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BIOSYNTHESIS OF MEDIUM-LONG-MEDIUM TYPE STRUCTURED LIPIDS USING TRICAPRYLIN AND TRILINOLENIN AS SUBSTRATES

by

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A thesis submitted to the Graduate and Post-Doctoral Studies Office in partial fulfillment of the requirements of the degree of Master of Science

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Short Title

BIOSYNTHESIS OF SELECTED MEDIUM-LONG-MEDIUM TYPE STRUCTURED LIPIDS

ABSTRACT

M.SC. SHAN BAI

Using tricaprylin (TC) and trilinolenin (TLN) as substrates, biosynthesis of medium-long-medium (MLM) type structured lipids (SLs), by Lipozyme IM from *Rhizomucor meihei* and Novozym 435 from *Candida antarctica*, was investigated to determine their capacity as biocatalysts for the biosynthesis of SLs. At 30°C, Lipozyme IM showed higher bioconversion yield (24.7%) and initial enzyme activity (6.3 μmol CLnC/g enzyme /min) as compared to that of 24.0% and 1.6 μmol CLnC/g enzyme/min, respectively, for the Novozym 435 at 50°C. As a result, Lipozyme IM was subsequently used for further investigations. The SLs were recovered and characterized by silver-ion exchange high-performance liquid chromatography and gas-liquid chromatography. The structural analyses indicated that the major products of the enzymatic reaction were 1,3-dicapryl-2-linolenyl glycerol (CLnC) and 1(3)-capryl-2,3(1)-dilinolenyl glycerol (CLnLn). In order to optimize the bioconversion yield of CLnC, selected parameters, including initial water activity and solvent type, lipase concentration (5 to 20 mg solid enzyme), substrate molar ratios (TC:TLN of 1:4 to 8:1) and molecular sieve (5 to 20 mg/mL, Type 3Å), were investigated. The experimental results showed that using hexane at initial a_w 0.06, 10 mg solid enzyme/mL and substrate molar ratio of TC to TLN of 6:1 resulted in the highest bioconversion yield of 73.2% of CLnC. However, the addition of molecular sieve to the reaction medium resulted in a 14.0% decrease in the bioconversion yield of CLnC. Using the optimized conditions, the effects of TLN concentration and other selective limiting parameters, including the denaturation of enzyme, a_w and the formation of glycerol layer, on the mass productivity (P_M), enzymatic productivity (P_E) and volumetric productivity (P_V) of the interesterification reaction were investigated. Using 80 mM TLN, the maximum P_M of 15.5 mg CLnC/g substrates/h was obtained; however, using 200 mM TLN, the maximum P_E and P_V were 0.07 mg/enzyme unit/h and 6.1 g CLnC/L/h, respectively. The addition of 3 mg Silica gel to the reaction medium resulted in 52.0, 37.3 and 37.3% increase in P_M, P_E and P_V, respectively.

RÉSUMÉ

La biosynthèse de lipides structurés (SLs) de type medium-long-medium (MLM), en utilisant l'acide tricaprylique (TC) et l'acide trilinoleique (TLN) comme substrat, par Lipozyme IM de *Rhizomucor meihei* et par Novozym 435 de *Candida antarctica*, a été étudiée afin de déterminer leur capacité de biocatalyseur dans la biosynthèse de SLs. A 30○C, Lipozyme IM montre un plus haut rendement de bioconversion (24.7%) et une plus haute activité enzymatique initiale (6.3 μmol CLnC/g enzyme/min) comparée à ceux de Novozym à 50°C, respectivement 24.0% et 1.6 μmol CLnC/g enzyme/min. Lipozyme IM a donc été choisi comme l'enzyme utilisé pour la suite de la recherche entreprise. Les SLs ont été prélevés et caractérisés par chromatographie liquide haute performance avec une colonne échangeuse d'ion argent et par chromatographie en phase gazeuse. L'analyse structurale indique que les produits majoritaires sont le 1,3-dicapryl-2-linolenyl glycerol (CLnC) and 1(3)-capryl-2,3(1)-dilinolenyl glycerol. Afin d'optimiser le rendement de bioconversion de CLnC, différents paramètres sélectionnés ont été étudiés, dont l'activité initiale de l'eau/type de solvant, concentration de lipase (5 à 20 mg d'enzyme solide), le ratio molaire en substrat (TC:TLN de 1:4 à 8:1) et l'ajout de tamis moléculaire (5 à 20 mg/ml, type 3Å). Les résultats expérimentaux montrent que l'utilisation d'hexane à une a_w initiale de 0.06, de 10 mg enzyme solide/mL et d'un ratio molaire en substrat de TC/TLN de 6:1 engendre le plus haut rendement de bioconversion soit 73.2% de CLnC. En revanche, l'ajout de tamis moléculaire au milieu réactionnel induit une diminution du rendement de bioconversion de 14.0%. L'utilisation des conditions optimales, les effets de la concentration en TLN et d'autres paramètres sélectionnés, incluant la dénaturation de l'enzyme, l' a_w et la formation d'une pellicule (d'une couche) de glycérol, sur la productivité massique (P_M), la productivité enzymatique (P_E) et la productivité volumétrique (P_V) de la réaction de transesterification ont été étudiés. Lors de l'utilisation de 80 mM de TLN, une PM maximum de 15.5 mg CLnC/g substrat/h a été obtenue, alors que l'utilisation de 200 mM de TLN aboutit aux P_E et PV maximum de 0.07 mg/unité enzymatique/h et 6.1 g CLnC/L/h, respectivement. L'addition de 3 mg de gel de silice au milieu de réaction entraine une augmentation de 52.0, 37.3 et 37.3% de P_M, P_E et P_V, respectivement.

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LIST OF ABBREVIATIONS

ω-3: Omega-3

ω-6: Omega-6

ω-9: Omega-9

AA: Arachidonic acid

APCI: Atmospheric pressure chemical ionization

a_w: Water activity

BY: Bioconversion yield

CA: Caprylic acid

CLA: Conjugated linoleic acid

CCLn: 1(3),2-dicapryl-3(1)-linolenyl glycerol

CLnC: 1,3-dicapryl-2-linolenyl glycerol

CLnLn: 1(3)-capryl-2,3(1)-dilinolenyl glycerol

DAGs: Diacylglycerols

DGLA: Dihomogamma-linolenic acid

DHA: Docosahexaenoic acid

DMAP: 4-Dimethylaminopyridine

EDCI: 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide

EFAs: Essential fatty acids

ELSD: Evaporative light scattering detector

EPA: Eicosapentaenoic acid

ESI: Electro-spray ionization

FAME: Fatty acid methyl esters

FAs: Fatty acids

FD: Fluorescence detector

FFAs: Free fatty acids

GC: Gas liquid chromatography

HDL: High density lipoproteins

HPLC: High-performance liquid chromatography

LA: Linoleic acid

LCFAs: Long-chain polyunsaturated fatty acids

LC-MS: Liquid chromatography-mass spectroscopy

LCTAG: Long chain triacylglycerols

LDLs: Low density lipoproteins

LLL: trilinoleoylglycerol

α-LNA: α-Linolenic acid

LnCLn: 2-capryl-1,3-dilinolenyl glycerol

MAGs: Monoacylglycerols

MCFAs: Medium-chain fatty acids

MLM-type SLs: Medium-long-medium- type structured lipids

NMR: Nuclear magnetic resonance

OA: Oleic acid

PA: Palmitic acid

PUFAs: Polyunsaturated fatty acids

R: Acyl group

RI: Refractive index

SA: Stearic acid

SCFAs: Short-chain fatty acids

SLs: Structured lipids

SLL: 1(3)-stearoyl-2,3(1)- dilinoleovlglycerol

SLS: 2-linoleoyl-1,3- distearoylglycerol

TAGs: Triacylglycerols

TLC: Thin-layer chromatography

UV: Ultraviolet

1. INTRODUCTION

Triacylglycerols (TAGs) are the major components of fats and oils; they are composed of the ester form of fatty acids (FAs) and glycerol. Depending on the carbon chain length, FAs are classified as short chain fatty acids (SCFAs), medium chain fatty acids (MCFAs) and long-chain fatty acids (LCFAs). Medium-chain fatty acids (MCFAs) are saturated fatty acids with a 6 to 12 carbon chain; they are preferentially transported via the portal vein to the liver because of their smaller size and greater solubility, and are readily absorbed via the portal route and metabolized as fast as glucose in the body with little being stored in the adipose tissue because they are not easily re-esterified into TAGs (Hayes *et al.*, 1994). MCFAs have also been used in total parental nutrition and formulas for preterm infants (Akoh, 2002). Polyunsaturated fatty acids (PUFAs) have more than one carbon-carbon double bonds and their carbon chain length is longer than 12. The importance of PUFAs in human nutrition and disease prevention has been and is still one of the major issues for the nutraceutical and pharmaceutical industries. Both ω-3 and ω-6 PUFAs are precursors of eicosanoids which are involved in a wide range of physiological functions and hormonal activities (Haraldsson *et al.*, 2001). The numerous health benefits of ω-3 PUFAs have been scientifically linked to the prevention of diseases, including cardiovascular, cancer and diabetes (Medina *et al.*, 1999; Kimura *et al.*, 2005).

Structured lipids (SLs) are the TAGs that have been modified by changing the FAs composition which could cause changes in their physico-chemical and biological properties, such as melting behavior, digestion, absorption, nutritional quality and therapeutic applications (Akoh, 2002). By having MCFA in *sn*-1,3 position and PUFAs in *sn*-2 position of glycerol backbone, medium-long-medium (MLM)-type SLs combine the benefits of both MCFAs and PUFAs.

