ENZYMATIC SYNTHESIS OF STRUCTURED LIPIDS AND PHYTOSTERYL ESTERS

AND THEIR DIETARY EFFECTS ON BLOOD LIPID PROFILES AND

CARDIOVASCULAR PARAMETERS IN SPONTANEOUSLY HYPERTENSIVE RATS

by

BYUNG HEE KIM

(Under the Direction of CASIMIR C. AKOH)

ABSTRACT

Structured lipids (SLs) were synthesized from roasted sesame oil and caprylic acid by *Rhizomucor miehei* lipase-catalyzed acidolysis and phytosteryl esters (PEs) were synthesized from phytosterols and oleic acid by *Candida rugosa* lipase-catalyzed esterification. The reactions were modeled by response surface methodology, respectively, and their optimal reaction conditions were established using the models, respectively. For SLs, the substrate molar ratio (caprylic acid/sesame oil) should be kept as high as possible (6.0) and relatively low temperature (45.0 ºC) was required to maximize total incorporation and minimize acyl migration. Furthermore, total incorporation should be kept below 55 mol% caprylic acid to prevent decrease in quality and yield of targeted SLs. The optimal reaction conditions for PEs were: temperature, 51.3 ºC; reaction time, 17.0 h; substrate molar ratio (oleic acid/phytosterols), 2.1; enzyme amount, 7.2%; and degree of esterification was 97.0 mol% under these conditions. The SLs were produced in a bench-scale continuous packed bed reactor under the optimal reaction conditions established above. Total incorporation and acyl migration of caprylic acid in the SLs were 42.5 mol% and 3.1 mol%, respectively. The SLs displayed different physicochemical properties:

lower viscosity, lower melting and crystallization temperature ranges, higher melting and crystallization enthalpies, higher smoke point, higher saponification value, and lower iodine value, in comparison to unmodified sesame oil. There was no difference in the contents of tocopherols and phytosterols. However, total sesame lignans content was decreased in SLs due to the loss of sesamol and most volatiles were removed from SLs during short-path distillation of SL. The dietary effects of SLs and PEs on the blood lipid profiles and cardiovascular parameters were investigated in spontaneously hypertensive rats. Rats fed sesame oil fortified with PEs or SLs fortified with PEs showed higher plasma high density lipoprotein (HDL) cholesterol levels and higher plasma HDL/total cholesterol ratios than those fed lard, sesame oil or SLs. There was no notable difference in plasma lipid profiles of rats fed SLs compared to those fed lard or sesame oil. Resting arterial blood pressures in the rats fed high-fat diets including SLs or PEs were not different from controls (rats fed normal diet); however, resting heart rates in the rats fed high-fat diets were higher than the controls.

INDEX WORDS: Acidolysis, *Candida rugosa* lipase, Caprylic acid, Cardiovascular parameters, Continuous packed bed reactor, Esterification, Lipid modification, Oleic acid, Phytosterols, Phytosteryl esters, Plasma lipid profiles, Response surface methodology, *Rhizomucor miehei* lipase, Roasted sesame oil, Spontaneously hypertensive rats, Structured lipids

ENZYMATIC SYNTHESIS OF STRUCTURED LIPIDS AND PHYTOSTERYL ESTERS

AND THEIR DIETARY EFFECTS ON BLOOD LIPID PROFILES AND

CARDIOVASCULAR PARAMETERS IN SPONTANEOUSLY HYPERTENSIVE RATS

by

BYUNG HEE KIM

B.Agr., Korea University, Korea, 1996

M.S., Korea University, Korea, 1998

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial

Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2006

ENZYMATIC SYNTHESIS OF STRUCTURED LIPIDS AND PHYTOSTERYL ESTERS

AND THEIR DIETARY EFFECTS ON BLOOD LIPID PROFILES AND

CARDIOVASCULAR PARAMETERS IN SPONTANEOUSLY HYPERTENSIVE RATS

by

BYUNG HEE KIM

Major Professor: Casimir C. Akoh

Committee: Ronald R. Eitenmiller
Yao-Wen Huang
William L. Kerr
Stephen J. Lewis

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2006

DEDICATION

This dissertation is dedicated to my parents:

Mr. Kyung-Je Kim and Ms. Young-Soon Hong

for their support and encouragement

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who gave me the opportunity to complete this dissertation. I am deeply indebted to my advisor, Dr. Casimir C. Akoh for his invaluable guidance, constant encouragement, and unreserved financial support throughout my entire study. My deep appreciation is extended to Drs. Ronald R. Eitenmiller, Yao-Wen Huang, William L. Kerr, and Stephen J. Lewis for serving on my advisory committee. Specially, I would like to thank Dr. Lewis for allowing me to work in his laboratory and for all of his assistance.

I would like to thank my colleagues, Brenda Jennings, Weiguang Yi, Stephen Enyam, and Anita Srivastava in Dr. Akoh's laboratory for their help throughout my doctoral program. I also would like to thank Anne Morris, Lin Ye, and Kevin Sandock for their technical assistance to my experiments. I also express my thanks to my seniors and friends, Drs. Sung-Gil Choi, Jiyeon Chun, Joonsoo Lee, Jee-Hoon Ryu, Hoikyung Kim, In-Hwan Kim, Kwang Soon Shin, and Heedon Choi, and Kiung Ryu, Insuk Kim, Jinhee Yi, Dong Sik Yang, Hyoungill Lee, Byong Kwon Yoo, Jiyoung Jung, Eui-Cheol Shin, Mi Jeong Kim, and Yoen Ju Park for their encouragement and friendships. I express my gratitude to Drs. Hong-Yon Cho, Sang-Yun Choi, and Dong-Hoon Shin for recommending me to the University of Georgia doctoral program.

My deepest thanks must go to my family. I am really grateful to my parents for their understanding and endless love throughout my life, and my sister and her family for supporting me to study abroad.

Finally, I thank my heavenly Father for all the blessing of my entire life.

TABLE OF CONTENTS

Page

CHAPTER

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Natural lipids are not always appropriate for the consumers' demands. The primary purpose of lipid modification is to endow the lipid with the nutritional or physicochemical attributes, which are partially or totally different from those of corresponding natural lipids, thus making the modified lipid fit the need of the consumers. For the last 100 years, lipid scientists and technologists have been engaged in improving the quality of lipids in nutritional and physicochemical terms by modifying them. As a result, partial hydrogenation, fractionation, interesterification, etc. have been successfully developed and extensively used to modify lipids (Wright and Marangoni 2006). These lipid modification techniques are being developed and improved continually and the scope of their application is also being extended from traditional fats/oils, (mainly triacylglycerols) to other types of lipids, such as diacylglycerols, monoacylglycerols, fatty acids, phospholipids, sterols, and waxes, as well as other organic substances, such as alcohols, esters, and sugars (Weete 2002).

Lipid modification is also one of the most important areas in food processing industry that demands novel economic and environment-friendly technologies. In this respect, the production of modified lipids by enzymatic procedures has a big potential in the future market, and hence microbial lipases are of immense importance in modifying lipids. Today, lipid modifications are generally performed by chemical procedures. However, this process is energy intensive and nonspecific. Lipase-mediated modifications would occupy a prominent place in fat/oil industry for modifying lipids since enzymatic modifications are specific and can be carried out under mild reaction conditions even though lipases are yet to be fully exploited as a commercial venture. Once the technologies are established, the demand for lipases in lipid industry is expected to increase tremendously in the near future for specific modifications of lipids to meet the changing consumers' dietary requirements.

The ultimate goals of this dissertation are to establish the optimal reaction conditions to synthesize two kinds of enzymatically modified lipids: structured lipids (SLs) and phytosteryl esters (PEs) and to investigate their dietary effects on blood lipid profiles and cardiovascular parameters in the animal model using spontaneously hypertensive (SH) rats.

There are four objectives:

1. To model the *Rhizomucor miehei* lipase-catalyzed acidolysis reaction between roasted sesame oil and caprylic acid to synthesize MLM-type SLs by response surface methodology (RSM) and to optimize the reaction conditions.

2. To produce the MLM-type SLs from roasted sesame oil and caprylic acid in large-scale using continuous packed bed reactor and to investigate the influence of SL synthesis processes on the characteristics of SL by comparing with those of the corresponding original roasted sesame oil.

3. To model the *Candida rugosa* lipase-catalyzed esterification reaction between phytosterols and oleic acid to synthesize PEs by RSM and to optimize the reaction conditions.

4. To investigate the dietary effects of roasted sesame oil-based MLM-SLs and PEs on the blood lipid profiles and cardiovascular parameters in SH rats.

References

Weete, J. D. Microbial lipase. In *Food Lipids—Chemistry, Nutrition, and Biotechnology*, 2nd ed.; Akoh, C. C., Min, D. B., Eds.; Marcel Dekker: New York, NY, 2002; pp 813-838.

Wright, A. J.; Marangoni, A. G. Physical properties of fats and oils. In *Handbook of Functional Lipids*, 1st ed.; Akoh, C. C., Ed.; CRC Press: New York, NY, 2006; pp 135-162.

CHAPTER 2

LITERATURE REVIEW

Sesame Oil

Characteristics and significance of sesame oil

Sesame (*Sesamum indicum* L.) is one of the world's most important and oldest oilseed crops and is the only cultivated *Sesamum* species (Sonntag 1981; Kochhar 2002). It has been cultivated for centuries, particularly in Asia and Africa, due to its high content of edible oil and protein (Salunkhe *et al.* 1991). However, despite its long history as an edible oil source, its production is far less than those of other major oil sources, such as soybean and rapeseed (Namiki 1995).

Fatty acids (FAs) of the sesame oil are comprised mainly of oleic (C18:1*n-9*) and linoleic acids (C18:2*n-6*), with small amounts of saturated FAs (SFAs), such as palmitic (C16:0) and stearic acids (C18:0), and with only trace amount of α-linolenic acid (C18:3*n-3*) (Namiki 1995). The secondary hydroxyl group of glycerol is esterified predominantly with unsaturated FAs (USFAs) similar to other common vegetable oils. Sesame oil lacks trans USFAs which are usually produced during the processing of other vegetable oils. As compared to other vegetable oils, sesame oil contains a relatively high amount of unsaponifiable matters, which include health-beneficial compounds, such as phytosterols, tocopherols, and sesame lignans (Kochhar 2002).

Although sesame oil has a low content of n-3 polyunsaturated FAs (PUFAs), such as α-linolenic acid, eicosapentaenoic acid (EPA; C20:5*n-3*), and docosahexaenoic acid (DHA; C22:6*n-3*), this would not place sesame oil much further behind other vegetable oils in nutritive and commercial values due to its characteristic flavor, its high oxidative stability, and the presence of health-beneficial phytochemicals as mentioned before. However, because its

production is relatively low and its market price is higher than that of other vegetable oils, sesame oil is often used commercially as a mixture with other fats and oils (Namiki 1995).

Roasted sesame oil and unroasted sesame oil

Two different types of sesame oil are produced: one is from roasted seed, and the other is from unroasted seed cooked with steam. The former (roasted sesame oil) is classified according to roasting temperature (140-200 ºC) and time (below 30 min). The roasted sesame oil is a crude oil, which are expelled from the seed and are simply filtered without further purification. Roasting of sesame seeds gives a dark brown color to the oil and develops a characteristic roasted flavor in the oil mainly due to thermochemical reactions, such as Maillard reactions (Kochhar 2002; Kim and Choe 2005). The intensities of color and flavor depend on the roasting conditions. The latter (unroasted sesame oil, also called sesame salad oil) is further processed by degumming, alkali washing, water washing, decolorization, and deodorization. The unroasted sesame oil is pale in color and nearly odorless. Roasted sesame oil is indispensable especially in Korean, Chinese and Japanese cooking due to its characteristic roasted flavor.

Flavor of roasted sesame oil

The characteristic roasted flavor of sesame oil is mainly developed by thermochemical reactions, such as Maillard reactions, during the roasting step of sesame seed at 180-200 ºC before expelling it from the seed (Namiki 1995). So far about 220 kinds of volatile compounds were identified in the oil. The major volatile compounds identified in roasted sesame oil include: pyrazines, furans, other nitrogen-containing compounds, carbonyl compounds, alcohols, lactones, esters, acids, hydrocarbons, and sulfur-containing compounds (Namiki 1995). Among these

numerous compounds, some pyrazines, which are generated by Maillard reactions, and some furan compounds are believed to contribute to the representative roasted flavors of sesame oil (Lee *et al.* 1993; Namiki 1995). Apparently, no single key compound responsible for the characteristic roasted flavor has been found yet (Namiki 1995).

Sesame lignans as an antioxidant in sesame oil

The oxidative stability of sesame oil is superior to that of other vegetable oils although it contains nearly 85% USFAs (Sonntag 1981). The remarkable stability of sesame oil may be due to the presence of tocopherols and unique components called sesame lignans (Abou-Gharbia *et al.* 2000). Lignans, which are low molecular weight compounds produced by oxidative coupling of *p*-hydroxyphenylpropane, are in minor amount but very important functional components of sesame oil. Sesame oil contains significant amounts of characteristic lignans, such as sesamin and sesamolin (Namiki 1995). On the other hand, the content of tocopherol in sesame oil is less than that found in soybean and corn oil. However, the tocopherol found in sesame oil is largely γ-tocopherol, and the α-tocopherol is very small (Speek *et al.* 1985; Fukuda *et al.* 1986; Kamal-Eldin and Appelqvist 1994). Generally γ-tocopherol is considered a more efficient antioxidant than α-tocopherol in edible fats and oils (Eitenmiller and Lee 2004). Furthermore, an effective suppressive synergistic effect by the tocopherols and sesame lignans present in sesame oil on lipid peroxidations is shown to exist and this explains the remarkable oxidative stability of sesame oil (Namiki 1995).

Physiological effects of sesame lignans

Interesting physiological effects of sesame lignans have been reported from the studies on microbial production of PUFAs, such as dihomo-γ-linolenic (C20:3*n-6*) and arachidonic acids (C20:4*n-6*), which are precursors of prostaglandins, as reported by Shimizu *et al.* (1989, 1991). They reported that sesame lignans specifically inhibit Δ5-desaturase in the process of formation of arachidonic acid from linoleic acid (C18:2*n-6*), resulting in increase in the dihomo-γ-linolenic acid content. This was also demonstrated in an animal study using rats by Sugano *et al.* (1990). Besides, several interesting physiological activities of sesame lignans in animal and human tests were also shown in the literature, such as hypocholesterolemic activity, suppressive activity of chemically induced cancer, and enhancing effect on various liver activities including detoxification of carbon tetrachloride and ethanol (Akimoto *et al.* 1993; Satchithanandam *et al.* 1993; Hirose *et al.* 1992; Hirose *et al.* 1991; Sugano *et al.* 1990).

Structured Lipids

Significance of structured lipids

Structured lipids (SLs) are defined as triacylglycerols (TAGs) that have been restructured to change the composition and positional distribution of FAs from the native state by chemical or enzymatic methods (Akoh 2002). Such structural changes can consequently endow the SLs with specific, desired nutritional or physicochemical attributes, which are partially or totally different from those of corresponding conventional TAGs. Thus, for the last decade SLs have received much attention as one of the important class of functional foods and nutraceuticals or 'tailor-made' fats and oils satisfying consumers' particular demands.

Significance of MLM-type structured lipids synthesis

Long chain TAGs (LCTs) are the predominant form of traditional edible oil and serve as a source of essential FAs (EFAs). However, LCTs are metabolized slowly and have high tendency to be deposited in human body (Akoh 2002). On the other hand, medium chain TAGs (MCTs) can provide quick delivery of energy via oxidation of the more hydrophilic medium chain FAs (MCFAs); have less-calories because of their shorter chain length as compared to long chain FAs (LCFAs); and have less tendencies to be deposited in the adipose tissue due to their predominant transportation via the portal vein to the liver rather than through the lymphatic system (Bray *et al.* 1980; Bach and Babayan 1982; Iwasaki and Yamane 2000). However, MCTs have the problem of EFAs deficiency. Therefore, the development of a specific SL may overcome the disadvantages of LCTs and MCTs while retaining the benefits of both TAGs. That is, MLM-type structured TAGs (MLM-SLs) are TAGs comprised of MCFAs esterified at *sn*-1,3 positions and LCFAs at *sn*-2 position of the glycerol backbone. These SLs can be expected to have unique and desirable nutritional characteristics because of the presence of both MCFAs and LCFAs in the same TAG molecules. While providing the benefits of MCTs described above, MLM-SLs can act as efficient carrier of LCFAs, such as monounsaturated FAs (MUFAs), PUFAs, and EFAs since the *sn*-2 monoacylglycerols (*sn*-2 MAGs) produced by pancreatic lipase digestion during metabolism are well absorbed through the intestinal wall (Iwasaki and Yamane 2000).

Lipase-catalyzed synthesis of MLM-type structured lipids

MLM-SL can be synthesized by chemical or enzymatic methods as mentioned before. Chemical synthesis of MLM-SL usually involves hydrolysis of a mixture of MCTs and LCTs

and then reesterification after the MCFAs and LCFAs were mixed randomly, by a process called transesterification (ester interchange). The most commonly used catalysts in the chemical transesterification are alkylates (methylate and ethylate) of sodium because they are inexpensive and simple to use and active at relatively low temperatures, and small quantities are required as compared to other types of chemical catalysts, such as metal salts. However, chemical transesterification induces the randomized distribution of FAs in glycerol molecule due to the lack of regioselectivity of chemical catalysts. Therefore, targeted MLM-SL as well as a number of unwanted structured TAGs species, such as MLL, LML, and MLM are formed by the chemical method. Moreover, this process usually requires high temperature and anhydrous conditions and also induces a problem of staining (i.e., development of undesired color).

To overcome such shortcomings of chemical methods, enzymatic methods using *sn*-1,3 specific lipases can be used for the production of MLM-SLs. Above all, the use of these lipases enables the formation of targeted MLM-SLs selectively even though a slight amount of undesired TAG species, as mentioned above, can also be generated because of a side reaction called acyl migration phenomenon (Xu *et al*. 1998; Yang *et al*. 2005). Furthermore, these enzymatic methods can be performed under mild reaction conditions, such as low temperature and do not cause staining of the SL products. Figure 2.1 shows the lipase-catalyzed synthesis methods of MLM-SLs.

Phytosteryl Esters

Phytosterols/phytostanols

Phytosterols (also called plant sterols) are sterols derived from plant sources and have similar structure to animal tissue sterol, cholesterol. Vegetable oils and cereal products are the major sources of phytosterols. More than 40 different phytosterol analogues have been identified in several plant sources and the most abundant phytosterols are β-sitosterol, stigmasterol, and campesterol (Wester 2000). These phytosterols have created interest to lipid scientists and nutritionists for several decades as one class of important health-beneficial phytochemicals. That is, although they possess very similar structure to cholesterol except for a difference in the side chain, phytosterols are known to have a hypocholesterolemic effect by lowering plasma total and low density lipoprotein (LDL) cholesterol levels without affecting the concentration of plasma high density lipoprotein (HDL) cholesterol (Pollak 1953; Beveridge *et al*. 1964; Lees *et al*. 1977). Furthermore, phytostanols, the saturated derivative of phytosterols, have also been found to have a cholesterol-lowering effect in humans (Heinemann *et al.* 1986; Heinemann *et al.* 1991; Becker *et al.* 1993).

Cholesterol lowering mechanism of phytosterols/phytostanols

The cholesterol lowering mechanisms of phytosterols/phytostanols are still not known in detail even though recently many studies have been carried out on their effects on cholesterol metabolism. The main mechanism responsible for the cholesterol lowering effect of phytosterols/phytostanols has been assumed to be the inhibition of intestinal cholesterol absorption arising from chemical structure similarities between phytosterols/phytostanols and

cholesterol (Trautwein *et al.* 2003). Plat and Mensink (2005) explains the principle of this main mechanism as follows: Since phytosterols/phytostanols have higher affinity and higher solubilization in dietary mixed micelles as compared to cholesterol, which are caused by their higher hydrophobicity than that of cholesterol, they displace cholesterol from mixed micelles. This results in reduced intestinal absorption of cholesterol and a higher fecal excretion of cholesterol. Besides the main mechanism described above, Trautwein *et al.* (2003) also suggested that the hypocholesterolemic effect of phytosterols/phytostanols may occur via other mechanisms such as co-crystallization with cholesterol to form insoluble mixed crystals and interference with the hydrolysis process by lipases and cholesterol esterases, etc.

Significance of phytosteryl/phytostanyl esters synthesis

The solubility of free phytosterols/phytostanols in edible oil is very low (below 1% at room temperature). Furthermore, free forms of phytosterols/phytostanols have very high melting point. For example, the melting point of β-sitostanol is 140-150 °C (Wester 2000). Therefore, such physical attributes of free phytosterols/phytostanols limit their application in food products even though they possess health beneficial effects as described before.

Phytosteryl/phytostanyl esters are defined as the derivatives of phytosterols/phytostanols, which have ester group formed from the linkage with FA at C-3 position instead of a hydroxyl group. Esterifying phytosterols/phytostanols with FAs can enhance their solubility and decrease their melting point. For example, the melting points of β-sitostanyl oleate and β-sitostanyl linoleate are 44-45 °C and below 20 °C, respectively, and have fat-like properties (Weber 2001). Due to these improved physical attributes, FA ester forms of phytosterols/phytostanols can be easily incorporated into a wide variety of food products and provide an easy means for intake of

the daily amount of phytosterols/phytostanols needed for optimal reduction of cholesterol absorption without changing the taste of the final product. In addition, many recent studies have shown that phytosteryl/phytostanyl esters are also able to effectively reduce plasma total and LDL cholesterol levels in a similar manner as the corresponding phytosterols/phytostanols (Jones *et al*. 1997; Weststrate and Meijer 1998; Law 2000; Neil *et al*. 2001). The use of FA esters of phytosterols/phytostanols instead of free phytosterols/phytostanols is based on the fact that they can be efficiently hydrolyzed by pancreatic cholesterol esterase (EC 3.1.1.13), resulting in the release of free form of phytosterols/phytostanols, the active forms responsible for reducing cholesterol absorption (Miettinen and Gylling 1997).

Lipase-catalyzed synthesis of phytosteryl/phytostanyl esters

Technical processes presently used for the preparation of phytosteryl/phytostanyl esters from vegetable oil deodorizer distillates involve hydrolysis or transestrification followed by fractional crystallization or co-crystallization for the recovery of phytosterols/phytostanols. Then, the corresponding FA esters of phytosterols/phytostanols are prepared by chemical esterification or transesterification (Daguet and Coïc 1999). However, the chemical methods have problems, such as the formation of a 3,5-diene derivative as a side product and staining (Negishi *et al*. 2003).

Recently, to solve these problems, enzymatic procedures have been developed for the preparation of phytosteryl/phytostanyl esters instead of chemical esterification (Shimada *et al*. 1999; Weber *et al*. 2001; Weber *et al*. 2002; Negishi *et al*. 2003; Villeneuve *et al*. 2005). These enzymatic procedures are used to prepare phytosteryl/phytostanyl esters from the corresponding phytosterols/phytostanols via lipase-catalyzed esterification with FAs and transesterification with

FA methyl/ethyl esters or TAGs (Weber *et al*. 2002). Figure 2.2 depicts the lipase-catalyzed synthesis methods of β-sitosteryl oleate. These reactions were performed in monophasic media of organic solvents to increase the solubility of the phytosterols/phytostanols in the oil substrate or in an oil/water two-phase media, and furthermore in oil itself with/without removing water (or alcohol), which is formed during the esterification between FAs and phytosterols, by the use of water (or alcohol)-trapping agents (e.g., KOH pellet and molecular sieve) or reduced pressure conditions.

In the 1980's the synthesis of cholesteryl oleate was reported using lipase and organic solvents, such as cyclohexane, benzene, and toluene (Negishi *et al*. 2003). Shimada *et al*. (1999) reported the synthesis of cholesteryl docosahexaenoate in a two-phase medium containing 30% water. Recently Weber *et al*. (2001) reported that phytosteryl/phytostanyl esters can be synthesized in high yield by lipase-catalyzed reaction under reduced pressure (20-40 mbar) at moderate temperature (40 ºC) to remove the water and alcohol from the reaction system. In their procedure, neither organic solvent nor water is required so that a drying reagent, such as a molecular sieve is not used. They found that at 40 ºC under atmospheric pressure, reacting β-sitostanol with oleic acid in the presence of lipase for 24 h led to only 49.9% conversion of β-sitostanol into β-sitostanyl oleate as compared to 93.9% conversion in the assay using the same lipase under reduced pressure. This result is consistent with the findings of Shimada *et al*. (1999) that water generated during the esterification should be removed to facilitate the reaction.

Shimada *et al*. (1999) tested several kinds of lipases for a suitable lipase for the synthesis of cholesteryl docosahexaenoate. *Rhizopus delemar* lipase did not synthesize cholesteryl docosahexaenoate, because it is a *sn*-1,3 specific lipase and does not act on secondary alcohol. Whereas, *Candida rugosa* lipase synthesized cholesteryl linoleate (conversion ratio, 73.7%)

because it is nonspecific toward ester bonds of TAGs and acts on secondary alcohol. However, cholesteryl docosahexaenoate was not synthesized with this lipase (conversion ratio, 3.0%). This result was shown to be due to its weak activity on docosahexaenoic acid (DHA). In the esterifcation reaction of DHA with cholesterol, a *Pseudomonas* species lipase, which is also nonspecific, showed the highest activity (conversion ratio, 62.5%) among the lipases tested. These results show that nonspecific lipase is more suitable for the enzymatic method to synthesize phytosteryl/phytostanyl esters than *sn*-1,3 specific lipase.

The lipase activity can also be affected by types of sterols/stanols even though the same types of lipase and FA are used (Weber *et al*. 2001). In their esterification reaction catalyzed by *Candida rugosa* lipase using oleic acid, stigmasteryl oleate and ergosteryl oleate were synthesized in very high yields (maximum conversions, 98.8% and 98.6%, respectively) from stigmasterol and ergosterol, respectively, after 16 h. However, the maximum conversion of β-stiosteryl oleate was 73.0% after 48 h and thiocholesterol could not be converted to thiocholesteryl oleate after 24 h (maximum conversion, 0%). These results indicate that the activity of lipase is affected by types of sterols/stanols used as substrates. In other words, the lipase-catalyzed esterification and transesterification in the synthesis of FA esters of phytosterols/phytostanols is a substrate specific enzyme reaction.

Application of phytosteryl/phytostanyl esters in food products

The use of phytosteryl/phytostanyl esters led to the first commercial application to a margarine called Benecol®, in Finland in 1995 (Weber 2000). Benecol® contains synthetic FA esters of phytosterols/phytostanols derived from tall oil. Another most popular cholesterol-lowering functional food currently available on the market is Take Control®, a table spread. In

Take control, synthetic FA esters of phytosterols/phytostanols derived from soybean oil were used (Hicks and Moreau 2001). The phytosteryl/phytostanyl esters are already being used in various types of fat-based food products, such as margarine, spread, salad dressing, milk, and edible oil.

Microbial Lipases

Significance of microbial lipase

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are defined as enzymes, which hydrolyze TAGs to diacylglycerols (DAGs), monoacylglycerols (MAGs), free FAs (FFAs), and glycerols (Jaeger and Eggert 2002; Weete 2002). Lipases are widely distributed in animals, plants, and microorganisms. Among them, microbial lipases have attracted the greatest attention as an important group of biocatalysts in the fields of fat/oil industry as well as organic chemical industry because of their versatility and availability as follows: (a) they usually display chemoselectivity, regioselectivity, and steroselectivity; (b) they are readily available in large quantities due to their production in high yields and extracellular secretion from microorganisms; (c) the crystal structures of many lipases have already been solved, facilitating the design of rational engineering strategies; and (d) they are often stable and active in a wide range of organic solvents and do not usually require cofactors (Bornscheuer *et al.* 2002; Jaeger and Eggert 2002).

Characteristics of microbial lipase

Lipases can catalyze the hydrolysis, as mentioned above as well as esterification similar to esterases (carboxyl esterases, EC 3.1.1.1); therefore, they can be used as the biocatalyst in the

transesterification of TAGs with FFAs (acidolysis), FFA ethyl esters (FFAEEs) (ester interchange), and other TAGs (ester interchange) or direct esterification of glycerol with FFAs for producing SLs (Akoh 2002). Lipases are also able to catalyze transesterification of phosphoacylglycerols (phospholipids) as well as to synthesize a variety of non-acylglycerol ester bonds, resulting in several kinds of modified lipids (Svensson 1990; Oguntimein *et al.* 1993; Isono *et al.* 1995; Weber *et al.* 2001).

However, unlike esterases acting on hydrophilic substrates, the lipases show catalytic activity against water-insoluble substrates and the phenomenon of "interfacial activation"; that is, their catalytic activity is enhanced only in the presence of a hydrophobic phase, a lipid droplet dispersed in water or an organic solvent (Sarda and Desnuelle 1958; Verger 1997; Bornscheuer *et al.* 2002). Furthermore, lipases require a minimum level of substrate before reaching high activity unlike esterases obeying typical Michaelis-Menten kinetics (Bornscheuer 2002). These phenomena are related to the presence of a hydrophobic oligopeptide (lid) covering the entrance to the active site of the lipases. That is, only under a hydrophobic environment (e.g., organic solvent) and the presence of a minimum concentration of substrate, the lid moves aside and the substrate enters the binding pocket.

Lipases can be classified into two major groups according to their positional specificity (i.e., regioselectivity): (a) nonspecific lipase, which shows no distinct specificity with respect to the position of the acyl group on the glycerol molecule; or to the specific nature of the FA of the substrate; and (b) *sn*-1,3 specific lipase, which shows marked preference for the ester bonds specifically at the first and third positions of the acylglycerols (Macrae 1983; Sonnet 1988). However, less common lipases display *sn*-2 specificity and some lipases show preference for a

specific FA or chain length range (Jensen 1974; Sugihara *et al*. 1991; Yamaguchi and Mase 1991; Mukherjee *et al*. 1993; Rogalska *et al*. 1993; Gulomova *et al*. 1996).

Structure and hydrolysis/esterification mechanism of microbial lipase

The three-dimensional (3D) structures of many lipases show that they possess the characteristic α/β-hydrolase fold, which is comprised of central, mostly parallel, several β-sheets with several α-helices on both sides of the sheets, and an active site region with a catalytic triad. The catalytic triad consists of Ser⋯Asp⋯His (Glu instead of Asp for some lipases, such as *Candida rugosa* lipase). Many lipases also share a consensus sequence motif of the pentapeptide, Gly-x-Ser-x-Gly (where x represents an arbitrary amino acid residue), which is found around the active site serine. Recently, however, a new subfamily of lipases showing a different consensus sequence motif, GDSL (Gly-Asp-Ser-Leu) with the active site serine located near the N-terminus have been found.

The mechanism of hydrolysis or esterification by lipases is composed of four steps as follows: firstly, the substrate is bound to the active site serine; secondly, a tetrahedral intermediate is formed and stabilized by the catalytic His and Asp residues; thirdly, the alcohol is released and an acyl-enzyme complex is formed; and finally, a nucleophile (i.e., water in hydrolysis; alcohol or ester in transesterification) attacks the acyl-enzyme complex and forms a tetrahedral intermediate again, which after resolution yields the reaction product (i.e., an acid or an ester) and free lipase (Bornscheuer 2002).

Candida rugosa lipase and Rhizomucor miehei lipase

Candida rugosa is a nonsporogenic, unicellular, nonpathogenic yeast having GRAS (generally regarded as safe) status (Benjamin and Pandey 1998; Akoh *et al*. 2004). *Candida rugosa* Type VII and ATCC 14830 are the wild-type strains most widely used to produce the extracellular lipases (Akoh *et al*. 2004). *Candida rugosa* lipase is generally classified as nonspecific lipases. Thus, they can catalyze hydrolysis or esterification at the *sn*-1,-2, and -3 positions of the glycerol molecule and other non-acylglycerol moieties, such as phospholipids, sterols, sugars, and polyhydric alcohols (polyols) (Akoh *et al*. 2004).

Rhizomucor miehei lipase is the representative biocatalyst for the enzymatic production of SLs (especially, MLM-SLs) due to its high *sn*-1,3 specificity. It is produced by *Aspergillus oryzae* containing the structural gene for the precursor of *Rhizomucor miehei* lipase (Broadmeadow *et al*. 1994). Currently the major proportion of *Rhizomucor miehei* lipase is commercially available as the form immobilized onto a weak anion exchange resin. This lipase was not shown to be mutagenic and did not cause chromosomal damage in humans according to the results of *in vitro* toxicological tests (Broadmeadow *et al.* 1994). Furthermore, rats fed for 13 weeks with diet having dietary concentrations up to 1600 mg/kg diet displayed no adverse effect (Broadmeadow *et al.* 1994).

Industrial applications of microbial lipase

Microbial lipases are currently used or have the potential for use in a wide range of applications. These include the use in flavor development of cheese, fermented products, and ice cream; in the oleochemical industry for hydrolysis, glycerolysis, and alcoholysis of fats and oils; in the synthesis of SLs, emulsifiers, fat substitutes, pharmaceuticals, agrochemicals, ingredients

of personal care products, and polymers; and in the stereoselective synthesis of desired enantiomers of racemic compounds (Bjorkling *et al*. 1991; Vulfson 1994). Table 2.1 lists some examples of representative industrial applications of microbial lipases.

Dietary Fats and Coronary Heart Disease

Lipoproteins as carriers of dietary fats

Lipids are not able to circulate freely in plasma having aqueous environment due to their hydrophobic structures. Lipids can be carried through blood vessels by binding with proteins known as lipoproteins. Lipoproteins are globular and macromolecular particles which are complex aggregates of lipid and protein molecules. The lipoproteins have the structure of a hydrophobic core, mainly containing TAGs and esterified cholesterols surrounded by a hydrophilic coat consisting of phospholipids and free cholesterols, interpolated with specific apolipoproteins (abbreviated as Apo) with the polar heads located at the surface of the macromolecule. Therefore, lipoproteins carry all types of circulating lipids, such as cholesterol in its free and esterified forms, TAGs, and phospholipids in plasma.

Metabolism of dietary fats

Lipoprotein complexes are transported via two types of pathway: (a) exogenous pathway; and (b) endogenous pathway. In the exogenous pathway, dietary lipids are transported from the intestine to the liver. Dietary TAGs are emulsified with the assistance of bile salts in the duodenum and are then hydrolyzed into DAGs, MAGs, FFAs, and glycerol by pancreatic lipase. The formed emulsion of lipids including the dietary cholesterol passes the mucous membrane of

the intestinal cells and further hydrolysis of lipids and reesterification of glycerol and cholesterol with FFAs occur, resulting in the formation of new TAGs and cholesteryl esters. The newly synthesized TAGs and cholesteryl esters are then incorporated into chylomicrons. The chylomicrons are the lipoproteins mainly consisting of TAGs with small amount of cholesterol. The chylomicrons enter the lymph and subsequently the blood circulation via the subclavian vein. In the blood, the TAGs in the core of chylomicron are hydrolyzed by lipoprotein lipase (LPL), which adheres to the endothelial cells of the blood vessels. LPL is activated by the ApoC-II in the surfaces of chylomicrons and very low density lipoproteins (VLDLs). The FFAs hydrolyzed from TAGs, which pass the endothelial cells and enter adipocytes or muscle cells, are stored as TAGs or oxidized FAs in these cells, respectively. The resultant chylomicron remnants, meaning empty chylomicron, are rapidly removed from the circulation by the hepatic remnant receptor, which has a high affinity for ApoE in the chylomicron surface.

In the endogenous pathway, dietary and *de novo* synthesized lipids are transported from the liver to extrahepatic tissues. TAGs and cholesterol in the liver are secreted into the circulation by the formation of VLDLs. Similar to chylomicrons, LPL releases FFAs and glycerol from TAGs in VLDL, resulting in the formation of VLDL remnants, which are also called intermediate density lipoproteins (IDLs). Some IDL particles are taken up by the liver, while the remaining IDLs are converted into LDLs in the circulation by the loss of TAGs and ApoE. LDLs are smaller lipoproteins, which are nearly devoid of TAGs and are rich in cholesteryl esters as compared to chylomicrons and VLDLs. LDLs are the main carriers of free and esterified cholesterols in the plasma. The cholesterol of LDL is used to build cell membranes and to synthesize steroid hormones by cells. Most LDLs are removed from the circulation by the hepatic or extrahepatic LDL receptors (ApoB:E receptors) recognizing ApoB-100 in the LDL

surface. The ApoB:E receptor-mediated pathway is the main pathway to uptake LDLs from the blood. This pathway is down-regulated when the concentration of cholesterol in the cell is too high. A part of plasma LDLs is removed via another pathway called scavenger pathway. This pathway is up-regulated when the concentration of LDL cholesterol in the blood is too high. Too much uptake of LDL via scavenger pathway from macrophages result in the formation of cells loaded with cholesterol called foam cells, which are frequently found in atherosclerotic lesions.

