shelf life still remain to be solved.

Surface-active phospholipids can be used to ameliorate bronchoconstriction in asthmatic adults (Kurashima et al., 1991). Surface-active phospholipids such as dipalmitoyl-PC and dioleoyl-PC are capable of forming a thin film or coating on the surface of the lungs. Therefore, they can be used as an antiasthma medicament that is capable of binding to the tissue surface of the airways, thereby masking receptors against stimulation by noxious agents or sensitization by allergenic stimuli.

PG is believed to enhance or potentiate the binding of the diacylphosphatidylcholine to the epithelial surface. Therefore, PG may be a preferred component of the surface-active phospholipids. PG has an important function to cause the surface-active phospholipids to form a dispersion of finely divided, dry powder in air. PG is a surfactant with a great hydrogen-bonding capacity, and surfactants containing PG have better alveolar stabilizing properties. In view of PG's effectiveness as a component of an artificial lung surfactant for respiratory distress syndrome and its relative rarity in nature, its economic production in large quantities is desired. PG can be produced via PLD-catalyzed transphosphatidylation reaction from egg lecithin or soy lecithin, and glycerol. Several reports were published for the enzymatic synthesis of PG (Lee et al., 1985; Barenholz and Amselem, 2000).

PE seems to be involved in the blood coagulation pathway (Klein et al., 2001). It was reported that blood coagulation activity is stimulated in a membrane containing PS by PE. The amine group of ethanolamine of PE is involved in protein binding. When PE is included in liposome, it makes protein bind to the liposome, and this property is useful for immunoliposome preparation and support of enzyme (Kung and Redemann, 1986).

Since the biological activity of the platelet activating factor (PAF), which is a mediator involved in mammalian allergic and inflammatory processes, has been known, a good deal of attention has been focused on alkylphospholipids and their effects. PAF is known to be a potent immunological regulator possessing a wide range of biological effects (Braquet et al., 1987). Ether lipids have shown to possess antineoplastic activity and a typical compound is 1-octadecyl-2-methyl-glycerophosphorylcholine. In addition, structural alterations of ether lipids have been tried to get elevated antitumor activity together with reduced side effects (Ukawa et al., 1989; Salari et al., 1992).

Lysophospholipids are small lipid molecules with various activities such as cell survival, proliferation, cytoskeletal, and electrophysiological effects. Lysophospholipids have excellent carcinostatic and immunostimulating activities. However, they also have a strong hemolysis and thus have a problem in safety in use as pharmaceutical agents. Treating with lysophospholipids mixed with phospholipids may solve this problem (Nakabayashi et al., 1983).

Among lysophospholipids, LPA has been noticed due to its various physiological activities. As described in the previous section, LPA shows a variety of physiological properties. Recently, many researches on LPA-receptor-mediated signaling have been reported. Determining receptor selectivity can deduce *in vivo* functions. Therefore, a number of papers about LPA receptors have been reported. In this section, we will describe the function of LPA in view of pharmacological aspects. It is known that LPA serves as a mediator involved in inflammation and plays roles in thrombosis, and as a factor involved in growth and contraction of smooth muscles and fibroblasts. Also, it is involved in induction of vascular cell adhesion molecules, together with sphingosine-1 phosphate (Tigyi and Parrill, 2003). It can be expected that LPA is involved in asthma, an inflammatory respiratory disease. Moreover, since it is involved in the expression and induction of vascular cell adhesion molecules, accordingly, it is considered to be closely linked to quick wound healing and formation of new blood vessels. LPA is rapidly generated by platelets and stimulates platelet aggregation and wound repair (Lee et al., 2000; Inoue et al., 2001). LPA also enhances binding of fibronectin to cells and regulate cell shape (Zhang et al., 1994). In addition, it enhances fibronectin binding to epithelial and endothelial cells in a skin wound (Mosher and Checovich, 1996). It also activates macrophages and inhibits necrosis in tumor cells (Weisenthal, 1992). LPA has been found to exhibit antiapotoptic activity and/or to preserve or restore cell, tissue, or organ function (Barr and Tomei, 1999). It was reported that LPA has therapeutic and preventive effects for sepsis and stroke (Cho and Huh, 2002). Uncontrolled cell hyperproliferation leads to numerous disease conditions. Some of the most severe consequences of hyperproliferation lead to malignant tumors. LPA could be used for the treatment or prevention of hyperproliferative condition (Piazza and Mazur, 1996).

Cyclic phosphatidic acid (cPA), which has a cyclic phosphate at the sn-2 and sn-3 positions of the glycerol backbone, also has various physiological activities. It shows specific biological functions including antimitogenic regulation of the cell cycle, regulation of actin stress fiber formation and rearrangement, inhibition of cancer cell invasion and metastasis, regulation of differentiation, and viability of neuronal cells (Murakami-Murofushi et al., 2002). Although cPA has a structure similar to that of LPA, the cellular effects seem to be different and are considered to be independent and cooperative mediators. It has high anti-invasive activity and no side effects. Therefore, this compound can be anticipated to be a promising anticancer agent.

Sphingolipids not only help define the structural properties of membranes, but also play important roles in cell-to-cell and cell-to-substratum interactions. They also help regulate growth and differentiation by a variety of mechanisms, such as inhibition of growth factor receptor kinases and effects on numerous cellular signal transduction systems (Spiegel and Merrill, 1996). Recently, pharmaceutical properties

of sphingolipids have been noticed. They also interact with cell surface receptors for growth factors, and the extracellular matrix. Their lipid backbones (ceramide, sphingosine, and sphingosine 1-phosphate) function as "second messengers" to affect protein kinases, phosphoprotein phosphatases, ion transporters, and other regulatory machinery. As examples, tumor necrosis factor-alpha, interleukin 1-beta, and nerve growth factor induce sphingomyelin hydrolysis to ceramide as a second messenger (Hannun, 1994).

Sphingosines and sphingolipids exert many effects on mammalian cells (Spiegel and Milstein, 1995) and are useful as inhibitors of protein kinase C (Radin, 2003), whereby such sphingosines and sphingolipids may be of therapeutic value in a number of disease states such as cancer, rheumatoid arthritis, diabetic complication, and central nervous system disorders. Merrill and Schimelz (2003) reported that sphingolipid derivatives were effective for the treatment of abnormal cell proliferation, including benign and malignant tumors, the promotion of cell differentiation, the induction of apoptosis, the inhibition of protein kinase C, and the treatment of inflammatory conditions, psoriasis, inflammatory bowel disease, as well as proliferation of smooth muscle cells in the course of development of plaques in vascular tissue. Further, they were also effective for treating bacterial infections causing colon cancer and other disorders of the intestine. Several reports related to methods for producing sphingolipids or sphingolipid derivatives were published (Merrill and Schmelz, 2000; Sueyoshi et al., 2003).

N-methyl-sphingosine and sphingosine are especially strong inhibitors of growth of many cells including tumor cells, cells involved in immune responses, and cells involved in inflammatory responses. This finding opens the possibility of using these compounds as effective growth inhibitors of cancer cells such as those mentioned above (Shitori and Ito, 1990). Recently, derivatization of sphingosine is being widely studied for the development of antitumor agent (Ghidoni et al., 1999).

19.6 CONCLUSIONS

The results of systematic investigations into the physicochemical properties and biological activity of phospholipids have led to wide practical application of these natural phospholipids both in the fundamental biological studies and in practical biotechnology, medicine, food industries, cosmetics, and some other commercial technological processes. Thousands of experimental studies show the nutraceutical benefits of phospholipids for the brain, liver, and circulation. It is expected that their new applications would be realized, as the ongoing active research reveals the new physiological and biological functions of the various phospholipids and modified phospholipids. Lots of commercial products from phospholipids are being sold and the market has been growing more and more. But the limitation of storage times of phospholipids, generally not exceeding 12 months, results in the restriction of their wide use. The growing demands of medical and other industrial fields require improving technologies for a specific and stable individual and mixture of phospholipid compounds. Therefore, stabilization technique for wide application and

economical purification technique for specific phospholipid species will be important and promising in the future in addition to searching new efficacy and application of phospholipids.

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20

APPLICATION OF PARTITION CHROMATOGRAPHIC THEORY ON THE ROUTINE ANALYSIS OF LIPID MOLECULAR SPECIES

KORETARO TAKAHASHI

Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate 041-8611, Japan

TSUGIHIKO HIRANO

Renesas Northern Japan Semiconductor, Inc., 145-1 Nakajima Nanae-cho Kameda-gun, Hokkaido 041-1133, Japan

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In making a discussion on the functionality of lipids, simple fatty acid compositional analyses of those lipids are not enough in modern life science. The combination of acyl moieties or alkenyl moieties or alkyl moieties, i.e., the individual lipid molecular species, should be discriminated so as to know the true dynamics of the functional lipids. But unfortunately, molecular species analysis is an extremely time-consuming work because it requires fractionation of the individual peaks that appear on chromatograms, and multiple chromatographic analyses on those fractionated peaks. For example, if one wants to know the composition of a certain plant source triacylglycerol (TG) molecular species, a reverse-phase high-performance liquid chromatography (HPLC) is usually used to fractionate and collect the individual molecular species peaks. The collected individual peaks are then subjected to total acyl carbon number analysis by using nonpolar-column equipped gas liquid chromatography (GLC) and fatty acid compositional analysis of the composing fatty acid moieties after cleaving off from the glycerol or glycerophospho- backbone, and then methyl esterified. For this fatty acid compositional analysis, polar column equipped GLC is used. Sometimes, mass spectrometry (MS) is also used to do the fractionated peak analysis. HPLC-MS is becoming one of the most powerful tools in analyzing lipid molecular species.

In this chapter, we will first demonstrate how we induced the universal chromatographic law on the analysis of lipid molecular species on reverse-phase HPLC. Then we show how to apply this law in the practical prediction of lipid molecular species on reverse-phase HPLC.

20.1 RELATIONSHIP BETWEEN THE STRUCTURE OF A LIPID MOLECULE AND THE SEQUENCE OF ELUTION: THE TRADITIONAL WAY OF PREDICTING THE RETENTION TIME ON REVERSE-PHASE HPLC

Reverse-phase HPLC has been the most useful tool in analyzing lipid molecular species (Lin and McKeon, 2005; Christie, 1987). The earlier workers in this field tried to characterize the chromatographic behavior such as partition number (PN) (Wada et al., 1977; Wada et al., 1978) or effective carbon number (EC) (Porter et al., 1979), which are defined as PN=CN–2×DB and EC=CN–DB, respectively, in the elution of TG or lecithin molecular species. CN is the total acyl carbon number, and DB is the number of total double bonds in the lipid molecule. These empirical equations were useful in predicting the approximate retention value (in practical cases, retention time or retention volume), although they lacked a theoretical background. In the same year with the proposal of PN, Plattner et al. (1977) proposed an empirically derived law on reverse-phase HPLC named equivalent carbon number (ECN), which has exactly the same definition as PN. Then in 1981, El-Hamdy and Parkins proposed a more precise way of prediction of retention time named theoretical carbon number (TCN) by introducing a correction term to the ECN (or PN). The generalized form of PN (or ECN) and EC was proposed by Compton et al. (1982) and defined as $I_u = I_s - C \times D_b$, where I_s is the carbon number of the standard alkane, D_b the total

double bonds in the molecule, and I_u the index observed in unsaturated TG and phospholipid molecular species by coefficient C.

20.2 RELATIONSHIP BETWEEN THE STRUCTURE OF A LIPID MOLECULE AND THE SEQUENCE OF ELUTION: PREDICTING THE RETENTION TIME OF INDIVIDUAL LIPID MOLECULAR SPECIES ON REVERSE-PHASE HPLC

In order to analyze the individual molecular species contained in marine phosphatidylcholine (PC), egg PC, and soy PC, the polar groups were first cleaved off with phospholipase C. Then, those acetate derivatives were prepared from the produced diacylglycerols that have the same acyl group combinations with the original PCs (Takahashi et al., 1982). The reason why we did this was to exclude the interaction between the polar head of the phospholipid and the remaining silanol on the stationary phase, which impairs the resolution of the individual molecular species. The prepared acetate derivatives, i.e., the acetyl diacylglycerols, that represent the original PCs were subjected to monomeric reverse-phase HPLC (conditions shown in the figure legend of Fig. 20.1). Peaks on the chromatograms were collected individually and those individual total acyl carbon number and fatty acid composition were analyzed using two kinds of GLC, one with nonpolar and other with polar columns. By plotting the relationship between the relative retention time (RRT) of the identified individual molecular species and the CN, or by plotting the relationship between the RRT of the individual molecular species and the DB of the identified individual molecular species, the following correlations depicted in Figure 20.1 were discovered.

$$\mathrm{CN} = P_1 \log(\mathrm{RRT}) + Q_1 \qquad \mathrm{CN} = \begin{vmatrix} x & d_1 \\ c_2 & d_2 \end{vmatrix} \tag{20.1}$$

$$\mathrm{DB} = P_2 \log(\mathrm{RRT}) + Q_2 \qquad \mathrm{DB} = \begin{vmatrix} c_1 & y \\ c_2 & d_2 \end{vmatrix} \tag{20.2}$$

were P is the slope and Q the intersection on the ordinate of the semilogarithmic plots of the RRTs of molecular species against CN or DB. c and d are acyl carbon number and number of double bonds in each group, respectively. x and y are variables of acyl carbon number and number of double bonds, respectively. (The expressions (20.1) and (20.2) do not follow the mathematical rules of matrix itself. This is an expedient way of expressing the correlations.) However, the more precise way of expressing the above Eqs. (20.1) and (20.2) should be:

$$\mathrm{CN} = P_1 \log(\mathrm{RRT}) + Q_1 \qquad \mathrm{CN} = \begin{vmatrix} x & d_1 \\ c_2 & d_2 \\ 2 & 0 \end{vmatrix} \tag{20.3}$$

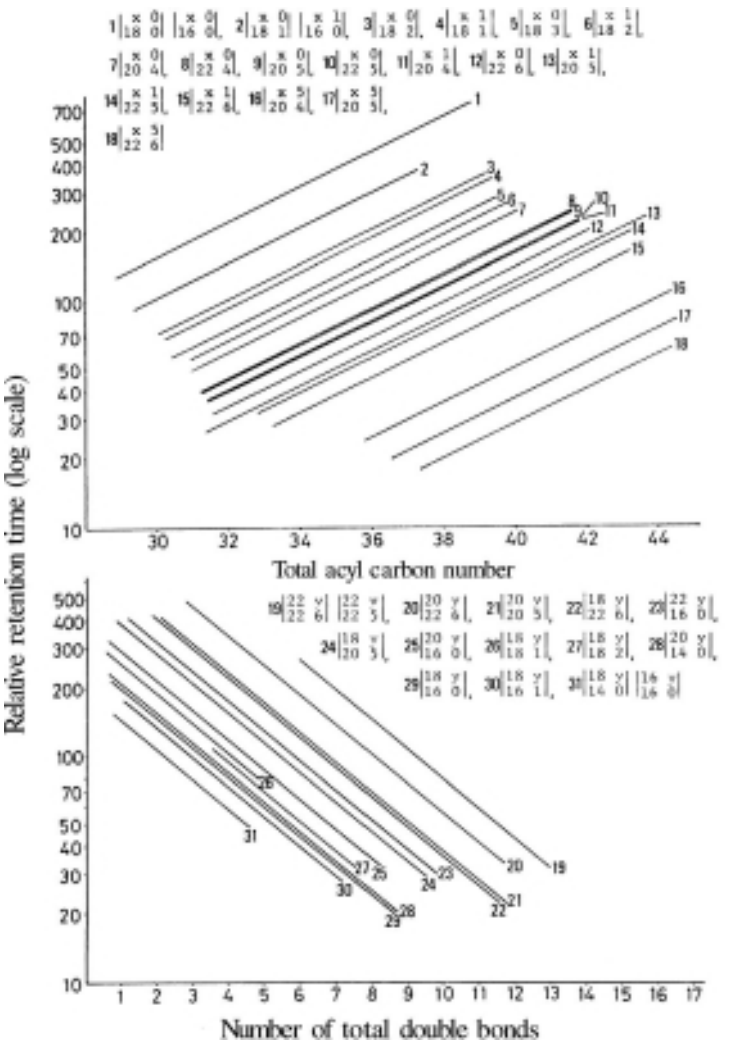

FIGURE 20.1 Relationship between relative retention time (RRT) and total acyl carbon number and relationship between RRT and total double bonds of phospholipid molecular species on reverse-phase HPLC (Takahashi et al., 1982). Acetyl diacylglycerols derived from marine PC, egg yolk PC, and soy PC were injected into Hitachi liquid chromatograph (HPLC) Model 638-50 equipped with twin 8×250 mm LiChrosorb RP-18 columns using Shodex RI Model SE-11 as a detector. The eluting solvent used was isopropanol/acetone/methanol/acetonitrile (1 : 1 : 3 : 4, v/v) at a flow rate of 1.5 mL/min (x and y are variables of acyl carbon number and number of double bonds, respectively. For example, x can take values 14, 16, 18, 20, 22. . . and y can take values 1, 2, 3, 4, 5. . .).

$$\mathrm{DB} = P_2 \log(\mathrm{RRT}) + Q_2 \qquad \mathrm{DB} = \begin{vmatrix} c_1 & y \\ c_2 & d_2 \\ 2 & 0 \end{vmatrix} \tag{20.4}$$

because acetyldiacylglycerol derived from PC was actually used. (2, 0) in position *sn*-3 of the glycerobackbone corresponds to the acetyl (acyl) group that has two carbons with no double bonds. This leads us to develop that the acetyl group (–CO–CH_3) in position *sn*-3 can be considered as the shortest form of the acyl group of TG, i.e., the following expressions should hold.

$$\mathrm{CN} = P_1 \log(\mathrm{RRT}) + Q_1 \qquad \mathrm{CN} = \begin{vmatrix} \mathrm{x} & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix} \tag{20.5}$$

$$\mathrm{DB} = P_2 \log(\mathrm{RRT}) + Q_2 \qquad \mathrm{DB} = \begin{vmatrix} c_1 & y \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix} \tag{20.6}$$

We can consider that TG∋acetyl diacylglycerol.

To bore out that Eqs. (20.5) and (20.6) should hold in natural occurring TG, linseed oil TG, rapeseed oil TG, olive oil TG, cacao butter TG, palm oil TG, and algae "Ogoniri" TG were subjected to monomeric reverse-phase HPLC (conditions shown in the figure legend of Fig. 20.2). Peaks on the chromatograms were collected and those individual CN and fatty acid compositions were analyzed using two kinds of GLC as aforementioned. By plotting the relationship between the RRT of the identified individual TG molecular species and the total acyl carbon number, or by plotting the relationship between the RRT of the individual TG molecular species and the DB of the identified individual TG molecular species, Eqs. (20.5) and (20.6) were borne out as can be seen in Figure 20.2 (Takahashi et al., 1983).

We tried to find out why these sets of oblique lines appear in the RRT versus CN plots or in the RRT versus DB plots. In partition chromatography, simple additional theorem of chemical potential of the composing functional groups should hold (Martin, 1950). This theorem can be explained as follows. Suppose that A and B are members of homologous series differing by the functional group X; the chemical potential of B, which is denoted as μ_B, can be expressed as a simple additional theorem of μ_A and μ_X (Fig. 20.3). Chemical potential in HPLC lipid analysis can be defined as the free energy necessary for 1 mol of lipid molecule or the composing group of its molecule to be transported from stationary phase to mobile phase. For example, supposing that A and B are TG molecular species that have the same two acyl moieties but differ only in one acyl moiety that corresponds to palmitate moiety for A and stearate moiety for B, X corresponds to one –CH_2– unit in the acyl moiety as illustrated in Figure 20.3. Or supposing that A and B are TG molecular species that

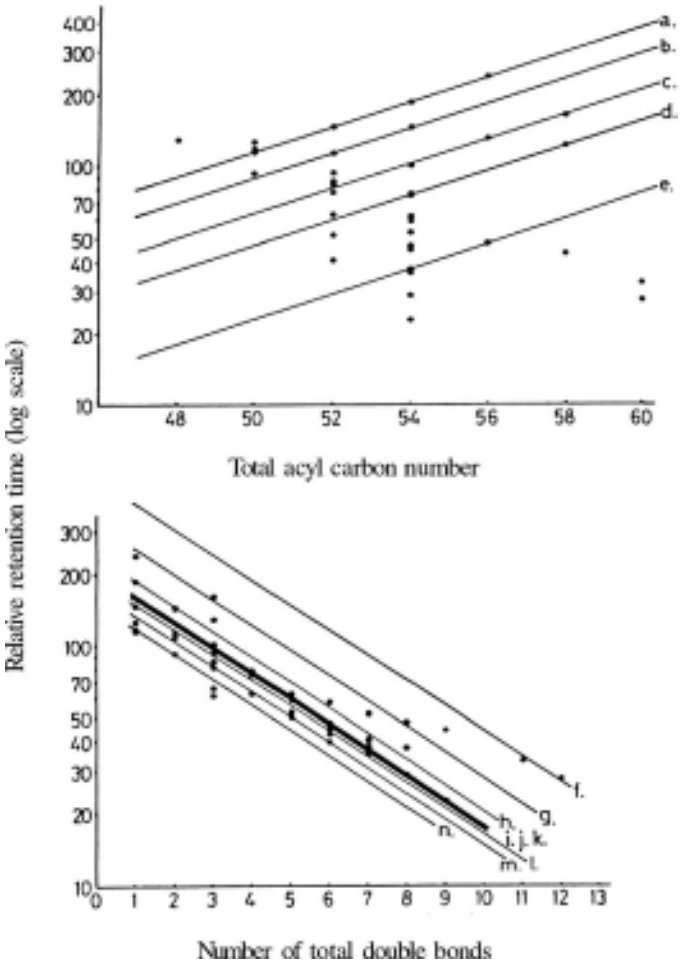

FIGURE 20.2 Relationship between RRT and total acyl carbon number and relationship between RRT and total double bonds of TG molecular species on reverse-phase HPLC (Takahashi et al., 1983). Natural occurring TG from plant sources were injected into the same HPLC system as in Figure 20.1 except that acetone/acetonitrile (3 : 1, v/v) was used as eluting solvent. 18 0 18 1 18 1 18 1 *x* 0 20 *y* 18 *y* a. 18 1, b. 18 1, c. 18 1, d. 18 2, e. 20 4, f. 20 4, g. 18 1, *x* 0 *x* 0 *x* 1 *x* 1 20 4 20 4 22 1 18 0 18 1 18 2 18 3 16 0 16 0 16 0 h. 18 0, i. 18 1, j. 18 2, k. 18 3, l. 18 0, m. 18 2, n. 16 0 18 *y* 18 *y* 18 *y* 18 *y* 18 *y* 18 *y* (*x* and *y* are variables of acyl carbon number and number of double bonds, respectively. For example, *x* can take 14, 16, 18. . . and *y* can take 1, 2, 3, 4. . .).

have the same two acyl moieties but differ only in one acyl moiety that corresponds to oleate moiety for A and linolate for B, X corresponds to one –CH=CH– unit in the acyl moiety that gives minus chemical potential in the chromatographic system as illustrated in Figure 20.4. Therefore, we can sum up by saying the general expression

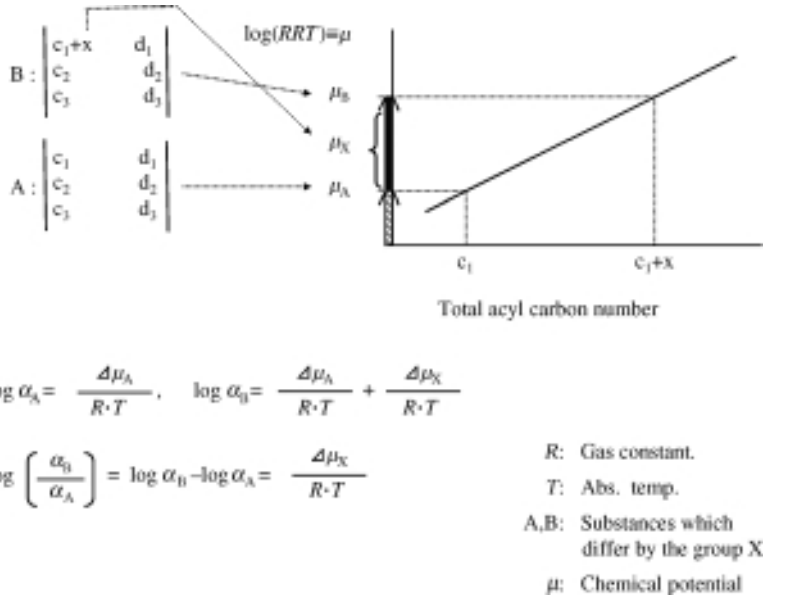

FIGURE 20.3 Relationship between the increased chemical potential of a certain glycerolipid molecular species (μ_B) and that of the original glycerolipid molecular species (μ_A) in accordance with the increase in chemical potential (μ_X) due to an increase in acyl carbon number.

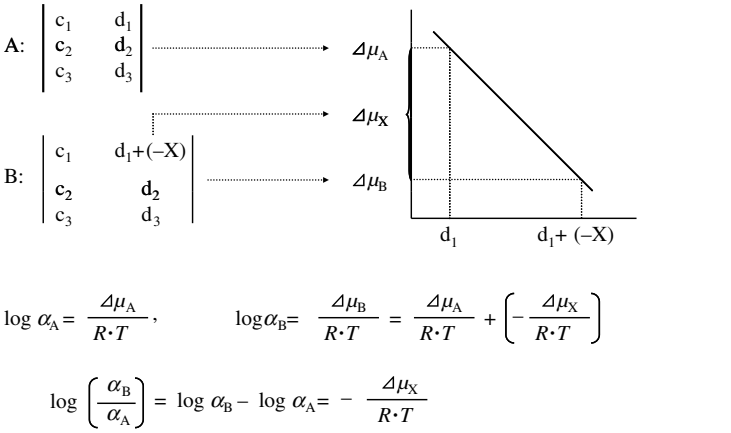

FIGURE 20.4 Relationship between the decreased chemical potential of a certain glycerolipid molecular species (μ_B) and that of the original glycerolipid molecular species (μ_A) in accordance with the decrease in chemical potential ($-\mu_X$) due to decreased number in acyl double bonds.

that covers the whole expressions from (20.1) to (20.6) is:

$$\mathrm{CN} = P_1\log(\mathrm{RRT}) + Q_1 \quad \mathrm{CN} = \begin{matrix} c_1 + x & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{matrix} \tag{20.7}$$

$$\mathrm{DB} = P_2\log(\mathrm{RRT}) + Q_2 \quad \mathrm{DB} = \begin{matrix} c_1 & d_1 + (-\mathrm{x}) \\ c_2 & d_2 \\ c_3 & d_3 \end{matrix} \tag{20.8}$$

By knowing the changes in log(RRT) in accordance with the increase or decrease in X units, we can predict the RRT of the desired molecular species by the forthcoming log(RRT) versus CN or log(RRT) versus DB plots. According to the law of Martine (1950), $\ln(\alpha_B/\alpha_A) = \ln(\mathrm{RRT}) = \Delta\mu_X/R \times T$ and $\log(\alpha_B/\alpha_A) = \log(\mathrm{RRT}) = \Delta\mu_X/2.303 \times R \times T$ should hold, where α, R, and T designate partition coefficient, gas constant, and absolute temperature, respectively. This shows that log(RRT) is proportional to the chemical potential of the solute or the functional group in the chromatographic system.

20.3 APPLICATION OF THE SIMPLE ADDITIONAL THEOREM OF CHEMICAL POTENTIAL IN THE CHROMATOGRAPHIC SYSTEM

The simple additional theorem of chemical potential on TG molecular species that appear as simple monotonous change in log(RRT) such as in Figures 20.1 and 20.2 led to the following possibility. Suppose palmitate moiety in tripalmitin is substituted with oleate moiety one by one, finally to form triolein, a linear relationship between the log (RRT) of those individual molecular species, i.e., tripalmitin, oleoyl dipalmitin, palmitoyl diolein, and triolein versus CN or DB of those molecular species should hold. In the same manner, suppose when palmitate moiety in tripalmitin is substituted with linoleic moiety or linolenic moiety one by one, and finally to form trilinolein or trilinolenin, a linear relationship between those log(RRT)s and CN or DB of those molecular species should hold. These predictions were true as illustrated in Figure 20.5 (Takahashi et al., 1986). From this result, the following equation should be derived.

$$(\Delta\mu TG/R \times T) = (\Delta\mu_{\mathrm{acyl1}}/R \times T) + (\Delta\mu_{\mathrm{acyl2}}/R \times T) + (\Delta\mu_{\mathrm{acyl3}}/R \times T) \tag{20.9}$$

Eq. (20.9) must also be applied as follows.

$$(\Delta\mu_{\mathrm{DG}}/R \times T) = (\Delta\mu_{\mathrm{acyl1}}/R \times T) + (\Delta\mu_{\mathrm{acyl2}}/R \times T)$$

$$(\Delta\mu_{\mathrm{PL}}/R \times T) = (\Delta\mu_{\mathrm{acyl1}}/R \times T) + (\Delta\mu_{\mathrm{acyl2}}/R \times T)$$

$$(\Delta\mu_{\mathrm{alkenylacyl}}/R \times T) = (\Delta\mu_{\mathrm{alkenyl}}/R \times T) + (\Delta\mu_{\mathrm{acyl}}/R \times T)$$

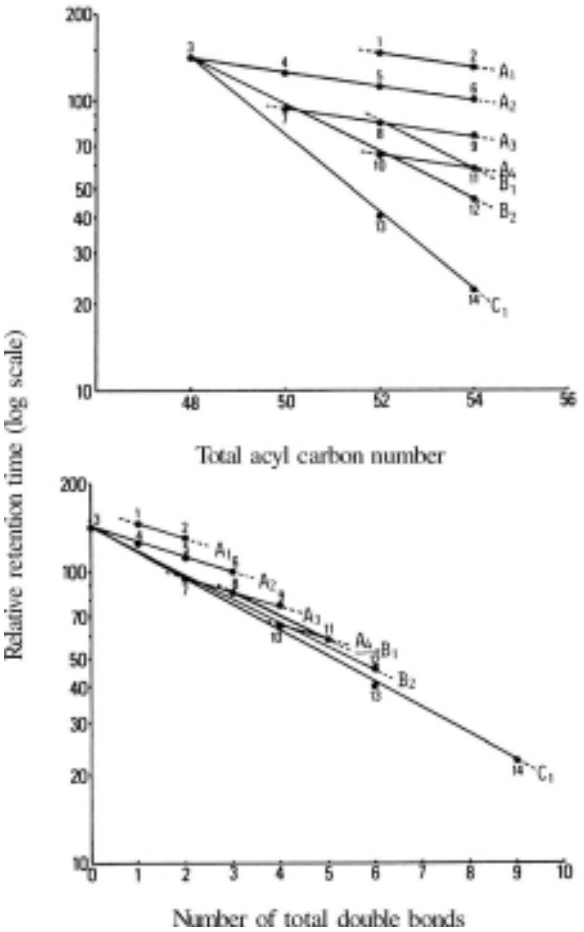

FIGURE 20.5 Relationship between RRT of a molecular species of a TG versus total acyl carbon number or number of total double bonds when the fatty acid residue is substituted (Takahashi et al., 1986). Lines from A_1 to A_4 demonstrate the lines of 16 : 0 → 18 : 1 substitution. Lines B_1 and B_2 demonstrate the lines of 16 : 0 → 18 : 2 substitution. Line C_1 demonstrates the line of 16 : 0 → 18 : 3 substitution. Lines are: A_1: (18 : 0, 16 : 0, 18 : 1n-9) (18 : 0, 18 : 1n-9, 18 : 1n-9) A_2: (16 : 0, 16 : 0, 16 : 0) → (16 : 0, 16 : 0, 18 : 1n-9) → (16 : 0, 18 : 1n-9, 18 : 1n-9) → (18 : 1n-9, 18 : 1n-9, 18 : 1n-9) A_3 : (16 : 0, 16 : 0, 18 : 2n-6) → (18 : 2n-6, 16 : 0, 18 : 1n-9) → (18 : 2n-6, 18 : 1n-9, 18 : 1n-9) A_4: (16 : 0, 18 : 2n-6, 18 : 2n-6) → (18 : 1n-9, 18 : 2n-6, 18 : 2n-6) B_1: (18 : 2n-6, 16 : 0, 18 : 1n-9) → (18 : 1n-9, 18 : 2n-6, 18 : 2n-6) B_2: (16 : 0, 16 : 0, 16 : 0) → (16 : 0, 16 : 0, 18 : 2n-6) → (16 : 0, 18 : 2n-6, 18 : 2n-6) → (18 : 2n-6, 18 : 2n-6, 18 : 2n-6) C_1: (16 : 0, 16 : 0, 16 : 0) → (16 : 0, 16 : 0, 18 : 3n-3) → (16 : 0, 18 : 3n-3, 18 : 3n-3) → (18 : 3n-3, 18 : 3n-3, 18 : 3n-3) Points are: 1.(18 : 0, 16 : 0, 18 : 1n-9), 2.(18 : 0, 18 : 1n-9, 18 : 1n-9), 3.(16 : 0, 16 : 0, 16 : 0), 4.(16 : 0, 16 : 0, 18 : 1n-9), 5.(16 : 0, 18 : 1n-9, 18 : 1n-9), 6.(18 : 1n-9, 18 : 1n-9, 18 : 1n-9), 7.(16 : 0, 16 : 0, 18 : 2n-6), 8.(18 : 2n-6, 16 : 0, 18 : 1n-9), 9.(18 : 2n-6, 18 : 1n-9, 18 : 1n-9), 10.(16 : 0, 18 : 2n-6, 18 : 2n-6), 11.(18 : 1n-9, 18 : 2n-6, 18 : 2n-6), 12.(18 : 2n-6, 18 : 2n-6, 18 : 2n-6), 13.(16 : 0, 18 : 3n-3, 18 : 3n-3), 14. (18 : 3n-3, 18 : 3n-3, 18 : 3n-3).

where TG, DG, and PL designate TG, diacylglycerol, and glycerophospholipids, respectively. In fact, in several empirical analyses (Takahashi et al., 1988; Takahashi and Hirano, 1987), these relations were borne out.

20.4 CALCULATION OF RELATIVE RETENTION POTENTIAL INDEX (RPI) ON HPLC

Since log(RRT) is proportional to the chemical potential of the functional group as aforementioned, Eq. (20.9) can be expressed as follows.

$$\log(\mathrm{RRT})_{\mathrm{TG}} = \log(\mathrm{RRT})\mathrm{acyl}_1 + \log(\mathrm{RRT})\mathrm{acyl}_2 + \log(\mathrm{RRT})\mathrm{acyl}_3 \qquad (20.10)$$

If we define the log(RRT) as "Relative Retention Potential Index (RPI)", the RPI of the individual acyl moiety in TG can be calculated from the authentic mono acid TG standards such as trimyristin, tripalmitin, triolein, trilinolein, trilinolenin, triarachidonin, and trieicosapentaenoin. By applying Eq. (20.10), the RPI per one acyl moiety (log(RRT)acyl) in mono acid TG can be calculated as:

$$\mathrm{RPI\,acyl} \equiv \log(\mathrm{RRT})\mathrm{acyl} = \log(\mathrm{RRT})_{\mathrm{TG}}/3$$

And from known RRT of di- or triacid triglycerides, the unknown RPI of one moiety can be calculated by subtracting the known RPIs from the RRT of that known TG molecule, i.e.:

$$\mathrm{RPI\,acyl}_1 \equiv \underset{(\text{unknown})}{\log(\mathrm{RRT})\mathrm{acyl}_1} = \underset{(\text{known})}{\log(\mathrm{RRT})_{\mathrm{TG}}} - \underset{(\text{known})}{\log(\mathrm{RRT})\mathrm{acyl}_2} - \underset{(\text{known})}{\log(\mathrm{RRT})\mathrm{acyl}_3} \qquad (20.11)$$

Table 20.1 shows the theoretically determined RPI of the individual fatty acid moiety derived from the standard TG molecular species. Each RPI value shown in this table was calculated as follows; For example, by defining the RRT of trieicosapentaenoin (20 : 5n-3, 20 : 5n-3, 20 : 5n-3) as 100 for convenience sake, the RPI of one 20 : 5n-3 moiety can be calculated as (log 100.0)/3 = 0.667. In the same way, supposing that the RRT of triolein is 952.8 in the same chromatographic condition,[1] the RPI of 18 : 1n-9 fatty acid residue can be calculated as (log 952.8)/3 = 0.993. The HPLC chromatogram of sand flounder muscle TG as an example of one of the most complicated TG molecular species is illustrated in Figure 20.6. Outstanding peaks were numbered in sequence of elution. Peak number 2 in Figure 20.6 was composed of two molar 20 : 5n-3 (EPA) and one molar 22 : 6n-3 (DHA), and these two fatty acids were absolutely dominant by inferring the results obtained by GLC. Therefore, this peak was identified as (22 : 6n-3, 20 : 5n-3, 20 : 5n-3).[2] The actual retention time of

[1]Chromatographic conditions: Hitachi Liquid Chromatograph (HPLC) Model 638-50 equipped with 4 × 250 mm Supersphere RP-18 columns using Shodex RI Model SE-11 as a detector. The eluting solvent used was acetone/acetonitrile (1 : 1, v/v) at a flow rate of 0.8 mL/min.

[2]The binding position of the fatty acid residues are not discriminated here.

TABLE 20.1 Contribution Potential toward log(RRT)* of the Individual Triacylglycerol (TG) Molecular Species on Reverse Reverse-Phase High High-Performance Liquid Chromatography (Takahashi and Hirano, 1988)

Fatty Acid Potential Residue RRT*	Contribution Toward Log
14 : 0	0.847
16 : 0	1.033
18 : 0	1.178
16 : 1n-9	0.861
18 : 1n-9	0.993
18 : 2n-6	0.861
18 : 3n-3	0.740
20 : 3n-3	0.834
20 : 4n-6	0.781
20 : 5n-3	0.667
22 : 6n-3	0.602

*We defined this as relative retention potential index (RPI) in the text.

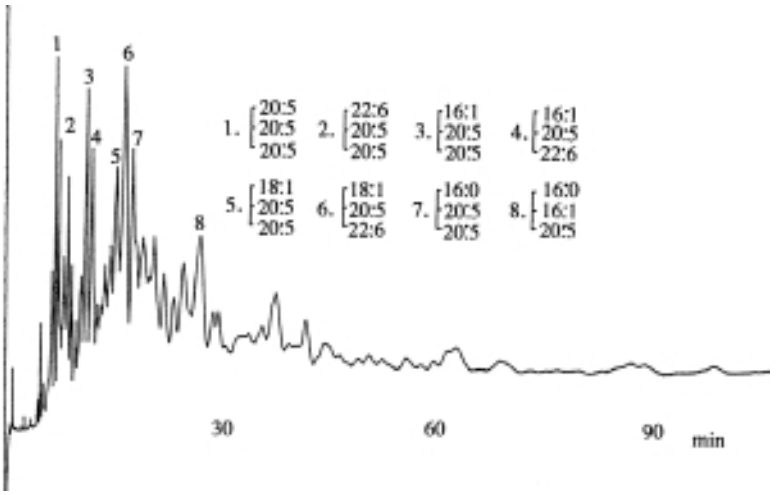

FIGURE 20.6 Comparison of predicted and the actual RRT of prominent molecular species of sand flounder muscle on reverse-phase HPLC (Takahashi et al., 1988). Same HPLC system was used as in Figure 20.2 except that single 4×250 mm Supersphere RP-18 non-end cap column and acetone/acetonitrile (1 : 1, v/v) were used.

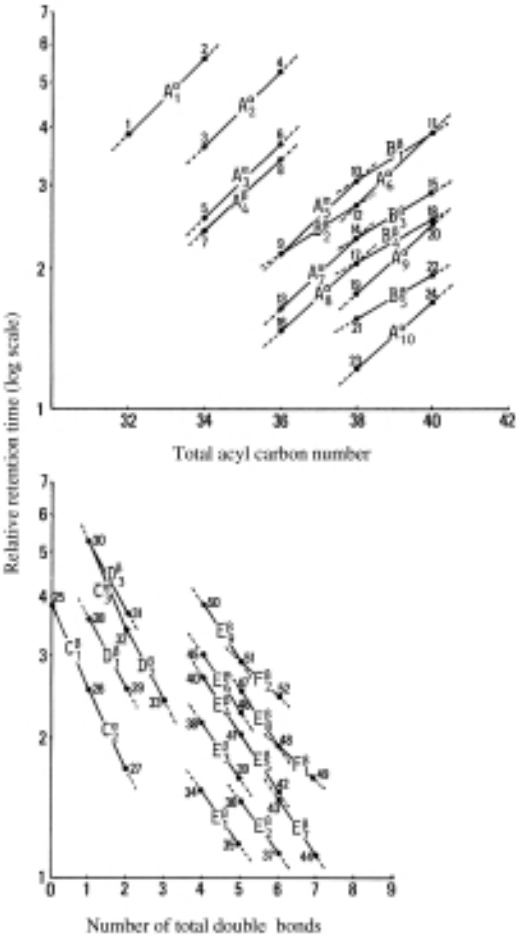

FIGURE 20.7 Relationship between RRT of molecular species of diacylglycerolipid in the form of dinitrobenzoyl derivative versus total acyl carbon number or number of total double bonds on reverse-phase HPLC (Takahashi and Hirano, 1991). Retention time of (12 : 0, 12 : 0) is regarded as 1.00. The superscript on the alphabet shows the acyl carbon number or number of double bonds substituted position (α for *sn*-1 and β for *sn*-2). Lines A and B are for the acyl carbon number substitutions, and lines from C to F are for the acyl double bonds substitutions. Lines with the same alphabet are parallel to each other, although there is a slight difference between that for *sn*-1 and that for *sn*-2 positions; The *sn*-1,2- and the *sn*-1,3- positional isomers are all discriminated here. Points are: 1.(16 : 0, 16 : 0), 2.(18 : 0, 16 : 0), 3.(16 : 0, 18 : 1n-9), 4. (18 : 0, 18 : 1n-9), 5. (16 : 0, 18 : 2n-6), 6.(18 : 0, 18 : 2n-6), 7.(18 : 1n-9, 16 : 1n-9), 8.(18 : 1n-9, 18 : 1n-9), 9.(16 : 0, 20 : 4n-6), 10.(18 : 0, 20 : 4n-6), 11.(18 : 0, 22 : 4n-6), 12.(16 : 0, 22 : 4n-6), 13.(16 : 0, 20 : 5n-3), 14.(18 : 0, 20 : 5n-3), 15.(18 : 0, 22 : 5n-3), 16.(16 : 1n-9, 20 : 4n-6), 17. (18 : 1n-9, 20 : 4n-6), 19.(16 : 0, 22 : 6n-3), 20.(18 : 0, 22 : 6n-3), 21.(18 : 1n-9, 20 : 5n-3), 22. (18 : 1n-9, 22 : 5n-3), 23.(16 : 1n-9, 22 : 6n-3), 24.(18 : 1n-9, 22 : 6n-3), 25.(16 : 0, 16 : 0), 26. (16 : 0, 16 : 1n-9), 27.(16 : 1n-9, 16 : 1n-9), 28.(16 : 0, 18 : 1n-9), 29.(16 : 0, 18 : 2n-6), 30.(18 : 0,

TABLE 20.2 Comparison of the Predicted and the Empirically Determined log(RRT) of the Individual Triacylglycerol Molecular Species on Reverse-Phase High-Performance Liquid Chromatography[1]

Peak Number	Molecular Species	Predicted Retention Time (min)	Empirically Determined Retention Time (min)	Relative Deviation (%)
1	(20 : 5n-3, 20 : 5n-3, 20 : 5n-3)*	—	7.06	—
2	(22 : 6n-3, 20 : 5n-3, 20 : 5n-3)*	—	7.46	—
3	(16 : 1n-9, 20 : 5n-3, 20 : 5n-3)*[3]	11.06	11.05	0.1
4	(16 : 1n-9, 20 : 5n-3, 22 : 6n-3)*[3]	11.72	11.77	0.4
5	(18 : 1n-9, 20 : 5n-3, 20 : 5n-3)*[3]	14.99	14.89	0.7
6	(18 : 1n-9, 20 : 5n-3, 22 : 6n-3)*[3]	15.88	15.94	0.4
7	(16 : 0, 20 : 5n-3, 20 : 5n-3)*[3]	16.44	16.89	2.7
8	(16 : 0, 16 : 1n-9, 20 : 5n-3)*[3]	25.69	25.70	0

*Standard (reference) peaks.

this molecular species was 7.49 on the HPLC employed, and the unknown $RPI_{22:6n-3}$ was calculated as follows.

Step 1. The RRT of (22 : 6n-3, 20 : 5n-3, 20 : 5n-3) was calculated as $100 \times 7.49/7.06^3 = 106.1$

Step 2. From step 1,7 the RPI of (22 : 6n-3, 20 : 5n-3, 20 : 5n-3) was calculated as $\log 106.1 = 2.026$.

Step 3. Thus, from Eq. (20.11), $RPI_{22:6n-3} = 2.026\text{-}0.667\text{-}0.667 = 0.692$ was obtained.

The RPIs obtained in the same manner shown in Table 20.1 is extremely useful in predicting the retention time of the desired molecular species appearing on the HPLC chromatograms with acyl combinations composed of the fatty acid residues shown in Table 20.1. Since 11 kinds of fatty acid residues are demonstrated in Table 20.1, the retention time of $11^3/3 = 443$ kinds (binding positions of the acyl moieties are not discriminated) of TG molecular species can be predicted theoretically. For example, first the RPI of (18 : 1, 20 : 5n-3, 22 : 6n-3) can be calculated as 0.993 + 0.667

FIGURE 20.7 *(Continued)* 18 : 1n-9), 31.(18 : 0, 18 : 2n-6), 32.(18 : 1n-9, 18 : 1n-9), 33.(18 : 1n-9, 18 : 2n-6), 34.(14 : 0, 20 : 4n-6), 35.(14 : 0, 20 : 5n-3), 36.(16 : 1n-9, 20 : 4n-6), 37.(16 : 1n-9, 20 : 5n-3), 38.(16 : 0, 20 : 4n-6), 39.(16 : 0, 20 : 5n-3), 40.(16 : 0, 22 : 4n-6), 41.(16 : 0, 22 : 5n-3) and (18 : 1n-9, 20 : 4n-6), 42.(18 : 1n-9, 20 : 5n-3), 43.(18 : 2n-6, 20 : 4n-6), 44.(18 : 2n-6, 20 : 5n-3), 45.(18 : 0, 20 : 4n-6), 46.(18 : 0, 20 : 5n-3), 47.(18 : 1n-9, 22 : 4n-6), 48.(18 : 1n-9, 22 : 5n-3), 49.(18 : 1n-9, 22 : 6n-3), 50.(18 : 0, 22 : 4n-6), 51.(18 : 0, 22 : 6n-3).

[3]Retention time of trieicosapentaenoin (20 : 5n-3, 20 : 5n-3, 20 : 5n-3) considered as standard of RRT for convenience sake.

$0.692 = 2.352$ by employing the forthcoming data in Table 20.1. The RRT of this molecular species can be calculated as $10^{2.352} = 224.9$, since RRT is the antilogarithm of RPI. The actual retention time on the HPLC chromatogram of (18 : 1, 20 : 5n-3, 22 : 6n-3) can be thus calculated as;[4]

$$224.9 \times 7.06/100 = 15.88\,(\text{min})$$

The actual retention time of the peak on HPLC that represented (18 : 1, 20 : 5n-3, 22 : 6n-3) in Figure 20.6 was 15.94 (min). Other numbered prominent peaks were also identified with GLC. The actual retention times of those molecular species representing peaks were compared with the theoretically calculated retention times that were obtained in the above-mentioned manner. As shown in Table 20.2, the theoretically predicted and the actual retention times on peaks in Figure 20.6 coincide well, showing that the proposed prediction method of lipid molecular species is practically viable, especially in routine analysis.

APPENDIXES

As mentioned earlier, acetate derivative of diacylglycerol prepared by cleaving off the polar head group of glycerophospholipid can be considered as a member of TG set that has the shortest acyl group (–CO–CH_3–) as one constituent moiety. Aizawa (2009) recently pointed out that in monomeric HPLC system (such as in this study), the reverse isomers of the acetate derivatives of diacylglycerols are impossible to be discriminated. This must be the reason why glossy parallel oblique lines shown in Figures 20.1 and 20.2 can be obtained. However, as illustrated in Figure 20.7, the plots of the RRT of the dinitrobenzoyl derivatives of glycerophospholipid molecular species versus those of CN or DB are approximately parallel but, strictly speaking, the slopes of the lines with very highly polyunsaturated fatty acid moieties such as pentaene or hexaene tend to lay more compared with the less unsaturated or saturated fatty acid moieties (Takahashi and Hirano, 1991). From this aspect, the modified groups with a structure completely different from that of the acyl chains such as with aromatic groups should affect the linearity of the RRT versus CN or the RRT versus DB plots.

At any rate, the simple additional theorem of RPI rests on the chemical potential of solute and its composing functional groups are no doubt eternal.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Ching T. Hou for providing the precious opportunity to show in part their classical but applicable theory to carry out lipid molecular species analysis on modern HPLC.