Over the last two decades, the literature showed increasing interest in the incorporation of MCFAs into PUFAs rich oil which could result from the biosynthesis of MLM-type SLs that possess both functional and nutritional benefits (Huang and Akoh, 1996; Fomuso and Akoh, 1998; Halldorsson *et al.*, 2000; Turan *et al.*, 2007). The synthesis of such bio-molecules could be obtained either by chemical synthesis or by using 1,3-specific lipase-

catalyzed transesterification reaction in non-conventional media. Chemical synthesis of SLs is catalyzed by alkali metals or alkali metal alkylates. It requires high temperature and anhydrous conditions. Moreover, only randomized products can usually be produced by chemical synthesis (Gupta *et al.*, 2003). Compared with chemical synthesis, the most significant advantage of enzymatic synthesis is the regio/sterero- specificity of lipases, which can result in products with are easily defined and have a more predictable structure than those of chemical synthesis (Akoh, 2002). Acidolysis reaction is a type of transesterification reaction involving the exchange of acyl groups between a TAG and a free fatty acid (FFA), while the interesterification reaction involves the exchange of acyl groups between one TAG and another TAG (Akoh, 2002). The literature reported extensive research work on the interesterification reaction (Gunstone, 1999; Balcao and Malcata, 1998; Caro *et al.*, 2000; Kim *et al.* 2001; Akimoto *et al.*, 2003) since it offers the faster initial reaction rate than the acidolysis reaction (Huang and Akoh, 1996).

The use of non-conventional media, in particular organic solvent media, offers numerous advantages in the transesterification reaction of biosynthesis of MLM-type SLs. The effect of solvent type on the biosynthesis of SLs has been reported in the literature (Fomuso and Akoh, 1998; Schmid *et al.* 1999; Negishi *et al.*, 2003). Organic solvents produce various physicochemical effects on enzyme molecules. Using organic solvent, the conformational changes of enzymes could result in the alteration of substrate specificity and substrates affinity (Zaks and Russell, 1988). The solubility of the hydrophobic substrate is enhanced in organic solvent medium. Many side-reactions that are water dependent are reduced in organic solvent medium (Lanne *et al.*, 1987). In addition, the enzymes show a higher thermal stability in organic solvent than in the aqueous media. The enzyme activity in non-aqueous media is correlated with the partition coefficient. Solvents with a Log P value of 2 to 4 are appropriate for enzymatic reaction systems (Lanne *et al.*, 1987; Gupta *et al.*, 2003).

The overall aim of this research is to establish and optimize a model system for the biosynthesis of MLM-type SLs in organic solvent media and for the characterization of the subsequent MLM-type SLs. In order to optimize the bioconversion yield, a wide range of parameters, including the choice of biocatalyst, lipase concentration, organic

solvent ratio, substrate molar ratio as well as the presence of molecular sieve, were investigated. To assess the productivity of the enzymatic synthesis of MLM-type SLs, the effect of substrate concentration, selected limiting factors and the addition of Silica gel were also investigated.

The specific objectives of this research were:

1. To investigate the biosynthesis of a lipase-catalyzed transesterification reaction, using tricaprylin and trilinolenin as substrates, as well as selected biocatalysts, including Lipozyme IM and Novozym 435.

2. To recover, separate and carry out the structural characterization of 1,3-dicapryl-2-linolenyl glycerol (CLnC).

3. To optimize the enzymatic system for the biosynthesis of CLnC, using tricaprylin and trilinolenin as substrates, with selected immobilized lipase as biocatalyst.

4. To investigate the productivity of CLnC of the interesterification reaction.

2. LITERATURE REVIEW

2.1. Lipids

Lipids are used to denote a chemically heterogeneous group of substances that have the property of being insoluble in water, but soluble in non-aqueous solvents such as chloroform, hydrocarbon and alcohol. Lipids are defined on the basis of their solubility properties rather than their chemical structures. Water insolubility is the major analytical property that separates lipids from proteins and carbohydrates. Some lipids are amphipathic molecules which contain both hydrophobic and hydrophilic moieties. Hence, they are polar and distinctly different from neutral lipids (Gurr *et al.*, 2002).

Based on their structures, lipids can be classified into three main classes: simple, derived or complex. Those composed of two entities, such as fatty acids (FAs) and alcohol, are called simple lipids. An example would be acylglycerols. Complex lipids contain two or more components, such as glycerolphospholipids and glyceroglycolipids. FAs and alcohols are considered as derived lipids since they can be obtained from simple and complex lipids. Triacylglycerols (TAGs) are FA esters of glycerol (Nelson and Cox, 2004).

2.1.1. Sources of Lipids

Lipids can be found throughout the living world from microorganisms to higher plants and animals. They exist in all cell types and contribute to cell structures, energy storage and participate in many biological processes, such as signal transductions and as carriers of the nutritionally essential fat-soluble vitamins (Nelson and Cox, 2004).

Dietary lipids are mainly obtained from structural and storage lipids that come from animals and plants. The major components of these dietary lipids are TAGs. In some

animals, such as seal and bear, TAGs, stored under the skin, serve not only as energy stores but also as insulation against low temperatures. The low density of TAGs constitutes another remarkable function of these compounds. In sperm whales, the storage of TAGs and waxes allows them to match the buoyancy of their bodies to that of their surroundings during deep dives in cold water (Nelson and Cox, 2004).

One of the important aspects of dietary lipids is that they contain different types of FAs which can be saturated, monounsaturated and polyunsaturated. Current research focuses on the polyunsaturated FAs (PUFAs), which are involved in many vital biological activities, such as immune and inflammatory process (Calder and Grimble, 2002).

2.1.2. Composition of Lipids

The major lipids coming from the diet are TAGs. They are the simplest lipid composed of FAs linked to glycerol backbone. TAGs are composed of three FAs in ester linkage with a single glycerol (Nelson and Cox, 2004). The FAs' composition and their positional distribution on glycerol backbone determine the properties of TAGs. Depends on the chain length, FAs are classified into three groups, short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs) and long-chain fatty acids (LCFAs).

SCFAs range from 2 to 6 carbons long, and are known as volatile FAs. The most common sources are the bovine milk and butter fats. Due to their water solubility nature, molecular size, and short chain length, they are more easily absorbed in stomach than other FAs (Akoh, 2002).

The primary sources of MCFAs, containing 6-12 carbon atoms, are coconut and kernel oils. These FAs are preferentially transported via the portal vein to the liver because of their smaller size and greater solubility. MCFAs are metabolized as fast as glucose in the body with little being stored in the adipose tissue since they are not easily re-esterified

into TAG (Hayes *et al.*, 1994). MCFAs are frequently used in the diet of patients with maldigestion and malabsorption. They have also been used in total parental nutrition and in the formulas developed for preterm infants. However, MCFAs cannot serve as the only source of dietary fat since they do not provide essential FAs (EFAs) required for human health (Senanayake and Shahidi, 2002), and can potentially raise serum cholesterol levels (Akoh, 2002).

LCFAs, ranging from C14 to C24, are common to animal fats, vegetable and marine oils. Due to their high hydrophobicity, they can not be absorbed or transported in the blood. Saturated LCFAs are believed to respond to the high level of serum cholesterol. However, stearic acid (SA 18:0) is neutral with respect to cholesterol levels in blood because its melting point is higher than the body temperature, and it is readily unsaturated to oleic acid (OA) in *vivo*.

Unsaturated LCFAs include monounsaturated and polyunsaturated FAs (PUFAs). In general, the more common compounds tend to have even number of carbon atom as well as a *cis* double bond at ω-9 position. *Trans* isomers are rare but do exist. When LCFAs, derived from monounsaturated FA, have additional double bond(s), it is called polyunsaturated double FAs (PUFAs) (Gurr *et al.*, 2002). Several types of PUFAs exist which are important in structured lipids production, and will be discussed in the following parts.

2.2. Polyunsaturated Fatty Acids

Lipids hydrolysis releases aliphatic carboxylic acids which are different in chemical structures. They can be divided into groups based on their chain length, number, position and configuration of their double-bonds. PUFAs contain two to six double-bonds in their

acyl residues (Nelson and Cox, 2004). The double-bonds are usually three carbon atoms apart and evenly spaced.

2.2.1. Classification

Omega-3 (ω-3), omega-6 (ω-6) and omega-9 (ω-9) PUFAs are the three major classes of PUFAs encountered in the diet; these classes of FAs are required for normal human health (Jump, 2002). The ω-3 PUFAs have their first double bond at the third carbon atom from the methyl end; the ω-6 PUFAs have the first double bond at the sixth carbon from the methyl end, whereas ω-9 FAs have their first double bond located at the ninth carbon from the methyl end, and are not considered as EFAs, but rather to play a moderate role in reducing plasma cholesterol in the body (Gottenbos, 1988).

2.2.2. ω-6 Polyunsaturated Fatty Acids

The ω-6 PUFAs cannot be synthesized by human body, and therefore are considered EFAs. Linoleic acid (LA 18:2 ω-6), found in most vegetable oils and plant seeds, is an EFA that can be desaturated further, and elongated to arachidonic acid (AA 20:4 ω-6). AA is a precursor for eicosanoid formation. ω-6 FAs give rise to the eicosanoid family of inflammatory mediators (prostaglandins, leukotrienes and related metabolites), and through these, ω-6 FAs regulate the activities of inflammatory cells, the production of cytokines and the various balances within the immune system (Calder and Grimble, 2002).