Cholesterol can also be carried out of tissues by reverse cholesterol transport system mediated by HDLs, lecithin-cholesteryl acyltransferase (LCAT), and cholesterol ester transfer protein (CETP). Free cholesterol from tissues is bound to HDL and is esterified by LCAT, which is associated with HDL containing ApoA-I, activator of LCAT. LCAT converts the free form of cholesterol into esterified cholesterol by transferring FAs from the *sn*-2 position of lecithin to free cholesterol. LCAT is involved in all the esterification of cholesterol from HDL as well as from other lipoproteins, such as chylomicron remnants, VLDL, IDL, and LDL. The acquired cholesteryl esters can be transferred with the help of CETP to the other lipoproteins, such as ApoB-100-containing lipoproteins. The large portion of HDL might also be taken up in the liver by a putative HDL receptor or lose a part of its content by the action of hepatic lipase, which hydrolyze TAGs from remnant lipoprotein particles, and then reenter the circulation.

Effect of dietary fats on coronary heart disease

Coronary heart disease (CHD), also termed coronary artery disease (CAD) or cardiovascular disease (CVD) is a leading cause of death of adults in developed countries. Many factors are associated with elevated risk for CHD as follows: family history of CHD; increasing age; male gender; menopause in female; cigarette smoking; lack of physical activity or exercise;

high blood pressure; diabetes; obesity; and high cholesterol levels in blood (specifically, high LDL cholesterol level) (Mensink and Plat 2002). Among these risk factors, plasma cholesterol levels are affected by changes in the sources and amount of dietary fats.

High level of plasma LDL is related to a high risk of CHD, whereas high concentration of plasma HDL reduces the risk. Thus, plasma LDL:HDL ratio is a good predictor for the risk of CHD. Since LDL carries most plasma cholesterol, the level of total plasma cholesterol also affects CHD. However, since high level of total plasma cholesterol sometimes arises from the high concentration of plasma HDL, the ratio of total cholesterol to HDL cholesterol in plasma might be the most efficient predictor for the risk of CHD. High level of TAGs, which are mainly found in the VLDLs in the fasting condition, is known to be associated with a high risk of CHD.

Saturated fats containing lauric (C12:0), myristic (C14:0), and palmitic acid are well known to raise the levels of plasma total and LDL cholesterols (Mensink and Plat 2002). Therefore, these three kinds of SFAs are generally classified into hypercholesterolemic FAs. Whereas, saturated fats containing MCFAs with less than 12 carbon atoms, as well as stearic acid have minimal or neutral effect on plasma cholesterol levels (Hayes 2000). However, it was shown that high intake of MCFAs increase plasma TAG levels even though modest amounts of MCFAs have comparable effects on the plasma total and LDL cholesterol levels and TAG level as have carbohydrates (McGandy *et al*. 1970). MUFA, such as oleic acid, has no effect on total cholesterol when exchanged for carbohydrate, but lowers the levels of plasma total and LDL cholesterols when exchanged for SFAs (Hayes 2000). n-6 PUFAs, such as linoleic acid, are shown to be hypocholesterolemic similar to oleic acid. Moderate intake of linoleic acid has similar effects on plasma HDL and LDL cholesterol to oleic acid (Mensink and Katan 1989; Valsta *et al*. 1992; Wahrburg *et al*. 1992). However, studies at higher intakes found that linoleic

acid lowers the level of plasma HDL cholesterol (Mattson and Grundy 1985). However, other recent studies also reported that linoleic acid has a small favorable effect on plasma LDL cholesterol as compared to oleic acid (Howard *et al.* 1995). Among n-3 PUFAs, α-linolenic acid is known to have similar effect on the plasma lipoprotein profile as linoleic acid (Chan *et al.* 1991). The other n-3 PUFAs from fish oils, such as EPA and DHA, do not affect plasma LDL and HDL cholesterol concentrations, but lower plasma TAG and VLDL cholesterol levels in normocholesterolemic subjects (Harris *et al.* 1983).

Spontaneously hypertensive rats as a model of coronary heart disease

Animal models have been used to study chronic CHD in various stages of the disease as well as to investigate the mechanisms of the pathogenesis and the effects of a specific diet or drug on the disease. An ideal animal model for any CHD in humans should meet several requirements as follows: (a) mimic the human disease as much as possible; (b) allow studies in chronic and stable disease; (c) produce predictable and controllable symptoms; (d) satisfy economical, technical, and animal welfare considerations; and (e) allow measurement of relevant cardiac, biochemical and hemo-dynamic parameters (Doggrell and Brown 1998; Muders and Elsner 2000).

Rat models have some limitations for CHD research as follows: (a) CHD, such as hypertension and heart failure in humans are usually slowly developing in a time course of years in contrast to the acute onset of symptoms of these diseases in rat models; (b) CHD is usually a disease of the elderly in humans, whereas, young or adult rats are used in animal models; and (c) the development of atherosclerosis is unusual in most strains of rats even though high levels of blood lipid are sustained, in contrast to humans where atherosclerosis is common and an

important risk factor in CHD. Despite these shortcomings described above, however, the use of rats as animal models for CHD research is feasible since their costs are relatively low and many techniques have been developed to measure relevant functional parameters.

Hypertension, either systolic/diastolic (>140 mmHg/>90 mmHg) or isolated systolic (>140 mmHg/<90 mmHg), is considered a major risk factor for CHD. Spontaneously Hypertensive rat (SHR) with Wistar Kyoto rat (WKY) as the normotensive control is the most commonly used rat model of chronic primary (or essential) hypertension (Doggrell and Brown 1998). The SHRs are descendants of an outbred Wistar male with spontaneous hypertension from a colony in Kyoto, Japan, mating with a female with an elevated blood pressure, and then brother×sister mating continued with selection for spontaneous hypertension, defined as a systolic blood pressure of over 150 mmHg lasting for more than 1 month (Okamoto and Aoki 1963).

Hypertension development in the SHRs leads to left ventricular hypertrophy and vascular wall widening by increased proliferation of smooth muscle cells in aorta (Mulvany *et al.* 1978; Makino *et al.* 1997; Perona *et al*. 2005). In these cells, the local accumulation of TAGs and cholesteryl esters, which is one of the prominent features of atherosclerotic lesion, occurs until they become foam cells (Perona *et al*. 2005). Furthermore, they have been shown to bind monocytes in thoracic aorta with greater avidity than their corresponding control WKY, which was concomitant to development of hypertriglyceridemia (Asagami *et al.* 1999).

References

Abou-Gharbia, H. A.; Shehata, A. A. Y.; Shahidi, F. Effect of processing on oxidative stability and lipid classes of sesame oil. *Food Res. Int.* **2000**, *33*, 331-340.

Akimoto, K.; Kitagawa, Y.; Akamatsu, T.; Hirose, N.; Sugano, M.; Shimizu, S. Yamada, H. Protective effects of sesamin against liver-damage caused by alcohol or carbon-tetrachloride in rodents. *Ann. Nutr. Metab.* **1993**, *37*, 218-224.

Akoh, C. C. Structured lipids. In *Food Lipids—Chemistry, Nutrition, and Biotechnology*, 2nd ed.; Akoh, C. C., Min, D. B., Eds.; Marcel Dekker: New York, NY, 2002; pp 877-908.

Akoh, C. C.; Lee, G. C.; Shaw, J. F. Protein engineering and applications of *Candida rugosa* lipase isoforms. *Lipids* **2004**, *39,* 513-526.

Akoh, C. C.; Mutua, L. N. Synthesis of alkyl glycoside fatty acid esters: Effect of reaction parameters and the incorporation of n-3 polyunsaturated fatty acids. *Enz. Microbial Technol.* **1994**, *16*, 115-119.

Asagami, T.; Reaven, G. M.; Tsao, P. S. Enhanced monocyte adherence to thoracic aortae from rats with two forms of experimental hypertension. *Am. J. Hyperntens.* **1999**, *12*, 890-893.

Bach, A. C.; Babayan, V. K. Medium-chain triglycerides: an update. *Am. J. Clin. Nutr.* **1982**, *36,* 950-962.

Becker, M.; Staab, D.; Von Bergmann, K. Treatment of severe familial cholesterolemia in childhood with sitosterol and sitostanol. *J. Pediatr.* **1993**, *122*, 292-296.

Benjamin, S.; Pandey, A. *Candida rugosa* lipases: molecular biology and versatility in biotechnology. *Yeast* **1998**, *14*, 1069-1087.

Beveridge, J. M.; Haust, H. L.; Connel, W. F. Magnitude of the hypocholesterolemic effect of dietary sitosterol in man. *J. Nutr.* **1964**, *83*, 119-122.

Bjorkling, F.; Godtfredsen, S. E.; Kirk, O. The future impact of industrial lipases. *Trends Biotechnol.* **1991**, *9*, 360-363.

Bloomer, S.; Adlercreutz, P.; Mattiasson, B. Triglyceride interesterification by lipases. Cocoa butter equivalents from fraction of palm oil. *J. Am. Oil Chem. Soc.* **1990**, *67*, 519-524.

Bornscheuer, U. T. Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS Microbiol. Rev.* **2002**, *26,* 73-81.

Bornscheuer, U. T.; Bessler, C.; Srinivas, R.; Krishna, S. H. Optimizing lipases and related enzymes for efficient application. *Trends Biotechnol.* **2002**, *20,* 433-437.

Bray, G. A.; Lee, M.; Bray, T. L. Weight gain of rats fed medium-chain triglycerides is less than rats fed long-chain triglycerides. *Int. J. Obes.* **1980**, *4*, 27-32.

Broadmeadow, A.; Clare, C.; De Boer, A. S. An overview of the safety evaluation of the *Rhizomucor miehei* lipase enzyme. *Food Addit. Contam.* **1994**, *11*, 105-119.

Chan, J. K.; Bruce, V. M.; McDonald, B. E. Dietary α-linolenic acid is as effective as oleic acid and linoleic acid in lowering blood cholesterol in normolipidemic men. *Am. J. Clin. Nutr.* **1991**, *53*, 1230-1234.

Chattopadhyay, S.; Sivalingam, G.; Madras, G. Lipase specificity for the hydrolysis of poly (vinyl acetate). *Polymer Degrad. Stability* **2003**, *80*, 477-483.

Chen, P. Y.; Wu, S. H.; Wang, K. T. Double enantioselective esterification of racemic acids and alcohols by lipase from *Candida cylindracea*. *Biotechnol. Lett.* **1993**, *15*, 181-184.

Daguet, D.; Coïc, J. P. Phytosterol extraction: State of the art. *Oléagineux Corps gras Lipides* **1999**, *6*, 25-28.

Doggrell, S. A.; Brown, L. Rat models of hypertension, cardiac hypertrophy and failure. *Cardiovasc. Res.* **1998**, *39*, 89-105.

Eitenmiller, R.; Lee, J. Oxidation and the role of vitamin E as an antioxidant in foods. In *Vitamin E—Food Chemistry, Composition, and Analysis*, 1st ed.; Eitenmiller, R., Lee, J., Eds.; Marcel Dekker: New York, NY, 2004; pp 89-135.

Fomuso, L. B.; Akoh, C. C. Enzymatic modification of triolein: Incorporation of caproic and butyric acids to produce reduced-calorie structured lipids. *J. Am. Oil Chem. Soc.* **1997**, *74*, 269-272.

Fomuso, L. B.; Akoh, C. C. Enzymatic modification of high-laurate canola to produce margarine fat. *J. Agric. Food Chem.* **2001**, *49*, 4482-4487.

Fomuso, L. B.; Akoh, C. C. Lipase-catalyzed acidolysis of olive oil and caprylic acid in a bench-scale packed bed bioreactor. *Food Res. Int.* **2002**, *35*, 15-21.

Fukuda, Y.; Nagata, M.; Osawa, T.; Namiki, M. Chemical aspects of the antioxidative activity of roasted sesame seed oil, and the effect of using the oil for frying. *Agric. Biol. Chem.* **1986**, *50*, 857-862.

Gulomova, K.; Ziomek, E.; Schrag, J. D.; Davranov, K.; Cygler, M. Purification and characterization of a *Penicillium* sp. lipase which discriminates against diglycerides. *Lipids* **1996**, *31*, 379-384.

Harris, W. S.; Connor, W. E.; McMurry, M. P. The comparative reductions of the plasma lipids and lipoproteins by dietary polyunsaturated fats: Salmon oil versus vegetable oil. *Metabolism*, **1983**, 32, 179-184.

Hayes, D. G.; Gulari, E. Formation of polyol-fatty acid esters by lipases in reverse micellar media. *Biotechnol. Bioeng.* **1992**, 40, 110-118.

Hayes, K. C. Invited commentary—Dietary fatty acids, cholesterol, and the lipoprotein profile. *British J. Nutr.* **2000**, *84*, 397-399.

Heinemann, T.; Leiss, O.; Von Bergmann, K. Effect of low-dose sitostanol on serum cholesterol in patients with hypercholesterolemia. *Atherosclerosis*, **1986**, *61*, 219-223.

Heinemann, T.; Kullak-Ublick, G. A.; Pietruck, B.; Von Bergmann, K. Mechanisms of action of plant sterols on inhibition of cholesterol absorption. Comparison of sitosterol and sitostanol. *Eur. J. Clin. Pharmacol.* **1991**, *40*, S59-63.

Hicks, K. B.; Moreau, R. A. Phytosterols and phytostanols: functional food cholesterol busters. *Food Technol.* **2001**, *55*, 63-67.

Hirose, N.; Doi, F.; Ueki, T.; Akazawa, K.; Chijiiwa, K.; Sugano, M.; Akimoto, K.; Shimizu, S.; Yamada, H. Suppressive effect of sesamin against 7,12-dimethylbenz[α]-anthracene induced rat mammary carcinogenesis. *Anticancer Res.* **1992**, *12*, 1259-1266.

Hirose, N.; Inoue, T.; Nishihara, K.; Sugano, M.; Akimoto, K.; Shimizu, S.; Yamada, H. Inhibition of cholesterol absorption and synthesis in rats by sesamin. *J. Lipid Res.* **1991**, *32*, 629-638.

Howard, B. V.; Hannah, J. S.; Heiser, C. C.; Jablonski, K. A.; Paidi, M. C.; Alarif, L.; Robbins, D. C.; Howard, W. J. Polyunsaturated fatty acids result in greater cholesterol lowering and less triacylglycerol elevation than do monounsaturated fatty acids in a dose-response comparison in a multiracial study group. *Am. J. Clin. Nutr.* **1995**, *62*, 392-402.

Huang, K.; Akoh, C. C. Lipase-catalyzed incorporation of n-3 polyunsaturated fatty acids into vegetable oils. *J. Am. Oil Chem. Soc.* **1994**, *71*, 1277-1280.

Isono, Y.; Nabetani, H.; Nakajima, M. Wax ester synthesis in a membrane reactor with lipase-surfactant complex in hexane. *J. Am. Oil Chem. Soc.* **1995**, *72*, 887-890.

Iwasaki, Y.; Yamane, T. Enzymatic synthesis of structured lipids. *J. Mol. Catal. B-Enz.,* **2000**, *10,* 129-140.

Jaeger, K. E.; Eggert, T. Lipase for biotechnology. *Current Opinion Biotech.* **2002**, *13,* 390-397.

Jennings, B. H.; Akoh, C. C. Designer lipids with different n-6/n-3 ratios. In *Essential Fatty Acids and Eicosanoids – Invited Papers from the Fifth International Congress*; Huang, Y. S., Lin, S. J., Huang, P. J., Eds.; American Oil Chemists' Society; Champaign, IL, 2002; pp 305-308.

Jensen, R. G. Characteristics of the lipase from the mold, *Geotrichum candidum*: a review. *Lipids* **1974**, *9*, 149-157.

Jones, P. J. H.; MacDougall, D. E.; Ntanios, F.; Vanstone, C. A. Dietary phytosterols as cholesterol-lowering agents in humans. *Can. J. Physiol. Pharmacol.* **1997**, *75*, 217-227.

Kamal-Eldin, A.; Appelqvist, L. A. Variations in the composition of sterols, tocopherols and lignans in seed oils from 4 *sesamum* species. *J. Am. Oil Chem. Soc.* **1994**, *71*,149-156.

Kim, I.; Choe, E. Effects of bleaching on the properties of roasted sesame oil. *J. Food Sci.* **2005**, *70*, 48-52.

Kochhar, S. P. Sesame, rice-bran and flaxseed oils. In *Vegetable Oils in Food Technology – Composition, Properties and Uses*, 1st ed.; Gunstone, F. D., Eds.; Blackwell: Oxford, UK, 2002; pp 297-322.

Law, M. Plant sterol and stanol margarines and health. *Br. Med. J.* **2000**, *320*, 861-864.

Lee, J. H.; Kim, M. R.; Kim, I. H.; Kim, H.; Shin, L.; Lee, K. T. Physiochemical and volatile characterization of structured lipids from olive oil produced in a stirred-tank batch reactor. *J. Food Sci.* **2004**, *69*, C89-C95.

Lee, Y. G.; Lim, S. U.; Kim, J. O. Influence of roasting conditions on the flavor quality of sesame seed oil. *Korean Agric. Chem. Soc.* **1993**, *36*, 407-415.

Lees, A. M.; Mok, H. Y.; Lees, R. S.; McCluskey, M. A.; Grundy, S. M. Plant sterols as cholesterol-lowering agents: clinical trials in patients with hypercholesterolemia and studies of sterol balance. *Atherosclerosis* **1977**, *28*, 325-338.

Lumor, S. E.; Akoh, C. C. Incorporation of gamma-linolenic and linoleic acids into a palm kernel oil/palm olein blend. *Eur. J. Lipid Sci. Technol.* **2005**, *107*, 447-454.

Macrae, A. R. Lipase-catalyzed interesterification of oils and fats. *J. Am. Oil Chem. Soc.* **1983**, *60*, 291-294.

Makino, N.; Sugano, M.; Otsuka, S.; Hata, T. Molecular mechanism of angiotensin II type I and type II receptors in cardiac hypertrophy of spontaneously hypertensive rats. *Hypertension*, **1997**, *30*, 796-802.

Mattson, F. H.; Grundy, S. M. Comparsion of effects of dietary saturated, monounsaturated, and polyunsaturated fatty acids on plasma lipids and lipoproteins in man. *J. Lipid Res.* **1985**, *26*, 194-202.

McGandy, R. B.; Hegsted, D. M.; Myers, M. L. Use of semisynthetic fats in determining effects of specific dietary fatty acids on serum lipids in man. *Am. J. Clin. Nutr.* **1970**, *23*, 1288-1298.

Mensink, R. P.; Katan, M. B. Effect of a diet enriched with monounsaturated or polyunsaturated fatty acids on levels of low-density and high-density lipoprotein cholesterol in healthy women and men. *N. Engl. J. Med.* **1989**, 321, 436-441.

Mensink, R. P.; Plat, J. Dietary fats and coronary heart disease. In *Food Lipids—Chemistry, Nutrition, and Biotechnology*, 2nd ed.; Akoh, C. C., Min, D. B., Eds.; Marcel Dekker: New York, NY, 2002; pp 603-636.

Miettinen, T. A.; Gylling, H. Sitostanol-ester margarine. In *New Technologies for Healthy Foods and Nutraceuticals*, 1st ed.; Yalpani, M., Eds.; ATL press: Shrewsbury, MA, 1997; pp 71-83.

Muders, F.; Elsner, D. Animal models of chronic heart failure. *Pharmacol. Res.* **2000**, *41*, 605-612.

Mukherjee, K. D.; Kiewitt, I.; Hills, M. J. Substrate specificities of lipases in view of kinetic resolution of unsaturated fatty acids. *Appl. Microbiol. Biotechnol.* **1993**, *40*, 489-493.

Mulvany, M. J.: Hansen, O. K.; Aalkjaer, C. Direct evidence that the greater contractility of resistance vessels in spontaneously hypertensive rats is associated with a narrowed lumen, a thickened media, and an increased number of smooth muscle cell layers. *Circ. Res.* **1978**, *43*, 854-864.

Mutua, L. N.; Akoh, C. C. Lipase catalyzed modification of phospholipids: Incorporation of *n-3* fatty acids into biosurfactants. *J. Am. Oil Chem. Soc.* **1993**, *70*, 125-128.

Namiki, M. The chemistry and physiological functions of sesame. *Food Rev. Int.* **1995**, *11*, 281-329.

Negishi, S.; Hidaka, I.; Takahashi, I.; Kunita, S. Transesterification of phytosterol and edible oil by lipase powder at high temperature. *J. Am. Oil Chem. Soc.* **2003**, *80*, 905-907.

Neil, H. A. S.; Meijer, G. W.; Roe, L. S. Randomised controlled trial of use by hypercholesterolaemic patients of a vegetable oil sterol-enriched fat spread. *Atherosclerosis* **2001**, *156*, 329-337.

Oba, T.; Witholt, B. Interesterification of milk fat with oleic acid catalyzed by immobilized *Rhizopus oryzae* lipase. *J. Dairy Sci.* **1994**, *77*, 1790-1797.

Oguntimein, G. B.; Erdmann, H.; Schmid, R. D. Lipase catalyzed synthesis of sugar ester in organic solvents. *Biotechnol. Lett.* **1993**, *15*, 175-180.

Okamoto, K.; Aoki, K. Development of a strain of spontaneously hypertensive rats. *Japan Circ. J.* **1963**, *27*, 282-293.

Perona, J. S.; Rodriguez-Rodriguez, R.; Ruiz-Gutierrez, V. Effects of oleic acid rich oils on aorta lipids and lipoprotein lipase activity of spontaneously hypertensive rats. *J. Agric. Food Chem.* **2005**, *53*, 7330-7336.

Plat, J.; Mensink, R. P. Plant stanol and sterol esters in the control of blood cholesterol levels: mechanism and safety aspects. *Am. J. Cardiol.* **2005**, *96*, 15D-22D.

Pollak, O. J. Successive prevention of experimental hypercholesteremia and cholesterol atherosclerosis in the rabbit. *Circulation* **1953**, *7*, 696-701.

Rahmatullah, M. S. K.; Shukla, V. K. S.; Mukherjee, K. D. Enrichment of GLA from borage and evening primrose oils via lipase-catalyzed hydrolysis. *J. Am. Oil Chem. Soc.* **1994**, *71*, 569-573.

Rogalska, E.; Cudrey, C.; Ferrato, F.; Verger, R. Stereoselective hydrolysis of triglycerides by animal and microbial lipases. *Chirality* **1993**, *5*, 24-30.

Rousseau, D.; Marangoni, A. G. Tailoring the textural attributes of butter fat/canola oil blends via *Rhizopus arrhizus* lipase-catalyzed interesterification. 2. Modifications of physical properties. *J. Agric. Food Chem.* **1998**, *46*, 2375-2381.

Salunkhe, D. K.; Chavan, J. K.; Adsule, R. N.; Kadam, S. S. Sesame. In *World Oilseeds: History, Technology, and Utilization*. Van Nostrand Reinhold: New York, NY, 1991; pp 371-402.

Sarda, L.; Desnuelle, P. Actions of pancreatic lipase on esters in emulsions. *Biochem. Biophys. Acta.* **1958**, *30*, 513-521.

Satchithanandam, S.; Reicks, M.; Calvert, R. J.; Cassidy, M. M.; Kritchevsky, D. Coconut oil and sesame oil affect lymphatic absorption of cholesterol and fatty acids in rats. *J. Nutr.* **1993**, *123*, 1852-1858.

Shieh, C. J.; Akoh, C. C.; Yee, L. N. Optimized enzymatic synthesis of geranyl butyrate with lipase AY from *Candida rugosa. Biotechnol. Bioeng.* **1996**, *51*, 371-374.

Shimada, Y.; Hirota, Y.; Baba, T.; Sugihara, A.; Moriyama, S.; Tominaga, Y.; Terai, T. Enzymatic synthesis of steryl esters of polyunsaturated fatty acids. *J. Am. Oil Chem. Soc.* **1999**, *76*, 713-716.

Shimizu, S.; Akimoto, K.; Shinmen, Y.; Kawashima, H.; Sugano, M.; Yamada, H. Sesamin is a potent and specific inhibitor of Δ-5 desaturase in polyunsaturated fatty acid biosynthesis. *Lipids* **1991**, *26*, 512-516.

Shimizu, S.; Akimoto, K.; Kawashima, H.; Shinmen, Y.; Yamada, H. Production of dihomo-gamma-linolenic acid by *Mortierella alpina* 1S-4. *J. Am. Oil Chem.* **1989**, *66*, 237-241.

Singh, C. P.; Shah, D. O.; Holmberg, K. Synthesis of mono- and diglycerides in water-in-oil microemulsions. *J. Am. Oil Chem. Soc.* **1994**, *71*, 583-587.

Sonnet, P. E. Lipase selectivities. *J. Am. Oil Chem. Soc.* **1988**, *65*, 900-904.

Sonntag, N. O. V. Composition and characteristics of individual fats and oils. In *Bailey's Industrial Oil and Fat Products*, 4th ed.; Swern, D., Eds.; Wiley: New York, NY, 1981; pp 289-477.

Speek, A. J.; Schrijver, J.; Schreurs, W. H. P. Vitamin-E composition of some seed oils as determined by high performance liquid-chromatography with fluorometric detection. *J. Food Sci.* **1985**, *50*, 121-124.

Sugano, M.; Inoue, T.; Koba, K.; Yoshida, K.; Hirose, N.; Shinmen, Y.; Akimoto, K.; Amachi, T. Influence of sesame lignans on various lipid parameters in rats. *Agric. Biol. Chem.* **1990**, *54*, 2669-2673.

Sugihara, A.; Shimada, Y.; Tominaga, Y. A novel *Geotrichum candidum* lipase with some preference for the 2-position on a triglyceride molecule. *Appl. Microbiol. Biotechnol.* **1991**, *35*, 738-740.

Svensson, I.; Adlercreutz, P.; Mattiasson, B. Interesterification of phosphatidylcholine with lipases in organic media. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 255-258.

Trautwein, E. A.; Duchateau, G. S. M. J. E.; Lin, Y. G.; Mel'nikov, S. M.; Molhuizen, H. O. F.; Ntanios F. Y. Proposed mechanisms of cholesterol-lowering action of plant sterols. *Eur. J. Lipid Sci. Technol.* **2003**, *105*, 171-185.

Valsta, L. M.; Jauhiainen, M.; Aro, A.; Katan, M. B.; Mutanen, M. Effects of a monounsaturated rapeseed oil and a polyunsaturated sunflower oil diet on lipoprotein levels in humans. *Arterioscler. Thromb.* **1992**, *12*, 50-57.

Verger, R. 'Interfacial activation' of lipases: facts and artifacts. *Trends Biotechnol.* **1997**, *15*, 32-38.

Villeneuve, P.; Turon, F.; Caro, Y.; Escoffier, R.; Barea, B.; Barouh, B.; Lago, R.; Piombo, G.; Pina, M. Lipase-catalyzed synthesis of canola phytosterols oleate esters as cholesterol lowering agents. *Enz. Microbial Technol.* **2005**, *37*, 150-155.

Vulfson, E. N. Industrial applications of lipases. In *Lipases: Their Structure, Biochemistry, and Application*; Woolley, P., Petersen, S. B., Eds.; Cambridge University Press: Cambridge, England, 1994; pp 271-288.

Wahrburg, U.; Martin, H.; Sandkamp, M.; Schulte, H.; Assmann, G. Comparative effects of a recommended lipid-lowering diet vs a diet rich in monounsaturated fatty acids on serum lipid profiles in healthy young adults. *Am. J. Clin. Nutr.* **1992**, *56*, 678-683.

Watanabe, T.; Suzuki, Y.; Sagesaka, Y.; Kohashi, M. Immobilization of lipases on polyethylene and application to perilla oil hydrolysis for production of alpha-linolenic acid. *J. Nutr. Sci. Vitaminol.* **1995**, *41*, 307-312.

Weber, N.; Weitkamp, P.; Mukherjee, K. D. Fatty acid steryl, stanyl, and steroid esters by esterification and transesterification in vacuo using *Candida rugosa* lipase as catalyst. *J. Agric. Food Chem.* **2001**, *49*, 67-71.

Weber, N.; Weitkamp, P.; Mukherjee, K. D. Cholesterol-lowering food additives: lipase-catalysed preparation of phytosterol and phytostanol esters. *Food Res. Int.* **2002**, *235*, 177-181.

Weete, J. D. Microbial lipase. In *Food Lipids—Chemistry, Nutrition, and Biotechnology*, 2nd ed.; Akoh, C. C., Min, D. B., Eds.; Marcel Dekker: New York, NY, 2002; pp 813-838.

Wester, I. Cholesterol-lowering effect of plant sterols. *Eur. J. Lipid Sci. Technol.* **2000**, *102*, 37-44.

Weststrate, J.A.; Meijer, G. W. Plant sterol-enriched margarines and reduction of plasma total and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur. J. Clin. Nutr.* **1998**, *52*, 334-343.

Xu, X.; Skands, A. R. H.; Høy, C. E.; Mu, H., Balchen, S.; Alder-Nissen, J. Production of specific-structured lipids by enzymatic interesterification: Elucidation of acyl migration by response surface design. *J. Am. Oil Chem. Soc.* **1998**, *75,* 1179-1186.

Yamaguchi, S.; Mase, T. High-yield synthesis of monoglyceride by monoacylglycerol and diacylglycerol lipase from *Penicillium camembertii* U-150. *J. Ferment. Bioeng.* **1991**, *72,* 162-167.

Yankah, V. V.; Akoh, C. C. Lipase-catalyzed acidolysis of tristearin with oleic or caprylic acids to produce structured lipids. *J. Am. Oil Chem. Soc.* **2000**, *77,* 495-500.

Yang, T.; Fruekilde, M. B.; Xu, X. Suppression of acyl migration in enzymatic production of structured lipids through temperature programming. *Food Chem.* **2005**, *92,* 101-107.

Table 2.1. Selected representative applications of microbial lipases in lipid and organic chemical industries

Application	Reaction type	Reference
Synthesis of MLM-type triacylglycerols (TAGs)	Esterification	Fomuso and Akoh (2002)
Synthesis of oleic acid-enriched TAGs	Esterification	Yankah and Akoh (2000)
Synthesis of EPA/DHA-enriched TAGs	Esterification	Huang and Akoh (1994)
Synthesis of γ-linolenic acid (GLA)-enriched TAGs	Esterification	Lumor and Akoh (2005)
Synthesis of conjugated linoleic acid (CLA)-enriched TAGs	Esterification	Lee *et al.* (2004)
Synthesis of n-3/n-6 FAs-balanced TAGs	Esterification	Jennings and Akoh (2002)
Synthesis of human milk fat substitutes	Esterification	Oba and Witholt (1994)
Synthesis of cocoa butter substitutes	Esterification	Bloomer *et al.* (1990)
Synthesis of low-calorie fats	Esterification	Fomuso and Akoh (1997)
Synthesis of cold-spreadable fats	Esterification	Rousseau and Marangoni (1998)
Synthesis of trans FAs-free margarine substitutes	Esterification	Fomuso and Akoh (2001)
Synthesis of mono- and diacylglycerols	Hydrolysis/esterification	Singh *et al.* (1994)
Modification of phospholipids	Esterification	Mutua and Akoh (1993)
Synthesis of sugar FA esters	Esterification	Oguntimein *et al.* (1993)
Synthesis of steryl, stanyl, and steroid FA esters	Esterification	Weber *et al.* (2001)
Synthesis of wax esters	Esterification	Isono *et al.* (1995)
Synthesis of polyol FA esters	Esterification	Hayes and Gulari (1992)
Synthesis of alkyl glucoside FA esters	Esterification	Akoh and Mutua (1994)
Synthesis of terpene or flavor esters	Esterification	Shieh *et al.* (1996)
Enantioselective hydrolysis or esterification of racemic acids and alcohols	Hydrolysis/esterification	Chen *et al.* (1993)
Enrichment of specific FAs (e.g., GLA, erucic acid) from oil sources	Hydrolysis	Rahmatullah *et al.* (1994)
FA production	Hydrolysis	Watanabe *et al.* (1995)
Bioremediation	Hydrolysis	Benjamin and Pandey (1998)
Polymer degradation	Hydrolysis	Chattopadhyay *et al.* (2003)

Source: Weete (2002); Akoh *et al.* (2004)

Figure 2.1. Lipase-catalyzed synthesis of MLM-type structured triacylglycerols (MLM-SLs).

1. Direct esterification

Glycerol (OH, OH, OH) + MCFA + LCFA →(Lipase)→ Structured Lipid (M, L, M) + Water
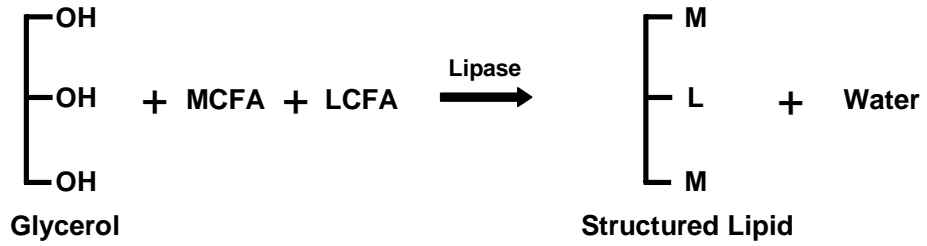
2. Transesterification (acidolysis)

LCT (L, L, L) + MCFA →(Lipase)→ Structured Lipid (M, L, M) + LCFA

3. Transesterification (ester interchange)

LCT (L, L, L) + MCT (M, M, M) →(Lipase)→ Structured Lipid (M, L, M)
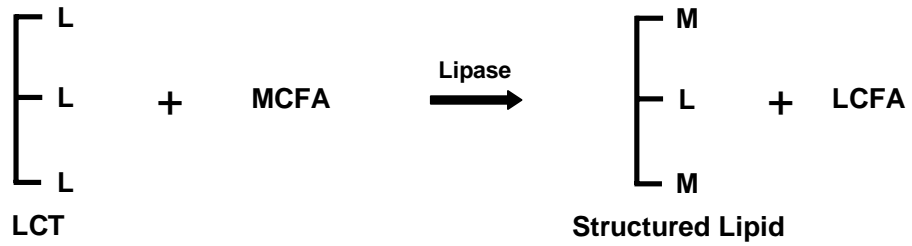
LCT (L, L, L) + MCFAEE →(Lipase)→ Structured Lipid (M, L, M) + LCFAEE
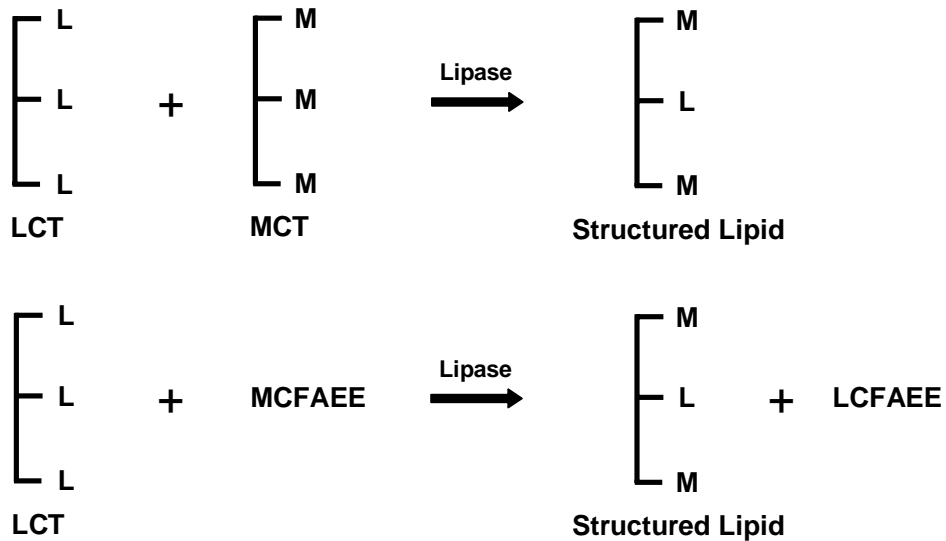
Figure 2.2. Lipase-catalyzed synthesis of β-sitosteryl oleate.
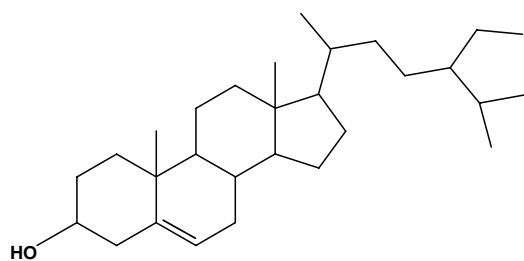
β-Sitosterol

+

Oleic acid (*for direct esterification*) or
Oleic acid ethyl ester (*for transesterification*)

Lipase
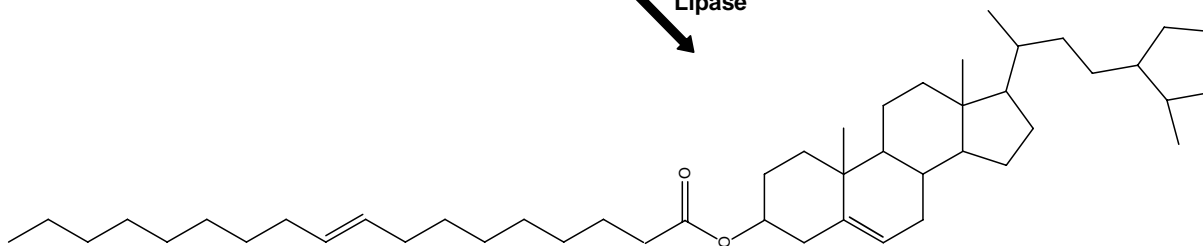
β-Sitosteryl oleate

+

Water (*in direct esterification*) or
Ethanol (*in transesterification*)

CHAPTER 3

MODELING OF LIPASE-CATALYZED ACIDOLYSIS OF SESAME OIL AND CAPRYLIC

ACID BY RESPONSE SURFACE METHODOLOGY: OPTIMIZATION OF REACTION

CONDITIONS BY CONSIDERING BOTH ACYL INCORPORATION AND MIGRATION[1]

ABSTRACT

Lipase-catalyzed acidolysis in hexane to produce structured lipids (SLs) from sesame oil and caprylic acid was optimized by considering both total incorporation (Y_1) and acyl migration (Y_2). Response surface methodology was applied to model Y_1 and Y_2, respectively, with three reaction parameters: temperature (X_1), reaction time (X_2), and substrate molar ratio (X_3). Well-fitting models for Y_1 and Y_2 were established after regression analysis with backward elimination, and verified by a chi-square test. All factors investigated positively affected Y_1. For Y_2, X_1 showed the greatest positive effect. However, there was no effect of X_3. We predicted the levels of Y_2 and acyl incorporation into *sn*-1,3 positions (Y_3) based on Y_1. The results showed that over the range of *ca.* 55 mol% of Y_1, Y_3 started to decrease, and Y_2 increased rapidly, suggesting that Y_1 should be kept below *ca.* 55 mol% to prevent decrease in quality and yield of targeted SLs.