[4]Retention time of trieicosapentaenoin (20 : 5n-3, 20 : 5n-3, 20 : 5n-3) considered as standard of RRT for convenience sake.

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21

DEHYDROGENASE-CATALYZED SYNTHESIS OF CHIRAL INTERMEDIATES FOR DRUGS

RAMESH N. PATEL

SLRP Associates, Biotechnology Consultation, 572 Cabot Hill Road, Bridgewater, NJ 08807, USA

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21.1 INTRODUCTION

The production of single enantiomers of chiral intermediates has become increasingly important in the pharmaceutical industry (Food and Drug Administration, 1992). Single enantiomers can be produced by chemical or chemoenzymatic synthesis. Biocatalysis often offers advantages over chemical synthesis as enzyme-catalyzed reactions are often highly enantioselective and regioselective. They can be carried out at ambient temperature and atmospheric pressure, thus avoiding the use of more extreme conditions that could cause problems with isomerization, racemization, epimerization, and rearrangement. Microbial cells and enzymes derived therefrom can be immobilized and reused for many cycles. In addition, enzymes can be overexpressed to make biocatalytic processes economically efficient, and enzymes with modified activity can be tailor-made. Directed evolution of biocatalysts can lead to increased enzyme activity, selectivity, and stability (Food and Drug Administration, 1992; Oliver et al., 2002; Kazlauskas, 2005; Schmidt et al., 2004; Reetz et al., 2004; Otey et al., 2006; Huisman and Lalonde, 2007; Alphand et al., 2003; DiCosimo, 2007; Patel, 2006). A number of review articles (Simeo et al., 2007; Simons et al., 2006; Turner, 2004; Robertson and Bornscheuer, 2005; Ishige et al., 2005; Stewart, 2005; Fessner and Jenneneein, 2007; Boyd and Bugg, 2006; Wandrey et al., 1981; Brunhuber and Blanchard, 1994) have been published on the use of enzymes in organic synthesis. This review provides some examples of the use of dehydrogenases in the synthesis of single enantiomers of key intermediates used in the pharmaceutical synthesis.

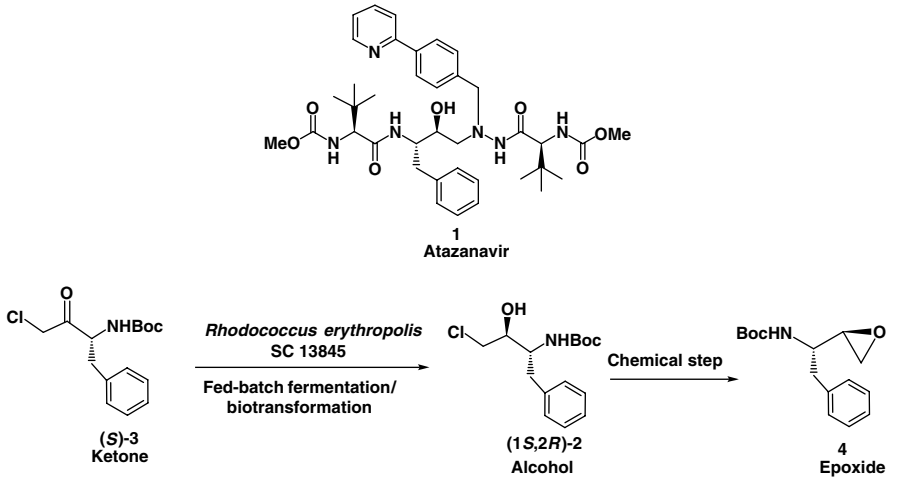

FIGURE 21.1 Enzymatic preparation of (1*S*,2*R*)-[3-chloro-2-hydroxy-1-(phenylmethyl) propyl]-carbamic acid, 1,1-dimethyl-ethyl ester.

21.2 DEHYDROGENASE-CATALYZED REDUCTIONS

21.2.1 Enzymatic Preparation of (1*S*,2*R*)-[3-Chloro-2-Hydroxy-1-(Phenylmethyl)Propyl]-Carbamic Acid, 1,1-Dimethyl-Ethyl Ester

Atazanavir **1** is an acyclic aza-peptidomimetic, a potent HIV protease inhibitor (Bold et al., 1998; Robinson et al., 2000) approved recently by the Food and Drug Administration for the treatment of auto immune diseases (AIDS). An enzymatic process has been developed for the preparation of (1*S*,2*R*)-[3-chloro-2-hydroxy-1-(phenylmethyl)propyl]carbamic acid, 1,1-dimethylethyl ester (**2**, Fig. 21.1), a key chiral intermediate required for the total synthesis of the HIV protease inhibitor atazanavir. Among microbial cultures evaluated for the diastereoselective reduction of (1*S*)-[3-chloro-2-oxo-1-(phenylmethyl)propyl]carbamic acid, 1,1-dimethylethyl ester **3**, three strains of *Rhodococcus* gave >90% yield with a diastereomeric purity of >98% and an enantiomeric excess (e.e.) of 99.4% (Patel et al., 2003). An efficient single-stage fed-batch fermentation-biotransformation process was developed for the reduction of ketone **3** with cells of *Rhodococcus erythropolis* SC 13845 to yield **2** in 95% with a diastereomeric purity of 98.2% and an e.e. of 99.4%. (1*S*,2*R*)-**2** was converted to epoxide **4** (Fig. 21.1) and used in the synthesis of atazanavir (Jajoo et al., 1989).

21.2.2 Enzymatic Preparation of 6-Hydroxybuspirone

Buspirone (Buspar®, **5**, Fig. 21.2) is a drug used for treatment of anxiety and depression that is thought to produce its effects by binding to the serotonin 5HT1A receptor (Mayol, 2000; Yevich et al., 1992; Yevich et al., 2003). It is extensively

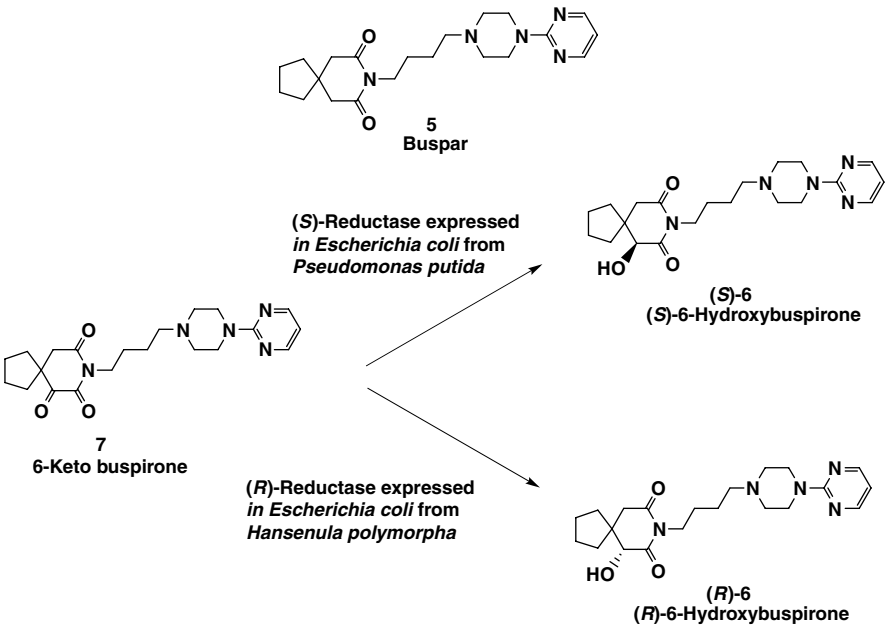

FIGURE 21.2 Enzymatic preparation of 6-hydroxybuspirone.

converted to various metabolites mainly as a result of hydroxylation reactions and blood concentrations return to low levels a few hours after dosing (Patel et al., 2005). A major metabolite, 6-hydroxybuspirone (**6**), produced by the action of liver cytochrome P450 CYP3A4, is present at much higher concentrations in human blood than buspirone itself. For the development of 6-hydroxybuspirone as a potential antianxiety drug, preparation and testing of the two enantiomers as well as the racemate was of interest. An enantioselective microbial reduction process was developed for reduction of 6-oxobuspirone (**7**) to either (*R*)- or (*S*)-6-hydroxybuspirone **6** (Goldberg et al., 2006). About 150 microorganisms were screened for the enantioselective reduction of **7**. *Rhizopus stolonifer* SC 13898, *Neurospora crassa* SC 13816, *Mucor racemosus* SC 16198, and *Pseudomonas putida* SC 13817 gave >50% reaction yields and >95% e.e.'s of (*S*)-6-hydroxybuspirone. The yeast strains *Hansenula polymorpha* SC 13845 and *Candida maltosa* SC 16112 gave (*R*)-6-hydroxybuspirone in >60% reaction yield and >97% e.e. The NADP-dependent (*R*)-reductase (RHBR) from *H. polymorpha* SC 13845 was cloned and expressed in *Escherichia coli*. To regenerate the cofactor NADPH required for reduction, the glucose-6-phosphate dehydrogenase gene from *Saccharomyces cerevisiae* was cloned and overexpressed in *E. coli*. Also, the NAD-dependent (*S*)-reductase (SHBR) from *Pseudomonas putida* SC 16269 was cloned and expressed in *E. coli*. To regenerate the cofactor NADH required for reduction, the formate dehydrogenase (FDH) gene from *Pichia pastoris* was cloned and overexpressed in *E. coli*. Recombinant *E. coli* expressing (*S*)-reductase and (*R*)-reductase were used to catalyze the

reduction of 6-ketobuspirone to (*S*)-6-hyrxybuspirone and (*R*)-6-hyrxybuspirone, respectively, in >98% yield and >99.9% e.e. (Goldberg et al., 2006).

21.2.3 Enzymatic Preparation of (*S*)-2-Chloro-1-(3-Chlorophenyl)Ethanol

The synthesis of the leading candidate compound **8** in an anticancer program (IGF-1 receptor inhibitors) (Wittman et al., 2005; Wittman et al., 2007) required (*S*)-2-chloro-1-(3-chlorophenyl)ethanol (**9**, Fig. 21.3) as an intermediate. Other possible candidate compounds used analogs of (*S*)-alcohol **9**. From microbial screen of the reduction of ketones **10** to (*S*)-alcohols **9**, two cultures namely *H. polymorpha* SC13824 (73.8% e.e.) and *Rhodococcus globerulus* SC SC16305 (71.8% ee) were identified that had the highest enantioselectivity. A ketoreductase from *H. polymorpha*, after purification to homogeneity, gave (*S*)-alcohol **9** with 100% ee. The ketoreductase was cloned and expressed in *E. coli* together with a glucose-6-phosphate dehydrogenase from *Saccharomyces cerevisiae* to allow regeneration of the NADPH required for the reduction process. An extract of *E. coli* containing the

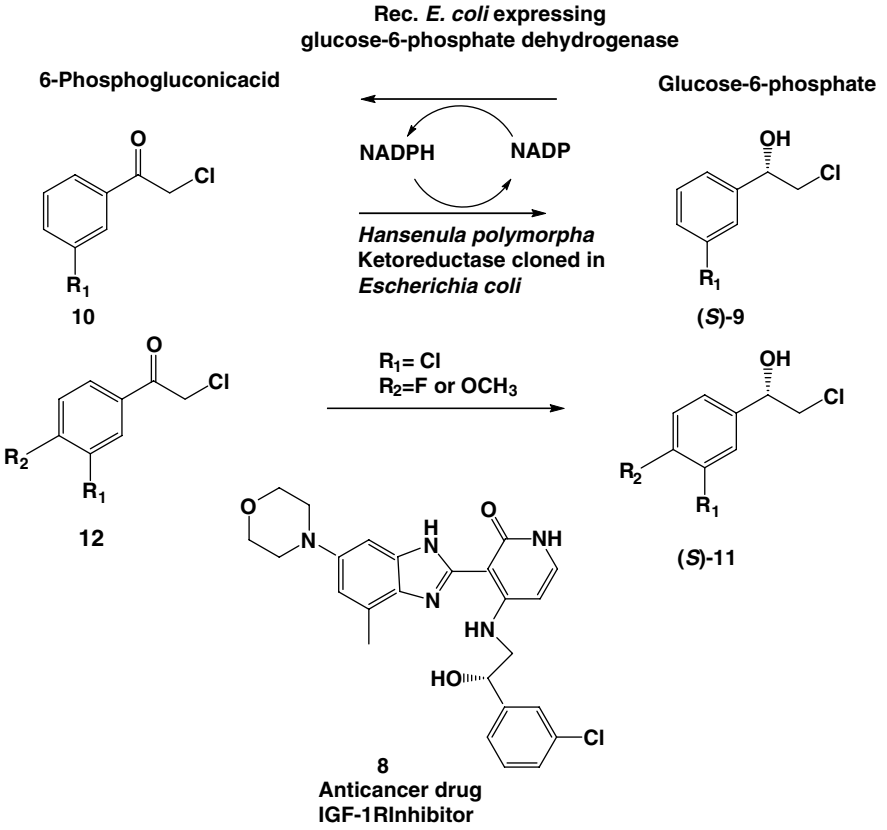

FIGURE 21.3 Enzymatic preparation of (S)-2-chloro-1-(3-chlorophenyl)ethanol.

two recombinant enzymes was used to reduce 2-chloro-1-(3-chloro-4-fluorophenyl)-ethanone **10**. Intact *E. coli* cells provided with glucose were used to prepare (*S*)-2-chloro-1-(3-chloro-4-fluorophenyl)ethanol **9** in 89% yield with 100% e.e. This extract also converted ketone **12** to corresponding (*S*)-alcohol **11** in 90% yield and 99.8% ee (Hanson et al., 2005).

21.2.4 Enzymatic Preparation of (3*S*,5*R*)-Dihydroxy-6-(Benzyloxy) Hexanoic Acid, Ethyl Ester

The enantioselective reduction of a diketone 3,5-dioxo-6-(benzyloxy) hexanoic acid, ethyl ester **13** to (3*S*,5*R*)-dihydroxy-6-(benzyloxy) hexanoic acid, ethyl ester **14** (Fig. 21.4) was demonstrated by cell suspensions of *Acinetobacter calcoaceticus* SC 13876 (Patel et al., 1993). Compound **14** is a key chiral intermediate required for the chemical synthesis of **15** (Patel et al., 1993) and Atorvastatin; both are anticholesterol drugs which act by inhibition of HMG CoA reductase (Roth, 2002). A reaction yield of 85% and an e.e. of 97% were obtained. Cell extracts of *A. calcoaceticus* SC 13876 in the presence of NAD^+, glucose, and glucose dehydrogenase reduced **13** to the corresponding monohydroxy compounds [3-hydroxy-5-oxo-6-(benzyloxy) hexanoic acid ethyl ester **16** and 5-hydroxy-3-oxo-6-(benzyloxy)

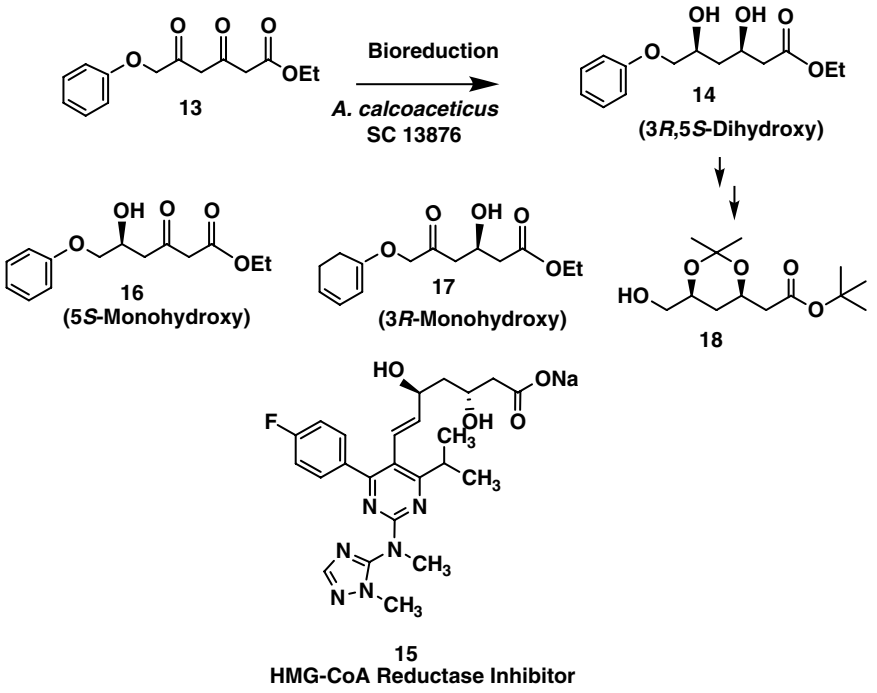

FIGURE 21.4 Enzymatic preparation of (3*S*,5*R*)-dihydroxy-6-(benzyloxy) hexanoic acid, ethyl ester.

hexanoic acid ethyl ester **17**]. Both **16** and **17** were further reduced to the (3*S*,4*R*)-dihydroxy compound **14** in 85% yield and 99% e.e. by cell extracts. (3*S*,5*R*)-**14** was converted to **18**, a key chiral intermediate for the synthesis of **15**. Three different ketoreductases were purified to homogeneity from cell extracts, and their biochemical properties were compared. Reductase I only catalyzes the reduction of ethyl diketoester **13** to its monohydroxy products whereas reductase II catalyzes the formation of dihydroxy products from monohydroxy substrates. A third reductase (III) was identified that catalyzes the reduction of diketoester **13** to *syn*-(3*R*,5*S*)-dihydroxy ester **14** (Guo et al., 2006), which now has been cloned and expressed in *E. coli*.

21.2.5 Enzymatic Preparation of (*R*)-2-Hydroxy-3,3-Dimethylbutanoic Acid

Thrombin is a trypsin-like protease enzyme that plays a critical role in intrinsic and extrinsic blood coagulation. As a result of the enzymatic activation of numerous coagulation factors, thrombin is activated to cleave fibrinogen, producing fibrin, which is directly responsible for blood clotting. An imbalance between these factors and their endogenous activators and inhibitors can give rise to a number of disease states such as myocardial infarction, unstable angina, stroke, ischemia, restenosis following angioplasty, pulmonary embolism, deep vein thrombosis, and arterial thrombosis (Vacca, 2000; Gladwell, 2002). Consequently, the aggressive search for a potent, selective, and bioavailable thrombin inhibitor is widespread (Fevig and Wexler, 1999). An intensive effort by Merck has led to the identification of thrombin inhibitor **19** (Williams et al., 2002). The synthesis of **19** (Fig. 21.5) required a key chiral intermediate (*R*)-hydroxy ester **20**. An enzymatic process was developed for the asymmetric reduction of ketoester **21** to (*R*)-**20** using commercially available

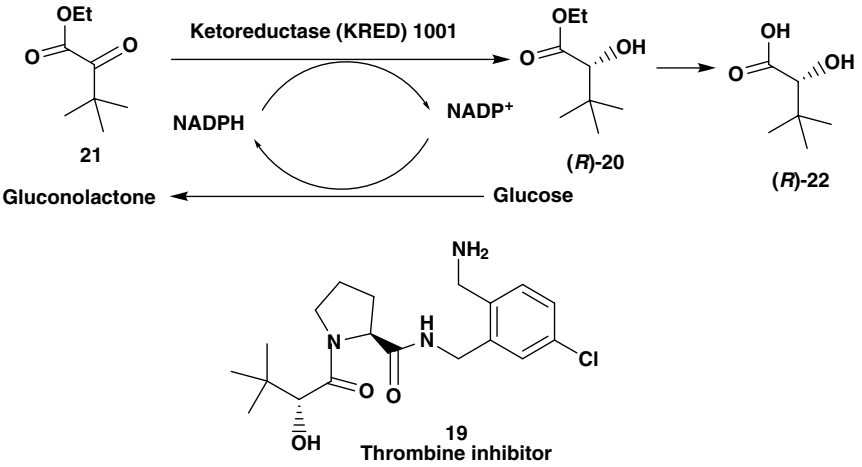

FIGURE 21.5 Enzymatic preparation of (*R*)-2-hydroxy-3,3-dimethylbutanoic acid.

ketoreductase KRED1001. The cofactor NADPH required for this reaction was regenerated using glucose dehydrogenase. The hydroxy ester (*R*)-**20** was isolated as an oil and then saponified to the corresponding enantiomerically pure hydroxy acid (*R*)-**22** without epimerization (Nelson et al., 2004). The enantiomerically pure (*R*)-**22** was obtained in 82% isolated yield (>99.5% e.e.).

21.2.6 Enantioselective Enzymatic Reduction of 5-Oxohexanoate and 5-Oxohexanenitrile

Ethyl-(*S*)-5-hydroxyhexanoate **23** and (*S*)-5-hydroxyhexanenitrile **24** (Fig. 21.6) are key chiral intermediates in the synthesis of anti-Alzheimer drugs (Prasad et al., 2004). Both chiral compounds have been prepared by the enantioselective reduction of ethyl-5-oxohexanoate **25** and 5-oxohexanenitrile **26** by *Pichia methanolica* SC 16116. Reaction yields of 80–90% and >95% e.e. were obtained for each chiral compound. In an alternate approach, the enzymatic resolution of racemic 5-hydroxyhexane nitrile **27** by enzymatic succinylation was demonstrated using immobilized lipase PS-30 to obtain (*S*)-5-hydroxyhexanenitrile **24** in 35% yield (maximum yield is 50%). (*S*)-5-Acetoxy-hexanenitrile **28** was prepared by enantioselective enzymatic hydrolysis of racemic 5-acetoxyhexanenitrile **29** by

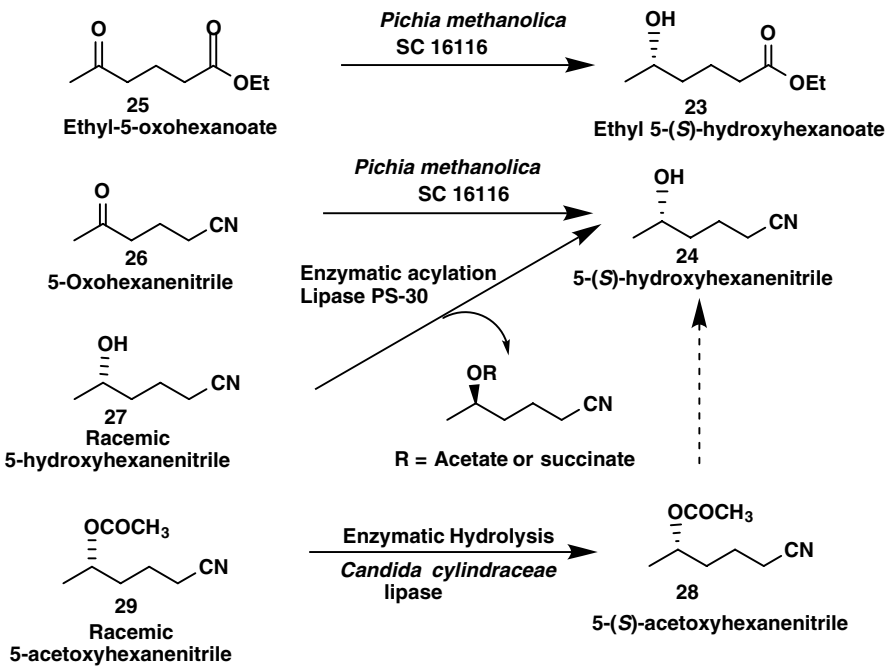

FIGURE 21.6 Enantioselective enzymatic reduction of 5-oxohexanoate and 5-oxohexanenitrile.

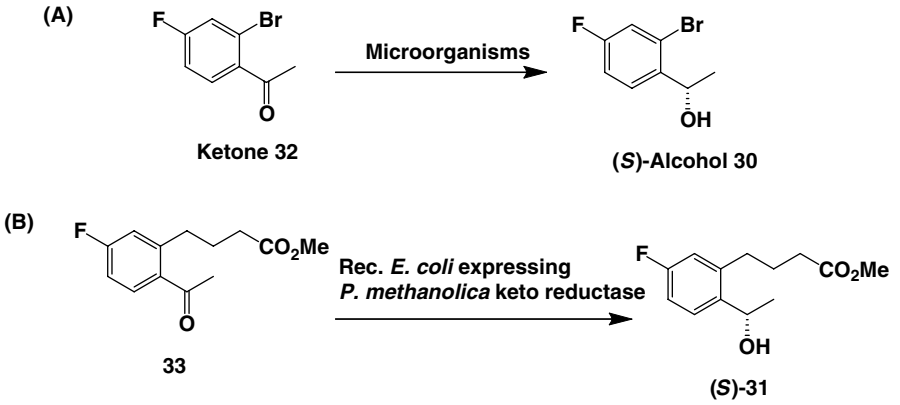

FIGURE 21.7 Enantioselective microbial reduction of substituted acetophenone.

Candida antarctica lipase. A reaction yield of 42% and an e.e. of >99% were obtained (Nanduri et al., 2002).

21.2.7 Enantioselective Microbial Reduction of Substituted Acetophenone

The chiral intermediates (*S*)-1-(2′-bromo-4′-fluorophenyl)ethanol **30** and (*S*)-methyl 4-(2′-acetyl-5′-fluorophenyl)-butanol **31** are potential intermediates for the synthesis of several potential anti-Alzheimer drugs (Prasad et al., 2004; Schenk et al., 2001). The chiral intermediate (*S*)-1-(2′-bromo-4′-fluoro phenyl)ethanol **30** (Fig. 21.7A) was prepared by the enantioselective microbial reduction of 2-bromo-4-fluoro acetophenone **32** (Patel et al., 2004). Organisms from genus *Candida*, *Hansenula*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Sphingomonas*, and Baker's yeast reduced **32** to **30** in >90% yield and 99% e.e.

In an alternate approach, the enantioselective microbial reduction of methyl-4-(2′-acetyl-5′-fluorophenyl) butanoates **33** (Fig. 21.7B) was demonstrated using strains of *Candida* and *Pichia*. Reaction yields of 40–53% and e.e.s of 90–99% were obtained for the corresponding (*S*)-hydroxy esters **32**. The reductase, which catalyzed the enantioselective reduction of ketoesters, was purified to homogeneity from cell extracts of *P. methanolica* SC 13825. It was cloned and expressed in *E. coli* and recombinant cultures were used for the enantioselective reduction of the keto-methyl ester **33** to the corresponding (*S*)-hydroxy methyl ester **32**. On a preparative scale, a reaction yield of 98% with an e.e. of 99% was obtained (Patel et al., 2004).

21.2.8 Enzymatic Preparation of 2-(*R*)-Hydroxy-2-(1′,2′,3′,4′-Tetrahydro-1′,1′,4′,4′-Tetramethyl-6′-Naphthalenyl)Acetate

Retinoic acid and its natural and synthetic analogs (retinoids) exert a wide variety of biological effects by binding to or activating a specific receptor or sets of receptors.

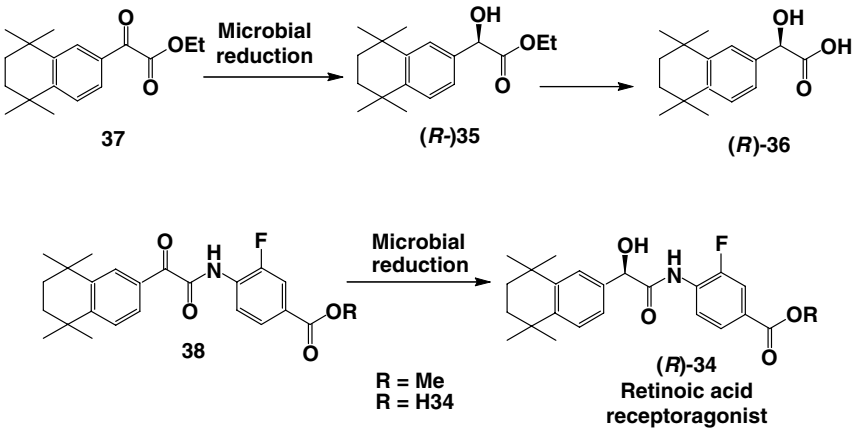

FIGURE 21.8 Enzymatic preparation of 2-(*R*)-hydroxy-2-(1′,2′,3′,4′-tetrahydro-1′,1′,4′,4′-tetramethyl-6′-naphthalenyl)acetate.

They have been shown to affect cellular growth and differentiation and are promising drugs for the treatment of cancers (Kagechika et al., 1989; Kagechika and Shudo, 1990). A few retinoids are already in clinical use for the treatment of dermatological diseases such as acne and psoriasis (Morriss-Kay, 1997). (*R*)-3-Fluoro-4-[[hydroxy-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-acetyl]amino]benzoic acid **34** (Fig. 21.8) is a retinoic acid receptor gamma-specific agonist potentially useful as a dermatological and anticancer drug (Moon and Mehta, 1986).

Ethyl 2-(*R*)-hydroxy-2-(1′,2′,3′,4′-tetrahydro-1′,1′,4′,4′-tetramethyl-6′-naphthalenyl)acetate **35** (Fig. 21.8) and the corresponding acid **36** were prepared as intermediates in the synthesis of the retinoic acid receptor gamma-specific agonist **34** (Patel et al., 2002). Enantioselective microbial reduction of ethyl 2-oxo-2-(1′,2′,3′,4′-tetrahydro-1′,1′,4′,4′-tetramethyl-6-naphthalenyl) acetate **37** to (*R*)-hydroxyester **35** was carried out using *Aureobasidium pullulans* SC 13849 in 98% yield and with an e. e. of 96%. At the end of the reaction, hydroxyester **35** was adsorbed onto XAD-16 resin and, after filtration, recovered in 94% yield from the resin with acetonitrile extraction. The recovered (*R*)-hydroxyester **35** was treated with Chirazyme L-2 or pig liver esterase to convert it to the corresponding (*R*)-hydroxyacid **36** in quantitative yield. The enantioselective microbial reduction of ketoamide **38** to the corresponding (*R*)-hydroxyamide **34** by *A. pullulans* SC 13849 was also demonstrated (Patel et al., 2002).

21.2.9 Enantioselective Microbial Reduction of Keto Ester and Chloroketone

Endothelin is present in elevated levels in the blood of patients with hypertension, acute myocardial infarction, and pulmonary hypertension. Two endothelin receptor subtypes have been identified which bind endothelin, thus causing vasoconstriction (Fukuroda

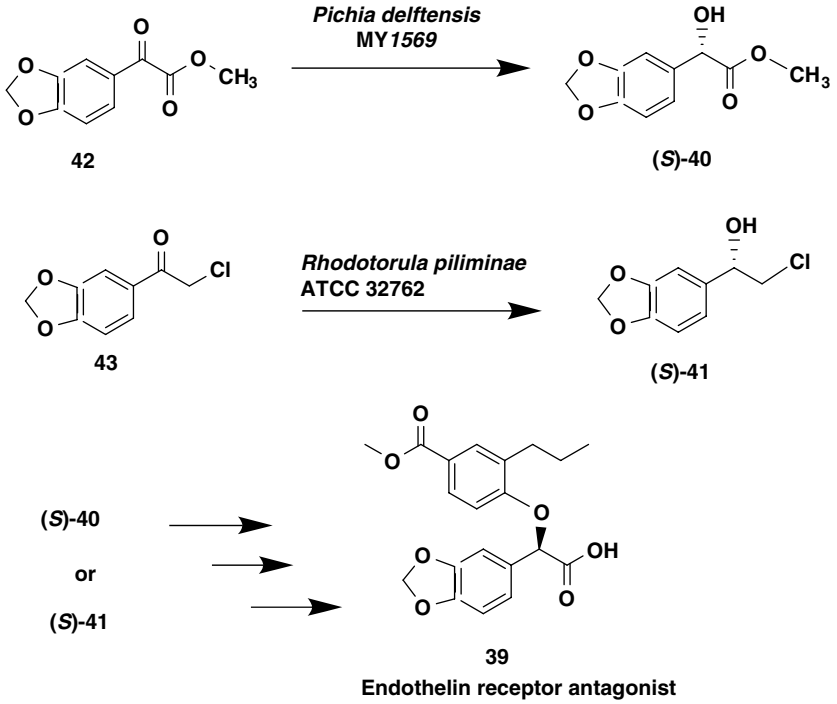

FIGURE 21.9 Enantioselective microbial reduction of keto ester and chloroketone.

and Nishikibe, 1998; Sumner et al., 1992). Endothelin receptor antagonists such as compound **39** (Fig. 21.9) have potential therapeutic value. Synthesis of compound **39** required two key chiral intermediates (*S*)-alcohols **40** and **41**. Enantioselective microbial reduction of a ketoester **42** and a chlorinated ketone **43** to their corresponding (*S*)-alcohols **40** and **41** was demonstrated using *Pichia delftensis* MY 1569 and *Rhodotorula piliminae* ATCC 32762 to afford desired products in >98% e.e. and >99% e.e, respectively (Krulewicz et al., 2001). Reductions were scaled up to 23 L to produce the desired (*S*)-alcohols in 88% and 97% yields, respectively.

21.2.10 Enzymatic Preparation of (2*R*,3*S*)-*N*-Benzoyl-3-Phenyl Isoserine Ethyl Ester

Among the antimitotic agents, paclitaxel (taxol®) (**44**, Fig. 21.10), a complex, polycyclic diterpene, exhibits a unique mode of action on microtubule proteins responsible for the formation of the spindle during cell division. Various types of cancers have been treated with paclitaxel and it was approved for use by the FDA for treatment of ovarian cancer and metastatic breast cancer (Holton et al., 1995; Patel, 1992). A key precursor for the paclitaxel semisynthetic process is the chiral

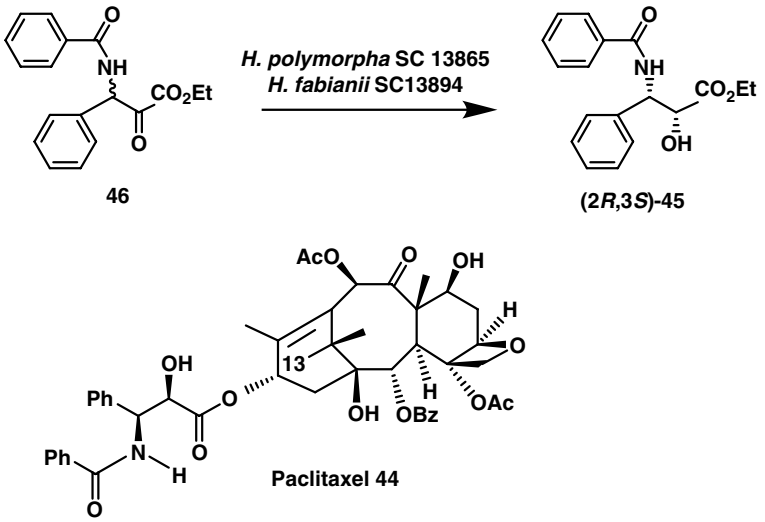

FIGURE 21.10 Enzymatic preparation of (2*R*,3*S*)-*N*-benzoyl-3-phenyl isoserine ethyl ester.

C-13 paclitaxel side-chain **45**. An enzymatic enantioselective microbial reduction of 2-keto-3-(*N*-benzoylamino)-3-phenyl propionic acid ethyl ester **46** to yield (2*R*,3*S*)-*N*-benzoyl-3-phenyl isoserine ethyl ester **45** was demonstrated using two strains of *Hansenula* (Patel et al., 1993). Preparative-scale bioreduction of ketone **46** was demonstrated using cell suspensions of *H. polymorpha* SC 13865 and *Hansenula fabianii* SC 13894 in independent experiments. In both batches, a reaction yield of >80% and e.e.s of >94% were obtained for (2*R*,3*S*)-**45**. In a single-stage bioreduction process, cells of *H. fabianii* were grown in a 15-L fermentor for 48 h, then the bioreduction process was initiated by addition of 30 g of substrate and 250 g of glucose and continued for 72 h. A reaction yield of 88% with an e.e. of 95% was obtained for (2*R*,3*S*)-**45**.

21.2.11 Enzymatic Reduction of 1-(4-Fluorophenyl)4-[4-(5-Fluoro-2-Pyrimidinyl)1-Piperazinyl]-1-Butanone

The sigma receptor system in the brain and endocrine tissue has been the target for development of new class of antipsychotic drugs (Junien and Leonard, 1989; Ferris et al., 1991). Compound (*R*)-**47** (Fig. 21.11) is a sigma ligand and has a high affinity for sigma binding site and antipsychotic efficacy. The enantioselective microbial reduction process was developed for the conversion of ketone **48** to both enantiomers of alcohols. Various microorganisms screened for the enatioselective reduction of 1-(4-fluorophenyl)4-[4-(5-fluoro-2-pyrimidinyl)1-piperazinyl]-1butanone **48**. From this screen, *Mortierella ramanniana* ATCC 38191 was identified to predominantly reduce compound **48** to (*R*)-**47**, while *Pullularia pullulans* ATCC 16623 was identified to predominantly reduce compound **48** to (*S*)-**47**. A single-stage fermen-

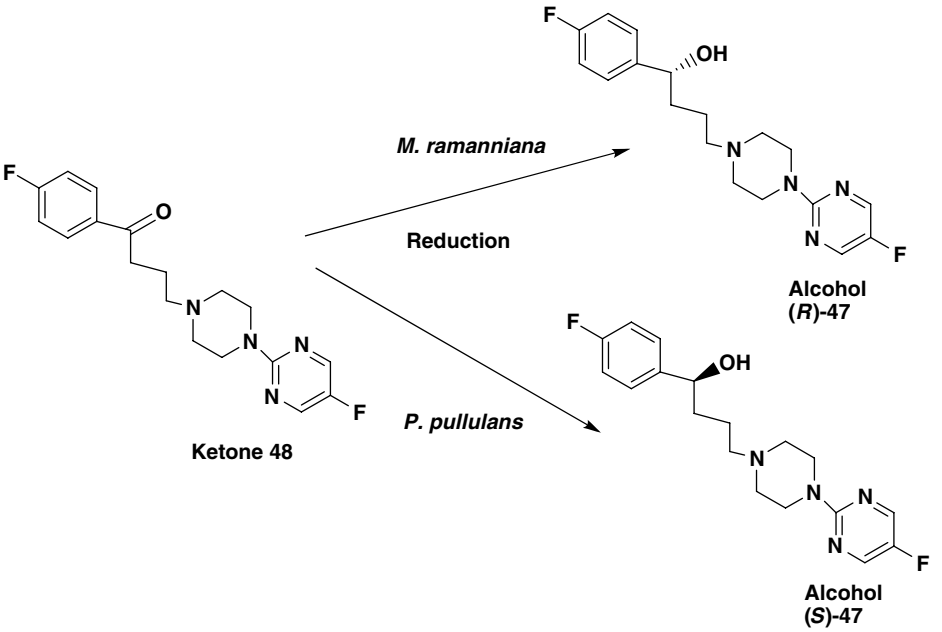

FIGURE 21.11 Enzymatic reduction of 1-(4-fluorophenyl)4-[4-(5-fluoro-2-pyrimidinyl)1-piperazinyl]-1-butanone.

tation/biotransformation process was developed. Cells of M. *ramanniana* were grown in a 20-L fermentor and after 40 h of growth period, the biotransformation process was initiated by addition of 40 g ketone **48** and 400 g glucose. The biotransformation process was completed in 24 h with a reaction yield of 100% and an e.e. of 98.9% for (*R*)-**47**. At the end of the biotransformation process, cells were removed by filtration and the product was recovered from the filtrate in overall 80% yield (Patel et al., 1993).

21.2.12 Microbial Reduction of 4-Benzyloxy-3-Methanesulfonylamino-2′-Bromoacetophenone

β3-Adrenergic receptors are found on the cell surface of both white and brown adipocytes and are responsible for lipolysis, thermogenesis, and relaxation of intestinal smooth muscle (Arch, 1997). Consequently, several research groups are engaged in developing selective β3 agonists for the treatment of gastrointestinal disorders, type II diabetes, and obesity (Bloom et al., 1989; Fisher et al., 1994). The biocatalytic syntheses of chiral intermediates required for the total synthesis of β3 receptor agonists **49** was investigated (Patel et al., 1998). The microbial reduction of 4-benzyloxy-3-methanesulfonylamino-2′-bromoacetophenone **50** (Fig. 21.12) to the corresponding (*R*)-alcohol **51** has been demonstrated (Patel et al., 1998) using *Sphingomonas paucimobilis* SC 16113. The growth of *S. paucimobilis* SC 16113 was carried out in a 750-L fermentor and cells (60 kg) harvested from the fermentor

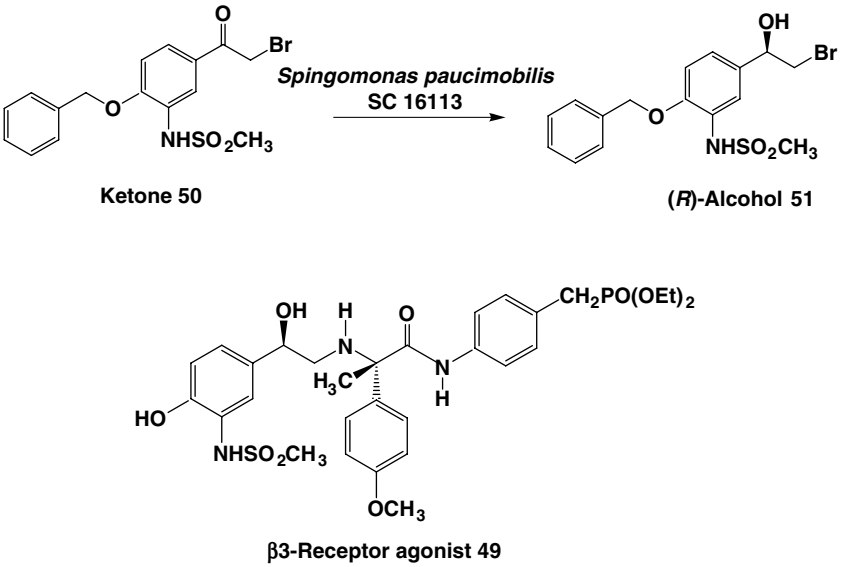

FIGURE 21.12 Microbial reduction of 4-benzyloxy-3-methanesulfonylamino-2′-bromoacetophenone.

were used to conduct the biotransformation in 10-L and 200-L preparative batches. The cells were suspended in 80 mM potassium phosphate buffer (pH 6.0) at 20% (wt/vol, wet cells) concentration and supplemented with compound **50** (2 g/L) and glucose (25 g/L) and the reduction was carried out at 37°C. In some batches, the fermentation broth was concentrated threefold by microfilteration and subsequently washed with buffer by diafilteration and used directly in the bioreduction process. In all the batches, reaction yields of >85% and e.e.s. of >98% were obtained. The isolation of alcohol **51** from the 200-L batch gave 320 g (80% yield) of product with an e.e. of 99.5%.

In an alternate process, frozen cells of *S. paucimobilis* SC 16113 were used with XAD-16 hydrophobic resin (50 g/L) adsorbed substrate at 10 g/L concentration. In this process, an average reaction yield of 85% and an e.e. of >99% were obtained for alcohol **51**. At the end of the biotransformation, the reaction mixture was filtered on a 100 mesh (150 μm) stainless steel screen, and the resin retained by the screen was washed with water. The product was then desorbed from the resin with acetonitrile and crystallized in an overall 75 M% yield and 99.8% e.e.

21.2.13 Enzymatic Preparation of (*R*)-1,3-Butanediol and (*R*)-4-Chloro-3-Hydroxybutonoate

(*R*)-1,3-butanediol **52** (Fig. 21.13) is a key starting material of azetidinone derivatives, which are key chiral intermediates for the synthesis of penem and carbapenem antibiotics (Iwata et al., 1990). From a microbial screen, the *Candida parapsilosis* strain IFO 1396 was identified which produced (*R*)-1,3-butanediol from the racemate.

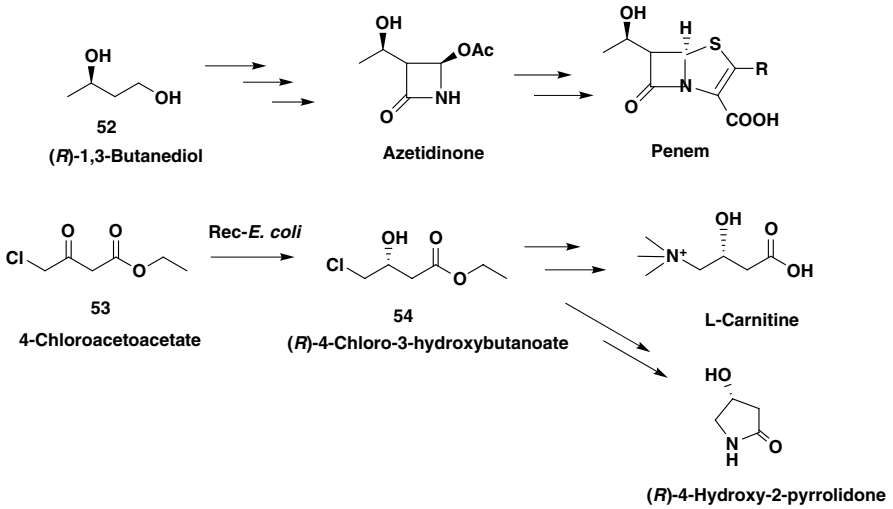

FIGURE 21.13 Enzymatic preparation of (*R*)-1,3-butanediol and (*R*)-4-chloro-3-hydroxybutonoate.

The (*S*)-1,3-butanediol oxidizing enzyme (CpSADH), which produced (*R*)-1,3-butanediol from the racemate, was cloned in *Escherichia coli*. The recombinant culture catalyzed the enantioselective oxidation of secondary alcohols and also catalyzed the asymmetric reduction of aromatic and aliphatic ketones to their corresponding (*S*)-secondary alcohols. Using the recombinant enzyme, (*R*)-1,3-butanediol was produced in 97% yield and 95% e.e. using 150 g/L input of the racemate. Recombinant enzyme (CpSADH) was also used for the reduction of ethyl 4-chloroacetoacetate **53** to produce (*R*)-4-chloro-3-hydroxybutonoate **54** in 95% yield and 99% e.e. using 36 g/L substrate input. Isopropanol was used to regenerate the NADH required for this reduction. (*R*)-4-Chloro-3-hydroxybutonoate is useful for the synthesis of L-carnitine and (*R*)-4-hydroxypyrrolidone (Matsuyama et al., 2002).

21.2.14 Enzymatic Preparation of Carbobenzoxy (Cbz)-(*S*)-Lysine

Ceranopril **55** is another ACE inhibitor (Karenewsky et al., 1988) which requires chiral intermediate carbobenzyloxy(Cbz)-L-oxylysine **56** (Fig. 21.14). A biotransformation process was developed by Hanson et al. (Hanson et al., 1992) to prepare the Cbz-L-oxylysine. *N*ε-carbobenzyloxy (Cbz)-L-lysine **57** was first converted to the corresponding keto acid **58** by oxidative deamination using cells of *Providencia alcalifaciens* SC 9036 which contained L-amino acid oxidase and catalase. The keto acid **58** was subsequently converted to **56** using L-2-hydroxy-isocaproate dehydrogenase (HIC) from *Lactobacillus confusus*. The NADH required for this reaction was regenerated using FDH from *C. boidinii*. The reaction yield of 95% with 98.5% e.e. was obtained in the overall process.

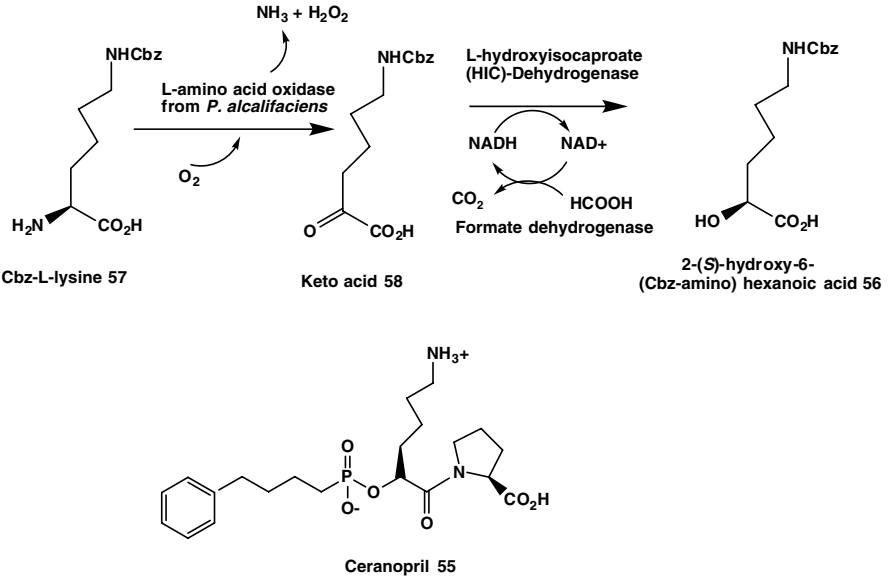

FIGURE 21.14 Enzymatic preparation of carbobenzoxy (Cbz)-(*S*)-lysine.