2.2.3. ω-3 Polyunsaturated Fatty Acids

Another type of EFAs is the ω-3 FAs. They are essential in growth and development throughout the human life cycle and should be included in the diet. ω-3 PUFAs have diverse effects on physiological processes impacting normal health and chronic diseases, such as the regulation of plasma lipid levels, cardiovascular and immune function, insulin action, neuronal development and visual function (Jump, 2002). They inhibit tissue

eicosanoid biosynthesis and reduce inflammation. Diets rich in ω-3 PUFAs also increase high density lipoproteins (HDLs), while decreasing low density lipoproteins (LDLs) and very low density lipoproteins (VLDLs) cholesterol levels (Osborn and Akoh, 2002).

There are three common types of ω-3 PUFAs, which are α-linolenic acid (LAN C18:3), eicosapentaenoic acid (EPA C20:5) and docosahexaenoic acid (DHA, C22:6). EPA shows highly beneficial effects, such as preventing coronary heart diseases, hyper-triglyceridemia, blood platelet aggregation and lowering blood cholesterol, thus reducing the risk of atherosclerosis, inflammation and various carcinomas (Medina *et al.*, 1999). DHA is selectively concentrated in synaptic plasma and retinal membranes and is thought to be related to visual function, brain development, behaviour and learning process (Kimura *et al.*, 2005).

2.2.4. Sources and Biological Functions of Polyunsaturated Fatty Acids

In western diet, saturated and monounsaturated FAs are particularly high (Shahidi and Wanasundara, 1998). The consumption of vegetable oil has steadily increased in the past 25 years, whereas the demand for animal fat declined due to the fact that it contains high level of saturated FAs. The intake of this kind of FAs increases the risk of cardiovascular diseases. However, humane body can not insert double-bonds between the methyl terminus and carbon number 9 in oleic acid. Thus it can not be converted to LA which is sequently converted to LNA (The enzymes responsible for this are called denaturases, and they are only found in plants). Plant tissue and plant oil are rich sources of LA and LNA which contribute to 50-80% of FAs in plant oil. Thus, the major dietary sources of PUFAs come from vegetable oils and fish oil.

ω-3 PUFA, such as EPA and DHA are abundant in fish oil. LA, as a ω-6 PUFA, is mainly found in flaxseed oil. In western diets, the major source of ω-6 PUFA is from

vegetable oils. Increased consumption of marine lipids (fish oil) has been suggested in order to increase the dietary intake of ω-3 PUFAs (Shahidi and Wanasundara, 1998).

The beneficial effects of ω-3 FAs have been associated with their ability to lower serum TAG and cholesterol levels, and to prevent and treat arthritis, hypertension, inflammatory and immune disorders (Senanayake and Shahidi, 2002). Consumption of ω-3 PUFAs can decrease the amount of AA in cell membranes that are available for eicosanoid production. Therefore, ω-3 PUFAs act as ω-6 PUFA antagonists. Cytokines, the key inflammatory components of natural and acquired immunity, can be affected by ω-3 PUFAs. Although some of the effects of ω-3 FAs may be brought about by modulation of the amount and types of eicosanoid made, it is possible that these FAs might elicit some of their effects by eicosanoid-independent mechanisms (Calder, 2001). This type of ω-3 FAs induced effects may be of use as a therapy for acute and chronic inflammation, and for immune disorders.

2.3. Structured Lipids

The new generation of fats, structured lipids (SLs), are considered as ''nutraceuticals'' or functional ingredients of potential therapeutically or/and health benefits (Akoh, 2002). SLs can be classified as any modified or synthetic oil and fat by any artificial means, such as hydrogenation, fractionation, blending, interesterification, esterification, and even from bioengineered plants (Xu, 2000). However, the common definition of SLs refers to the TAGs that have been modified by changing the FA composition and/or their positional distribution on glycerol backbone by chemical and/or enzyme-catalyzed reaction and/or genetic engineering (Xu, 2000). By changing the positional distribution and/or composition of the FAs on the glycerol backbone, the physiological, nutritional and functional properties of the lipids could be modulated and optimized; SLs are used in a number of nutritional applications such as: reduced-calorie fats, infant formulas, enteral

and parenteral nutrition (Kanjilal *et al.*, 1999; Akoh, 2002).

2.3.1. Development and Marketed Products

Due to the possibility to combine the benefits of the component FAs into one TAG molecule, SLs play a more and more important role in nutrition, food and health application. The typical examples are cocoa butter and breast milk fat.

Cocoa butter is an important major constituent of the chocolate formulations (Xu, 2000) and it is composed of symmetrical TAGs with oleic acid in the *sn*-2 position, mainly palmitic-oleic-palmitic (POP), palnitic-oleic-stearic (POSt) and setaric-oleic-palmitic (StOP). Coca butter equivalents in which the *sn*-2 position is mainly occupied by oleic acid are closest to cocoa butter in composition and property and are totally compatible with cocoa butter (Xu, 2000).

Breast milk fat is one of the main sources of nutrients and energy for infants. The main saturated FA in all human milks is PA, which represents 25% of the total milk FAs and more than 10% of the babies' total energy intake. PA mostly occupies the *sn*-2 position of the glycerol backbone while other unsaturated FAs are mainly found in *sn*-1,3 positions. *Betapol* is a brand of a human milk fat substitute from *Loders Croklaan (Unilever)*. In *Betapol*, 35% of PA is attached to the *sn*-1 and *sn*-3, and 65% to the *sn*-2 position of glycerol backbone. This product has a nutritive value similar to that of breast milk (Xu, 2000).

2.3.2. Medium-long-medium (MLM) Type SLs

2.3.2.1 Definition of MLM-Type SLs

MLM-type SLs refer to any TAGs containing both PUFAs at *sn*-2 position and MCFAs on *sn*-1,3 positions (Xu *et al.*, 1999). Figure 1 shows the stereo-chemical configuration of a MLM-type SL with *sn* notation.

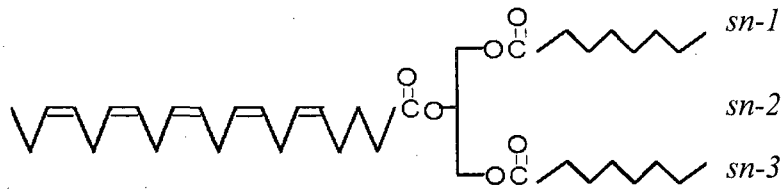


Figure 1. Stereo-chemical configuration of triacylglycerols (TAGs) or structured lipids (SLs) with *sn* notation indicating stereo-chemical numbering of the carbon atoms of glycerol moiety.

2.3.2.2. Metabolism Pathway and Nutritional Function of MLM-Type SLs

The MFCAs at the *sn*-1,3 positions of dietary SLs are rapidly hydrolyzed by the enzymes known as pancreatic and gastric lipases present in the intestines. These FFAs are then released and oxidized in the liver as readily available energy. They are not normally deposited in adipose tissue (Hayes *et al.*, 1994; Akoh, 2002). Approximately 75% of the FAs on *sn*-2 position of TAG, *sn*-2 monoacylglycerols (MAGs), remain in the *sn*-2 MAGs. Because the *sn-2* MAGs are the main carriers of FAs through the intestinal wall, the FAs at *sn-2* position are more efficiently absorbed than those at *sn*-1 and *sn*-3 positions. They are then absorbed and converted to new TAGs in mucosal cells (Christensen and Høy, 1995). Therefore, MLM-type SLs have the advantage of efficiently providing EFAs and a quick source of energy (Hita *et al.*, 2007).

In recent years, research has focused on the variation in TAGs composition within the postprandial period and on the differences in the TAG structure-dependent formation and the clearance of chylomicrons and very-low density lipoprotein (Linderborg and Kallio, 2005). The nutraceutical and pharmaceutical application of MLM-type SLs have been extensively investigated by a lot of researchers (Hayes *et al.*, 1994; Christensen and Høy, 1995; Zock *et al.*, 1996; Christophe *et al.*, 2000; Mu and Høy, 2000; Nagata *et al.*, 2003; Fernie *et al.*, 2004; Yagi *et al.*, 2004; Linderborg and Kallio, 2005; Chambrier *et al.*, 2006; Vistisen *et al.*, 2006). Although some of these research works are done with human

subjects, most of them are carried out with animals, mainly rats. Their results support that a diet composed of MLM-type SLs can be applied as carriers of desired FAs as nutraceuticals, functional lipids, and pharmaceuticals to target specific diseases, metabolic conditions or optimal nutrition.

Normally there are two ways to synthesize MLM-type SLs either chemically or enzymatically. Chemical synthesis is a mature technology in industry and only randomized products can usually be produced. Enzymatic synthesis of SLs refers to the reactions that are catalyzed by lipases, which are widely used in production of SLs in recent years (Xu, 2000).

2.4. Lipase

Lipase (EC 3.1.1.3) is defined as carboxylic ester or glycerol ester hydrolases that can hydrolyze TAGs, MAGs and DAGs at the interface between oil and water (Belit and Grosch, 1987). Although lipases are widely spread in nature, microbial lipases have been the most attractive ones since they are stable without co-lipase requirements. With genetic engineering, recombination and mutation, lipase could be produced in a suitable host microorganism with desired properties and efficient expression (Xu, 2000). Lipase-catalyzed reactions can be classified into three groups: hydrolysis, esterification and transesterification (Gupta *et al.*, 2003).