Keywords: acidolysis; acyl migration; caprylic acid; Lipozyme RM IM; response surface methodology; sesame oil; structured lipids

INTRODUCTION

Structured lipids (SLs) are triacylglycerols (TAGs) that have been restructured to change the composition and positional distribution of fatty acids (FAs) from the native state by chemical or enzymatic methods (*1*). Among several types of SLs, MLM-type structured TAGs (MLM-SLs), in which medium-chain FAs (MCFAs) are esterified at *sn*-1,3 positions and long-chain FAs (LCFAs) at *sn*-2 position of the glycerol backbone have attracted much attention and many research works have been conducted over the past decade because of their unique and desirable nutritional characteristics. MLM-SLs can provide quick delivery of energy via oxidation of the more hydrophilic MCFAs located at *sn*-1,3 positions (*2*). They can also act as efficient carrier of LCFAs, such as monounsaturated FAs (MUFAs), polyunsaturated FAs (PUFAs), and essential FAs because the 2-monoacylglycerols (*sn*-2 MAGs) produced by pancreatic lipase digestion during metabolism are well absorbed through the intestinal wall (*3, 4*).

MLM-SLs can be prepared by enzymatic methods using *sn*-1,3 specific lipase (*5, 6*). Lipase-catalyzed acidolysis, in which MCFAs (in this study, caprylic acid) are used as acyl donors and vegetable oils are used as the source of glycerol backbone and LCFAs, is one of the most commonly used methods to produce MLM-SLs (*7-9*). Sesame oil obtained from sesame seed (*Sesamum indicum* L.) mainly consists of unsaturated FAs, such as oleic and linoleic acids, with small amounts of saturated FAs, such as palmitic and stearic acid and with only trace amounts of linolenic acid (*10, 11*). In our preliminary work, *sn*-2 position of TAGs in sesame oil is mostly comprised of the unsaturated FAs as other common vegetable oils (> 97 mol% by GC analysis). Therefore, sesame oil could be chosen as the substrate for the production of MLM-SLs.

The enzymatic methods can be expected to have several advantages, such as selectivity (i.e., incorporation of desirable FAs into specific position of TAGs) and few or no unwanted

side-reactions or by-products (*1*). However, in spite of using *sn*-1,3 specific lipase, undesirable side-reactions (especially, acyl migration) are known to occur in the overall process of SLs production. Acyl migration in the synthesis of MLM-SLs includes both migration of incorporated MCFAs from *sn*-1,3 to *sn*-2 position and migration of LCFAs or re-migration of MCFAs from *sn*-2 to *sn*-1,3 positions *(14, 15)*. Therefore, acyl migration plays a major role in the quality deterioration of MLM-SLs, such as the formation of undesirable TAG products (MML, LMM, LML, and MMM) or the loss of original LCFAs at *sn*-2 position. Recently, several studies have been attempted to elucidate the parameters of the lipase-catalyzed reaction that influence acyl migration and to reduce it during laboratory-scale or plot-scale production of SLs (*4, 8, 9, 12-15*). Because acyl migration is an undesirable but unavoidable side-reaction in the enzymatic production of specific SLs, the minimization of acyl migration is a key to improving the quality of targeted MLM-SLs. Therefore, acyl migration as well as acyl incorporation should be considered together in the SLs production from the standpoints of both yield and quality of SLs.

The objective of our study was to optimize the conditions of lipase-catalyzed acidolysis reaction between sesame oil and caprylic acid to produce MLM-SLs by considering both acyl incorporation and migration. The effects of three reaction parameters (temperature, reaction time, and substrate molar ratio) on the total incorporation and acyl migration were evaluated, respectively, and quadratic polynomial model equations for the total incorporation and acyl migration were also established, respectively, by response surface methodology (RSM), and then the optimized reaction conditions were proposed by using both models.

MATERIALS AND METHODS

Materials. Roasted and unrefined sesame oil was obtained from a grocery store. Caprylic acid (C8:0, purity > 98%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Lipozyme RM IM, a *sn*-1,3 specific immobilized lipase from *Rhizomucor miehei* was obtained from Novozymes North America Inc. (Franklinton, NC) and pancreatic lipase (EC 3.1.1.3) was obtained from Sigma-Aldrich Co. (St. Louis, MO). n-Hexane and anhydrous diethyl ether were purchased from J.T. Baker (Philipsburg, NJ). All other reagents used were of analytical or enzymatic grades and purchased from Fisher Scientific (Fair Lawn, NJ).

Experimental Design for RSM. Factors considered important were reaction temperature (X_1 = 45-65 ºC), reaction time (X_2 = 18-30 h), and substrate molar ratio; i.e., caprylic acid to total TAGs molar ratio (X_3 = 4-8). RSM was used to optimize reaction parameters. Central composite design (CCD) was adopted in this study. CCD is a 2^k factorial design with star points and center points. Twenty three experimental settings consisting of 6 star points (star distance is 1.682) and 9 center points were generated with 3 factors and 5 levels by the principle of RSM using commercial software, *Modde* 5.0 (Umetrics, Umeå, Sweden). The quadratic polynomial regression model was assumed for predicting individual *Y* variables (Y_1 = total incorporation; Y_2 = acyl migration). The model proposed for each response of *Y* fitted equation (1) is as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad (1)$$

Where *Y* is response variable (C8:0 mol%). β_0, β_i, β_{ii}, and β_{ij} are constant coefficients of intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are independent variables.

Acidolysis Reaction. One hundred milligrams of sesame oil was mixed with caprylic acid at different levels of caprylic acid to total TAGs (in sesame oil) molar ratio generated by

RSM, in screw-capped test tubes, and then Lipozyme RM IM and 3 mL of n-hexane were added to the reaction mixtures. The amounts of Lipozyme RM IM added to the reaction mixtures were maintained at 10% (w/w) of the sum of the above two substrates. The reaction was carried out in an orbital shaking water bath at 200 rpm at different temperatures and for different time periods generated by RSM, as indicated.

Separation of Structured TAGs. The reactions were stopped by filtering Lipozyme RM IM through anhydrous sodium sulfate column. Aliquots (300 µL) of the reactants were then separated by thin-layer chromatography (TLC) on silica gel G plates (Fisher Scientific, Norcross, GA) developed with petroleum ether/diethyl ether/acetic acid (80:20:0.5, v/v/v). After the TLC plates were dried in air, and sprayed with 0.2% 2´,7´-dichlorofluorescein in methanol, the bands were visualized under ultraviolet light. The bands corresponding to TAGs were scraped from the plates, and then extracted three times with 4 mL of anhydrous diethyl ether. The structured TAGs were obtained after evaporating the diethyl ether under nitrogen, and were used for *sn*-2 positional analysis.

Methylation and GC analysis. The bands corresponding to TAGs scraped from the TLC plate were methylated in 3 mL of 6% HCl in methanol at 75 ºC for 2 h. The FA methyl esters (FAMEs) were extracted and analyzed by gas chromatography (GC). An Agilent Technologies 6890N gas chromatograph (Agilent Technologies Inc., Palo Alto, CA), equipped with a flame ionization detector (FID) and a fused silica capillary column (AT-225, 30 m × 0.25 mm i.d., Alltech Associates, Inc., Deerfield, IL) was used. The carrier gas was helium and the total gas flow rate was 23 mL/min. The injector and detector temperatures were maintained at 250 and 260 ºC, respectively. The column was initially held at 40 ºC for 3 min and programmed to increase to 130 ºC at the rate of 10 ºC/min. After held at 130 ºC for 3 min, the column was then

programmed to increase to 215 ºC at the rate of 20 ºC/min. The FAMEs were identified and their relative contents were calculated as mol% with heptadecanoic acid (C17:0) as an internal standard.

Pancreatic Lipase-Catalyzed *sn*-2 Positional Analysis. The structured TAGs were used to analyze the FAs which are esterified at the *sn*-2 position according to the pancreatic lipase hydrolysis procedure (*16-18*). One milliliter of 1 M Tris butter (adjusted to pH 8.0 with HCl) was first added to the test tubes containing the TAG samples. Also added were 0.25 mL 0.05% sodium cholate solution and 0.1 mL 2.2% calcium chloride solution, and then the mixtures were vortexed thoroughly to emulsify the samples. Then 20 mg of pancreatic lipase was subsequently added and mixed well. The test tubes were immediately placed in a water bath maintained at 40 ºC. After 3 min the tubes were vortexed for exactly 2 min, and then 0.5 mL of 6 N HCl solution and 4 mL of anhydrous diethyl ether were added. The mixtures were vortexed, centrifuged, and then diethyl ether layers containing pancreatic lipase hydrolysates were passed through sodium sulfate column. Four milliliters of diethyl ether was added two more times to extract the pancreatic lipase hydrolysates thoroughly. After the extraction, diethyl ether was completely evaporated under nitrogen. The pancreatic lipase hydrolysates were dissolved in 2 mL of anhydrous diethyl ether, transferred to small vials, and then diethyl ether was concentrated up to 300 μL for spotting on TLC plates. After the plates were developed with hexane/diethyl ether/formic acid (60:40:1.6, v/v/v), the plates were dried in air, and sprayed with 0.2% 2´,7´-dichlorofluorescein in methanol. The bands corresponding to the *sn*-2 MAGs were scraped from the plates, methylated and analyzed by GC, as previously described. For identification of TLC bands of *sn*-2 MAGs, 2-monoolein was used as a standard.

Statistical analysis. All data were analyzed with the assistance of commercial software, Modde 5.0 (Umetrics, Umeå, Sweden). The significant second-order coefficients were selected by regression analysis with backward elimination. Then, the fit of the model was evaluated by coefficients of determination (R^2 and Q^2 values) and a test for lack of fit, which was performed by comparing mean square (MS) lack of fit to MS pure experimental error, from the analysis of variance (ANOVA). The model equation established was finally proposed after verification by a chi-square test.

RESULTS AND DISCUSSION

FA Composition of Sesame Oil. Sesame oil used as the substrate for the synthesis of MLM-SLs contained high amount of oleic (C18:1*n-9*) and linoleic (C18:2*n-6*) acids, which constitute *ca.* 80 mol% of the total FAs of TAGs in the oil (**Table 3.1**). **Table 3.1** also shows that *sn*-2 position of TAGs of sesame oil is mostly comprised of these two unsaturated FAs (*ca*. 97 mol%). Therefore, as expected, sesame oil was suitable as a good source to provide glycerol backbone and LCFAs for the production of MLM-SLs in this study.

Model Fitting. RSM was applied to model total incorporation (Y_1) and acyl migration (Y_2), respectively, with 3 reaction parameters: temperature (X_1), reaction time (X_2), and substrate molar ratio (X_3). RSM enabled us to obtain sufficient information for statistically acceptable results using reduced number of experimental sets, and is efficient method to evaluate the effects of multiple parameters, alone or in combination, on response variables (*14, 19, 20*). **Table 3.2** shows the levels of Y_1 and Y_2 at each 23 experimental set generated by the principles of RSM used in this study. Results of all experimental sets were used for modeling Y_1; however, for Y_2, 4 outliers (experiments 10, 12, 13, and 14) were excluded to enhance the fit of the model. Then,

the best-fitting models were determined by multiple linear regression (MLR) and backward elimination. The fits of the models were evaluated by coefficients of determination (R^2 and Q^2 values) and a test for lack of fit from ANOVA. In the model for Y_1, R^2 (i.e., the fraction of the variation of the response explained by the model) and Q^2 (i.e., the fraction of the variation of the response predicted by the model) values were 0.965 and 0.798, respectively (**Table 3.3**). **Table 3.4** showed the values of R^2 (0.981) and Q^2 (0.879) in the model for Y_2. ANOVA also showed that the probabilities for the regression of each model were significant ($p < 0.001$), meaning that the models were statistically good, and the models had no lack of fit at 95% level of significance (data not shown). As the results, well-fitting models for Y_1 and Y_2 were successfully established, respectively.

Effects of Parameters. Table 3.3 showed that Y_1 was affected positively by all 3 reaction parameters investigated. Among them X_3 showed the greatest effect on Y_1. The second and third significant ($P < 0.05$) parameters were X_1 and X_2, respectively. However, squared term of X_3 negatively affected Y_1. Whereas, X_1 showed the greatest positive effect on Y_2, and all squared and interaction terms also positively affected it as shown in **Table 3.4**. The result was shown to be in accordance with the previous report of Xu et al. (*14*) that reaction temperature was the most important factor affecting acyl migration, and the interactions with or between temperatures were also more significant than the others that were not interacting with temperatures. X_2 also showed a positive effect on Y_2; however, there was no significant ($P < 0.05$) effect of X_3.

As described above, reaction temperature was positively related with both total incorporation and acyl migration in our study; however, the effect of temperature was shown to be greater on the latter than the former. **Figure 3.1 (a)** shows that within the given range (45-65

ºC) of X_1, Y_2 showed an approximately exponential increase with the increase of X_1, unlike Y_1 which increased linearly. Xu et al. (*14*) reported that because acyl migration is a thermodynamic process following the general rule of the Arrhenius equation, the acyl migration rate was faster at higher than at lower temperature. This suggests that a relatively lower temperature would be a key factor for optimized reaction conditions to suppress acyl migration even though there might be some decrease in total incorporation. Unlike the temperature effect on acyl migration which was exponential, the effect of reaction time on acyl migration was linear and similar to that of total incorporation as illustrated in **Figure 3.1 (b)**. Our results also suggest that substrate molar ratio could be an important factor for the optimization of SLs production. That is, relatively higher substrate molar ratio was shown to result in higher total incorporation without affecting acyl migration significantly (**Figure 3.1 (c)**).

Model Verification. A chi-square test using 8 additional experimental sets chosen from the given ranges of reaction parameters was performed to examine the adequacies of the models established. The chi-square test for Y_1 indicated that there were no significant ($P < 0.05$) difference between the observed and predicted values since the chi-square value (0.44) was much smaller than 14.07, cutoff points at $\alpha = 0.05$ and df = 7 (**Table 3.5**). The chi-square test for Y_2 also showed that observed values were not significantly ($P < 0.05$) different from the values predicted by the model because of smaller chi-square value (4.49) than cutoff points (11.07) at $\alpha = 0.05$ and df = 5. However, for Y_2, the experiments 7 and 8 were excluded from the process of model verification because reasonable data could not be obtained. However, it is known that *sn*-2 positional analysis method by pancreatic lipase cannot be applied unreservedly to oils containing substantial amounts of MCFAs with 12 or fewer carbon atoms (*8, 16*). Therefore, we surmise that the failure in obtaining reasonable data for the experiments 7 and 8 was due to high amount

of caprylic acid present in SLs synthesized at the conditions of the two experimental sets or other unknown factors. Similar failure to obtain reasonable experimental data was also found in the process of model establishment for Y_2; that is, among 4 outliers, experiments 10, 12, and 14 showing that high levels of Y_1 were excluded because the data were shown to be overestimated and had poor-precisions (**Table 3.2**).

Optimization of Reaction Conditions. The possible minimum levels of Y_2 were predicted based on Y_1 at 1 mol% interval, and the suitable conditions enabling the levels were generated by optimizer function of Modde 5.0 (**Table 3.6**). As expected, X_3 should be kept at the highest level (i.e., 8) to minimize Y_2 at all given levels of Y_1. Therefore, increasing substrate molar ratio seemed to be the best way to suppress acyl migration while achieving targeted total incorporation during SLs production. However, this may have a limitation from the standpoint of industrial production of SLs because using high amount of substrates demands more efforts, such as higher temperature and longer time, to purify the SLs. Xu et al. (*13*) reported that acyl migration can occur during the purification stage in the conventional batch deodorizer as well as reaction stage, and the higher distillation temperature and longer time during purification were the main factors that increase acyl migration. Therefore, suitable substrate molar ratio should be determined carefully by considering overall process of SLs production including purification step as well as reaction step.

The incorporation of caprylic acid into *sn*-1,3 positions (Y_3) was also predicted in an effort to optimize reaction conditions, and the calculated values of Y_3 were given in **Table 3.6**. Y_3 could be calculated by the following equation (2):

$$Y_3 \text{ (mol\%)} = [3 \times Y_1 \text{ (mol\%)} - Y_2 \text{ (mol\%)}] / 2 \tag{2}$$

Then, a plot to predicted levels of Y_2 and Y_3 based on Y_1 was generated by using data in **Table 3.6 (Figure 3.2)**. **Table 3.6** showed that below the range of *ca.* 55 mol% of Y_1, Y_1 could be increased by only increasing X_2 at the lowest level of X_1 (i.e., 45 ºC) and highest X_3 (i.e., 8) while suppressing Y_2; whereas, the range of Y_1 above *ca.* 55 mol% could be achieved by increasing X_1. However, **Table 3.6** and **Figure 3.2** showed that over the range of *ca.* 55 mol% of Y_1, Y_3 started to decrease, and Y_2 increased rapidly. From the predicted results above, we could assume that considerable portion of total incorporation over *ca.* 55 mol% was attained by acyl migration, not by incorporation into *sn*-1,3 positions. Therefore, on the supposition that 55 mol% of total incorporation is a critical point at which maximum incorporation into *sn*-1,3 positions can be achieved, we surmise that the value is 75 mol%. In other words, the content of targeted MLM-SLs also cannot exceed 75 mol% of total TAG products. This result was shown to be in close agreement with other reports. Xu et al. (*4*) predicted that even though there was no acyl migration, the maximum incorporation into *sn*-1,3 position was only 75 mol% during solvent-free acidolysis to produce MLM-SLs in a batch-type reactor. Negishi et al. (*21*) could obtain only *ca.* 76% content of targeted MLM-SLs and their TAG isomers in total reaction mixture by the solvent-free interesterification reaction. Finally, the overall levels of both Y_1 and Y_2 were evaluated and found to be relatively higher (38.3-60.7 mol% for Y_1 and 9.6-49.8 mol% for Y_2; from **Table 3.2**) than expected; this may be caused by hexane as the reaction media. In the SL synthesis, organic solvent systems are known to cause higher rates of acyl migration as well as acyl incorporation than solvent-free systems because of the lower viscosity of substrates (*14, 22*). In addition, hexane will add to the cost of the industrial scale production of SL. Therefore, for food application and cost reduction, it would be preferable to produce SL in solvent-free systems. However, both total incorporation and acyl migration are expected to be relatively decreased.

In conclusion, substrate molar ratio should be kept as high as possible and relatively low temperature was required to maximize total incorporation and to minimize acyl migration. Total incorporation was kept below *ca*. 55 mol% to prevent decrease in quality and yield of targeted MLM-SLs.

LITERATURE CITED

(1) Akoh, C. C. Structured Lipids. In *Food lipids – Chemistry, Nutrition, and Biotechnology*, 2nd ed.; Akoh, C. C., Min, D. B., Eds.; Marcel Dekker: New York, NY, 2002; pp 877-908.

(2) Bach, A. C.; Babayan, V. K. Medium-chain triglycerides: an update. *Am. J. Clin. Nutr.* **1982**, *36,* 950-962.

(3) Iwasaki, Y.; Yamane, T. Enzymatic synthesis of structured lipids. *J. Mol. Catal. B-Enz.,* **2000**, *10,* 129-140.

(4) Xu, X.; Balchen, S.; Høy, C. E.; Alder-Nissen, J. Pilot batch production of specific-structured lipids by lipase-catalyzed interesterification: Preliminary study on incorporation and acyl migration. *J. Am. Oil Chem. Soc.* **1998**, *75,* 301-308.

(5) Fomuso, L. B.; Akoh, C. C. Structured lipids: lipase-catalyzed interesterification of tricaproin and trilinolein. *J. Am. Oil Chem. Soc.* **1998**, *75,* 405-410.

(6) Fomuso, L. B.; Akoh, C. C. Enzymatic modification of triolein: incorporation of caproic and butyric acids to produce reduced-calorie structured lipid. *J. Am. Oil Chem. Soc.* **1997**, *74,* 269-272.

(7) Fomuso, L. B; Akoh, C. C. Lipase-catalyzed acidolysis of olive oil and caprylic acid in a bench-scale packed bed bioreactor. *Food Res. Int.* **2002**, *35,* 15-21.

(8) Mu, H.; Kurvinen, J. P.; Kallio, H.; Xu, X.; Høy C. E. Quantitation of acyl migration during lipase-catalyzed acidolysis, and of the regioisomers of structured triacylglycerols formed. *J. Am. Oil Chem. Soc.* **2001**, *78,* 959-964.

(9) Mu, H.; Xu, X.; Høy C. E. Production of specific-structured triacylglycerols by lipase-catalyzed interesterification in a laboratory-scale continuous reactor. *J. Am. Oil Chem. Soc.* **1998**, *75,* 1187-1193.

(10) Kochhar, S. P. Sesame, rice-bran and flaxseed oils. In *Vegetable Oils in Food Technology: Composition, Properties, and Uses*; Gunstone, F. D. Ed.; CRC press; Blackwell; 2002; pp 297-326.

(11) Namiki, M. The chemistry and physiological functions of sesame. *Food Rev. Int.* **1995**, *11,* 281-329.

(12) Xu, X.; Mu, H.; Skands, A. R. H.; Høy, C. E.; Alder-Nissen, J. Parameters affecting diacylglycerol formation during the production of specific-structured lipids by lipase-catalyzed interesterification. *J. Am. Oil Chem. Soc.* **1999**, *76,* 175-181.

(13) Xu, X.; Skands, A.; Alder-Nissen, J. Purification of specific structured lipids by distillation: Effects on acyl migration. *J. Am. Oil Chem. Soc.* **2001**, *78,* 715-718.

(14) Xu, X.; Skands, A. R. H.; Høy, C. E.; Mu, H., Balchen, S.; Alder-Nissen, J. Production of specific-structured lipids by enzymatic interesterification: Elucidation of acyl migration by response surface design. *J. Am. Oil Chem. Soc.* **1998**, *75,* 1179-1186.

(15) Yang T.; Fruekilde, M. B.; Xu, X. Suppression of acyl migration in enzymatic production of structured lipids through temperature programming. *Food Chem.* **2005**, *92,* 101-107.

(16) AOCS. *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 5th ed.; AOCS press: Champaign, IL, 1998; Ch 3-91.

(17) Gunstone, F. D.; Harwood, J. H.; Palley, F. B. Triacylglycerol analyses by pancreatic lipase hydrolysis. In *The Lipid Handbook*, 2nd ed.; Gunstone, F. D., Harwood, J. H., Palley, F. B., Eds.; Chapman and Hall; New York, NY, 1994; pp 337-338.

(18) Luddy, F. E.; Barford, R. A.; Herb, S. F.; Magidman, P; Riemenschneider, R. W. Pancreatic lipase hydrolysis of triglycerides by a semimicro technique. *J. Am. Oil Chem. Soc.* **1963**, *41,* 693-696.

(19) Shieh, C. J.; Akoh, C. C.; Koehler, P. E. Four-factor response surface optimization of the enzymatic modification of triolein to structured lipids. *J. Am. Oil Chem. Soc.* **1995**, *72,* 619-623.

(20) Huang, K. H.; Akoh, C. C. Optimization and scale-up of enzymatic synthesis of structured lipids using RSM. *J. Food Sci.* **1996**, *61,* 137-141.

(21) Negishi, S.; Arai, Y.; Arimoto, S.; Tsuchiya, K.; Takahashi, I. Synthesis of 1,3-dicapryloyl-2-docosahexaenoylgylcerol by a combination of nonselective and sn-1,3-selective lipase reactions. *J. Am. Oil Chem. Soc.* **2003**, *80,* 971-974.

(22) Goh, S. H.; Yeong, S. K.; Wang, C. W. Transesterification of cocoa butter by fungal lipases: Effect of solvent on 1,3-specificity. *J. Am. Oil Chem. Soc.* **1993**, *70,* 567-570.

Table 3.1. Fatty Acid Composition of Total TAG and s*n*-2 Position of TAG of Sesame oil (mol%)[a]

Fatty acid	Total TAG	*sn*-2 position
C12:0	Trace[b]	-
C14:0	Trace	-
C16:0	15.4 ± 0.1	1.8 ± 0.0
C16:1	Trace	0.2 ± 0.0
C18:0	3.9 ± 0.1	0.3 ± 0.0
C18:1*n-9*	35.7 ± 0.3	41.9 ± 0.1
C18:2*n-6*	44.6 ± 0.3	55.5 ± 0.0
C18:3*n-3*	0.4 ± 0.0	0.3 ± 0.0

[a] Mean ± SD, n = 2. [b] < 0.05 mol%.

Table 3.2. Central Composite Design Arrangement and Responses for the Lipozyme RM IM-Catalyzed Total Incorporation of Caprylic Acid into TAG of Sesame Oil and Migration of Caprylic Acid into sn-2 Position of TAG of Sesame Oil[a]

Exp. No.	Factors			Responses	
	X_1 (°C)	X_2 (h)	X_3	Y_1 (mol%)	Y_2 (mol%)
1	45	18	4	41.9 ± 0.8[b]	14.5 ± 0.2
2	65	18	4	45.1 ± 2.0	33.0 ± 4.8
3	45	30	4	43.0 ± 0.7	16.8 ± 1.8
4	65	30	4	51.1 ± 0.0	45.8 ± 0.8
5	45	18	8	48.1 ± 1.7	9.6 ± 1.6
6	65	18	8	56.6 ± 1.1	40.5 ± 0.4
7	45	30	8	53.6 ± 0.7	13.6 ± 1.9
8	65	30	8	60.7 ± 0.9	49.8 ± 0.8
9	38.18	24	6	46.3 ± 2.6	12.5 ± 0.4
10	71.82	24	6	57.8 ± 2.1	-[c]
11	55	13.91	6	47.6 ± 0.1	18.8 ± 1.4
12	55	34.09	6	58.7 ± 0.0	-
13	55	24	2.64	38.3 ± 0.2	-
14	55	24	9.36	57.9 ± 0.7	-
15	55	24	6	52.9 ± 0.7	26.8 ± 0.3
16	55	24	6	53.3 ± 0.4	27.9 ± 1.2
17	55	24	6	53.1 ± 0.6	32.9 ± 2.2
18	55	24	6	52.4 ± 0.3	29.2 ± 1.8
19	55	24	6	54.2 ± 2.5	28.8 ± 1.4
20	55	24	6	55.1 ± 0.5	31.6 ± 1.4
21	55	24	6	52.5 ± 0.1	27.0 ± 3.1
22	55	24	6	53.7 ± 0.8	30.0 ± 1.7
23	55	24	6	51.8 ± 0.6	26.8 ± 2.5

[a] X_1 = reaction temperature; X_2 = reaction time; X_3 = substrate molar ratio (caprylic acid to sesame oil); Y_1 = total incorporation of caprylic acid into TAG; Y_2 = migration of caprylic acid into sn-2 position. [b] Mean ± SD, n = 2. [c] Outlier excluded from the model establishment.

Table 3.3. Significant Regression Coefficients and Coefficients of Determination of the Second-Order Polynomials After Backward Elimination for Total Incorporation of Caprylic Acid into TAG of Sesame Oil[a]

Variables	Coefficients	P-Values
Intercept	53.243	5.582×10^{-21}
X_1	3.386	5.550×10^{-7}
X_2	2.590	1.032×10^{-4}
X_3	5.189	3.544×10^{-9}
X_3^2	-2.010	6.135×10^{-5}
R^2	0.965	
Q^2	0.798	

[a] See Table 3.2 for description of abbreviations.

Table 3.4. Significant Regression Coefficients and Coefficients of Determination of the Second-Order Polynomials After Backward Elimination for Migration of Caprylic Acid into sn-2 Position of TAG of Sesame Oil[a]

Variables	Coefficients	P-Values
Intercept	29.000	1.660×10^{-11}
X_1	14.325	1.488×10^{-8}
X_2	3.550	1.142×10^{-3}
X_1^2	2.684	1.679×10^{-2}
$X_1 \times X_2$	1.975	2.841×10^{-2}
$X_1 \times X_3$	2.450	1.026×10^{-2}
R^2	0.981	
Q^2	0.879	

[a] See Table 3.2 for description of abbreviations.

Table 3.5. Model Verification by Chi-square (χ^2) Test[a]

Exp. No.	Factors			Responses			
	X_1 (°C)	X_2 (h)	X_3	Y_1 (mol%)		Y_2 (mol%)	
				Observed	Predicted	Observed	Predicted
1	50	21	5	48.9 ± 0.8[b]	47.3	13.2 ± 2.1	20.6
2	50	21	7	50.3 ± 2.0	52.0	20.5 ± 1.3	20.0
3	50	27	5	50.2 ± 1.0	49.5	29.2 ± 0.7	23.3
4	50	27	7	52.9 ± 0.8	54.6	21.4 ± 3.7	22.4
5	60	21	5	51.7 ± 0.7	50.2	33.5 ± 4.2	32.8
6	60	21	7	54.0 ± 1.0	55.5	31.5 ± 2.5	34.5
7	60	27	5	51.5 ± 1.6	52.8	-[c]	37.4
8	60	27	7	55.4 ± 0.9	58.4	-	38.9
				χ^2 = 0.44[d]		χ^2 = 4.49	

[a] See Table 3.2 for description of abbreviations. [b] Mean ± S.D., n = 2. [c] Outlier excluded from the model verification. [d] $\chi^2 = \Sigma$ [(Observed value – Predicted value)^2/ Predicted value]; cutoff points are 11.07 at α = 0.05, df = 5, and 14.07 at at α = 0.05, df = 7, respectively.

63

Table 3.6. Predicted Suitable Conditions to Minimize Levels of Acyl Migration Based on the Predicted Total Incorporation[a]

Responses			Factors		
Y_1(mol%)	Y_2(mol%)	Y_3(mol%)[b]	X_1 (°C)	X_2 (h)	X_3
49.2	10.3	68.7	45.0	18.0	8.0
50.0	11.3	69.4	45.0	19.6	8.0
51.0	12.4	70.3	45.0	21.8	8.0
52.0	13.1	71.5	45.0	24.2	8.0
53.0	13.4	72.8	45.0	26.7	8.0
54.0	13.0	74.5	45.0	29.7	8.0
55.0	15.0	75.0	46.5	30.0	8.0
56.0	17.8	75.1	48.4	30.0	8.0
57.0	21.0	75.0	50.4	30.0	8.0
58.0	24.5	74.8	52.5	30.0	8.0
59.0	28.5	74.3	54.7	30.0	8.0
60.0	32.9	73.6	57.0	30.0	8.0
61.0	38.2	72.4	59.6	30.0	8.0
62.0	44.1	71.0	62.3	30.0	8.0
62.9	50.5	69.1	65.0	30.0	8.0

[a] See table 3.2 for description of abbreviations. [b] Incorporation of caprylic acid into *sn*-1,3 positions; Y_3 (mol%) = [3 × Y_1 (mol%) – Y_2 (mol%)] / 2.

Figure 3.1. Prediction plots for the acyl incorporation into total TAG and acyl migration into *sn*-2 position by the effects of main parameters during acidolysis between sesame oil and caprylic acid: (**A**) temperature; (**B**) reaction time; (**C**) substrate molar ratio. Factors setup: reaction time 24 h, substrate molar ratio 6 for (**A**); temperature 55 ℃, substrate molar ratio 6 for (**B**); temperature 55 ℃, reaction time 24 h for (**C**).
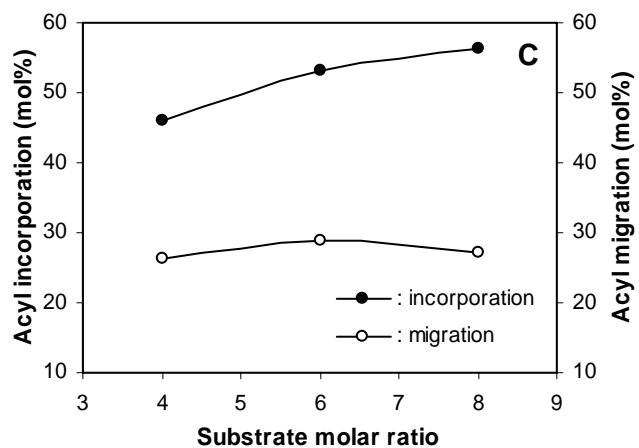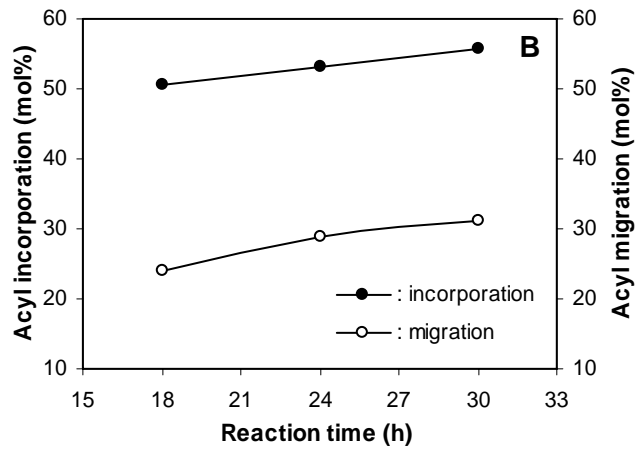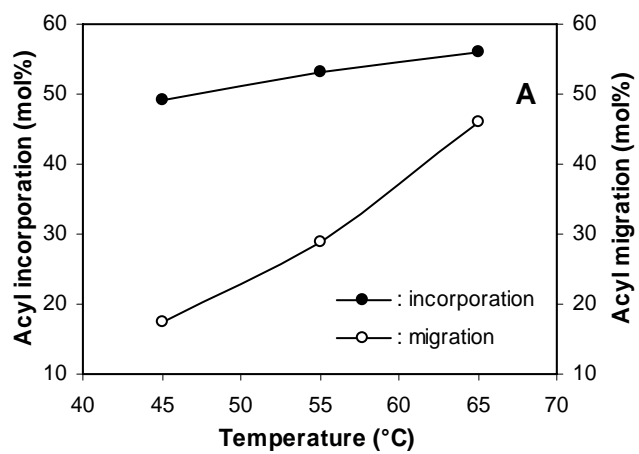
Figure 3.2. Predicted plot for acyl incorporation into *sn*-1,3 positions and acyl migration into *sn*-2 position based on total acyl incorporation during acidolysis between sesame oil and caprylic acid.
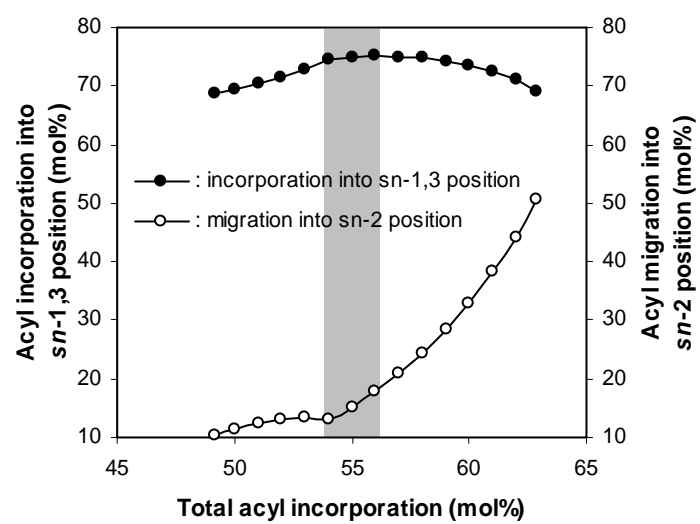
CHAPTER 4

CHARACTERISTICS OF STRUCTURED LIPID PREPARED BY LIPASE-CATALYZED

ACIDOLYSIS OF ROASTED SESAME OIL AND CAPRYLIC ACID IN A BENCH-SCALE

CONTINUOUS PACKED BED REACTOR[1]

ABSTRACT

Structured lipid (SL) was prepared from roasted sesame oil and caprylic acid (CA) by *Rhizomucor miehei* lipase-catalyzed acidolysis in a bench-scale continuous packed bed reactor. Total incorporation and acyl migration of CA in the SL were 42.5 mol% and 3.1 mol%, respectively, and the half-life of the lipase was 19.2 days. The SL displayed different physical and chemical properties: less saturated dark brown color, lower viscosity, lower melting and crystallization temperature ranges, higher melting and crystallization enthalpies, higher smoke point, higher saponification value, and lower iodine value, in comparison to unmodified sesame oil. The oxidative stability of purified SL was lower than that of sesame oil. There were no differences in the contents of unsaponifiables including tocopherols and phytosterols. However, total sesame lignans content was decreased in SL due to the loss of sesamol when compared to sesame oil. Most of the 70 volatiles present in roasted sesame oil were removed from SL during short-path distillation of SL. These results indicate that the characteristics of SL are different from those of original sesame oil in several aspects except for the contents of tocopherols and phytosterols.