21.2.15 Enzymatic Synthesis of [(3*R*-cis)-1,3,4,5-Tetrahydro-3-Hydroxy-4-(4-Methoxyphenyl)-6-(Trifluromethyl)-2H-1-Benzazepin-2-one]

Diltiazem **59** (Fig. 21.15), a benzothiazepinone calcium channel blocking agent that inhibits influx of extracellular calcium through L-type voltage-operated calcium channels, has been widely used clinically in the treatment of hypertension and angina (Chaffman and Brogden, 1985). Since diltiazem has a relatively short duration of action (Kawai et al., 1981), an 8-chloroderivative recently has been introduced into the clinic as a more potent analog (Isshiki et al., 1988). Lack of extended duration of action and little information on structure–activity relationships in this class of compounds led Floyd et al. (1990) to prepare isosteric 1-benzazepin-2-ones; this led to identification of [(*cis*)-3-(acetoxy)-1-[2-(dimethylamino)ethyl]-1,3,4,5-tetrahydro-4-(4-methoxyphenyl)trifluoromethyl)-2H-1-benzazepin-2-one] **60** as a longer lasting and more potent antihypertensive agent. A key intermediate in the synthesis of this compound was [(3*R*-*cis*)-1,3,4,5-tetrahydro-3-hydroxy-4-(4-methoxyphenyl)-6-(trifluromethyl)-2H-1-benzazepin-2-one] **61**. A enantioselective microbial process (Fig. 21.15) was developed for the reduction of 4,5-dihydro-4-(4-methoxyphenyl)-6-(trifluoromethyl)-1H-1benzazepin-2,3-dione **62**, which exists predominantly in the achiral enol form in rapid equilibrium with the two enantiomeric keto forms. Reduction of **62** could give rise to the formation of four possible alcohol stereoisomers. Remarkably, conditions were found under which only the single alcohol isomer **61** was obtained. Among various cultures evaluated, microorganisms from the genera *Nocardia, Rhodococcus*, *Corynebacterium*, and *Arthobacter* reduced compound **61**

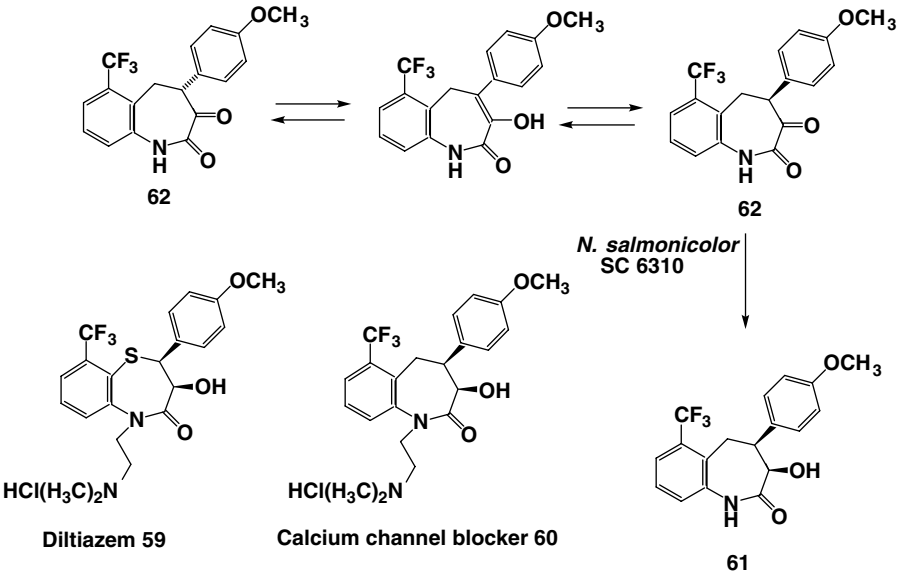

FIGURE 21.15 Enzymatic synthesis of [(3*R*-cis)-1,3,4,5-tetrahydro-3-hydroxy-4-(4-methoxyphenyl)-6-(trifluromethyl)-2H-1-benzazepin -2-one].

with 60–70% conversion yield at 1 g/L substrate concentration. The most effective culture, *Nocardia salmonicolor* SC 6310, catalyzed the bioconversion of **62** to **61** in 96% reaction yield with 99.8% e.e. at 2 g/L substrate concentration. A preparative-scale fermentation process for growth of *N. salmonicolor* and a bioreduction process using cell suspensions of the organism were demonstrated (Patel et al., 1991).

21.2.16 Enzymatic Preparation of (*R*)-4-Cyano-3-Hydroxybutyrate

An enzymatic process has been developed for the preparation of 4-halo-3-hydroxybutyric acid derivatives by ketoreductase-catalyzed conversion of 4-halo-3-ketobutyric acid derivatives (Davis et al., 2004). The genes encoding halohydrin dehydrogenase from *Agrobacterium tumefaciens*, ketoreductase from *Candida magnoliae*, glucose dehydrogenase from *Bacillus subtilis*, and FDH from *Candida boidinii* were separately cloned into *E. coli* BL21. Each enzyme was then produced by fermentation, isolated and characterized. Then ethyl (*R*)-4-cyano-3-hydroxybutyrate (**63**) (Fig. 21.16) was prepared from ethyl 4-chloroacetoacetate (**64**) by the following procedure: Ethyl 4-chloroacetoacetate **64** was incubated at pH 7.0 with ketoreductase, glucose dehydrogenase, and NADP for 40 h to produce ethyl (*S*)-chloro-3-hydroxybutyrate (**65**). The ethyl (*S*)-chloro-3-hydroxybutyrate **65** was extracted with ethyl acetate, dried, filtered, and concentrated to yield ~97% pure ester. The dried ethyl (*S*)-chloro-3-hydroxybutyrate (**64**) was dissolved in phosphate buffer and mixed with halohydrin dehalogenase and sodium cyanide at pH 8.0. After 57 h, essentially pure

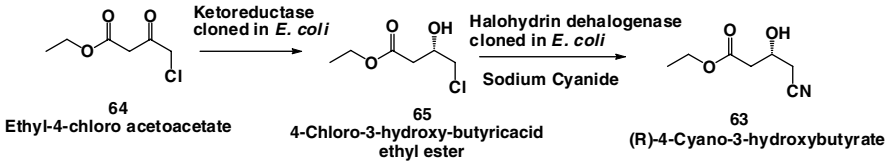

FIGURE 21.16 Enzymatic preparation of (*R*)-4-cyano-3-hydroxybutyrate.

(*R*)-4-cyano-3-hydroxybutyrate (**63**), an intermediate used in many HMG-CoA reductase inhibitors syntheses, was recovered (Davis et al., 2004).

21.3 DEHYDROGENASE-CATALYZED REDUCTIVE AMINATIONS

21.3.1 Enzymatic Synthesis of (*S*)-β-Hydroxyvaline

The preparation of enantiomerically pure non-natural (*S*)-amino acids is of wide importance due to pharmaceutical applications thereof. Reductive amination of ketoacids using amino acid dehydrogenases has long been known to be a useful method for the synthesis of natural and unnatural amino acids (Wandrey et al., 1981; Brunhuber and Blanchard, 1994; Bommarius, 2002).

(*S*)-β-hydroxyvaline **66** (Fig. 21.17), is a key chiral intermediate required for the total synthesis of orally active monobactam (Hanson et al., 1990), Tigemonam **67**.

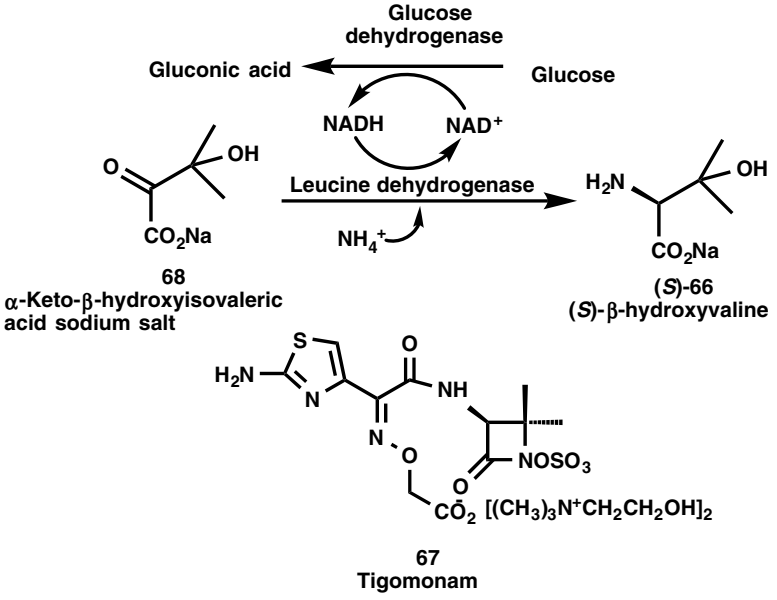

FIGURE 21.17 Enzymatic synthesis of (*S*)-β-hydroxyvaline.

The synthesis of (*S*)-β-hydroxyvaline **66** from α-keto-β-hydroxyisovalerate **68** by reductive amination using leucine dehydrogenase from *Bacillus sphaericus* ATCC 4525 has been demonstrated (Hanson et al., 1990). The NADH required for this reaction was regenerated by either FDH from *C. boidinii* or glucose dehydrogenase from *Bacillus megaterium*. The required substrate **68** was generated either from α-keto-β-bromoisovalerate or its ethyl esters by hydrolysis with sodium hydroxide *in situ*. In this process, an overall reaction yield of 98% and an e.e. of 99.8% were obtained for the L-β-hydroxyvaline **66**.

21.3.2 Enzymatic Synthesis of (*S*)-6-Hydroxynorleucine

Vanlev **69** (Fig. 21.18) is an antihypertensive drug that acts by inhibiting angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP) (Robl et al., 1997). (*S*)-6-Hydroxynorleucine **70** (Fig. 21.18) is a key intermediate in the synthesis of Vanlev. The synthesis and complete conversion of 2-keto-6-hydroxyhexanoic acid **71** to (*S*)-6-hydroxynorleucine **70** was demonstrated by reductive amination using beef liver glutamate dehydrogenase (Hanson et al., 1999). As depicted, compound **71**, in equilibrium with 2-hydroxytetrahydropyran-2-carboxylic acid sodium salt **72**, was converted to **70**. The reaction requires ammonia and NADH. NAD produced during the reaction was recycled to NADH by the oxidation of glucose to gluconic acid using glucose dehydrogenase from *B. megaterium*. The reaction was complete in about 3 h at 100 g/L substrate input with a reaction yields of 92% and e.e. of 99.8% for (*S*)-6-hydroxynorleucine. The synthesis and isolation of keto acid **71** required several steps.

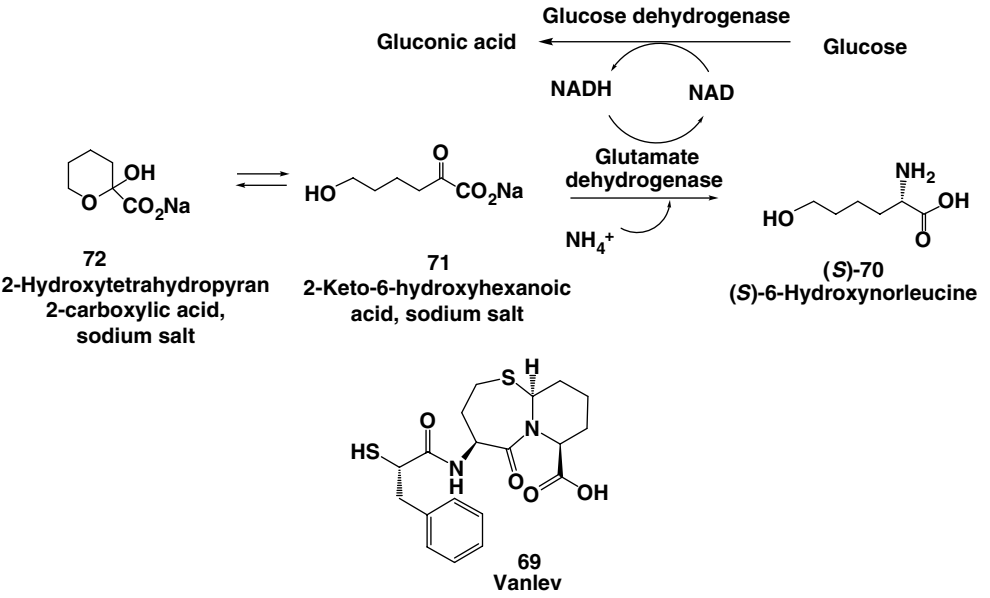

FIGURE 21.18 Enzymatic synthesis of (*S*)-6-hydroxynorleucine from keto acid.

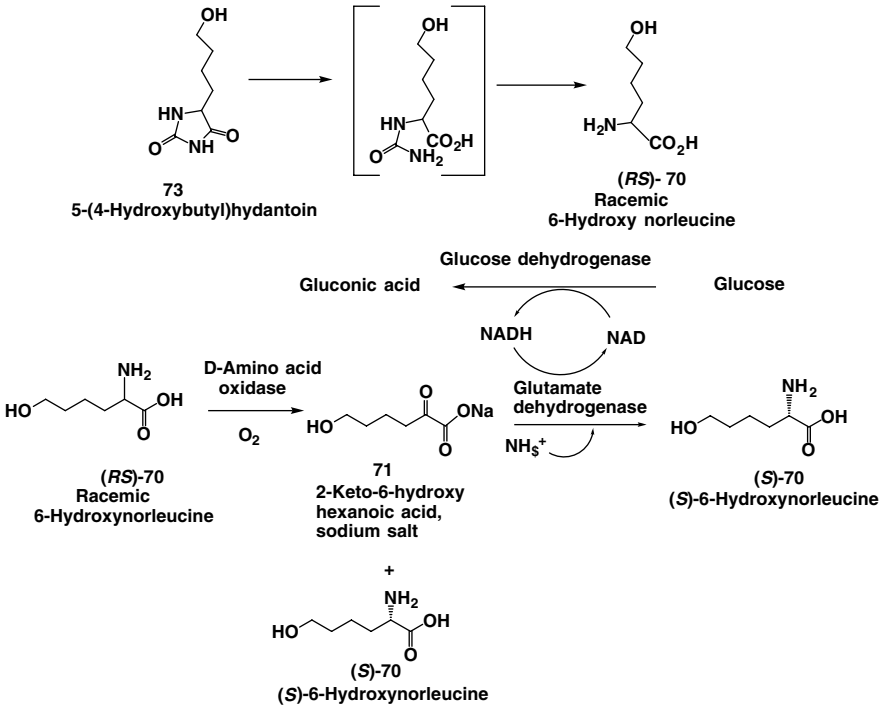

FIGURE 21.19 Enzymatic synthesis of (*S*)-6-hydroxynorleucine from hydratoin.

In a second, more convenient process the ketoacid was prepared by the treatment of racemic 6-hydroxy norleucine **70** [produced by hydrolysis of 5-(4-hydroxybutyl) hydantoin **73**] with (*R*)-amino acid oxidase (Fig. 21.19) After the e.e. of the unreacted (*S*)-6-hydroxynorleucine had risen to 99.8%, the reductive amination procedure was used to convert the mixture containing the 2-keto-6-hydroxyhexanoic acid entirely to (*S*)-6-hydroxynorleucine in 97% yield with 99.8% e.e. from racemic 6-hydroxynorleucine at 100 g/L substrate input (Hanson et al., 1999). The (*S*)-6-hydroxynorleucine prepared by the enzymatic process was converted chemically to Valev **69** (Patel, 2001).

21.3.3 Enzymatic Synthesis of Allysine Ethylene Acetal

(*S*)-2-Amino-5-(1,3-dioxolan-2-yl)-pentanoic acid [(*S*)-allysine ethylene acetal] (**74**, Fig. 21.20) is one of three building blocks used in an alternative synthesis of Vanlev **69**. Synthesis of **74** was demonstrated by reductive amination of ketoacid acetal **75** using phenylalanine dehydrogenase (PDH) from *Thermoactinomyces intermedius* (Hanson et al., 2000). The reaction required ammonia and NADH; NAD produced during the reaction was recycled to NADH by the oxidation of formate to CO_2 using FDH. *T. intermedius* PDH was cloned and expressed in *E. coli* and the recombinant culture was used as a source of PDH. *P. pastoris* (Schütte et al., 1976; Hou et al., 1982)

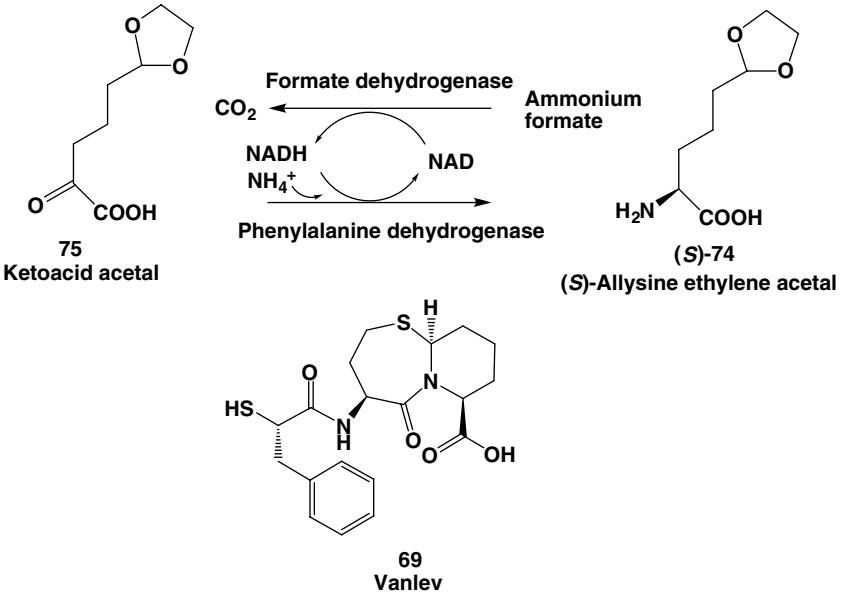

FIGURE 21.20 Enzymatic synthesis of (*S*)-allysine ethylene acetal.

grown on methanol are also useful sources of FDH. Expression of *T. intermedius* PDH in *P. pastoris*, inducible by methanol, allowed generation of both enzymes in a single fermentation. A total of 197 kg of **74** was produced in three 1600-L batches using a 5% concentration of substrate **75** with an average yield of 91M% and e.e. >98% (Hanson et al., 2000). (*S*)-allysine ethylene acetal was converted to Vanlev **69** (Patel, 2001).

21.3.4 Enzymatic Reductive Amination of 2-(3-Hydroxy-1-Adamantyl)-2-Oxoethanoic Acid

Dipeptidyl peptidase 4 (DPP-4) is a ubiquitous proline-specific serine protease responsible for the rapid inactivation of incretins, including glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide. To alleviate the inactivation of GLP-1, inhibitors of DPP-IV are being evaluated for their ability to provide improved control of blood glucose for diabetics (Gallwitz, 2005; Sinclair and Drucker, 2005; Augeri et al., 2005). Januvia developed by Merck is a marketed DPP4 Inhibitor (Sinclair, 2005).

Saxagliptin **76** (Gallwitz, 2005; Augeri et al., 2005) (Fig. 21.21), a DPP-IV inhibitor under development by Bristol-Myers Squibb, requires (*S*)-*N*-boc-3-hydroxyadamantylglycine **77** as an intermediate. A process for conversion of the keto acids **78** to the corresponding amino acid **79** using (*S*)-amino acid dehydrogenases was developed. A modified form of a recombinant PDH cloned from *T. intermedius* and expressed in *Pichia pastoris* or *Escherichia coli* was used for this process. NAD

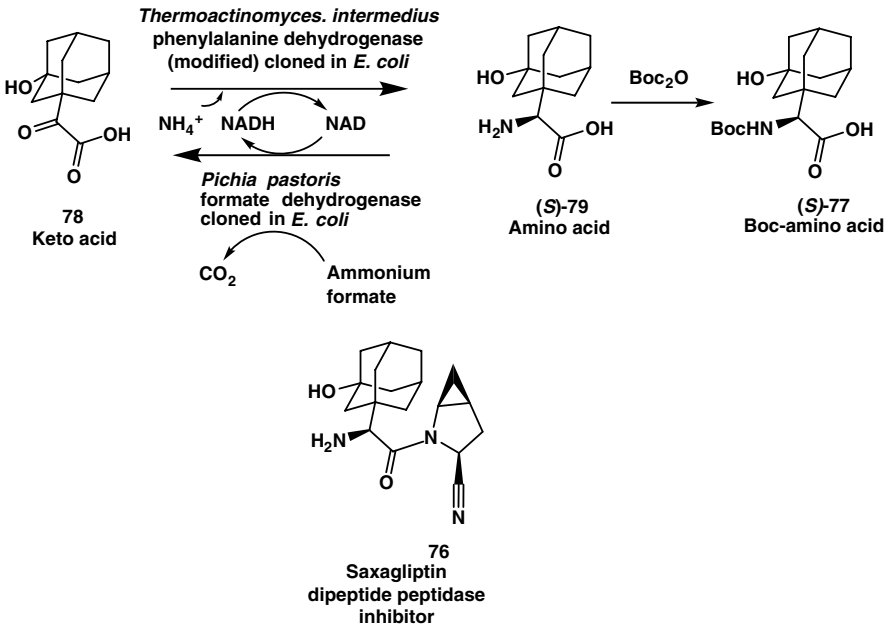

FIGURE 21.21 Enzymatic synthesis of (*S*)-3-hydroxyadmantylglycine.

produced during the reaction was recycled to NADH using FDH. The modified PDH contains two amino acid changes at the C-terminus and a 12 amino acid extension of the C-terminus (Vu et al., 2004; Hanson et al., 2007).

Production of multi-kg batches was originally carried out with extracts of *P. pastoris* expressing the modified PDH from *T. intermedius* and endogenous FDH. The reductive amination process was further scaled up using a preparation of the two enzymes expressed in single recombinant *E. coli*. The amino acid **79** was directly protected as its boc derivative without isolation to afford an intermediate. Yields before isolation were close to 98% with 100% e.e. (Vu et al., 2004; Hanson et al., 2007).

Reductive amination was also conducted using cell extracts from *E. coli* strain SC16496 expressing PDHmod and cloned FDH from *P. pastoris*. Cell extracts after polyethyleneamine treatment, clarification, and concentration were used to complete the reaction in 30 h with >96% yield and >99.9% e.e. of product **79**. This process has now been used to prepare several hundred kg of boc-protected amino acid **77** to support the development of Saxagliptin.

Use of PDH in the production of (*S*)-phenylalanine and other related (*S*)-amino acids was demonstrated by Asano (2005). PDH was engineered by directed evolution to use in the organic solvent to carry out enzymatic reactions in biphasic and homogeneous systems was developed for the preparation of (*S*)-amino acids (Cainelli et al., 2005).

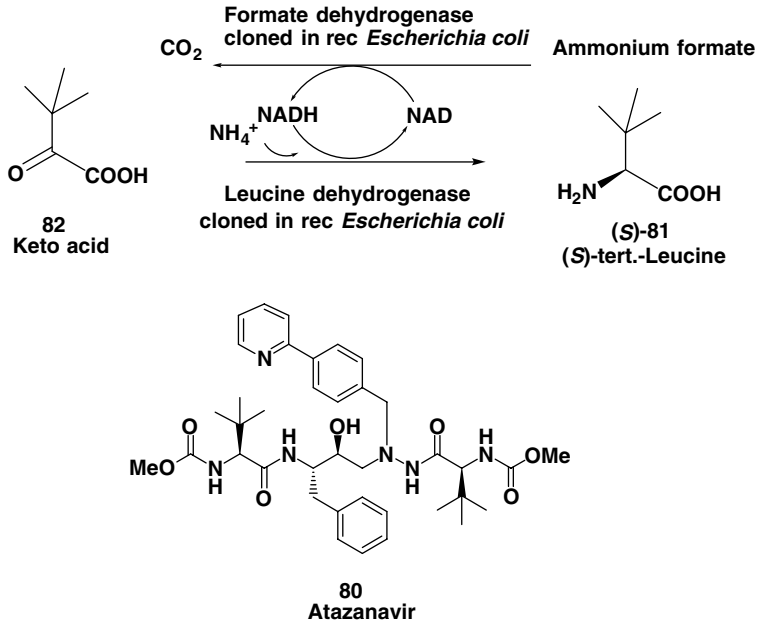

FIGURE 21.22 Enzymatic synthesis of (*S*)-tertiary leucine.

21.3.5 Enzymatic Synthesis of (*S*)-Tertiary-Leucine

Atazanavir **80** is an acyclic aza-peptidomimetic, a potent HIV protease inhibitor (Bold et al., 1998; Robinson et al., 2000). Synthesis of atazanavir required (*S*)-tertiary leucine (**81**, Fig. 21.22). An enzymatic reductive amination of ketoacid **82** to amino acid **81** by recombinant *E. coli* expressing leucine dehydrogenase from *T. intermedius* has been demonstrated. The reaction required ammonia and NADH as a cofactor. NAD produced during the reaction was converted back to NADH using recombinant *E. coli* expressing FDH from *P. pastoris*. A reaction yield of >95% with an e.e. of >99.5% was obtained for **81** at 100 g/L substrate input (R. Hanson, S. Goldberg, R. Patel, unpublished results). Leucine dehydrogenase from *Bacillus* strain has also been cloned and expressed and used in reductive amination process (Stoyan et al., 1997; Menzel et al., 2004).

21.3.6 Enzymatic Synthesis of (*S*)-Neopentylglycine

A "second-generation process" for the enantioselective synthesis of (*S*)-neopentylglycine **83** (Fig. 21.23) has been developed by Groeger et al. (2006). Recombinant whole cell containing leucine dehydrogenase and FDH was used in the reductive amination of the corresponding α-keto acid **84**. The desired (*S*)-neopentylglycine was obtained with >95% conversion and a high enantioselectivity of >99% e.e. at

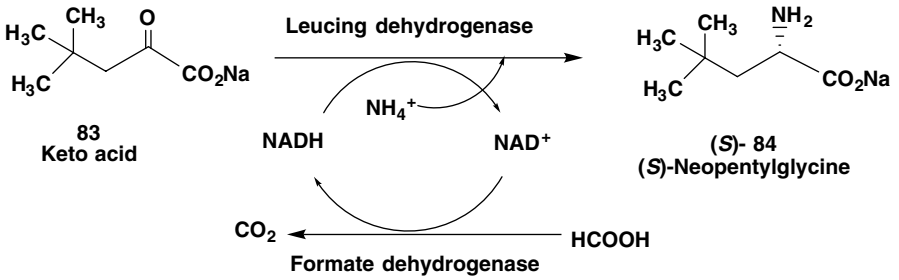

FIGURE 21.23 Enzymatic synthesis of (*S*)-neopentylglycine.

substrate concentrations of up to 88 g/L. Spiroheterocyclic compounds (morpholine-4-carboxylic acid amides of heterocyclic cyclohexylalanine and neopentylglycine derivatives and their analogs) are useful as reversible inhibitors of cysteine proteases such as cathepsin S useful in the treatment of a variety of autoimmune diseases (Emmanuel et al., 2001).

21.3.7 Enzymatic Synthesis of *N*-Alkyl-(*S*)-Amino Acids

A novel *N*-methyl-(*S*)-amino acid dehydrogenase activity in various bacterial strains, such as *P. putida* and *Bacillus alvei* was reported (Mihara et al., 2005). This enzyme was cloned from *P. putida* ATCC12633 into *E. coli* and used in the synthesis of *N*-alkyl-(*S*)-amino acids from the corresponding α-oxo acids (e.g., pyruvate, phenylpyruvate, and hydroxypyruvate) and alkylamines (e.g. methylamine, ethylamine, and propylamine). Ammonia was used as an amino donor and the NADPH was more than 300 times more efficient than NADH as a hydrogen donor in the enzymatic reductive amination.

Kato et al. discovered an opine dehydrogenase (ODH) from *Arthrobacter* sp. that used pyruvate as a amino acceptor and was active toward short-chain aliphatic (*S*)-amino acids and those substituted with acyloxy, phosphonooxy, and halogen groups (Kato et al., 1996). The enzyme was named *N*-[1-(*R*)-(carboxyl) ethyl]-(*S*)-norvaline: NAD+ oxidoreductase (*S*-norvaline forming). Other substrates for the enzyme were 3-aminobutyric acid and (*S*)-phenylalaninol. Optically pure opine-type secondary amine carboxylic acids were synthesized from amino acids and their analogs such as (S)-Met, (*S*)-Ile, (*S*)-Leu, (*S*)-Val, (*S*)-Phe, (*S*)-Ala, (*S*)-Thr, (*S*)-Ser, and (*S*)-phenylalaninol, and α-keto acids such as glyoxylate, pyruvate, and 2-oxobutyrate using the enzyme. The regeneration of NADH was carried out by FDH from *Moraxella* sp.C-1. The absolute configuration of the asymmetric center of the opines was of the (*R*) stereochemistry with >99.9% e.e. One-pot synthesis of *N*-[1-(*R*)-(carboxyl)ethyl]-(S)-phenylalanine **85** from phenylpyruvate **86** and pyruvate (Fig. 21.24) by using ODH, FDH, and PDH has been described (Kato et al., 1996).

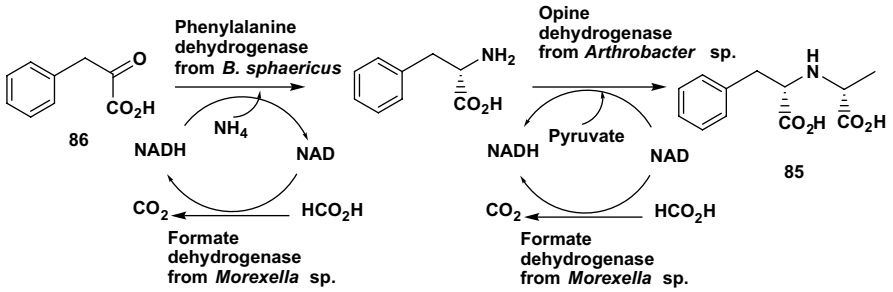

FIGURE 21.24 Enzymatic synthesis of *N*-[1-(*R*)-(carboxyl)ethyl]-(S)-phenylalanine.

21.3.8 Preparation of (*R*)-Amino Acid

(*R*)-Amino acids are increasingly becoming important building blocks in the production of pharmaceuticals and fine chemicals, and as chiral directing auxiliaries and chiral synthons in organic synthesis. Applications of (*R*)-amino acids include their use as key components in β-lactam antibiotics, fertility drugs, and anticoagulants (Merviel et al., 2005; Hauptmann, 2002).

Two important (*R*)-amino acids used in semisynthetic antibiotics, (*R*)-phenylglycine (ampicillin), and *p*-hydroxy-(*R*)-phenylglycine (amoxicillin) are currently produced on a tons scale per year. In addition, there are more than 20 (*R*)-amino acids currently produced at pilot- or full-scale levels. In many of these cases, the (*R*)-enantiomer is not only frequently more potent than the corresponding (*S*)-enantiomer but also often more stable *in vivo* against enzyme degradation (Straathof et al., 2002; Bommarius et al., 1998).

Using both rational and random mutagenesis, Rozzell and Novick (Vedha-Peters et al., 2006) have created the broad substrate range, nicotinamide-cofactor dependent, and highly stereoselective (*R*)-amino acid dehydrogenase. This new enzyme is capable of producing (*R*)-amino acids via the reductive amination of the corresponding 2-keto acid with ammonia. This biocatalyst was the result of three rounds of mutagenesis and screening performed on the enzyme *meso*-diaminopimelate (*R*)-dehydrogenase from *Corynebacterium glutamicum*. The first round targeted the active site of the wild-type enzyme and produced mutants that were no longer strictly dependent on the native substrate. The second and third rounds produced mutants that had an increased substrate range including straight- and branched-aliphatic amino acids and aromatic amino acids. The very high selectivity toward the (*R*)-enantiomer (95–>99% e.e.) was shown to be preserved for three rounds of mutagenesis and screening (Vedha-Peters et al., 2006). This new enzyme was active against a variety of amino acids and could complement and improve upon current methods for (*R*)-amino acid synthesis. The synthesis of (*R*)-cyclohexylalanine **87** (Fig. 21.25) was developed by reductive amination of cyclohexylpyruvate **88** to yield (*R*)-**87** in 98% yield and >99% e.e. (*R*)-**87** is a potential chiral intermediate for the synthesis of thrombin inhibitor Inogatran **89** (Gustafsson et al., 1996).

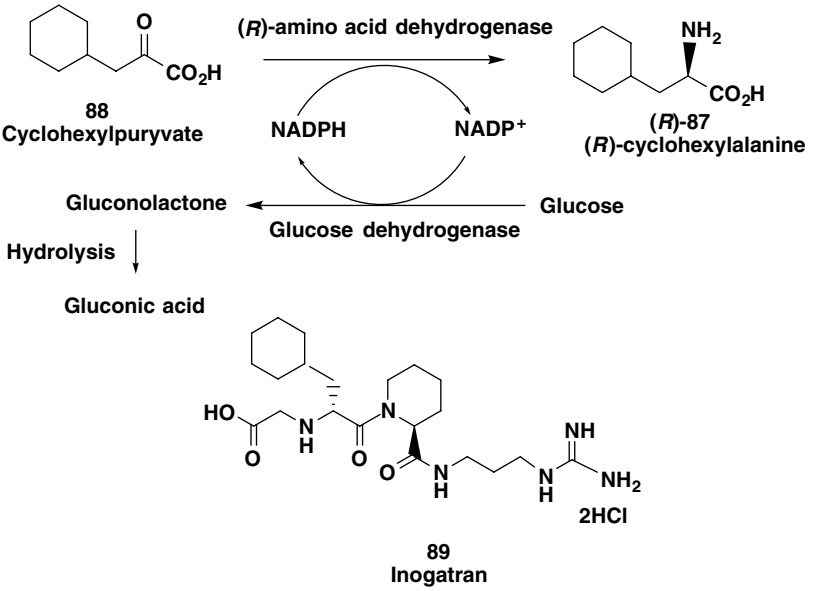

FIGURE 21.25 Enzymatic synthesis of (*R*)-cyclohexylalanine.

21.4 CONCLUSIONS

The production of single enantiomers of drug intermediates is becoming increasingly important in the pharmaceutical industry. Biocatalysis provides organic chemists an alternate opportunity to prepare pharmaceutically important chiral compounds. The examples presented in this review are only from a few selected articles that demonstrate the interest of process scientists for the use of biotransformations in the preparation of chiral compounds. Different types of biocatalytic reactions are capable of generating a wide variety of chiral compounds useful in the development of drugs. Hydrolytic enzymes such as lipases, esterases, proteases, dehalogenases, acylases, amidases, nitrilases, epoxide hydrolases, and decarboxylases are used for the resolution of a variety of racemic compounds and in the asymmetric synthesis of enantiomerically enriched chiral compounds. Dehydrogenases and aminotransferases have been successfully used along with cofactors and cofactor-regenerating enzymes for the synthesis of chiral alcohols, aminoalcohols, amino acids, and amines.

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22

ENGINEERING OF BACTERIAL CYTOCHROME P450 MONOOXYGENASE AS BIOCATALYSTS FOR CHEMICAL SYNTHESIS AND ENVIRONMENTAL BIOREMEDIATION

JUN OGAWA*, QING-SHAN LI AND SAKAYU SHIMIZU

*Research Division of Microbial Sciences, Kyoto University, Kitashirakwa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan

Divison of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakwa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan

VLADA URLACHER AND ROLF D. SCHMID

Institut für Technische Biochemie, Universität Stuttgart, Allmandring 31, D-70459 Stuttgart, Germany

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22.1 INTRODUCTION

Cytochrome P450s act on the inactive carbon–hydrogen bonds of alkanes, fatty acids, terpenes and steroids, and some exhibit high regio- and stereoselective monooxygenation activity. Therefore, cytochrome P450s are expected to be potential catalysts for fine chemical synthesis. However, they are intrinsically not very active, exhibit poor stability, and require associated factors such as ferredoxin, flavin mononucleotide reductase, and NAD(P)H for electron transfer. Bacterial P450s are relatively stable and highly active, and can often be prepared in large amounts using recombinant expression systems. Cytochrome P450 BM-3 from *Bacillus megaterium* is one of the best-studied bacterial P450s, and it is a natural fusion of a P450 and a mammalian-like diflavin NADPH-P450 reductase (Narhi and Fulco, 1986; Ruettinger et al., 1989). Its properties are closer to application requirements than those of other P450s. However, this P450 still has some problems for practical purposes, such as cofactor (NADPH) requirement and a well-defined substrate specificity for long-chain unsaturated fatty acids (Narhi and Fulco, 1986; Ruettinger et al., 1989; Boddupalli et al., 1990; Graham-Lorence et al., 1997; Li and Poulos, 1999).

The need for electron-donating cofactor NADPH is one of the main barriers prohibiting the practical use of cytochrome P450 BM-3. Peroxide shunt reactions of P450s have attracted much attention recently mainly due to their attractive potential of substituting NADPH by cofactors. In this chapter, we describe the creation of the cytochrome P450 BM-3 mutants with improved peroxide shunt reaction. The mutant described here showed H_2O_2-supported substrate hydroxylation activity (Li et al., 2001a).

To obtain activity toward substrates other than long-chain fatty acids, protein engineering of cytochrome P450 BM-3 has been widely investigated (Li et al., 2000; Ost et al., 2000; Cirino and Arnold, 2002; Li et al., 2001b, 2001c; Sulistyaningdyah et al., 2004; Sulistyaningdyah et al., 2005; Li et al., 2005). In particular, the effect of phenylalanine 87 (Phe87) on the substrate specificity of P450 BM-3 has received much attention (Graham-Lorence et al., 1997; Li and Poulos, 1999; Li et al., 2001a; Li et al., 2000; Li et al., 2001b, 2001c; Oliver et al., 1997; Li et al., 2001d). The structure of cytochrome P450 BM-3 heme domain revealed that the phenyl side chain of Phe87 extends into the heme pocket and is positioned above the porphyrin plane (Li and Poulos, 1997). Site-directed mutagenesis studies have proven that Phe87 plays a key role in the control of the selectivity of reactions (Graham-Lorence et al., 1997; Oliver et al., 1997). For example, the Phe87Val mutation (F87V) significantly affects the stereoselectivity of arachidonic acid epoxidation. It has also been reported that F87V shows much higher activity toward indole than the wild-type P450 BM-3 (Li et al., 2000; Li et al., 2005).

In this chapter, we also describe the creation of cytochrome P450 BM-3 mutants acting on polycyclic aromatic hydrocarbons (PAHs), arylalkyl compounds, and phenolic compounds. These mutants are expected to be novel catalysts for environmental bioremediation and chemical synthesis.

22.2 H_2O_2-DEPENDENT SUBSTRATE HYDROXYLATION ACTIVITY AND H_2O_2 INACTIVATION OF MUTANT CYTOCHROME P450 BM-3

Cofactor (NADPH) requirement is one of the problems of cytochrome P450 BM-3 for practical use. Peroxidase shunt reactions of cytochrome P450 BM-3 have attracted much attentions, due to their potentials of substituting NADPH. But, there have been few reports of relatively high cytochrome P450 BM-3 activities supported by a hydroperoxide. We found that Phe87 played an important role in H_2O_2-supported hydroxylation (Li et al., 2001a).

Replacement of Phe87 with Ala or Gly (mutant F87A or F87G) greatly increased the H_2O_2-supported substrate hydroxylation activity of cytochrome P450 BM-3, whose original H_2O_2-supported activity is hardly detectable. On the other hand, replacement of Phe87 with Val (mutant F87V) did not. In the oxidation of *p*-nitrophenoxydodecanoic acid (12-pNCA), the turnover numbers of the mutant F87A in the presence of NADPH and O_2 or H_2O_2 were 439 and 162 nmol/min/nmol, respectively. The H_2O_2-supported F87A hydroxylation activity was further confirmed with a free fatty acid, myristate, as substrate. A product of myristate hydroxylation, ω-hydroxymyristate, was obviously observed in the reaction with H_2O_2 by mutant F87A (Fig. 22.1B) as well as in the reaction with NADPH by wild-type cytochrome P450 BM-3 (WT) (Fig. 22.1A). However, only a trace amount of the product was found if F87A was replaced by WT in the H_2O_2-supported reaction (Fig. 22.1C). Moreover, the stability of F87A in H_2O_2 solutions also largely increased. The order of the stability of WT, F87A, and their substrate (12-pNCA)-binding complexes in H_2O_2 solutions listed from high to low was F87A, WT, F87A substrate-binding complex, and WT substrate-binding complex. We proposed that the free space size in the vicinity of the heme iron significantly influences cytochrome P450 BM-3 H_2O_2-supported activity and H_2O_2 inactivation.

22.3 ENGINEERING CYTOCHROME P450 BM-3 FOR OXIDATION OF POLYCYCLIC AROMATIC HYDROCARBONS

PAHs are ubiquitously present in the environment and have harmful effects on humans (Cerniglia, 1992; Johnson et al., 1985). Recently, the potentials of P450 enzymes to transform PAHs have been evaluated to develop novel strategies in environmental bioremediation (Harford-Cross et al., 2000; Joo et al., 1999; Shou et al., 1996). Hydroxylation of PAHs by P450 enables the PAHs being more biodegradable. Hydroxylated PAHs are more water-soluble and are the substrates for oxidases such as peroxidase and laccase, which decompose phenolic compounds.

Cytochrome P450 BM-3 has been engineered into a catalyst for the oxidation of PAHs (Li et al., 2001b). The activities of a triple mutant with replacements of Ala74 with Gly, Phe87 with Val, and Leu188 with Gln (A74G/F87V/L188Q) toward naphthalene, fluorene, acenaphthene, acenaphthylene, and 9-methylanthracene were

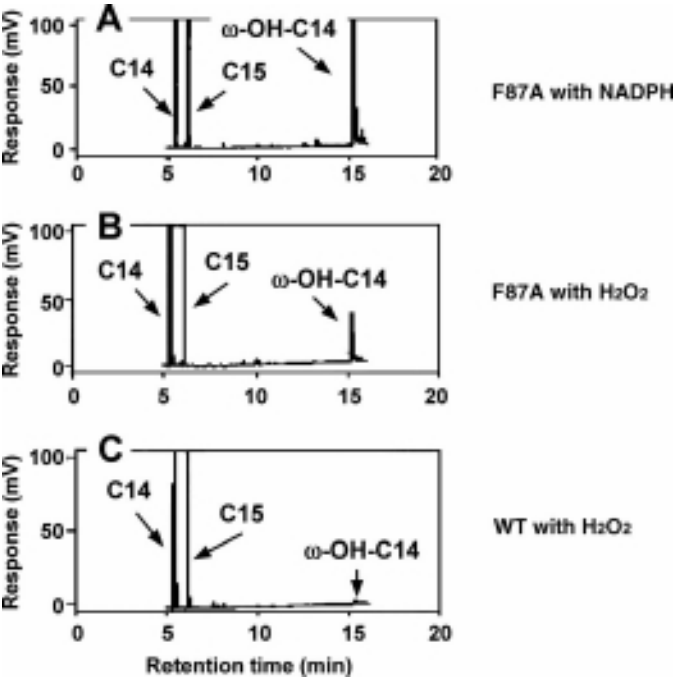


FIGURE 22.1 Gas–liquid chromatography analysis of the products formed in NADPH-supported F87A hydroxylation of myristate (A), H_2O_2-supported F87A hydroxylation of myristate (B), and H_2O_2-supported cytochrome P450 BM-3 wild-type (WT) hydroxylation of myristate (C). C14, myristate; C15, pentadecanoic acid (internal standard); ω-OH-C14, ω-hydroxymyristate.

160, 53, 109, 287, and 22 nmol/min/nmol P450, respectively (Table 22.1). Compared with the activities of the wild type toward these PAHs, those of the mutant were improved by up to four orders of magnitude. The coupling efficiencies of the mutant toward naphthalene, fluorene, acenaphthene, acenaphthylene, and 9-methylanthracene were 11, 26, 5.4, 15, and 3.2%, respectively, which were also improved several to hundreds fold. The products derived from PAHs by the mutant are summarized in

TABLE 22.1 The Rates of Oxidation of Polycyclic Aromatic Hydrocarbons Catalyzed by the Wild-Type Cytochrome P450 BM-3 (WT) and Mutants

	Substrate Oxidation Rate (nmol/min/nmol P450)			
Substrate	WT	F87V	F87V/L188Q	A74G/F87V/L188Q
Naphthalene	0.76	111	115	160
Fluorene	0.014	2.8	22.7	52.8
Acenaphthene	0.0028	2.2	76.2	109
Acenaphthylene	0.12	71.3	149	287
9-Methylanthracene	0.0019	0.71	9.2	21.8

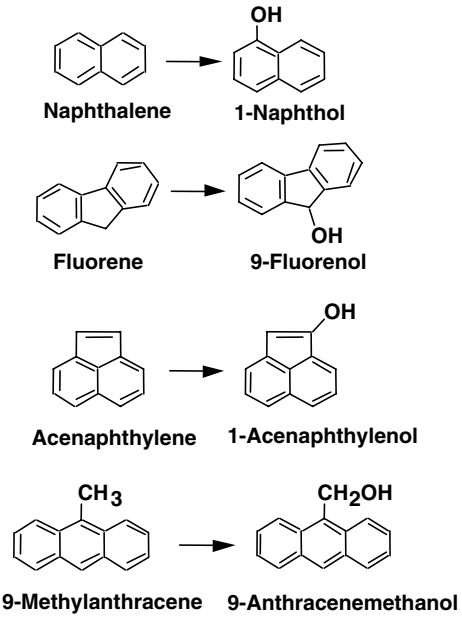


FIGURE 22.2 Proposed reactions of PAHs hydroxylation catalyzed by a cytochrome P450 BM-3 mutant F87V.

Figure 22.2. The high activities of the mutant toward PAHs indicate the potential of engineering cytochrome P450 BM-3 for the biodegradation of these compounds in the environment (Li et al., 2001b).

22.4 METABOLISM OF POLYCHLORINATED DIBENZO-*p*-DIOXINS BY CYTOCHROME P450 BM-3 AND ITS MUTANT

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are environmental contaminants with extreme toxicity of some components. The toxicity of PCDDs is mainly caused by chlorination at lateral positions (C-2, 3, 7, and 8), and 2,3,7,8-tetrachlorodibenzo-dioxin (2,3,7,8-tetraCDD) is known to be the most toxic one (Van Leeuwen et al., 2000). Potential reactions for bioremediation of PCDDs catalyzed by mammalian cytochrome P450s (CYPs) have been reported. The recombinant yeast microsomal fraction containing each of rat or human CYP1 family members (belonging to the CYP group I) and yeast NADPH-P450 reductase metabolize PCDDs via multiple reactions such as hydroxylation at an unsubstituted position, hydroxylation with migration of the chloride substituent, hydroxylation with elimination of the chloride substituent, and opening of the dioxin ring (Inouye et al., 2002; Sakaki et al., 2002; Shinkyo et al., 2003). All of these reactions appear to lead to the detoxication of PCDDs.

TABLE 22.2 Activities of the Wild-Type Cytochrome P450 BM-3 (WT) and its Mutant, A74G/F87V/L188Q, in Polychlorinated Dibenzo-*p*-Dioxin Decomposition

	Activity ($\times 10^{-3}$ nmol/min/nmol P450)		
Substrate	WT	A74G/F87V/L188Q	Ratio (A74G/F87V/L188Q/WT)
1-MCDD	43.7	525	12.0
2,3-DCDD	6.48	12.3	1.9
2,3,7-triCDD	0.08	0.86	10.7

Note: 1-MCDD, 1-monochloro-dibenzo-*p*-dioxin; 2,3-DCDD, 2,3-dichloro-dibenzo-*p*-dioxin; 2,3,7-triCDD, 2,3,7-trichloro-dibenzo-*p*-dioxin.

The metabolism of PCDDs by cytochrome P450 BM-3 WT and its triple mutant A74G/F87V/L188Q was examined (Sulistyaningdyah et al., 2004). Both purified enzymes metabolized 1-monochloro-, 2,3-dichloro-, and 2,3,7-trichloro-dibenzo-*p*-dioxin, but not 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin. The mutant A74G/F87V/L188Q showed 2–12 times higher activity than WT toward PCDDs (Table 22.2). The products were less toxic derivatives with lower than 10% toxicity of the original compounds, and hydroxylated at an unsubstituted position and/or showing migration of the chloride (Fig. 22.3) (Sulistyaningdyah et al., 2004). Although the activity of A74G/F87V/L188Q toward PCDDs was lower than those of mammalian P450s, the results indicated the possibilities of constructing a mutant bacterial P450 with high catalytic activity against PCDDs, and of using bacterial P450s for the bioremediation of soil contaminated by PCDDs.

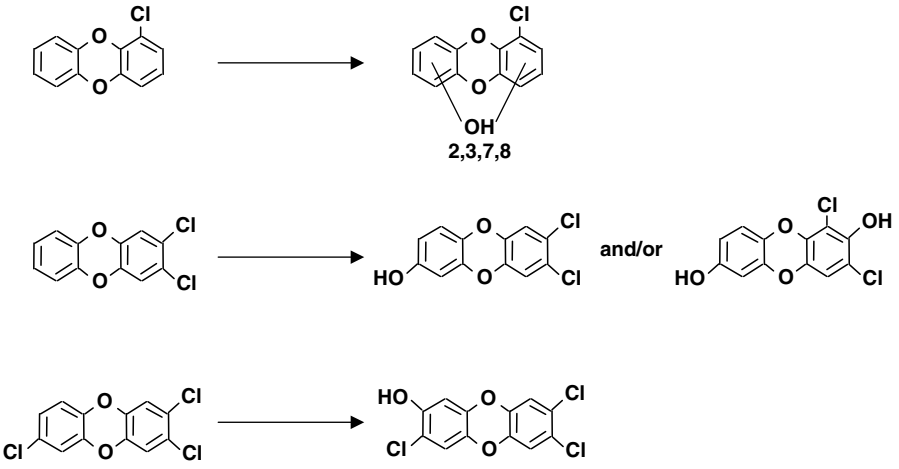


FIGURE 22.3 Proposed reactions of polychlorinated dibenzo-*p*-dioxins hydroxylation catalyzed by a cytochrome P450 BM-3 mutant A74G/F87V/L188Q.