2.4.1. Hydrolysis

Hydrolysis happens under the presence of water. The acyl group (R) is replaced by hydroxyl group. The hydrolysis of lipids by lipase produces free FAs (FFAS), MAGs, DAGs and glycerol (Macrae, 1983) as shown in Figure 2. This reaction can be used to produce FFAs from natural oil, especially for the selective hydrolysis of PUFAs (Shahidi and Wanasundara, 1999; Rakshit *et al.*, 2000; Laszlo and Compton, 2006). It is also widely used in analysis of positional distribution of FAs in TAGs combined with

methylation followed by gas-liquid chromatography (GC) analysis (Bergana and Lee, 1996; Kim and Akoh, 2006; Turan *et al.*, 2007).

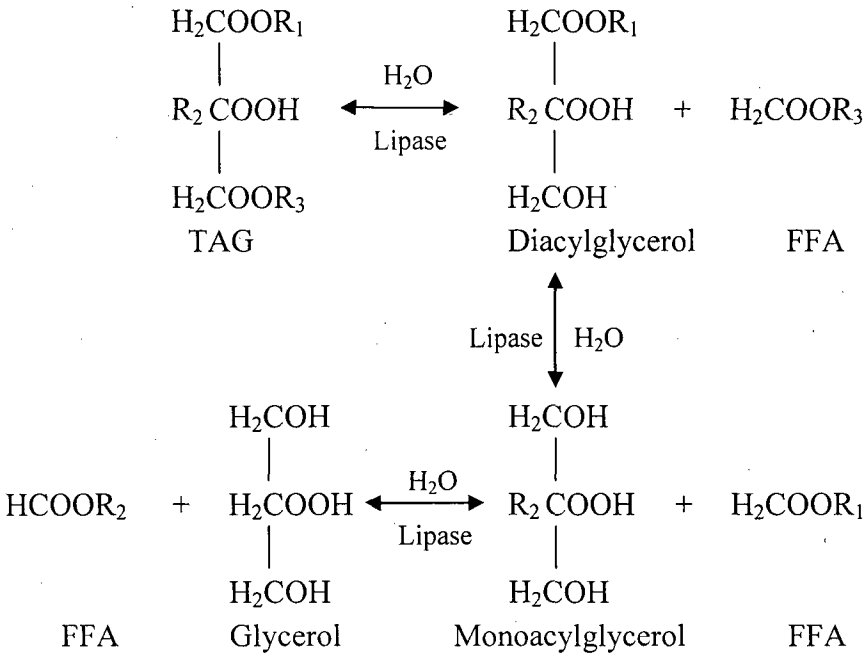


Figure 2. Hydrolysis of triacylglycerols (TAGs) where R_1, R_2 and R_3 are different acyl groups.

2.4.2. Esterification

Esterification is the reverse reaction of hydrolysis, and refers to the reaction between acids and alcohol. Lipase can catalyze such reaction. The products of esterification are normally an ester and water as shown in Figure 3. This process is affected by the amount of water in the reaction system. The availability of water is an important parameter since it affects not only the reaction equilibrium but also the enzymatic activity in organic solvent (Medina *et al.*, 1999; Caro *et al.*, 2000; Lue *et al.*, 2005).

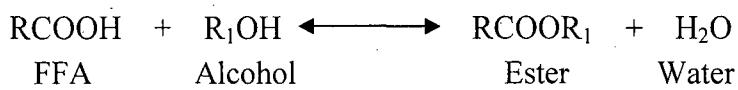


Figure 3. Lipase-catalyzed esterification reaction (Gandhi, 1997)

2.4.3. Transesterification

During the synthesis of esters via esterification reactions, water is produced as a by-product. Transesterification reactions such as alcoholysis, acidolysis and interesterification, give rise respectively to alcohols, acids or esters containing compounds. These reactions are exchange processes of acyl group between two molecules. Figure 4 shows the scheme of transesterification (Kvittingen, 1994).

(a) Alcoholysis

$$\underset{\text{Ester}}{R_1COOR_2} + \underset{\text{Alcohol}}{R_3\text{-}OH} \xleftrightarrow[\text{Lipase}]{} \underset{\text{Ester}}{R_3COOR_2} + \underset{\text{Alcohol}}{R_1\text{-}OH}$$

(b) Acidolysis

$$\underset{\text{Ester}}{R_1COOR_2} + \underset{\text{Acid}}{R_3COOH} \xleftrightarrow[\text{Lipase}]{} \underset{\text{Ester}}{R_1COOR_3} + \underset{\text{Acid}}{R_2COOH}$$

(c) Interesterifcation

$$\underset{\text{Ester}}{R_1COOR_2} + \underset{\text{Ester}}{R_3COOR_4} \xleftrightarrow[\text{Lipase}]{} \underset{\text{Ester}}{R_1COOR_3} + \underset{\text{Ester}}{R_2COOR_4}$$

Figure 4. Lipase-catalyzed transesterification reactions

The first reaction illustrated in Figure 4, alcoholysis (a), refers to an ester that reacts with an alcohol to produce a new ester with a different alkyl group. In this case, the alcohol could be either monohydric (i.e. ethanol) or polyhydric (i.e. glycerol). The second reaction, acidolysis (b), is an ester exchange reaction of an ester with an acid. It is one of the most frequently used reactions to incorporate novel FAs into TAGs in several research (Jennings and Akoh, 2000; Rao *et al.*, 2002; Senanayake and Shahidi, 2002). The last synthesis reaction, interesterification (c), is the reaction between two esters to produce two new esters. It is a combination process of hydrolysis and esterification. The first step is the hydrolysis of TAG to FFAs and glycerol, and the second step is the esterification between the FFAs and glycerol (Irimescu *et al.*, 2001). Interesterification is

another main strategy to incorporate PUFAs into TAGs. The literature reported extensive research work on the interesterification reaction (Balcao and Malcata, 1998; Gunstone, 1999; Caro *et al.*, 2000; Kim *et al.* 2001; Akimoto *et al.*, 2003).

2.4.4. Structure and Mechanism of Lipase Action

All lipases consist of a catalytic triad of His-Ser-Asp (Glu). In addition, an oxyanion hole is formed stabilizing the charge distribution and reducing the ground state energy of the tetrahedral intermediate (Derewenda, 1994). The catalytic triad of lipases is buried under a "lid" of a surface loop which undergoes a conformational change to open a channel for the active site accessible of substrate. This repositioning of the "lid" is caused by interfacial activation (Wong, 1995).

The structural studies of lipases greatly improved the understanding of their catalytic mechanism. The formation of an acyl lipase intermediate has been detected by Derewenda (1994). The mechanism can be divided into two steps. The first step is an acylation step in which a covalent acyl-enzyme is formed by nucleophilic attack on the carbonyl carbon of the substrate by the essential Ser-OH. Deacylation of the acyl-enzyme is the second step at which the water being the nucleophile, giving the product and enzyme. The oxyanion developed in the tetrahedral intermediates in both acylation and deacylation are stabilized by hydrogen bond with the residues in the oxyanion hole (Derewenda, 1994).

Immobilized lipases normally are more stable than their normal free states (Nagao *et al.*, 2001). They can be used at high temperature, especially in micro-aqueous system, such as organic solvents (Caro *et al.*, 2000; Rao *et al.*, 2002; Senanayake and Shahidi, 2002; Akimoto *et al.*, 2003), solvent free systems (Jennings and Akoh, 2000; Zhou, *et al.*, 2001; Laszlo and Compton, 2006) and ionic liquids (Itoh *et al.*, 2006). Recently,

lipase-catalyzed transesterification are also carried out under supercritical CO_2 (Liu *et al.*, 2007). However, the disadvantages of immobilized enzymes are also obvious; mainly the shape of the active site may be changed and the enzyme may not be an effective catalyst. Enzymes are sometimes detached from their solid supports; the cost of developing immobilized enzymes can be very high (Katchalski-Katzir, 1993).

2.4.5. Specificity of Lipase

The meaning of specificity of enzyme generally refers to the ability of an enzyme to discriminate several substrates that compete for active sites, including the desired and undesired substrates competing for one enzyme, and the relative reaction rate of the mixture of undesired and desired substrates. Lipases can be divided into three groups based on their specificity: non-specific lipases, acyl-group specific lipases, and regio/stereo-specific lipases (Xu, 2000).

2.4.5.1. Non-Specific Lipases

Interesterification catalyzed by a non-specific lipase, such as lipases from *Candida Antarctica A* and *Arthrobacter* sp. (Chandler, 2001), yields TAGs in which FAs are randomly distributed on the three positions of glycerol backbone, as shown in Figure 5. Nonspecific lipases act randomly on TAGs molecule and hence are of little use for fat and oil modification (Gupta *et al.*, 2003).

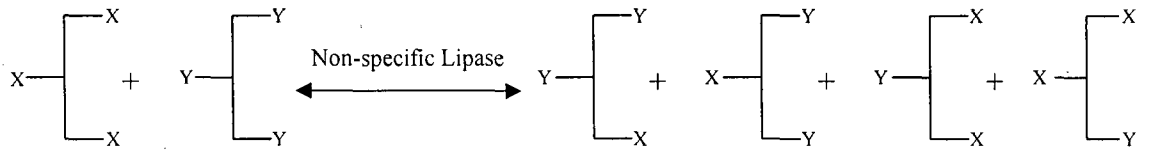


Figure 5. Reaction scheme for non-specific lipase-catalyzed reactions.

2.4.5.2. Acyl-Group Specific Lipases

Acyl-group specificity (also called typo-selectivity) of lipases means that they can selectively release a certain type of FA family, and exchange with another FA without changing any other FAs position on the glycerol backbone. Table 1 shows the acyl-group specificity of several lipases.