Keywords: acidolysis; caprylic acid; continuous packed bed reactor; Lipozyme RM IM; roasted sesame oil; sesame lignans; short-path distillation; structured lipids

INTRODUCTION

Structured lipids (SLs) are restructured fats or oils in which the composition and positional distribution of fatty acids (FAs) are modified from the native state by chemical or enzymatic methods (*1*). In the SLs synthesis, the enzymatic methods have several advantages over chemical methods, such as selectivity and few or no formation of undesirable by-products (*1*). However, despite these benefits, the application of the enzymatic methods for the industrial production of SLs has been slow. The main disadvantage of the enzymatic methods in industrial scale application is the high cost of the lipase as compared to chemical methods. To overcome this problem, the use of immobilized lipase is preferred because it would allow the recovery and reutilization of the enzyme.

Continuous packed bed reactors are the most commonly used reactors for immobilized lipase in industrial scale applications because of their ease of construction and operation, resulting in relatively low cost and labor. The potential for reutilization of immobilized lipase is much higher in the continuous packed bed reactors as compared to stirred batch reactors because rupture of the supporting materials for immobilized lipase can be avoided in the former. Because such rupture is known to facilitate acyl migration, which is one of the most significant problems in the SL synthesis, such undesirable side-reactions can also be reduced in the continuous packed bed reactors (*2, 3*). Moreover, because the ratio between substrate and enzyme is much lower in a continuous packed bed reactor than is the case with a stirred batch reactor during enzymatic reactions, the lower substrate/enzyme ratio results in shorter reaction time and higher reaction rate, thereby reducing acyl migration (*4*). In these respects, continuous packed bed reactors would be the most desirable and feasible reactor type for the industrial scale production of SLs.

In the present work, we produced SLs from roasted sesame oil in a continuous packed bed reactor. Sesame oil used in this study is an edible oil obtained from the seed of sesame (*Sesamum indicum* L.), which is one of the world's oldest oilseed crops and has been cultivated mainly in Asia and Africa for centuries (*5, 6*). Two different types of sesame oil are commercially produced: one is refined, bleached, and deodorized (RBD) unroasted sesame oil (also called sesame salad oil) and the other is unrefined roasted sesame oil. Unlike RBD unroasted sesame oil prepared from sesame seeds cooked with steam, roasted sesame oil is produced by expelling sesame seeds roasted at about 180-200 ºC followed by filtration without further refining process. Roasting of sesame seeds gives a dark brown color to the oil and develops a characteristic roasted flavor in the oil mainly due to thermochemical reactions, such as Maillard reactions (*6, 7*). Roasted sesame oil is indispensable in Eastern Asian dishes because of such characteristic roasted flavor specially preferred by Eastern Asians, such as Korean, Chinese, and Japanese.

Roasted sesame oil consists mainly of neutral triacylglycerols (TAGs) containing about 80% unsaturated FAs (USFAs), such as oleic and linoleic acids, with relatively larger quantity of free FAs (FFAs) (1-3%) than other RBD vegetable oils because of the absence of refining processes (*6*). Compared to other vegetable oils, sesame oil also contains a relatively large amount of unsaponifiable matters (1-3%) that includes phytosterols, tocopherols, and unique compounds called sesame lignans including sesamol, sesamin, and sesamolin (*6*). Roasted sesame oil is known to have superior resistance to oxidation despite its relatively high concentration of FFAs as compared to other vegetable oils (*6*). The remarkable oxidative stability of sesame oil is reported to be due to the presence of sesame lignans as well as tocopherols (*5, 6*). Sesame lignans are also demonstrated to display several interesting

72

physiological effects, such as antioxidant, anticarcinogen, and hypocholesterolemic activity in animal models (*5*).

So far numerous studies on SL synthesis from diverse kinds of vegetable oils have placed too much importance on RBD oils; whereas, there were few studies on crude oils, such as roasted sesame oil (*8-13*). Since roasted sesame oil possesses several special attributes as compared to other vegetable oils, such as distinctive flavor, superior oxidative stability, and existence of unique and beneficial minor components (sesame lignans, phytosterols), therefore, it might be meaningful to use the roasted sesame oil as the source oil for SL synthesis to evaluate the characteristics of the resultant SL.

The objectives of our study were to compare the characteristics of roasted sesame oil-based MLM-type SL with those of original roasted sesame oil and to investigate the effect of SL synthesis processes on the attributes and minor components. In this study, we produced the SL by lipase-catalyzed acidolysis of roasted sesame oil with caprylic acid (CA) in a bench-scale continuous packed bed reactor. First, we evaluated the levels of incorporation and acyl migration of CA in the SL and the stability of the *sn*-1,3 specific lipase from *Rhizomucor miehei* under the acidolysis reaction conditions used in our work. Then, we investigated several physical and chemical properties, oxidative stability of the SL, and the compositions of several components present in the SL, such as FAs, minor components (phytosterols, tocopherols, sesame lignans), and volatile compounds.

MATERIALS AND METHODS

Materials. Unrefined roasted sesame oil was purchased from Spectrum Organic Product, Inc. (Petaluma, CA). CA (C8:0, purity > 98%) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Lipozyme RM IM, a *sn*-1,3 specific immobilized lipase from *Rhizomucor miehei* was provided by Novozymes North America Inc. (Franklinton, NC). Tocopherols, sesamol, and sesamin were from Sigma-Aldrich Co. (St. Louis, MO). Plant sterol mixture and 5β-cholestane-3β-ol were products of Matreya Inc. (Pleasant Gap, PA). All other reagents were analytical or HPLC grades.

Continuous Packed Bed Reactor Setup. Figure 4.1 depicts the cross section of the continuous packed bed reactor used in this study. The reactor has a jacketed stainless steel column (50 cm × 4.7 mm i.d.). The column was packed with 250 g of Lipozyme RM IM and the upper and lower ends of the column were layered with glass wool at the thickness of 3 and 7 cm, respectively. The column temperature was maintained at a constant level by a circulating water bath. The substrate mixture was fed upwards through the column using FMI Lab pump (model QV, Fluid Metering Inc., Oyster Bay, NY) for the time period to elute one bed volume (*ca*. 700 mL) to condition the enzyme bed before collecting the reaction products for analysis.

Acidolysis Reaction. The SL synthesis was performed by acidolysis reaction between sesame oil and CA in the continuous packed bed reactor. The reaction was carried out under the following conditions: substrate flow rate, 1.15 mL/min; column temperature, 45 °C; substrate molar ratio 1:6 (sesame oil: CA).

Evaluation of Lipase Stability. The stability of Lipozyme RM IM during acidolysis reaction in the continuous packed bed reactor was evaluated by estimating half-life of the lipase. Five milliliters of reaction products eluted from the outlet of the reactor were sampled at 2 L

intervals from 0 to 8 L of total elution volumes after collection of products started. The sampled reaction products at each elution volume were used to measure the total incorporation of CA and half-life of the lipase was calculated from the change in the total incorporation over the reaction time.

Short-Path Distillation. Short-path distillation was used to purify the synthesized SL with a KDL-4 unit (UIC Inc., Joliet, IL) under the following conditions: holding temperature, 25 ºC; heating oil temperature, 185 ºC; cooling water temperature, 15 ºC; and vacuum pressure, < 50 mbar. The SL was passed through the unit five times to reduce the free FA (FFA) content to the same level of original sesame oil.

Methylation and GC Analysis. Fifty milligrams of oil sample was methylated in 3 mL of 6% HCl solution (in methanol) at 75 ºC for 2 h. The FA methyl esters (FAMEs) were extracted and analyzed by gas chromatography (GC). An Agilent Technologies 6890N gas chromatograph (Agilent Technologies Inc., Palo Alto, CA), equipped with a flame ionization detector (FID) and a fused silica capillary column (AT-225, 30 m × 0.25 mm i.d., Alltech Associates, Inc., Deerfield, IL) was used. The carrier gas was helium and the total gas flow rate was 23 mL/min. The injector and detector temperatures were maintained at 250 and 260 ºC, respectively. The column was initially held at 40 ºC for 3 min and programmed to increase to 130 ºC at the rate of 10 ºC/min. After holding at 130 ºC for 3 min, the column was then programmed to increase to 215 ºC at the rate of 20 ºC/min. The FAMEs were identified and their relative contents were calculated as mol% with heptadecanoic acid (C17:0) as an internal standard.

Pancreatic Lipase-Catalyzed *sn*-2 Positional Analysis. Fifty milligrams of oil sample was used to analyze the FAs which were esterified at the *sn*-2 position according to the pancreatic lipase hydrolysis procedure described by Luddy *et al.* (*14*).

Color and Viscosity Analysis. The color of oil sample was determined by measuring CIE *L** (lightness), *a** (redness), and *b** (yellowness) values, and *C* (chroma) and *h°* (hue angle) with a Minolta chroma meter (model CR-300, Minolta Co. Ltd., Osaka, Japan). Viscosity of oil sample was measured at 10 ºC intervals from 5 to 35 ºC using a Brookfield Digital Viscometer (model DV-E, Brookfield Engineering Lab. Inc., Middleboro, MA).

Melting and Crystallization Profile Analysis. The melting and crystallization profiles of oil sample were determined using Perkin-Elmer differential scanning calorimeter (DSC) (model DSC 7, Perkin-Elmer Co., Norwalk, CT) according to AOCS recommended procedure Cj 1-94 (*15*) with a slight modification of the temperature program. Normal standardization was performed with Indium (m.p. 156.60 ºC, *ΔH* 28.45 J/g) as a reference standard. Dry ice was used as the coolant. A sample of 6-8 mg was hermetically sealed in a 30 μL capacity aluminum pan (Perkin-Elmer, Norwalk, CT), with an empty sealed pan used as a reference. Oil sample was rapidly heated from room temperature to 80 ºC and held at this temperature for 10 min to destroy any previous crystalline structure, before being cooled to -60 ºC at a rate of 5 ºC/min to obtain the crystallization profiles. After holding for 10 min at -60 ºC, samples were heated to 80 ºC at a rate of 5 ºC/min to generate melting profiles. However, the crystallization profiles of SL, which could not be obtained by the operating conditions above, was generated as follows: SL was rapidly heated from room temperature to 80 ºC and held at that temperature for 10 min and then cooled to -60 ºC at a rate of 1 ºC/min. The profiles were analyzed by the software provided with the DSC (Pyris software, Perkin-Elmer, Shelton, CT).

Chemical Properties Analysis. FFAs content, unsaponifiable matter content, smoke point, saponification value and iodine value of oil sample were determined by AOCS official methods (*15*), respectively.

Tocopherol Analysis. Tocopherols of oil sample were analyzed by normal phase HPLC following the method described by Ye *et al.* (*16*).

Phytosterol Analysis. Phytosterols of oil sample were analyzed by GC according to the method described by Jekel *et al*. (*17*) with a slight modification as described below. Oil sample (1.0 g) was weighed into a screw-capped tube and added to 1 mL of internal standard solution (100 μg/mL of 5α-cholestane-3β-ol in toluene). After flushing with nitrogen, the sample was saponified with 0.5 mL of saturated KOH in water at 80 ºC for 30 min in the presence of 8 mL of 3% pyrogallol in ethanol. After cooling to room temperature, the sample was added to 20 mL of hexane and 12 mL of water. The tube was shaken for 5 min to extract the unsaponifiables. As layers separated, an aliquot of 10 mL was taken from the top layer and then dried under nitrogen. The dried unsaponifiables were added to 1 mL of hexane and 1 μL of this solution was injected into the GC. A Hewlett Packard 5890 Series II gas chromatograph (Agilent Technologies Inc., Palo Alto, CA), equipped with a FID and a fused silica capillary column (SAC-5, 30 m × 0.25 mm i.d., Supelco Inc., Bellefonte, PA) was used. The carrier gas was nitrogen and the total gas flow rate was 20 mL/min. The injector and detector temperatures were maintained at 270 and 300 ºC, respectively. The column was initially held at 155 ºC for 3 min and programmed to increase to 275 ºC at the rate of 20 ºC/min. The phytosterols of oil sample were identified by comparing with GC chromatogram of plant sterol mixture and their contents were calculated as mg/100 g with 5β-cholestane-3β-ol as an internal standard.

77

Sesame Lignan Analysis. Sesamol, sesamin, and sesamolin in oil sample were analyzed by HPLC following the method of Han *et al.* (*18*). The oil sample (0.2 mL) was injected into a Waters sep-pak C18 cartridge (Waters Co., Milford, MA) activated with methanol and eluted with 5 mL of methanol. The eluate (20 μL) was injected into a Hewlett Packard 1100 series HPLC equipped with a Beckman Ultrasphere C18 ODS column (25 cm × 4.6 mm i.d.; Beckman Coulter Inc., Fullerton, CA) and a UV detector at 288 nm. The isocratic mobile phase was a mixture of methanol and water (70:30, v/v) at a flow rate of 0.8 mL/min. Concentration of each lignan compound in the oil was determined from the calibration curve of standard sesamol and sesamin.

Oil Stability Index (OSI) Analysis. The induction period of oxidation of oil sample was determined according to AOCS official method Cd 12b-92 (*15*) by using oxidative stability instrument (Omnion Inc., Rockland, MA).

Weight Gain. Oil sample (2 ± 0.01 g) was weighed into glass vials and then incubated in a dry oven kept at 60 ± 2 ºC. The weight gain of the oil sample was monitored at 5 days intervals for 40 days.

Volatile Compound Analysis. Thirty grams of oil sample was weighed into a 250 mL Erlenmeyer flask and the top of flask was sealed with a silicon stopper. The flask containing the sample was placed in a water bath maintained at 40 ºC for 1 h. A stainless-steel sorbent tube packed with 100 g 60/80 mesh Tenax TA (Alltech Associates Inc., Deer field, IL) was connected to the top of flask through a silicon tube to absorb the volatile compounds, which were generated from the sample, in the headspace of flask. Purified air was passed into the flask through a column packed with activated charcoal during the extraction of volatile compounds from the sample. Extracted volatile compounds were desorbed at 250 ºC for 5 min using an automated

short-path thermal desorption/cryofocusing system (Model TD-5, Scientific Instrument Services Inc., Ringoes, NJ) that sits directly on the injector area of the GC-MS. An Agilent Technologies 6890N gas chromatograph (Agilent Technologies Inc., Palo Alto, CA), equipped with a fused silica capillary column (HP-5ms, 30 m × 0.25 mm i.d., Agilent Technologies Inc., Palo Alto, CA) and an Agilent Technologies 5973 mass selective detector (Agilent Technologies Inc., Palo Alto, CA) was used to analyze the volatile compounds. Helium gas was used as the carrier gas at a flow rate of 1mL/min. The injection system was split 1:5. The column was initially held at 35 ºC for 1 min, increased to 120 ºC at the rate of 1.5 ºC/min, held at 120 ºC for 1 min, and then increased to 280 ºC at the rate of 20 ºC/min. The injector temperature was maintained at 225 ºC. The volatile compounds were identified by comparing the mass spectral data with the reference in mass spectral libraries, NIST 02 and Wiley 7.

Statistical Analysis. Statistical analysis was conducted with the SAS software package (*19*). One-way analysis of variance (ANOVA) was performed to determine the differences in sesame oil before and after acidolysis to produce SL. When F values for the ANOVA were significant, differences in means was determined using Duncan's multiple range test as a procedure of mean separation ($P < 0.05$).

RESULTS AND DISCUSSION

Lipase Stability. Figure 4.2 shows the changes in total incorporation of CA into the TAG of sesame oil over the elution volume of reaction products during the acidolysis reactions and the regression equation on the relationship between them. Total incorporation of CA in the SL was shown to decrease linearly ($R^2 = 0.9048$) as the elution volume increased, indicating that the activity of lipase decreased as the reaction time increased. The half-life of the lipase was

79

estimated from the regression equation in **Figure 4.2**, as a stability indicator of the lipase under the reaction conditions used in this study. The half-life of an enzyme is defined as the time which it takes for the activity to reduce to half of the initial activity. Therefore, the total incorporation of caprylic acid at the time when the elution volume is zero was designated as the initial activity (i.e., 40.2 mol%). As a result, total incorporation was expected to decrease to 20.1 mol% after 31.8 L of reaction products would have eluted. Because the flow rate of substrates fed to the reactor was 1.15 mL/min, therefore, the half-life of *ca.* 19.2 days could be obtained for this lipase under the SL synthesis conditions used in this study.

FA Composition. FA compositions of sesame oil and SL were given in **Table 4.1**. Total incorporation of CA into TAG of sesame oil was 42.5 mol%. The level of acyl migration (CA found at *sn*-2 position) in the SL was 3.1 mol%. Acyl migration is a major side-reaction occurring during lipase-catalyzed acidolysis and is well known to be affected by several factors: reaction temperature, reaction time, reactor type, and reaction system (*20, 21*). Kim and Akoh (*22*) showed that relatively lower temperature and higher substrate molar ratio (FA/target oil) were required to reduce the acyl migration as well as to minimize the decrease in total incorporation. From the standpoint of reactor type and reaction systems, a continuous packed bed reactor and solvent-free systems are preferred to batch-type reactor and solvent systems (e.g., hexane) for the prevention of acyl migration (*20, 23, 24*). In these respects, we have used higher substrate molar ratio (sesame oil: CA) of 1:6 and lower reaction temperature of 45 °C as the reaction conditions for our current work as compared to the reaction conditions (e.g., 1:5 and 65 °C) of our previous works (*25-27*) to synthesize SLs by acidolysis of several vegetable oils with caprylic acid. When compared with SLs prepared in the previous studies mentioned above, the SL synthesized in the current study showed relatively lower level of acyl migration. Because of

this suppression of acyl migration, two major unsaturated FAs (oleic and linoleic acid) at the *sn*-2 position of original sesame oil remained almost intact at that position in the SL (**Table 4.1**). The FA profile at *sn*-1,3 positions was also obtained by calculation using the equation: *sn*-1,3 (mol%) = [3 × Total (mol%) − *sn*-2 (mol%)] / 2. The result showed that the incorporation of CA into *sn*-1,3 positions, as we intended, by using Lipozyme RM IM (a *sn*-1,3 specific lipase), reached 62.3 mol% (**Table 4.1**).

Therefore, the results above indicate that we successfully produced MLM-type SL, in which *sn*-1,3 positions predominantly consist of CA, medium-chain FAs (MCFAs) and *sn*-2 position mostly comprised of long-chain FAs (LCFAs) originally present in sesame oil.

Physical Properties. The color of SL was compared with that of original sesame oil by measuring CIE color values (**Table 4.2**). Both oils have low *L** (lightness) value and positive *a** (redness) and *b** (yellowness) values with *h*˚ (hue angle) value of over 45˚, meaning that they have dark reddish yellow (i.e., dark brown) colors. However, *a** value of SL was significantly ($P < 0.05$) smaller than that of sesame oil, resulting in significant ($P < 0.05$) decrease in *C* (chroma) value of SL. This result suggests that some reddish pigments might have been removed from sesame oil during SL synthesis or purification steps. We surmise that the short-path distillation step to reduce the FFAs content of SL, also removed some Maillard reaction compounds, which were generated during the roasting step of sesame seeds and responsible for red color of sesame oil.

The viscosities of sesame oil and SL at different temperatures are shown in **Table 4.3**. As the temperature increased, the viscosities of both oils decreased, respectively. SL was significantly ($P < 0.05$) less viscous than sesame oil over the range of temperature investigated.

The lower viscosity of SL is due to the decrease in the molecular weight of SL arising from the replacement of some of the original LCFAs in sesame oil with CA (MCFA).

Melting and crystallization behaviors of sesame oil and SL were evaluated by DSC thermal profiles (**Figure 4.3**). Edible oil is a complex molecular system comprising predominantly of diverse TAG species with a few diacylglycerols (DAGs), monoacylglycerols (MAGs), and FFAs. Therefore, the melting and crystallization of oils containing mixed FAs do not occur at a particular temperature, but over a wide temperature range. **Figure 4.3 (a)** shows the DSC melting curve of sesame oil and SL. Sesame oil had four melting peaks overlapping each other; whereas, SL showed only two melting peaks. The smaller number of melting peaks of SL is shown to be due to the decrease in the diversity of TAG species of SL arising from the replacement of several kinds of LCFA in sesame oil with considerable amount of CA (42.5 mol% from **Table 4.1**). This substitution is also shown to induce the formation of one distinct big melting peak 2 in SL unlike sesame oil with no big difference in the peak sizes. The melting ranges of sesame oil and SL were also compared to each other. First, each melting peak was labeled as 1, 2, 3, and 4 (for sesame oil) and 1 and 2 (for SL), in the order of low to high temperatures, respectively, as shown in **Figure 4.3 (a)**. Then, the melting onset temperature (T_o) of peak 1 and melting completion temperature (T_c) of peak 4 were considered as the temperatures at which the melting starts and ends, respectively, for sesame oil. In a similar manner, T_o of peak 1 and T_c of peak 2 were considered as the temperatures of melting starting and ending, respectively, for SL. **Table 4.4** shows that both sesame oil and SL started to melt at almost the same temperature (*ca.* -35 ºC), but the melting of SL was completed at significantly ($P < 0.05$) lower temperature than that of sesame oil. **Table 4.4** also shows that SL exhibited about 3 times higher overall melting enthalpy (ΔH) than sesame oil, indicating that more energy was necessary

to drive the melting of SL. We concluded that the higher ΔH value of SL is related to the higher saturation degree of SL than that of sesame oil. This is because the most abundant FA in SL is CA (SFA) having linear structure unlike the sesame oil, which is predominantly comprised of USFA having bended structure. TAG molecules were packed closer in SL than sesame oil. This compact arrangement of TAG molecules in SL means larger amount of energy was required for SL melting. The DSC crystallization curves of sesame oil and SL are presented in **Figure 4.3 (b)**. Both oils showed only one crystallization peak. Therefore, the crystallization T_o and T_c of each peak 1 could be considered as the temperatures at which the crystallization starts and ends, respectively, for sesame oil and SL, respectively. **Table 4.4** shows that SL was crystallized (or solidified) at significantly ($P < 0.05$) lower temperature than sesame oil. Both **Figure 4.3 (b)** and **Table 4.4** also show that the solidifying process of SL occurred at narrower temperature range as compared to that of sesame oil which occurred at a much wider temperature range. This narrower temperature range of SL crystallization is also shown to be related to the smaller diversity in TAG species of SL as mentioned above.

  Chemical Properties. Several important chemical characteristics of sesame oil and SL are listed in **Table 4.5**. Original sesame oil contained 0.7% FFA (**Table 4.5**). Roasted sesame oil is known to contain relatively large amount of FFAs as compared to other RBD vegetable oils since the refining steps were absent (*6*). In the acidolysis reaction to produce SL, the purification step to remove the FFAs, is indispensable since large amount of FFAs remain in the SL after the reaction. In our current work, the FFA content of SL was reduced to 0.7%, which was the same level as that of original sesame oil, using short-path distillation technique.

  Table 4.5 also shows that no significant ($P < 0.05$) difference was found in unsaponifiable matter content between sesame oil and SL. This result indicates that the short-

path distillation step removed few unsaponifiable matters from SL. The unsaponifiable matter of sesame oil is known to consist mostly of tocopherols, phytosterols, and sesame lignans (*6*). The effect of short-path distillation step on each unsaponifiable component above is discussed later in this paper.

The smoke points of sesame oil and SL were compared in **Table 4.5**. SL had significantly ($P < 0.05$) higher smoke point (*ca.* 176 ℃) compared to original sesame oil (*ca.* 167 ℃). RBD oils usually have higher smoke points than unrefined oils since pigments and unsaponifiable matters are removed during refining process (*7*). The short-path distillation step in the current study is a kind of physical refining. Therefore, the higher smoke point of SL might be due to the removal of some unsaponifiable matters during the short-path distillation of SL. However, we noted that the unsaponifiable matter content of SL was not significantly ($P < 0.05$) different from original sesame oil as mentioned. On the other hand, it is known that in order for an edible oil to be used as frying oil, its smoke point should be over 170 ℃ (*7, 28*). Therefore, based on smoke point values, this result indicates that the SL may be used as frying oil unlike roasted sesame oil. Because the SL contained a considerable amount of CA, which has pungent odor and unpleasant rancid taste, sensory aspects of SL would also be evaluated before its use as a frying oil.

Saponification value (SV) was measured as an indication of the average molecular weight of oil sample (**Table 4.5**). According to the official standards of sesame oil adopted by Codex Alimentations Commission, sesame oil should have 187-195 SV (*23*). SV of sesame oil used in this study was 193.7 and within the Codex range; whereas, as expected, SL showed SV of 236.5, significantly ($P < 0.05$) higher than that of sesame oil, meaning that the molecular weight of SL is lower than that of sesame oil.

Iodine value (IV) was measured as an indication of the instauration degree of oil sample (**Table 4.5**). Sesame oil IV of 118.0 was within the Codex range of 104-120 (*29*); whereas, SL showed significantly ($P < 0.05$) lower IV (93.2), meaning that the SL was more saturated than sesame oil.

Minor Components. Sesame oil contains a relatively large amount of unsaponifiable matter mostly tocopherols, phytosterols, and sesame lignans as mentioned before (*6*). In this section we examined the change in the content of each minor component between sesame oil and SL and discussed the effect of short-path distillation step, which was our purification process for SL, on the minor components content during the production of SL.

Table 4.6 shows the tocopherol contents of sesame oil and SL. α-Tocopherol was the most abundant in both oils, followed by γ- and δ-tocopherols in that order. β-Tocopherol and tocotrienol isomers were not detected in both oils. No significant differences ($P < 0.05$) were found in the total content of tocopherols between sesame oil and SL. There were significant ($P < 0.05$) differences in the contents of γ- and δ-tocopherols; these differences arose from very high precision (i.e., standard deviation was 0) of our analysis technique and were shown to be negligible in absolute terms. This result indicates that the tocopherol content was not changed during short-path distillation step for SL purification. Tocopherols are known to be retained throughout the refining process of edible oils, although there is slight loss during the deodorization step (*30*). Therefore, our result indicates that short-path distillation also has no effect on the tocopherol content of oil similar to commercial refining process. On the other hand, total tocopherol content of roasted sesame oil is known to be generally in the range of 40 to 70 mg/100g (*5, 6*). In addition, it was reported that the tocopherols found in sesame oil are predominantly γ-tocopherol (96-98%) and α-tocopherol (2-3%) with trace amounts of β- and δ-

tocopherols (*5, 6*). However, our oil samples showed higher total tocopherol content (*ca*. 110 mg/100 g) and the content of α-tocopherol was much higher than γ-tocopherol as compared to other reports above (**Table 4.6**). We assumed that the reason was that extra α-tocopherol may have been added as an antioxidant to the sesame oil by the manufacturer during packaging.

Table 4.6 lists the phytosterol contents of sesame oil and SL. β-Sitosterol was the most abundant in both oils, followed by campesterol and stigmasterol. There were no significant differences ($P < 0.05$) in the content of the phytosterol analogues as well as the total phytosterol content of sesame oil and SL. This result indicates that the phytosterol content was also not affected by short-path distillation process as for tocopherol.

Table 4.6 also shows the contents of representative sesame lignan compounds, such as sesamol, sesamin, and sesamolin of sesame oil and SL. Sesamin was found most abundantly in both oils, followed by sesamolin and sesamol. There were no significant differences ($P < 0.05$) in the content of sesamin and sesamolin between sesame oil and SL. However, SL showed significantly ($P < 0.05$) lower content of sesamol than sesame oil. This result indicates that sesamin and sesamolin were not removed during short-path distillation step; whereas, sesamol was lost under the conditions of short-path distillation process used in our study. Such loss of sesamol can be explained by the fact that sesamol is a relatively lower molecular weight compound (M.W. 138.1) than sesamin (M.W. 354.4) and sesamolin (M.W. 370.3). Namiki (*5*) also mentioned that negligible amounts of sesamol were found in the RBD sesame oil because commercial deodorizing process tends to remove most of sesamol in sesame oil. On the other hand, our roasted sesame oil sample was shown to contain relatively smaller amounts of sesame lignans as compared to other roasted sesame oils used in another published work (*7*). For example, the roasted sesame oil used in our study contained only 22.1 mg of sesamolin in 100 g

86

of oil; whereas, Kim and Choe (*7*) reported that the concentration of sesamolin in their oil sample was *ca.* 200 mg/100 g oil. The sesamin content of our oil sample was also lower than that of their oil sample (75.6 vs. 466.0 mg/100 g). Such smaller sesame lignans content of our oil samples may be related to their relatively decreased oxidative stability, as discussed below, compared to the superior oxidative stability of roasted sesame oil reported in other published works (*5, 6*).

Oxidative Stability. Roasted sesame oil has been known to be very stable to oxidative deterioration compared to other vegetable oils due to the existence of well-known antioxidants, such as sesame lignans and tocopherols, as well as potential and unidentified antioxidants, such as brown color pigments generated by Maillard reaction during the roasting step of sesame seeds (*5*).

The induction period of oxidation in SL, which was measured by traditional weight gain method (**Figure 4.4**) and oil stability index (OSI) (**Table 4.7**), was shorter than that of sesame oil. **Figure 4.4** also showed that SL displayed smaller weight gain than sesame oil after *ca.* 25 days despite the shorter induction period. This could be related to the fact that SL was more saturated and contained MCFAs than sesame oil as indicated in **Table 4.1** and **Table 4.5**. These results indicate that the oxidative stability of SL was lower than that of sesame oil. We believe that such lower oxidative stability of SL, when compared to original sesame oil, arose from two possibilities: First, the loss of sesamol during short-path distillation process, as mentioned above, might decrease the oxidative stability of SL even though some researchers questioned the antioxidative activity of sesamol and reported that other kinds of sesame lignan compounds, such as sesamolinol and sesaminol were more efficient antioxidants than sesamol in sesame oil (*5*). Secondly, we may have removed some reddish pigments which may have antioxidative activity

during short-path distillation process as seen from the results of color measurement (**Table 4.2**). On the other hand, our roasted sesame oil had shorter OSI induction period of 3.2 h at 120 ºC than 6.0 h of Codex standard at the same temperature (*29*) (**Table 4.7**). This result is also due to the lower levels of sesame lignans content in our oil sample compared to those in other researchers' oil sample (*7*).

Volatile Compounds. Roasted sesame oil is used widely in Eastern Asian countries as important flavoring agents due to its characteristic roasted flavor (*6, 7*). The characteristic roasted flavor of the oil is mainly developed by thermochemical reactions, such as Maillard reactions, during the roasting step of sesame seed at 180-200 ºC, before expelling it from the seed (*5*). So far about 220 kinds of volatile compounds were identified in the oil. These compounds include: pyrazines, furans, other nitrogen-containing compounds, carbonyl compounds (aldehydes and ketones), alcohols, lactones, esters, acids, hydrocarbons, and sulfur-containing compounds. Among these compounds, some pyrazines, which are generated by Maillard reactions, and some furan compounds are believed to contribute to the representative roasted flavors of sesame oil (*5, 31*). Apparently, no single key compound responsible for the characteristic roasted flavor has been found yet (*5*).

Table 4.8 lists 70 volatile compounds identified from our sesame oil: 11 pyrazines, 5 furans, 10 other nitrogen-containing compounds, 13 aldehydes and ketones, 6 alcohols, 1 acid, 15 hydrocarbons, 2 phenols, and 7 sulfur-containing compounds. Most of these compounds including pyrazines and furans were not found in SL except for a few compounds, such as aldehydes, ketones, alcohols, etc., which are probably not critical contributors to the characteristic flavor of sesame oil (**Table 4.8**). Moreover, **Figure 4.5** shows that the content of volatile compounds in SL was negligible compared to that of sesame oil. These results indicate

that as expected, most volatile compounds were removed from SL during short-path distillation step, resulting in the loss of characteristic sesame oil flavor in SL. Therefore, we suggest that SL would not be able to substitute for original sesame oil in dishes that require the characteristic flavor of sesame oil. However, the flavorless character of SL might make it easy to be used as an ingredient for incorporation into other food products.

In conclusion, the characteristics of roasted sesame oil-based SL, which was produced in a bench-scale continuous packed bed reactor, were different from those of original sesame oil in many aspects, such as physical and chemical properties, the composition of several components (FAs, sesame lignans, volatile compounds), and oxidative stability except for the contents of some components (tocopherols, phytosterols).

LITERATURE CITED

(1) Akoh, C. C. Structured lipids. In *Food Lipids–Chemistry, Nutrition, and Biotechnology*, 2nd ed.; Akoh, C. C., Min, D. B., Eds.; Marcel Dekker: New York, NY, 2002; pp 877-908.

(2) Xu, X. Enzymatic production of structured lipids: Process reaction and acyl migration. *inform* **2000**, *11,* 1121-1131.

(3) Torres, C. F.; Munir, F.; Lessard, L. P.; Hill, C. G. Lipase-mediated acidolysis of tristearin with CLA in a packed-bed reactor: A kinetic study. *J. Am. Oil Chem. Soc.* **2002**, *79*, 655-661.

(4) Mu, H.; Xu, X.; Høy, C. E. Production of specific-structured triacylglycerols by lipase-catalyzed interesterification in a laboratory-scale continuous reactor. *J. Am. Oil Chem. Soc.* **1998**, *75,*1187-1193.

(5) Namiki, M. The chemistry and physiological functions of sesame. *Food Rev. Int.* **1995**, *11,* 281-329.

(6) Kochhar, S. P. Sesame, rice-bran and flaxseed oils. In *Vegetable Oils in Food Technology – Composition, Properties and Uses*, 1st ed.; Gunstone, F. D., Eds.; Blackwell: Oxford, UK, 2002; pp 297-322.

(7) Kim, I.; Choe, E. Effects of bleaching on the properties of roasted sesame oil. *J. Food Sci.* **2005**, *70*, 48-52.

(8) Sahin, N.; Akoh, C. C.; Karaali, A. Lipase-catalyzed acidolysis of tripalmitin with hazelnut oil fatty acids and stearic acid to produce human milk fat substitutes. *J. Agric. Food Chem.* 2005, *53*, 5779-5783.

(9) Lee, J. H.; Shin, J. A.; Lee, J. R.; Lee, K. T. Production of lipase-catalyzed structured lipids from safflower oil with conjugated linoleic acid and oxidation studies with rosemary extracts. *Food Res. Int.* **2004**, *37*, 967-974.

(10) Timm-Heinrich, M.; Xu, X. B.; Nielsen, N. S.; Jacobsen, C. Oxidative stability of structured lipids produced from sunflower oil and caprylic acid. *Eur. J. Lipid Sci. Tech.* 2003, *105*, 436-448.

(11) Xu, X. B.; Fomuso, L. B.; Akoh, C. C. Synthesis of structured triacylglycerols by lipase-catalyzed acidolysis in a packed bed bioreactor. *J. Agric. Food Chem.* **2000**, *48*, 3-10.

(12) Jennings, B. H.; Akoh, C. C. Lipase-catalyzed modification of rice bran oil to incorporate capric acid. *J. Agric. Food Chem.* **2000**, *48*, 4439-4443.

(13) Khare, S. K.; Nakajima, M. Immobilization of *Rhizopus japonicus* lipase on celite and its application for enrichment of docosahexaenoic acid in soybean oil. *Food Chem.* **2000**, *68*, 153-157.

(14) Luddy, F. E.; Barford, R. A.; Herb, S. F.; Magidman, P.; Riemenschneider, R. W. Pancreatic lipase hydrolysis of triglycerides by a semimicro technique. *J. Am. Oil Chem. Soc.* **1964**, *41*, 693-696.

(15) AOCS. *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 5th ed.; American Oil Chemists' Society: Champaign, IL, 1998, Ca 5a-40; Ca 6a-40; Cj 1-94; Cc 9a-48; Cd 3-25; Cd 1-25; Cd 12b-92.

(16) Ye, L.; Landen, W. O. Jr.; Lee, J.; Eitenmiller, R. R. Vitamin E content of margarine and reduced fat products using a simplified extraction procedure and HPLC determination. *J. Liquid Chrom. Related Tech.* **1998**, *21,* 1227-1238.