22.5 STEREOSELECTIVITY IN PROPYLBENZENE AND 3-CHLOROSTYRENE OXIDATION BY CYTOCHROME P450 BM-3 AND ITS MUTANT

Cytochrome P450s act on the inactive carbon–hydrogen bonds and some exhibit high regio- and stereoselective monooxygenation activity. Therefore, cytochrome P450s are expected to be potential catalysts for fine chemical synthesis. Cytochrome P450 BM-3 WT and a mutant F87G showed high activities toward propylbenzene and 3-chlorostyrene with considerable stereoselectivity (Fig. 22.4) (Li et al., 2001c). Furthermore, reaction analysis using cytochrome P450 BM-3 mutants revealed that the stereoselectivity is under the control of the residue size at position 87 of this enzyme (Li et al., 2001c).

We reported oxidation of propylbenzene and 3-chlorostyrene by wild-type cytochrome P450 BM-3 with high turnover (479 nmol 1-phenyl-1-propanol/min/nmol P450 and 300 nmol 3-chlorostyrene oxide/min/nmol P450). Furthermore, the residue size at position 87 of P450 BM-3 was found to play critical roles in determining stereoselectivity in oxidation of propylbenzene and 3-chlorostyrene. Replacement of Phe87 with Val, Ala, and Gly resulted in decreases in optical purity of produced (*R*)-(+)-1-phenyl-1-propanol from 90.0 to 37.4, 26.0, and −15.6% e.e., respectively, and in increases in those of produced (*R*)-(+)-3-chlorostyrene oxide from −61.0 to −38.0, 67.0, and 94.6% e.e., respectively (Li et al., 2001c). These results indicated the high potential of cytochrome P450 BM-3 and its mutants for the preparation of useful chiral compounds.

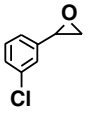
Substrate	 **Propylbenzene**	 **3-Chlorostyrene**
Product	 **1-Phenyl-1-propanol**	 **3-Chlorostyrene oxide**
Enzyme	**WT**	**F87G**
Product distribution (%)	**99.2**	**100**
Optical purity of (*R*)-product (% *e.e.*)	**90.0**	**94.6**

FIGURE 22.4 Regio- and stereoselective transformation of propylbezene and 3-chlorostyrene by cytochrome P450 BM-3 WT and a mutant F87G.

22.6 APPLICATION OF CYTOCHROME P450 BM-3 MUTANT TO THE SYNTHESIS OF HYDROQUINONE DERIVATIVES FROM PHENOLIC COMPOUNDS

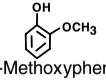
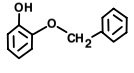
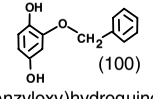
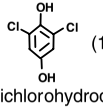
We further investigated the activity of F87V toward various kinds of aromatic compounds and found that it showed significant activity toward phenolic compounds (Sulistyaningdyah et al., 2005). Furthermore, we paid attention to the regioselective hydroxylation activity of F87V toward a variety of phenolic compounds, and applied the activity to the enzymatic synthesis of useful hydroquinone derivatives from phenolic compounds (Sulistyaningdyah et al., 2005).

The apparent initial reaction rates of F87V as to benzothiophene, indan, 2,6-dichlorophenol, and 2-(benzyloxy)phenol were 227, 204, 129, and 385 nmol/min/nmol P450, which are 220, 66, 99, and 963 fold those of the WT, respectively. These results indicate that Phe87 plays a critical role in the control of the substrate specificity of cytochrome P450 BM-3.

Because the hydroxylation of 2,6-dichlorophenol and 2-(benzyloxy)phenol by F87V proceeded well with high regioselectivity as to the *para* position, the hydroxylation of various phenolic compounds was further investigated from the view point of application. The hydroxylation activity was measured under practical conditions for 24 h with an NADPH-regenerating system using glucose dehydrogenase with glucose. The hydroxylation activities of WT and F87V toward various phenolic compounds, and the structures of the major products identified on GC-MS analysis (in comparison with authentic samples) are shown in Figure 22.5 (Sulistyaningdyah et al., 2005). Each product obtained with WT and F87V from phenol, *o*-cresol, *m*-cresol, *o*-methoxyphenol, *m*-methoxyphenol, 2-(benzyloxy)phenol, 2,6-dichlorophenol, *o*-chlorophenol, and *o*-bromophenol was identified as the corresponding *p*-hydroquinone compound, while from each of *m*-chlorophenol and *m*-bromophenol a mixture of two products was obtained. *o*- and *m*-aminophenol did not act as substrates. F87V showed higher hydroxylation activities toward all substrates than WT. The hydroxylation activity of F87V toward 2-(benzyloxy)phenol was particularly high, the value being 35.3 nmol/min/nmol P450, i.e. about 88 times higher than that of WT. With F87V as the catalyst, 0.71 mg/ml 2-(benzyloxy)hydroquinone, an intermediate for pharmaceuticals, was produced from 1.0 mg/ml 2-(benzyloxy)phenol in 4 h with an NADPH-regenerating system, the molar yield being 66% (Sulistyaningdyah et al., 2005).

22.7 CONCLUSIONS

Bioprocesses, which involve biocatalysts for the production of useful compounds and decomposition of toxic compounds, are expected to become a leading player in green chemistry and environmental conservation. The first step in bioprocess development is the creation of useful catalysts. This review introduces some examples of catalyst design stemming from mutation analysis of a versatile enzyme, cytochrome P450 BM-3. Recently, rational methods for creating new biocatalysts have been rapidly developed. Modern gene technology, crystal structure analysis, and bioinformatics

Substrate	Activity (nmol/min/nmol P450) WT	F87V	Ratio of F87V/WT	Product (%)
 Phenol	0.428	7.04	16.5	 (100) Hydroquinone
 *o*-Cresol	0.642	8.91	13.9	 (100) 2-Methylhydroquinone
 *m*-Cresol	0.737	10.41	14.1	
 *o*-Methoxyphenol	0.234	11.9	51.0	 (100) 2-Methoxyhydroquinone
 *m*-Methoxyphenol	0.102	1.06	10.4	
 2-(Benzyloxy)phenol	0.399	35.3	88.6	 (100) 2-(Benzyloxy)hydroquinone
 2,6-Dichlorophenol	6.48	24.6	3.79	 (100) 2,6-Dichlorohydroquinone

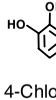
Substrate	Activity (nmol/min/nmol P450) WT	F87V	Ratio of F87V/WT	Product (%)
 *o*-Chlorophenol	2.10	8.37	3.98	 (100) 2-Chlorohydroquinone
 *m*-Chlorophenol	2.31	23.0	9.95	 (42) 2-Chlorohydroquinone  (58) 4-Chlorocatechol
 *o*-Bromophenol	2.10	8.37	3.98	 (100) 2-Bromohydroquinone
 *m*-Bromophenol	2.31	23.0	9.95	 (90) 2-Bromohydroquinone Unknown (10)
 *o*-Nitrophenol	1.56	1.60	1.02	Unknown (100)
 *m*-Nitrophenol	0.789	1.22	1.55	

FIGURE 22.5 Hydroxylation activities of cytochrome P450 BM-3 WT and a mutant F87V toward phenolic compounds.

make possible the modulation of enzyme functions through site-directed mutagenesis, DNA shuffling, and so on. In this chapter, we summarized some examples of modification of the substrate specificity of a fatty acid monooxygenase, cytochrome P450 BM-3, as described above, and proved the potentials of the mutant enzymes for chemical synthesis and environmental bioremediation.

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Sulistyaningdyah, W.T., Ogawa, J., Li, Q.-S., Shinkyo, R., Sakaki, T., Inouye, K., Schmid, R.D., and Shimizu, S. (2004) Metabolism of polychlorinated dibenzo-*p*-dioxins by cytochrome P450 BM-3 and its mutant. *Biotechnol. Lett.* 26, 1857–1860.

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23

GLYCOSYNTHASES FROM INVERTING HYDROLASES

MOTOMITSU KITAOKA

National Food Research Institute, National Agriculture and Food Research Organization, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

23.1 RETAINING AND INVERTING GLYCOSIDE HYDROLASES

Enzymatic synthesis of glycosides is a key method in glycotechnology because unlike organic synthesis, it does not require selective protection–deprotection processes of hydroxyl groups. Hydrolytic enzymes for glycosides (i.e., glycoside hydrolases, GH) are considered to be practical catalysts in the preparation of glycosides because of the wide variety available. GHs are generally categorized into retaining and inverting based on changes in the anomeric configurations during their reactions

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(Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat and Bairoch, 1996; Davies and Henrissat, 1995; Sinnott, 1990).

Many retaining GHs exhibit transglycosylation activity in which glycosides are transferred to another acceptor molecule. These GHs are used in the production of various glycosides. In contrast, inverting GHs do not show transglycosylation activity, thereby limiting their usage in hydrolysis. The difference in transglycosylation activity can be explained by the difference in reaction mechanisms. Typically, both types of GHs catalyze hydrolysis using two acidic residues that act as a general acid (a proton donor) and a general base (a nucleophile) as illustrated in Figure 23.1.

In the case of retaining GHs, the acid residue donates a proton to the glycosyl oxygen atom and the base residue directly attacks the anomeric center in concert, producing a covalently bound intermediate at the base residue with a Walden inversion, followed by an inverting hydrolysis of the intermediate, which results in anomeric retention during the overall reaction. If the glycosyl–enzyme intermediate of the retaining GH is attacked by another alcohol instead of water, transglycosylation occurs. It should be noted that no water molecule participates in the reaction, making transglycosylation possible in water.

The reaction of inverting GHs differs in the nucleophilic reagent attacking the anomeric center. In this case, a water molecule activated by the base residue attacks the anomeric center to hydrolyze the glycoside with anomeric inversion. Considering the transglycosylation reaction by an inverting GH, a dehydration step must be present because the reaction is initiated by the hydration of the glycosyl linkage. Since the enzymatic reaction must be performed in water, the presence of abundant molecules of water means that the dehydration step does not occur. Thus, inverting GHs theoretically do not exhibit transglycosylation activity and their use is limited in hydrolysis, but not in the production of glycosides by transglycosylation. Because approximately one-third of GHs are inverting enzymes, their use in the synthesis of glycosides has been expected.

23.2 REACTIONS OF GH WITH THE GLYCOSYL FLUORIDE OF THE OPPOSITE ANOMER

β-Amylase, an inverting GH, has been reported to hydrolyze β-maltosyl fluoride (the opposite anomer of the glycosides to be hydrolyzed) into maltose and F^- (Hehre et al., 1979). Later, various inverting enzymes were found to hydrolyze the glycosyl fluorides of the opposite anomer (Konstantinidis et al., 1993; Becker et al., 2000; Hehre, 2000; Williams and Withers, 2000), suggesting that the reaction is common among inverting GHs. Instead of simple hydrolysis, the reaction mechanism consists of two steps. In the first step, a new glycoside of the correct anomer forms from the glycosyl fluoride of the opposite anomer and an acceptor with a Walden inversion. The new glycoside is immediately hydrolyzed with anomeric inversion at the same site on the enzyme before it is released from the active center, which is the normal reaction of an inverting GH (Fig. 23.2) (Hehre, 2000; Williams and Withers, 2000).

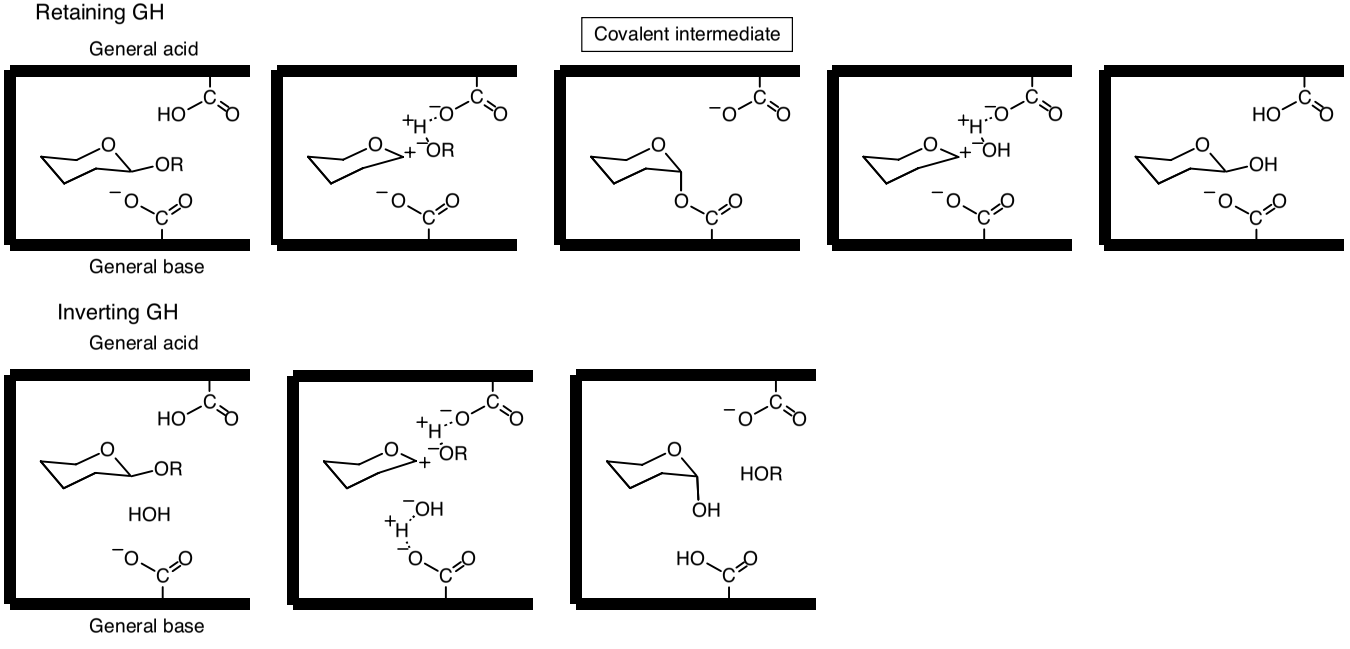
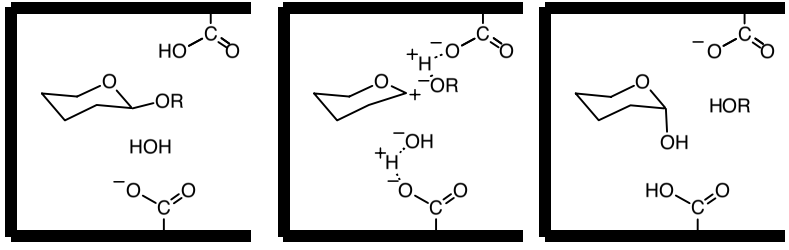


FIGURE 23.1 Typical reaction mechanisms of retaining and inverting glycoside hydrolases (GH).

Hydrolysis



Hehre resynthesis-hydrolysis reaction

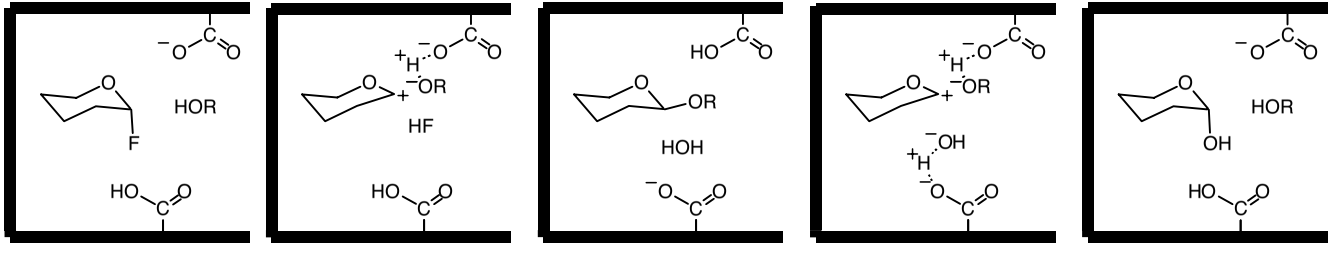


FIGURE 23.2 Reactions of inverting GHs.

The mechanism was confirmed by analyzing the reaction of trehalase, which hydrolyzed an α-1, α-1 linkage with anomeric inversion (Hehre et al., 1982).

The mutant GH enzymes that catalyze the synthesis of glycosides from the glycosyl fluoride of the opposite anomer are called glycosynthases (Fig. 23.3). In 1998, Withers' group reported the first glycosynthase, in which a mutant retaining enzyme, GH 1 β-glucosidase from *Agrobacterium* sp. with a mutation at its nucleophilic residue (E358) catalyzed the synthesis of various β-glucosides using α-glucosyl fluoride as a donor and various *p*-nitrophenyl-β-glycosides as acceptors (Mackenzie et al., 1998). Since then, various retaining GHs have been converted into glycosynthases by substituting their nucleophilic residues (Malet and Planas, 1998; Trincone et al., 2000; Fort et al., 2000; Mayer et al., 2000; Nashiru et al., 2001; Okuyama et al., 2002; Hrmova et al., 2002; Ducros et al., 2003; van Lieshout et al., 2004; Drone et al., 2005; Sugimura et al., 2006; Kim et al., 2006; Hommalai et al., 2007).

Williams and Withers commented that the glycosynthase technique was developed by mimicking the Hehre resynthesis–hydrolysis mechanism of inverting GHs (Williams and Withers, 2000). However, no glycosynthase mutant derived from an inverting GH has been reported. Thus, we attempted to convert an inverting GH into glycosynthase, which is expected to expand the usage of inverting GHs in the synthesis of glycosides.

23.3 THE MODEL INVERTING GH: REDUCING-END-XYLOSE RELEASING EXO-OLIGOXYLANASE (Rex)

GH8 is a family of inverting hydrolases whose main members are cellulase, chitinase, and chitosanase. A GH8 endo β-1,4 xylanase (pXyl) was found in a culture supernatant of *Pseudoalteromonas haloplanktis* and was characterized (Collins et al., 2002). The 3D structures of two GH8 enzymes were found to have $(\alpha/\alpha)_6$ barrel structure (clan GH-M) (Guerin et al., 2002; Van Petegem et al., 2003). The GH8 xylanase, pXyl, has the highest amino acid identity (32.6%) with the protein encoded by the BH2105 gene (GenBank accession number:BAB05824) of *Bacillus halodurans* C-125, an alkalophilic bacterium whose genomic sequence is available (Takami et al., 2000; Collins et al., 2002).

The recombinant BH2105 protein was expressed in *Escherichia coli* and purified to yield a 45-kDa protein on SDS-PAGE. The sequence information suggested that the enzyme had no signal peptide. This enzyme did not hydrolyze birch wood xylan or any other polymeric substrate for GH8 enzymes (chitosan, lichenan, curdlan, and carboxymethyl cellulose) but hydrolyzed xylooligosaccharides (X_n) producing initially X_1 and X_{n-1} and finally X_1 and X_2, when $n \geq 3$. It hydrolyzed X_2 only to a small extent. On the other hand, the enzyme exhibited no activity on 4-nitrophenyl-β-xyloside and 4-nitrophenyl-β-xylobioside, suggesting the possibility of hydrolysis at the reducing end. The reducing-end specificity was confirmed by the hydrolysis product of β-1,4 linked D-glucose- and D-xylose-based trisaccharides (Shintate et al., 2003), e.g., the formation of G–X and X_1 from G–X–X. Anomeric analysis

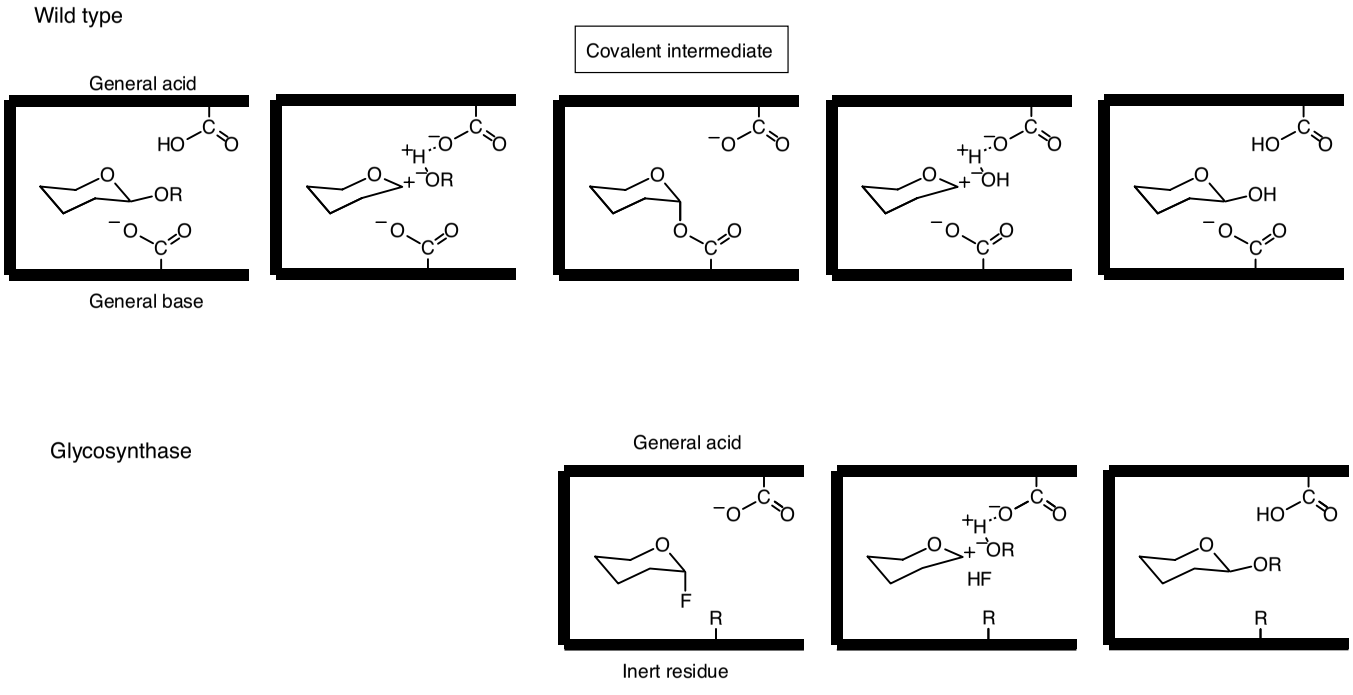


FIGURE 23.3 Glycosynthases derived from retaining GHs.

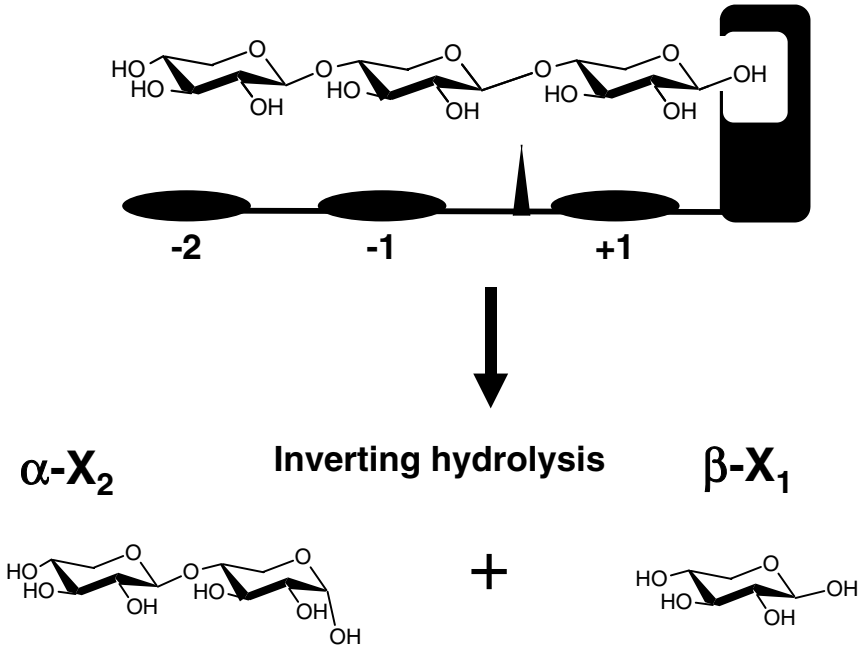


FIGURE 23.4 Substrate recognition and reaction of Rex.

of the product revealed that the enzyme hydrolyzed only the β-anomer of X_3 with anomeric inversion to form α-X_2 and β-X_1 (Fig. 23.4). The reducing-end specificity was completely different from that of β-xylosidase, which liberates xylose from the nonreducing end. We propose that the name of the enzyme be reducing-end-xylose releasing exo-oligoxylanase (Rex) (Honda and Kitaoka, 2004). It has been registered as a new enzyme and designated EC 3.2.1.156 in the Enzyme Nomenclature. The enzyme had no transglycosylation activity as is usual for inverting enzymes.

To understand the mechanism of the reducing-end-specific exolytic activity, structural analyses of Rex were carried out (Honda et al., 2005; Fushinobu et al., 2005). The crystal structures of Rex in unliganded and complex forms at 1.35–2.20 Å resolution (PDB accession numbers: –1WU4, 1WU5, and 1WU6[0]) were determined in order to reveal the structural aspects of its three subsites ranging from -2 to $+1$ (Fushinobu et al., 2005). The structure of Rex is very similar to that of pXyl (Van Petegem et al., 2003), the GH8 endo-xylanase with the $(\alpha/\alpha)_6$ barrel structure. The catalytic machinery of Rex is well conserved with pXyl. The most significant difference between the structures of Rex and pXyl was found at the binding cleft. Subsite $+2$ of Rex is blocked by a barrier formed by a kink in the loop before helix α_{10}, and H319 in the loop forms a direct hydrogen bond with the β-hydroxyl of xylose residue bound at subsite $+1$, both contributing to the specific recognition of the reducing-end xylose.

23.4 HEHRE RESYNTHESIS-HYDROLYSIS OF Rex

The Hehre resynthesis–hydrolysis mechanism is often difficult to detect because many enzymes recognize glycosyl fluoride not only as the donor but also as the acceptor (Konstantinidis et al., 1993; Becker et al., 2000; Williams and Withers, 2000; Hehre, 2000), such as β-maltosyl fluoride for β-amylase and β-glucosyl fluoride for glucoamylase. Reliable experimental evidence for the mechanism was reported for trehalase using β-glucosyl fluoride as the donor and glucose or xylose as the acceptor (Hehre et al., 1982; Kasumi et al., 1986). However, experimental difficulty persists because the donor β-glycosyl fluoride is hydrolyzed into the acceptor glucose both spontaneously and enzymatically, making the reaction complicated.

We notice that Rex is a suitable enzyme to examine the Hehre resynthesis–hydrolysis mechanism. As Rex hydrolyzes X_3 to release X_1 from the reducing end, it is expected to use α-xylobiosyl fluoride (α-X_2F) as the donor and X_1 as the acceptor. It should be mentioned that the hydrolytic product of α-X_2F, X_2, will never act as the acceptor molecule due to the reducing-end exo-specificity. Thus, the donor and acceptor are considered to be completely independent during the reaction (Fig. 23.5).

Rex was inactive on α-X_2F in the absence of X_1, but produced X_2 from α-X_2F in the presence of X_1. The result suggests that the reaction was not simple hydrolysis of α-X_2F but Hehre resynthesis–hydrolysis. No X_3 was detected during the reaction. Most of the resulting X_3 was supposed to be hydrolyzed into X_1 and X_2 before it escaped from the active center because X_3 was generated at the exact position where it was hydrolyzed (Fig. 23.5).

23.5 CONVERSION OF Rex INTO GLYCOSNYTHASE BY MUTATING BASE RESIDUE

To convert Rex into glycosynthase, initially the base residue D263 was mutated as performed for retaining GHs. A saturation mutagenesis library of Rex was constructed at D263 (Honda and Kitaoka, 2006). From the D263X mutant library, 120 colonies were randomly picked and each mutant enzyme was purified by His-tag affinity column chromatography. During the screening of α-X_2F-consuming activity in the presence of X_1 and the accumulation of X_3 on TLC, 71 of the 120 proteins, with 9 kinds of substitution, exhibited activity. The wild type exhibited the highest α-X_2F consumption without accumulation of X_3. D263C exhibited the highest production of X_3, and D263N the second highest among the mutants. Thus, we selected D263C and D263N as targets for further analysis of glycosynthase properties.

The F^--releasing activity from 10 mM α-X_2F in the presence of 10 mM X_1 and the hydrolytic activities to 2.6 mM X_3 of the wild type and D263 mutants are summarized in Table 23.1. Both D263C and D263N showed approximately 1/10 of the F^--releasing activity of the wild type. The mutations caused marked decreases in the hydrolytic activity (4.5×10^{-4} for D263C, 2.9×10^{-3} for D263N). It should be noted that the remaining hydrolytic activity of D263N was 6.4 times higher than that of D263C, whereas their F^--releasing activities were almost identical, resulting in

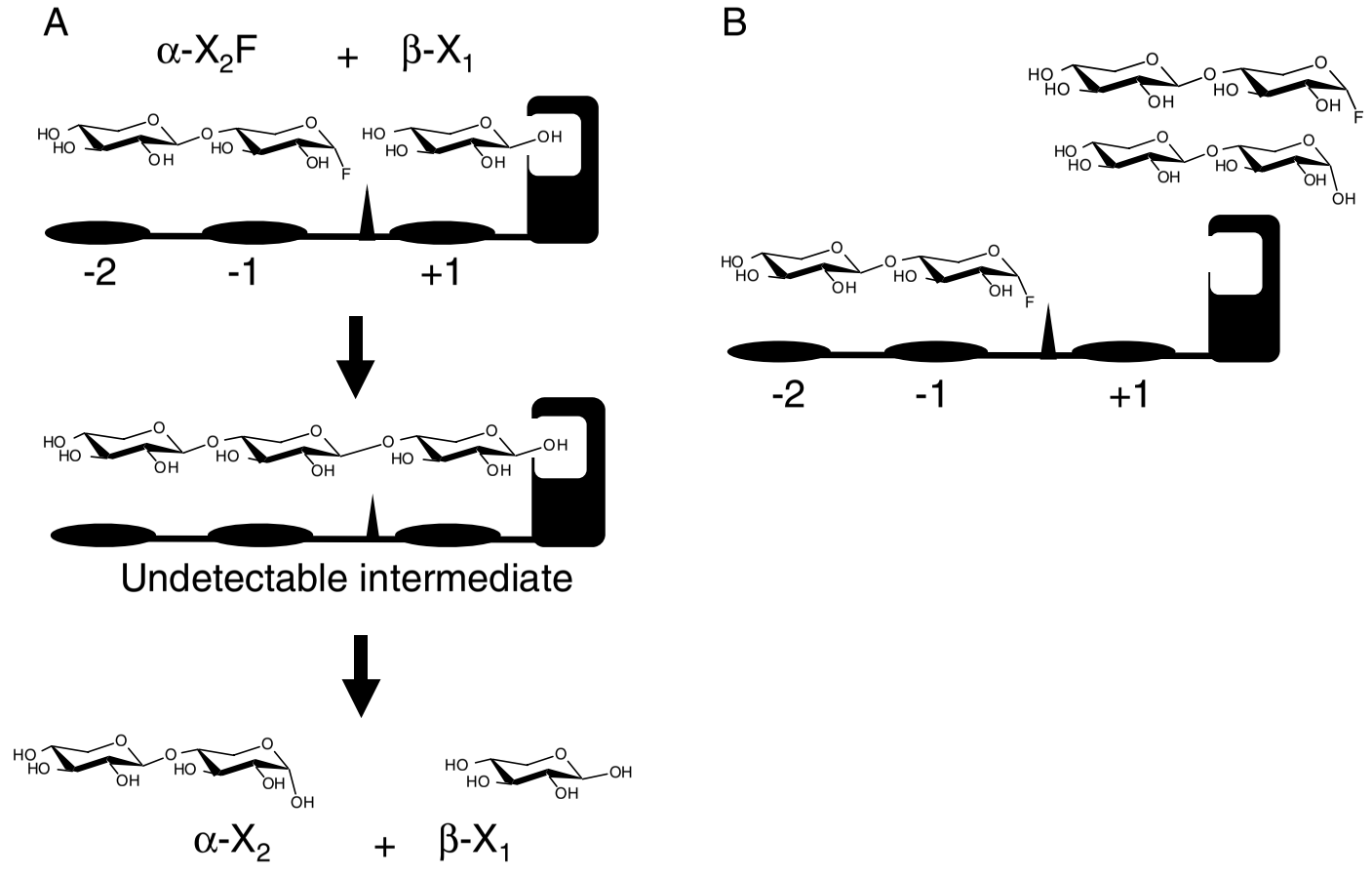


FIGURE 23.5 Model of Hehre resynthesis–hydrolysis reaction by Rex. (A) In the presence of X_1, the reaction proceeds through an undetectable intermediate. (B) In the absence of X_1, the reaction does not occur because neither α-X_2F nor X_2 can enter subsite $+1$ due to the reducing-end specificity.

TABLE 23.1 Activities of each mutant of Rex

	F^- Release* (*F*)	X_3 Hydrolysis** (*H*)	*F*/*H*
Wild type	3.1	31.2	0.1
D263C	0.29	0.014	21
D263N	0.32	0.09	3.6
Y198F	4.7	0.06	78
Y198F/D263C	0.03	0.0006	50
Y198F/D263N	0.23	0.0016	144

*F^--releasing activity from 10 mM α-X_2F and 10 mM X_1. Values are given as s^{-1}.
**Hydrolytic activity on 2.6 mM X_3. Values are given as s^{-1}.

a difference in the ratio of F^--releasing activity to hydrolytic activity (*F*/*H* ratio). The time courses of α-X_2F and X_1 reactions by the enzymes were examined. In the wild-type reaction, F^- and X_2 concentrations increased in parallel but X_3 was not detected. On the other hand, X_3 was detected in reactions catalyzed by the mutants. In the case of D263N, X_2 concentration was higher than X_3 in the reaction mixture, indicating that the hydrolytic reaction was higher than the transfer reaction. In contrast, X_3 concentration was higher than X_2 in the reaction with D263C, indicating that the transfer activity was higher than the hydrolytic activity. The difference agrees with the remaining hydrolytic activities of the mutants.

23.6 CONVERSION OF Rex INTO GLYCOSNYTHASE BY MUTATING THE RESIDUE SUPPORTING THE NUCLEOPHILIC WATER MOLECULE

Conversion of Rex into glycosynthase was successfully performed by mutating the base residue. However, the D263 mutants retained significant hydrolytic activity that decreased the yield of the synthetic product. Moreover, the F^--releasing activities of the mutants were much lower than that of the wild type (Table 23.1). The mutation at the general base residue of the inverting GH could not remove the hydrolytic activity completely, probably because the water molecule retained some activity as a nucleophilic reagent without the aid of the residue (Honda and Kitaoka, 2006). It is therefore still important to reduce the hydrolytic activity of glycosynthase obtained from inverting GHs.

Collins et al. (2002, 2005) found that a mutation at a conserved tyrosine residue (Y203) in pXyl, which shares 32.6% amino acid sequence identity with Rex, causes a drastic decrease in the hydrolytic activity of the enzyme toward xylan. The residue formed a hydrogen bond with the nucleophilic water molecule that had another hydrogen bond with the general base residue (D281). The pH dependency of the Y203F mutant indicated that the tyrosine residue was not a general base, but was important in locating the nucleophilic water at the proper position (Collins et al., 2005). Thus, we attempted to mutate the corresponding residue of Rex, Y198 (Fig. 23.6), confirmed by both the alignment of amino acid sequences and structural analysis of Rex (Fushinobu et al., 2005).

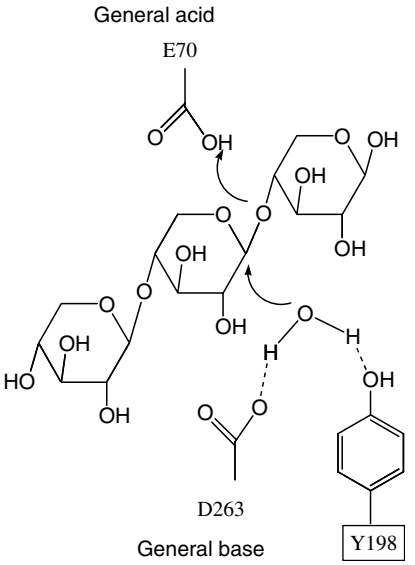


FIGURE 23.6 Schematic diagram of the catalytic residues of Rex.

The F^--releasing activity and the hydrolytic activities of the mutants at Y198 and/or D263 are given in Table 23.1. The mutation at Y198 drastically decreased the hydrolytic activity (from 31.2 to 0.06 s^{-1}), with a small increase in the F^--releasing activity. The F^--releasing activity of Y198F was more than 10 times that of the mutants at the general base residue (D263). The *F*/*H* ratio was 78, approximately four times that of D263C, the best glycosynthase mutant at the general base residue. Double mutation was also tested. Y198F/D263N showed 20 times less F^--releasing activity than Y198F, which was comparable to that of the corresponding single mutant, D263N. The *F*/*H* ratio (144) of D263N was two times higher than that of Y198F. The Y198F/D263C mutant showed scarce F^--releasing activity.

Much greater accumulation of X_3 (glycosynthase product) and much less formation of X_2 (hydrolytic product from X_3) were observed in the reactions catalyzed by Y198F and Y198F/D263N than in those catalyzed by D263C. The ratio of X_3 in the products $\{X_3/(X_2 + X_3)\}$ when half of α-X_2F was consumed was 0 (wild type), 0.59 (D263C), 0.19 (D263N), 0.93 (Y198F), and 0.96 (Y198F/D263N). Although Y198F/D263N showed a slightly larger X_3 ratio than Y198F, the F^--releasing activity of Y198F was much higher than that of Y198F/D263N. We judged that Y198F was the best glycosynthase among the mutants (Honda et al., 2008).

23.7 COMPARISON OF GLYCOSYNTHASE CONVERSION FROM RETAINING AND INVERTING GHs

The relationship between the F^--releasing activity and the hydrolytic activity of Rex during acquiring the glycosynthase activity was completely different from that of

retaining enzymes. In the case of retaining enzymes, the parent enzyme does not possess F^--releasing activity (Williams and Withers, 2000; Sugimura et al., 2006), and the mutation at the base residue completely removes the hydrolytic activity because the base residue directly attacks the C1 of the hydrolyzed glycoside. Thus, while converting retaining GHs into glycosynthases, it is important to acquire F^--releasing activity by the mutation.

In the case of inverting GHs, however, the water molecule activated by the base residue attacks the C1 of the glycoside. Thus, mutation of the base residue cannot remove the hydrolytic activity completely because the water molecule retains some activity without the aid of the catalytic residue. Furthermore, the parent enzyme possesses F^--releasing activity and the mutations at the base residue cause a decrease in this activity. Thus, while converting inverting GHs into glycosynthases, it is important to minimize the decrease in F^--releasing activity while maximizing the decrease in hydrolytic activity. We here conclude that an effective glycosynthase is generated from an inverting GH by mutating a residue holding the nucleophilic water molecule with the general base residue while keeping the general base residue intact.

23.8 GLYCOSYNTHASE FROM AN INVERTING GH WITH A TYPICAL REACTION MECHANISM

The second example of a glycosynthase derived from an inverting GH was recently reported to be GH95 α-1,2-L-fucosidase from *Bifidobacteriumbifidum* (AfcA) (Wada et al., 2008); this is the first report of a glycosynthase derived from an inverting α-glycosidase. This enzyme hydrolyzes α-1,2-linked L-fucose specifically at the nonreducing end (Katayama et al., 2004) and is classified as GH95. Because α-1,2-fucosylated sugars are often found in biologically functional sugar chains, the enzymatic production of such compounds using AfcA is desired. However, AfcA is an inverting enzyme with no transglycosylation activity. Structural analysis of AfcA revealed that its reaction mechanism was different from that of typical inverting GHs (Nagae et al., 2007). AfcA has an acidic proton donor residue, E566, similar to typical inverting GHs. However, the base residue activating the nucleophilic water is not an acidic amino acid, but an asparagine residue N423 activated by an acidic residue D766. The catalytic water residue is held by another asparagine residue N421 (Fig. 23.7).

These three residues were individually mutated to glycine and the glycosynthase activities were examined using β-fucosyl fluoride as the donor and lactose as the acceptor. The product was detected significantly only in the D766G mutant. The structure of the product was determined to be 2′-fucosyllactose, as expected from the specificity of AfcA (Wada et al., 2008). In this case, the residue that was mutated (D766) was not the residue holding the catalytic water molecule with the base residue (N421). The mutation of the residue activating the base residue was changed, while the base residue N423 was kept intact. Though the catalytic mechanism of AfcA is

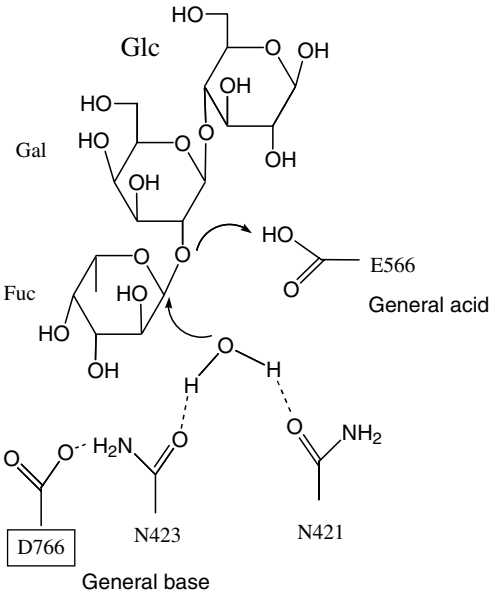


FIGURE 23.7 Schematic diagram of the catalytic residues of AfcA.

different from that of other typical inverting GHs, it should be noted that the base residue should be intact to obtain the best glycosynthase mutant.

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24

MOLECULAR SPECIES OF DIACYLGLYCEROLS AND TRIACYLGLYCEROLS CONTAINING DIHYDROXY FATTY ACIDS IN CASTOR OIL

JIANN-TSYH LIN

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, California 94710, USA

24.1 INTRODUCTION

Ricinoleate (OH18 : 1, Fig. 24.1A), a monohydroxy fatty acid, has many industrial uses such as in the manufacture of aviation lubricant, plastic, paint, and cosmetics. Ricinoleate occurs as acylglycerols (AG) in castor oil, and about 70% of castor oil

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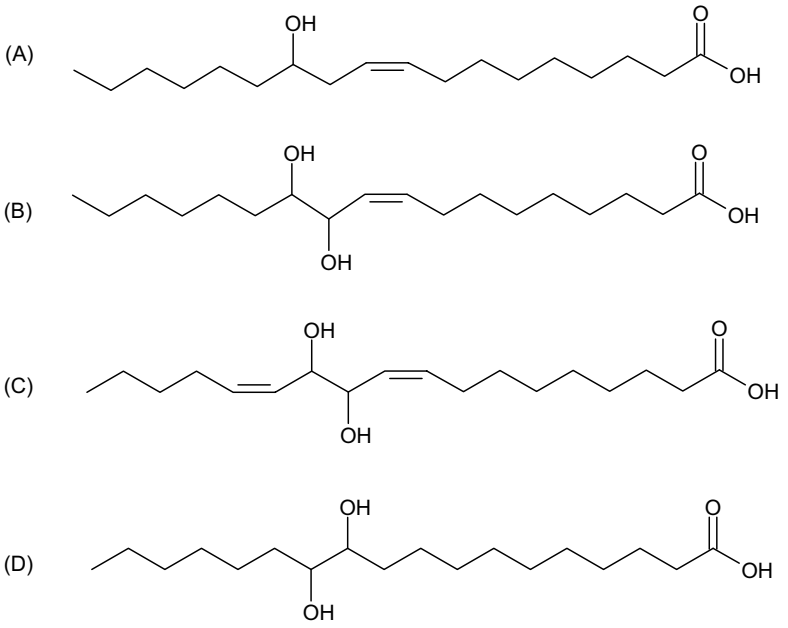

FIGURE 24.1 The structure of ricinoleate and the proposed structures of dihydroxy fatty acids in castor oil. (A) ricinoleate. (B) 11,12-dihydroxy-9-octadecenoic acid. (C) 11,12-dihydroxy-9,13-octadecadienoic acid. (D) 11,12-dihydroxyoctadecanoic acid.

is triricinolein (triricinoleoylglycerol) (Lin et al., 2003). Castor oil is the only commercial source of ricinoleate. We have previously identified and quantified 14 molecular species of AG containing ricinoleate in castor oil using high-performance liquid chromatography (HPLC) and mass spectrometry (MS) methods (Lin et al., 2003, 2006, 2007, 2008a). The identification of 12 molecular species of AG containing dihydroxy fatty acids in castor oil is reported here. These dihydroxy fatty acids are proposed as 11,12-dihydroxy-9-octadecenoic acid (11,12-dihydroxyoleic acid, diOH18 : 1, Fig. 24.1B), 11,12-dihydroxy-9,13-octadecadienoic acid (diOH18 : 2, Fig. 24.1C), and 11,12-dihydroxyoctadecanoic acid (11,12-dihydroxystearic acid, diOH18 : 0, Fig. 24.1D). These fatty acids and the AG containing these fatty acids (Fig. 24.2) have not been previously reported. Dihydroxy fatty acids in higher plants also have not been previously reported. Oleic acid could convert to 7(*S*),10(*S*)-dihydroxy-8(*E*)-octadecenoic acid (Hou et al., 1991) and ricinoleic acid could convert to 10,12-dihydroxy-8(*E*)-octadecenoic acid (Kim et al., 2000) in a microbial culture. 8*R*-Hydroxyoctadecadienoic acid could convert to some dihydroxyoctadecadienoic acids in microbial cultures (Garscha and Oliw, 2008). Dihydroxy fatty acids occur as eicosanoids in animals (acting as localized hormones) and can not be used in industry as a source due to their low contents (Liu and Huang, 2005).

The presence of a hydroxyl group on fatty acid drastically changes the physical properties of the oil, e.g., viscosity, pour point, melting point, heat of fusion, solubility,

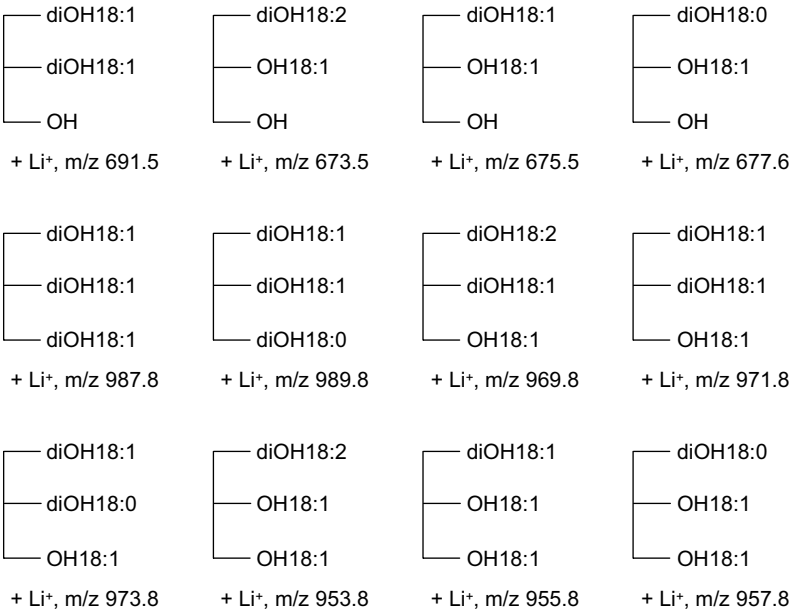

FIGURE 24.2 The structures of 12 acylglycerols containing dihydroxy fatty acids in castor oil. The calculated *m*/*z* of the lithium adducts of acylglycerols are also shown. The stereospecific locations of the fatty acids were not determined.

crystal structure, and polymorphism (Foubert et al., 2007). Because of the physical and chemical changes from the normal fatty acids, many industrial uses of ricinoleate have been found. The physical and chemical properties of dihydroxy fatty acids and AG containing dihydroxy fatty acids are different from those of monohydroxy fatty acids and normal fatty acids. The dihydroxy fatty acids and AG containing dihydroxy fatty acids can be used in industry different from or similar to those of ricinoleate. These dihydroxy fatty acids have not been used in industry to date as there has been no practical source but, in the future it may be isolated from castor oil or produced by a transgenic oil seed plant.