Caro *et al.* (2000) determined that lipases from *Euphorbia characias* latex had a high selectivity to short chain FA in hydrolysis reaction, and in interesterification reaction, whereas the lipases from crude papain had no typo-selectivity. Fajardo *et al.* (2003) pointed out that lipase QLM from *Alcaligenes* sp. discriminated against EPA when compared with DHA. Fu and Parkin (2004) studied the selectivity of FAs incorporation into acylglycerols in esterification reactions using lipases from *Rhizomucor miehei* and *Burkholderia cepacia*. They found that lipases from *R. miehei* were not very selective at all among the group of FAs C8 to C18, while lipases from *B. cepacia* have the acyl-group specificity towards saturated FAs compared with PUFAs.

2.4.5.3. Regio- and Stereo-Specific Lipases

Lipase region-selectivity is defined as the ability to distinguish between primary (*sn*-1,3) and secondary (*sn*-2) ester functionalities in a TAG molecule, which is very important in the production of MLM-type SLs. Therefore, lipases can be classified according to their selectivity for the acyl position on the glycerol backbone. Figure 6 shows the reaction scheme catalyzed by *sn*-1,3 specific lipase.

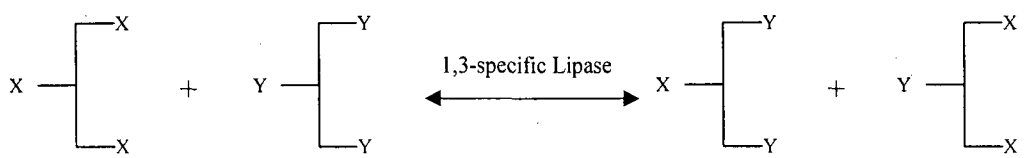


Figure 6. Reaction scheme for *sn*-1,3 specific lipase-catalyzed reactions, where the X and Y are different fatty acids.

Chandler (2001), Kim *et al.* (2001) and Hita *et al.* (2007) studied the positional specificity of several lipases from different microorganisms. According to their results, Lipozyme from *R. miehei*, lipase N from *Rhizopus niveus*, lipase D from *R. oryzae*, lipase PS-C from *Pseudomonas. Cepacia*, lipase Rd from *R. delemar*, and lipolase from *Humicola* sp. showed high 1,3- specificity.Beside *sn*-1,3 specific lipases, there are few lipases having *sn*-2 specificity, such as Lipase P from *Pseudomonas* sp. and *Arthrobacter* sp. (Chandler, 2001) or *sn*-1 and *sn*-3 specificity (Mangos *et al.*, 1999). A list of regiospecific lipases is given in Table 1. 1,3-specific lipases have been intensively used in bio-synthesis of MLM-type SLs in recent years for both research purposes and food industry sectors (Kawashima *et al.*, 2001; Zhou *et al.*, 2001; Kawashima *et al.*, 2002; Kim and Akoh, 2005 and 2006).

Table 1. Specific lipases for the production of specific SLs (Xu, 2000).

Lipase Source	Acyl group specificity	Regio-specificity (*sn*-)
Aspergillus niger	S, M, L	1, 3 >> 2
C. lipolytica	S, M, L	1, 3 > 2
Humicola lanuginosa	S, M, L	1, 3 >> 2
Mucor javanicus	M, L >> S	1, 3 > 2
R. miehei	S > M, L	1 > 3 >> 2
Pancreatic	S > M, L	1, 3
Pre-gastric	S, M >> L	1, 3
Penicillium camembertii	MAGs, DAGs> TAGs	1, 3
Penicillium roquefortii	S, M >> L	1, 3
R. delemar	M, L >> S	1, 3 >> 2
R. javanicus	M, L > S	1, 3 > 2
R. japonicus	S, M, L	1, 3 > 2
R. niveus	M, L > S	1, 3 > 2
R. oryzae	M, L > S	1, 3 >>> 2
P. fluofescens	M, L > S	1, 3 > 2
Pseudomonas sp.	S, M, L	1, 3 > 2
R. arrhizus	S, M > L	1, 3

Abbreviations: MAGs, monoacylglycerols; DAGs, diacylglycerols; TAG, triacylglycerols; L, long-chain FA; M, medium-chain FA; S, short-chain FA.

2.5. Enzymatic Synthesis of MLM-type SLs in Organic Solvent Media

2.5.1. Lipase in Organic Solvent Media

It is accepted that lipases could catalyze reactions in organic solvent with minimal water, and this has numerous advantages (Kvittingen, 1994). For example, it is possible to transform substrates that are unstable or poorly soluble in water into the reaction medium and prevent many side-reactions that are water dependent, including the denaturation of enzymes which show higher thermal stability. Moreover, in absence of water, the synthesis by lipases of ester and amide bonds can be favoured over hydrolysis. By varying the organic solvent, it is also possible to modify the substrate specificity and the regio-selectivity of a given lipase (Carrea *et al.*, 1995; Huang and Akoh, 1996). Table 2 lists advantages and disadvantages of using lipases as biocatalysts in organic solvents for the production of SLs compared with those in aqueous media.

Table 2. Effects of organic solvents on biocatalysts (Sellek and Chaudhuri, 1999).

Advantages
-Higher solubility of hydrophobic species
-Altered substrate-, region/stereo-specificity
-Reduced water activity (alters hydrolytic equilibrium)
-Reduced incidence of the side reactions found in water
-High thermal stability of enzymes in nonpolar media
Disadvantages
-Solvent tend to reduce enzyme activity: demand higher enzyme concentration
-Polar solvents can act as denaturants
-Interfacial inactivation two –phase systems
-Water activity control needed for processes involving condensation reactions

However, the disadvantages of organic solvents are also obvious. Solvents tend to reduce enzyme activity. As a result, higher concentration of biocatalysts is often required; polar solvents can act as denaturants since they compete for water with enzyme in the system. With increasing concern of environmental problems, synthesis of SLs in solvent free system (Rocha-Uribe and Hernandez, 2004) and ionic liquids stem (Lee *et al.*, 2004; Guo and Xu, 2005) have also been studied.

2.5.2. Synthesis of MLM-type SLs

The two ways to synthesize SLs are chemical and enzymatic by transesterification reactions. Chemical interesterification is widely used in industry and only randomized products can usually be produced. Enzymatic transesterification with *sn*-1,3 specific lipases has also been used by industry for production of coca butter-like fats, human milk fat substitutes and so on (Quinlan and Moore, 1993).

2.5.2.1. Chemical Synthesis

Chemical synthesis of SLs is the first method of the production of SLs, which is inexpensive and easy to scale up to. However, it lacks specificity of positional distribution of FAs in the final product (Willis and Marangoni, 1999). This process usually includes the esterification of LCFAs with glycerol to produce long chain TAGs (LCTAGs). Re-esterification after random mixing of the MCFAs and LCTAGs has occurred by the transesterification reaction, which results in desired randomized TAG and number of unwanted products that are difficult to remove (Akoh, 2002), thus losing the heath properties of PUFAs. Moreover, this process is also energy intensive and non-specific (Gupta *et al.*, 2003).

However, Halldorsson *et al.* (2000) and Haraldsson *et al.* (2001) reported that by using chemoenzymatic method, they synthesized high purity SLs via a two-steps reaction; the

literature indicated that *sn*-1,3 DAG is firstly synthesized via Lipozyme from *R. miehei* catalyzed-esterification reaction. In the subsequent step, 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide (EDCI) and 4-Dimethylaminopyridine (DMAP) are used as chemical catalysts to insert PUFAs into *sn*-2 position at room temperature.

2.5.2.2. Enzymatic Synthesis

Strategies for enzymatic synthesis of structured lipids include: interesterification of medium-chain triacylglycerols with long-chain triacylglycerols; acidolysis of long-chain triacylglycerols and MCFAs, and interesterification of long-chain triacylglycerols with the MCFA ester (Huang and Akoh, 1996). The main advantage of enzymatic synthesis is that it offers greater control over the positional distribution of FAs in the final product.

The enzymatic synthesis of MLM-type SLs by acidolysis reaction, a type of transesterification reaction, has been widely investigated. As compared to other transesterification reactions products, the SLs can be easily recovered by distillation or by other convenient techniques (Akoh, 2002). Figure 7 shows the reaction scheme of the acidolysis reaction in the synthesis of MLM-type SLs from caprylic acid and triolein. Shimada *et al.* (1999) have studied the synthesis of SLs, containing DHA and caprylic acid, by *R. delemar* lipase-catalyzed acidolysis reaction. Kim *et al.* (2001) investigated the acidolysis reaction of Perilla oil and caprylic acid, using Lipozyme RM IM from *R. miehei* and Lipozyme TL IM from *T. lanuginose* as biocatalysts. The enzymatic synthesis of SLs byacidolysis reaction of borage oil and caprylic acid was reported by Kawashima *et al.* (2002), using lipase from *R. oryzae.* Hita *et al.* (2007) reported the acidolysis reaction of tuna oil and caprylic acid, using immobilized lipases from *Rhizopus oryzae* from *R. delemar.*

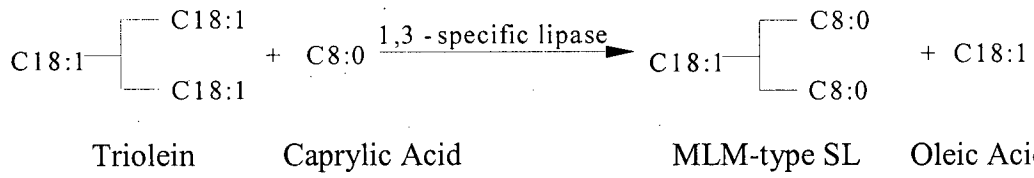


Figure 7. The reaction scheme of acidolysis reaction in the synthesis of structured lipids from caprylic acid and triolein.