(17) Jekel, A. A.; Vaessen, H. A. M. G.; Schothorst, R. C. Capillary gas-chromatographic method for determining non-derivatized sterol–Some results for duplicate 24 h diet samples collected in 1994. *Fresenius J. Anal. Chem.* **1998,** *360,* 595-600.

(18) Han, J. S.; Moon, S. Y.; Ahn, S. Y. Effects of oil refining process on oxidative stability and antioxidative substrances of sesame oil. *Korean J. Food Sci. Technol.* **1997**, *29*, 15-20.

(19) SAS Institute. SAS Statistics Software, release 8.2; SAS Institute: Cary, NC, 2000.

(20) Xu, X.; Skands, A. R. H.; Høy, C. E.; Mu, H.; Balchen, S.; Alder-Nissen, J. Production of specific-structured lipids by enzymatic interesterification: Elucidation of acyl migration by response surface design. *J. Am. Oil Chem. Soc.* **1998**, *75,* 1179-1186.

(21) Xu, X.; Balchen, S.; Høy, C. E.; Alder-Nissen, J. Production of specific-structured lipids by enzymatic interesterification in a pilot continuous enzyme bed reactor. *J. Am. Oil Chem. Soc.* **1998**, *75,* 1573-1579.

(22) Kim, B. H.; Akoh, C. C. Modeling of lipase-catalyzed acidolysis of sesame oil and caprylic acid by response surface methodology: Optimization of reaction conditions by considering both acyl incorporation and migration. *J. Agric. Food Chem.* **2005,** *53*, 8033-8037.

(23) Torres C. F.; Munir, F.; Lessard, L. P.; Hill, C. G. Lipase-mediated acidolysis of tristearin with CLA in a packed-bed reactor: A kinetic study. *J. Am. Oil Chem. Soc.* **2002**, *79,* 655-661.

(24) Goh, S. H.; Yeong, S. K.; Wang, C. W. Transesterification of cocoa butter by fungal lipases: Effect of solvent on 1,3-specificity. *J. Am. Oil Chem. Soc.* **1993,** *70,* 567-570.

(25) Fomuso, L. B.; Akoh, C. C. Lipase-catalyzed acidolysis of olive oil and caprylic acid in a bench-scale packed bed bioreactor. *Food Res. Int.* **2002**, *35*, 15-21.

(26) Kim, B. H.; Akoh, C. C. Chemical and physical properties of butterfat-vegetable oil blend spread prepared with enzymatically transesterified canola oil and caprylic acid. *J. Agric. Food Chem.* **2005**, *53,* 4954-4961.

(27) Lai, O. M.; Low, C. T.; Akoh, C. C. Lipase-catalyzed acidolysis of palm olein and caprylic acid in a continuous bench-scale packed bed bioreactor. *Food Chem.* **2005**, *92,* 527-533.

(28) Onal, B.; Ergin, G. Antioxidative effects of alpha-tocopherol and ascorbyl palmitate on thermal oxidation of canola oil. *Nahrung* **2002**, *46,* 420-426.

(29) Codex Alimentarius Commission, Codex standard for named vegetable oils; Codex-Stan 210 (Adopted 1999; Amended 2003), http://www.codexalimentarius.net/web/index_en.jsp (accessed Aug. 2005).

(30) Reische, D. W.; Lillard, D. A.; Eitenmiller, R. R. Antioxidants. In *Food Lipids–Chemistry, Nutrition, and Biotechnology*, 2nd ed.; Akoh, C.C., Min D.B., Eds.; Marcel Dekker: New York, NY, 2002; pp 489-516.

(31) Lee, Y. G.; Lim, S. U.; Kim, J. O. Influence of roasting conditions on the flavor quality of sesame seed oil. *Korean Agric. Chem. Soc.* **1993**, *36,* 407-415.

Table 4.1. Fatty Acid Composition of Total, sn-2, and sn-1,3 Positions of TAG of Sesame Oil and SL (mol%)[a]

FA	Total		sn-2		sn-1,3[b]	
	Sesame oil	SL[c]	Sesame oil	SL	Sesame oil	SL
C8:0	-	42.5 ± 0.1	-	3.1 ± 0.1	-	62.3 ± 0.2
C12:0	trace[d]	-	-	-	trace	-
C14:0	trace	-	-	-	trace	-
C16:0	10.0 ± 0.0	2.7 ± 0.0	1.1 ± 0.0	1.2 ± 0.0	14.4 ± 0.0	3.5 ± 0.0
C16:1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
C18:0	3.6 ± 0.0	1.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	5.3 ± 0.0	1.6 ± 0.0
C18:1n-9	37.0 ± 0.0	21.8 ± 0.1	40.2 ± 0.6	39.1 ± 0.1	35.4 ± 0.3	13.1 ± 0.1
C18:2n-6	48.4 ± 0.0	31.1 ± 0.0	57.3 ± 0.7	55.2 ± 0.0	44.0 ± 0.3	19.0 ± 0.0
C18:3n-3	0.9 ± 0.0	0.6 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.8 ± 0.0	0.4 ± 0.0
SFA[e]	13.6 ± 0.0	46.5 ± 0.1	1.5 ± 0.1	4.6 ± 0.1	19.7 ± 0.0	67.4 ± 0.2
USFA[f]	86.4 ± 0.0	53.5 ± 0.1	98.5 ± 0.1	95.4 ± 0.1	80.3 ± 0.0	32.6 ± 0.2

[a] Mean ± SD, n = 2. [b] sn-1,3 (mol%) = [3 × Total (mol%) − sn-2 (mol%)] / 2 [c] Structured lipid prepared by acidolysis of sesame oil with caprylic acid in a bench-scale continuous packed bed reactor. [d] < 0.05 mol%. [e] Saturated FA. [f] Unsaturated FA.

94

Table 4.2. CIE $L^*a^*b^*$ Color of Sesame Oil and SL[a]

Parameter	Sesame oil	SL[b]
L^*	24.27 ± 0.08A	24.31 ± 0.04A
a^*	0.54 ± 0.03A	0.28 ± 0.02B
b^*	1.90 ± 0.06A	1.79 ± 0.04A
C	1.97 ± 0.07A	1.81 ± 0.04B
$h°$	74.27 ± 0.68B	81.30 ± 0.85A

[a] Mean ± SD, n = 3; Means with the same letter in the same row are not significantly different ($P < 0.05$). [b]

Structured lipid prepared by acidolysis of sesame oil with caprylic acid in a bench-scale continuous packed

bed reactor.

Table 4.3. Viscosity of Sesame Oil and SL (mPa·s)[a]

Temperature (°C)	Sesame oil	SL[b]
5	110.4 ± 0.1A	105.6 ± 0.1B
15	76.0 ± 0.2A	73.6 ± 0.2B
25	54.5 ± 0.1A	53.0 ± 0.2B
35	40.1 ± 0.1A	36.5 ± 0.2B

[a] Mean ± SD, n = 3; Means with the same letter in the same row are not significantly different ($P < 0.05$). [b] Structured lipid prepared by acidolysis of sesame oil with caprylic acid in a bench-scale continuous packed bed reactor.

Table 4.4. Comparison of DSC Melting and Crystallization Properties of Sesame Oil and SL[a]

Property	Sesame oil	SL[b]
Melting		
T_o (°C)[c]	-35.2 ± 1.8A	-34.9 ± 0.4A
T_c (°C)[d]	-5.4 ± 0.3A	-18.6 ± 0.5B
ΔH (J/g)[e]	21.4 ± 2.1B	66.1 ± 1.1A
Crystallization		
T_o (°C)[f]	-10.7 ± 0.1A	-36.7 ± 0.4B
T_c (°C)[g]	-30.6 ± 2.1A	-40.5 ± 0.5B
ΔH (J/g)[h]	-10.2 ± 1.9A	-49.1 ± 1.1B

[a] Mean ± SD, n = 3; Means with the same letter in the same row are not significantly different ($P < 0.05$). [b] Structured lipid prepared by acidolysis of sesame oil with caprylic acid in a bench-scale continuous packed bed reactor. [c] Melting onset temperature; melting onset temperature of peak 1 was used for sesame oil and melting onset temperature of peak 1 was used for SL as shown in Figure 4.3 (a). [d] Melting completion temperature; melting completion temperature of peak 4 was used for sesame oil and melting completion temperature of peak 2 was used for SL as shown in Figure 4.3 (a). [e] Melting enthalpy; overall melting enthalpy of peaks 1, 2, 3, and 4 were used for sesame oil and overall melting enthalpy of peaks 1 and 2 were used for SL as shown in Figure 4.3 (a). [f] Crystallization onset temperature. [g] Crystallization completion temperature. [h] Crystallization enthalpy.

Table 4.5. Chemical Properties of Sesame Oil and SL[a]

Property	Sesame oil	SL[b]
Free fatty acid (%, oleic acid)	$0.7 \pm 0.0A$	$0.7 \pm 0.0A$
Unsaponifiable matter (%)	$1.2 \pm 0.3A$	$1.1 \pm 0.1A$
Smoke point (ºC)	$167.3 \pm 1.2B$	$176.3 \pm 4.0A$
Saponification value	$193.7 \pm 2.5B$	$236.5 \pm 0.1A$
Iodine value	$118.0 \pm 2.1A$	$93.2 \pm 0.9B$

[a] Mean $\pm$ SD, n = 2 (for smoke point, n = 3); Means with the same letter in the same row are not significantly different ($P < 0.05$). [b] Structured lipid prepared by acidolysis of sesame oil with caprylic acid in a bench-scale continuous packed bed reactor.

Table 4.6. Tocopherol, Phytosterol, and Sesame Lignan Contents of Sesame Oil and SL (mg/100 g)[a]

Component	Sesame oil	SL[b]
Tocopherol		
Alpha	93.7 ± 2.5A	93.0 ± 3.5A
Beta	-	-
Gamma	15.8 ± 0.0A	15.3 ± 0.0B
Delta	0.6 ± 0.0A	0.5 ± 0.0B
Total	110.1 ± 2.5A	108.8 ± 3.5A
Phytosterol		
Brassicasterol	9.4 ± 0.5A	9.6 ± 0.2A
Campesterol	65.6 ± 5.4A	74.6 ± 5.8A
Stigmasterol	25.1 ± 2.2A	28.2 ± 2.4A
β-Sitosterol	260.5 ± 18.6A	293.1 ± 25.7A
Δ^5-Avenasterol	20.3 ± 1.6A	24.8 ± 1.3A
Δ^7-Stigmasterol	5.0 ± 0.2A	5.2 ± 0.6A
Δ^7-Avenasterol	9.3 ± 1.1A	8.6 ± 0.2A
Total	395.2 ± 27.3A	440.0 ± 34.3A
Sesame lignan		
Sesamol	18.7 ± 0.8A	2.6 ± 0.1B
Sesamin	75.6 ± 4.3A	75.9 ± 5.1A
Sesamolin	22.1 ± 1.3A	21.7 ± 1.3A
Total	118.7 ± 4.1A	100.1 ± 6.2B

[a] Mean $\pm$ SD, n = 3 (for tocopherol, n = 2); Means with the same letter in the same row are not significantly different ($P < 0.05$). [b] Structured lipid prepared by acidolysis of sesame oil with caprylic acid in a bench-scale continuous packed bed reactor.

Table 4.7. Oil Stability Index of Sesame Oil and SL[a]

	Oil Stability Index (h, at 120 ºC)
Sesame oil	3.2 ± 0.1A
SL[b]	2.4 ± 0.0B

[a] Mean $\pm$ SD, n = 3; Means with the same letter in the same column are not significantly different ($P < 0.05$).

[b] Structured lipid prepared by acidolysis of sesame oil with caprylic acid in a bench-scale continuous packed bed reactor.

Table 4.8. Volatile Compounds of Sesame Oil

Pyrazines
  2-Methylpyrazine
  2-Isopropylpyrazine
  2-Ethyl-5(or 6)-methylpyrazine
  Trimethylpyrazine
  2-Propylpyrazine
  Acetylpyrazine
  2-Isopropenylpyrazine
  3-Ethyl-2,5-dimethylpyrazine
  2-Acetyl-5-methylpyrazine
  2-Acetyl-6-methylpyrazine
  5H-5-Methyl-6,7-dihydrocyclopentapyrazine
Furans
  2-Furfural
  5-Methyl-2-furfural*
  Methyl 2-furoate
  2-Pentylfuran
  2-Furylmethyl acetate
Nitrogen-containing compounds
  2-Methylpyridine
  2-Ethylpyridine
  4,6-Dimethylpyrimidine
  Trimethyloxazole
  N,N-Dimethylethanamide
  3,4-Dimethylpyridine
  2,3(or 4)-Dimethylpyridine
  3-Ethylpyridine
  2-Hexylpyridine
  Indole
Aldehydes and ketones
  Hexanal*
  2-Hexenal*
  Heptanal*
  2-Heptenal
  Benzaldehyde*
  2-Octenal*
  Nonanal*
  2-Nonenal*
  2,4-Decadienal
  2-Heptanone

Aldehydes and ketones (continued)
  2-Octanone*
  Acetophenone
  2-Nonanone
Alcohols
  Pentanol
  2(or 3)-Furanmethanol
  Hexanol*
  1-Octen-3-ol*
  2-Ethyl-1-hexanol*
  3,5-Octadien-2-ol
Acids
  Octanoic acid*
Hydrocarbons
  Undecane*
  Ethylbenzene
  Xylene
  Styrene
  1,3-Dimethylbenzene
  Propylbenzene
  1,2,3(or 4)-Trimethylbenzene
  Limonene
  3-Ethyl-2-methyl-1,3-hexadiene
  3,7-Dimethyl-1,3,6-octatriene
  Butylbenzene
  Pentylbenzene
  Dodecane
  Tridecane
  Tetradecane
Phenols
  2-Methoxyphenol*
  2-Methoxy-4-vinylphenol
Sulfur-containing compounds
  4(or 5)-Methylthiazole
  4-Methylthiazole
  2,4(or 5)-Dimethylthiazole
  5-Ethylthiazole
  4,5-Dimethylthiazole
  2-Acetyl-4-methylthiazole
  Benzothiazole*

* Volatile compound also found in structured lipid.

Figure 4.1. Cross section of the bench-scale continuous packed bed reactor used in our study.
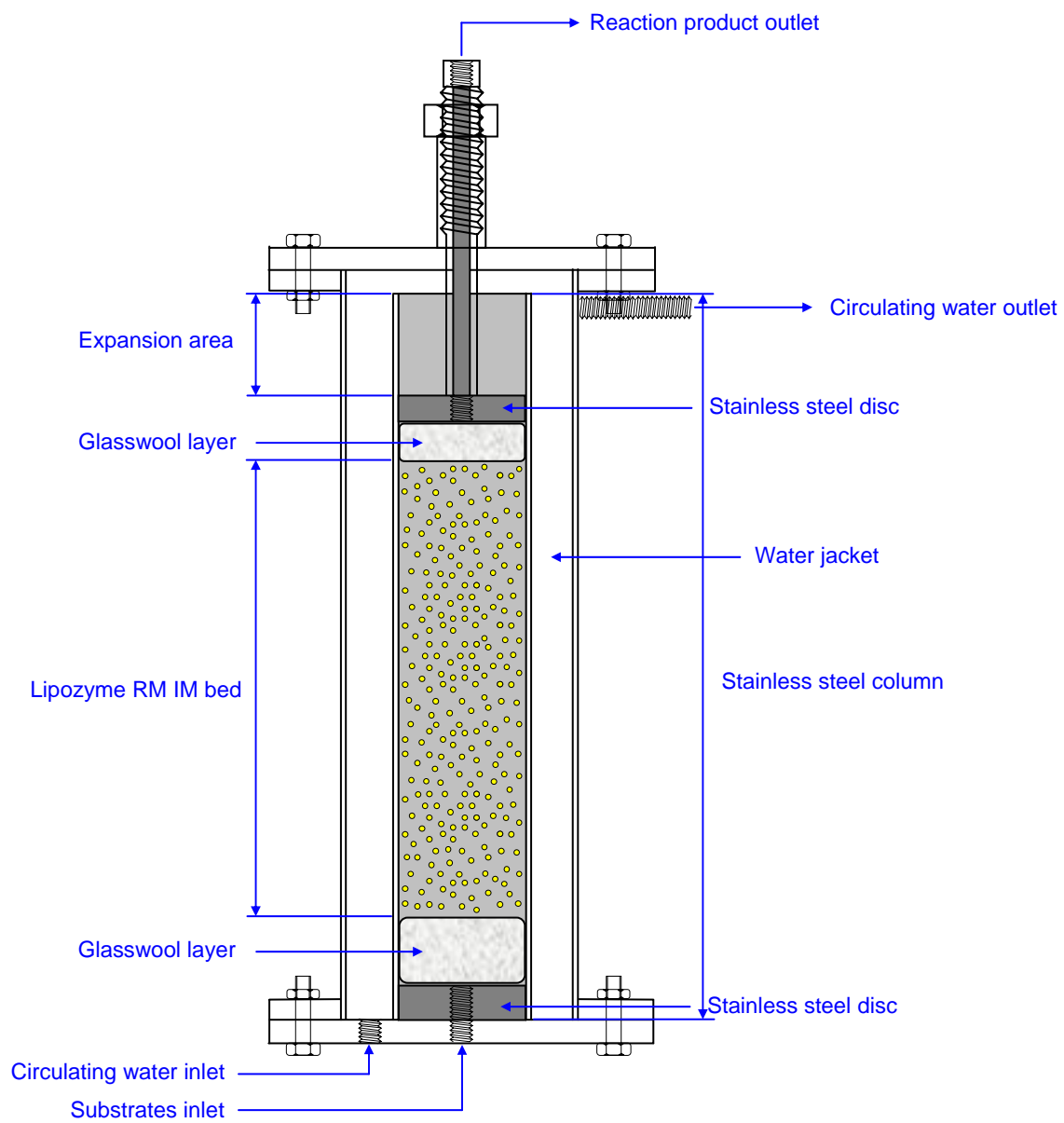
Reaction product outlet

Circulating water outlet

Expansion area

Stainless steel disc

Glasswool layer

Water jacket

Lipozyme RM IM bed

Stainless steel column

Glasswool layer

Stainless steel disc

Circulating water inlet

Substrates inlet

Figure 4.2. Changes in activity of Lipozyme RM IM over elution volume of reaction products during acidolysis of sesame oil with caprylic acid in a bench-scale continuous packed bed reactor.

y = -0.6313x + 40.175
$R^2 = 0.9048$
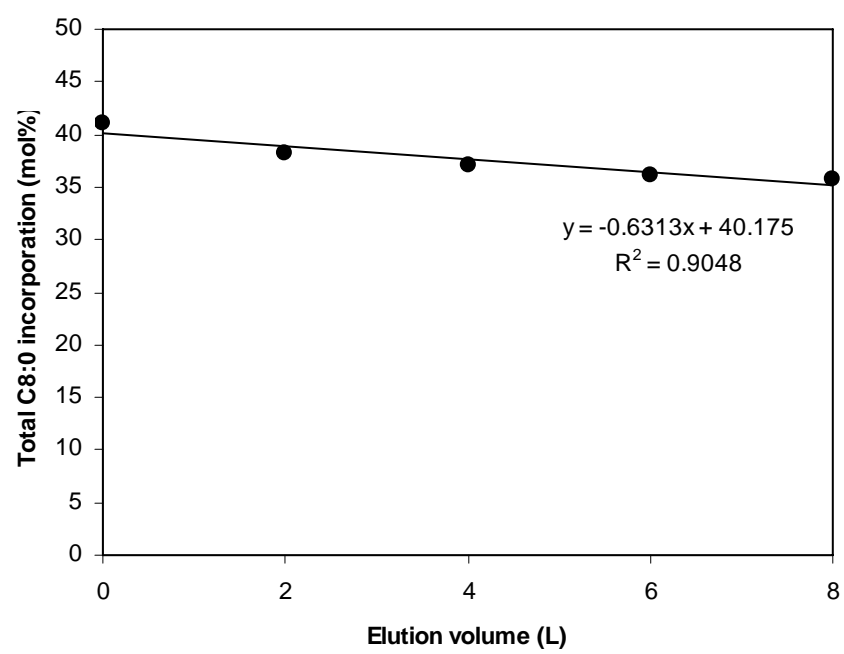
Figure 4.3. DSC melting (**A**) and crystallization (**B**) profiles of sesame oil and SL; SL indicates the structured lipid prepared by acidolysis of sesame oil with caprylic acid in a bench-scale continuous packed bed reactor.
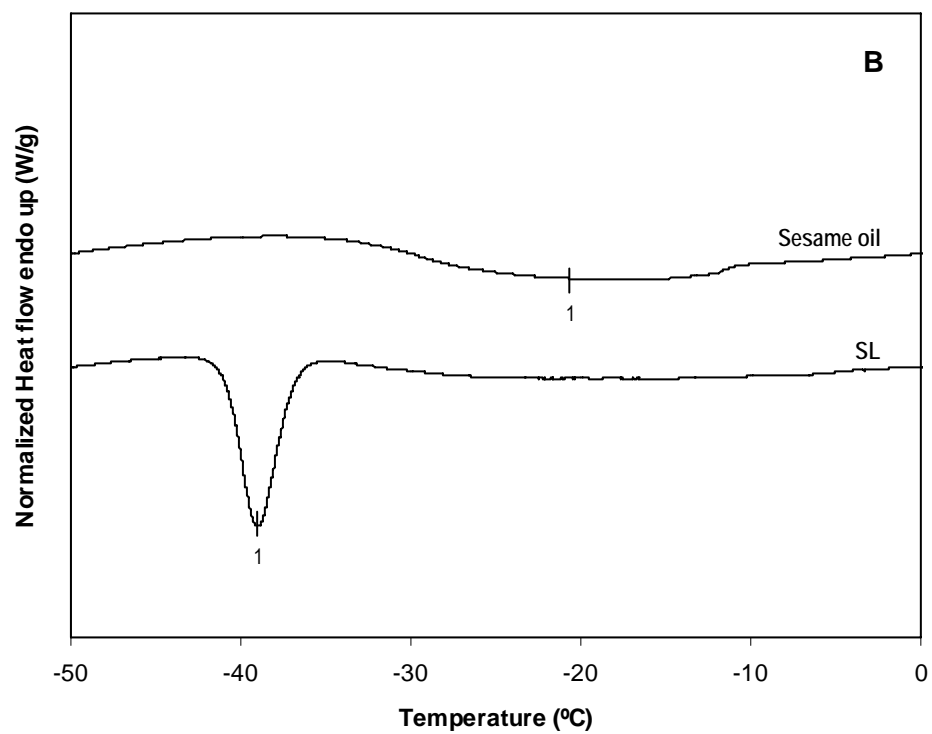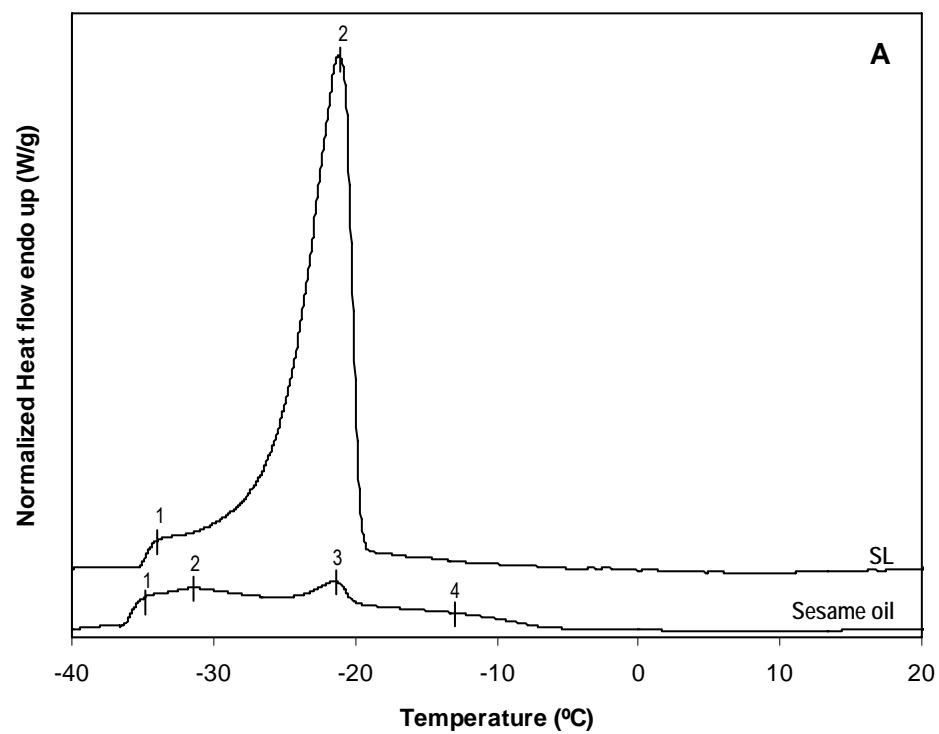
107

Figure 4.4. Oxidative stability of sesame oil and SL (weight gain method); SL indicates the structured lipid

prepared by acidolysis of sesame oil with caprylic acid in a bench-scale continuous packed bed reactor.
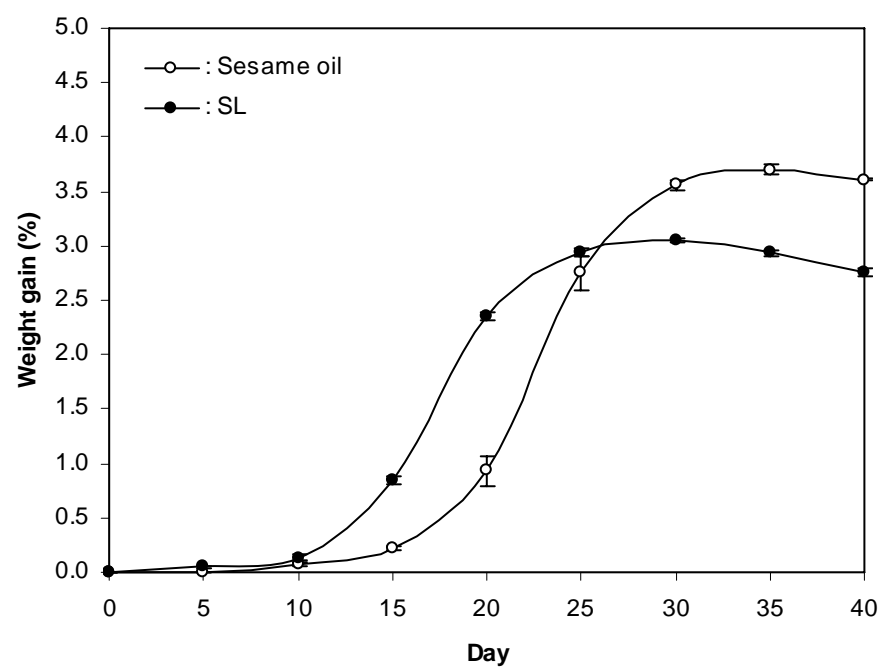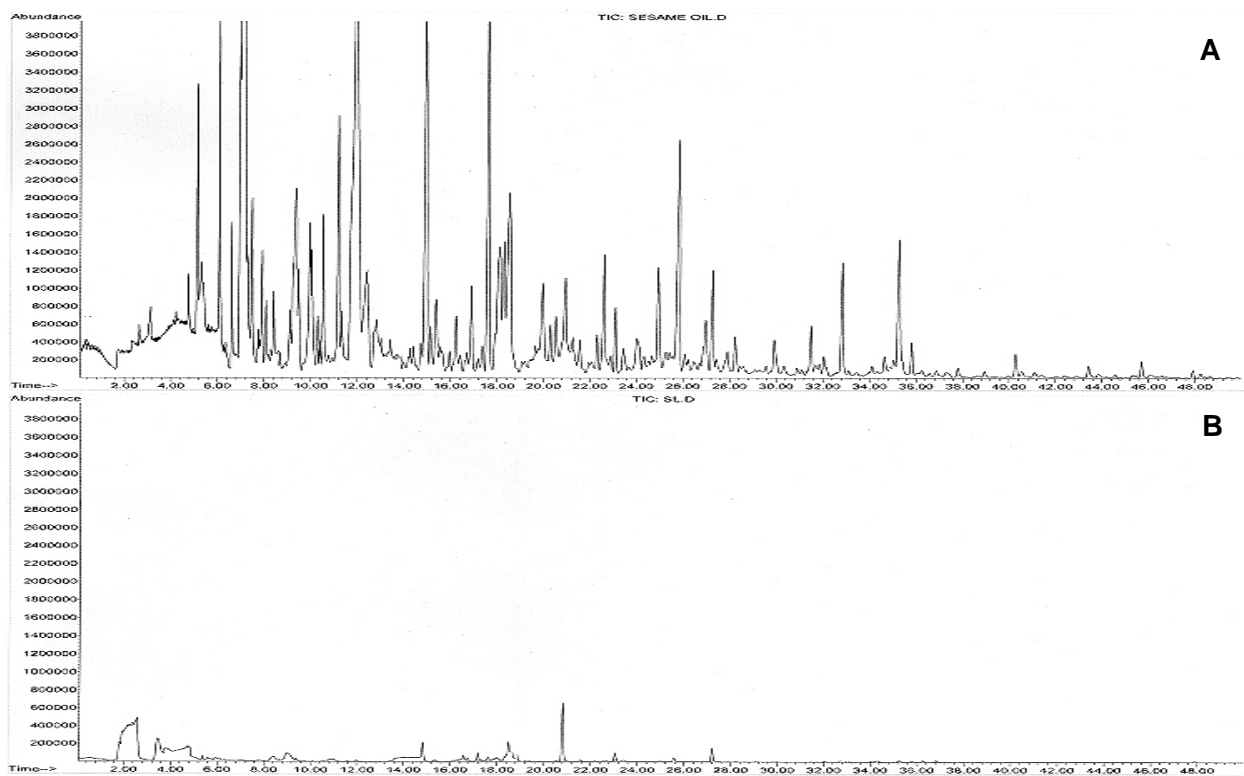
Figure 4.5. Total ion chromatograms of volatile compounds of sesame oil (A) and SL (B); SL indicates the structured lipid prepared by acidolysis of sesame oil with caprylic acid in a bench-scale continuous packed bed reactor.

TIC: SESAME OIL.D

TIC: SL.D

111

CHAPTER 5

MODELING AND OPTIMIZATION OF LIPASE-CATALYZED SYNTHESIS OF

PHYTOSTERYL ESTERS OF OLEIC ACID BY RESPONSE SURFACE METHODOLOGY [1]

Abstract

Enzymatic esterification of phytosterols with oleic acid to produce phytosteryl esters was performed in hexane. Response surface methodology was used to model the reaction. *Candida rugosa* lipase was the biocatalyst for the reaction. The reaction factors investigated were temperature (*Te* = 35-55 ℃), reaction time (*t* = 4-24 h), substrate molar ratio (*Sr* = 1-3, oleic acid:phytosterols), and enzyme amount (*En* = 2-10%). Well-fitting quadratic polynomial regression model for degree of esterification (*DE*) was established after regression analysis with backward elimination and verified by a χ^2 test. All factors investigated positively affected *DE*, with *t* having the greatest effect followed by *En*, *Sr*, and *Te*. The quadratic terms of *t*, *Sr*, and *En* showed negative effects on *DE*, whereas, that of *Te* had no effect on *DE*. Optimal reaction conditions were: *Te*, 51.3 ℃; *t*, 17.0 h; *Sr*, 2.1; *En*, 7.2% and *DE* was 97.0 mol% under these conditions.

Keywords: Candida rugosa lipase; esterification; phytosterols; phytosteryl esters of fatty acids; response surface methodology

1. Introduction

Phytosterols are sterols derived from plant sources, such as vegetable oils and cereals and have similar structure with animal tissue sterol, cholesterol. However, phytosterols are known to have a hypocholesterolemic effect by lowering plasma total and low density lipoprotein (LDL) cholesterol levels without affecting plasma high density lipoprotein (HDL) cholesterol concentration (Beveridge, Haust, & Connel, 1964; Lees, Mok, Lees, McCluskey, & Grundy, 1977; Pollak, 1953; Wester, 2000). It is generally believed that plasma total and LDL cholesterol levels can be reduced up to 10-20% with a dose of 1.5-3.0 g phytosterols/day in humans (Hendriks, Weststrate, van Vliet, & Meijer, 1999; Jones, MacDougall, Ntanios, & Vanstone, 1997; Katan, Grundy, Jones, Law, Miettinen, & Paoletti, 2003; Ling & Jones, 1995; Miettinen & Gylling, 2004; Miettinen, Puska, Gylling, Vanhanen, & Vartiainen, 1995; Nestel, Cehun, Pomeroy, Abbey, & Weldon, 2001; Noakes, Clifton, Ntanios, Shrapnel, Record, & McInerney, 2002; Weststrate & Meijer, 1998).

Because of such health beneficial attributes of phytosterols, their application as a dietary supplement has been recently attempted in various types of food products such as margarine, spread, salad dressing, edible oil, and milk. However, phytosterols have limitations in usage as a dietary supplement since they possess very low solubility in edible oil and have very high melting point. Therefore, to overcome such problems of free forms of phytosterols, phytosteryl esters (i.e., fatty acid (FA) ester forms of phytosterols), are preferred in food formulations. Since phytosteryl esters have much greater solubility in oils and much lower melting point as compared to the corresponding phytosterols, they can be easily incorporated into a wide variety of diets and fat-based food products and provide an easy means of intake of the daily amount of phytosterol

needed for sufficient reduction of cholesterol absorption without changing the taste of the final product. Moreover, many studies in recent years have shown that phytosteryl esters can also effectively reduce plasma total and LDL cholesterol levels in a similar manner as phytosterols (Jones, et al., 2003; Law, 2000; Neil, Meijer, & Roe, 2001; Weststrate, et al., 1998).

Phytosteryl esters of FAs are presently synthesized by chemical esterification and transesterification. However, the chemical method involves some problems such as the formation of side products (e.g., dehydrated or oxysterols) and staining (Negishi, Hidaka, Takahashi, & Kunita, 2003; Villeneuve, et al., 2005). Hence, in recent years, several enzymatic procedures using lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) obtained from diverse kinds of microbial sources for the preparation of phytosteryl esters of FAs have been developed to overcome such shortcomings of chemical method (Negishi, et al., 2003; Shimada, et al., 1999; Villeneuve, et al., 2005; Vu, Shin, Lim, & Lee, 2004; Weber, Weitkamp, & Mukherjee, 2002; Weber, Weitkamp, & Mukherjee, 2001). The lipase-catalyzed esterification and transesterification were carried out successfully in monophasic media (organic solvents) (Villeneuve, et al., 2005; Vu, et al., 2004) or in multiphasic media (oil/water two-phase) (Shimada, et al., 1999), and furthermore in oil itself (Negishi, et al., 2003; Weber et al., 2002; Weber, et al., 2001), with/without removing water, which is generated during the esterification between FAs and phytosterols, by the use of water-trapping agents (e.g., KOH pellet and molecular sieve) or reduced pressure conditions. However, for future industrial scale enzymatic production of phytosteryl esters of FAs, it would be beneficial to simplify the reaction conditions as much as possible. In our current study, we successfully performed small scale synthesis of phytosteryl esters of FAs in high yields without removal of water generated during the reactions. The synthesis with free phytosterols and FAs was carried out in hexane under mild reaction

115

conditions (low temperature ~55 ℃ and short reaction time ~24 h). So far there are few studies on elucidating the effect of several reaction parameters on phytosteryl ester synthesis as well as modeling the reactions catalyzed by lipases.

The objective of our study was to model the lipase-catalyzed esterification reaction between phytosterols and oleic acid to produce phytosteryl esters of oleic acid and to optimize the reaction conditions. The effects of four reaction parameters (temperature, reaction time, substrate molar ratio, and enzyme amount) on the degree of esterification were evaluated, and quadratic polynomial model equations for the degree of esterification were also established by response surface methodology (RSM), and then the optimal reaction conditions were proposed.

2. Materials and methods

2.1. Materials

β-Sitosterol (purity > 40%) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The β-sitosterol was analyzed and found to contain three major phytosterol analogues (β-sitosterol 42.7 mol%, campesterol 27.1 mol%, dihydrobrassicasterol 25.2 mol%; total, 95.0 mol%) by GC in our laboratory. Oleic acid (C18:1, purity > 99%) was also purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Powdered *Candida rugosa* lipase (CRL) type VII (EC 3.1.1.3) was the product (Cat. No.: L 1754) of Sigma-Aldrich Co. (St. Louis, MO, USA). n-Hexane and anhydrous diethyl ether were purchased from J.T. Baker (Philipsburg, NJ, USA). Plant sterol mixture and Corowise^{TM} phytosteryl esters were products of Matreya Inc. (Pleasant

116

Gap, PA, USA) and Cargill Inc. (Minneapolis, MN, USA), respectively. All other reagents used

were of analytical grades and purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Experimental design for RSM

Factors considered important were temperature (Te = 35-55 °C), reaction time (t = 4-24

h), substrate molar ratio; i.e., oleic acid to total phytosterols molar ratio (Sr = 1-3), and enzyme

amount; i.e., weight percent of total substrates (En = 2-10%). RSM was used to optimize reaction

parameters. Central composite design (CCD) was adopted in this study. CCD is a 2^k factorial

design with star points and center points. Twenty seven experimental settings consisting of 8 star

points (star distance is 0) and 3 center points were generated with 4 factors and 3 levels by the

principle of RSM using commercial software, *Modde* 5.0 (Umetrics, Umeå, Sweden). The

quadratic polynomial regression model was assumed for predicting Y variable (DE = degree of

esterification of phytosterols with oleic acid). The model proposed for the response of Y fitted

equation (1) as follows:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \tag{1}$$

Where, Y is response variable (DE, mol%). β_0, β_i, β_{ii}, and β_{ij} are constant coefficients of intercept,

linear, quadratic and interaction terms, respectively, and X_i and X_j are independent variables (Te,

t, Sr, and En).