We have identified and quantified the regiospecific TAG in castor oil (Lin and Arcinas, 2007, 2008a) and olive oil (Lin and Arcinas, 2008b) using the fragment ions of MS of lithium adducts from the loss of the fatty acid at the *sn*-2 position as α,β-unsaturated fatty acid (Hsu and Turk, 1999). The regiospecific (*sn*-2) identification and quantification of TAG (AAB, with two identical fatty acids), as well as the quantification of the three different fatty acids at the *sn*-2 position of TAG (ABC) containing dihydroxy fatty acids by MS, is presented here. Regiospecific and stereospecific structures of AG affect the physical property of AG for industrial uses (Foubert et al., 2007). The regiospecific and stereospecific identification can help to understand the biosynthesis of AG for the development of transgenic oil seed plant to produce dihydroxy fatty acids.

24.2 HPLC FRACTIONATION OF THE MOLECULAR SPECIES OF ACYLGLYCEROLS IN CASTOR OIL

The fractionation of the molecular species of AG in castor oil was as previously reported (Lin et al., 2003). Chromatographic fractionation was performed using a Waters HPLC and a C_{18} analytical column (Gemini, 250 × 4.6 mm, 5 μ, C18, Phenomenex, Torrance, CA, USA). One milligram of castor oil was chromatographed at 22°C (room temperature) with a linear gradient from 100% methanol to 100% 2-propanol in 40 min, at a 1 mL/min flow rate, and detected at 205 nm. One-half minute fractions were collected and corresponding fractions were pooled from 15 HPLC runs. Fractions eluted before triricinolein (fraction #20, retention time 9.5–10.0 min) were used for MS studies. The final sample solutions were prepared for direct infusion into the mass spectrometer by combining approximately one fourth of each fraction with 50 μL of a methanol solution of 100 mM lithium acetate and diluting to a total volume of 200 μL.

24.3 PROPOSED STRUCTURES OF DIHYDROXY FATTY ACIDS

Figure 24.3 shows the MS^2 spectrum of diacylglycerol, dihydroxyoleoyl-ricinoleoyl-glycerol (diOH18 : 1-OH18 : 1), in fraction #9. The spectrum shows the fragment ions from the losses of dihydroxyoleate and ricinoleate as $[M + Li - diOH18:1]^+$ at *m/z* 361.2 and $[M + Li - OH18:1]^+$ at *m/z* 377.2. The two free fatty acids are also shown as $[diOH18:1 + Li]^+$ at *m/z* 321.2 and $[OH18:1 + Li]^+$ at *m/z* 305.3. Two

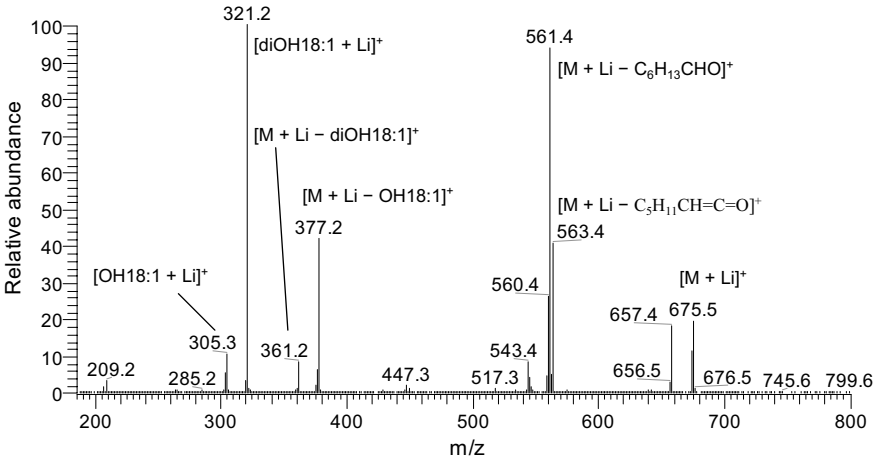

FIGURE 24.3 Ion trap mass spectrum of ESI-MS^2 of dihydroxyoleoyl-ricinoleoyl-glycerol $[M + Li]^+$ at *m/z* 675.5 in the HPLC fraction 9 of castor oil (collision energy 37%). Abbreviations: diOH18 : 1 is dihydroxyoleate, OH18 : 1 is ricinoleate, $C_5H_{11}CH{=}C{=}O$ is a ketene, $C_6H_{13}CHO$ is an aldehyde.

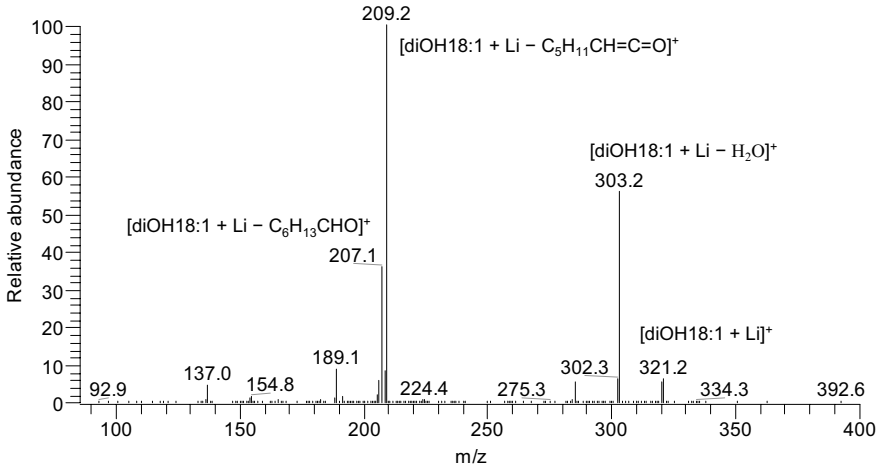

FIGURE 24.4 Ion trap mass spectrum of ESI-MS^3 of dihydroxyoleate $[diOH18:1 + Li]^+$ at *m/z* 321.2 (collision energy 36%). This was from $[diOH18:1 + Li]^+$ at *m/z* 321.2 shown in Fig. 24.3. For abbreviations, see Fig. 24.3. For proposed fragmentation pathways, see Fig. 24.5A and 24.5B.

other significant ions are $[M + Li - C_6H_{13}CHO]^+$ at *m/z* 561.4 and $[M + Li - C_5H_{11}CH{=}C{=}O]^+$ at *m/z* 563.4. The fragment ions from the loss of $C_6H_{13}CHO$ and $C_5H_{11}CH{=}C{=}O$ are also shown in Fig. 24.4.

Figure 24.4 is the MS^3 spectrum of dihydroxyoleate $[diOH18:1 + Li]^+$ at *m/z* 321.2. Three significant fragment ions are $[diOH18:1 + Li - H_2O]^+$ at *m/z* 303.2, $[diOH18:1 + Li - C_5H_{11}CH{=}C{=}O]^+$ at *m/z* 209.2, and $[diOH18:1 + Li - C_6H_{13}\,CHO]^+$ at *m/z* 207.1. The loss of $C_6H_{13}CHO$ (or shown earlier as $C_7H_{14}O$) has been previously reported (Lin and Arcinas, 2007, 2008a; Byrdwell and Neff, 1998) from the cleavage between C-11 and C-12 of the ricinoleoyl chain for TAG containing ricinoleate. The proposed fragmentation pathway of this ion at *m/z* 207.1 is as shown in Fig. 24.5A. The fragmentation showed the cleavage was between C-11 and C-12 of diOH18 : 1 and the two hydroxyl groups were at both sides of the cleavage. The fragment ion at *m/z* 207.1 had gained an extra hydrogen atom from the hydroxyl group at C-12 position as shown in Fig. 24.5A. The location of this 12-hydroxyl group of diOH18 : 1 is the same as that of the 12-hydroxyl group of ricinoleate (Fig. 24.1A). The loss of ketene, $C_5H_{11}CH{=}C{=}O$, also has been previously reported (Lin and Arcinas, 2008a, 2008b). The proposed fragmentation pathway of the ion at *m/z* 209.2 is shown as Fig. 24.5B. The fragment ion at *m/z* 209.2 had gained three extra hydrogen atoms from the other side of the carbon–carbon cleavage between C-11 and C-12 of diOH18 : 1 as shown in Figure 24.5B. Comparing between the MS spectra of diOH18 : 1 (Fig. 24.4) and ricinoleate (Fig. 24.6), the spectrum of ricinoleate $[OH18:1 + Li]^+$ at *m/z* 305.3 showed only the significant fragment ions of $[OH18:1 + Li - C_6H_{13}CHO]^+$ at *m/z* 191.1 and $[OH18:1 + Li - H_2O]^+$ at *m/z* 287.2, the ion from the loss of ketene $[OH18:1 + Li - C_5H_{11}CH{=}C{=}O]^+$ at *m/z* 193 was not detected. Apparently, the hydroxyl

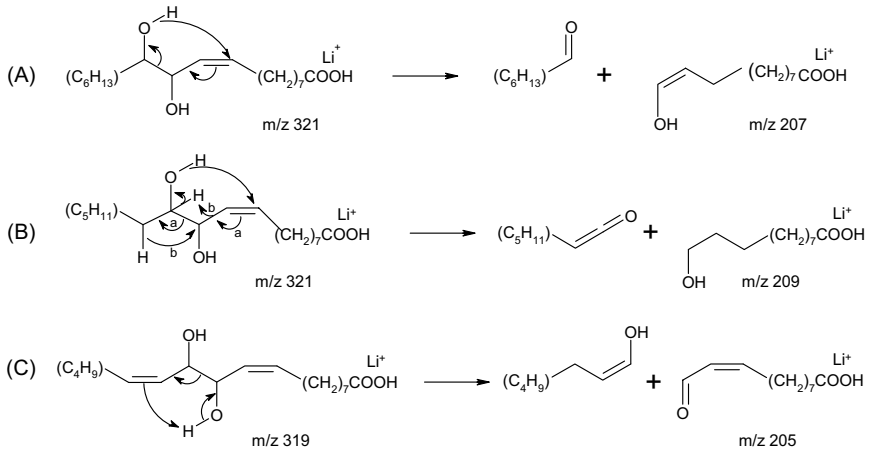

FIGURE 24.5 Proposed fragmentation pathways of dihydroxy fatty acids. (A) $[diOH18:1 + Li - C_6H_{13}CHO]^+$ at *m/z* 207.1 shown in Fig. 24.4. (B) $[diOH18:1 + Li - C_5H_{11}CH{=}C{=}O]^+$ at *m/z* 209.2 shown in Fig. 24.4. The cleavage of the bond between C-11 and C-12, the electron pair transfer *a*, was after the formation of the double bond at C-10 position (*a*). The electron pair transfer *b* to the hydrogen atom at C-12 is after the migration of the hydrogen atom at C-13 to C-11 (*b*). (C) $[diOH18:2 + Li - C_5H_{11}CH{=}CHOH]^+$ at *m/z* 205.2 shown in Fig. 24.7.

group at C-11 position facilitated the fragment ion at *m/z* 209.2 (Fig. 24.4). The location of this hydroxyl group other than at C-11 could not cause the cleavage between C-11 and C-12 as well as the fragmentation from the loss of ketene. We propose the structure of diOH18 : 1 as 11,12-dihydroxy-9-octadecenoic acid (Fig. 24.1B) and the two hydroxyl groups are attached to adjacent carbon atoms at C-11 and C-12 positions.

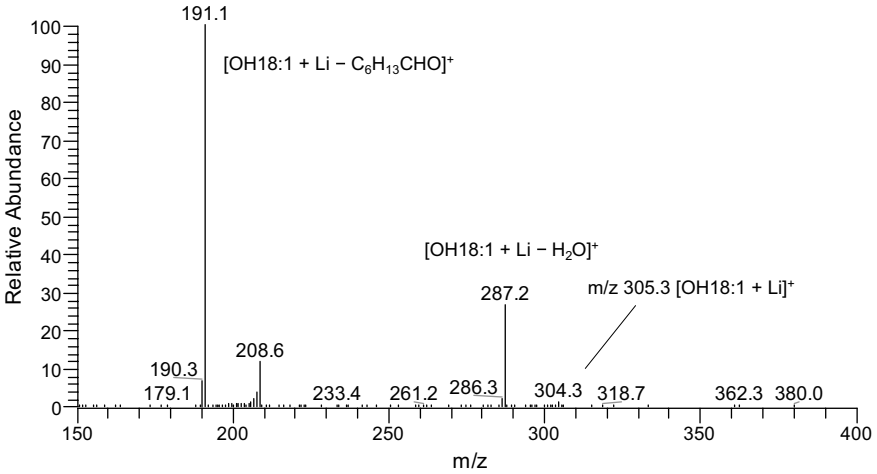

FIGURE 24.6 Ion trap mass spectrum of ESI-MS^2 of ricinoleate standard (collision energy 36%).

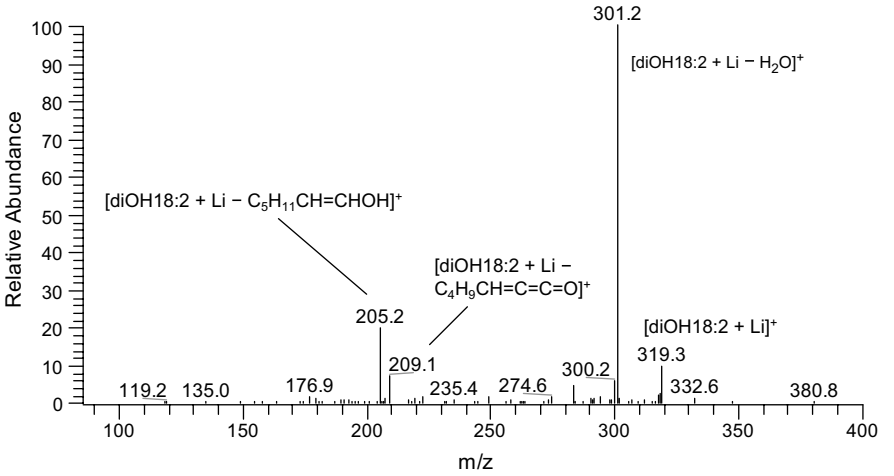

FIGURE 24.7 Ion trap mass spectrum of ESI-MS^4 of dihydroxyoctadecadienoic acid $[diOH18:2 + Li]^+$ at *m/z* 319.3 (collision energy 39%). This was from the MS^2 (collision energy 35%) of $[diOH18:2\text{-}OH18:1\text{-}OH18:1 + Li]^+$ at *m/z* 953.6 from the HPLC fraction #15 of castor oil and then the MS^3 (collision energy 38%) of $[diOH18:2\text{-}OH18:1\text{-}OH18:1 + Li - OH18:1]^+$. For proposed fragmentation pathways, see Fig. 24.5B and 24.5C.

Figure 24.7 is the MS^4 spectrum of dihydroxyoctadecadienoic acid $[diOH18:2 + Li]^+$ at *m/z* 319.3. This originated from the MS^2 of dihydroxyloctadecadienoyl-diricinoleoyl-glycerol $[M + Li]^+$ at *m/z* 953.6 from the HPLC fraction 15 of castor oil and then MS^3 of $[M + Li - OH18:1]^+$ at *m/z* 655.5. Figure 24.7 shows three significant fragment ions, $[diOH18:2 + Li - H_2O]^+$ at *m/z* 301.2, $[diOH18:2 + Li - C_4H_9CH{=}C{=}C{=}O]^+$ at *m/z* 209.1, and $[diOH18:2 + Li - C_5H_{11}CH{=}CHOH]^+$ at *m/z* 205.2. The proposed fragmentation pathway to *m/z* 205.2 is as shown in Fig. 24.5C. The proposed fragmentation pathway to ion at *m/z* 209.1 is the same as that of Fig. 24.5B. We propose the structure of diOH18 : 2 as 11,12-dihydroxy-9,13-octadecadienoic acid (Fig. 24.1C).

Figure 24.8 is the MS^4 spectrum of dihydroxyoctadecanoic acid $[diOH18:0 + Li]^+$ at *m/z* 323.3. This was originated from MS^2 of dihydroxyloctadecanoyl-diricinoleoyl-glycerol $[M + Li]^+$ at *m/z* 957.7 from the HPLC fraction 15 of castor oil and then MS^3 of $[M + Li - OH18:1]^+$ at *m/z* 659.5. Figure 24.8 shows only one significant fragment ion. The ion at *m/z* 305.2 was the dehydration of the precursor ion $[diOH18:0 + Li - H_2O]^+$. The locations of the two hydroxyl groups of diOH18 : 0 were likely to be the same as those of the diOH18 : 1 and diOH18 : 2 (Figs. 24.1B and 24.1C). The standard 11,12-dihydroxyoctadecanoic acid is not commercially available. Instead we used the standard, 9,10-dihydroxyoctadecanoic acid, for MS^2 (spectrum not given here) to compare with Figure 24.8 showing diOH18 : 0 from castor oil. These two MS spectra were similar when the same collision energy 37% was used including the relative abundances of the precursor ions (about 23%). The MS spectrum of standard 12-hydroxyoctadecanoic acid (not given here) at the same collision energy 37% also showed the only one significant fragment ion $[OH18:0 + Li - H_2O]^+$ at *m/z* 289.2. However, the relative abundance of the

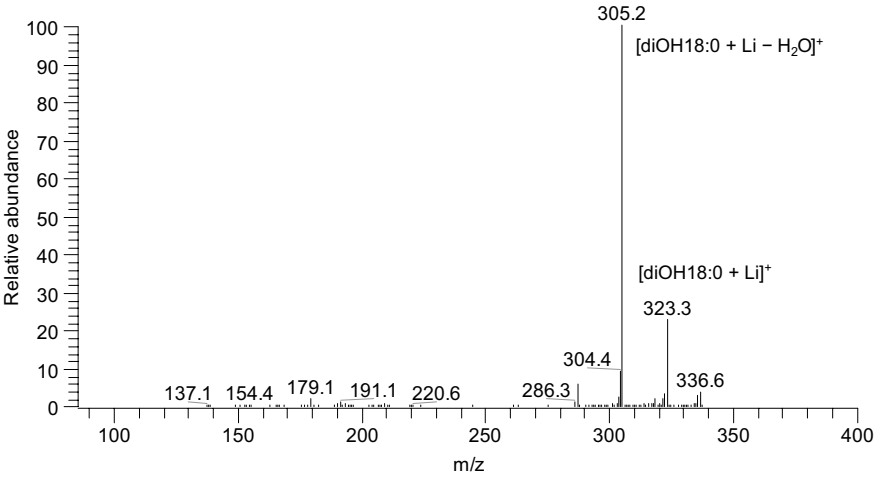

FIGURE 24.8 Ion trap mass spectrum of ESI-MS^4 of dihydroxyoctadecanoic acid [diOH18 : 0 + Li]$^+$ at *m/z* 323.3 (collision energy 37%). This was originated from MS^2 (collision energy 36%) of [diOH18 : 0-OH18 : 1-OH18 : 1 + Li]$^+$ at *m/z* 957.7 from the HPLC fraction 15 of castor oil and then MS^3 (collision energy 39%) of [diOH18 : 0-OH18 : 1-OH18 : 1 + Li – OH18 : 1]$^+$ at *m/z* 659.5.

precursor ion was only 1.5%. This suggested that the diOH18 : 0 in castor oil contained two hydroxyl groups attached to adjacent carbon atoms. The dehydration [diOH18 : 0 + Li – H_2O]$^+$ with two hydroxyl groups attached to adjacent carbon atoms on the fatty acid chain required more collision energy than that of [OH18 : 0 + Li – H_2O]$^+$ with monohydroxyl group on the chain. We propose here the structure of diOH18 : 0 as 11,12-dihydroxyoctadecanoic acid (Fig. 24.1D).

24.4 STRUCTURES OF ACYLGLYCEROLS CONTAINING DIHYDROXY FATTY ACIDS

Figure 24.2 shows the four diacylglycerols and eight triacylglycerols containing dihydroxy fatty acids in castor oil identified by MS. The stereospecific locations of fatty acids on the glycerol backbones were not determined. The mass *m/z* of AG lithium adducts are also given in Fig. 24.2. Similar to Fig. 24.3, AG molecular species in Fig. 24.2 were identified by MS^2 of the individual AG lithium adducts. The MS^2 spectra (not shown here) showed the fragment ions from the loss of fatty acids, diOH18 : 2 *m/z* 312, diOH18 : 1 *m/z* 314, diOH18 : 0 *m/z* 316, and OH18 : 0 *m/z* 298. The MS^3 (from diacylglycerols) and MS^4 (from triacylglycerols) spectra (not shown here) of the dihydroxy fatty acids showed that these dihydroxy fatty acids were the same as those of the Figs. 24.4, 24.7 and 24.8.

In this HPLC fractionation, some AG detected by MS were eluted in many fractions. The HPLC resolutions of these AG containing dihydroxy fatty acids were

not good and the accurate quantifications of these AG were difficult. However, according to the HPLC chromatogram of castor oil detected by evaporative light-scattering detector using the same HPLC conditions published earlier (Lin et al., 2003), the individual AG containing dihydroxy fatty acids were about 0.5% of castor oil or less. The total AG containing dihydroxy fatty acids were about 2.5% of castor oil. These AG were eluted before triricinolein. Among these 12 AG (Fig. 24.2), the contents of triacylglycerols, diOH18 : 1-OH18 : 1-OH18 : 1, diOH18 : 0-OH18 : 1-OH18 : 1, diOH18 : 1-diOH18 : 1-OH18 : 1, diOH18 : 1-diOH18 : 0-OH18 : 1, and diacylglycerol, diOH18 : 1-OH18 : 1 were higher than those of the other AG in castor oil.

24.5 REGIOSPECIFIC QUANTIFICATION OF TRIACYLGLYCEROLS

Figure 24.9 shows the structures of the most abundant regioisomers of the triacylglycerols containing dihydroxy fatty acids in castor oil. MS^1 of the lithium adducts of Fraction 12 (retention time 5.5–6.0 min, the spectrum not shown here) showed the ion at *m/z* 971.6 (lithium adduct of TAG, diOH18 : 1-OH18 : 1-diOH18 : 1, ABA, with two identical fatty acids, Fig. 24.9) and the ion at *m/z* 987.6 (lithium adduct of TAG, diOH18 : 1-diOH18 : 1-diOH18 : 1, AAA, with three identical fatty acids, Fig. 24.9). The ion at *m/z* 971.6 was the most prominent ion. Figure 24.10 is the MS^2 spectrum of diOH18 : 1-OH18 : 1-diOH18 : 1 $[M + Li]^+$ at *m/z* 971.6. The two prominent fragment ions were from the losses of ricinoleate OH18 : 1, $[M + Li - OH18 : 1]^+$ at *m/z* 673.5, and diOH18 : 1, $[M + Li - diOH18 : 1]^+$ at *m/z* 657.5.

Figure 24.11 is the MS^3 spectrum of $[M + Li - diOH18 : 1]^+$ at *m/z* 657.5 from Fig. 24.10. The fragment ions from the loss of fatty acid specific at the *sn*-2 position as

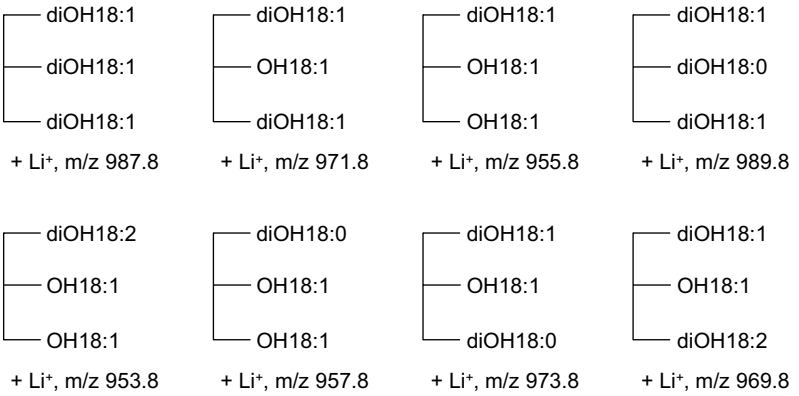

FIGURE 24.9 The structures of the most abundant regioisomers of the triacylglycerols containing dihydroxy fatty acids in castor oil. The calculated *m/z* of the lithium adducts of triacylglycerols are also shown.

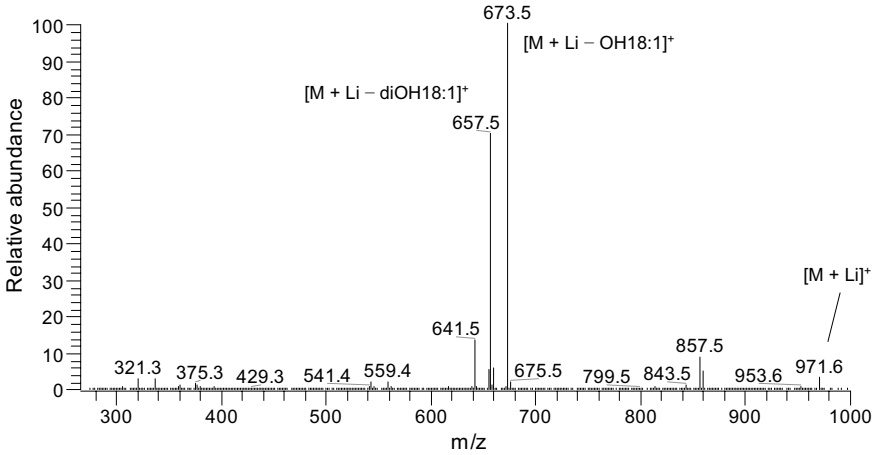

FIGURE 24.10 Ion trap mass spectrum of ESI-MS^2 of diOH18 : 1-OH18 : 1-diOH18 : 1 $[M + Li]^+$ at *m/z* 971.6 in HPLC fraction 12 of castor oil (collision energy 36%). Abbreviations: diOH18 : 1 is dihydroxyoleate, OH18 : 1 is ricinoleate.

α,β-unsaturated fatty acid were $[M + Li - diOH18:1 - OH18:1 + 2]^+$ at *m/z* 361.2 and $[M + Li - diOH18:1 - diOH18:1 + 2]^+$ at *m/z* 345.2. Both (OH18 : 1–2) and (diOH18 : 1–2) were α,β-unsaturated fatty acids. The abundances of these two fragment ions *m/z* 361.2 and *m/z* 345.2 represented the relative contents of fatty acids at the *sn*-2 position (Lin and Arcinas, 2007). The content of regiospecific (or stereospecific) diOH18 : 1-OH18 : 1-diOH18 : 1 was 89% in the total of the three stereospecific diOH18 :

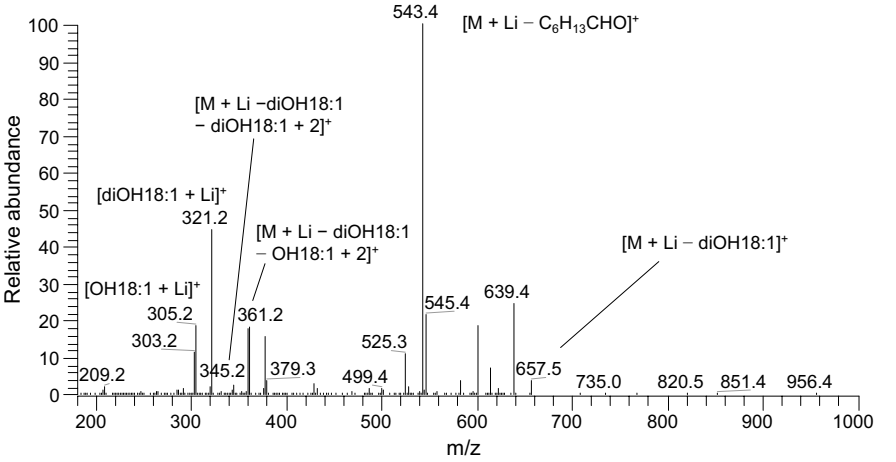

FIGURE 24.11 Ion trap mass spectrum of ESI-MS^3 of diOH18 : 1-OH18 : 1-diOH18 : 1. This was from $[M + Li - diOH18:1]^+$ at *m/z* 657.5 of Fig. 24.10 (collision energy 38%). Abbreviations: see Fig. 24.10. $C_6H_{13}CHO$ is an aldehyde.

1-OH18 : 1-diOH18 : 1, diOH18 : 1-diOH18 : 1-OH18 : 1, and OH18 : 1-diOH18 : 1-diOH18 : 1 combined. The same contents in other HPLC fractions were 94% (fraction 13), 92% (fraction 14), 94% (fraction 15), 89% (fraction 16), 89% (fraction 17), 94% (fraction 18). The contents were similar at the average of 92%. The resolution of TAG containing dihydroxy fatty acids was not good for this HPLC system. Also the regioisomers could not be separated by this HPLC system. The base ion at *m/z* 543.4 (Fig. 24.11) was from the loss of $C_6H_{13}CHO$. The loss of $C_6H_{13}CHO$ (or shown earlier as $C_7H_{14}O$) has been previously reported (Lin and Arcinas, 2007, 2008a; Byrdwell and Neff, 1998) from the cleavage between C-11 and C-12 of the ricinoleoyl chain for TAG containing ricinoleate.

The contents of the other regioisomeric AAB were estimated as described as that of diOH18 : 1-OH18 : 1-diOH18 : 1. The content of regioisomer diOH18 : 1-OH18 : 1-OH18 : 1 (ABB), $[M + Li]^+$ at *m/z* 955.7, was estimated as follows: 95% (fraction 13), 95% (fraction 14), 95% (fraction 15), 88% (fraction 16), 86% (fraction 17), and 92% (fraction 18), at the average of 92%. The content of regio-isomer diOH18 : 1-diOH18 : 0-diOH18 : 1 (ABA), $[M + Li]^+$ at *m/z* 989.7, was 91% (fraction 13). The content of regioisomer diOH18 : 2-OH18 : 1-OH18 : 1 (ABB), $[M + Li]^+$ at *m/z* 953.7, was 80% (fraction 15). The content of regioisomer diOH18 : 0-OH18 : 1-OH18 : 1 (ABB), $[M + Li]^+$ at *m/z* 957.8, was estimated as 97% (fraction 15) and 94% (fraction 16), at the average of 96%.

24.6 RATIOS OF FATTY ACIDS AT THE *SN*-2 POSITION OF TRIACYLGLYCEROLS

The ratio of the three different fatty acids at the *sn*-2 position of TAG (ABC) also can be estimated by the abundances of the fragment ions (MS^3) from the loss of the fatty acids at the *sn*-2 position as α,β-unsaturated fatty acids (Lin and Arcinas, 2007). For diOH18 : 1-OH18 : 1-diOH18 : 0 (ABC, see Fig. 24.9) in fraction 13 (6–6.5 min), the MS^2 spectrum (not shown here) of $[M + Li]^+$ at *m/z* 973.6 showed the fragment ions from the losses of the three fatty acids as follows: $[M + Li - diOH18 : 0]^+$ at *m/z* 657.4, $[M + Li - diOH18 : 1]^+$ at *m/z* 659.4 and $[M + Li - OH18 : 1]^+$ at *m/z* 675.4. The MS^3 spectrum (not shown here) of $[M + Li - diOH18 : 0]^+$ at *m/z* 657.5 showed the fragment ions from the losses of fatty acids at the *sn*-2 position as α,β-unsaturated fatty acids, $[M + Li - diOH18 : 0–OH18 : 1 + 2]^+$ at *m/z* 361.2 and $[M + Li - diOH18 : 0–diOH18 : 1 + 2]^+$ at *m/z* 345.3. The ratio of the abundances of these two fragment ions was 88 : 12. So the ratio of contents of OH18 : 1 (ricinoleate) and diOH18 : 1 at the *sn*-2 position was about 88 : 12. The MS^3 spectrum (not shown here) of $[M + Li - diOH18 : 1]^+$ at *m/z* 659.4 showed the fragment ions from the losses of fatty acids at the *sn*-2 position as α,β-unsaturated fatty acids, $[M + Li - diOH18 : 1–OH18 : 1 + 2]^+$ at *m/z* 363.2 and $[M + Li - diOH18 : 1 - diOH18 : 0 + 2]^+$ at *m/z* 345.2. The ratio of the abundances of these two fragment ions was 81 : 19. So the ratio of the contents of OH18 : 1 (ricinoleate) and diOH18 : 0 at the *sn*-2 position was about 81 : 19. From these two ratios, 88 : 12 (OH18 : 1: diOH18 : 1 at the *sn*-2 position) and 81 : 19 (OH18 : 1: diOH18 : 0 at the *sn*-2

position), the ratios of these three fatty acids at the *sn*-2 position were calculated as about 73 : 17 : 10 (OH18 : 1 : diOH18 : 0 : diOH18 : 1). Using the same method, the ratios of the three different fatty acids (ABC) at the *sn*-2 position of diOH18 : 1-OH18 : 1-diOH18 : 2, $[M + Li]^+$ at *m*/*z* 969.6 (see Fig. 24.9) in fraction 15 (7.0–7.5 min) were estimated as about 71 : 23 : 6 (OH18 : 1 : diOH18 : 2 : diOH18 : 1). The ratios of contents of three fatty acids at the *sn*-2 position of TAG (ABC) by MS were first reported.

24.7 CONCLUSIONS

The identification of four molecular species of diacylglycerols and eight molecular species of triacylglycerols containing dihydroxy fatty acids in castor oil was reported. The structures of the three dihydroxy fatty acids were proposed as 11,12-dihydroxy-9-octadecenoic acid, 11,12-dihydroxy-9,13-octadecadienoic acid, and 11,12-dihydroxyoctadecanoic acid. Ricinoleate was predominately at the *sn*-2 position of TAG (AAB and ABC) containing dihydroxy fatty acids and the dihydroxy fatty acids were mainly at the *sn*-1,3 position in castor oil. The AG containing dihydroxy fatty acids in castor oil can be isolated for industrial uses although the total content is low (about 2.5%). The regiospecific identification of TAG affects the physical (Foubert et al., 2007) and chemical properties of the oil and it will help in the industrial uses of the oil.

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25

BIOCATALYTIC PRODUCTION OF LACTOBIONIC ACID

HIROFUMI NAKANO, TAKAAKI KIRYU, TARO KISO, AND HIROMI MURAKAMI

Osaka Municipal Technical Research Institute, 1-6-50, Morinomiya, Joto-ku, Osaka 536-8553, Japan

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25.1 INTRODUCTION

Lactobionic acid (β-*O*-D-galactosyl D-gluconic acid) is an aldobionic acid derived from the oxidation of lactose. Lactose, which is obtained from a dairy by-product, cheese whey, is one of the most useful carbohydrates available cheaply, abundantly, and renewably. It is estimated, however, that 3–4 million tons of lactose are wasted in the form of whey, which corresponds to approximately 40% of its annual production. The conversion of lactose to lactobionic acid and its salts, therefore, seems to be a promising approach for exploiting such an underutilized resource. Since Fischer and Meyer (1889) first described the oxidation of lactose with bromine, chemical and electrochemical production methods have been developed (Sen Gupta et al., 1968; Druliolle et al., 1995; Fuertes and Fleche, 1991). At present, lactobionic acid is produced by the chemical method for use as a pharmaceutical ingredient. In spite of the reaction optimization, however, the yield still remains low, as little as 30%, mainly due to the cleavage of the glycosidic linkage (Yang and Montgomery, 2005). Moreover, the health-promoting activities of the acid and its salts have been revealed in recent years, which suggest their potential uses as functional foods. Therefore, production of lactobionic acid using the biocatalytic approach, which has become a subject of interest, has general advantages such as high selectivity, high efficiency, simple reaction systems, mild reaction conditions, and avoidance of poisonous chemicals. In the bioconversion, lactose is oxidized by specific enzymes to form lactobiono-δ-lactone (1,5-lactone) and hydrogen peroxide (H_2O_2) using electron acceptors such as molecular oxygen (O_2), in which the lactone is subsequently hydrolyzed to lactobionic acid (Fig. 25.1). The hydrolysis of the aldonate lactone occurs spontaneously in aqueous solutions, or in some microorganisms, the participation of specific enzymes, lactonases, is also suggested (Nishizuka and Hayaishi, 1962). For the practical production of lactobionic acid, several microorganisms and their enzymes have been investigated as selective and efficient biocatalysts. This article reviews the practical and potential uses of lactobionic acid

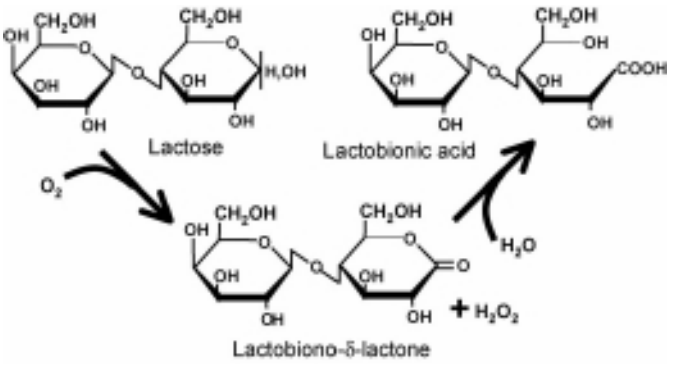

FIGURE 25.1 Scheme of bioconversion of lactose to lactobionic acid. Lactose is oxidized to produce lactobiono-δ-lactone, which is subsequently hydrolyzed to lactobionic acid.

and its salts, the occurrence in foods, and the biocatalytic production. Special focus is given in our studies on the production of the calcium salt (Kiryu et al., 2007; Murakami et al., 2008) by a fungal carbohydrate oxidase (Kiryu et al. 2008).

25.2 PRACTICAL AND FEASIBLE APPLICATIONS

25.2.1 Uses in the Pharmaceutical and Cosmetic Industries

Lactobionic acid possesses practical importance mainly in the pharmaceutical industry: one of its largest commercial uses is as an ingredient of erythromycin lactobionate, which is 50–100 times more soluble than the free antibiotic (Hoffhine, 1956; Shi et al., 1989). The acid is also added to the preservative fluid for transplanting organs. Because of its chelating ability for metal ions, oxidative damage to tissues is reduced during the storage and the subsequent reperfusion, allowing organs to be preserved outside of the body for up to two days (Southard and Bezer, 1995; Sumimoto et al., 1992). Calcium lactobionate stabilizes the supersaturated solution of calcium gluconate, a traditional medicine for calcium supplementation. The acid is currently added to skin care cosmetics. Lactobionic acid is a hygroscopic compound. Thus its excellent water retention and absorbing properties are considered to contribute to the skin conditioning benefits. In addition to such uses as expensive specialty chemicals, the following applications are proposed, some of which require bulk production at prices competitive to those of other polyhydroxyl organic acids such as gluconate, citrate, and lactate: materials for biodegradable polymers, agents for the inhibition or prevention of metal rust, scale inhibitor for boilers, and builders for detergents (Gerling, 1998).

25.2.2 Uses in the Food Industry

Lactobionic acid has both mild sourness and sweetness. The mineral salts of the acid also have no peculiar taste. For example, copper and iron salts of lactobionic acid can be added to milk without imparting oxidized flavors and odors (Hagenauer et al., 1979). Lactobionic acid shows resistance to the hydrolysis by the intestinal digestive enzymes (Harju, 1990), even though it is hydrolyzed very slowly compared to lactose by certain microbial β-galactosidases (Nakano et al., 1998). Therefore, the acid may undergo fermentation by the intestinal microbial flora.

Several applications in the food industry have been considered (Gerling, 1998): reduction of souring and ripening time for the production of cheese and yogurt, enhancement of flavors, and improvement of bitter or sour taste of foods (Booij, 1985). Lactobionic acid coagulates protein solutions, as is observed in gluconic acid or its lactone. Therefore, the acid is registered with the U.S. Food and Drug Administration as a thickening food additive for pudding mixes. Moreover, health-promoting functions have been revealed to explore methods for its feasible application in the food industry. Lactobionate promotes intestinal absorption of minerals (Suguri et al., 1995a; Oe et al., 2008) to improve bone metabolic balance. Possible use as

TABLE 25.1 Water Solubility of Several Calcium Salts

Calcium Salt	Solubility ($g\,dL^{-1}$)
Oxalate	0.001
Carbonate	0.0014
Diphosphate	1.8
Citrate	0.25–0.96
Lactate	5.0–9.6
Gluconate	3.3–3.8
Lactobionate	62

a prebiotic to stimulate the proliferation of Bifidobacteria has also been suggested (Suguri et al., 1995b). Similarly, it has been suggested that poultry feed supplemented with calcium lactobionate can strengthen egg shells to reduce the cracking ratio (Kimura and Dompou, 2004).

The excellent solubility of lactobionate, especially that of calcium salt as shown in Table 25.1, should be noted. Owing to two glycosyl moieties and a carboxyl group, calcium lactobionate dissolves at more than $60\,g\,dL^{-1}$, which is 10–60 times higher than those of gluconate, lactate, and citrate. Lactobionate is approximately 28,000 times more soluble than $CaCO_3$ that is commonly added to various foods for the purpose of calcium supplementation. Other soluble calcium salts, for example, $CaCl_2$, have a bitter taste, while calcium lactobionate has no such unfavorable taste. A relatively high threshold concentration as well as the good taste may allow the addition of sufficient minerals, for example, to cold beverages of a high clarity.

25.2.3 Occurrence in Foods

In spite of the wide distribution of lactose in mammalian milk, the occurrence of lactobionic acid in foods has not been demonstrated. The acid was first discovered in a Caucasian traditional yogurt, which has been produced domestically as well as commercially in Japan for years under the common name of "Caspian Sea yogurt" (Kiryu et al., 2009). *Acetobacter orientalis* and *Lactococcus lactis* ssp. *cremoris* participate in the fermentation (Ishida et al., 2005). The latter lactic acid bacterium plays the main role in the fermentation, while the former aerobic acetic acid bacterium that grows in the upper layer of the yogurt is responsible for the production of lactobionic acid. Therefore, lactobionic acid is detected predominantly in the upper layer as shown in Figure 25.2. The washed cells of *Acetobacter* oxidize D-glucose to D-glucono-δ-lactone at a considerable rate, whereas the activities for lactose, maltose, and cellobiose were much lower. The occurrence of lactobionic acid in a traditional food helps its application in the food industry, because it indicates that people have unconsciously consumed the acid for a long time. This also encouraged us to investigate its production by acetic acid bacteria, microorganisms that are generally recognized as safe. Further investigations are now in progress to optimize the bioconversion of lactose by acetic acid bacteria (Oe et al., 2008).

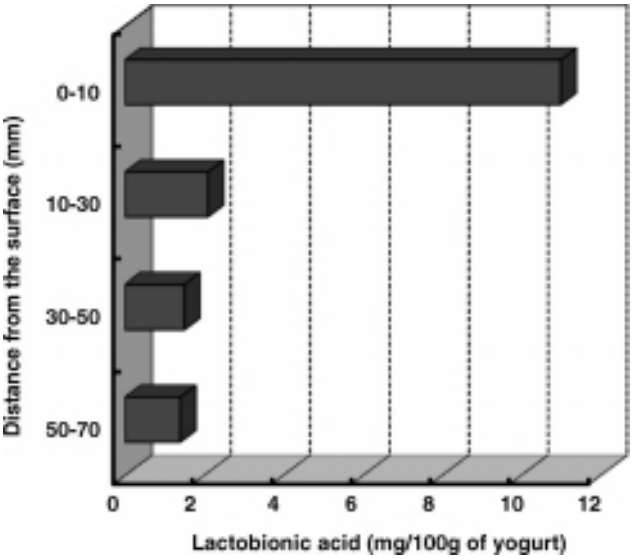

FIGURE 25.2 Production of lactobionic acid in various layers of Caucasian yogurt. Lactobionic acid distributes more in the upper layer of the yogurt, where *Acetobacter orientalis*, an aerobe, grows well and oxidizes lactose.

25.3 BIOCATALYTIC PRODUCTION METHODS

25.3.1 Production by Microbial Processes

Several authors have repeatedly confirmed the lactose-oxidizing activity in *Pseudomonas* species (Kluyver et al., 1951; Nishizuka and Hayaishi, 1962; Miyamoto et al., 2000). Bioconversion of lactose to lactobionic acid has also been observed in other microorganisms such as *Halobacterium sacharovorum* (Tomlinson et al., 1978) and *Penicillium chrysogenum* (Cort et al., 1956). We performed screening of microorganisms that possessed not only a high lactose-oxidizing activity, but no β-galactosidase activity that decomposes the substrate and the product (Murakami et al., 2002). A strain of *Burkholderia cepacia* was found to produce lactobionate considerably. The bacterium was used for fermentative production (Murakami et al., 2002, 2003), in which the oxidation of lactose occurs along with the cell growth, and then for the bioconversion with the resting cells (Murakami et al., 2006a, 2006b). The lactose-oxidizing activity is localized in the membrane fraction of the disrupted cells. The productivity of lactobionate as well as other aldonic acids was improved by the selection of a mutant strain that had a tolerance to high lactose concentrations. The resting cells were subjected to repeated batch conversion (Murakami et al., 2006a, 2006b), which not only saved the catalyst but also facilitated the subsequent refining of the product. Recently, Oe et al. (2008) reported resting cells of a strain of acetic acid bacteria, *Gluconobacter*, converted 300 g L^{-1} lactose and 30 g L^{-1} $CaCO_3$ to calcium lactobionate in 80% yield within 48 h at 40°C.

25.3.2 Production by Enzymes

Several redox enzymes serve as catalysts for the oxidation of lactose. A flavin-containing enzyme designated as lactose dehydrogenase from *Pseudomonas graveolens* mediates effective oxidation (Nishizuka and Hayaishi, 1962). Practically, however, difficulties exist in the extraction of the membrane-bound enzyme, and in the requirement not of O_2 as a preferable electron acceptor, but of the toxic and expensive redox mediators such as 2,6-dichlorophenolindophenol (DCIP), methylene blue, or ferrocyanide. Cellobiose dehydrogenase from *Sclerotium rolfsii* was studied in view of the production of lactobionic acid (Baminger et al., 2001; Ludwig et al., 2004). The enzyme also shows low specificity for O_2. Therefore, the conversion was conducted in the presence of redox mediators such as benzoquinones, catechol, and DCIP as well as *Trametes* laccase for reoxidizing the mediator using O_2 as the terminal electron acceptor. The use of such chemical mediators may be unfavorable for the practical production of the acid, especially for those of the food grade in view of the safety.

The use of a glucose-fructose oxidoreductase from *Zymomonas mobilis* provides a new production scheme (Nidetzky et al., 1997; Satory et al., 1997). The enzyme catalyzes the transhydrogenation, in which the oxidation of various aldoses to the corresponding lactone is coupled with the reduction of D-fructose to sorbitol. The conversion was conducted in batch, fed-batch, and continuous reaction modes to achieve a productivity of 110 g $(L\,d)^{-1}$ (Satory et al., 1997). The following disadvantages are suggested in this approach: the requirement of a pair of substrates, which leads to two products, lactobionic acid and sorbitol; the low affinity for lactose; and the moderate enzyme stability.

Some carbohydrate oxidases produce lactobionic acid. Hexose oxidases from red algae have a broad substrate spectrum (Sullivan and Ikawa, 1973). A cloned enzyme from *Chondrus crispus* is commercially available for food-manufacturing uses. However, the operational stability may not be satisfactory as described later. A FAD-containing carbohydrate oxidase designated as glucooligosaccharide oxidase from *Acremonium strictum* has been found to oxidize not only lactose but also various oligosaccharides with 1,4-glycosidic linkages (Lin et al., 1991; Lee et al., 2005). The enzyme shows optimum activity in the alkaline region (pH 10), where isomeration of lactose to lactulose can occur to reduce the yield. Moreover, it is difficult to maintain such alkaline region using $CaCO_3$ as a neutralization agent. A carbohydrate oxidase from *Microdochium nivale*, which is cloned and expressed in *Fusarium*, has high activity on lactose and other oligosaccharides (Xu et al., 2001). The effect of dissolved oxygen (DO) on the reaction kinetics as well as the operational stability of the enzyme has been investigated in a reaction system at constant pH and temperature in a stirred tank reactor with a working volume of 1 L (Nordkvist et al., 2007). It was reported that a weak base (ammonia) solution was effective for the neutralization of lactobionic acid to increase the operational stability of the enzyme and thus the productivity. The conversion was then performed in a 600-L pilot-scale reactor equipped with a rotary jet head to ensure sufficient mixing and mass transfer (Hua et al., 2007). In such a system, lactobionate was obtained at

more than 98% yields from 50 g L^{-1} lactose and whey permeate of the same lactose concentration as well.

As a potent producer of lactobionic acid, we isolated a fungal strain identified as *Paraconiothyrium* that belongs to Ascomycetes (Kiryu et al., 2008). The fungus produced an extracellular carbohydrate oxidase abbreviated as PCOX. In the following few sections, the results of our studies on the PCOX-catalyzed production of calcium lactobionate are described.

25.4 PRODUCTION BY *Paraconiothyrium* OXIDASE

25.4.1 Enzyme Properties

A carbohydrate oxidase from *Paraconiothyrium*, PCOX, was purified to an apparent homogeneity from the culture supernatant (Kiryu et al., 2008). The enzyme is a FAD-containing protein with an approximate molecular mass of 54 kDa. The inexistence of heme was indicated by spectrometric analysis. The enzyme is most active at around pH 5.5 and 50°C and stable at pH 2.0–7.0. O_2 and DCIP serve as good electron acceptors, while cytochrome C, methylene blue, and $FeCl_3$ are ineffective. This means lactose can be oxidized simply by aeration. Figure 25.3 illustrates the substrate (electron-donor) specificity of PCOX. Lactose of the α-anomeric type did not act as a substrate, but of the β-type did, as is often observed in other carbohydrate oxidases. PCOX exhibited the activity on various aldoses including mono- and oligosaccharides having several degrees of polymerization. A loose recognition at the C4 position of the reducing-end residues was suggested by the oxidation of substrates such as cellobiose, maltose, and D-galactose. On the contrary, the enzyme appeared to strictly recognize the C2 and C3 positions, which was suggested by no activities on oligosaccharides substituted at these positions or the epimers of D-glucose of these positions. Substrates harboring bulky groups at the C6 position may be unacceptable, which was deduced from no oxidation of glucuronic acid and 1,6-linked disaccharides. As for the specificity on oligosaccharides, therefore, effective substrates are restricted to 1,4-linked types, cellooligosaccharides and maltooligosaccharides.