Interesterification is another type of transesterification reaction that is used for the synthesis of MLM-type SLs. As compared to carboxylic acids, the use of carboxylic ethyl esters as acyl donors could lead to a faster reaction rate for the synthesis of SLs as well as to more diglyceride formation; in addtion, the initial reaction rate of SL synthesis was found higher in order of magnitude in the presence of fatty acid glycerol esters > fatty acid methyl esters> fatty acids (Huang and Akoh, 1996). Figure 8 shows the reaction scheme of the interesterification reaction in the synthesis of MLM-type SLs from triolenin and caprylic acid ethyl ester. The enzymatic synthesis of MLM-type SLs using triolein and caprlic acid ethyl ester as substrates were reported by Huang and Akoh (1996), using lipases from *C. Antarctica* and *R. Miehei.* Fomuso and Akoh (1998) reported the synthesis of the SLs by Lipozyme IM-60-catalyzed interesterification of tricaproin with trilinolein. Irimescu *et al.* (2001) compared the yield of MLM-type SLs obtained by Lipozyme IM-catalyzed acidolysis and interesterification reactions, using caprylic acid and ethyl caprylate as acyl donors, and trieicosapentaenoylglycerol as acyl acceptor; these authors concluded that the interesterification reaction was more efficient than the acidolysis reaction for the enzymatic synthesis of MLM-type SLs.

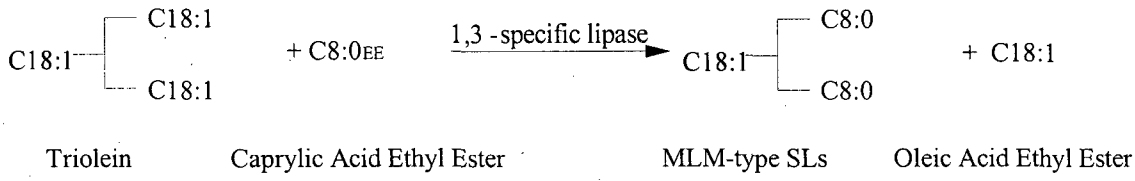


Figure 8. The reaction scheme of the interesterification reaction for the synthesis of MLM-type SLs from triolenin and caprylic acid ethyl ester.

Compared with chemical synthesis, the most significant advantage of enzymatic synthesis is the regio/sterero- specificity of lipases, which can result in easily defined products and have a more predictable chemical structure than those of chemical synthesis (Akoh, 2002). Table 3 lists some of the advantages of enzymatic synthesis of SLs.

Table 3. Advantages of enzymatic synthesis of structured lipid (Akoh, 2002).

- Position-specific SL (i.e., desirable FAs can be incorporated at specific positions of TAG.
- Enzymes exhibit positional/region-selectivity, optical activity and acyl group specificity.
- Can design SL on case-by-case basis to target specific food or therapeutic use- synthesis.
- Products with defined structure can be produced.
- Novel products not possible by conventional plant breeding and genetic engineering can be obtained (e.g., by inserting specific FAs at the *sn*-2 position of glycerol molecule).
- Mild reaction conditions.
- Few or no unwanted side reactions or products.
- Can control the overall process.
- Ease of product recovery.
- Add value to fats and oils.
- Improve functionality and properties of fats.

2.5.3. Parameters Affecting the Enzymatic Synthesis of MLM-type SLs

Enzymatic synthesis of MLM-type SLs can be approached by using 1,3-specific lipase as biocatalysts. However, during the reaction process, there are several parameters that can affect the bioconversion yield (BY) of the SLs either by enhancing acyl migration, or by changing enzyme specificity.

2.5.3.1 Water Activity (a_w)

It is now well accepted that water is essential for enzymatic catalysis. It has an effect on enzyme activity and the reaction direction. Water is responsible for maintaining the three dimensions active conformation of lipase. Different enzymes need different absolute amount of water, and this varies from one solvent to another (Zak and Russell, 1988; Halling, 1994; Wehtje and Adlercreutz, 1997; Schmid *et al.*, 1999).

However, due to the different interactions between water and other components in the reaction system, changing these conditions may alter the optimal water requirements for enzyme catalysis (Halling, 1994). Because of these potential interactions, it is suitable in organic reaction system to eliminate any variable influence of water relations on enzyme behavior by conducting reactions at constant a_w.

From the thermodynamic point of view, it has been increasingly accepted to use a_w, instead of water content or concentration which has been used in many studies (Huang and Akoh, 1996; Irimescu *et al.*, 2001; Paez *et al.*, 2002; Hamam and Shahidi, 2006), to characterize the relationship between water and the other components in the micro-aqueous reaction system. Research have been done on the effect of water content and/or a_w on enzyme activities and bioconversion yield (Schmid *et al.*, 1999; Secundo and Carrea, 2003; Guo and Xu, 2005; Lue *et al.*, 2005).

a_w can be measured via the vapor phase above the reaction mixture and can be expressed as the partial pressure of the solution over the partial pressure of pure water measured through the vapor phase (Pomeranz and Meloan, 1994; Halling, 2002). At equilibrium, a_w values are the same for all phases, regardless of the critical water contents of individual phases.

Most of lipases can catalyze reactions at a_w less than 0.1 (Chamouleau *et al.*, 2001; Secundo and Carrea, 2003; Lue *et al.*, 2005). For the same enzyme, under same reaction conditions, as a_w increases, the bioconversion yield is limited by hydrolysis reaction, whereas as a_w decreases, the bioconversion yield is limited by hydration of enzyme (Lue *et al.*, 2005).

2.5.3.2. Solvent Type

In addition to the importance of a_w, another essential parameter is the solvent hydrophobicity. Enzyme activity in nonaqueous media is correlated with the partition coefficient, log *P*. It is a quantitative measurement of solvent polarity. Solvents with a log *P* value of less than 2 are not appropriate for enzymatic reaction systems, whereas solvents with a log *P* greater than 4 can maintain the activity of the biocatalysts (Laane *et al.*, 1987; Gupta *et al.*, 2003). Lipases in higher log *P* values are stabilized as in aqueous solutions by a series of weak interactions by which van der Waals' interactions, enable them to exhibit strong stabilizing force (Lehninger *et al.*, 1993).

Schmid *et al.* (1999) studied the effect of solvent types on the bioconversion yield of alcoholysis of tripalmintin. They found that Ethyl methyl ketone as well as 2-propanol gave good yields of 2-monopalmitin (MP), but in hexane only a small amount of 2-MP was formed, while in methanol MP was not found; Negishi *et al.* (2003) also reported that lipases, which possess stronger binding forces to water, exert a higher catalytic activity.

These authors have concluded that the hydrophobic solvents are more appropriate for enzymatic biocatalysts than those of hydrophilic solvents. Therefore, proper choice of solvent can greatly affect the reaction.

2.5.3.3. Reaction Temperature

Temperature changes can have a direct effect on enzyme stability, affinity of enzyme for substrate, and preponderance of competing reactions (Akoh, 2002). Thermostability of enzymes is one of the major factors that need to be considered. Enzymes are more thermo-stable in water-restricted environments such as organic solvents (Dordick, 1989). Several studies have studied the effect of reaction temperature on the lipase activity. Caro *et al.* (2000) reported that the lipase from *Euphorbia characias* latex and commercially available crude preparation of bromelain showed the highest activity at 45 and 55°C, respectively. Irimescu *et al.* (2001) observed that using Lipozyme IM as a biocatalyst, the acylglycerol proportion of MLM-type SLs was remained relatively constant (49.9 to 51.2%) when the reaction temperature was changing from 40 to 60°C in the acidolysis reaction. While in the interesterification reaction, the highest yield (81%) of MLM-type SL was observed at 30°C. Turon *et al.* (2003) concluded that in lipase (from *Carica papaya)* catalyzed biosynthesis of butyl laurate, the maximum enzyme-catalyzed yields reached 9.8 and 14.6% in esterification and transesterification reactions, respectively. Lue *et al.* (2005) also reported that the highest initial enzyme activity of Novozym 435 was observed at 55°C. Moreover, an increase in reaction temperature not only results the reaction reaches its equilibrium faster, but also shifts toward hydrolysis (Lumor and Akoh, 2002).

2.5.3.4. Enzyme Concentration

Normally, as enzyme concentration increases, the reaction equilibrium will be shifted towards the synthesis direction quickly. After reaching certain enzyme concentration, the

bioconversion yield is constant. Kim *et al.* (2001) concluded that in the Lipase-catalyzed incorporation of CLA into TC, the optimal enzyme concentrations were 5% for Novozym 435, and Lipozyme IM and 10% for Lipase PS-C. Zhao *et al.* (2006) studied the effect of the concentration of Lipozyme TL IM on the acidolysis reaction of lard with caprylic acid (CA). Within their testing range (5 to 25%), they noted a gradual increase in CA incorporation in lard when enzyme load was increased up to 15%. Above this enzyme concentration, the incorporation of caprylic acid did not increase. Carrín and Crapiste (2008) found that in Lipozyme IM-catalyzed acidolysis of sunflower oil with a PA and SA mixture, the extents of PA and SA incorporations were enhanced by increasing the amount of enzyme in the reaction mixtures, but a significant increase was not observed when the enzyme was present at greater than 8% by weigh of substrates.