2.3. Lipase-catalyzed esterification

Fifty milligrams of phytosterols was mixed with different milligrams of oleic acid corresponding to the different substrate molar ratios generated by RSM, in screw-capped test tubes. Different amounts of lipase, which were generated by RSM, and then 1.5 ml of n-hexane were subsequently added. The reaction was performed in an orbital shaking water bath at 200 rpm at different temperatures and for different time periods generated by RSM, as indicated in Table 5.1. The reaction was stopped by cooling in running cold water, and then 3 ml of anhydrous diethyl ether was added. The mixtures were vortexed for 30 sec, centrifuged at 2000 rpm for 3 min, and then the solvent containing reactants were passed through sodium sulfate column to remove the lipase and water generated during reaction. Diethyl ether (4 ml) was added two more times to recover the reaction products thoroughly. After the extraction, the solvents were completely evaporated under the flow of nitrogen. The sample was redissolved in chloroform (4 ml) and then 1 µl of the solution was used for gas chromatographic (GC) analysis.

2.4. GC analysis

The composition of the reaction products was analyzed by GC. A Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard Co., Avondale, PA, USA), equipped with a FID and a fused silica capillary column (DB-5ht, 30 m × 0.25 mm i.d., Agilent Technologies., Deerfield, IL, USA) was used. The carrier gas was helium and the total gas flow rate was 23 ml/min. The injector and detector temperatures were maintained at 350 ºC, respectively. The column was heated initially at 180 ºC and programmed to increase to 300 ºC at the rate of 3

ºC/min. The column temperature was then programmed to increase to 380 ºC at the rate of 10 ºC/min and held at 380 ºC for 5 min. The phytosterols and phytosteryl esters of oleic acid were identified using plant sterol mixture and Corowise[TM] phytosteryl esters (major phytosterols: sitosterol *ca.* 58 mol%, campesterol *ca.* 28 mol%; major FAs: oleic acid *ca.* 56 mol%, linoleic acid *ca.* 20 mol%) and their relative contents were calculated as mol%. With the GC operation conditions above, the retention time of each compound was as follows: oleic acid (9.53 min); campesteryl ester of oleic acid (27.41 min); dihydrobrassicasteryl ester of oleic acid (28.13 min); β-sitosteryl ester of oleic acid (29.39 min); campesterol (32.26 min); dihydrobrassicasterol (32.97 min) and β-sitosterol (34.22 min).

2.5. Calculation of degree of esterification

The degree of esterification (mol%) of phytosterols with oleic acid to form phytosteryl esters of oleic acid was calculated from the GC profile of reactants using the following equation (2):

$$\text{Degree of esterification } (DE, \text{mol\%}) = \frac{B}{1.638 \times A + B} \times 100 \qquad (2)$$

Where, A = peak area of total phytosterols (i.e., campesterol + dihydrobrassicasterol + β-sitosterol); B = peak area of total phytosteryl esters of oleic acid (i.e., campesteryl ester of oleic acid + dihydrobrassicasteryl ester of oleic acid + β-sitosteryl ester of oleic acid); 1.638 = ratio of average molecular weight of total phytosteryl esters of oleic acid to average molecular weight of total phytosterols.

2.6. Statistical analysis

All data were analyzed with the assistance of commercial software, Modde 5.0 (Umetrics, Umeå, Sweden) and were presented as the mean ± SD. The significant second-order coefficients were selected by regression analysis with backward elimination. Then, the fit of the model was evaluated by coefficients of determination (R^2 and Q^2 values) and a test for lack of fit, which was performed by comparing mean square (MS) lack of fit to MS pure experimental error, from the analysis of variance (ANOVA). The model equation established was finally proposed after verification by a chi-square test.

3. Results and discussion

The ultimate goal of our study was to model the degree of esterification (*DE*) of total phytosterols with oleic acid when CRL was used as the biocatalyst for the esterification reaction. In our study, we used phytosterol mixture containing 3 kinds of major phytosterols (β-sitosterol, campesterol, dihydrobrassicasterol) as the substrate. Weber et al. (2001) reported that the activity of CRL can be affected by types of phytosterols in the synthesis of phytosteryl esters of FAs. This indicates that the CRL-catalyzed esterification to synthesize FA esters of phytosterols is a substrate dependent enzyme reaction. In our study, β-sitosterol and campesterol showed similar *DE* with oleic acid at all reaction conditions investigated; whereas dihydrobrassicasterol showed relatively lower *DE*. For example, in the experiment No. 1 (from Table 5.1), *DE* of each phytosterol analogue was as follows: β-sitosterol 49.7 mol%, campesterol 52.1 mol%, and dihydrobrassicasterol 38.6 mol%. However, in the experimental sets showing very high *DE* of

total phytosterols, all the 3 kinds of phytosterols showed almost the same level of *DE* (e.g., experiment No. 27 from Table 5.1: β-sitosterol 97.4 mol%, campesterol 94.8 mol%, and dihydrobrassicasterol 97.0 mol%). Thus, we succeeded in modeling *DE* of individual phytosterol analogues as well as *DE* of total phytosterols. We note that the *DE* of total phytosterols was the main focus of this paper.

3.1. Model fitting

RSM was applied to model the *DE* of phytosterols with oleic acid to produce phytosteryl esters of oleic acid, with 4 reaction parameters: temperature (*Te*), reaction time (*t*), substrate molar ratio (*Sr*), and enzyme amount (*En*). RSM enabled us to obtain sufficient information for statistically acceptable results using reduced number of experimental sets, and is an efficient method to evaluate the effects of multiple parameters, alone or in combination, on response variables (Huang & Akoh, 1996; Shieh, Akoh, & Koehler, 1995; Xu, Skands, Høy, Mu, Balchen, & Alder-Nissen, 1998). Table 5.1 lists the levels of *DE* at each of the 27 experimental sets generated by the principles of RSM used in this study and the levels ranged from as low as 47.2 to as high as 97.2 mol%. The best-fitting models were determined by multiple linear regression (MLR) and backward elimination. The fits of the models were evaluated by coefficients of determination (R^2 and Q^2 values) and a test for lack of fit from ANOVA (Table 5.2). According to the ANOVA, R^2, which means the fraction of the variation of the response explained by the model, and Q^2, which indicates the fraction of the variation of the response predicted by the model, were 0.973 and 0.884, respectively (Table 5.2). Table 5.2 also showed that the probabilities for the regression of the model were significant ($P < 0.001$), meaning that the

models were statistically good, and the models had no lack of fit at 95% level of significance (Table 5.2). The normal probability plot (data not shown) and the observed values vs. predicted values plot (Fig. 5.1) also showed almost linear distribution, which is indicative of a good model. As a result, well-fitting models for *DE* were successfully established.

3.2. Effects of parameters

Table 5.3 lists the significant ($P < 0.05$) regression coefficients of the established model equation. Although among them, two terms (*Sr*Sr* and *Te*Sr*) were significant at 90% level, they were not removed by backward elimination in the process of model fitting described before to enhance the fitness of model. All 4 reaction parameters investigated positively affected *DE*, with *t* having the greatest effect followed by *En*, *Sr*, and *Te*. The quadratic terms of *t*, *Sr*, and *En* showed negative effects on *DE*; whereas, the quadratic term of *Te* had no significant ($P < 0.05$) effect on *DE*. All the significant ($P < 0.05$) interaction terms negatively affected *DE* and the order of effect is as follows: *t*En*, *t*Sr*, *Sr*En = Te*En*, and *Te*Sr*.

Fig. 5.2(a) shows that within the given range (35-55 ºC) of *Te, DE* increased almost linearly with the increase in *Te*. Tenkanen, Kontkanen, Isoniemi, Spetz and Holmbom (2002) reported that CRL had optimal activity below 55 ºC at pH 5-7 when applied to the hydrolysis of phytosteryl esters. Therefore, our esterification result was in close agreement with their hydrolysis result. However, for better clarification of optimal temperature of CRL in the esterification reaction between phytosterols and oleic acid, further investigation on the synthesis of phytosteryl esters of oleic acid above 55 ºC would be required. Fig. 5.2(b), (c), and (d) illustrate that the rate of increase in *DE* was decreased, respectively, after center points of each

parameter (*t*, 14 h; *Sr*, 2; *En*, 6%) even though overall *DE* continued to increase. It is possible that equilibrium was reached at these center points.

3.3. Model verification

A chi-square test using eight additional experimental sets chosen from the given ranges of reaction parameters was performed to examine the adequacies of the model established. The chi-square test for *DE* indicated that there were no significant ($P < 0.05$) difference between the observed and predicted values since the chi-square value (1.87) was much smaller than 14.07, cutoff point at $\alpha = 0.05$ and df $= 7$ (Table 5.4).

3.4. Optimization of reaction conditions

Contour plot is generally used to evaluate the relationships between parameters and to predict the result under given conditions. However, it is complicated to analyze the interaction between parameters in this study due to the existence of many interaction terms as shown in Table 5.3.

Instead, we used contour plots for optimizing the conditions of esterification reaction. Six contour plots between four parameters were constructed and minimum level of each parameter which would enable us to reach the maximum level (theoretically 100%) of *DE* was predicted and pointed by using dotted arrows on each contour plot (Fig. 5.3). From the result, it can be concluded that optimal conditions for synthesis of phytosteryl esters of oleic acid were: *Te*, 51.3 ºC; *t*, 17.0 h; *Sr*, 2.1; *En*, 7.2%. When one additional esterification reaction was conducted under

the established optimal conditions, as expected, *DE* (97.0 ± 0.5 mol%) near 100 mol% was achieved.

4. Conclusion

The modeling of CRL-catalyzed esterification reaction to synthesize phytosteryl esters of oleic acid as a possible cholesterol lowering agent was successfully performed. By using the established model, the degrees of effect of four main reaction parameters (temperature, reaction time, substrate molar ratio, and enzyme amount) were elucidated at given ranges and optimized reaction conditions were obtained. In addition, very high *DE* (up to 97.2 mol%) of phytosterol with oleic acid was achieved even though our reaction conditions are shown to be simple and mild (i.e., monophasic media of hexane, low temperature ~55 ºC, short reaction time ~24 h, and without the use of water-trapping agents or reduced pressure system) compared to other previous works (Negishi, et al., 2003; Shimada, et al., 1999; Weber, et al., 2002; Weber, et al., 2001; Vu, et al., 2004). The phytosteryl esters of oleic acid produced in this study are expected to have lower melting point and greater solubility in oils compared to the corresponding phytosterols with free hydroxyl groups.

References

Beveridge, J. M., Haust, H. L., & Connel, W. F. (1964). Magnitude of the hypocholesterolemic effect of dietary sitosterol in man. *Journal of Nutrition, 83*, 119-122.

Hendriks, H. F. J., Weststrate, J. A., van Vliet, T., & Meijer, G. W. (1999). Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects. *European Journal of Clinical Nutrition, 53*, 319-327.

Huang, K. H., & Akoh, C. C. (1996). Optimization and scale-up of enzymatic synthesis of structured lipids using RSM. *Journal of Food Science, 61*, 137-141.

Jones, P. J. H., MacDougall, D. E., Ntanios, F., & Vanstone, C. A. (1997). Dietary phytosterols as cholesterol-lowering agents in humans. *Canadian Journal of Physiology and Pharmacology, 75*, 217-227.

Katan, M. B., Grundy, S. M., Jones, P., Law, M., Miettinen, T., & Paoletti, R. (2003). Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. *Mayo Clinic Proceedings, 78*, 965-978.

Law, M. (2000). Plant sterol and stanol margarines and health. *British Medical Journal, 320*, 861-864.

Lees, A. M., Mok, H. Y., Lees, R. S. McCluskey, M. A., & Grundy, S. M. (1977). Plant sterols as cholesterol-lowering agents: clinical trials in patients with hypercholesterolemia and studies of sterol balance. *Atherosclerosis, 28*, 325-338.

Ling, W. H., & Jones, P. J. H. (1995). Dietary phytosterols: a review of metabolism, benefits and side effects. *Life Sciences, 57*, 195-206.

Miettinen, T. A., & Gylling, H. (2004). Plant stanol and sterol esters in prevention of cardiovascular diseases. *Annals of Medicine, 36*, 126-134.

Miettinen, T. A., Puska, P., Gylling, H., Vanhanen, H., & Vartiainen, E. (1995). Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. *New England Journal of Medicine, 333*, 1308-1312.

Negishi, S., Hidaka, I., Takahashi, I., & Kunita, S. (2003). Transesterification of phytosterol and edible oil by lipase powder at high temperature. *Journal of the American Oil Chemists Society, 80*, 905-907.

Neil, H. A. S., Meijer, G. W., & Roe, L. S. (2001). Randomised controlled trial of use by hypercholesterolaemic patients of a vegetable oil sterol-enriched fat spread. *Atherosclerosis, 156*, 329-337.

Nestel, P., Cehun, M., Pomeroy, S., Abbey, M., & Weldon, G. (2001). Cholesterol-lowering effects of plant sterol esters and non-esterified stanols in margarine, butter and low-fat foods. *European Journal of Clinical Nutrition, 55*, 1084-1090.

Noakes, M., Clifton, P., Ntanios, F., Shrapnel, W., Record, I., & McInerney, J. (2002). An increase in dietary carotenoids when consuming plant sterols or stanols is effective in maintaining plasma carotenoid concentrations. *American Journal of Clinical Nutrition, 75*, 79-86.

Pollak, O. J. (1953). Successive prevention of experimental hypercholesteremia and cholesterol atherosclerosis in the rabbit. *Circulation, 7*, 696-701.

Shieh, C. J., Akoh, C. C., & Koehler, P. E. (1995). Four-factor response surface optimization of the enzymatic modification of triolein to structured lipids. *Journal of the American Oil Chemists Society, 72*, 619-623.

Shimada, Y., Hirota, Y., Baba, T., Sugihara, A., Moriyama, S., Tominaga, Y., & Terai, T. (1999). Enzymatic synthesis of steryl esters of polyunsaturated fatty acids. *Journal of the American Oil Chemists Society, 76*, 713-716.

Tenkanen, M., Kontkanen, H., Isoniemi, R., Spetz, P., & Holmbom, B. (2002). Hydrolysis of steryl esters by a lipase (Lip3) from *Candida rugosa*. *Applied Microbiology and Biotechnology, 60*, 120-127.

Villeneuve, P., Turon, F., Caro, Y., Escoffier, R., Barea, B., Barouh, B., Lago, R., Piombo, G., & Pina, M. (2005). Lipase-catalyzed synthesis of canola phytosterols oleate esters as cholesterol lowering agents. *Enzyme and Microbial Technology, 37*, 150-155.

Vu, P. L., Shin, J. A., Lim, C. H., & Lee, K. T. (2004). Lipase-catalyzed production of phytosteryl esters and their crystallization behavior in corn oil. *Food Research International, 37*, 175-180.

Weber, N., Weitkamp, P., & Mukherjee, K. D. (2002). Cholesterol-lowering food additives: lipase-catalysed preparation of phytosterol and phytostanol esters. *Food Research International, 235*, 177-181.

Weber, N., Weitkamp, P., & Mukherjee, K. D. (2001). Fatty acid steryl, stanyl, and steroid esters by esterification and transesterification in vacuo using *Candida rugosa* lipase as catalyst. *Journal of Agricultural and Food Chemistry, 49*, 67-71.

Wester, I. (2000). Cholesterol-lowering effect of plant sterols. *European Journal of Lipid Science and Technology, 102***, 37-44.

Weststrate, J. A., & Meijer, G. W. (1998). Plant sterol-enriched margarines and reduction of plasma total and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. *European Journal of Clinical Nutrition, 52*, 334-343.

Xu, X., Skands, A. R. H., Høy, C. E., Mu, H., Balchen, S., & Alder-Nissen, J. (1998). Production of specific-structured lipids by enzymatic interesterification: Elucidation of acyl migration by response surface design. *Journal of the American Oil Chemists Society, 75,* 1179-1186.

Table 5.1. Central composite design arrangement and response for the *Candida rugosa* lipase (CRL)-catalyzed esterification to synthesize phytosteryl esters of oleic acid[a]

Exp. No.	Factors				Response
	Te (°C)	*t* (h)	*Sr*	*En* (%)	*DE* (mol%)
1	35	4	1	2	47.8 ± 0.3[b]
2	55	4	1	2	60.4 ± 4.1
3	35	24	1	2	76.2 ± 3.8
4	55	24	1	2	92.2 ± 0.4
5	35	4	3	2	69.4 ± 3.2
6	55	4	3	2	77.5 ± 3.2
7	35	24	3	2	89.7 ± 3.8
8	55	24	3	2	96.6 ± 0.3
9	35	4	1	10	76.3 ± 3.4
10	55	4	1	10	87.0 ± 0.4
11	35	24	1	10	95.5 ± 0.0
12	55	24	1	10	94.5 ± 0.8
13	35	4	3	10	91.6 ± 1.8
14	55	4	3	10	93.3 ± 1.1
15	35	24	3	10	96.4 ± 1.2
16	55	24	3	10	96.4 ± 0.5
17	35	14	2	6	96.5 ± 0.8
18	55	14	2	6	97.1 ± 0.2
19	45	4	2	6	86.6 ± 1.8
20	45	24	2	6	96.5 ± 0.3
21	45	14	1	6	91.1 ± 0.7
22	45	14	3	6	96.7 ± 0.6
23	45	14	2	2	89.0 ± 1.0
24	45	14	2	10	97.1 ± 0.3
25	45	14	2	6	95.8 ± 0.3
26	45	14	2	6	96.6 ± 0.3
27	45	14	2	6	97.2 ± 0.9

[a] *Te* = temperature; *t* = reaction time; *Sr* = substrate molar ratio (oleic acid to phytosterols); *En* = enzyme amount; *DE* = degree of esterification of phytosterol with oleic acid to form phytosteryl esters of oleic acid.

[b] Mean $\pm$ SD, n = 2.

Table 5.2. ANOVA table[a]

	df	SS	MS	*F*-values	*P*-values
Total	27	214072	7928.6		
Constant	1	209969	209969		
Total corrected	26	4103.2	157.82		
Regression	12	3991.2	332.60	41.564	< 0.001
Residual	14	112.03	8.002		
Lack of fit (model error)	12	111.04	9.2534	18.757	0.052
Pure error (Replicate error)	2	0.9867	0.4933		
R^2	0.973				
Q^2	0.884				

[a] Abbreviations: df, degrees of freedom; SS, sum of squares; MS, mean square

Table 5.3. Significant ($P < 0.05$) regression coefficients of the second-order polynomials after backward elimination for the *Candida rugosa* lipase (CRL)-catalyzed esterification to synthesize phytosteryl esters of oleic acid[a]

Variables	Coefficients	P-values
Intercept	96.880	5.069×10^{-21}
Te	3.089	3.876×10^{-4}
t	8.006	9.285×10^{-9}
Sr	4.811	4.454×10^{-6}
En	7.183	3.683×10^{-8}
t*t	-5.631	4.949×10^{-3}
Sr*Sr[b]	-3.281	7.269×10^{-2}
En*En	-4.131	2.839×10^{-2}
Te*Sr[b]	-1.350	7.698×10^{-2}
Te*En	-2.013	1.296×10^{-2}
t*Sr	-2.475	3.537×10^{-3}
t*En	-4.063	5.073×10^{-5}
Sr*En	-2.013	1.296×10^{-2}

[a] See Table 1 for description of abbreviations.

[b] Regression coefficient which is significant at $P < 0.1$.

Table 5.4. Model verification by chi-square (χ^2) test[a]

Exp. No.	Factors				Responses	
					DE (mol%)	
	Te (ºC)	t (h)	Sr	En (%)	Observed	Predicted
1	35.9	4.7	1.2	2.1	50.6 ± 4.9[b]	55.0
2	40.0	7.5	1.1	2.6	71.9 ± 1.0	65.0
3	50.6	5.7	1.4	3.5	80.5 ± 4.3	75.0
4	50.1	4.0	1.0	8.0	77.4 ± 0.6	80.0
5	37.1	18.9	1.2	3.7	84.8 ± 1.8	85.0
6	38.6	21.4	1.1	5.2	93.6 ± 2.0	90.0
7	48.6	8.9	1.7	9.0	92.0 ± 3.6	95.0
8	55.0	9.8	2.5	8.2	96.6 ± 0.0	99.0
					$\chi^2 = 1.87$[c]	

[a] See Table 1 for description of abbreviations.

[b] Mean $\pm$ SD, n = 2.

[c] $\chi^2 = \Sigma$ [(Observed value – Predicted value)^2 / Predicted value]; cutoff point was 14.07 at α = 0.05, df = 7.

Fig. 5.1. Plot showing relationships between observed values and values predicted by the model. Numbers inside the graph represent experimental numbers. The almost linear distribution of the experimental numbers is indicative of a good model.

$y = x + 1.000 \times 10^{-5}$
$R^2 = 0.973$
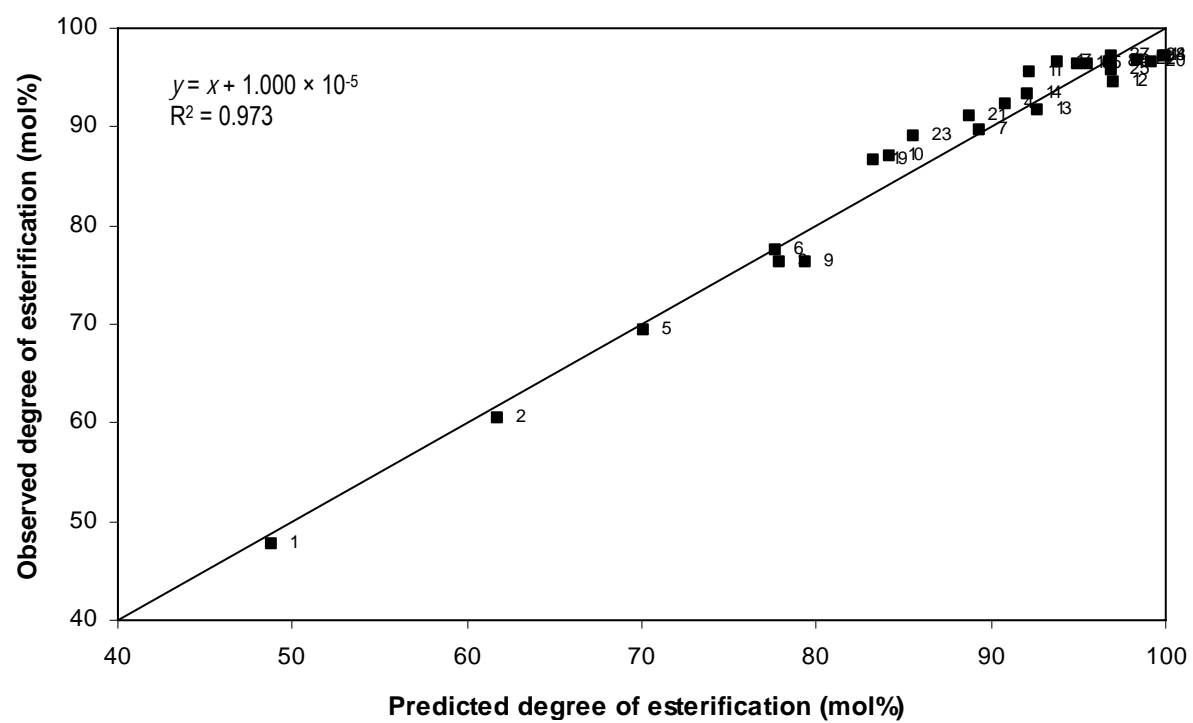
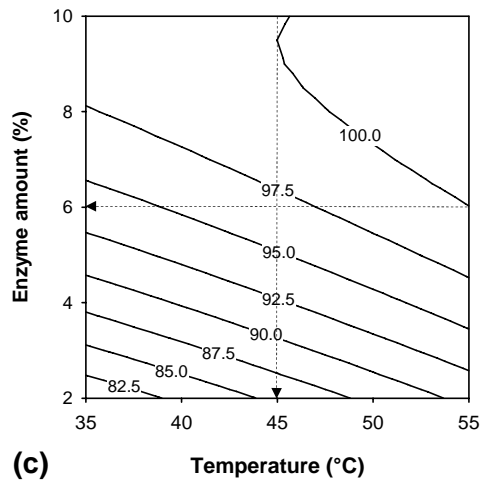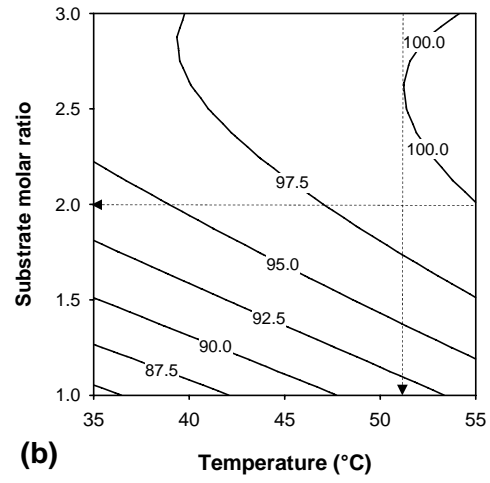Fig. 5.2. Prediction plots for degree of esterification of phytosterols with oleic acid to produce phytosteryl esters of oleic acid by the effects of main parameters: (a) temperature (*Te*); (b) reaction time (*t*); (c) substrate molar ratio (*Sr*); (d) enzyme amount (*En*). Factors setup: *t* 14 h, *Sr* 2, *En* 6% for (a); *Te* 45 ºC, *Sr* 2, *En* 6% for (b); *Te* 45 ºC, *t* 14 h, *En* 6% for (c); *Te* 45 ºC, *t* 14 h, *Sr* 2 for (d).
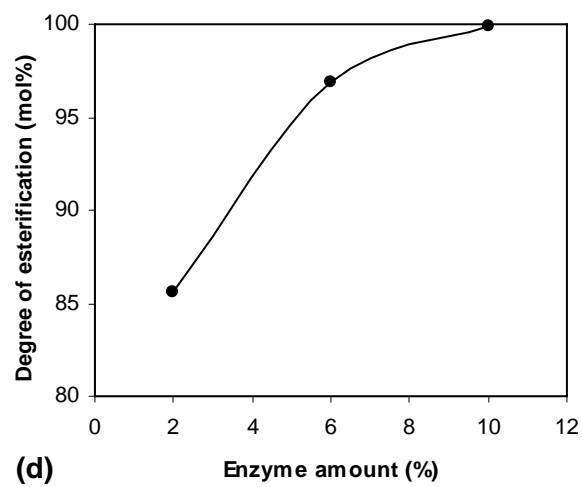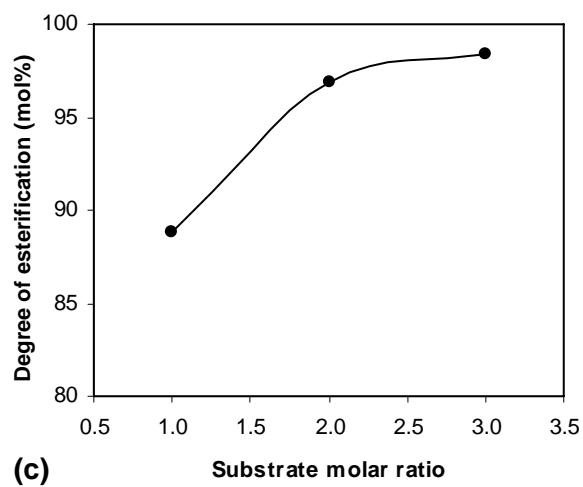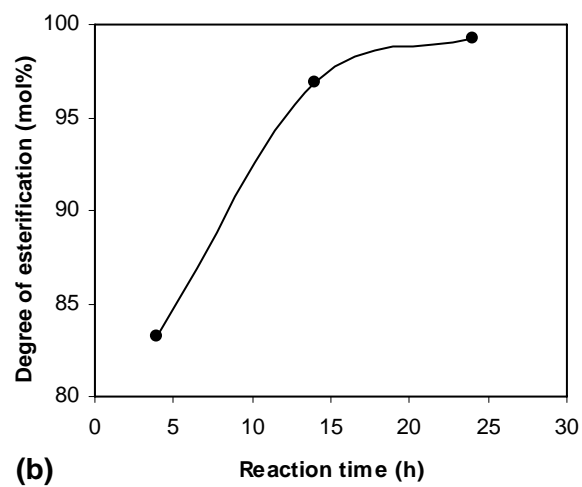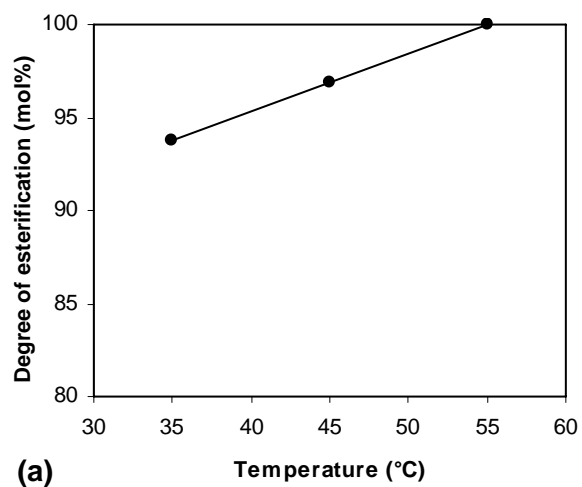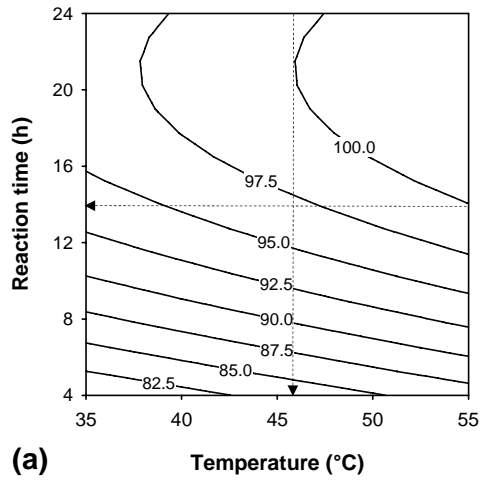
Fig. 5.3. Contour plots between two parameters for degree of esterification of phytosterols with oleic acid to produce phytosteryl esters of oleic acid: (a) temperature (*Te*) and reaction time (*t*); (b) *Te* and substrate molar ratio (*Sr*); (c) *Te* and enzyme amount (*En*); (d) *t* and *Sr*; (e) *t* and *En*; (f) *Sr* and *En*. Factors setup: *Sr* 2, *En* 6% for (a); *t* 14 h, *En* 6% for (b); *t* 14 h, *Sr* 2 for (c); *Te* 45 ºC, *En* 6% for (d); *Te* 45 ºC, *Sr* 2 for (e); *Te* 45 ºC, *t* 14 h for (f).

(a)

Temperature (°C)

(b)

Temperature (°C)

(c)

Temperature (°C)

(d)

Reaction time (h)

(e)

Reaction time (h)

(f)

Substrate molar ratio

138
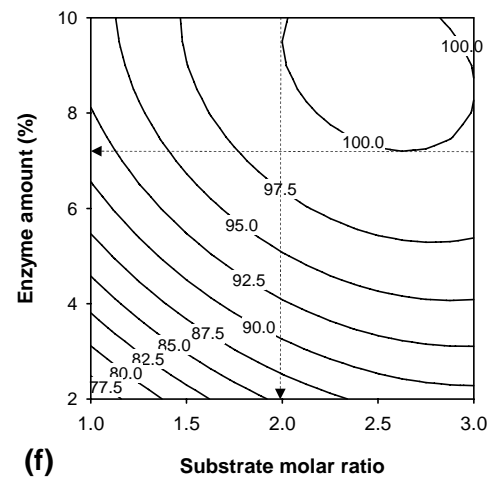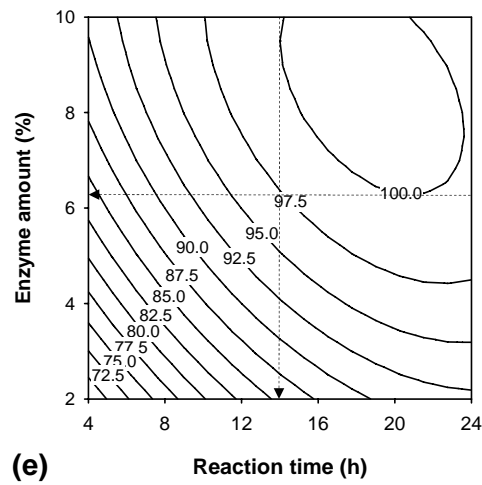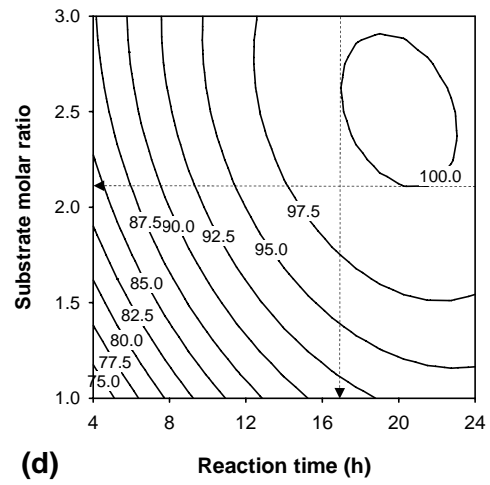
CHAPTER 6

DIETARY EFFECTS OF SESAME OIL-BASED STRUCTURED LIPIDS AND

PHYTOSTERYL ESTERS ON BLOOD LIPID PROFILES AND CARDIOVASCULAR

PARAMETERS IN SPONTANEOUSLY HYPERTENSIVE RATS[1]

---

[1] Kim, B.H., K.D. Sandock, T.P. Robertson, S.J. Lewis, and C.C. Akoh. To be submitted to *Hypertension*.

Abstract

This study examined the dietary effects of enzymatically modified sesame oil with caprylic acid (structured lipid, SL) and phytosteryl esters (PE) on the blood lipid profiles and cardiovascular parameters of spontaneously hypertensive rats fed high-fat (20% of diet weight) and high-cholesterol (0.56% of diet weight) diets. There were six dietary groups: control (rats fed normal diet, CO), lard (LA), sesame oil (SO), SL, SO fortified with PE (OP), and SL fortified with PE (LP). The fortified level of PE in diets was 0.54% of diet weight. After feeding for 9 weeks, rats fed SO, SL, OP, and LP gained less weight than rats fed LA despite similar amount of food intake. The plasma total and LDL cholesterol levels were similar in all high-fat dietary groups but were higher than control rats. However, the plasma HDL cholesterol levels and plasma HDL/total cholesterol ratios in the rats fed OP and LP were higher than those in the other high-fat dietary groups. The plasma triacylglycerol levels in high-fat dietary groups were similar to each other but were lower than that in control rats. As expected, body weight gains and final body weights of the high-fat diet groups were substantially higher than the controls. Liver weights and liver weight/body weight ratios in all high-fat dietary groups were higher than the controls. Systolic arterial blood pressures under pentobarbital anesthesia were lower in the high fat-fed rats than in controls whereas other parameters were variously affected in the different groups. Finally, resting arterial blood pressures in conscious high fat-fed rats were not different to control rats; however, resting heart rates in all high fat-fed groups of rats were higher than the controls.

Keywords: Cardiovascular parameters; phytosteryl esters; plasma lipid profile; sesame oil; spontaneously hypertensive rats; structured lipid

INTRODUCTION

Long chain triacylglycerols (LCTs), the predominant form of traditional edible oils, serve as a source of essential fatty acids (EFAs). However, LCTs are metabolized slowly and have high tendency to be deposited in body tissues (Akoh, 2002). In contrast, medium chain triacylglycerols (MCTs) provide quick delivery of energy via oxidation of the more hydrophilic medium chain fatty acids (MCFAs); have less-calories because of their shorter chain length compared to long chain fatty acids (LCFAs); and have less tendency to be deposited in the adipose tissue due to their predominant transportation via the portal vein to the liver rather than through the lymphatic system (Akoh, 2002; Iwasaki and Yamane, 2000; Xu et al., 1998; Bach and Babayan, 1982; Bray et al., 1980). However, one major disadvantage of MCTs is the lack of EFAs. To overcome each disadvantage of LCTs and MCTs while retaining the benefits of both triacylglycerols (TAGs), structured lipids have attracted much attention over the past decade.