Table 25.2 summarizes the properties of PCOX and the related enzymes. PCOX that lacks heme in the structure is obviously discriminated from cellobiose

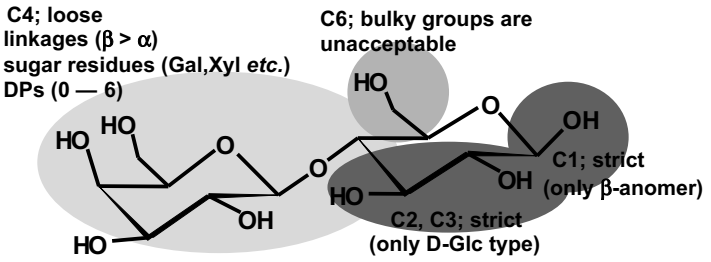

FIGURE 25.3 Summary of substrate (donor) specificity of PCOX.

TABLE 25.2 Some Properties of Enzyme from *Paraconiothyrium* and Related Enzymes

	Paraconiothyrium sp. This enzyme	*Microdochium nivale* COX	*Spotoyrichum pulverolentum* CBQ	*Acremonium strictum* GOOX	*Phanerochaete chrysosporium* CDH
Optimum pH	5.5 (O_2), 4.5 (DCIP)	5.5 (O_2)	6.0 (DCIP)	10.0 (O_2)	5.0 (DCIP)
Anomeric specificity	β-form	ND	ND	β-form	β-form
Absorption drived from					
FAD	+	+	+	+	+
Heme	−	−	−	−	+
Molecular mass (kDa)	54 (SDS-PAGE)	55	60	61	90
Substrate specificity (k_{cat}/K_m)					
Lactose ($mM^{-1}\,S^{-1}$)	57 (O_2), 23 (DCIP)	ND	17–15 (DCIP)	210 (O_2)	12 (DCIP)
Cellobiose ($mM^{-1}\,S^{-1}$)	69 (O_2), 86(DCIP)	0.21, 0.20* (O_2)	350–330 (DCIP)	110 (O_2)	140 (DCIP)
Maltose ($mM^{-1}\,S^{-1}$)	0.18 (O_2), 0.16 (DCIP)	0.55* (O_2)	0.26–0.27 (DCIP)	3.6 (O_2)	0.0048 (DCIP)
D-Glucose ($mM^{-1}\,S^{-1}$)	0.084 (O_2), 0.0023 (DCIP)	0.095* (O_2)	0.0030–0.0032 (DCIP)	1.1 (O_2)	0.0016 (DCIP)
Electron acceptor					
O_2	+	+	+	+	
DCIP	+	+	+	ND	
Cytochrome C	−	−	−	ND	
Reference	Kiryu et al. (2008)	Xu et al. (2001)	Morpeth and Jones (1986) Raices et al. (2002)	Lin et al. (1991); Lee et al. (2005)	Henriksson et al. (1998, 2000)

Note: ND, not determined; −, not detected.

*The values were obtained with recombinant enzyme.

dehydrogenases (EC 1. 1. 99. 18, CDH) (Henriksson et al., 1998, 2000). CDH and cellobiose, quinone oxidoreductases (EC 1. 1. 5. 1, CBQ) (Morpeth and Jones, 1986; Raices et al., 2002) have similar amino acid sequences, and CBQ is considered to be produced by the proteolysis of CDH (Raices et al., 2002). The enzymes termed as glucooligosaccharides oxidases (GOOX) (Lin et al., 1991; Lee et al., 2005), and carbohydrate acceptor oxidoreductase (COX) (Xu et al., 2001), however, are similar to PCOX in the properties of, for instance, molecular mass of 55–60 kDa, broad substrate specificity, and the presence of FAD as a prosthetic group. We also suggested a high homology (40.8%) in the amino acid sequences of COX and GOOX as well as a high similarity in the N-terminal sequences between PCOX and COX (Kiryu et al., 2008). Thus we concluded PCOX should be included in the enzyme group of COX and GOOX.

25.4.2 Small-Scale Production

We attempted the PCOX-mediated production of calcium lactobionate. Small-scale batch reactions were performed in L-shaped tubes with the aeration by reciprocal shaking (Murakami et al., 2008). On this scale, the productivity of calcium lactobionate was compared with a hexose oxidase from a red alga, *C. crispus* (Sullivan and Ikawa, 1973) (Fig. 25.4). The yield from 100 g L^{-1} lactose with the algal enzyme was as high as 15% in the absence of solid $CaCO_3$, where the enzyme was inactivated quickly due to the pH decline. Then in the presence of $CaCO_3$, pH shifted to 7.0–9.0, which was inadequate for the action of the hexose oxidase. PCOX was more stable and active in a wider pH region. A higher yield of approximately 75% was obtained even

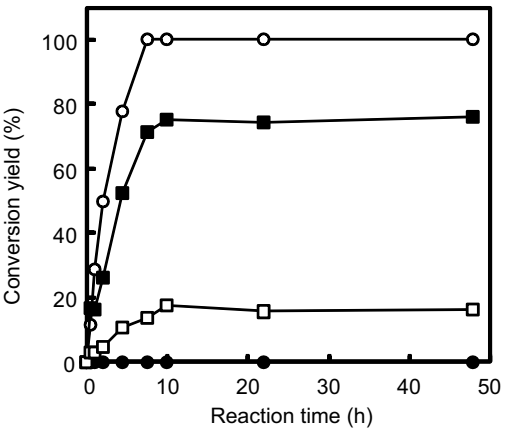

FIGURE 25.4 Conversion of lactose by PCOX and an algal hexose oxidase. Lactose (100 g L^{-1}) was reacted with PCOX and the algal hexose oxidase at 40°C with reciprocal shaking at 120 rpm. Closed circles, the algal enzyme in the presence of 1.4% $CaCO_3$; open squares, algal enzyme without $CaCO_3$; open circles, PCOX in the presence of 1.4% $CaCO_3$; closed squares, PCOX without $CaCO_3$.

in the absence of $CaCO_3$, where pH decreased to 3.0 along with the production of the acid. By the addition of solid $CaCO_3$, pH was maintained in a moderate range, pH 6.9–7.1, and calcium lactobionate was produced in 100% yield within 10 h.

25.4.3 Production in a Stirred Tank Reactor

Lactobionate was produced using an initial working volume of 0.5 L contained in a 2.0-L stirred tank reactor equipped with monitoring electrodes for pH and DO (Murakami et al., 2008). Base solution to neutralize the acidic product was fed as required to maintain a constant pH. Typical reaction courses are given in Figure 25.5 that shows the conversion of 150 g L^{-1} lactose at 40°C and pH maintained 5.5 either with 250 g L^{-1} NaOH or with 10% (w/w) $CaCO_3$ slurry. The neutralization with $CaCO_3$, commercial slurry in which fine $CaCO_3$ particles were suspended and stabilized, afforded complete conversion after 13 h. The pH stat reaction using a concentrated NaOH solution, however, decreased the yield to 70%, which was attributable to gradual inactivation of the enzyme by the strong base solution during the dilution. $CaCO_3$ is advantageous not only as a source of calcium ion that enables the one-pot production of calcium lactobionate, but also as a mild neutralization reagent.

As a result of further optimization of the working conditions, calcium lactobionate can be produced at an approximate rate of 9–11 g $(h\,L)^{-1}$, in which 100–150 g L^{-1} lactose were converted completely to calcium lactobionate within 10–20 h. For 200 g L^{-1} lactose, however, the conversion rate decreased almost by half, because

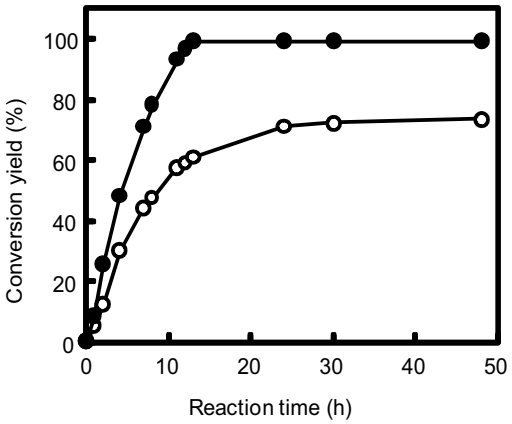

FIGURE 25.5 Conversion of lactose in a 2-L reactor under pH control with NaOH and $CaCO_3$. Reaction mixtures (0.5 L) containing PCOX (500 U L^{-1}), 150 g L^{-1} lactose, 50 mM acetate buffer (pH 5.5), *Aspergillus niger* catalase (100,000 U L^{-1}), and antifoam reagent (2.0 g L^{-1}) were incubated at 40°C with aeration (0.5 L min^{-1}) and agitation (450 rpm). pH was adjusted at 5.5 with either 250 g L^{-1} NaOH (open circles) or 10% (w/w) $CaCO_3$ slurry (closed circles).

of the substrate inhibition. The reaction rate was also affected greatly by the supply of O_2, so that constant aeration and vigorous mixing were required. The supplementation of catalase was found to be effective in increasing the DO level by decomposing H_2O_2. These operational conditions obtained in the 0.5-L scale can be applied to the reaction in the working volume of 10-L using a 30-L reactor to achieve 100% yield within 24 h. A syrup sample of 71% (w/w) calcium lactobionate was prepared by the concentration of the reaction mixture after treatment with activated carbon. A white powder obtained by spray drying meets the requirement of the US Pharmacopeias.

25.4.4 Production by Immobilized Enzyme

PCOX was immobilized on a cation exchange resin, CM-Sepharose, followed by the treatment with carbodiimide (Kiryu et al., 2007). The free and immobilized enzymes showed similar profiles in the optimum reaction conditions and the stability. In small-batch reactions with reciprocal shaking, the immobilized enzyme oxidized 185 g L^{-1} lactose completely to calcium lactobionate in the presence of solid $CaCO_3$. In the oxidation of an increased substrate concentration of 370 g L^{-1} lactose, however, the yield decreased to less than 50% due to the substrate inhibition as was observed in the reactions with the free enzyme. In addition, the repeated use of the immobilized enzyme for several batch reactions resulted in a significant decrease of the activity, which was ascribed not to the liberation of the enzyme from the carrier, but to the inactivation by H_2O_2. Therefore, difficulty in the continuous conversion of the immobilized enzyme still remains to be investigated.

25.5 CONCLUSIONS

Lactobionic acid and its salts are attractive as new and value-added food materials because of the good taste, the health-promoting functions, as well as the excellent solubility. The recent finding on the existence in a traditional yogurt may be important for more widespread applications. Efficient and safer processes for the production are therefore increasingly desired. As overviewed in this chapter, bioconversion seems promising. However, more efforts have to be made particularly on the increase in the entries of enzymes and microorganisms having high biological safety, improvement in the activity and stability, construction of a cost-effective operating system to secure a high oxygen level and avoid the inhibition at high substrate concentrations, and hopefully adoption of the continuous or repeated use processes to save the valuable biocatalysts.

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26

RECENT ADVANCES IN ALDOLASE-CATALYZED SYNTHESIS OF UNNATURAL SUGARS AND IMINOCYCLITOLS

MASAKAZU SUGIYAMA[a], ZHANGYONG HONG[b], WILLIAM A. GREENBERG[b], AND CHI-HUEY WONG[b]

[a]*AminoScience Laboratories, Ajinomoto Co. Inc., 1-1 Suzukicho, Kawasakiku, Kawasakishi, Japan, 210-8681*

[b]*Department of Chemistry, The Scripps Research Institute, 10550, N. Torrey Pines Rd., La Jolla, CA 92037, USA*

26.1 INTRODUCTION

The aldol reaction has been regarded as one of the most useful tools for organic synthesis due to its ability to form new carbon–carbon bonds. The control of stereogenic centers in the aldol reaction highly attracts organic chemists to fashion a broad range of both natural and novel polyhydroxylated compounds. Many efforts

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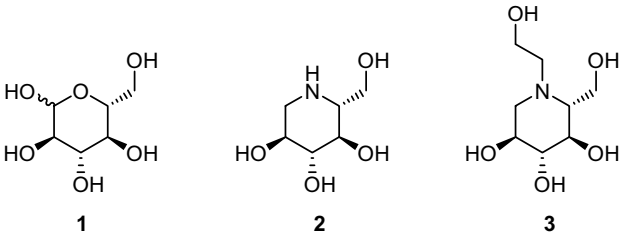


FIGURE 26.1 Iminocyclitols. **1**, D-glucose; **2**, 1-deoxynijirimycine (DNJ); **3**, miglitol (GLYSET™).

have been reported on the development of stereoselective aldol reactions using chiral auxiliaries, chiral Lewis acids, organocatalysts, and catalytic antibodies. Among many methods of catalytic asymmetric aldol reactions, chemoenzymatic synthesis using aldolases provides access to multiple scaffolds in highly stereoselective manners (Dean et al., 2007; Samland and Sprenger, 2006).

The aldolase family of enzymes is often divided into four groups based on their nucleophilic donor substrate specificities. One important class of aldolases utilizes dihydroxyacetone phosphate (DHAP) as the donor substrate and, in contrast, catalyzes reactions with a relatively broad range of different acceptor aldehydes. Well-known members of this class include fructose 1,6-diphosphate (FDP) aldolase and L-rhamnulose 1-phosphate (RhaD) aldolase. The synthetic utility of these DHAP-dependent aldolases has been thoroughly demonstrated with a wide array of novel acceptor aldehydes, and have been applied for carbohydrate synthesis such as unnatural sugars or iminocyclitols.

Iminocyclitols are potent glycosidase and glycosyltransferase inhibitors. The replacement of oxygen on the sugar rings by nitrogen results in compounds (Fig. 26.1) that are positively charged at physiological pH, and bind to carbohydrate-processing enzymes due to their mimicry of the transition state of the enzymatic reaction. Glycosidases and glycosyltransferases play key roles in metabolism, lysosomal catabolism, and glycoprotein processing. Glycoconjugates on the cell surface and proteins regulate many significant biological events such as viral infection, cell–cell recognition, and inflammation. As a result, iminocyclitols have been attractive as drug candidates for a number of diseases such as cancer, viral infection, lysosomal storage disorders, and diabetes.

The DHAP-dependent aldolases catalyze the addition of DHAP to acceptor aldehydes, and are capable of accepting aldehydes containing azide or *N*-Cbz-amine substitutions, providing the corresponding azido- or *N*-Cbz-amino polyhydroxy ketones. After the removal of the phosphate group with phosphatase, reduction of the azide or deprotection of *N*-Cbz group followed by intramolecular reductive animation affords a series of iminocyclitols (Fig. 26.2). However, DHAP-dependent aldolases possess the drawback of strict donor substrate specificity toward DHAP. Nonphosphorylated dihydroxyacetone (DHA) cannot be used, and the high cost and instability of DHAP (Schumperli et al., 2007), as well as the requirement of a phosphatase to remove the phosphate ester, make these aldolases less than ideally practical. Here we report recent efforts directed toward eliminating the need for

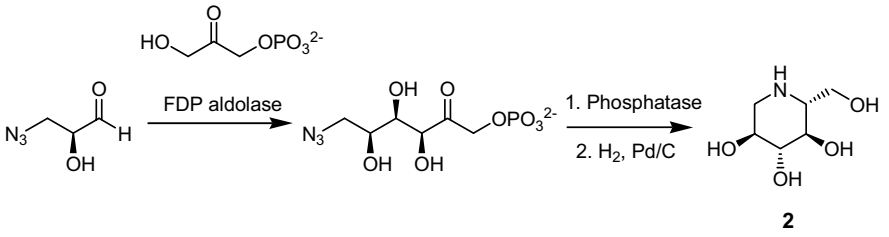


FIGURE 26.2 Synthesis of 1-deoxynojirimycine using FDP aldolase.

DHAP, by directed evolution, reaction engineering, or exploitation of newly discovered enzymes, respectively.

26.2 DIRECTED EVOLUTION OF L-RHAMNULOSE 1-PHOSPHATE ALDOLASE USING *IN VIVO* SELECTION

The unnatural noncaloric sweetener L-fructose has been the target of several chemical and chemoenzymatic syntheses, and we recently reported a one-pot synthesis of L-fructose from DHAP and DL-glyceraldehyde using RhaD aldolase and acid phosphatase (Fig. 26.3) (Franke et al., 2003). Our next step was to develop an aldolase-catalyzed synthesis starting from DHA, and the strategy was to alter the donor substrate specificity of RhaD by means of directed evolution to be capable of accepting DHA (Fig. 26.4) (Sugiyama et al., 2007). We developed an *in vivo* selection system for the directed evolution of RhaD to alter its donor substrate specificity from DHAP to DHA. The selection system allowed us a high-throughput screening for desired enzyme activity, evaluating large library sizes of up to 10^7–10^8. Use of an *Escherichia coli* deletion mutant of L-rhamnose metabolic genes as a selection host gives us an *in vivo* selection for the desired mutated RhaD that can cleave unphosphorylated L-rhamnulose, allowing rapid screening of mutated aldolases utilizing DHA as a donor substrate. L-Rhamnose, which is actively incorporated into *E. coli* cells, is first isomerized to the corresponding ketose, L-rhamnulose, by L-rhamnose isomerase (RhaA). Next, L-rhamnulose is phosphorylated by rhamnulose kinase (RhaB) to give the natural substrate of RhaD, L-rhamnulose-1-phosphate. RhaD splits rhamnulose-1P by a retroaldol reaction into two three-carbon carbohydrates, DHAP and L-lactaldehyde, which are then metabolized through glycolysis or the TCA cycle (Fig. 26.5). Since the wild-type RhaD cannot cleave L-rhamnulose, the disruption of

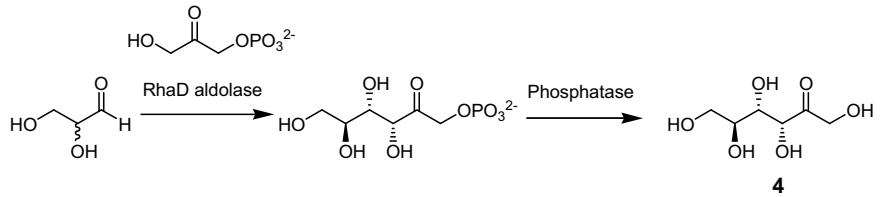


FIGURE 26.3 Synthesis of L-fructose **4** using L-rhamnulose 1-phosphate aldolase (RhaD).

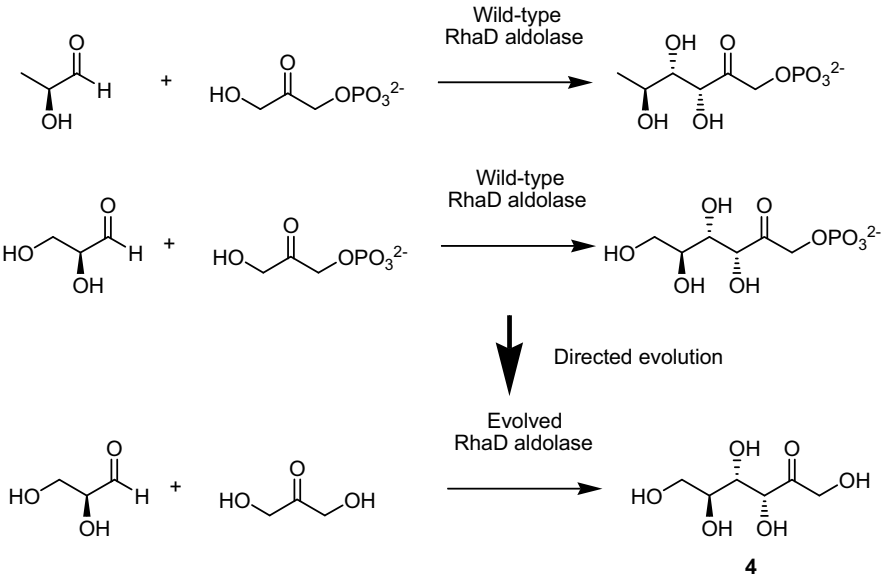


FIGURE 26.4 Directed evolution of RhaD aldolase toward the one-step synthesis of L-fructose **4**.

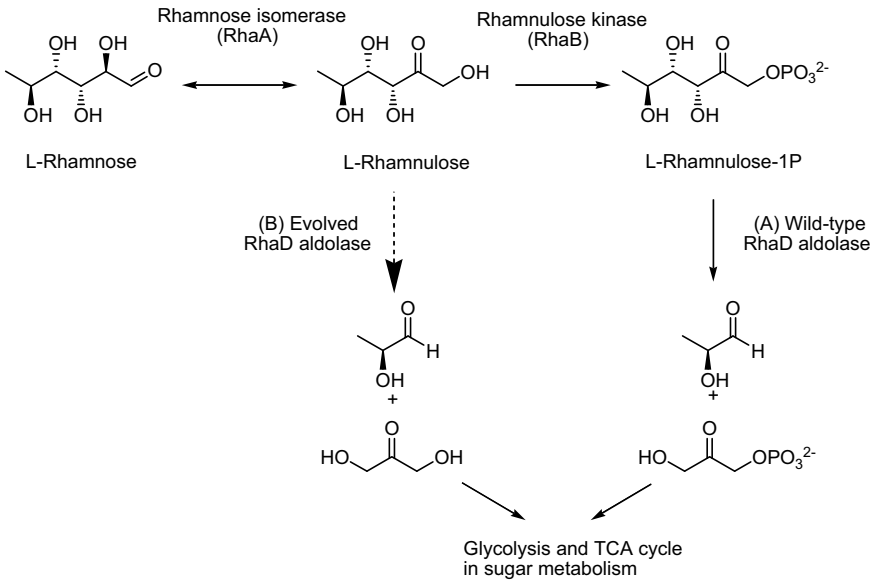


FIGURE 26.5 Strategy for the construction of *in vivo* selection. (A) The metabolic pathway of L-rhamnose in the wild-type *E. coli*; (B) an altered bypass in the rhamnulose kinase knockout strain with the evolved RhaD aldolase.

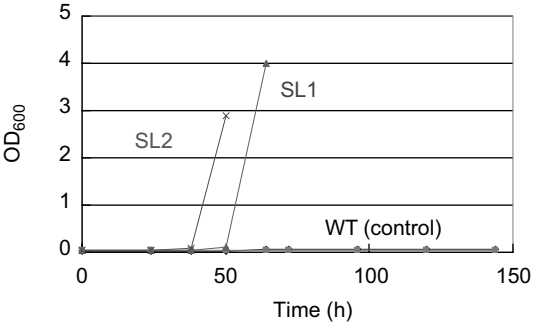


FIGURE 26.6 Growth of *E. coli* BW25113 (DE3) harboring the selected library and the wild type plasmid pETDRhaAD in minimal media containing L-rhamnose as a sole carbon source. SL1, Selected library 1; SL2, selected library 2; WT (control), pETDRhaAD.

the rhamnulose kinase gene (*rhaB*-) leads to the inability to grow on minimal media supplemented with L-rhamnose as a carbon source (MMRha). Use of the disruptant as a selection host allowed us to screen for mutated RhaD, which are capable of accepting unphosphorylated L-rhamnulose by selecting the survivors in MMRha. We constructed *E. coli* BW25113 (DE3) as a selection host strain that lacks all of the *rhaBAD* genes. An ep-PCR library of *rhaD* gene was constructed on the coexpression vector pETDRhaAD, and introduced into the selection strain. *E. coli* BW25113 (DE3) harboring the wild-type pETDRhaAD cannot grow in the MMRha media. In contrast, transformants from the ep-PCR library grew in MMRha media after 3 days incubation, suggesting that some mutants within the ep-PCR library allowed the selection host to survive in MMRha (Fig. 26.6). After the enrichment, the plasmids were

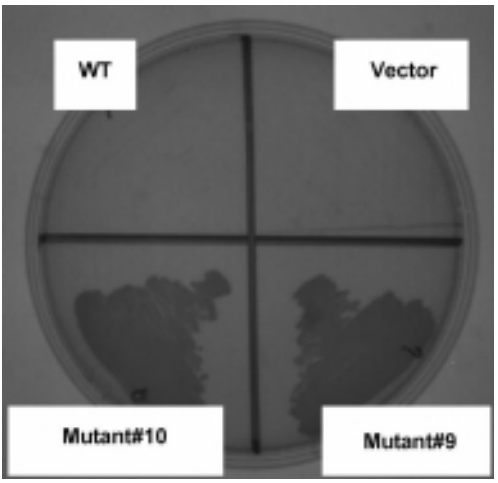


FIGURE 26.7 *In vivo* selection on a minimal plate containing L-rhamnose as a sole carbon source.

extracted and introduced into the selection host again, and survivors on MMRha plates were selected (Fig. 26.7). We obtained two individual mutated plasmids that enable the selection strain to grow in the MMRha media. The RhaD genes of these mutated plasmids were sequenced, showing that both plasmids possessed the same two amino acid substitutions (C142Y, T158S) in the protein sequence. Characterization of these mutants and further rounds of screening are in progress. Further work on the evolution of RhaD is in progress to develop a DHA-dependent aldolase, and to understand how we can alter the donor substrate specificity of aldolases in general.

Although altering the donor substrate specificity of aldolases is desirable for the advancement of these enzymes as biocatalysts for organic synthesis, there has been no report so far describing the alteration of donor substrate specificity of aldolases by directed evolution. The selection strategy demonstrated here could be applied for directed evolution of other DHAP-dependent aldolases.

26.3 USE OF BORATE AS A PHOSPHATE ESTER MIMIC IN ALDOLASE-CATALYZED REACTIONS: PRACTICAL SYNTHESIS OF L-FRUCTOSE AND L-IMINOCYCLITOLS

During the trial of directed evolution of RhaD aldolase, we also took another approach to develop a concise synthesis of L-fructose from DHA instead of DHAP (Sugiyama et al., 2006). Previous efforts to overcome the DHAP dependence of aldolases have involved *in situ* formation of arsenate or vanadate esters of DHA, which act as phosphate ester mimics (Schoevaart et al., 2001). Although arsenate has been used effectively for synthetic applications, the high toxicity of arsenate is an issue from a practical standpoint. Vanadate can also be used even at lower concentrations than arsenate, but vanadate may have problematic redox activity and its expense limits its practical utility.

While exploring other DHAP surrogates that can be accepted by DHAP aldolases and are also feasible for commercial production, we found that using inorganic borate buffer allows DHA to be accepted as a substrate by RhaD aldolase, presumably by reversibly forming a borate ester with DHA *in situ* (Fig. 26.8). We have used this discovery to develop a practical, inexpensive one-step synthesis of L-fructose on a gram scale.

His-tagged RhaD aldolase was overexpressed in *E. coli* and purified as reported previously. Alternatively, *E. coli* BL21 (DE3) cells harboring pETDRhaD were also used directly as a whole-cell biocatalyst without enzyme purification. A typical

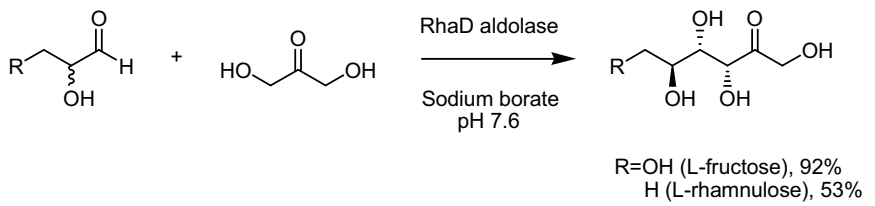


FIGURE 26.8 One-step synthesis of L-fructose and L-rhamnulose from DHA and DL-glyceraldehyde or lactaldehyde using RhaD aldolase in the presence of borate.

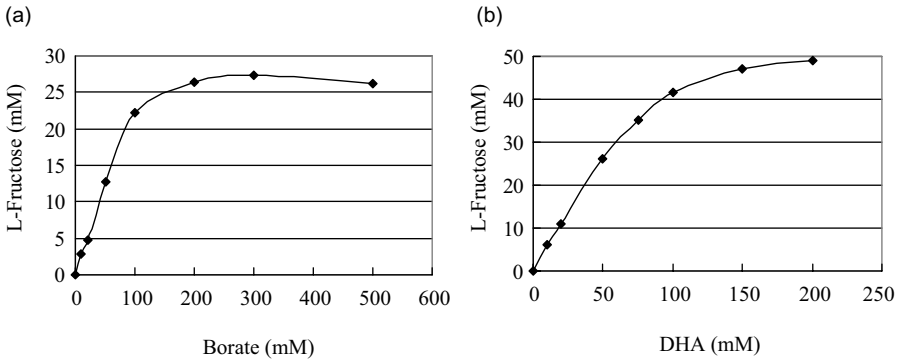


FIGURE 26.9 L-Fructose synthesis from DHA and DL-GA. (a) Borate-dependent formation of L-fructose. (b) DHA-dependent formation of L-fructose.

reaction mixture contained borate buffer (100 mM, pH 7.6), DHA (50 mM), DL-glyceraldehyde (100 mM), and RhaD aldolase. To confirm that this is an aldolase-catalyzed reaction, an omission test was performed under these conditions. The results showed that all components (borate, DHA, glyceraldehyde, and RhaD) were necessary for the formation of L-fructose, indicating that this is a borate-dependent RhaD-catalyzed aldol reaction. We believe that inorganic borate reversibly forms esters with DHA *in situ* in aqueous solution, and thus formed DHAB can be accepted by RhaD aldolase as a donor substrate. After the aldol reaction with L-glyceraldehyde to give L-fructose borate ester, the metastable borate esters were readily hydrolyzed during reaction workup.

The effect of borate concentration on the aldol reaction with constant DHA (50 mM) and DL-glyceraldehyde (100 mM) was examined (Fig. 26.9). The reaction reached maximum yield at about 200 mM borate. Next, DHA concentrations were varied at a constant concentration of 100 mM DL-glyceraldehyde in the presence of 200 mM borate. L-Fructose production was increased as the initial DHA concentra-tion increased, and reached a maximum at four equivalents of DHA relative to L-glyceraldehyde.

Enzyme kinetics in the presence of borate were determined in both the forward and reverse directions, and compared to the kinetics for the phosphorylated substrates (Table 26.1). The retroaldol activity of RhaD for L-rhamnulose in the presence of

TABLE 26.1 Activities of RhaD with Nonphosphorylated Substrates in the Presence of Borate

Substrate	V_{max} (μmol/min/mg)	K_m (mM)
L-Rhamnulose-1-phosphate	2.2	0.96
L-Fructose-1-phosphate	0.71	3.2
L-Rhamnulose + borate	0.035	
L-Fructose + borate	Not detected	
DL-Lactaldehyde + DHAP	33	
DL-Lactaldehyde + DHA + borate	1.0	
DL-Glyceraldehyde + DHAP	23	
DL-Glyceraldehyde + DHA + borate	0.48	

100 mM borate was approximately 60 times lower than that for the natural substrate L-rhamnulose-1-phosphate. Moreover, the retroaldol activity for L-fructose in the presence of borate was undetectable. These results suggest that L-fructose borate esters are not active substrates for the retroaldol reaction, while DHA-borate can be efficiently accepted by RhaD to go in the synthetic direction. It has been suggested by ^{11}B-, ^{13}C- NMR studies and thermodynamic studies that D-fructose forms relatively stable 1:1 and 1:2 borate–β-D-fructofuranose complexes. Thus, the L-fructose product may be thermodynamically trapped in such complexes and prevented from undergoing retroaldol reaction.

Next, we applied our method to the facile synthesis of a series of L-iminocyclitols by using azido aldehyde acceptors to efficiently synthesize several L-iminocyclitols (Fig. 26.10). The azido ketone aldol products underwent diasteroselective reductive cyclization to produce the iminocyclitols in only two steps. Recent reports have indicated that the L-enantiomers of known D-iminocyclitol glycosidase inhibitors can also be potent glycosidase inhibitors, acting in a noncompetitive mode and with unique specificity profiles (Yu et al., 2004).

We developed a simple one-step synthesis of L-fructose from racemic glyceraldehyde and DHA. We have also reduced the cost of producing L-fructose

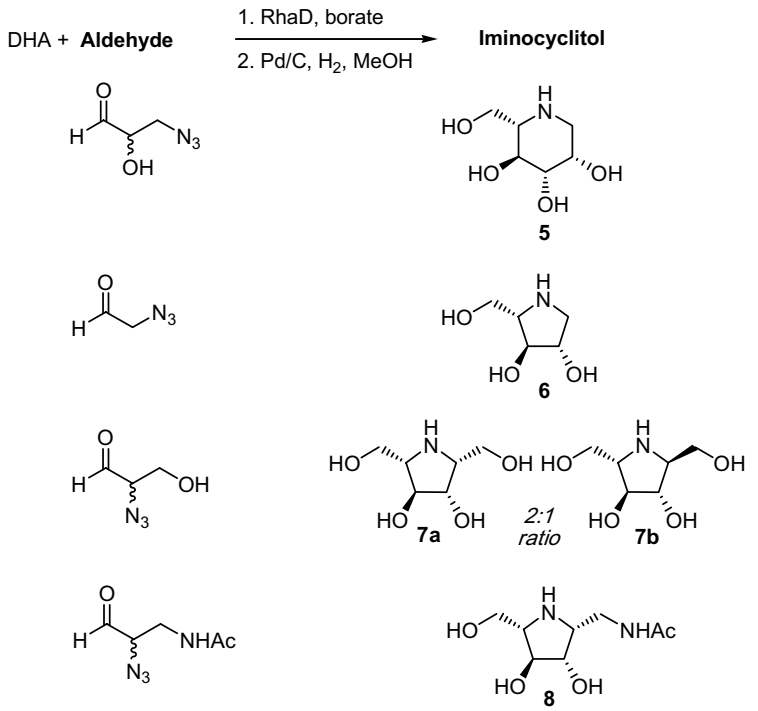


FIGURE 26.10 Two-step synthesis of iminocyclitols **5–8** from dihydroxyacetone using RhaD aldolase in the presence of borate, followed by reductive cyclization.

dramatically. Whereas DHAP is commercially available for ~$2200/g, DHA costs only $0.20/g.

26.4 ONE-POT SYNTHESIS OF D-IMINOCYCLITOLS USING D-FRUCTOSE 6-PHOSPHATE ALDOLASE

Our next approach was the application of a recently discovered enzyme, D-fructose 6-phosphate aldolase (FSA) from *E. coli*, for practical synthesis of a broad range of iminocyclitols in a one-pot procedure that avoids the need for phosphorylated substrate (Sugiyama et al., 2007). Previously, FSA has been found to catalyze the reversible aldol reaction from D-fructose 6-phosphate to D-glyceraldehyde 3-phosphate and the nonphosphorylated DHA (Schurmann and Sprenger, 2001; Schurmann et al., 2002). We applied this enzyme to synthesize iminocyclitols and related compounds using DHA instead of DHAP as the donor substrate. We were able to use FSA to synthesize iminocyclitols from DHA and a range of acceptor substrates. In addition, alleviating the need for DHAP has allowed us to develop a practical one-pot synthesis of iminocyclitols by performing the aldol and reductive amination reactions in a single vessel.

First, we synthesized 1,4-dideoxy-1,4-imino-D-arabitol, a naturally occurring five-membered iminocyclitol that inhibits α-glucosidases (Fig. 26.11). FSA accepted azidoacetaldehyde and DHA to give only one diastereomer of the aldol product. The aldol product was subjected to reductive amination to afford **12**. The NMR spectra of the product matched that of authentic **12**, and no peaks from other diastereomers were

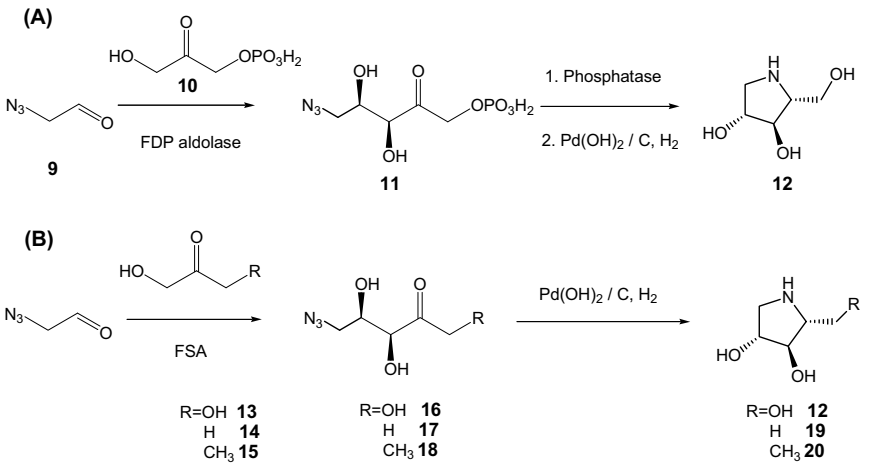


FIGURE 26.11 Aldolase-catalyzed synthesis of iminocyclitols. (A) Original synthesis with D-fructose-1,6-diphosphate aldolase; (B) synthesis with D-fructose 6-phosphate aldolase (FSA).

observed, confirming that FSA possesses the same (*3S, 4R*) stereoselectivity as that of D-fructose-diphosphate (FDP) aldolase.

Next, we investigated the donor and acceptor substrate specificity of FSA to determine whether a broader range of substrates was tolerated. FSA can accept not only DHA, but also hydroxyacetone or 1-hydroxybutan-2-one as donor substrate, to give methyl or ethyl derivatives. The broad donor substrate specificity of FSA is unusual, whereas most aldolases have very strict donor specificity. This allowed us to make a range of new iminocyclitols not previously available through aldolase catalysis.

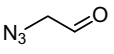
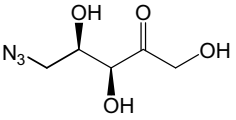
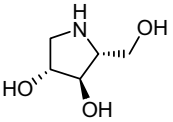
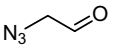
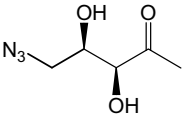
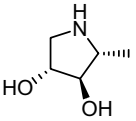
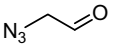
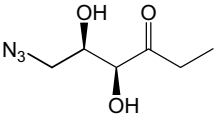
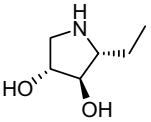
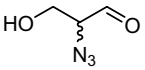
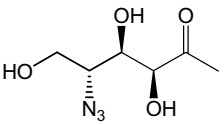
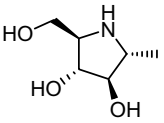
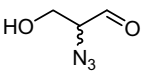
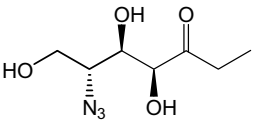
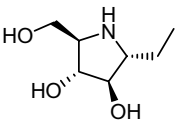
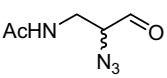
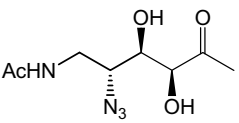
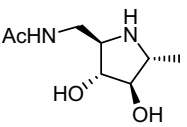
FSA also can utilize a wide range of acceptor aldehydes (Tables 26.2 and 26.3). In order to synthesize 1-deoxynojirimycin (DNJ) derivatives or 1-deoxymannojirimycin (mannoDNJ) derivatives, both pure enantiomers of 3-[(benzyloxycarbonyl)-amino]-2-hydroxy-propionaldehyde, **38** and **43** were prepared. DNJ and its derivatives constitute an important class of iminocyclitols, including *N*-butyl-DNJ (Miglustat), which is a potent glucosylceramide synthase inhibitor. It is used clinically for the treatment of the glycolipid storage disorder, type I Gaucher disease.

We further optimized our syntheses of iminocyclitols by developing methods for performing all operations in a single pot (Fig. 26.12). FSA-catalyzed aldolase reactions were performed in sodium borate buffer (pH 7.6), followed by the reductive amination in the presence of excess equivalents of diethylamine. The addition of diethylamine protected the secondary amine of the resulting iminocyclitols from further intermolecular reductive amination with any residual carbonyl compound. This meant that we could now complete the iminocyclitol synthesis in a one-pot fashion without any isolation of intermediates. All the iminocyclitols listed in Tables 26.2 and 26.3 were synthesized in the one-pot procedure.

Iminocyclitol **19**, **25**, **28**, **30**, **37**, **42**, and **47** are previously unreported compounds, and preliminary inhibitory activities toward a series of glycosidases were determined (Table 26.4). Compounds **19** and **20** were found to be slightly more potent inhibitors of jack bean α-mannosidase than the known inhibitor **12**, with 26% and 14% inhibition at 1 μM, respectively. While not exceptionally potent, **20** is quite specific for α-mannosidase, whereas 12 inhibits both α- and β-glucosidases as well as α-mannosidase. Compounds **28** and **30** are potent and specific inhibitors of β-*N*-acetylglucosaminidases from jack bean as well as human placenta. These structures may serve as readily available cores for the optimization in discovering new treatments for osteoarthritis.

We have developed a one-pot chemoenzymatic method for the synthesis of a variety of iminocyclitols from readily available, nonphosphorylated donor substrates by using FSA. It was found that in addition to DHA, hydroxyacetone and 1-hydroxybutan-2-one could also be accepted in the FSA-catalyzed aldol reactions with both azido- and Cbz-amino aldehyde acceptors. This broad substrate tolerance allowed us to synthesize a number of known as well as novel iminocyclitols with relatively high yields, in a concise fashion.

TABLE 26.2 Synthesis of Iminocyclitols from Azidoaldehydes

Acceptors	Donors	Aldol Products	Iminocyclitols	Yield (%)	
				Two-step	One-pot
 **9**	DHA	 **16**	 **12**	57	73
 **9**	HA	 **17**	 **19**	64	72
 **9**	HB	 **18**	 **20**	62	83
 **21**	HA	 **22**	 **23**	27	32
 **21**	HB	 **24**	 **25**	27	33
 **26**	HA	 **27**	 **28**	39	57

(*continued*)

TABLE 26.2 (*Continued*)

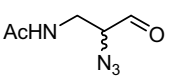
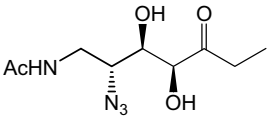
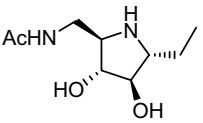
Acceptors	Donors	Aldol Products	Iminocyclitols	Yield (%)	
				Two-step	One-pot
 **26**	HB	 **29**	 **30**	31	53

TABLE 26.3 Synthesis of Iminicyclitols from Cbz-Protected Aminoaldehydes

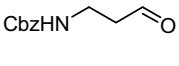
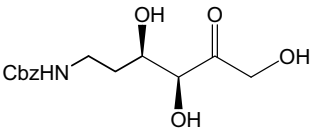
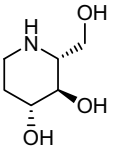
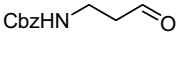
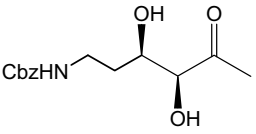
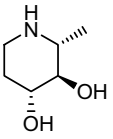
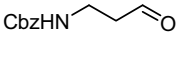
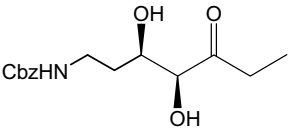
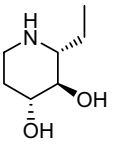
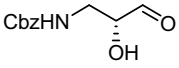
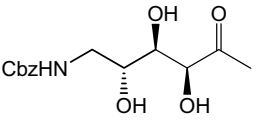
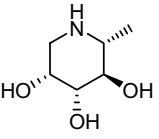
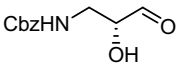
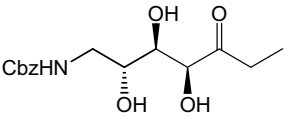
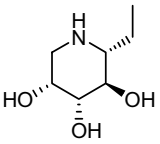
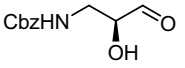
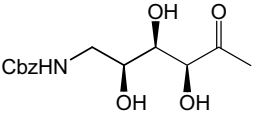
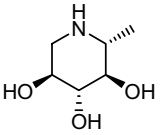
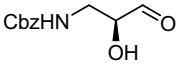
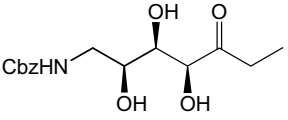
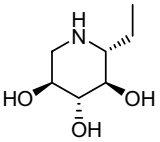
Acceptors	Donors	Aldol Products	Iminocyclitols	Yield (%)	
				Two-step	One-pot
 **31**	DHA	 **32**	 **33**	65	55
 **31**	HA	 **34**	 **35**	78	64
 **31**	HB	 **36**	 **37**	78	82
 **38**	HA	 **39**	 **40**	40	48

TABLE 26.3 (*Continued*)

Acceptors	Donors	Aldol Products	Iminocyclitols	Yield (%)	
				Two-step	One-pot
 **38**	HB	 **41**	 **42**	51	20
 **43**	HA	 **44**	 **45**	60	53
 **43**	HB	 **46**	 **47**	47	57

26.5 CONCLUSIONS

We discussed in this chapter three approaches toward facile synthesis of carbohydrates using DHAP aldolase and FSA. Directed evolution and reaction (or substrate) engineering will continue to be applied to overcome problems for synthetic

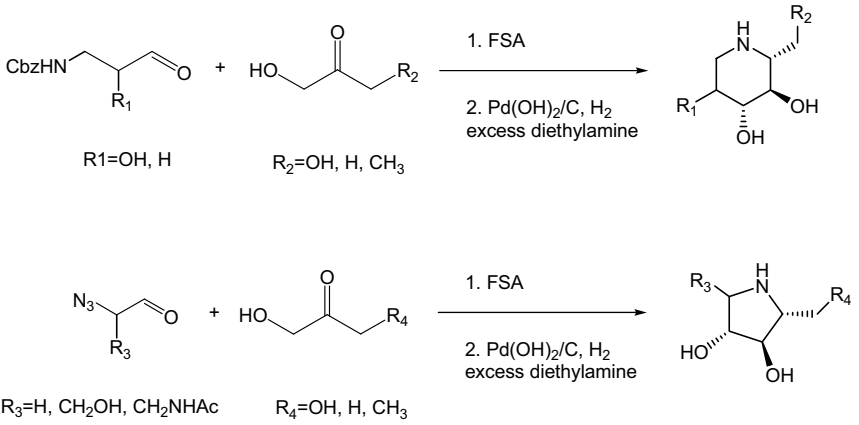


FIGURE 26.12 One-pot synthesis of iminocyclitols.

TABLE 26.4 Glycosidase Inhibition Activities of Synthesized Iminocyclitols

Enzyme	12	19	20	23	25	28	30	33	40	42
α-Glu	100 (58)	54 (4)	4	39	3	0	0	3	6	0
β-Glu	84 (4)	55 (4)	69 (0)	50	40	0	6	0	11	0
α-Man	81 (9)	95 (26)	95 (14)	25	11	11	8	14	85 (2)	42
β-Man	38	10	20	47	49	0	0	25	6	0
β-Gal	47	0	4	13	19	0	0	34	4	6
β-*N*-GlcNAc[1]	0	0	0	29	3	100 (61) **0.32**	100 (37) **0.76**	6	9	21
β-*N*-GlcNAc[2]	0	0	0	0	0	100 (59) **0.51**	100 (68) **0.29**	0	11	22

Note: Values represent percent inhibition at 200 μM. For the most potent inhibitors, percent inhibition was also determined at 1 μM, shown in parentheses. α-Glu, α-glucosidase from jack beans; β-Glu, β-glucosidase from almonds; α-Man, α-mannosidase from jack beans; β-Man, β-mannosidase from snail; β-Gal, β-galactosidase from *Aspergillus oryzae*; β-*N*-GlcNAc[1], β-*N*-acetylglucosaminidase from jack beans; β-*N*-GlcNAc[2], β-*N*-acetylglucosaminidase from human placenta. For compounds 28 and 30, K_i values against the two β-*N*-acetylglucosaminidase are shown in bold (mM).

application of aldolases, such as broadening the scope of available substrates and conditions that are tolerated, including cosolvents, pH, and temperature. In addition, it is likely that more aldolases will be discovered from genome sequence data, metabolite-degradation pathways, and extreme environments, and that these novel aldolases will increase the breadth of molecules accessible by enzymatic catalysis. Aldolases will surely play more important roles in fine chemical synthesis.

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27

PRODUCTION OF VALUE-ADDED PRODUCTS BY LACTIC ACID BACTERIA

SIQING LIU AND KENNETH M. BISCHOFF

Renewable Product Technology Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, 1815 N. University St., Peoria, IL61604, USA

YEBO LI AND FENGJIE CUI

Department of Food, Agricultural, and Biological Engineering, The Ohio State University/ Ohio Agricultural Research and Development Center, 1680 Madison Avenue, Wooster, OH 44691-4096, USA

HASSAN AZAIZEH AND AHMED TAFESH

Institute of Applied Research (Affiliated with University of Haifa), The Galilee Society, P.O. Box 437, Shefa-Amr 20200, Israel

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27.1 INTRODUCTION

Lactic acid bacteria (LAB) are a group of facultative anaerobic, catalase-negative, nonmotile, and nonspore-forming Gram-positive bacteria. Most LAB utilize high-energy C sources including monomer sugars to produce energy to maintain cellular structure and function. This anaerobic fermentation process also produces low-energy end products, primarily lactic acid. Based on the metabolic routes of given substrates and corresponding end products, LAB can be classified as homofermentative and heterofermentative.