2.5.3.5. Effect of Substrate Molar Ratio

Substrate molar ratio plays a role in the reaction equilibrium. With an increasing of the concentration of substrates, the reaction equilibrium will be shift toward the synthesis direction. Therefore, the bioconversion yield of SLs is increased. Jennings and Akoh (2000) reported that using substrate molar ratio 1:8 (capric acid to rice brain oil), the incorporation of capric acid into rice brain oil increased from 29.6 to 53.1%. Can and Özçelik (2005) pointed out that the incorporation rate was improved with higher substrate molar ratio of PUFAs to hazelnut oil and the reaction time could be shortened. However, Jenning and Akoh (2001) concluded that the high amount of substrate could cause the substrate inhibition of the enzyme.

2.5.3.6. Addition of Adsorbents

The promotion of the enzymatic synthesis of SLs can be obtained by shifting the thermodynamic reaction towards synthesis rather than hydrolysis; this can be achieved by addition of adsorbents to the reaction medium to reduce water content. Medina *et al.*

(1999) reported that the addition of molecular sieves increased the reaction rate and the bioconversion level due to their effect on sequestering the water layer surrounding the enzyme molecule which was essential for the water-enzyme interaction. However, Karboune *et al.* (2005) reported a decrease in the maximum bioconversion yield upon the addition of 10 mg/mL of molecular sieves to the lipase-catalyzed biosynthesis of cinnamoylated lipids.

Silica gel, an amorphous form of silicon dioxide, functions as a desiccator. The advantage of using Silica gel as an adsorbent is the physical adsorption of bounded water into its internal pores (Castillo *et al.,* 1997). No chemical side-reactions take place, which make Silica gel appealing. Halling (2002) indicated that Silica gel added to a reaction medium resulted in an enhancement in lipase-catalyzed esterification reactions. Karboune *et al.* (2005) reported that an increase in the maximum bioconversion yield of phenolic lipids was obtained by the addition of Silica gel as an adsorbent.

2.6. Analysis and Characterization of Structured Lipids

In order to characterize the structural properties and to monitor the produced MLM-type SL molecules, various separation and characterization techniques have been used. These include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), HPLC-Mass Spectrometry (LC-MS), gas liquid chromatography (GC) and nuclear magnetic resonance (NMR) spectroscopy. The choice of purification technique depends on substrate or reaction type, products formed, overall cost and whether a small-scale or large scale synthesis is employed (Akoh, 2002).

2.6.1. Thin-Layer Chromatography

Thin-layer chromatography (TLC) separation has been used widely for qualitative analysis of SLs and for preparative separation of products, which can be further analyzed

by HPLC and GC methods. Separation is generally applied in the form of spots at a level that is slightly above the mobile phase solvent and the plate is placed inside a tank and sealed. The solvent migrates up the plate by capillary action. The fractions of a sample are separated based on the partitioning within the mobile phase (Gurr *et al.*, 2002). The separated fractions can be visualized by eye if the spots are coloured or by spraying with 20% sulphuric acid (Sabally *et al.*, 2005) or 12.5% ethanol solution of phosphomolybdic acid (Yesiloglu and Kilic, 2004) or 0.2% 2, 7-dichlororescein in methanol (Jennings and Akoh, 2000; Kim *et al.*, 2001; Zhao *et al.*, 2006), which react with organic compounds to form dark products. Alternatively, plates with fluorescence incorporated into their stationary phase can be visualized under UV light after the fraction separation. The separated fractions quench the fluorescence of the plate and show the darker area under UV light.

The TLC analysis of the SLs with short and medium chains as well as PUFAs are mostly carried on silica-gel 60 TLC plate (Xu *et al.*, 1998; Can and Özçelik 2005) or Silica gel G (Rao *et al.*, 2002). In recent years, boric acid impregnated Silica gel plate are used with mobile phase of petroleum ether/ethyl ether/acetic acid mixture, (Jennings and Akoh, 2000; Kim *et al.*, 2001; Zhao *et al.*, 2006), hexane/diethyl ether/acetic acid mixture (Senanayake and Shahidi, 2002) and chloroform/acetone/methanol (Yesiloglu and Kilic, 2004).

2.6.2. High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry. HPLC of reverse phase and silver ion are generally used for the qualitative and quantitative analysis of TAGs from a mixture (Kuksis and Itabashi, 2005).

2.6.2.1. Reverse Phase HPLC

Reverse phase high-performance liquid chromatography HPLC (RP-HPLC) is used predominantly in the separation of SLs. It consists of a non-polar stationary phase and an aqueous, moderately polar mobile phase. The stationary phase of RP-HPLC is generally made up of hydrophobic alkyl chains ($-CH_2-CH_2-CH_2-CH_3$) that interact with the analytes. Most separation is based on the fatty acyl group and the saturation of the lipids. The octadecylsilylsilca also called C18 is commonly used in the stationary phase for the separation of SLs (Arcos *et al.*, 2000; Kuksis and Itabashi, 2005). Separation of SLs on C18 reverse phase column also depends on using gradient mobile phase. Mobile phase mainly used are acetone, acetonitrile, hexane, iso-proanol in different volume ratio and different concentration gradient (Mu *et al.*, 2001; Kawashima, 2001; Zhou *et al.*, 2001; Otero *et al.*, 2006).

2.6.2.2. Silver-Ion HPLC

Silver-ion HPLC, containing silver ion bonded Silica or similar substrates, has been becoming a tremendously powerful technique for the analytic and semi-preparative separation/isolation of FAs methyl ester (FAME) and positional isomers of SLs on *sn*-1,3 and *sn*-2 positions (Chandler, 2001; Irimescu *et al.*, 2001; Kawashima *et al.*, 2001; Adlof and List, 2004; Dugo *et al.*, 2004; Adlof, 2007). The separation principle of silver-ion HPLC is dependent on the fact that the π-electrons of double-bonds in the fatty acyl residues of lipids react reversibly with silver ions to form polar complexes (Adlof, 1997). The greater the number of double-bonds, the stronger the complexion effect. The separation of positional isomers of SLs is closely related to the specific positions of unsaturated FAs on the glycerol molecule (Adlof, 1997).

Because sample components have their own characteristics, several different types of detectors are used, including ultraviolet (UV) detector, refractive index (RI) detector,

fluorescence (FD) detector and evaporative light scattering detector (ELSD). However, for the analysis of TAGs, with EFAs and MCFAs which do not have multiple double-bonds, ELSD is generally adopted by most research (Zhou *et al.*, 2001; Irimescu *et al.*, 2001; Otero *et al.*, 2006; Vu *et al.*, 2008).

2.6.3. High-Performance Liquid Chromatography/Mass Spectrometry

Mass spectrometry (MS) is a sensitive method for molecular analysis, which can provide information on the molecular mass and structure of the analyte. It has been used for the identification of TAGs (Jakab *et al.*, 2003). By combining the advantages of HPLC and MS, LC/MS is becoming a very useful tool for analytical chemists due to its high selectivity and sensitivity (Mu *et al.*, 2001; Simal-Gandara *et al.*, 2002; Dugo *et al.*, 2004; Kuksis and Itabashi, 2005; Kalo *et al.*, 2006).

MS can generate protonated molecular ions $[M + H]^+$ and DAG fragment ions $[MH - RCOOH]^+$ from TAGs, hence can provide the location of FAs in the primary and secondary position of the glycerol backbone (Simal-Gandara *et al.*, 2002). The major challenge in coupling the HPLC with MS is due to the fact that gas-phase ions must be produced in order to obtain a mass spectrum. Therefore, prior to pass through MS, the separated TAG portions and the mobile phase from HPLC, have to be separated by the vaporization of mobile phase. There are several types of LC-MS interface currently available, including continuous flow fast-atom bombardment (CF-FAB), moving-belt (MB), chemical ionization (CI), thermospray (TSP), particle beam (PB), ion spray (ISP), electro spray (ES), atmospheric pressure chemical ionization (APCI), inductively coupled plasma (ICP) and plasma spray (PSP) interfaces (Simal-Gandara *et al.*, 2002).

Among the interfaces, APCI and ESI are the most frequently used ionization technique for TAGs analysis, because of easy coupling to non-aqueous mobile phase systems and high ionization efficiency for non-polar species (Kalo *et al.*, 2006; Krist *et al.*, 2006).

2.6.4. Gas-Liquid Chromatography

2.6.4.1. Generality

Gas-liquid chromatography (GC) is used for the detection of volatilized compounds, such as FAs, directly or by the derivation. The mobile phase consists of an inert gas, normally helium (Kim and Akoh, 2006) or nitrogen (Rocha-Uribe and Hernandez, 2004), which passes sample through a column. In order to increase the stability and volatility of the organic compounds mixtures during the analysis process, the samples have to be methylated before injection. During the process of analysis of the reaction mixtures, the temperature of the injector and the detector can be programmed to improve the resolution (Can and Özçelik, 2005).

2.6.4.2. Determination of FA in sn-2 Position of TAGs by GC

The FA compositions of TAGs are usually determined by GC, as regio- and stereo-selective analyses, are subsequent to partial hydrolysis and purification processes (Gunstone, 1999). The analysis of common FAs is normally carried out in the form of fatty acid methyl esters (FAMEs). To determine the positional distribution of FAs on the glycerol backbone, pancreatic lipase of *sn*-1,3 specificity, is often used (Kim and Akoh, 2005) to hydrolyze the TAG fraction of the reaction products. The hydrolyzed mixture is then separated by preparative TLC or preparative HPLC. The bands corresponding to MAGs are scraped and methylated followed by GC analysis (Kim and Akoh, 2006; Turan *et al.*, 2007). Although there are several types of detectors that can be used in GC where they are different in their particular measurement applications, detection limit required, matrix interferences, and/or regulatory guidelines, the detection of SLs is generally

carried out by FID (Mu *et al.*, 2001; Weber and Mukherjee, 2004; Yoshida *et al.*, 2005; Vu *et al.*, 2008).