Structured lipids (SLs) are defined as TAGs that have been restructured by incorporation of new fatty acids (FAs) to change the composition and positional distribution of FAs from the native state by chemical or enzymatic methods (Akoh, 2002). Among several types of structured lipids, MLM-type structured lipids are TAGs comprised of MCFAs esterified at *sn*-1,3 positions and LCFAs at *sn*-2 position of the glycerol backbone. MLM-structured lipids can be synthesized by attaching MCFA into LCTs with the help of *sn*-1,3 regiospecific lipase. These structured lipids can be expected to have unique and desirable nutritional characteristics because of the presence of both MCFAs and LCFAs in the same TAG molecules. While providing the benefits of MCTs described above, MLM-type structured lipids can act as efficient delivery system for LCFAs, such as monounsaturated FAs (MUFAs), polyunsaturated FAs (PUFAs), and EFAs

since the *sn*-2 monoacylglycerols (*sn*-2 MAGs) produced by pancreatic lipase digestion during metabolism are well absorbed through the intestinal wall (Iwasaki and Yamane, 2000; Xu et al., 1998). Therefore, theoretically MLM-type structured lipids containing specific and proper balances of MCFA, EFA (e.g., linoleic acid), and MUFAs (e.g., oleic acid) or PUFAs (e.g., linolenic acid, EPA, DHA) known as hypocholesterolemic FAs can be expected to have a beneficial effect on the plasma lipids and lipoproteins, which relates to coronary heart disease (CHD) as well as meet the EFA requirement. However, although MLM-type structured lipids were theoretically evaluated better than pure LCTs, MCTs, and/or the physical mixtures of LCTs and MCTs, so far there were only a few studies to evaluate their dietary effects, especially the effect on cardiovascular systems of animals and humans. Therefore, it would be meaningful to evaluate the dietary effect of MLM-type structured lipids on cardiovascular system empirically.

On the other hand, phytosterols are known to have hypocholesterolemic effect by lowering plasma total and low density lipoprotein (LDL) cholesterol levels without affecting plasma high density lipoprotein (HDL) cholesterol concentration (Wester, 2000; Lees et al., 1977; Beveridge et al., 1964; Pollak, 1953) in humans and some animals, such as rabbits. The cholesterol-lowering mechanisms of phytosterols are still not known in detail. Recently, Trautwein et al. (2003) proposed that the main mechanism responsible for the hypocholesterolemic effect of phytosterols is the inhibition of intestinal cholesterol absorption arising from chemical structure similarities between phytosterols and cholesterol. Besides this main mechanism, they also suggested other mechanisms as follows: competition with cholesterol for solubilization in dietary mixed micelles, co-crystallization with cholesterol to form insoluble mixed crystals, interference with the hydrolysis process by lipases and cholesterol esterases, etc. However, phytosterols have limitations in usage as a dietary supplement since they have very

low solubility in edible oil and have very high melting point. Therefore, to overcome such problems of free phytosterols, phytosteryl esters (fatty ester forms of phytosterols) are preferred in food formulation. Since phytosteryl esters have much greater solubility in oils and much lower melting point compared to phytosterols, they can be easily incorporated into a wide variety of diets and fat-based food products. Moreover, many studies in recent years have shown that phytosteryl esters can also effectively reduce plasma total and LDL cholesterol levels similar to phytosterols (Neil et al., 2001; Law, 2000; Weststrate and Meijer, 1998; Jones et al., 1997).

The aim of our study was to investigate the dietary effects of sesame oil-based MLM-type structured lipids and phytosteryl esters on the blood lipid profile and cardiovascular parameters of spontaneously hypertensive (SH) rats. For this study, MLM-type structured lipids were synthesized from sesame oil and caprylic acid by lipase-catalyzed acidolysis and commercial phytosteryl esters prepared from vegetable oil deodorizates were used.

MATERIALS AND METHODS

Materials

Lard and sesame oil were the products of Hatfield Quality Meats (Hatfield, PA, USA) and Spectrum Organic Product, Inc. (Petaluma, CA, USA), respectively. Caprylic acid (C8:0, purity > 98%) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Phytosteryl esters (from vegetable oil deodorizates) were kindly donated by Cargill, Inc. (Minneapolis, MN, USA). Lipozyme RM IM, a *sn*-1,3 regiospecific immobilized lipase from *Rhizomucor miehei* was provided by Novozymes North America, Inc. (Franklinton, NC, USA).

Structured lipid synthesis

The sesame oil-based structured lipid was synthesized by acidolysis reaction between sesame oil and caprylic acid in a bench-scale continuous packed bed reactor packed with 250 g of Lipozyme RM IM. The reaction was carried out under the following conditions: substrate flow rate, 1.15 mL/min; column temperature, 45 ºC; substrate molar ratio 1:6 (sesame oil: caprylic acid). Short-path distillation was carried out to remove the unreacted FAs in the synthesized SL with a KDL-4 unit (UIC Inc., Joliet, IL, USA). Consequently, about five kilograms of purified structured lipid were produced and used to prepare rat diets.

Total and positional FA profiles of fat source

Fifty milligrams of each fat sample was methylated in 3 mL of 6% HCl solution (in methanol) at 75 ºC for 2 h. The FA methyl esters (FAMEs) were extracted and analyzed by gas chromatography (GC). An Agilent Technologies 6890N gas chromatograph (Agilent Technologies Inc., Palo Alto, CA), equipped with a flame ionization detector (FID) and a fused silica capillary column (AT-225, 30 m × 0.25 mm i.d., Alltech Associates, Inc., Deerfield, IL, USA) was used. The carrier gas was helium and the total gas flow rate was 23 mL/min. The injector and detector temperatures were maintained at 250 and 260 ºC, respectively. The column was initially held at 40 ºC for 3 min and programmed to increase to 130 ºC at the rate of 10 ºC/min. After holding at 130 ºC for 3 min, the column was then programmed to increase to 215 ºC at the rate of 20 ºC/min. The FAMEs were identified and their relative contents were calculated as mol% with heptadecanoic acid (C17:0) as an internal standard. For the analysis of FA profile at *sn*-2 position of oil sample, *sn*-2 MAGs were prepared according to the pancreatic lipase hydrolysis procedure described by Luddy et al. (1964). The obtained *sn*-2

monoacylglycerols were methylated and analyzed by GC according to the procedure described above. The FA profile at *sn*-1,3 positions was also obtained by calculation using the following equation: *sn*-1,3 (mol%) = [3 × Total (mol%) – *sn*-2 (mol%)] / 2.

Rats

All animal studies were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. All protocols were approved by the Animal Care and Use Committee of the University of Georgia. Four-week old male spontaneously hypertensive rats (SH rats; n = 36) were obtained from Harlan (Madison, WI, USA). They were randomly assigned to one of six different dietary groups described below (n = 6 per group). The rats were individually housed in cages under controlled temperature (21 ± 1 ºC) and lighting (12 h light/dark cycle). The rats were fed diets and water *ad libitum* for 9 weeks. Diet intakes were recorded daily and body weights were measured weekly.

Diets

Normal diet for the control rats (CO) was purchased from Labdiet (Brentwood, MO, USA) and high-fat and high-cholesterol diets were prepared and pelleted by Research Diets (New Brunswick, NJ, USA). Five different kinds of high-fat diets were made for five different dietary groups: lard (LA), sesame oil (SO), structured lipid (SL), sesame oil fortified with phytosteryl esters (OP), and structured lipid fortified with phytosteryl ester (LP) (Table 6.1). The five formulations for high-fat dietary groups were identical in composition, except for the type of fat. The high-fat diets contained LA, SO, SL, OP, or LP as a fat source (20% of diet weight), respectively. Dietary cholesterol was adjusted to 0.56% of diet weight in all high-fat diets. The

amount of PE added in the high-fat diets for two groups (OP and LP) was adjusted to 0.54% of diet weight (1.20 g/1000 kcal) approximately corresponding to a therapeutic level (1.5-3.0 g/day) for hypocholesterolemic effect in humans (Miettinen and Gylling, 2004; Katan et al., 2003; Noakes et al., 2002; Nestel et al., 2001; Hendriks et al., 1999; Weststrate and Meijer, 1998; Jones et al., 1997; Ling and Jones, 1995; Miettinen et al., 1995). All high-fat diets were almost isoenergetic (4.54 kcal/kg for LA, SO, and SL; and 4.50 kcal/kg for OP, and LP).

Surgeries, blood collection and cardiovascular measurements

Twelve hours before the surgeries were performed, all rats were deprived of food. Water was freely available. All rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and a catheter (PE-50) was placed in a carotid artery or a femoral artery in order to record systolic arterial blood pressure (SBP), diastolic arterial blood pressure (DBP), pulse pressure (PP, systolic minus diastolic arterial pressure) and mean arterial blood pressure (MAP, diastolic arterial blood pressure plus one-third pulse pressure). The catheters were exteriorized to the back of the neck and all wounds were liberally coated with triple antibiotic (neomycin, polymixin B and bacitracin) ointment (Fougera, NY, USA) and then sutured. The arterial catheter was connected to a pressure transducer that was coupled to a computerized data acquisition system (PowerLab Chart, version 4.0.4, ADI Instruments, Colorado Springs, CO, USA) in order to record the resting blood pressure parameters described above, and heart rate (HR), which was derived from PP by way of an internal cardio-tachometer in the data acquisition system. Once the resting cardiovascular parameters were recorded, 2 mL of blood was taken via the arterial catheter for subsequent determination of plasma lipid profiles and glucose levels. The rats were returned to their home cages in a room maintained on a 12h light/dark cycle. Food and water

were freely available. The rats were given two days to recover from the above surgeries. On the day of data recording in the conscious rats, the arterial catheter of each conscious rat was connected to the pressure-transducer and recording equipment as described above.

Plasma lipid profiles and glucose level

Blood plasma samples were analyzed to determine total, HDL, and LDL cholesterols, TAG, and glucose levels. Plasma total and HDL cholesterol levels were determined by cholesterol oxidase method with Equal reagents (Catalog Nos. E46998 for total cholesterol and 5122 for HDL cholesterol, Equal Diagnostics, Exton, PA, USA), respectively. Plasma TAG level was determined by glycerol phosphate oxidase method with Amresco reagents (Code No. 7130, Amresco Inc., Solon, OH, USA). All testing for lipid profiles was performed on a Roche/Hitachi 717 analyzer (Roche Diagnostics, Indianapolis, IN, USA). Plasma LDL cholesterol was calculated using the Friedwald calculation, which is: LDL cholesterol = Total cholesterol − (TAG/5 + HDL cholesterol). Plasma glucose levels were determined by the hexokinase method with the Roche reagents (Catalog No. 1876899, Roche Diagnostics, Indianapolis, IN, USA) using a Roche/Hitachi 912 analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Statistics

The data are presented as mean ± S.E.M. and were analyzed by one way or repeated-measures analysis of variance (ANOVA) (Winer, 1971) followed by Student's modified t-test with the Bonferroni correction for multiple comparisons between means using the error mean square (EMS) terms from the ANOVAs (Wallenstein et al., 1980). A value of $P < 0.05$ denoted statistical significance.

RESULTS

FA composition of fat sources

FA compositions of lard, sesame oil, and structured lipid used as the fat sources of high-fat diets are given in Table 6.2. The FA compostion of control diet is also shown in Table 6.2. Major FAs of control diet were oleic acid (27.2 mol%), linoleic acid (26.5 mol%), plamitic acid (20.2 mol%), and linolenic acid (17.6 mol%). This result means that apparently the fat of control diet was originated from a mixture of animal fat and vegetable oil (see Table 6.1 legend). The most abundant FA in lard was oleic acid (46.4 mol%) followed by palmitic acid (23.4 mol%) and linoleic acid (16.4 mol%). Two major FAs of sesame oil were oleic acid (37.0 mol%) and linoleic acid (48.4 mol%). The content of palmitic acid in sesame oil was 10.0 mol%. However, they showed different positional distribution of FAs from each other. Oleic acid in lard predominantly existed at *sn*-1,3 positions (62.1 mol%) compared to *sn*-2 position (15.2 mol%); whereas sesame oil showed slightly higher content of oleic acid at *sn*-2 position (40.2 mol%) than *sn*-1,3 position (35.4 mol%). In lard, palmitic acid were mostly located at *sn*-2 position (68.5 mol%) compared to *sn*-1,3 position (0.9 mol%); whereas, palmitic acid in sesame oil mostly existed at *sn*-1,3 position (14.4 mol% at *sn*-1,3 position versus 1.1 mol% at *sn*-2 position). Total caprylic acid content of structured lipid was 45.8 mol%. The content of caprylic acid found at *sn*-2 position of the SL was 3.7 mol%. Two major USFAs (oleic and linoleic acids) at the *sn*-2 position of original sesame oil remained almost intact at that position in the structured lipid. The incorporation of caprylic acid into *sn*-1,3 positions, as we intended by using Lipozyme RM IM (a *sn*-1,3 specific lipase) in the synthesis of structured lipid, reached 66.8 mol%. These results indicate that MLM-type structured lipid was successfully produced.

Food intake, weight gain and food efficiency ratio

The weekly intake of food (grams/week), the weight gain (grams/week) and Food-Efficiency ratio (FER, weight gain per week/food intake per week, gram/gram) of the rats on control and high-fat diets are summarized in Table 6.3. The diets began when the rats were at 4 weeks of age and continued until they were 13 weeks of age. In essence, the rats in each group ate similar amounts of food. However, the rats fed high fat diets put on more weight for the first few weeks than the control rats. As such, the FER values for the high fad-fed rats were higher than controls for the first few weeks. The total intake of food, total weight gain and FERs are summarized in Table 6.4. Although the rats in each group ate similar amounts of food, the rats fed high fat diets put on more weight, and accordingly had higher FER ratios.

Body and organ weights

The body weights of the rats that received control or high-fat diets are summarized in Fig. 6.1. As can be seen, the body weights of the rats fed lard (LA), sesame oil (SO), structured lipid (SL), sesame oil fortified with phytosteryl esters (OP), or structured lipid fortified with phytosteryl ester (LP) were substantially greater than the rats fed control (CO) diet ($P < 0.05$, for all comparisons).

The liver weights (LW), heart weights (HW), kidney weights (KW), and the liver weight/body weight (LW/BW), heart weight/body weight (HW/BW), and kidney weight/body weight (KW/BW) ratios are summarized in Fig. 6.2. As can be seen, the liver weights and LW/BW ratios of the rats fed lard (LA), sesame oil (SO), structured lipid (SL), sesame oil fortified with phytosteryl esters (OP), or structured lipid fortified with phytosteryl ester (LP) were substantially greater than the rats fed control (CO) diet ($P < 0.05$, for all comparisons). The

149

heart weights, heart weight/body weight ratios, kidney weights, and kidney weight/body weight ratios of the rats fed high fat were not different to those of the control rats ($P > 0.05$, for all comparisons).

Plasma lipid profiles and glucose levels

Plasma lipid profiles and glucose levels in the six dietary groups are shown in Figs. 6.3. and 6.4. As seen in Fig. 6.3, the plasma total cholesterol, HDL cholesterol, and LDL cholesterol levels in the rats fed lard (LA), sesame oil (SO), structured lipid (SL), sesame oil fortified with phytosteryl esters (OP), or structured lipid fortified with phytosteryl ester (LP), were substantially higher than in the control rats ($P < 0.05$, for all comparisons). The plasma total cholesterol and HDL cholesterol levels in the rats fed OP or LP tended to be higher than those fed LA, SO or SL. The plasma LDL cholesterol levels were similar in all high-fat dietary groups (i.e., all groups other than control group). As a result, the plasma HDL/total cholesterol ratios were diminished (as compared to controls) in the rats fed LA, SO or SL. Moreover, the plasma LDL/total cholesterol ratios were higher in all high-fat dietary groups as compared to the control group. However, the plasma LDL/total cholesterol ratios of the rats fed OP or LP, tended to be lower than the other high-fat dietary groups. The plasma HDL/LDL cholesterol ratios were less in all high-fat dietary groups than in the control group. The ratios in the rats fed OP or LP were higher than those fed LA, SO or SL.

As seen in Fig. 6.4, the plasma TAG levels in the rats fed sesame oil (SO), structured lipid (SL), sesame oil fortified with phytosteryl esters (OP), or structured lipid fortified with phytosteryl ester (LP), were lower than that in control rats. However, the plasma TAG levels in rats fed lard (LA) were not different from those of control rats. There was no difference in the

plasma TAG levels between groups SO, SL, OP, and LP ($P > 0.05$, for all comparisons). An important finding was that the plasma glucose levels in the rats fed the high-fat diets were all substantially lower than those in control rats ($P < 0.05$, for all comparisons).

Resting cardiovascular parameters in anesthetized rats

Resting cardiovascular parameters in the dietary groups recorded under pentobarbital anesthesia are summarized in Fig. 6.5. Resting parameters in the control (CO) group of rats are similar to those reported previously (see Lewis et al., 2005, 2006a,b). As can be seen, resting systolic arterial blood pressure (SBP) values in the high-fat dietary groups., i.e., those fed lard (LA), sesame oil (SO), structured lipid (SL), sesame oil fortified with phytosteryl esters (OP), or structured lipid fortified with phytosteryl ester (LP), were lower than those in the control (CO) group ($P < 0.05$, for all comparisons). Resting diastolic arterial blood pressure (DBP) values in the rats fed lard (LA) or structured lipid (SL) were lower than those in the control (CO) group ($P < 0.05$, for both comparisons). The pulsatile pressure (PP) values in the rats fed lard (LA), sesame oil fortified with phytosteryl esters (OP), or structured lipid fortified with phytosteryl ester (LP) were lower than those in the control (CO) group ($P < 0.05$, for both comparisons). Resting heart rate (HR) of the rats fed sesame oil (SO) were higher than the control (CO) rats ($P < 0.05$, for all comparisons).

In essence, the major effects of the high-fat diets were (1) to lower systolic blood pressure and to tend to reduce diastolic and mean arterial blood pressures, and (2) the sesame oil (SO)- or structured lipid (SL)-fed rats displayed a tachycardia. However, it should be noted that the lard (LA)-fed rats showed distinct reductions in systolic, diastolic, pulse and mean arterial blood pressures that were not associated with a substantial tachycardia.

Resting cardiovascular parameters in conscious rats

Resting values for the cardiovascular parameters in the control (CO) SH rats and those fed lard (LA), sesame oil (SO), structured lipid (SL), sesame oil fortified with phytosteryl esters (OP), or structured lipid fortified with phytosteryl ester (LP) are summarized in Fig. 6.6. Resting parameters in the control (CO) rats are similar to those reported previously (see Lewis et al., 2005, 2006a,b). As can be seen, resting HR values in all high-fat dietary groups were substantially higher than in the control rats ($P < 0.05$, for all comparisons). However, resting blood pressure values, i.e., systolic arterial blood pressure (SBP), diastolic arterial blood pressure (DBP), pulsatile arterial blood pressure (PP) and mean arterial blood pressure (MAP) in all high-fat dietary groups were similar to those in the control group ($P > 0.05$, for all comparisons), with one exception. Specifically, the pulse pressure (PP) value for the group fed structured lipid fortified with phytosteryl ester (LP) was lower than that of the control group ($P < 0.05$).

DISCUSSION

Effects of positional distribution of fatty acid on plasma lipid profiles

Some previous studies proposed that a specific FA had a different effect on cholesterolemia and atherosclerosis according to its position in TAG molecule. Kritchevsky et al. (2000, 1998a,b) found that increased presence of palmitic acid known as hypercholesterolemic FA at the *sn*-2 position of a fat enhanced the atherogenic properties of that fat in rabbits. The FAs at the *sn*-2 position of the TAG are preferentially absorbed and stored in human bodies compared to the FAs located at the *sn*-1,3 positions since TAGs are hydrolyzed into FAs and 2-

152

monoacylglycerols (2-MAGs) by lipoprotein lipase and 2-MAGs readily form mixed micelles with bile salts (Kubow, 1996; Jandacek et al., 1987). The difference in metabolic aspects of FAs by positional distribution in TAG also suggests that lower content of palmitic acid at the *sn*-2 position may reduce its hypercholesterolemic or atherogenic effects. However, in the studies of Kritchevsky et al. described above, the plasma lipid profiles were not affected by the difference in the positional distribution of palmitic acid.

Our study showed that the palmitic acid was mostly located at the *sn*-2 position in lard; whereas, few palmitic acids were found at that position in sesame oil and structured lipid. Moreover, sesame oil and structured lipid had lower total palmitic acid content than lard. On the other hand, oleic acid is known to lower the levels of plasma total and LDL cholesterols when exchanged for SFAs (Hayes, 2000). Lard had higher total content of oleic acid than sesame oil and structured lipid. However, oleic acid present in lard is predominantly located at *sn*-1,3 positions. On the contrary, sesame oil and structured lipid showed higher contents of oleic acid at *sn*-2 position than lard even though their total oleic acid contents were lower than that of lard. Nonetheless the plasma lipid profiles in the rats fed lard were not substantially different from the rats fed sesame oil or structured lipid. Our results suggest that there were no significant effects of positional distribution of FA on the plasma lipid profiles in SH rats.

Effects of medium chain fatty acid on plasma lipid profiles

Cater et al. (1997) suggested that MCFAs including caprylic acid might be hypercholesterolemic FA since it tended to increase the plasma LDL cholesterol level in humans relative to oleic acid even though it slightly lowered the plasma LDL cholesterol level compared to palmitic acid. In addition, high amount (43% of diet calorie) of MCFAs slightly increased the

153

plasma TAG level compared to high oleic acid oil. However, in our study, no significant differences were found between the plasma lipid profiles in rats fed structured lipid or structured lipid fortified with phytosteryl ester and those in rats fed sesame oil or sesame oil fortified with phytosteryl ester.

On the contrary, structured lipid and structured lipid fortified with phytosteryl ester tended to decrease the plasma TAG levels compared to control diet and lard. The caprylic acid content of our experimental diets for SL and LP dietary groups were approximately 18% of diet calorie.

Effects of phytosteryl esters on plasma lipid profiles

The diets containing phytosteryl esters (i.e., sesame oil fortified with phytosteryl esters or structured lipid fortified with phytosteryl esters) significantly increased plasma HDL cholesterol levels in the SH rats. As a result, plasma total cholesterol levels were shown to increase slightly in rats fed sesame oil fortified with phytosteryl esters or structured lipid fortified with phytosteryl esters compared to those fed other high-fat diets; however, the differences were not significant. Moreover, the plasma HDL/total cholesterol ratios in rat fed phytosteryl esters were similar to that in control rats. These results indicate that phytosteryl esters have beneficial effects on the blood cholesterol profiles as reported by others. However, the beneficial effects of phytosteryl esters in SH rats were caused by increased plasma HDL cholesterol concentration as opposed to its hypocholesterolemic effects from reduced plasma total and LDL cholesterol levels in humans as mentioned before.

Body and organ weights

As expected, the body weights of the rats fed the high fat diets were substantially higher than the control rats. Moreover, the liver weights and liver weight/body weight ratios were higher in all high-fat diet groups compared to control rats. In contrast, the heart weights and kidney weights and organ weight/body weight ratios of the high fat diet groups were not different from the controls. The heart weights of SH rats were higher than normotensive control rats (see Lewis et al., 2005, 2006a,b). This hypertrophy is a direct result of the higher blood pressures in SH rats (see Lewis et al., 2005, 2006a,b). Accordingly, the lack of effect of the high fat diets on heart weights in SH rats is in line with the lack of major effects of the diets in conscious SH rats (see below).

Effects of high fat diets on cardiovascular parameters

The finding that resting systolic arterial blood pressures of the high-fat dietary groups was lower than that of the controls under *pentobarbital anesthesia* suggests that arterial compliance was *increased* by these diets under these conditions. This is a highly provocative finding and suggests that the timing and duration of high fat feeding as well as the state of consciousness of the animal plays a major role in the expression of the changes in cardiovascular status. The finding that resting arterial blood pressures of the *conscious* high-fat dietary groups was not different to the control group is also a highly provocative finding since there is some, although not convincing evidence that high fat diets increase arterial pressures in normotensive and SH rats. The finding that the resting heart rates of the high-fat dietary groups were higher than those of the control rats suggests that these diets have an important effect on cardiac pacemaker activity. Whether this is due to direct effects on the pacemaker cells (i.e., effects on

155

intrinsic pacemaker activity) or effects on the release of neurotransmitters (i.e., norepinephrine from cardiac sympathetic nerves and acetylcholine from cardiovagal nerves) and/or their signaling mechanisms (i.e., β-adrenoceptors for norepinephrine and muscarinic receptors for acetylcholine) remains to be determined.

References

Akoh, CC. Structured Lipids. In *Food lipids–Chemistry, Nutrition, and Biotechnology*, 2nd ed.; Akoh, CC, Min DB, Eds.; Marcel Dekker: New York, NY, 2002; pp. 877-908.

Bach, AC, Babayan, VK. Medium-chain triglycerides: an update. *Am. J. Clin. Nutr.* 1982, *36,* 950-962.

Beveridge, JM, Haust, HL, Connel, WF. Magnitude of the hypocholesterolemic effect of dietary sitosterol in man. *J. Nutr.* 1964, *83*, 119-122.

Bray, GA, Lee, M, Bray, TL. Weight gain of rats fed medium-chain triglycerides is less than rats fed long-chain triglycerides. *Int. J. Obes.* 1980, *4*, 27-32.

Cater, NB, Heller, HJ, Denke, MA. Comparison of the effects of medium-chain triacylglycerols, palm oil, and high oleic sunflower oil on plasma triacylglycerol fatty acid and lipid and lipoprotein concentrations in humans. *Am. J. Clin. Nutr.* 1997, 65, 41-45.

Hayes, DG, Gulari, E. Formation of polyol-fatty acid esters by lipases in reverse micellar media. *Biotechnol. Bioeng.* 1992, 40, 110-118.

Hendriks, HFJ., Weststrate, JA, van Vliet, T, Meijer, GW. Spreads enriched with three different levels of vegetable oil sterols and the degree of cholesterol lowering in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur. J. Clin. Nutr.* 1999, *53*, 319-327.

Iwasaki, Y, Yamane, T. Enzymatic synthesis of structured lipids. *J. Mol. Catal. B-Enz.,* 2000, *10,* 129-140.

Jandacek, RJ, Whiteside, JA, Holcombe, BN, Volpenhein, RA, Taulbee, JD. The rapid hydrolysis and efficient absorption of triglycerides with octanoic acid in the 1 and 3-positions and long chain fatty acid in the 2-position. *Am. J. Clin. Nutr.* 1987, *45*, 940-945.

Jones, PJH, MacDougall, DE, Ntanios, F, Vanstone, CA. Dietary phytosterols as cholesterol-lowering agents in humans. *Can. J. Physiol. Pharmacol.* 1997, *75*, 217-227.

Katan, MB, Grundy, SM, Jones, P, Law, M, Miettinen, T, Paoletti, R. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. *Mayo Clin. Proc.* 2003, *78*, 965-978.

Kritchevsky, D, Tepper, SA, Kuksis, A, Wright, S,Czarnecki, SK. Cholesterol vehicle in experimental atherosclerosis. 22. Refined, bleached, deodorized (RBD) palm oil, randomized palm oil and red palm oil. *Nutr. Res.* 2000, *20*, 887-892.

Kritchevsky, D, Tepper, SA, Kuksis, A, Eghtedary, K, Klurfeld, DM. Cholesterol vehicle in experimental atherosclerosis. 21. Native and randomized lard and tallow. *J. Nutr. Biochem.* 1998a, *9*, 582-585.

Kritchevsky, D, Tepper, SA, Wright, S, Kuksis, A, Hughes, TA. Cholesterol vehicle in experimental atherosclerosis. 20. Cottonseed oil and randomized cottonseed oil. *Nutr. Res.* 1998b, *18*, 259-264.

Kubow, S. The influence of positional distribution of fatty acids in native, interesterified and structure-specific lipids on lipoprotein metabolism and atherogenesis. *J. Nutr. Biochem.* 1996, *7*, 530-541.

Law, M. Plant sterol and stanol margarines and health. *Br. Med. J.*, 2000, *320*, 861-864.

Lees, AM, Mok, HY, Lees, RS, McCluskey, MA, Grundy, SM. Plant sterols as cholesterol-lowering agents: clinical trials in patients with hypercholesterolemia and studies of sterol balance. *Atherosclerosis* 1977, *28*, 325-338.

Lewis, SJ, Bhopatkar, MY, Walton TM, Bates, JN. Role of voltage-sensitive calcium-channels in nitric oxide-mediated vasodilation in spontaneously hypertensive rats. *Eur. J. Pharmacol.*

2005, *528*, 144-9.

Lewis, SJ, Hashmi-Hill MP, Owen, JR, Sandock, K, Robertson, TP, Bates, JN. The vasodilator potency of the endothelium-derived relaxing factor, L S-nitrosocysteine, is impaired in Spontaneously Hypertensive rats. Relevance to the identity of EDRF. *Vascul. Pharmacol.*, 2006a, in press.

Lewis, SJ, Hashmi-Hill, MP, Owen, JR, Sandock, K, Robertson, TP, Bates, JN. ACE inhibition restores the vasodilator potency of the endothelium-derived relaxing factor, L-S-nitrosocysteine, in Spontaneously Hypertensive rats. *Vascul. Pharmacol.*, 2006b, in press.

Ling, WH, Jones, PJH. Dietary phytosterols: a review of metabolism, benefits and side effects. *Life Sci.* 1995*, 57*, 195-206.

Luddy, FE, Barford, RA, Herb, SF, Magidman, P, Riemenschneider, RW. Pancreatic Lipase Hydrolysis of Triglycerides by a Semimicro Technique. *J. Am. Oil Chem. Soc.* 1964, *41*, 693-696.

Miettinen, TA, Gylling, H. Plant stanol and sterol esters in prevention of cardiovascular diseases. *Ann. Med.* 2004*, 36*, 126-134.

Miettinen, TA, Puska, P, Gylling, H, Vanhanen, H, Vartiainen, E. Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population. *N. Engl. J. Med.* 1995*, 333*, 1308-1312.

Neil, HA, Meijer, GW, Roe, LS. Randomised controlled trial of use by hypercholesterolaemic patients of a vegetable oil sterol-enriched fat spread. *Atherosclerosis*, 2001, *156*, 329-337.

Nestel, P, Cehun, M, Pomeroy, S, Abbey, M, Weldon, G. Cholesterol-lowering effects of plant sterol esters and non-esterified stanols in margarine, butter and low-fat foods. *Eur. J. Clin. Nutr.* 2001*, 55*, 1084-1090.

Noakes, M, Clifton, P, Ntanios, F, Shrapnel, W, Record, I, McInerney, J. An increase in dietary carotenoids when consuming plant sterols or stanols is effective in maintaining plasma carotenoid concentrations. *Am. J. Clin. Nutr.* 2002*, 75*, 79-86.

Pollak, OJ. 1953. Successive prevention of experimental hypercholesteremia and cholesterol atherosclerosis in the rabbit. *Circulation* 1953, *7*, 696-701.

Trautwein, EA, Duchateau, GSMJE, Lin, YG, Mel'nikov, SM, Molhuizen, HOF, Ntanios FY. Proposed mechanisms of cholesterol-lowering action of plant sterols. *Eur. J. Lipid Sci. Technol.* 2003, *105*, 171-185.

Wallenstein, S; Zucker, CL; Fleiss, JC. Some statistical methods useful in circulation research. *Circ. Res.* 1980, *47*, 1-9.

Wester, I. Cholesterol-lowering effect of plant sterols. *Eur. J. Lipid Sci. Technol.* 2000, *102*, 37-44.

Weststrate, JA, Meijer, GW. Plant sterol-enriched margarines and reduction of plasma total- and LDL-cholesterol concentrations in normocholesterolaemic and mildly hypercholesterolaemic subjects. *Eur. J. Clin. Nutr.* 1998, *52*, 334-343.

Winer, BJ. *Statistical Principles of Experimental Design*; McGraw-Hill, New York, NY, 1971, pp 752-809.

Xu, X, Balchen, S, Høy, CE, Alder-Nissen, J. Pilot batch production of specific-structured lipids by lipase-catalyzed interesterification: Preliminary study on incorporation and acyl migration. *J. Am. Oil Chem. Soc.* 1998, *75,* 301-308.

Table 6.1. Composition of Experimental Diets

Ingredient	CO		High-fat diet									
			LA		SO		SL		OP		LP	
	Weight	Calorie	Weight	Calorie	Weight	Calorie	Weight	Calorie	Weight	Calorie	Weight	Calorie
	g/kg	%	g/kg	%	g/kg	%	g/kg	%	g/kg	%	g/kg	%
Protein	234	28	227	20	227	20	227	20	227	20	227	20
Carbohydrate	499	60	459	40	459	40	459	40	459	41	459	41
Fat	45	12	200	40	200	40	200	40	194.6	39	194.6	39
(kcal/g)	(3.34)		(4.54)		(4.54)		(4.54)		(4.50)		(4.50)	
Casein			224		224		224		224		224	
L-Cystine			3		3		3		3		3	
Corn starch			112		112		112		112		112	
Maltodextrin			112		112		112		112		112	
Sucrose	Laboratory Rodent		224		224		224		224		224	
Cellulose	Diet 5001 (Labdiet,		56		56		56		56		56	
Lard (LA)	Brentwood, MO,		200		-		-		-		-	
Sesame oil (SO)	USA)[a]		-		200		-		194.6		-	
Structured lipid (SL)			-		-		200		-		194.6	
Phytosteryl ester (PE)			-		-		-		5.4		5.4	
Cholesterol			5.6		5.6		5.6		5.6		5.6	
Minerals[b]			50		50		50		50		50	
Vitamins[c]			13		13		13		13		13	

Abbreviations: CO = control; LA = lard; SO = sesame oil; SL = structured lipid; OP = sesame oil fortified with phytosteryl ester; LP = structured lipid fortified with phytosteryl ester.

[a] Ingredients: ground corn, dehulled soybean meal, dried beet pulp, fish meal, ground oats, brewers dried yeast, cane molasses, dehydrated alfalfa meal, dried whey, wheat germ, porcine meat meal, wheat middlings, animal fat preserved with BHA, salt, calcium carbonate, choline chloride, cholecalciferol, vitamin A acetate, folic acid, pyridoxine hydrochloride, DL-methionine, thiamin mononitrate, calcium pantothenate, nicotinic acid, dl-alpha tocopheryl acetate, cyanocobalamin, riboflavin, ferrous sulfate, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, cobalt carbonate.

[b] Comprised of RD-96 Salt Mix without calcium, phosphorus, and potassium, 11 g/kg; dicalcium phosphate, 14 g/kg; calcium carbonate, 6 g/kg; potassium citrate monohydrate, 19 g/kg. RD-96 Salt Mix without calcium, phosphorus, and potassium contains the following (g/kg): sodium chloride, 259.0; magnesium oxide, 41.9; magnesium sulfate, 257.6; chromium potassium sulfate, 1.925; cupric carbonate, 1.05; sodium fluoride, 0.2; potassium iodate, 0.035; ferric citrate, 21.0; manganous carbonate, 12.25; ammonium molybdate, 0.3; sodium selenite, 0.035; zinc carbonate, 5.6; sucrose, 399.105.

[c] Comprised of Vitamin Mix for AIN-76A Rodent Diet, 11 g/kg; choline bitartrate 2 g/kg. Vitamin Mix for AIN-76A Rodent Diet contains the following (g/kg): vitamin A palmitate (500,000 IU/g), 0.8; vitamin D_3 (100,000 IU/g), 1.0; vitamin E acetate (500 IU/g), 10.0; menadione sodium bisulfite, 0.08; biotin (1%), 2.0; cyancocobalamin (0.1%), 1.0; folic acid, 0.2; nicotinic acid, 3.0; calcium pantothenate, 1.6; pyridoxine HCl, 0.7; riboflavin, 0.6; thiamin HCl, 0.6; sucrose, 978.42.