Homofermentative LAB use the Embden–Meyerhof pathway to generate lactate as the sole fermentation product, while heterofermentative LAB use the pentose phosphoketolase pathway producing a mixture of lactate, ethanol, CO_2, and acetate. LAB is widely used in agriculture and food-processing industries as well as in medicine and immunological research. In this chapter, we focus on production of lactate, antibacterial peptides, and antifungal agents by LAB. Potential sustainable, renewable, and commercially viable production of value-added products by LAB is discussed.

27.2 LACTATE FERMENTATION

Lactic acid is a natural organic acid, and has a Generally Recognized as Safe (GRAS) status. It has been used in pharmaceuticals, in the leather industry as a green solvent, and in the food industry as a flavor enhancer and preservative agent. Recently, it is used for the preparation of biodegradable plastics (http://www.natureworksllc.com/). Approximately half of the world's supply of lactate is produced by fermentation (Demirci et al., 1993). The current global production for lactic acid is estimated at 130,000–150,000 metric tons/year, and the projected demand for lactic acid is more than 500,000 metric tons per year by 2010 (Wee et al., 2006). Chemical synthesis usually produces a racemic mixture whereas a specific isomer can be produced by biological fermentation. Along with the threat of global warming, limited petrochemical resources, and increased environmental concerns, the sustainable production of lactate via fermentation is predicted to significantly replace petroleum-based polymers.

The traditional bioproduction of lactate is based on microbial fermentation of the refined sugars glucose and sucrose (Zhou et al., 2003). Efforts have been made to convert raw starchy materials to lactate. However, the natural starch substrates need to be gelatinized, liquefied, and saccharified prior to microbial fermentation and these processes involve high temperatures followed by enzymatic hydrolysis and fermentation which are feasible but not competitive to petroleum-based production (John et al., 2007). To eliminate the pretreatment steps, high lactic acid producing strains that also produce amylolytic enzymes were developed (Reddy et al., 2008). The amylolytic strains such as *Lactobacillus amylophilus* GV6 (a lactic acid yield of 0.78 g/g from wheat bran starch and 0.96 g/g from soluble starch) (Vishnu et al., 2000), *L. amylophilus* B4437 (a yield of 0.64 g/g corn starch) (Mercier et al., 1992), and

L. amylovorus B4542 (a yield of 0.77 g/g liquefied starch) were reported as being capable of converting starch into lactic acid (Cheng et al., 1991; Zhang and Cheryan, 1991). However, most LAB require yeast extract as a nitrogen source to grow. Inexpensive nitrogen sources including ammonium sulfate, malt sprout, soybean meal, cotton seeds, mustard power, date juice, silkworm larvae, shrimp and fish waste were tested to replace yeast extract (John et al., 2007). To date, only the *L. amylophilus* GV6 was reported capable of utilizing inexpensive red lentil flour and baker's yeast cells as nitrogen sources instead of peptone and yeast extract (John et al., 2006). Moreover, continuously large-scale consumption of starch could lead to competition with the food and feed industries. Thus, the bioproduction of lactate utilizing renewable cellulosic biomass materials and low-valued dairy industry waste cheese whey has received extensive attention (Patel et al., 2005; Shahbazi et al., 2005).

27.2.1 From Lignocellulosic Biomass

As an alternative to traditional starchy feedstocks, lignocellulosic biomass is an inexpensive, abundant, and renewable carbon source. LAB have been used to convert lignocellulosic agricultural residues for lactic acid production. Commonly exploited raw materials for lactic acid production include corn stover (Zhu et al., 2007), wheat straw (Maas et al., 2008), spent grain (Mussatto et al., 2008), rice bran (Gao et al., 2008), vine trimming waste (Bustos et al., 2004), cassava bagasse (John et al., 2006a), sugar cane bagasse (Patel et al., 2005), and wheat bran (John et al., 2006b).

Lignocellulose consists primarily of cellulose and hemicelluloses, which are polysaccharides built up of hexose and pentose sugars, embedded in a matrix of the phenolic polymer lignin. To release simple sugars contained in lignocellulosic biomass feedstocks, pretreatment steps including physical (grinding and irradiation), chemical (dilute acid and alkali), and physicochemical (steam pretreatment and autohydrolysis) processes are needed initially to reduce particle size, to modify and/or remove lignin, to increase solubilization of hemicelluloses, and to enhance the accessibility of the polysaccharides to cellulolytic and xylanolytic enzymes in the subsequent enzymatic hydrolysis step (Hendriks and Zeeman, 2009).

Dilute sulfuric acid ($<4\%$ H_2SO_4) with elevated temperatures between 120 and 200°C has been used for pretreatment of lignocellulosic biomass feedstocks. It is an inexpensive and time-saving process (2–30 min) with up to 90% hemicellulose and glucose yields during the enzymatic hydrolysis (Bustos et al., 2004; Lloyd and Wyman, 2005). However, a large volume of waste stream is generated after dilute acid pretreatment, which would be a serious pollutant if directly disposed in the environment. Exploring new uses and new products from the waste stream are essential in order to increase the value and the overall economics of the biomass conversion.

Alkali pretreatment with NaOH, lime, or ammonia can reduce the degree of polymerization and crystallinity, increase the internal surface area, disrupt the lignin structure, and swell the pores of the cellulosic biomass at lower temperatures (25– 130°C) and pressures compared to other pretreatment technologies (Lloyd and Wyman, 2005; Maas et al., 2008). Lime as a pretreatment agent has promising

potential because it is inexpensive, safe, and its use hardly results in sugar degradation products such as furfural and hydroxymethyl furfural. Nevertheless, alkaline pretreatment results in relatively high pH value (>10); thus, lowering the pH is essential to achieve an efficient enzymatic (optimal enzyme activities at lower pH) hydrolysis of the polysaccharides. Furthermore, lowering the pH of the pretreated material with sulfuric acid can result in the formation of the low-value by-product gypsum.

Hydrolysis and fermentation can be carried out separately or simultaneously. During separate hydrolysis and fermentation (SHF), enzymatic hydrolysis and fermentation are run in two different vessels, each with its own optimal conditions (Wyman, 1999). However, the cellobiose and glucose produced in the hydrolysis step strongly inhibit the cellulase activities, and this two-step process involving consecutive enzymatic hydrolysis and microbial fermentation increases the total treatment time, thus the SHF is expensive to operate (Lynd et al., 2005). To reduce production time and cut operation expenses, the simultaneous saccharification and fermentation (SSF) process has received more attention (Punnapayak and Emert, 1986). In SSF configuration, the enzymatic hydrolysis and fermentation are completed in the same vessel, and the continuous removal of hydrolyzed sugars via fermentation minimizes the product inhibition of hydrolytic enzymes; thus, SSF offers great potential with high lactic acid productivity and reduced operation cost (Lee et al., 1997).

However, in order to convert lignocellulosic biomass feedstocks into lactate using the SSF process, new microbial strains need to be developed. Since biomass hydrolysates contain a mixture of sugars, selected strains from the heterofermentative LAB group capable of utilizing both hexoses and pentoses have been investigated. *Lactobacillus pentosus* ATCC 8041was used to convert corn stover hydrolysates in a fed-batch SSF into lactic acid with a maximum efficiency of 92%, productivity 0.70 g/L/h and a yield of 0.65 g/g. In this study, the corn stover was first pretreated with aqueous ammonia, which removed most of the structural lignin, and then with cellulase (Spezyme-CP) hydrolysis. The resulting hydrolysate was fermented to lactic acid. A maximum lactic acid concentration of 75.0 g/L was achieved when cheaper corn steep liquor was used instead of yeast extract (Zhu et al., 2007). Another *L. pentosus* strain CECT-4023 was reported converting glucose, xylose, and arabinose into lactic acid from the hydrolysates of trimming vine shoots, barley bran husks, and corn cobs. A productivity of 0.84 g/L/h and a yield of 0.77 g/g (glucose + xylose consumed) were achieved from dilute sulfuric acid pretreated trimming vine shoots (Bustos et al., 2004). The bioconversion of diluted acid pretreated biomass hydrolysates into lactic acid by *L. pentosus* was also reported from rice bran (Gao et al., 2008), brewer's spent grain (Mussatto et al., 2008), corncob, vine trimming wastes, spruce chips, and triticale straw (Bustos et al., 2004; Moldes et al., 2006). When barley bran hydrolysate was fermented by *L. pentosus,* the microbe converted all the hemicellulosic sugars to lactic acid, producing 33 g/L of lactic acid with a yield of 0.57 g/g (Moldes et al., 2006). Lactic acid productivity of 0.94 g/L/h and a cellulose conversion ratio of 67% was achieved from *Lactobacillus* sp. RKY2 from NaOH pretreated wood with SSF in a fed-batch operation supplied with fresh nutrients and enzymes (Yun et al., 2004).

Lactobacillus bifermentans DSM 20003 was used to convert glucose, arabinose, and xylose to lactic acid from a hemicellulosic hydrolysate of wheat bran with maximum lactic acid yield of 0.83 g/g, productivity of 1.17 g/L/h, and sugar utilization of 76% (Givry et al., 2008).

27.2.2 From Dairy Industry Waste

The cheese manufacturing industry represents a significant portion of the food industry and generates huge amounts of liquid waste, including cheese whey, one of the major by-products. Cheese whey is generated after separating the coagulum from whole milk, cream, or skim milk and represents 85–90% of the milk volume while retaining 55% of the milk nutrients. In the United States, approximately 1.2×10^9 tons of cheese whey are generated yearly (Shahbazi et al., 2005). It is estimated that as much as 40–50% of the whey produced was disposed as sewage or farmland fertilizer. Typically, 100 g of milk yields 10 g of cheese and 90 g of liquid whey. Cheese whey contains about 4.5–5% lactose, 0.6–0.8% soluble proteins, 0.4–0.5% (w/v) lipids, and varying concentrations of mineral salts (Siso Gonzalez, 1996).

The amount of organic nitrogen in whey-based media cannot fully support the growth of lactic acid producing bacteria, therefore, yeast extracts and peptones need to be supplemented. This is mainly because most LAB lack a complete proteolytic system, and require free amino acids and peptides instead of proteins found in milk-derived products (Kunji et al., 1996). The enzymatically hydrolyzed whey protein was found to be a good nitrogen source for *Lactobacillus casei* that is comparable to yeast extract in a recycle batch reactor system (Senthuran et al., 1999). In addition, corn steep liquor was found capable of replacing the expensive N-sources (Kim et al., 2006). The hydrolysates of cod viscera (*Gadus morhua L.*) were also tested as a complex nitrogen source to replace peptones and yeast extracts in supporting the growth of LAB (Aspmo et al., 2005).

Various fermentation processes have been tested with selected LAB strains for conversion of cheese whey into lactate. A conversion ratio of 79% and a yield of 0.84 g/g of lactose from cheese whey were achieved by using immobilized *Lactobacillus helveticus* in sodium alginate beads on a spiral-sheet bioreactor. Similarly, a conversion ratio of 69% and a yield of 0.51 g/g were obtained by immobilized *Bifidobacterium longum* (Shahbazi et al., 2005). A cell-recycle repeated batch system can improve fermentation efficiency significantly when compared with a batch fermentation using the strain *Lactobacillus* sp. RKY2, which converted whey lactose to lactic acid with a yield of 0.98 g/g. When the free cell and integrated membrane reactor were compared using *B. longum* (NCFB 2259), higher lactic acid productivity was achieved from cheese whey with the integrated membrane system that can harvest lactic acid products during the course of fermentation (Li et al., 2007). An optimized immobilization system showed 94.37% conversion of whey lactose to lactic acid (32.95 g/L). The lactose to lactic acid conversion rate remained constant for up to 16 batches, demonstrating the system sustainability and viability of the immobilized bacterial cells (Panesar et al., 2007).

27.3 PRODUCTION OF ANTIBACTERIAL PEPTIDES AND PROTEINS

Many conventional antibiotics are not easily degraded, and residues may accumulate in the environment and promote the emergence of drug-resistant strains. The contributing factors to dissemination of drug resistance include the improper and excessive use of broad-spectrum antibiotics, the introduction of antibacterials in household cleaning supplies, and the use of antibiotics as feed additives for livestock. Consequently, the emergence of drug-resistant strains limits the effectiveness of conventional synthetic antibiotics, which may prove life threatening to patients if treatment is nonresponsive to available drugs.

New biodegradable antibacterial agents are needed. Many LAB are capable of producing antibacterial peptides known as bacteriocins, which are synthesized in the ribosomes. Bacteriocins are proteinaceous biodegradable compounds, differing from antibiotics that are not ribosomally synthesized (Papagianni and Anastasiadou, 2009). Bacteriocins can inhibit or eliminate the growth of sensitive bacterial species, especially Gram-positive pathogens including *Listeria monocytogenes*, *Staphylococcus aureus*, and *Enterococcus faecalis*. Peptide bacteriocins appear to be effective in very low concentrations. The potential exists for commercial production of biodegradable bacteriocins for effective food and feed preservatives. Generally speaking, the bacteriocins produced by LAB can be divided into four groups: Class I, a group of modified cationic peptide, also called type A lantibiotics; Class II, the unmodified, heat-stable nonlantibiotics; Class III, a group of large heat-labile bacteriocins; and Class IV, a group of complex molecules with lipid and carbohydrate moieties (Papagianni and Anastasiadou, 2009). The most extensively studied are the Class I and Class II bacteriocins.

27.3.1 Class I bacteriocin

Nisin belongs to the Class I bacteriocins, since it contains lanthionine and methyllanthionine groups. Nisin, the most studied bacteriocin, is an oligopeptide of approximately 3.5 kDa, which is ribosomally synthesized, post-translationally modified, and produced by *Lactococcus lactis* (Buchman et al., 1988; Delves-Broughton et al., 1996). Nisin is rich in sulfur-containing unusual amino acids. More studies suggested that the sugar utilization and nisin production varies from species to species. Man Rogosa Sharpe (MRS) broth containing glucose was considered an ideal media for bacteriocin production, while xylose is the most efficient carbon source for the strain *L. lactis* IO-1 to produce nisin Z with activity of 810 AU/ml (Chinachoti et al., 2008). Sucrose could be a potentially cost-effective substrate for nisin production by *L. lactis* strain NIZO 22186, since a higher nisin activity (2208 AU/ml) was obtained from sucrose instead of glucose (Pongtharangkul and Demirci, 2006). However, when mixed sugars are used, LAB still prefer glucose to produce bacteriocin before consuming other sugars including xylose and sucrose (Luesink et al., 1999). Inexpensive feedstocks such as whey permeate, cull potatoes, and mussel processing wastes and diluted skim milk have been used as substrates for nisin production (Jozala et al., 2007; Liu et al., 2005a; Lv et al., 2005; Pongtharangkul and

Demirci, 2006). The highest nisin production of 51,000 AU/ml from whey permeate was obtained by immobilized *L. lactis* subsp. *lactis* ATCC 11545 (Liu et al., 2005a).

Optimization of the operating parameters (media composition, aeration, and pH) can reduce bacteriocin production costs. Nutrient-rich media such as yeast extract, peptone or tryptone, peptone from meat, peptone from soy, and corn steep solid have been used effectively for bacteriocin production (Liu et al., 2005a; Lv et al., 2004). In addition, inorganic KH_2PO_4, considered a good phosphate source when added at 0.3–5%, increased nisin production by *L. lactis* subsp. *lactis* ATCC 11454(Lv et al., 2004), but at a concentration from 1 to 3%, showed no significant production by *L. lactis* subsp. *lactis* NIZO 22186 (Pongtharangkul and Demirci, 2006). $MgSO_4 \cdot 7H_2O$ showed a positive effect on nisin production at an optimal concentration range (0.025–0.057%), while higher or lower concentrations reduced the product activity (Liu et al., 2005a; Pongtharangkul and Demirci, 2006).

Although an anaerobic environment is required for lactic acid production and cell growth of LAB, aeration has been shown to have a significant effect on nisin production, since the oxygen tolerance of LAB is associated with different metabolic pathways. An oxygen-enriched atmosphere (50–100%) enhanced nisin production, with activity reaching a maximum level of 41,000 AU/ml (Amiali et al., 1998; Cabo et al., 2001). During the fermentation process, by-products such as lactic acid and acetic acid can inhibit bacteriocin production and cell growth by decreasing the broth pH. Direct removal of lactic acid from the broth using an anionic exchange resin contributed to a two-fold increase of nisin production compared to a pH-adjusted fermentation process (Yu et al., 2002). Biological removal of lactic acid was conducted with a mixed-culture system such as *L. lactis* and yeast *Kluyveromyces marxianus* or *Saccharomyces cerevisiae* (Liu et al., 2006; Shimizu et al., 1999), in which the lactate produced by *L. lactis* is assimilated by yeast strains to produce CO_2 and H_2O.

Nisin production can be performed with batch, fed-batch, or continuous fermentation processes. Conventional fermentation methods using free cells in a batch process have several disadvantages including limited cell growth and low production yields and productivity rates. Fed-batch, continuous culture, and cell immobilization technologies overcome the nutrient limitations, product inhibition, and batch-to-batch variation. So far, the combination of cell immobilization and continuous cultures such as calcium alginate immobilized *L. lactis* in a continuous packed-bed reactor (Wan et al., 1995), or fiber-attached *L. lactis* in a continuous bioreactor (Liu et al., 2005) has been explored.

The Class I bacteriocin remains a potent antibacterial agent with huge therapeutic potential due to its broad spectrum of activities. The FDA approval of nisin as a food additive demonstrated potential applications of bacteriocin in the food and feed industry. Currently, nisin is used as a food preservative in more than 50 countries (Delves-Broughton et al., 1996); however, its commercial development has been limited by the cost of the fermentation processes, the effects of the activities by structure, and post-translational modifications. More than a decade of efforts has been dedicated to develop a cost-effective manufacturing process. The large-scale production of nisin is expensive, and so far, nisin is only manufactured by Aplin & Barrett in Beaminster, UK (Delves-Broughton et al., 1996). Thus, new technologies and

fermentation processes are needed to facilitate the affordable and profitable commercial production of the biodegradable antibacterial agents like nisin and similar products (Smith and Hillman, 2008).

27.3.2 Class II bacteriocins

The success of nisin has attracted more research in the production, purification, and genetics of other bacteriocins in recent years. Among a growing range of potential biopreservatives reported, pediocins, belonging to the Class II bacteriocins, have been recognized as another promising antibacterial agent (Papagianni and Anastasiadou, 2009). The Class II unmodified bacteriocins are small and unmodified peptides synthesized as inactive peptides until the N-terminal leader sequences are removed. These bacteriocins contain high antilisterial activity, with an YGNGV motif and a disulfide bridge in their N-terminal halves, and kill target cells by permeabilizing the membrane structures (Fimland et al., 2000). Most pediocins are initially translated as an inactive peptide, and the N-terminal (18–20 amino acids) is cleaved post-translationally to generate an active peptide (35–50 amino acids). The enzymatic removal of the leader peptide occurs at pH 4.0 or lower (Papagianni and Anastasiadou, 2009).

Pediocins are produced by bacterial strains that belong to the genus *Pediococcus.* Several species include *P. acidilactici* can produce pediocin PA-1(strain PAC1.0); pediocin AcH (Strain PAC1.0); pediocin SA-1(NRRL B5627); *P. pentosaceus* producing pediocin SM-1(SM-1); and *P. damnosus* producing pediocin PD-1. Other Class II bacteriocin producers include *L. amylovorus* DCE 471 producing amylovorin L471 (Delves-Broughton et al., 1996); *Lactobacillus curvarus* CWBI-B28 producing curvacin A (Ghalfi et al., 2006, 2007); *Leuconostoc gelidum* for leucocin A-UAL 187 (Hastings et al., 1991); *Lenconostoc mesenteroides* for mesentericin Y105 (Hechard et al., 1992); and *Lactobacillus sake* Lb706 for sakacin A (Holck et al., 1992).

Although current results regarding the efficiency of bacteriocins as biopreservatives are remarkable and promising, most work has been conducted in laboratories. So far only one pediocin is marketed under the commercial name of ALTA 2341, which showed inhibition of *Listeria* cell growth on packaged smoked salmon and frankfurters (Chen et al., 2004; Szabo and Cahill, 1999). Nevertheless, there is substantial reluctance by the industry to commit financially to developing commercial bacteriocin preparations, largely due to safety regulations, low production rates, unstable products, and expensive downstream processing. More research on developing new strains via genetic engineering and designing novel bioprocessing methods is needed to increase yields and reduce production costs, which will enable cost-effective and profitable production of bacteriocins.

27.4 OTHER APPLICATIONS

27.4.1 Biofuels

The success in cloning the pentose utilization pathway of *L. brevis* and *L. pentose* into *L. plantarum* demonstrates the feasibility of the metabolic engineering of this

microorganism for applications in converting biomass-derived sugars into fuels and chemicals (Chaillou et al., 1998a, 1998b). Exogenous ethanol production pathway genes from *Zymomonas mobilis, Zymobacter palmae*, and *Sarcina ventriculi* have been introduced into selected species of LAB for ethanol production (Gold et al., 1996; Liu et al., 2005b, 2007; Nichols et al., 2003). The resulting recombinant LAB strains produce ethanol at various levels but not sufficient enough for industrial-scale production. A recent survey indicated that selected LAB species including *L. delbrueckii* and *L. brevis* can grow in up to 3% butanol (Knoshaug and Zhang, 2009) so there is potential to engineer LAB for butanol production.

27.4.2 Probiotics

Several *Lactobacilli* strains including *L. johnsonii* La1 and GG, *L. rhamnosus* strain HN001, *L. acidophilus* strain LB, *L. casei* GG, *Bifidobacterium* spp. strains CA1 and F9, *B. lactis* strain HN019, *B. breve* strain Yakult, *B. longum spp*. Strain, and *L. plantarum* strain 299v have been used *in vivo* in mice to exclude pathogens from colonizing the gastrointestinal tract and to improve animal health (Servin and Coconnier, 2003). *L. rhamnosus* GG was effective in preventing and reducing diarrhea in infants (Guarino et al., 2009). The *L. pentosus* S-PT84 contributed to the control of human microflora by mechanisms such as pathogen growth inhibition, prevention of pathogen adherence, production of lactic acid, bacteriocins and H_2O_2, immune-modulation, and anti-inflammation (Nonaka et al., 2009). The *Lactobacilli* CNCM I-1984, CNCM I-1985, CNCM I-1986, and CNCM I-1987 were patented for their abilities to adhere to the pellicle of the teeth and to produce antibacterial bacteriocins. These strains have potential applications to reduce dental plaque, to treat or prevent root caries and other infections caused by *Actinomyces naeslundii* in mammals (Comelli et al., 2009).

27.4.3 Antifungal Activity

Recently, strains of LAB including *L. citreum*, *L. Rossiae,* and *L. plantarum* have been reported to inhibit the growth of fungal species *Aspergillus niger*, *Penicillium roqueforti,* and *Endomyces fibuliger*, which are common bakery contaminants (Valerio et al., 2009). Several compounds, which were isolated from the fermentation broth of LAB, have been characterized with antifungal activity including oligopeptides from *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 (Magnusson and Schnurer, 2001), organic acids (lactic acid, acetic acid, caproic acid, and propionic acid) from *Propionibacterium thoenii* (Lind et al., 2005), 3-phenyllactic acid from *L. plantarum* strain 21B (Lavermicocca et al., 2000, 2003), 4-hydroxyphenyllactic acid from *L. plantarum* MiLAB 14 (Sjogren et al., 2003), and cyclic dipeptides including cyclo (L-Phe-L-Pro) and cyclo (L-Phe-*trans*-4-OH-L-Pro) from a strain of *L. coryniformis* MiLAB 393 (Strom et al., 2002). However, most of the antifungal activities detected from LAB species displayed a narrow spectrum. One recent isolate of *L. paracasei* ssp. *tolerans* was reported to completely inhibit the growth of *Fusarium proliferatum* M5689, M5991, and

F. graminearum R 4053, and demonstrated potential applications as a biocontrol agent to reduce mold growth, and consequently prevent the production of mycotoxins (Hassan and Bullerman, 2008).

27.5 PERSPECTIVES

LAB are generally considered as beneficial microbes. Selected species have been used as natural preservatives and/or probiotics in dairy products, processed meats, beverages, vegetable fermentations, and silage. The fermentation products of LAB, including flavor enhancers, sweeteners, acidifiers, vitamins, and various antimicrobial agents, are highly desired; thus, more research is needed for cost-effective biochemical processes toward profitable and sustainable production.

Genetic engineering and biotechnology have been used in LAB to develop cost-effective and robust new strains to produce lactic acid, B-vitamins, low-calorie sugar alcohols such as sorbitol and mannitol (Hugenholtz and Kleerebezem, 1999; Hugenholtz et al., 2002), nisin Z (Chinachoti et al., 2008), value-added protein dextransucrase (Neubauer et al., 2003), and other secreted vaccines or antimicrobial oligopeptides (Le Loir et al., 2005). Moreover, since the LAB have GRAS status, the genetically modified strains may face fewer regulatory obstacles for applications including delivery of antigen or other proteins of interest in foodstuff or in the digestive tract of human and animals (Nouaille et al., 2003).

The fast-growing list of finished genome sequences of the LAB genera (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), and the ongoing sequencing progress (http://www.jgi.doe.gov) promise a thorough understanding of metabolic pathways (http://www.genome.jp/kegg/pathway.html) and identifying new products via proteomics and metabolomics (http://www.metabolomicscentre.nl/). Collective experiments and data from bioinformatics will allow new practical tools to be developed for genetic modifications of LAB for efficient fermentative products and for applications in the medical and pharmaceutical industry.

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28

ENZYMATIC SYNTHESIS OF GLYCOSIDES USING ALPHA-AMYLASE FAMILY ENZYMES

Kazuhisa Sugimoto, Takahisa Nishimura, Koji Nomura, Hiromi Nishiura, and Takashi Kuriki

Biochemical Research Laboratory, Ezaki Glico Co., Ltd., 4-6-5 Utajima, Nishiyodogawa-ku, Osaka 555-8502, Japan

28.1 INTRODUCTION

Glycosylation is an important method for the structural modification of bioactive compounds. It can be used to improve physicochemical and biological properties of many compounds, and it is expected that glycosylation makes compounds more useful for industrial applications. Enzymatic synthesis of glycoside is a more convenient method than chemical synthesis because it is a single-step, highly stereo- and site-specific reaction without using protecting groups. We have developed the systems to produce glycosides and glucose polymers (Yanase et al., 2007;

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Takata et al., 1996; Kakutani et al., 2007; Takaha et al., 1996; Fujii et al., 2003), and also improved enzymes used for systems based on the concept of alpha-amylase family (Takata et al., 1992; Kuriki, 1992) as a rational tool for designing and engineering the enzymes (Kuriki et al., 1996, 2006). Here, this article describes the enzymatic syntheses of several glycosides using transglycosylation reaction of alpha-amylase family enzymes. Some properties and industrial application of the glycosides are also presented.

28.2 ENZYMATIC SYNTHESIS OF ALPHA-ARBUTIN AND ITS MELANOGENESIS INHIBITION

Many kinds of biologically active compounds are used in food and cosmetic materials, and many include phenolic parts in their structures. Some of these compounds have undesirable properties such as strong pungency, low water solubility, and instability. Glycosylation of phenolic compounds was thought to be a good method to improve those properties. However, there had been a few reports on the glycosylation of phenolic compounds by microbial enzymes. Thus, 600 strains of soil microorganisms were screened for the production of hydroquinone glycosylating enzyme as a model of the enzyme that effectively catalyzes the glycosylation of phenolic compounds (Nishimura et al., 1994). Hydroquinone glycosylating enzyme was successfully isolated from a strain of *Bacillus subtilis*. Hydroquinone, resorcinol, catechins, and kojic acid were good acceptors for the transglycosylation reaction of the enzyme. However, several alcohols were not glycosylated (Table 28.1) (Nishimura et al., 1994). This property is thought to be useful for performing reactions in alcohol containing solutions when the acceptor molecule dissolves more in alcohol-containing solutions than in water. Enzymatic synthesis of hydroquinone-alpha-glucoside, i.e., alpha-arbutin (Fig. 28.1), was examined in detail (Nishimura et al., 1994). Soluble starch, dextrin, and maltopentaose were effective glucosyl donor molecules. In the initial part of the reaction, hydroquinone monoglucoside and hydroquinone oligoglucosides were produced. And, as the reaction continued, hydroquinone oligoglucosides gradually decreased, while hydroquinone monoglucoside accumulated. The enzyme-catalyzed alpha-anomer-selective transglycosylation toward hydroquinone, and arbutin, i.e., hydroquinone-beta-glucoside, was not detected at all. Furthermore, the enzyme sustained high transglycosylation activity even with a high concentration of hydroquinone, so the yield of alpha-arbutin in the reaction mixture reached more than 100 g/L. The potency of alpha-arbutin as a skin-lightening cosmetic ingredient was predicted because its anomer, arbutin, had been used for that purpose for years. Efficacy of arbutin had been studied by many researchers (Tomita et al., 1990; Sugai, 1992; Maeda and Fukuda, 1996; Chakraborty et al., 1998), and the major mechanism of the inhibitory effect of arbutin on melanin synthesis was thought to be the inhibition of tyrosinase, which is a key enzyme involved in melanin synthesis. Therefore, the inhibitory effect of alpha-arbutin on human tyrosinase was examined (Sugimoto et al., 2003). The inhibitory effect of alpha-arbutin on human tyrosinase was much stronger than that of arbutin. The 50%

TABLE 28.1 Acceptor Specificity of Hydroquinone Glucosylating Enzyme from *B. subtilis* X-23

Acceptor	Glycosylation	Acceptor	Glycosylation
Hydroquinone	+++	*o*-Methoxyphenol	+
Resorcinol	+++	*m*-Methoxyphenol	+
Catechol	++	*p*-Methoxyphenol	+
(–)Epigallocatechin gallate	+++	Gallic acid	+
(–)Epigallocatechin	+++	Tyrosine	–
(–)Epicatechin gallate	++	Kojic acid	+++
(+)Catechin	+++	Maltol	–
(–)Epicatechin	+++	Caffeic acid	++
3,4-Dimetoxyphenol	+	Methanol	–
3,5-Dimetoxyphenol	++	Ethanol	–
2,3-Dimetoxyphenol	+	1-Hexanol	–
2,6-Dimetoxyphenol	–	Geraniol	–
3,4,5-Trimetoxyphenol	–	Benzyl alcohol	–
Vanillin	+	2-Propanol	–
Capsaicin	–	2-Butanol	–
Acetaminophen	+	3-Butanol	–

Note: The reaction mixture containing the enzyme, acceptor, and maltopentaose was incubated at 40°C for 16 hours. After the reaction, glycosylated compounds were analyzed by TLC. The spots of glucosides were as follows: +++, strong; ++, medium; +, weak; –, undetected. Adapted from Nishimura et al. (1994).

inhibitory concentration (IC_{50}) of alpha-arbutin was 2.0 mM, whereas that of arbutin was more than 30 mM (Table 28.2). Furthermore, the inhibitory effects of alpha-arbutin on melanin biosynthesis in cultured human melanoma cells and in a three-dimensional cultured human skin model that is an epidermal equivalent containing normal human melanocytes and keratinocytes were examined (Sugimoto et al., 2004). The melanin production in cultured human melanoma cells and in a 3D human skin model was inhibited by alpha-arbutin in a dose-dependent manner. Additionally, alpha-arbutin exhibited no significant cytotoxicity at effective concentrations.

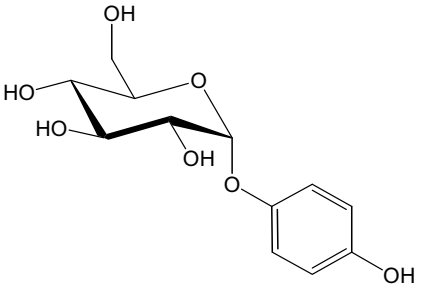

FIGURE 28.1 Structure of alpha-arbutin.

TABLE 28.2 Inhibitory Effects of Hydroquinone-Glycosides on Human Tyrosinase

Compound	IC_{50} (mM)	*K*i
Alpha-arbutin	2.1[a]	0.2[a]
Alpha-arbutin-alpha-glucoside	4.9[b]	0.6[b]
Alpha-arbutin-alpha-maltoside	13.9[b]	2.8[b]
Arbutin	>30.0[a]	4.2[a]
Arbutin-alpha-glucoside	5.7[a]	0.7[a]
Arbutin-alpha-maltoside	6.1[a]	0.9[a]

[a] Sugimoto et al. (2003).
[b] Sugimoto et al. (2005).

Figure 28.2 shows the macroscopic and inverted microscopic views of the human skin model cultivated with and without alpha-arbutin. Darkening of the human skin model was significantly inhibited by alpha-arbutin and alpha-arbutin-treated tissues had fewer darkened melanocytes than nontreated tissue. Standard, well-defined safety tests have been performed with alpha-arbutin, and it was concluded that alpha-arbutin is an effective and safe ingredient for skin-lightening cosmetics. It is expected that alpha-arbutin will contribute to enhance the QOL for many people.

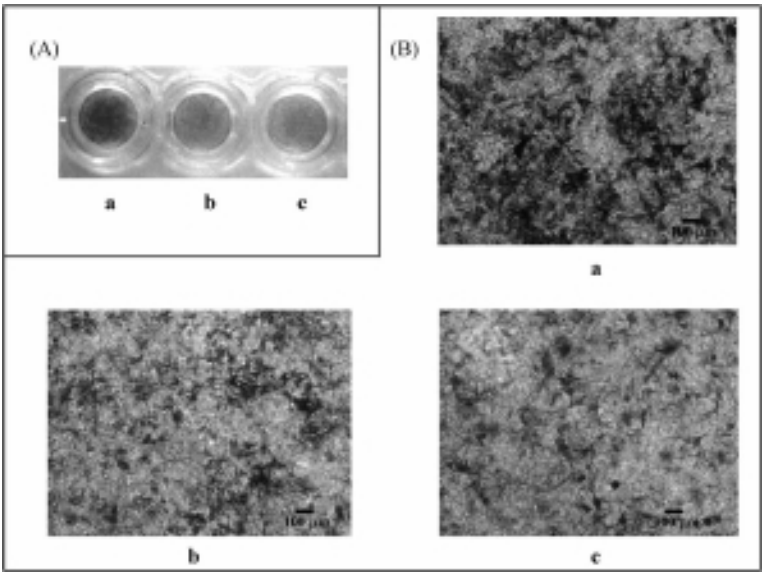

FIGURE 28.2 Macroscopic and microscopic views of a cultured human skin model with and without alpha-arbutin treatment. The human skin model was cultivated for 13 days. Alpha-arbutin dissolved in 0.1 ml of ultrapure water was applied to the surface of the tissues on days 0, 2, 4, 6, 8, and 10. (A) Macroscopic view of the cell cultures. (B) Inverted microscopic view of the cell cultures. a, control; b, 125 µg/tissue; c, 250 µg/tissue. Adapted from Sugimoto et al. (2004).

28.3 ENZYMATIC SYNTHESES OF HYDROQUINONE GLYCOSIDES AND THEIR INHIBITORY EFFECTS ON HUMAN TYROSINASE

The relationship between the inhibitory effects of hydroquinone-glycosides on human tyrosinase and the glycon structures was of interest. So, the synthesis of arbutin-alpha-glycosides (Sugimoto et al., 2003) and alpha-arbutin-alpha-glycosides (Sugimoto et al., 2005) using cyclomaltodextrin glucanotransferase from *Bacillus macerans* was examined. Alpha-arbutin or arbutin was used as the acceptor molecule and soluble starch was used as the donor molecule. Both alpha-arbutin and arbutin were good acceptor molecules because more than 70% of alpha-arbutin or arbutin supplied was estimated to be glycosylated. The two major glycosylated products of alpha-arbutin and those of arbutin were isolated. The structural analyses using ^{13}C-NMR and ^{1}H-NMR proved that the glycosylated products obtained from alpha-arbutin were alpha-arbutin-alpha-glucoside and alpha-arbutin-alpha-maltoside and those obtained from arbutin were arbutin-alpha-glucoside and arbutin-alpha-maltoside. Inhibitory effects of isolated inhibitors on human tyrosinase were examined. Kinetic analysis showed that all of these inhibitors were competitive inhibitors of the enzyme. The IC_{50} values and K_i values of the inhibition on human tyrosinase are shown in Table 28.2. The inhibitory effect of alpha-arbutin became lower by further glycosylation. On the other hand, the inhibitory effects of arbutin-alpha-glycosides were stronger than that of arbutin. It was very interesting to note that the inhibitory effect of alpha-arbutin was the highest and that of arbutin was the lowest among these inhibitors, and that the effect of glycosylation of arbutin on human tyrosinase inhibition was strikingly different from that of alpha-arbutin. The 3D-structure of human tyrosinase is still unknown. To estimate the relationship between structure and inhibitory activity, optimized structures of obtained hydroquinone glycosides were calculated using density functional theory (DFT), and electrostatic potential calculations were carried out on DFT-optimized structures (Sugimoto et al., 2005). A comparison of ESPs of these inhibitors revealed that significant negative ESPs existed around the benzene ring of arbutin but not on arbutin-glucoside and the other inhibitors. It is speculated that the difference of ESPs is one reason why the inhibitory effect of arbutin is the lowest among these inhibitors and that glycosylation affects the inhibitory effects of hydroquinone glycosides on human tyrosinase by changing their electrostatic nature.

28.4 TRANSGLYCOSYLATION TO CARBOXYLIC ACID BY SUCROSE PHOSPHORYLASE

Besides phenolic compounds, there are many biologically active compounds that have carboxyl group in their molecules. But, no one has reported that carbohydrate active enzymes catalyze transglycosylation to carboxylic acid without using nucleotide-activated sugars. Several carbohydrate active enzymes were screened for their ability to glycosylate carboxylic acid compounds, and it was found that sucrose phosphorylases (EC 2.4.1.7) catalyze the transglycosylation reaction to carboxylic groups (Nomura et al., 2006). Sucrose phosphorylases catalyze the phosphorolysis of

sucrose and produces glucose-1-phosphate and fructose (Doudoroff, 1943). It has been known that the enzyme also catalyzes transglycosylation reactions in many compounds that have hydroxyl groups, such as saccharides, alcohols, and phenolic compounds, using sucrose as a glucosyl donor molecule (Kitao and Sekine, 1994; Mieyal and Abeles, 1972; Kitaoka et al., 1994). Transglycosylation reaction of the enzyme to benzoic acid, a model of carboxylic acid compounds, was examined in detail (Sugimoto et al., 2007). Two well-known sucrose phosphorylases from *Streptococcus mutans* and *Leuconostoc mesenteroides* catalyze the transglycosylation reaction to benzoic acid, particularly under acidic conditions (Table 28.3). The optimum pH and the pH–activity profile of the transglycosylation activity of sucrose phosphorylase from *S. mutans* toward benzoic acid were quite different from those of the phosphorolytic activity (Fujii et al., 2006). The pK_a of benzoic acid is known to be 4.2, and the concentration of undissociated form of benzoic acid around neutral pH is very low. It was thought that these results support the hypothesis that undissociated form of carboxylic acid could be used as an effective acceptor molecule for the enzyme reaction. Transglycosylation reaction to benzoic acid by the enzyme and the formation of the products in the reaction mixture were examined in detail. HPLC analysis of the reaction mixture and subsequent analyses of purified products revealed that three compounds, 1-*O*-benzoyl alpha-D-glucopyranose, 2-*O*-benzoyl alpha-D-glucopyranose, and 2-*O*-benzoyl beta-D-glucopyranose, were produced. From the result of the production pattern of these compounds during the reaction, it was concluded that 1-*O*-benzoyl alpha-D-glucopyranose was produced initially by the enzyme reaction, and thereafter the other two compounds were nonenzymatically produced by intramolecular acyl migration reaction. The acceptor specificity of *S. mutans* sucrose phosphorylase is summarized in Table 28.4. The enzyme had broad acceptor specificity, and it catalyzed the transfer of glucosyl moiety of sucrose not only to benzoic acid but also to various carboxylic acids such as short-chain fatty

TABLE 28.3 Effect of pH on Transglucosylation Efficiency of Sucrose Phosphorylases

Enzyme (pH)	Glycosylation Ratio (%)
Leuconostoc mesenteroides	
3.9	0.0
5.1	10.0
6.1	4.0
7.1	0.5
Streptococcus mutans	
3.9	55.0
5.1	8.0
5.9	1.0
7.1	0.5

Note: Sucrose phosphorylase from *S. mutans* or *L. mesenteroides* (100 U) was added to the substrate mixture (1 ml) containing 0.8% benzoic acid and 30% sucrose in distilled water. The reaction mixture was incubated at 40°C for 16 hours and then analyzed by HPLC with UV detection at 280 nm. The glycosylation ratio was expressed as the percentage of the peak area of the transfer product against the total peak area of the transfer product and benzoic acid. Adapted from Sugimoto et al. (2007).

TABLE 28.4 Acceptor Specificity of Sucrose Phosphorylase from *S. mutans* Toward Various Carboxylic Compounds

Acceptor Molecules	Glycosylation
Aromatics	
Benzoic acid	+++
Ferulic acid	++[a]
Fatty acid	
Formic acid	++
Acetic acid	++++
Propionic acid	+++
Butyric acid	+++
Valeric acid	+++
Hexanoic acid	+
Octanoic acid	–
Hydroxy acid	
Lactic acid	+++[a]
Malic acid	++[a]
Dicarboxylic acid	
Malonic acid	++++
Maleic acid	++
Fumaric acid	+++

Note: The glycosylation ratios for each glucoside are as follows: ++++, >90%; +++, 50∼90%; ++, 10∼50%; +, < 10%; and –, undetected.

[a] Represents the glycosylation toward both carboxyl and hydroxyl groups. Adapted from Sugimoto et al. (2007).

acids, hydroxy acids, dicarboxylic acids, and acetic acid. Glycosylation of acetic acid by the enzyme and characterization of the product were also examined in detail (Nomura et al., 2008). The acidic taste of acetic acid was markedly reduced by glycosylation. It is expected that the sucrose phosphorylase should be a very useful enzyme for producing various monoacyl-glucoses.

28.5 CONCLUSIONS

As described above, several systems to produce glycosides of various compounds like phenolic compounds, glycosides, and carboxylic acid have been developed. They are probably very useful for improving the characteristics of biologically active compounds for use in food additives, cosmetics, and drugs. The development of new functional glycosides is expected to make further progress.

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29

BIOLOGICAL SYNTHESIS OF GOLD AND SILVER NANOPARTICLES USING PLANT LEAF EXTRACTS AND ANTIMICROBIAL APPLICATION

BEOM SOO KIM AND JAE YONG SONG

Department of Chemical Engineering, Chungbuk National University, Cheongju, Chungbuk 361-763, Republic of Korea

29.1 INTRODUCTION

Noble metal nanoparticles such as of gold, silver, and platinum are widely applied to products for human use such as shampoos, soaps, detergents, shoes, cosmetic products, and toothpastes as well as medicinal and pharmaceutical products. Therefore, there is a growing need to develop environmentally friendly processes of nanoparticles synthesis that do not use toxic chemicals. Biological methods of

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nanoparticle synthesis using microorganisms, enzymes, and plant or plant extracts have been suggested as possible ecofriendly alternatives to chemical and physical methods (Mohanpuria et al., 2008; Sharma et al., 2009).

Using plants for nanoparticles synthesis can be advantageous over other biological processes because it eliminates the elaborate process of maintaining cell cultures and can also be suitably scaled up for large-scale synthesis of nanoparticles (Shankar et al., 2004a). Gardea-Torresdey et al. (2002, 2003) demonstrated the synthesis of gold and silver nanoparticles within live alfalfa plants from solid media. Extracellular nanoparticles synthesis using plant leaf extracts rather than the whole plant would be more economical due to the easier downstream processing. The pioneering works of nanoparticles synthesis using plant extracts have been carried out by Sastry group (Shankar et al., 2003a, 2003b, 2004a, 2004b; Rai et al., 2006, 2007; Chandran et al., 2006). They reported that the rates of nanoparticles synthesis using plant extracts could be comparable to those of chemical methods. The shape of the nanoparticles plays a crucial role in modulating their optical properties. Gold nanotriangles were formed when lemongrass (*Cymbopogon flexuosus*) leaf extract was reacted with aqueous $AuCl_4^-$ ions (Shankar et al., 2004b). Gold and silver nanotriangles, in particular, are promising, as they could find potential applications in cancer hyperthermia and infrared-radiation-absorbing optical coatings (Chandran et al., 2006).

Recent reports on phytosynthesis of silver and gold nanoparticles employed coriander leaf (Narayanan and Sakthivel, 2008), sun-dried *Cinnamomum camphora* leaf (Huang et al., 2007), phyllanthin extract (Kasthuri et al., 2009a), and purified apiin compound extracted from henna leaf (Kasthuri et al., 2009b). The marked difference of shape control between gold and silver nanoparticles was attributed to the comparative advantage of protective biomolecules and reductive biomolecules. With sun-dried *C. camphora* leaf, the polyol components and the water-soluble heterocyclic components were mainly found to be responsible for the reduction of silver ions or chloroaurate ions and the stabilization of the nanoparticles, respectively (Huang et al., 2007). In phyllanthin-assisted biosynthesis of silver and gold nanoparticles, the rate of reduction of $HAuCl_4$ was greater than that of $AgNO_3$ at a constant amount of phyllanthin extract (Kasthuri et al., 2009a).

The size and shape of the nanoparticles could be controlled by varying the concentration of phyllanthin extract, thereby tuning their optical properties. Low concentration of the extract with $HAuCl_4$ led to the formation of hexagonal- or triangular-shaped gold nanoparticles, while the shape changed to spherical particles by increasing the initial concentration of phyllanthin extract.

In most reports on nanoparticles synthesis using plant extracts, the times required for conversion of Ag^+ and Au^{3+} ions to Ag and Au nanoparticles were several hours, which was longer than that for chemical synthesis (Shankar et al., 2004a). If biological synthesis of nanoparticles can compete with chemical methods, there is a need to achieve faster synthesis rates. We carried out engineering approaches such as rapid nanoparticles synthesis using plant extracts and size and shape control of the synthesized nanoparticles (Song and Kim, 2009). Several plant leaf extracts were screened and compared for their synthesis of gold and silver nanoparticles by

monitoring the conversion using UV–visible spectroscopy. The effects of reaction conditions such as temperature and composition of the reaction mixture were also investigated to control the size and shape of nanoparticles.

In this article, we summarize the biological synthesis of gold, silver, and gold/silver bimetal nanoparticles using several plant leaf extracts and the characterization of the nanoparticles. We also report antimicrobial application of the biosynthesized silver nanoparticles by coating on latex foam products.

29.2 SYNTHESIS OF SILVER NANOPARTICLES USING PLANT LEAF EXTRACTS

Silver has long been recognized as having an inhibitory effect toward many bacterial strains and microorganisms commonly present in medical and industrial processes (Jiang et al., 2004). The most widely used and known applications of silver and silver nanoparticles are in the medical industry. These include topical ointments and creams containing silver to prevent infection of burns and open wounds (Becker, 1999). Other widely used applications are medical devices and implants prepared with silver-impregnated polymers (Silver, 2003). In addition, silver-containing consumer products such as colloidal silver gel and silver-embedded fabrics are now used in sporting equipment.

We screened several plant leaf extracts and compared their synthesis of silver nanoparticles by monitoring the conversion using UV–visible spectroscopy (Song and Kim, 2009). Five plant leaves were collected and dried for 2 days at room temperature. They were pine (*Pinus desiflora*), persimmon (*Diopyros kaki*), ginkgo (*Ginko biloba*), magnolia (*Magnolia kobus*), and platanus (*Platanus orientalis*). The plant leaf broth solution was prepared by taking 5 g of thoroughly washed and finely cut leaves in a 300 mL Erlenmeyer flask with 100 mL of sterile distilled water and then boiling the mixture for 5 min before finally decanting it. They were stored at 4°C and used within a week. The experimental procedure is summarized in Figure 29.1.

Typically, 10 mL of leaf broth was added to 190 mL of 1 mM aqueous $AgNO_3$ solution for the reduction of Ag^+ ions. The effects of temperature on synthesis rate and particle size of the prepared silver nanoparticles were studied by carrying out the reaction in water bath at 25–95°C with reflux. The concentrations of $AgNO_3$ solution and leaf broth were also varied at 0.1–2 mM and 5–50% by volume, respectively. The silver nanoparticle solution thus obtained was purified by repeated centrifugation at 15,000 rpm for 20 min followed by redispersion of the pellet in deionized water. After freeze drying of the purified silver particles, the structure and composition were analyzed by scanning electron microscopy (SEM), field emission transmission electron microscopy (FE-TEM), energy-dispersive X-ray spectroscopy (EDS), and X-ray photoelectron spectroscopy (XPS). Silver concentrations and conversions were determined using inductively coupled plasma spectrometry (ICP). Average particle size and distribution were measured using particle analyzer.