Table 6.2. Fatty Acid Composition of Total, s*n*-2, and *sn*-1,3 Positions of TAG of Lard, Sesame Oil and Structured Lipid (mol%)

FA	Total				sn-2				sn-1,3[a]			
	Control	Lard	Sesame oil	SL[b]	Control	Lard	Sesame oil	SL	Control	Lard	Sesame oil	SL
C8:0	-	-	-	45.8 ± 0.2	-	-	-	3.7 ± 0.1	-	-	-	66.8 ± 0.2
C10:0	-	0.2 ± 0.0	-	-	-	0.3 ± 0.0	-	-	-	0.2 ± 0.0	-	-
C12:0	-	0.2 ± 0.0	trace[c]	-	-	0.3 ± 0.0	-	-	-	0.1 ± 0.0	trace	-
C14:0	1.8 ± 0.1	2.1 ± 0.0	trace	-	-	4.7 ± 0.0	-	-	2.7 ± 0.1	0.7 ± 0.0	trace	-
C14:1	-	0.1 ± 0.0	-	-	-	0.2 ± 0.0	-	-	-	-	-	-
C16:0	20.2 ± 0.4	23.4 ± 0.4	10.0 ± 0.0	3.1 ± 0.1	34.7 ± 1.1	68.5 ± 0.2	1.1 ± 0.0	1.5 ± 0.0	12.9 ± 0.1	0.9 ± 0.5	14.4 ± 0.0	4.0 ± 0.1
C16:1	3.0 ± 0.0	3.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	-	3.6 ± 0.1	0.1 ± 0.0	Trace	4.4 ± 0.0	3.1 ± 0.2	0.1 ± 0.0	0.1 ± 0.0
C18:0	3.7 ± 0.1	7.2 ± 0.3	3.6 ± 0.0	1.1 ± 0.0	3.7 ± 0.3	1.7 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	3.6 ± 0.3	9.9 ± 0.4	5.3 ± 0.0	1.5 ± 0.0
C18:1*n-9*	27.2 ± 0.4	46.4 ± 0.1	37.0 ± 0.0	19.4 ± 0.1	19.2 ± 1.2	15.2 ± 0.1	40.2 ± 0.4	36.4 ± 0.0	31.3 ± 0.0	62.1 ± 0.1	35.4 ± 0.2	11.0 ± 0.1
C18:2*n-6*	26.5 ± 0.6	16.4 ± 0.1	48.4 ± 0.0	30.0 ± 0.0	34.5 ± 0.3	5.1 ± 0.4	57.3 ± 0.5	57.2 ± 0.1	22.5 ± 0.8	22.1 ± 0.1	44.0 ± 0.2	16.3 ± 0.0
C18:3*n-3*	17.6 ± 1.6	0.7 ± 0.0	0.9 ± 0.0	0.5 ± 0.0	7.9 ± 2.2	0.4 ± 0.0	1.0 ± 0.0	0.8 ± 0.0	22.5 ± 1.2	0.9 ± 0.0	0.8 ± 0.0	0.3 ± 0.0
SFA[d]	25.6 ± 0.6	33.1 ± 0.1	13.6 ± 0.0	50.1 ± 0.1	38.4 ± 0.8	75.5 ± 0.1	1.5 ± 0.1	5.6 ± 0.1	19.2 ± 0.5	11.8 ± 0.1	19.7 ± 0.0	72.3 ± 0.1
USFA[e]	74.4 ± 0.6	66.9 ± 0.1	86.4 ± 0.0	49.9 ± 0.1	61.6 ± 0.8	24.5 ± 0.1	98.5 ± 0.1	94.4 ± 0.1	80.8 ± 0.5	88.2 ± 0.0	80.3 ± 0.0	27.7 ± 0.1

Data are presented as mean ± SEM; n = 2.

[a] *sn*-1,3 (mol%) = [3 × Total (mol%) – *sn*-2 (mol%)] / 2

[b] Structured lipid prepared by acidolysis of sesame oil with caprylic acid in bench-scale continuous packed bed reactor.

[c] < 0.05 mol%.

[d] Saturated FA.

[e] Unsaturated FA.

Table 6.3. Diet Intake, Weight Gain and Food Efficiency Ratio

	Diet group	Week								
		1	2	3	4	5	6	7	8	9
Diet intake (g/day)	CO	6 ± 1	10 ± 1	12 ± 1	13 ± 1	18 ± 1	19 ± 1	20 ± 1	21 ± 1	22 ± 1
	LA	7 ± 1	10 ± 1	13 ± 1	14 ± 1	16 ± 1	16 ± 1	16 ± 1	17 ± 2	17 ± 1
	SO	6 ± 1	10 ± 1	12 ± 1	14 ± 1	15 ± 1	16 ± 1	16 ± 1	17 ± 1	16 ± 1
	SL	6 ± 1	10 ± 1	12 ± 1	14 ± 1	15 ± 1	16 ± 1	16 ± 1	16 ± 1	16 ± 1
	OP	7 ± 1	10 ± 1	11 ± 1	13 ± 1	15 ± 1	15 ± 1	15 ± 1	16 ± 1	15 ± 1
	LP	7 ± 1	11 ± 1	13 ± 1	15 ± 1	16 ± 1	16 ± 1	16 ± 1	16 ± 1	16 ± 1
Weight gain (g/week)	CO	22 ± 2	29 ± 2	32 ± 2	36 ± 2	36 ± 2	36 ± 2	28 ± 2	22 ± 2	22 ± 2
	LA	29 ± 2*	49 ± 4*	42 ± 2*	46 ± 2*	45 ± 4	34 ± 2	28 ± 2	27 ± 2	20 ± 2
	SO	28 ± 3*	44 ± 2*	37 ± 2	46 ± 2*	42 ± 3	35 ± 3	28 ± 2	27 ± 2	17 ± 2
	SL	29 ± 3*	47 ± 4*	37 ± 3	46 ± 3*	37 ± 2	33 ± 2	25 ± 3	25 ± 2	18 ± 3
	OP	30 ± 4*	41 ± 3*	35 ± 2	44 ± 2*	39 ± 4	31 ± 2	25 ± 2	24 ± 2	16 ± 2
	LP	32 ± 4*	48 ± 3*	38 ± 2*	46 ± 3*	38 ± 2	32 ± 2	26 ± 2	22 ± 2	15 ± 2
FER[a]	CO	53 ± 2	42 ± 4	38 ± 2	41 ± 2	29 ± 2	27 ± 2	21 ± 2	15 ± 3	14 ± 2
	LA	64 ± 2*	70 ± 10*	47 ± 3*	46 ± 4	40 ± 3	30 ± 1	24 ± 1	23 ± 2	17 ± 2
	SO	63 ± 1*	65 ± 9*	45 ± 2*	49 ± 1*	39 ± 2	32 ± 2	25 ± 2	23 ± 3	15 ± 2
	SL	64 ± 2*	66 ± 12*	43 ± 3	47 ± 3	35 ± 2	30 ± 2	23 ± 2	22 ± 2	17 ± 2
	OP	62 ± 3*	60 ± 7*	43 ± 2	48 ± 3	37 ± 3	30 ± 2	24 ± 2	22 ± 3	14 ± 2
	LP	64 ± 4*	64 ± 9*	42 ± 2	45 ± 3	35 ± 3	29 ± 3	23 ± 3	20 ± 2	14 ± 3

Data are presented as mean ± SEM; n = 6.

Abbreviations: CO = control; LA = lard; SO = sesame oil; SL = structured lipid; OP = sesame oil fortified with phytosteryl ester; LP = structured lipid fortified with phytosteryl ester.

[a] Food efficiency ratio; FER = (Weight gain for 1 week / Diet intake for 1 week) × 100.

* $P < 0.05$, high fat-fed rats *versus* control.

Table 6.4. Total Diet Intake, Weight Gain and Food Efficiency Ratio

	Diet group					
	CO	LA	SO	SL	OP	LP
Diet intake (g/day)	16 ± 1	16 ± 1	15 ± 1	15 ± 1	15 ± 1	16 ± 1
Weight gain (g/9 weeks)	263 ± 4	$319 \pm 9*$	$303 \pm 8*$	$296 \pm 9*$	$284.0 \pm 7*$	$296 \pm 7*$
FER[a]	16 ± 1	$36 \pm 1*$	$36 \pm 1*$	$35 \pm 1*$	$35 \pm 1*$	$34 \pm 1*$

Data are presented as mean $\pm$ SEM; n = 6.

Abbreviations: CO = control; LA = lard; SO = sesame oil; SL = structured lipid; OP = sesame oil fortified with phytosteryl ester; LP = structured lipid fortified with phytosteryl ester.

[a] Food efficiency ratio; FER = Weight gain for 9 weeks / Diet intake for 9 weeks.

* $P < 0.05$, high fat-fed rats *versus* control.

Fig. 6.1. Dietary effects on final body weights. Abbreviations: CO = control; LA = lard; SO = sesame oil; SL = structured lipid; OP = sesame oil fortified with phytosteryl ester; LP = structured lipid fortified with phytosteryl ester. * $P < 0.05$, *versus* CO.
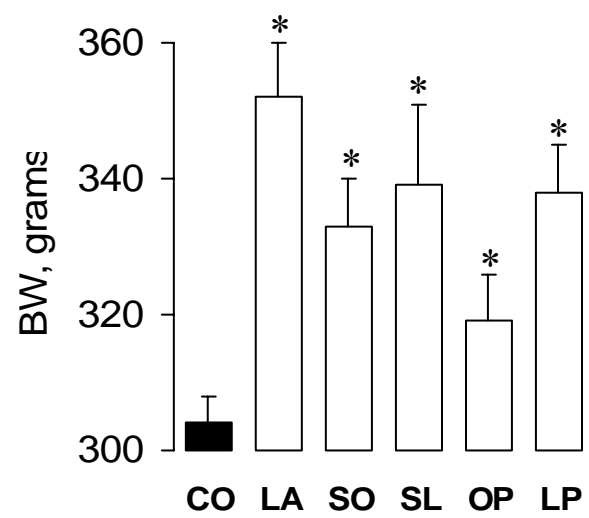
Fig. 6.2. Dietary effects on organ weights and organ weight/body weight ratios: (a) liver weight (LW); (b) liver weight/body weight ratio (LW/BW); (c) heart weight (HW); (d) heart weight/body weight ratio (HW/BW); (e) kidney weight (KW); (f) kidney weight/body weight ratio (KW/BW). Abbreviations: CO = control; LA = lard; SO = sesame oil; SL = structured lipid; OP = sesame oil fortified with phytosteryl ester; LP = structured lipid fortified with phytosteryl ester. * $P < 0.05$, *versus* CO.
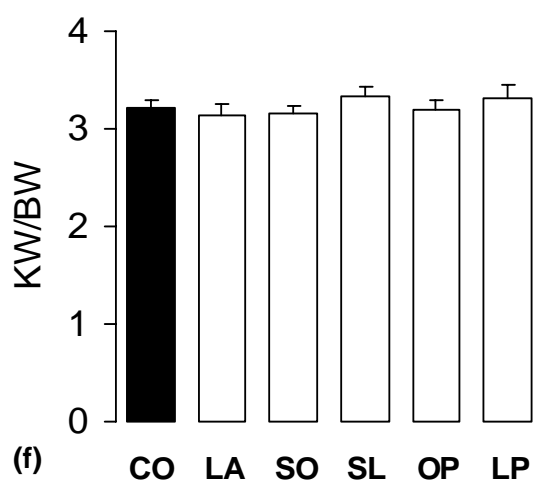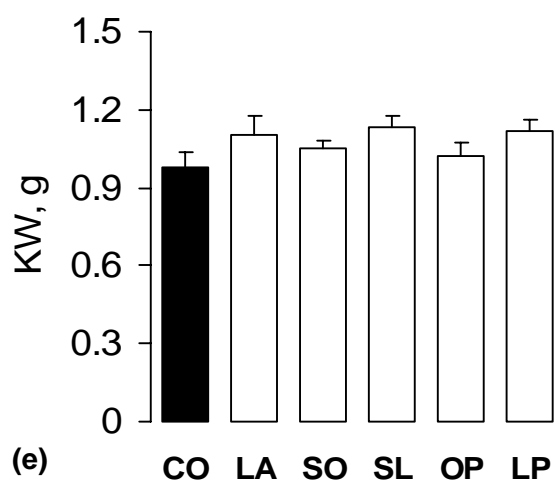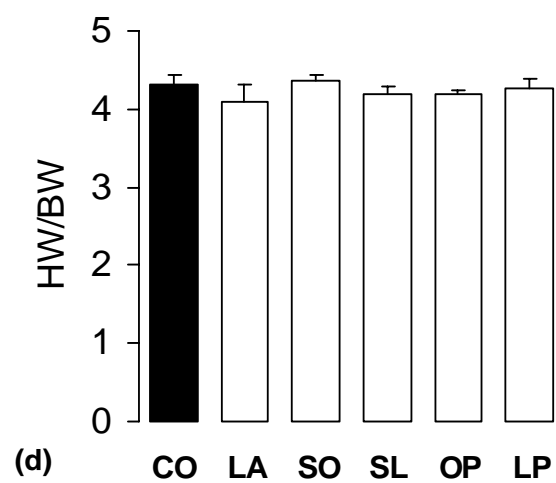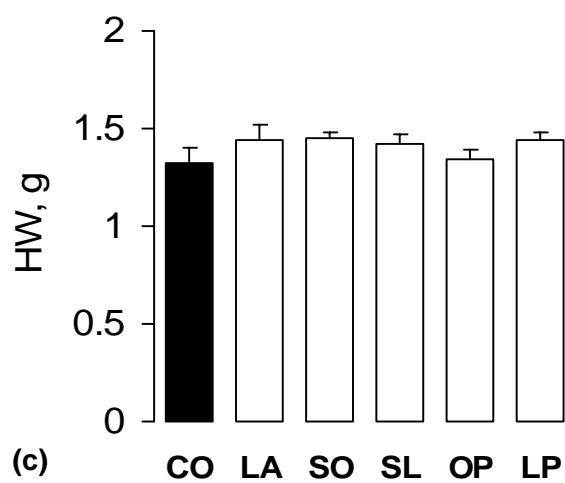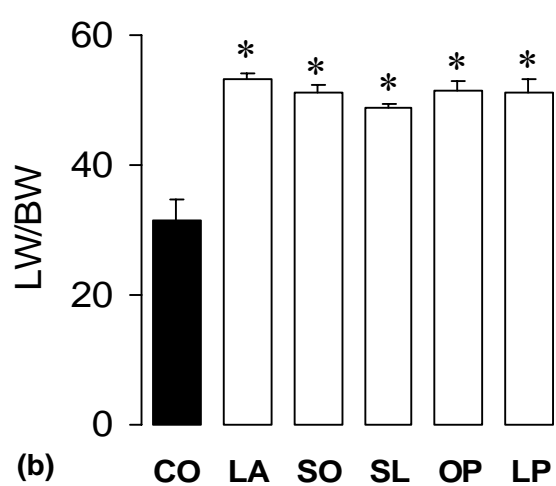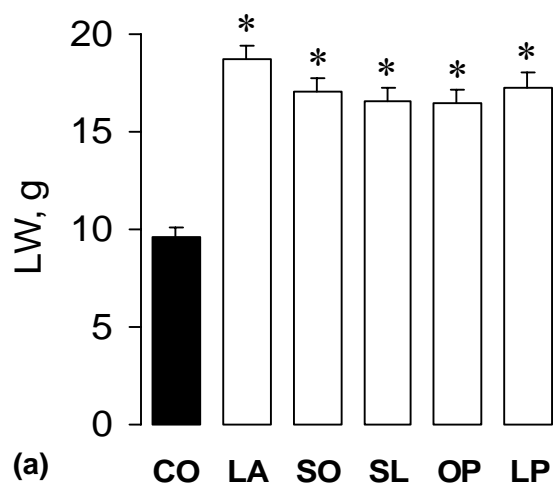
Fig. 6.3. Dietary effects on plasma cholesterol: (a) total cholesterol; (b) HDL cholesterol; (c) LDL cholesterol; (d) HDL/total cholesterol ratio; (e) HDL/LDL cholesterol ratio; (f) LDL/total cholesterol ratio. Abbreviations: CO = control; LA = lard; SO = sesame oil; SL = structured lipid; OP = sesame oil fortified with phytosteryl ester; LP = structured lipid fortified with phytosteryl ester. * $P < 0.05$, *versus* CO. † $P < 0.05$, *versus* LA, SO, and SL.
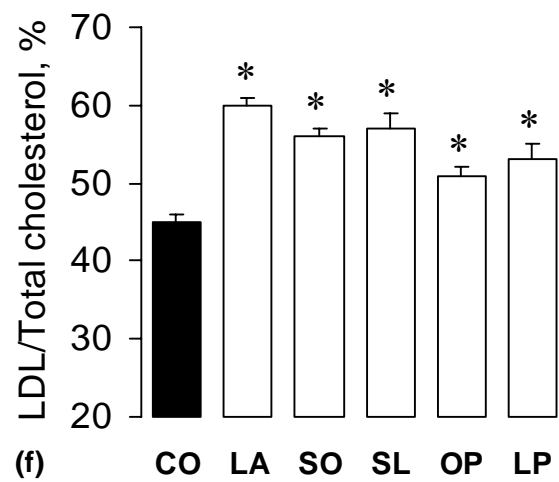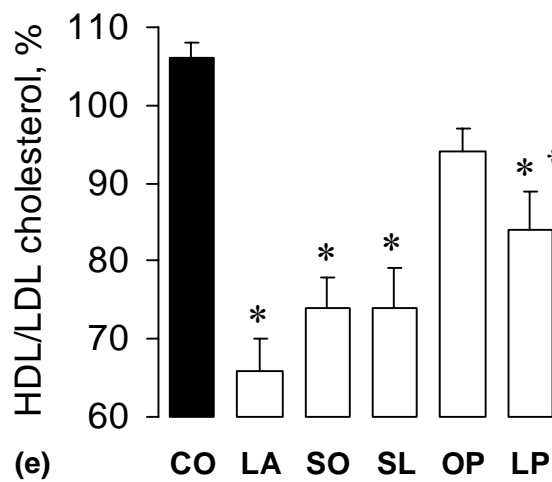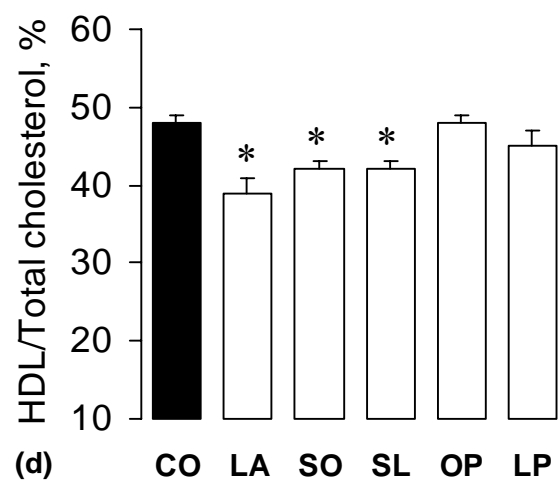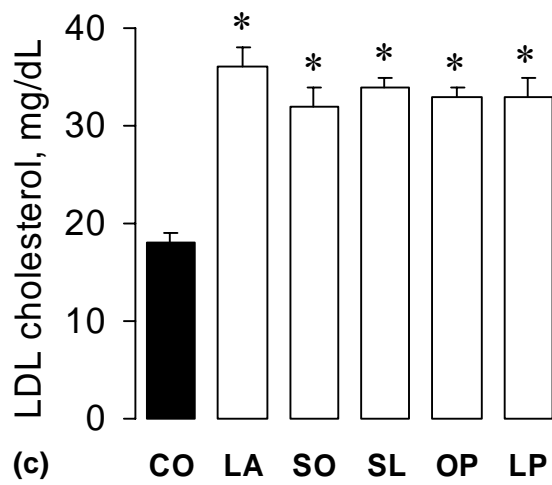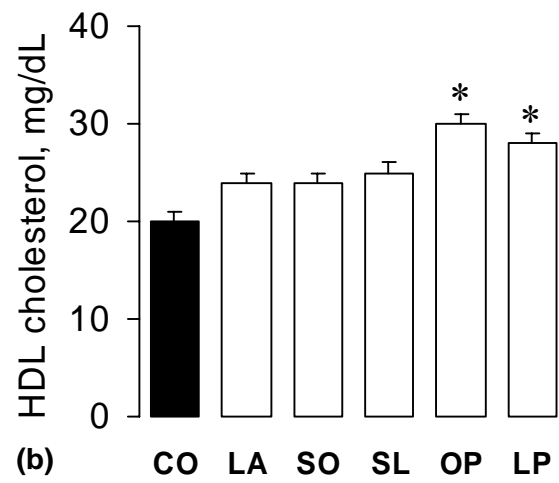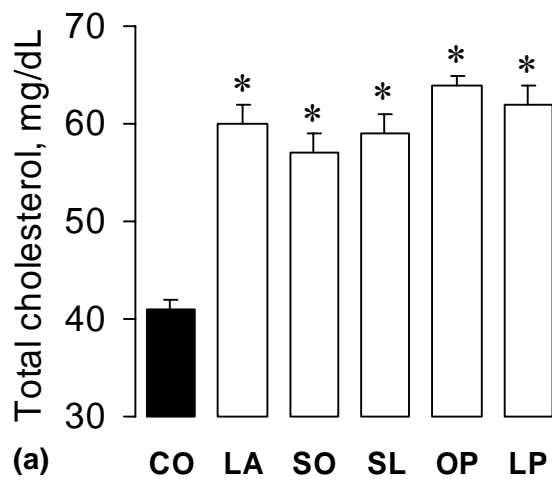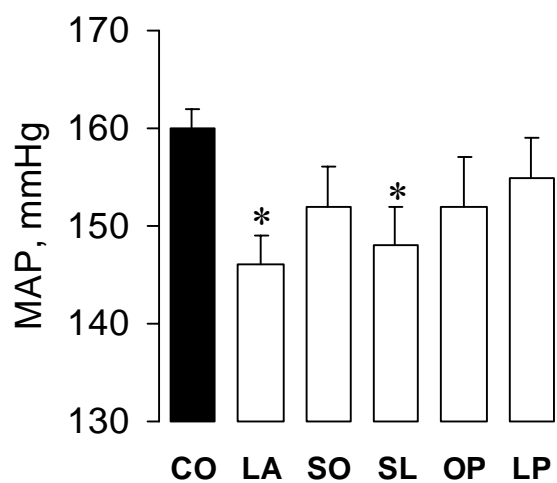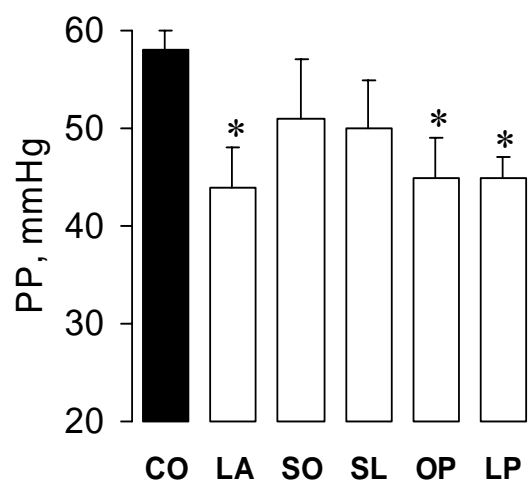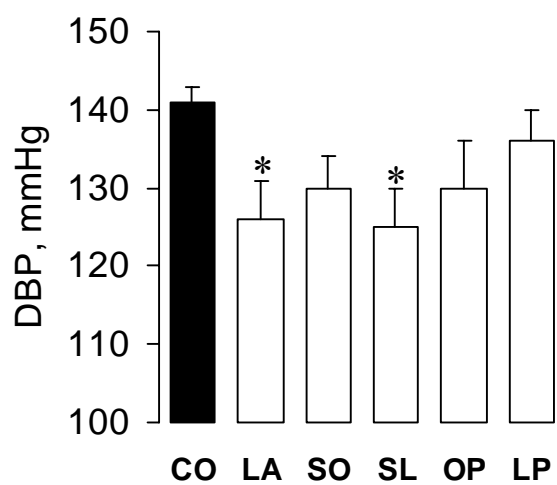
Fig. 6.4. Dietary effects on plasma triacylglycerol and glucose: (a) triacylglycerol; (b) glucose. Abbreviations: CO = control; LA = lard; SO = sesame oil; SL = structured lipid; OP = sesame oil fortified with phytosteryl ester; LP = structured lipid fortified with phytosteryl ester. * $P < 0.05$, *versus* CO.
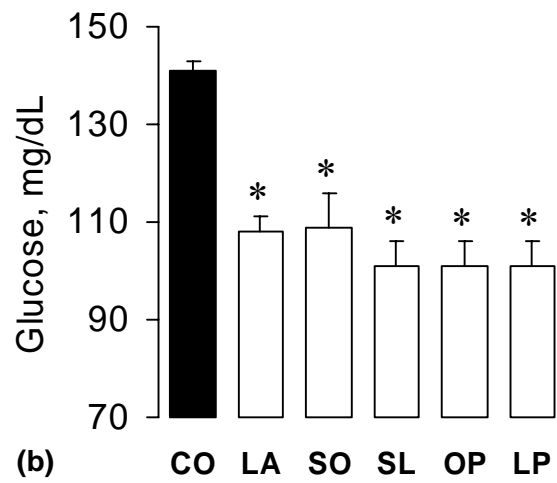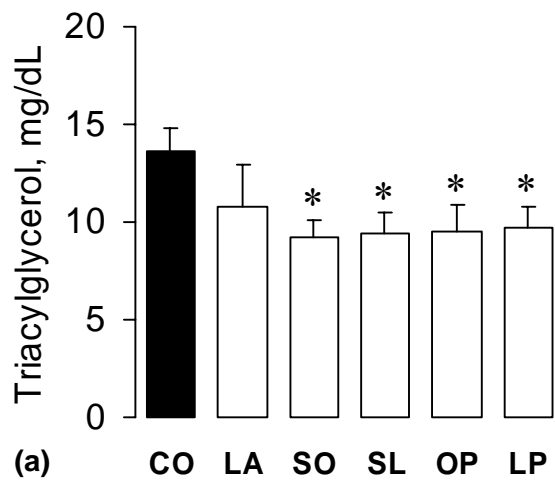
Fig. 6.5. Dietary effects on resting cardiovascular parameters in *anesthetized rats*: (a) systolic arterial blood pressure (SBP); (b) diastolic arterial blood pressure (DBP); (c) pulsatile pressure (PP); (d) mean arterial blood pressure (MAP); (e) heart rate (HR). Abbreviations: CO = control; LA = lard; SO = sesame oil; SL = structured lipid; OP = sesame oil fortified with phytosteryl ester; LP = structured lipid fortified with phytosteryl ester. * *P* < 0.05, *versus* CO.
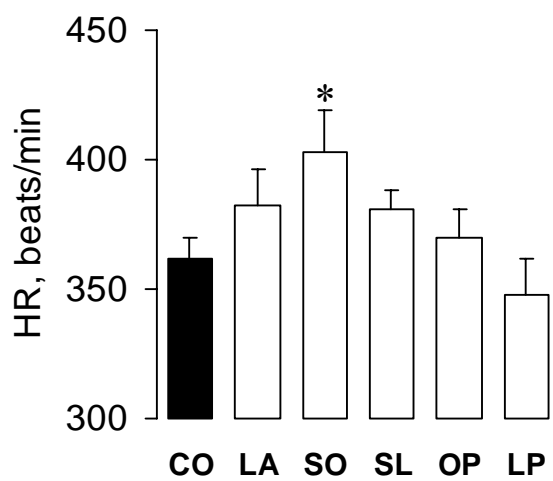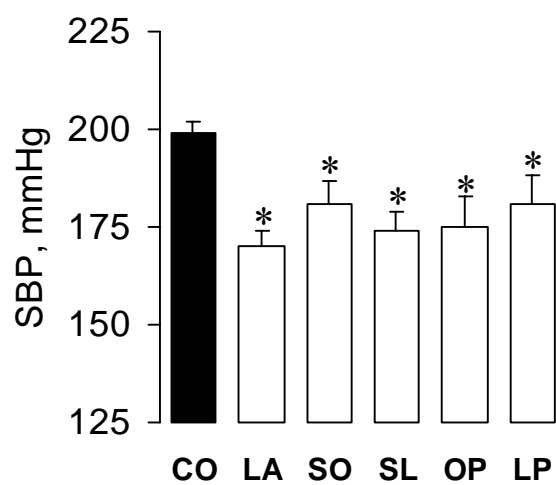
Fig. 6.6. Dietary effects on resting cardiovascular parameters in *conscious rats*: (a) systolic arterial blood pressure (SBP); (b) diastolic arterial blood pressure (DBP); (c) pulsatile pressure (PP); (d) mean arterial blood pressure (MAP); (e) heart rate (HR). Abbreviations: CO = control; LA = lard; SO = sesame oil; SL = structured lipid; OP = sesame oil fortified with phytosteryl ester; LP = structured lipid fortified with phytosteryl ester. * $P < 0.05$, *versus* CO.
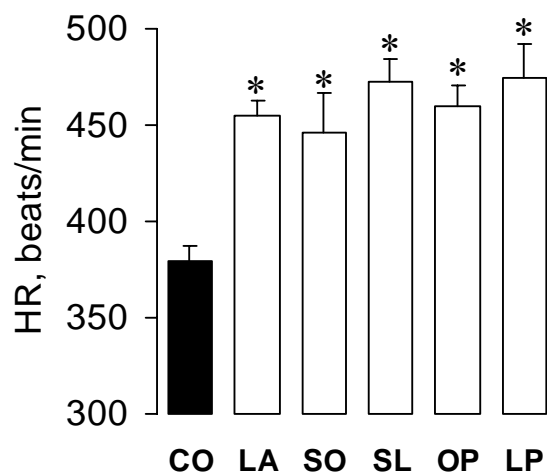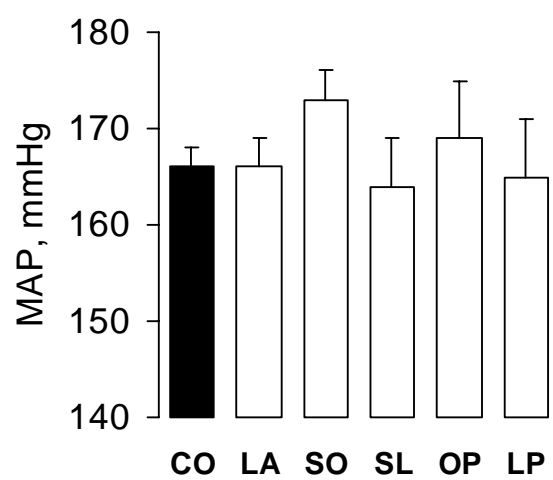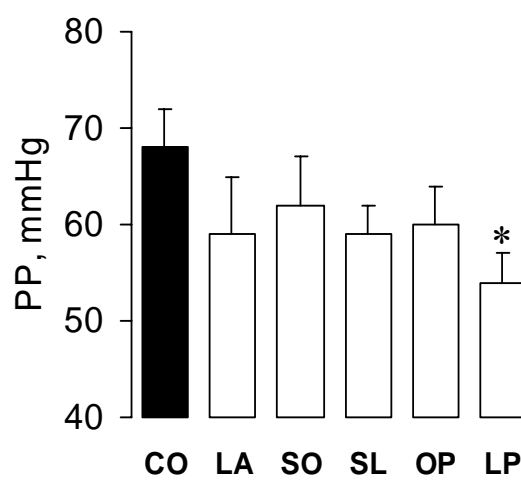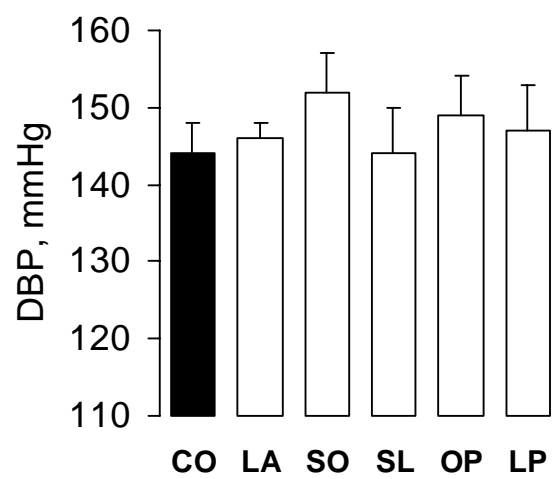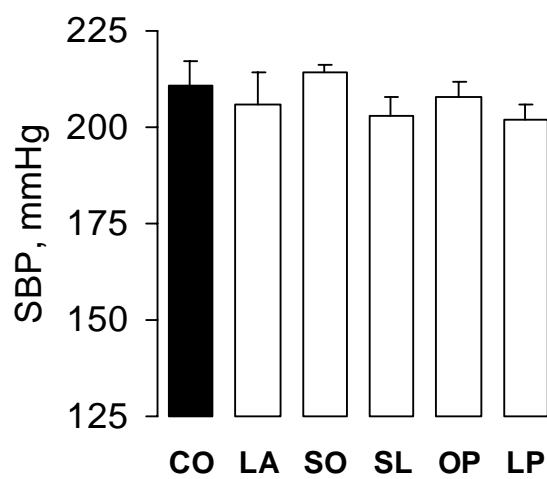
CHAPTER 7

SUMMARY AND CONCLUSIONS

In this study two kinds of modified lipids were synthesized by enzymatic methods: one is structured lipids (SLs) and the other is phytosteryl esters (PEs). The reactions were modeled by response surface methodology, respectively, and their optimal synthesis conditions were established using the models, respectively. Then, their dietary effects on the blood lipid profiles and cardiovascular parameters were investigated in an animal model using spontaneously hypertensive (SH) rats.

The SLs were synthesized from roasted sesame oil and caprylic acid by acidolysis reaction catalyzed by a *sn*-1,3 specific lipase from *Rhizomucor miehei*. The SLs were the MLM-type structured TAGs (MLM-SLs) where medium chain FAs (MCFAs) were esterified at *sn*-1,3 positions and long chain FA (LCFAs) were located at *sn*-2 position of the glycerol backbone. The reaction factors investigated for modeling the reaction were temperature (45-65 ℃), reaction time (18-30 h), and substrate molar ratio (4-8; caprylic acid/sesame oil). All factors investigated positively affected total incorporation of caprylic acid into sesame oil. For controlled acyl migration of caprylic acid in the SL, which is a side-reaction forming untargeted SLs, such as MML-, LMM-, LML-, and MMM-SLs, temperature showed the greatest positive effect. However, there was no effect of substrate molar ratio, indicating that the substrate molar ratio should be kept as high as possible (i.e., 6.0) and relatively low temperature (i.e, 45.0 ℃) was required to maximize total incorporation and to minimize acyl migration. Furthermore, beyond *ca.* 55 mol% of total incorporation, acyl incorporation into *sn*-1,3 positions started to decrease, and acyl migration increased rapidly, suggesting that total incorporation should be kept below *ca.* 55 mol% to prevent decrease in quality and yield of targeted MLM-SLs.

The SLs were further produced in a bench-scale continuous packed bed reactor under the optimal reaction conditions established above. Total incorporation and acyl migration of caprylic

178

acid in the SL were 42.5 mol% and 3.1 mol%, respectively, and the half-life of the lipase was 19.2 days. The SLs displayed different physical and chemical properties: less saturated dark brown color, lower viscosity, lower melting and crystallization temperature ranges, higher melting and crystallization enthalpies, higher smoke point, higher saponification value, and lower iodine value, in comparison to unmodified sesame oil. The oxidative stability of purified SL was lower than that of sesame oil. There were no differences in the contents of unsaponifiables including tocopherols and phytosterols. However, total sesame lignans content was decreased in SLs due to the loss of sesamol when compared to sesame oil. Most of the 70 volatiles present in roasted sesame oil were removed from SLs during short-path distillation of SLs. These results indicate that the characteristics of SLs are different from those of original sesame oil in several aspects except for the contents of tocopherols and phytosterols.

The PEs were synthesized from phytosterols and oleic acid by esterification reaction catalyzed by a nonspecific lipase, *Candida rugosa* lipase. The PEs had ester group formed by the linkage with oleic acid at C-3 position of phytosterols. The reaction factors investigated for modeling the reaction were temperature (35-55 ºC), reaction time (4-24 h), substrate molar ratio (1-3; oleic acid/phytosterols), and enzyme amount (2-10%). All the reaction factors investigated positively affected degree of esterification of phytosterols with oleic acid, with reaction time having the greatest effect followed by enzyme amount, substrate molar ratio, and temperature. Optimal reaction conditions were: temperature, 51.3 ºC; reaction time, 17.0 h; substrate molar ratio, 2.1; enzyme amount, 7.2%; and the degree of esterification was 97.0 mol% under these conditions.

The dietary effects of SLs and PEs were investigated on the blood lipid profiles and cardiovascular parameters of SH rats fed high-fat (20% of diet weight) and high-cholesterol

(0.56% of diet weight) diets. There were six dietary groups: control (rats fed normal diet), lard, sesame oil, SL, sesame oil fortified with PE, and SL fortified with PE. The fortified level of PE in diets was 0.54% of diet weight. After feeding for 9 weeks, the plasma total and LDL cholesterol levels were similar in all high-fat dietary groups but were higher than control rats. However, the plasma HDL cholesterol levels and plasma HDL/total cholesterol ratios in the rats fed sesame oil fortified with PE or SL fortified with PE were higher than those in the other high-fat dietary groups. There was no notable difference in plasma lipid profiles of rats fed SL compared to those fed lard or sesame oil. The plasma triacylglycerol levels in high-fat dietary groups were similar to each other but were lower than that in control rats. Systolic arterial blood pressures under pentobarbital anesthesia were lower in the high fat-fed rats than in controls. Finally, resting arterial blood pressures (systolic, diastolic, and mean blood pressures) in conscious high fat-fed rats were not different to control rats; however, resting heart rates in all high fat-fed groups of rats were higher than the controls.

Although the optimal synthesis conditions for PEs were established successfully in test tube-scale, the large-scale production of PEs was not carried out in the current study. Instead, chemically synthesized commercial PEs were used for the preparation of rat diets. Therefore, further study on the large-scale production of PEs by enzymatic method under the optimal conditions established in this study would be necessary in the future.