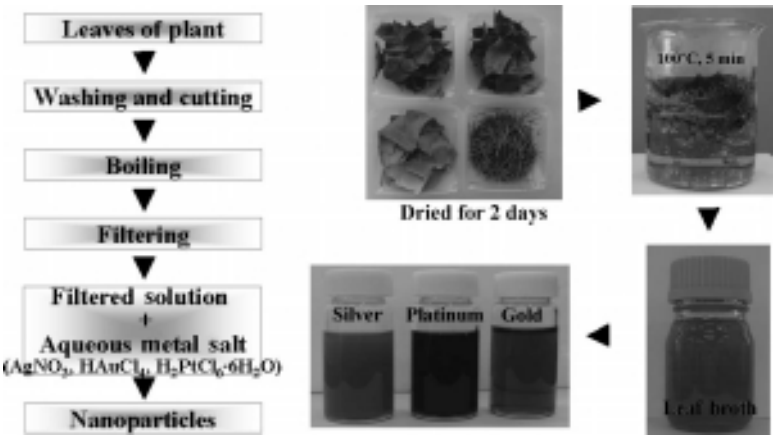

FIGURE 29.1 Schematic diagram of experimental procedure on nanoparticles synthesis using plant extracts.

Reduction of the silver ion to silver nanoparticles during exposure to the plant leaf extracts could be followed by color change to yellowish-brown and thus UV–visible spectroscopy. The maximum absorbance occurred at ca. 430 nm and steadily increased in intensity as a function of reaction time. Since the peak wavelength did not shift during the reaction, we could quantitatively monitor the concentrations of silver nanoparticles and thus conversion by measuring the absorbance at 430 nm. A linear relationship was obtained between the silver concentration determined by ICP and the absorbance at 430 nm.

As the reaction temperature increased, both the synthesis rate and final conversion to silver nanoparticles increased. With Persimmon leaf broth, the final conversion at 25°C was 60% and reached almost 100% at more than 55°C. The average particle size decreased from 50 nm at 25°C to 30 nm at 55°C and 16 nm at 95°C. The reason for decrease in particle size with temperature can be explained as follows. As the reaction temperature increases, the reaction rate increases and thus most silver ions are consumed in the formation of nuclei, stopping the secondary reduction process on the surface of the preformed nuclei.

EDS profile recorded from the silver nanoparticles showed strong silver signals along with a weak oxygen and carbon peak, which may originate from the biomolecules that are bound to the surface of the silver nanoparticles. TEM images in Figure 29.2 show that the silver particles synthesized using plant extracts are surrounded by a thin layer of some capping material and thus were stable in solution after their synthesis.

In order to screen plants with high production capability of silver nanoparticles, we compared several plant extracts for their synthesis rate of silver nanoparticles. The synthesis rate was highest with Magnolia leaf broth. Only 11 min were required for more than 90% conversion at 95°C, which was faster than, or comparable to, the synthesis rate of chemical methods. The average particle size could be controlled

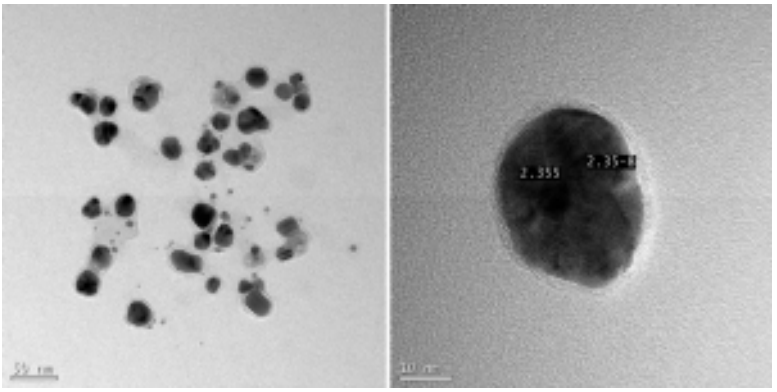

FIGURE 29.2 TEM image of silver nanoparticles formed with 1 mM $AgNO_3$ and 5% *Magnolia kobus* leaf broth at 95°C.

from 15 to 500 nm by changing the reaction temperature, leaf broth concentration, and $AgNO_3$ concentration (Song and Kim, 2009).

29.3 SYNTHESIS OF GOLD NANOPARTICLES USING PLANT LEAF EXTRACTS

Gold has a long history of use. Red colloidal gold has been used as medicine for revitalization in China and India (Bhattacharya and Murkherjee, 2008). Gold nanoparticles have found use in diagnostic and drug delivery applications (Bhumkar et al., 2007). Magnolia leaf broth as reducing agent, which was screened for high production rate of silver nanoparticles, also showed high synthesis rate for gold nanoparticles. Only 3 min were required for more than 90% conversion to gold nanoparticles at 95°C, which was faster than 11 min for the silver nanoparticles formation using the same leaf broth due to the higher reduction potential of Au^{3+} than Ag^{+} ion. The final conversion to gold nanoparticles was almost 100% at all the reaction temperatures, although the synthesis time was longer at room temperature.

UV–visible spectra recorded as a function of reaction time and TEM image are shown in Figure 29.3 obtained with 5% Magnolia leaf broth and 1 mM $HAuCl_4$ solution at 25°C. It is observed that the maximum absorbance occurs at ca. 540 nm and increases to saturation in intensity as a function of reaction time. A mixture of plates (triangle, pentagon, and hexagon) and spheres is shown with a wide size range of 5–100 nm. The shape of gold nanoparticles changed to mainly spheres with some plates at a higher temperature of 95°C. The change of morphology with reaction temperature can be explained in relation to the reaction rate. At higher temperature, most gold ions form the first nuclei and the secondary growth of particles stops because the reaction rate is too fast. The particle size and shape were further controlled

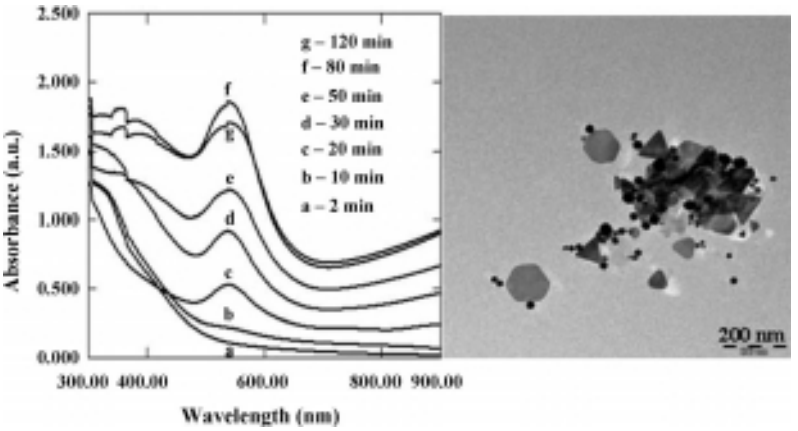

FIGURE 29.3 UV–visible spectra recorded as a function of reaction time and TEM image of gold nanoparticles formed with 1 mM $HAuCl_4$ and 5% *Magnolia kobus* leaf broth at 25°C.

by changing the composition of the reaction mixture. Compared with 5% leaf broth concentration as in Figure 29.3, large plate structures were not observed and most particles were spherical at leaf broth concentrations higher than 10%. The particle size decreased on increasing the leaf broth concentration.

Control of the shape and size of metallic nanoparticles enables tuning of their optical, electronic, magnetic, and catalytic properties. Gold nanoparticles synthesized by chemical methods are usually spherical but nanostructures with triangular morphology are rare. It was reported that the average edge length of the gold nanotriangles decreases with an increase in the amount of *Aloe vera* extract added (Chandran et al., 2006). The role of halide ions and temperature on the morphology of biogenic gold nanotriangles was also studied by Rai et al. (2006). They suggested that the presence of Cl^- ions during the synthesis promoted the growth of nanotriangles, whereas the presence of I^- ions distorted the nanotriangle morphology and induced the formation of aggregated spherical nanoparticles. Physical control of nanoparticle shape and size was reported using a granular medium with percolative microcavities that facilitate nearly similar grain size/shape-dependent reaction zones (Ogale et al., 2006).

29.4 SYNTHESIS OF GOLD/SILVER BIMETAL NANOPARTICLES USING PLANT LEAF EXTRACTS

In addition to pure metallic nanoparticles, bimetallic nanoparticles in the form of either alloys or core–shell nanostructures are being increasingly investigated because of their unusual electronic/optical and catalytic properties (Rai et al., 2007). We reported the synthesis of bimetallic Au/Ag nanoparticles by the competitive reduction of Au^{3+} and Ag^+ ions using Persimmon leaf broth and suggested that bimetallic Au core/Ag shell structure was formed through the characterization data (Song and Kim, 2008).

For synthesis of Au/Ag bimetallic nanoparticles, 190 mL of a 1 : 1 1 mM solution of $AgNO_3$ and $HAuCl_4$ was taken along with 10 mL of Persimmon broth. The reduction reaction was carried out in a water bath at 95°C with reflux. Large Au/Ag bimetallic particles with size 50–500 nm were formed with some cubic structure, while relatively spherical and uniform silver nanoparticles were formed with diameters of 15–90 nm. A possible reason of larger particles formation in Au/Ag bimetals is as follows. Gold nanoparticles are initially formed because the reduction rate of gold ion is faster than that of silver ion. Silver nanoparticles then assembled onto the surface of the gold nanoparticles due to interactions such as hydrogen bonds and electrostatic interactions between the bioorganic capping molecules bound to the gold and silver nanoparticles.

Shankar et al. (2004a) reported that bimetallic Au core/Ag shell nanoparticles were formed by competitive reduction of Au^{3+} and Ag^{+} ions in the 1 : 1 molar mixture by Neem leaf broth. Although it is plausible that Au core/Ag shell nanostructures would be formed, since the reduction rate of gold ion is faster than that of silver ion, no quantitative proofs on the core/shell nanostructures have been provided. We measured atomic contents of gold and silver in the bimetallic Au/Ag nanoparticles by EDS and XPS analysis. The atomic Ag content from EDS analysis was 36 wt%, which was correlated with the theoretical atomic Ag content in the bimetallic Au/Ag nanoparticles synthesized from 1 : 1 molar mixture of Au^{3+} and Ag^{+} ions. Meanwhile, the atomic Ag content from XPS analysis was 71 wt%, higher than that from EDS analysis. This shows that silver nanoparticles are richer near the surface of bimetallic Au/Ag nanoparticles because XPS is a surface chemical analysis technique that can be used to analyze from the top 1 to 10 nm of a material (Song and Kim, 2008).

29.5 ANTIMICROBIAL APPLICATION OF SILVER NANOPARTICLES SYNTHESIZED USING PLANT LEAF EXTRACTS

We studied antimicrobial application of silver nanoparticles prepared using *Magnolia kobus* leaf extract. Silver nanoparticles were coated on natural latex foam products that are used as materials to prepare pillows and mattresses. Five grams of latex foams (Latex Korea Co.) were put in 100 mL of three different concentrations of silver nanocolloidal solution at 0.2, 0.5, and 1 mM and incubated in a shaker for 1 h. The color of the treated foams changed to dark brown on increasing the silver nanoparticles concentration due to the coating of silver nanoparticles on the surface of foams, while the untreated foams were white. SEM image of the treated foam shows that silver nanoparticles are coated on the surface of latex foam (Fig. 29.4).

Table 29.1 summarizes the effects of concentration and synthesis temperature of silver nanoparticles used for the treatment of latex foam. Average sizes of silver nanoparticles used were in the range of 15–50 nm, depending on the synthesis temperature at 25–90°C. It is shown that the coated amounts of silver nanoparticles depend on the synthesis temperature and concentration of silver nanoparticles. Higher silver nanoparticles concentration gave higher coated amounts

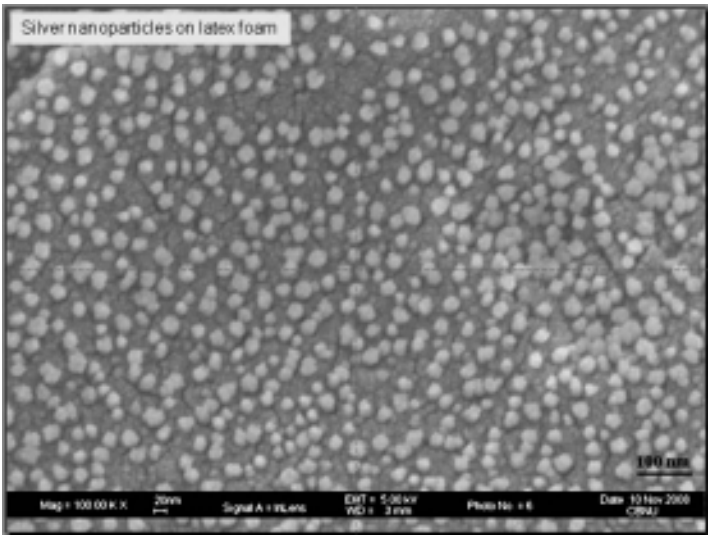

FIGURE 29.4 SEM image of latex foam after coating with silver nanoparticles.

of silver nanoparticles on latex foam. The coated amounts of smaller nanoparticles prepared at 95°C were lower than those of larger nanoparticles prepared at 25 and 60°C. The highest silver nanoparticles content coated on latex foam was 0.21% when the concentration and synthesis temperature of silver nanoparticles were 1 mM and 25°C, respectively.

For antimicrobial test, *Escherichia coli* cells were grown in LB media containing latex foams for 24 h at 37°C. Then 100 μL of cultured solution was transferred to agar medium and incubated for another 24 h. Figures 29.5a and 29.5b show the effects of synthesis temperature (particle size) and concentration of silver nanoparticles on the growth of *E. coli*. It is shown that the growth of *E. coli* was significantly suppressed by treating with silver nanoparticles compared with control (culture solution containing

TABLE 29.1 Effect of Concentration and Synthesis Temperature of Silver Nanoparticles on Coated Amounts

Nanoparticles concentration (mM)	1			0.5			0.2		
Synthesis temperature (°C)	95	60	25	95	60	25	95	60	25
Average size (nm)	50	30	16	50	30	16	50	30	16
Coated amount (mg/5 g latex foam)	4.0	10.5	10.4	0.53	2.9	4.6	0.38	1.0	0.96
Nanoparticles content on latex foam (wt%)	0.08	0.20	0.21	0.011	0.058	0.092	0.008	0.020	0.019

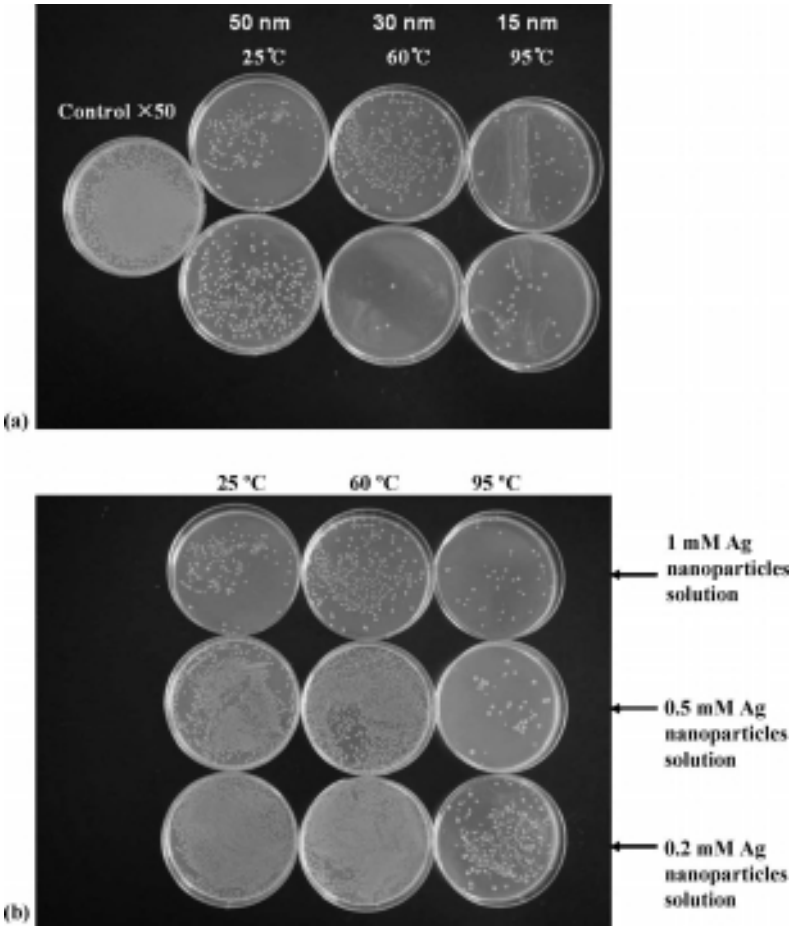

FIGURE 29.5 Effects of (a) synthesis temperature (nanoparticle size) and (b) concentration of silver nanoparticles on the growth of *E. coli*. Five grams of latex foams were put in 100 mL of 1 mM silver nanocolloidal solution and incubated in shaker for 1 h. *E. coli* cells were grown in LB media containing silver nanoparticles-treated latex foams for 24 h at 37°C. Then 100 µL of cultured solution was transferred to agar medium and incubated for another 24 h. Control is culture solution containing untreated latex foams.

untreated latex foams). Smaller nanoparticles showed higher antimicrobial activity possibly due to the larger specific surface area (Fig. 29.5a). The growth of *E. coli* decreased with an increase in the silver nanoparticles concentration (Fig. 29.5b).

Regarding the mechanism of antimicrobial activity of silver nanoparticles, several studies propose that silver nanoparticles attach to the surface of cell membrane disturbing permeability and respiration function of the cell (Sharma et al., 2009; Rai et al., 2009). The damage to the cell may be caused by the interaction of silver nanoparticles with phosphate- or sulfur-containing compounds such as DNA. Since the action of silver nanoparticles is not species-dependent, silver nanoparticles can possibly be used for the

treatment of multidrug-resistant bacteria. Recently, there is a concern that silver nanoparticles may be harmful to human health. However, no sufficient information is available on the adverse effects of nanoparticles on human health.

29.6 CONCLUSIONS

Nanoparticles were rapidly synthesized using *Magnolia kobus* leaf broth. Only 11 and 3 min were required for more than 90% conversion to silver and gold nanoparticles, respectively. The size and shape of the particles could be controlled by changing the temperature and composition of the reaction mixture. Gold/silver bimetal nanoparticles were also synthesized with core/shell structure using plant extracts. The growth of *E. coli* in shake flask culture was significantly suppressed by treatment of silver nanoparticles to the latex foams. This environmentally friendly method of biological metal nanoparticles production can potentially be used in various areas such as consumer goods, foods, medical and pharmaceutical applications.

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30

POTENTIAL APPROACH OF MICROBIAL CONVERSION TO DEVELOP NEW ANTIFUNGAL PRODUCTS OF OMEGA-3 FATTY ACIDS

VIVEK K. BAJPAI AND SUN-CHUL KANG

Department of Biotechnology, Daegu University, Kyoungsan, Kyoungbook 712-714, Republic of Korea

HAK-RYUL KIM

Department of Animal Science and Biotechnology, Kyoungpook National University, Daegu 702-701, Republic of Korea

CHING T. HOU

Microbial Genomic and Bioprocessing Research Unit, National Centre for Agricultural Utilization Research, ARS, USDA, Peoria IL 61604, USA

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30.1 INTRODUCTION

Omega-3/(ω-3) or *n*-3 fatty acids are a family of unsaturated fatty acids that have in common a final carbon–carbon double bond in the *n*-3 position. The term *n*-3 signifies that the first double bond exists as the third carbon–carbon bond from the terminal methyl end (*n*) of the carbon chain.

n-3 fatty acids that are important in human nutrition are: α-linolenic acid (18:3, *n*-3; ALA), eicosapentaenoic acid (20:5, *n*-3; EPA), and docosahexaenoic acid (22:6, *n*-3; DHA). These three polyunsaturates have either three, five, or six double bonds in a carbon chain of 18, 20, or 22 carbon atoms, respectively. All double bonds are in the *cis*-configuration, i.e., the two hydrogen atoms are on the same side of the double bond.

Most naturally produced fatty acids (created or transformed in animal or plant cells with an even number of carbon in chains) are in *cis*-configuration where they are more easily transformable. The *trans*-configuration results in much more stable chains that are very difficult to further break or transform, forming longer chains that aggregate in tissues and lacking the necessary hydrophilic properties. This *trans*-configuration can be the result of the transformation in alkaline solutions, or of the action of some bacteria that are shortening the carbonic chains. Natural transforms in vegetal or animal cells more rarely affect the last *n*-3 group itself.

A 1992 article by biochemist William E.M. Lands provides an overview of the research into *n*-3 fatty acids, and is the basis of this section (Lands, 1992). A small amount of *n*-3 in the diet (∼1% of total calories) enabled normal growth in children and animals, and increasing the amount had little to no additional effect on growth. These preliminary findings led researchers to concentrate their studies on *n*-3, and it was only in recent decades that *n*-3 has become of interest.

The U.S. Food and Drug Administration gave "qualified health claim" status to EPA and DHA *n*-3 fatty acids, stating that "supportive but not conclusive research shows that consumption of EPA and DHA [*n*-3] fatty acids may reduce the risk of coronary heart disease (United States Food and Drug Administration, 2004). DHA and EPA are made by microalgae that live in seawater. These are then consumed by fish and accumulate to high levels in their internal organs. People with certain circulatory problems, such as varicose veins, benefit from fish oil because it contains the EPA and DHA derived from microalgae. Fish oil stimulates blood circulation, increases the breakdown of fibrin, a compound involved in clot and scar formation, and additionally has been shown to reduce blood pressure (Morris et al., 1993; Mori et al., 1993). There is strong scientific evidence that *n*-3 fatty acids reduce blood triglyceride levels (Harris, 1997; Sanders et al., 1997; Roche and Gibney, 1996; Davidson et al., 2007) and regular intake reduces the risk of secondary and primary heart attack (Bucher et al., 2002; Burr et al., 1994; Willett et al., 1993; Stone, 1996).

Several studies report possible anticancer effects of *n*-3 fatty acids particularly breast, colon, and prostate cancer (Augustsson et al., 2003; De Deckere, 1999; Caygill and Hill, 1995). ω-3 fatty acids reduced prostate tumor growth, slowed histopathological progression, and increased survival (Yong, 2007). In 1999, the GISSI-Prevenzione Investigators reported in the *Lancet*, the results of a major clinical

study in 11,324 patients with a recent myocardial infarction. Treatment with 1 g/d of *n*-3 fatty acids reduced the occurrence of death, cardiovascular death, and sudden cardiac death by 20, 30, and 45% respectively (GISSI-Prevenzione, 1999).

Another study examining whether ω-3 exerts neuroprotective action in Parkinson's disease found that it did, using an experimental mice model, exhibit a protective effect, much like it did for Alzheimer's disease as well (Marchioli, 2002). The scientists found that high doses of ω-3 given to the experimental group completely prevented the neurotoxin-induced decrease of dopamine that ordinarily occurs. Since Parkinson's is a disease caused by disruption of the dopamine system, this protective effect exhibited could show promise for future research in the prevention of Parkinson's disease.

Further clinical studies indicated that *n*-3 fatty acids are essential, i.e., humans must consume them in the diet. *n*-3 competes for the metabolic enzymes; thus, *n*-3 will significantly influence the ratio of the ensuing eicosanoids (hormones) (e.g. prostaglandins, leukotrienes, thromboxanes etc.), and will alter the body's metabolic function (Tribole, 2006).

Due to recent research activities, we are now beginning to learn more on how the microbial conversion of *n*-3 fatty acids is compatible in developing new value-added bioconverted industrial products of *n*-3 fatty acids by *Pseudomonas aeruginosa* PRB to combat against plant fungal diseases. We have confirmed that how these bioconverted industrial products of ω-3/*n*-3 fatty acids could effectively inhibit the growth of certain important plant pathogenic fungi that cause severe losses to food and agriculture industries. However, there is not much research on the antifungal properties of bioconverted products of ω-3 fatty acids. Therefore, the aim of this review is to present the progress as to what is known of the biological activities of these bioconverted industrial products of *n*-3 fatty acids with particular emphasis on EPA and DHA bioconverted products as well as their possible applications in food and agriculture industries to control certain important plant pathogenic fungi.

30.2 DEVELOPMENT OF NEW ANTIFUNGAL PRODUCTS OF OMEGA-3 FATTY ACID BY THE MICROBIAL CONVERSION

30.2.1 Microbial Conversion of Omega-3 Fatty Acids

Microbial conversion "green technology" is a biological process that converts fatty acids into entirely new chemical compounds with antimicrobial or biomedical properties (Bajpai et al., 2008). These properties give bioconverted products to be used in a wide range of industrial applications (Hagemann and Rothfus, 1991). Industrial products are many and include both cells and substances made by cells. Primary and secondary metabolites are produced during active cell growth or near the onset of stationary phase. Many economically valuable microbial products are secondary metabolites. Microbial bioconversion employs microorganisms to biocatalyze a specific step or steps in an otherwise strictly chemical synthesis (Bajpai et al., 2009; Carballeira, 2008). An industrial microorganism can produce the product

of interest in high yield, grow rapidly on inexpensive culture media available in bulk quantities, be amenable to genetic manipulation, and, if possible, be nonpathogenic. However, microbial conversion of unsaturated fatty acids has been widely exploited to produce new, value-added hydroxy products (Hou and Bagby, 1991; Kim, 2001). The bioconversion reactions by *P. aeruginosa* PR3 have been cited extensively among microbial systems that produce mono-, di-, and trihydroxy fatty acid derivatives from unsaturated fatty acids (Hou and Bagby, 1991; Kim, 2001; Kuo, 1998; Kuo, 2001). Strain PR3, isolated from a wastewater stream on a pig farm in Morton, Illinois, was found to convert oleic acid to a novel compound, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) (Hou and Bagby, 1991), and to convert ricinoleic acid to a novel compound, 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD) (Kuo, 1998). Microbial oxidation of unsaturated fatty acids was recently reviewed (Hou, 1995). Among those unsaturated fatty acids used for microbial production of hydroxy fatty acids oleic acid, linoleic acid and linolenic acid were well studied as substrates to produce mono-, di-, and trihydroxy fatty acids (Wallen, 1962; De Andres, 1994; Hou, 1996). Various value-added hydroxy fatty acids and their derivates for industrial applications can be produced by using the unique reaction specificities of microbial enzymes. *Clavibacter* sp. ALA2 converted linoleic acid to 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid (12,13,17-THOA) and 12,13,16-THOA (Bajpai, 2009). Recently, the EPA and DHA were converted to 15, 18-dihydroxy-14, 17-epoxy-5(*Z*), 8(*Z*), 11(*Z*)-eicosatrienoic acid and 17, 20-dihydroxy-16, 19-epoxy-4(*Z*), 7(*Z*), 10(*Z*), 13(*Z*)-docosatetraenoic acid with *Clavibacter* sp. ALA2 (Hosokawa, 2003). More recently our research group reported the microbial conversion of ω-3 fatty acids. Some polyunsaturated fatty acids were tested for the production of hydroxyl fatty acids by *P. aeruginosa* PR3 because polyunsaturated fatty acids contained a 1,4-*cis, cis*-diene unit that could serve as a key structural element for the hydroxylation by lipoxygenase. When EPA and DHA carrying 1,4-*cis,cis*-diene unit were used as substrates for bioconversion by PR3, new products were detected on TLC analysis as shown in Figure 30.1. Although new products were not clearly separated into spots, there were noticeable differences in band patterns between the bioconverted fatty acids and substrate fatty acids.

30.2.2 Introduction to Antifungal Diseases

Fungi cause more plant diseases than any other group of plant pest with over 8000 species shown to cause disease. Fungi are eukaryotic organisms that lack chlorophyll and thus do not have the ability to photosynthesize their own food. They obtain nutrients by absorption through tiny thread-like filaments called hyphae that branch in all directions throughout a substrate. A collection of hyphae is referred to as mycelium (pl., mycelia). The hyphae are filled with protoplasm containing nuclei and other organelles. Mycelia are the key diagnostic sign associated with diseases caused by fungi. Most of us have seen mycelium growing on old bread or rotten fruit or vegetables and may have referred to these organisms collectively as molds. The disease cause of fungi as agent of plants, human diseases, and decomposers has spurred scientists worldwide to control such pathogens through bioindustrial

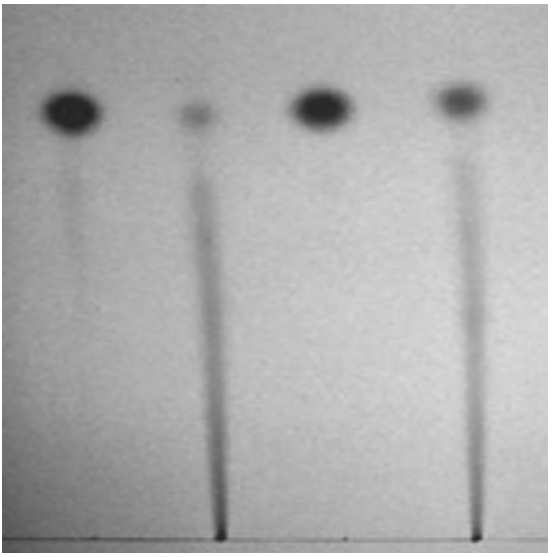

FIGURE 30.1 Thin layer chromatography analysis of products from the bioconversion of fatty acids by *P. aeruginosa* PR3. Lane numbers consecutively represented standard docosahexaenoic acid (DHA), bioconverted DHA (bDHA), standard eicosapentaenoic acid (EPA), and bEPA.

value-added products (Bajpai et al., 2008; Bajpai et al., 2009; Bajpai et al., 2004; Bajpai et al., 2006). The impact that fungi have with regards to plant health, food loss, and human nutrition is staggering. Some of the world's great famines and human suffering can be blamed on plant pathogenic fungi. Wheat crops of the Middle Ages were commonly destroyed when the grains became infected with a dark, dusty powder now known to be the spores of the fungus called bunt or stinking smut. The potato blight in Ireland and northern Europe, rampant during two successive seasons (1845–1846 and 1846–1847), was caused by the fungus-like organism *Phytophthora infestans* (Ellis, 2007). The genus itself, *Phytophthora*, was named by Anton de Bary in 1876 as "plant destroyer." This single organism caused the death of more than 1 million people by starvation and initiated one of the largest human migrations on the planet. Among these pathogens, wilt disease of cotton caused by *Fusarium oxysporum* has been reported to be very destructive for crop under field conditions in China, where about 4 million hectares of cotton, accounting for 20% of the world's total production, is grown annually (Dong, 2006). In the 1870s, an epidemic of downy mildew, caused by the fungus *Plasmopara viticola*, struck the grape vineyards of central Europe, causing great losses to grape growers and wine makers (Ellis, 2007). Also gray mold disease caused by *B. cinerea* was reported to be very destructive on crops under greenhouse conditions (Elad, 1998). In the United States alone, hundreds of millions of bushels of wheat have been lost in epidemic years to stem rust of *Puccinia graminis tritici*. In addition to being agents of pre- and postharvest diseases and rots, fungi produce highly toxic, hallucinogenic, and carcinogenic chemicals that

not only affected the lives of millions historically, but continue to be problems today. Dozens of dogs perished from food tainted with aflatoxin, a chemical produced by several *Aspergillus* species (Wilson, 1966). These fungi can grow on corn and fill the seed with the toxin that not only attacks the liver, but is one of the most carcinogenic substances known. Because of the sheer number of plant diseases caused by fungi and the huge diversity in how plant pathogenic fungi cause disease, it is impossible and beyond the scope of this publication to provide details about specific disease cycles and integrated fungal disease management strategies. But similar to all other groups of plant pathogens, fungal pathogens have developed ways to survive periods of unfavorable environmental conditions or in the absence of a susceptible host, spread, infect, grow, and reproduce on and within plants (Burdon, 1993).

30.3 BIOLOGICAL ACTIVITIES OF MICROBIALLY CONVERTED NEW ANTIFUNGAL PRODUCTS OF OMEGA-3 FATTY ACIDS

30.3.1 Antifungal Activities

Since the bioconverted industrial products of ω-3 fatty acids such as bioconverted docosahexaenoic acid (bDHA) and bioconverted eicosapentaenoic acid (bEPA) showed strong oxidative activities toward fish oil (Kim et al., 2006), and potential antibacterial effect against food spoilage and foodborne pathogenic bacteria (Shin et al., 2007), further, antifungal effect of bioconverted products of ω-3 fatty acids was evaluated against certain important plant pathogenic fungi (Bajpai et al., 2008; Bajpai et al., 2009). As shown in Table 30.1, the bDHA exhibited remarkable antifungal activity against *Botrytis cinerea* (55.30%), *Colletotrichum capsici* (60.30%), *Fusarium oxysporum,* (65.90%), *Fusarium solani* (62.90%), *Phytophthora capsici* (64.10%), *R. solani* (65.60%), and *Sclerotinia sclerotiorum*

TABLE 30.1 Antifungal Activity of Bioconverted Products of Omega-3 Fatty Acids Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA)

Fungal Pathogen	Radial Growth Inhibition (%)[a]	
	bDHA[b]	bEPA[c]
Botrytis cinerea (KACC40573)	55.3 ± 0.01	57.1 ± 0.76
Colletotrichum capsici (KACC40978)	60.3 ± 0.02	52.3 ± 1.04
Fusarium oxysporum (KACC41083)	65.9 ± 0.01	58.5 ± 0.86
Fusarium solani (KACC41092)	62.9 ± 0.02	57.7 ± 0.28
Phytophthora capsici (KACC40157)	64.1 ± 0.07	55.2 ± 1.0
Rhizoctonia solani (KACC40111)	65.6 ± 0.01	60.3 ± 0.50
Sclerotinia sclerotiorum (KACC41065)	56.7 ± 0.01	34.7 ± 0.57

[a] Radial growth inhibition percentage;
[b] bioconverted docosahexaenoic acid;
[c] bioconverted eicosapentaenoic acid.
Values are given as mean ± S.D. of three experiments.

TABLE 30.2 Determination of Minimum Inhibitory Concentration of the Bioconverted Products of Omega-3 Fatty Acids Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA)

Fungal Pathogen	MIC[a]	
	bDHA[b]	bEPA[c]
Botrytis cinerea (KACC40573)	500	250
Colletotrichum capsici (KACC40978)	250	500
Fusarium oxysporum (KACC41083)	125	250
Fusarium solani (KACC41092)	500	250
Phytophthora capsici (KACC40157)	250	500
Rhizoctonia solani (KACC40111)	na[d]	na
Sclerotinia sclerotiorum (KACC41065)	250	250

[a] Minimum inhibitory concentration (values in μg/ml);
[b] bioconverted docosahexaenoic acid;
[c] bioconverted docosahexaenoic acid;
[d] not applicable.
Values are given as mean ± S.D. of three experiments;

(56.70%) (Bajpai et al., 2009). Also the bEPA had a potential antifungal effect against *R. solani* (60.3%), *B. cinerea* (57.1%), *F. oxysporum,* (58.5%), *F. solani* (57.7%), *P. capsici* (55.2%), *S. sclerotiorum* (34.7%), and *C. capsici* (52.3%) (Bajpai et al., 2008). As shown in Table 30.2, the minimum inhibitory concentrations (MICs) defined as the lowest concentrations of bioconverted DHA that resulted in complete growth inhibition of *Botrytis cinerea, C. capsici, F. oxysporum, F. solani*, *P. capsici,* and *S. sclerotiorum* were found to be 500, 250, 125, 500, 250, and 250 μg/ml, respectively (Bajpai et al., 2009). However the MIC values of bEPA against the employed plant pathogenic fungi were noted to be as 250, 500, 250, 250, 500, and 250 μg/ml, respectively (Table 30.2) (Bajpai et al., 2008).

The results obtained for bDHA and bEPA from the spore germination assay of each of the test fungi are shown in Figures 30.2 and 30.3, respectively (Bajpai et al., 2008; Bajpai et al., 2009). There was a significant inhibition of fungal spore germination by different concentrations of bDHA and bEPA. A 100% inhibition of fungal spore

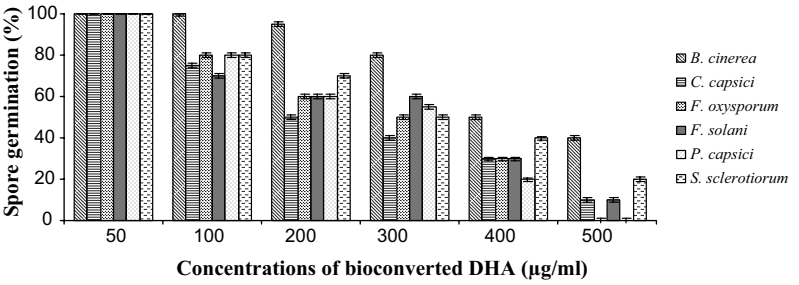

FIGURE 30.2 Inhibitory effect of different concentrations (μg/ml) of bDHA on spore germination of tested plant pathogenic fungi.

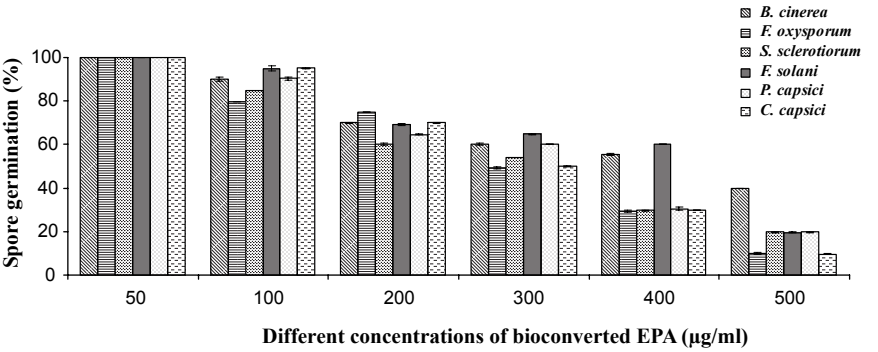

FIGURE 30.3 Inhibitory effect of different concentrations (µg/ml) of bEPA on spore germination of tested plant pathogenic fungi.

germination was observed in *F. oxysporum* and *P. capsici* at 500 µg/ml bDHA (Bajpai et al., 2009). bDHA also exhibited a potent inhibitory effect on the spore germination of *B. cinerea*, *C. capsici, F. solani*, and *S. sclerotiorum* in the range of 50–90% at concentrations ranging from 400 to 500 µg/ml. However, bEPA displayed 60–90% spore germination inhibition against the tested plant pathogens at the concentration of 400–500 µg/ml (Bajpai et al., 2008).

TABLE 30.3 ***In Vivo*** **Antifungal Activity of Bioconverted Omega-3 Product of Docosahexaenoic Acid (DHA)**

Material	Percentage of Inhibition (%)[a]			
	Concentration[b]	LSP[c]	LSHP[d]	WNT[e]
bDHA[f]	1500	100 ± 0.00	100 ± 0.00	100 ± 0.00
	500	100 ± 0.00	100 ± 0.00	100 ± 0.00
	300	40 ± 1.15	25 ± 1.52	48 ± 1.52
PC[g]	—	00 ± 0.00	00 ± 0.00	00 ± 0.00
DMSO[h]	0.5%	00 ± 0.00	00 ± 0.00	00 ± 0.00
Tween 20[i]	0.1%	00 ± 0.00	00 ± 0.00	00 ± 0.00
DMSO + Tween 20[j]	0.5 + 0.1%	00 ± 0.00	00 ± 0.00	00 ± 0.00
Water[k]	0.1%	00 ± 0.00	00 ± 0.00	00 ± 0.00

[a] 100%: Complete inhibition; 0%: no inhibition;
[b] µg/ml;
[c] LSP, leaf spot of pepper (*Colletotrichum capsici*);
[d] LSHP, leaf scorch of pepper (*Phytopthora capsici*);
[e] WNT, wilt/necrosis of tomato (*Fusarium oxysporum*);
[f] bioconverted docosahexaenoic acid;
[g] no treatment;
[h] dimethyl sulfoxide (negative control);
[i] Tween 20 (negative control);
[j] dimethyl sulfoxide + Tween 20;
[k] water.
Values are mean ± S.E.M. of triplicate experiments.

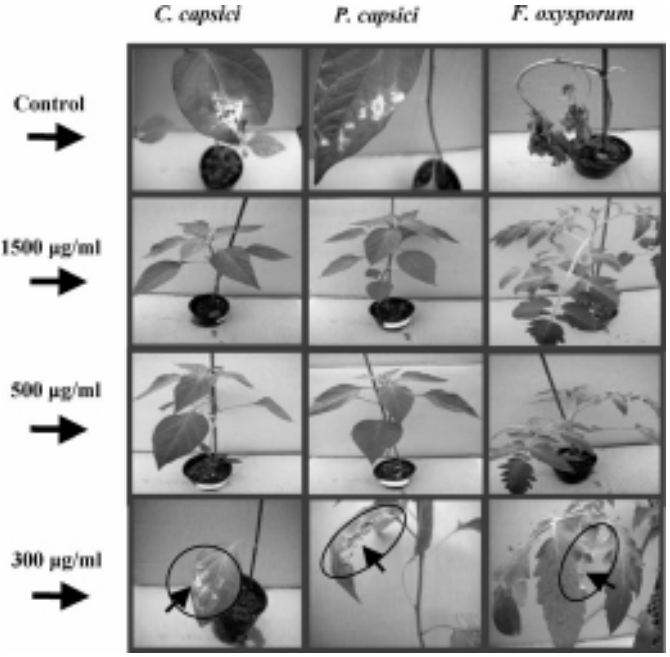

FIGURE 30.4 *In vivo* antifungal activity of bDHA against the tested plant pathogens. Arrows show antifungal effect at respective concentrations of 1500 and 500 µg/ml as 100% inhibitory effect, whereas at 300 µg/ml, arrows show moderate severity of the diseases caused by *Colletotrichum capsici, Phytophthora capsici*, and *Fusarium oxysporum*, respectively

Further elaborative *in vivo* antifungal study of bioconverted products of ω-3 fatty acids was carried out on selected plant pathogens such as *C. capsici, F. oxysporum*, and *P. capsici* to prove the industrial potential of bioconverted products of DHA and EPA. bDHA and bEPA exhibited a wide range of antifungal activity against all the plant pathogens tested, whereas DHA and EPA as negative controls had no antifungal effect. As shown in Table 30.3 and Figure 30.4, at the initial concentration of 1500 µg/ml bDHA exhibited 100% antifungal effect against leaf scorch of pepper caused by *P. capsici*, wilt/necrosis of tomato caused by *F. oxysporum* and leaf spot of pepper caused by *C. capsici* (Bajpai et al., 2009). Further dilutions of bDHA applied to the plants were 500 and 300 µg/ml. Also at the concentration of 500 µg/ml, strong antifungal effect of bDHA was observed against all the plant pathogenic fungi with 100% antifungal effect, whereas bDHA at the concentration of 300 µg/ml had a moderate antifungal effect against *C. capsici, F. oxysporum*, and *P. capsici* with their respective inhibition percentage of 40, 48, and 25%. It was observed that the antifungal effect of bDHA was rapid and exhibited remarkable antifungal effect at a higher concentration against the tested plant pathogens. Also, the bEPA showed a wide range of antifungal activity against the tested plant pathogenic fungi. As shown in Table 30.4 and Figure 30.5, at the initial concentration of 3000 µg/ml bioconverted EPA exhibited 100% antifungal effect against all the tested plant pathogenic fungi

TABLE 30.4 ***In Vivo*** **Antifungal Activity of Bioconverted Omega-3 Product of Eicosapentaenoic Acid (EPA)**

Material	Percentage of Inhibition (%)[a]			
	Concentration[b]	LSP[c]	LSHP[d]	WNT[e]
bEPA[f]	3000	100 ± 0.00	100 ± 0.00	100 ± 0.00
	1500	70 ± 1.75	100 ± 0.00	100 ± 0.00
	500	3 ± 0.57	94 ± 2.36	100 ± 0.00
	300	00 ± 0.00	34 ± 0.57	42 ± 0.57
PC[g]	—	00 ± 0.00	00 ± 0.00	00 ± 0.00
DMSO[h]	0.5%	00 ± 0.00	00 ± 0.00	00 ± 0.00
Tween 20[i]	0.1%	00 ± 0.00	00 ± 0.00	00 ± 0.00
DMSO + Tween 20[j]	0.5 + 0.1%	00 ± 0.00	00 ± 0.00	00 ± 0.00
Water[k]	0.1%	00 ± 0.00	00 ± 0.00	00 ± 0.00

[a] 100%: Complete inhibition; 0%: no inhibition;
[b] µg/ml;
[c] LSP, leaf spot of pepper (*Colletotrichum capsici*);
[d] LSHP, leaf scorch of pepper (*Phytopthora capsici*);
[e] WNT, wilt/necrosis of tomato (*Fusarium oxysporum*);
[f] bioconverted eicosapentaenoic acid;
[g] no treatment;
[h] dimethyl sulfoxide (negative control);
[i] Tween 20 (negative control);
[j] dimethyl sulfoxide + Tween 20;
[k] water.
Values are mean ± S.E.M. of triplicate experiments.

(Bajpai et al., 2008). Further dilutions applied to the plants were 1500, 500, and 300 µg/ml. A dilution of 1500 µg/ml exhibited 100% antifungal effect against leaf scorch of pepper caused by *P. capsici* and wilt/necrosis of tomato caused by *F. oxysporum*, whereas 70% antifungal action of bEPA was observed against leaf spot of pepper caused by *C. capsici.* Also, the bEPA has potential antifungal effect against *P. capsici* and *F. oxysporum* at the concentration of 500 µg/ml with their respective inhibition percentage values of 94 and 100%. However, bEPA had a mild to moderate antifungal effect against the tested plant pathogenic fungi at the concentration of 300 µg/ml (Bajpai et al., 2008).

30.4 CONCLUSIONS

From what we have reviewed herein it is clear that microbial conversion of ω-3 or *n*-3 fatty acids has been widely exploited to produce a diverse range of new value-added industrial products to serve as potential antifungal agents (Bajpai et al., 2008; Bajpai et al., 2009). Bioconverted products of ω-3 fatty acids hold promise to control plant pathogenic fungi, which cause severe losses to food and agriculture industries. In general, information on the antifungal effects of bioconverted products of ω-3

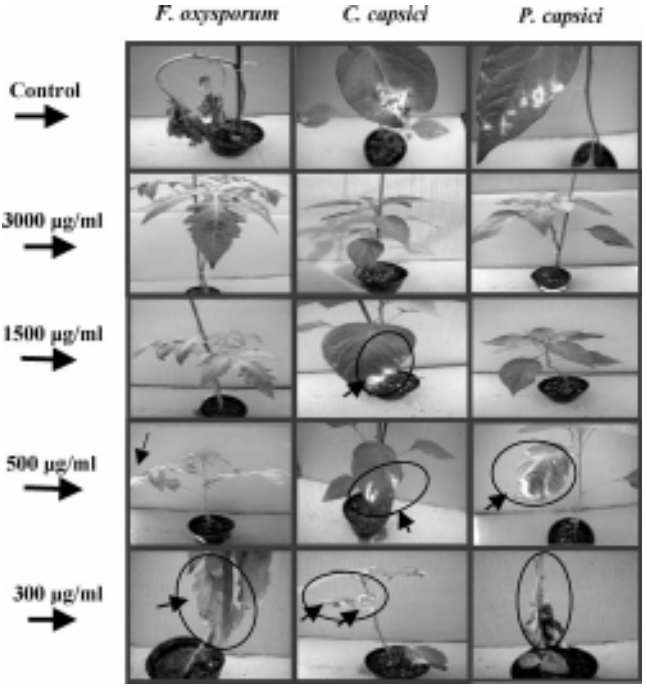

FIGURE 30.5 *In vivo* antifungal activity of bEPA against the tested plant pathogens. Concentration of 3000 µg/ml has 100% antifungal effect. Arrows show antifungal effect at 1500, 500, and 300 as strong, moderate, and mild antifungal effect against *Colletotrichum capsici*, *Phytophthora capsici,* and *Fusarium oxysporum*, respectively.

fatty acids such as bDHA and bEPA is scant, and these results showed for the first time that bioconverted products of ω-3 fatty acids (bDHA and bEPA) possessed substantial antifungal effects in both *in vitro* and *in vivo* models against certain important plant pathogenic fungi. These pathogens cause serious economic losses in various parts of the world and control measures are of limited effectiveness. Thus, as a result, the data reviewed herein would be worthy as an important industrial approach in improving food safety standards as well as in controlling plant fungal diseases. However, to enhance the potential of microbial conversion as an industrial approach to generate new value-added bioconverted products as potential antifungal agents, it is necessary to improve the productivity and efficiency by developing better microbial strains and more efficient bioconversion processes concerning conversion rate and process cost. Thus from the above review, it can be concluded that the use of bioconverted products of ω-3 fatty acids (bDHA and bEPA) can be considered as antifungal availability for trials in controlling the standards of food and agro industries. Therefore, bioconverted products of ω-3 fatty acids can be applied as potential industrial products in food and agriculture industries against plant pathogenic fungi where these pathogens cause sever damages by hampering the quality of food and agro materials and consumer demands.

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