2.6.5. Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) is a spectroscopic technique that reveals information about the environment of magnetically active nuclei (Bergana and Lee, 1996). The most important applications for the structure analysis of SLs are ^{1}H NMR and ^{13}C NMR spectroscopy (Bergana and Lee, 1996; Haraldsson *et al.*, 2001; Negishi *et al.*, 2003).

Regarding the ^{1}H NMR spectroscopy, the protons belonging to the glycerol backbone of the acylglycerols are highly useful for characterizing the products because these protons resonate very characteristically in individual acylglycerols, such as 1- and 2-MAGs, 1,2- and 1,3-DAGs, and TAGs (Haraldsson *et al.*, 2001; Laszlo and Compton, 2006),. The ^{13}C NMR spectroscopy is particularly used for the monitoring of the region-control of the SLs due to the fact that the two distinctive resonance signals from the carbonyl group carbon of each FA depend on the location of their acyl groups at the end positions or at the mid-position of the glycerol backbone (Negishi *et al.*, 2003). The mixture of transesterification reaction normally is firstly separated by silver-ion HPLC. By using the ^{13} NMR magnified spectral method, the peaks corresponding to the carbonyl carbon atoms of the *sn*-2 PUFAs and MCFAs could be measured where the FA composition is determined on the basis of their peak heights (Bergana and Lee, 1996).

3. MATERIALS AND METHODS

3.1. Materials

Commercially immobilized lipases Novozym 435 from *Candida antarctica* (with an activity of 10,000 propyl laurate units/g solid enzyme at pH 8.0 and 40°C), Lipozyme IM from *Mucor miehei* (with an activity of 86.8 stearic acid unit/g solid enzyme at pH 8.0 and 70°C) and Type VI-S porcine pancreatic lipase (with an activity of 100,000 unit/g solid enzyme, where one unit corresponds to the hydrolysis of 1.0 microequivalent of fatty acid from a triacylglycerol in 1 h at pH 7.7 at 37°C using olive oil as substrate) were purchased from Sigma Chemical Co. (St-Louis, MO). Tricaprylin (TC) was also purchased from Sigma Chemical Co. Trilinolenin (TLN), dilinolenin, monolinolenin, caprylic and linolenic ether esters were purchased from Nu-Chek Prep Inc. (Elysian, MN). Molecular sieve 3Å (rod) and Silica gel were purchased from Sigma Chemical Co. All solvents were of HPLC grade or higher and were purchased, along with ACS grade salts used for pre-equilibration of water activity, from Fisher Scientific (Fair Lawn, NJ).

3.2. Methods

3.2.1. Lipase-Catalyzed Interesterification of Tricaprylin with Trilinolenin

3.2.1.1. Preparation of Substrate Solutions

Prior to each enzymatic reaction, stock solutions of 40 mM of tricaprylin (TC) and 40 mM of trilinolenin (TLN) were freshly prepared in hexane.

3.2.1.2. Interesterification Reaction

Defined quantities of TC and TLN stock solutions were diluted with the appropriate amount of hexane in 50 mL-Erlenmeyer flasks to a yield final concentration of 20 mM for each substrate.

Enzymatic reactions were initiated by the addition of 20 mg of either Lipozyme IM or Novozym 435. The Erlenmeyer flasks were incubated under vacuum at 50°C with continuous shaking at 150 rpm in an orbital shaker (New Brunswick Scientific Co., Inc., Edison, NJ) in obscurity to avoid light. Control reactions without lipase were carried out in tandem with the enzymatic trials under the same conditions.

3.2.1.3. Analysis of Reaction End Products

Sample mixtures were re-solubilized in appropriate amount of hexane (0.1 mL) and subjected to high-performance liquid chromatography (HPLC) analysis for quantification of substrates and end products, according to a modification of the method described by Adlof (2007).

HPLC analysis was carried out with a ChromSpher 5 Lipids column (250×4.6 mm, Varian Inc., Middleburg, Netherlands), using Beckman HPLC system (Model 126, Beckman Instruments Inc., San Ramon, CA). This system was equipped with an Evaporative light scatter detector (Varex Corporation, Rockville, ML) with computerized data handling and integration analysis (System Gold software, version 8.0, Beckman). The conditions of the ELSD were a pressure of 20 psi, 50 mL/min air flow and 75°C temperature. The injected sample was 20 μL, with a flow rate of mobile phase of 1 mL/min. The mobile phase, consisting of A, hexane and acetonitrile (100:1, v/v) and solvent B, hexane/toluene (50:50, v/v), was initially at 50% of A gradient to 100% of A, held for 30 min. At the end of the run, the column was equilibrated, for the next analysis, with the 50% mobile phase A for 15 min.

Calibration curves were constructed from a wide range of concentrations (5 to 30 mM) of substrates and the SLs products. Because of the lack of the availability of commercial standards of the MLM and LML-type SLs, the standard curves were constructed by their recovery from HPLC.

3.2.2. Calculation of the Bioconversion Yield and Initial Enzymatic Activity

The bioconversion yield (BY) was defined as the concentration of the TAG products divided by the concentration of limiting substrate (acyl acceptor) in the blank at time *t*, multiplied by 100. The initial enzymatic activity was calculated from the slope of the linear portion of the plot of the concentration of 1,3-dicapryl-2-linolenyl glycerol (CLnC) versus the reaction time and it was expressed as μmol of CLnC per g of solid enzyme per min of reaction.

3.2.3. Characterization of Positional Distribution of FAs on MLM-type SLs

The positional distribution of the FAs on the glycerol backbone of the interesterified end products was investigated by their regio-selective hydrolysis with pancreatic lipase. The separation of the hydrolysis products was carried out by thin-layer chromatography (TLC). The monoacylglycrols (MAGs) FAs composition was investigated by gas-liquid chromatography (GC) analysis.

3.2.3.1. Selective Hydrolysis

The positional distribution of fatty acids on glycerol backbone was determined, according to the method described by Kim and Akoh (2006), using the type VI-S porcine pancreatic lipase for the hydrolysis of MLM-type and LML-type SLs. One mg of SL was mixed with 1 mL of 1 M Tris-HCl buffer (pH 7.6), 0.25 mL of 0.05% bile salt, 0.1 mL of 2.2% $CaCl_2$ and 1 mg porcine pancreatic lipase. The mixture was incubated in a water bath at 37°C for 3 min and extracted twice with 3 mL diethyl ether.

3.2.3.2. Separation of Monoacylglycerol on Thin-Layer Chromatography

The hydrolysis products were separated by Silica gel 60 plates with fluorescent indicator (Whatman, Fisher Scientific). The mobile phase for the TLC analysis was a mixture of chloroform/acetone/acetic acid (96:4:1, v/v/v) as described by Hita *et al.* (2007). After drying, the bands corresponding to MAGs were visualized under short wavelength UV light (254 nm) in a fluorescence analysis cabinet (Model CX20, Spectronics Corporation, Westbury, N.Y.).

3.2.3.3. Methylation

The bands corresponding to TAGs were scraped from TLC and extracted by isopropanol. Methylation of the MAGs was accomplished according to a modification of the method described by Rocha-Uribe and Hernandez (2004). One mg of MAGs was diluted in 0.6 mL of hexane and 2 M sodium methoxide in 20% methanol. The mixture was incubated in reciprocal shaking water-bath (Model 25, Precision Scientific, Chicago, IL) at 65°C. After 20 min of incubation, 10% sulfuric acid solution prepared in methanol was added followed by its incubation in a water-bath at 85°C for 30 min. The methylated FAs were recovered by two times extraction with 2 mL of hexane. The upper layer was collected

and recovered for GC analysis.

3.2.3.4. GC analysis

The methyl ester of FAs was solubilized in hexane for GC analysis. The analysis was performed in a gas chromatograph (Agilent 6890, Agilent Technologies, Wilmington, DE). The chromatograph was equipped with a HP-INNOWax (Agilent Technologies, Wilmington, DE) column (30 m × 0.25 mm × 0.25 μm) and a flame ionization detector (FID). Helium was used as the carrier gas at 63 mL/min flow rate. The injector and detector temperatures were set at 150 and 230°C, respectively. The initial column temperature was 150°C for 1 min, and then increased by 10°C/min to 180°C followed by 1°C/min to 220°C for 30 min and held for an additional 5 min.

3.2.4. Optimization of Lipase-Catalyzed Interesterification Reaction

3.2.4.1. Selection of Biocatalyst

The selection of a biocatalyst for the interesterification reaction was investigated, using two different sources of enzymes, Lipozyme IM and Novozym 435. Aliquots of the enzymatic reaction medium were recovered during an 8-h incubation period. The interesterification reactions were carried out under a wide range of temperature (20 to 50°C) and well-defined conditions including solid enzyme amount (20 mg), substrate molar ratio (1:1), agitation speed (150 rpm) and reaction volume (2 mL of hexane).

3.2.4.2. Effect of Initial Water Activity/Solvent Type

The effect of initial water activity (a_w) of the reaction system on the enzymatic synthesis of SLs was investigated. The determination of a_w was performed according to the method developed in our lab (Lue *et al.*, 2005). The selected lipase and solvents, including iso-octane (Log P = 4.5), heptane (Log P = 4.0), hexane (Log P = 3.5) and cyclohexane (Log P = 3.2) were pre-equilibrated separately up to the required a_w. The equilibration was carried out at 4°C for the solid enzyme and at room temperature for the solvents, in sealed containers using saturated salts with characteristic a_w values, including LiCl (a_w = 0.11), $MgCl_2$ (a_w = 0.33), $Mg(NO_3)_2$(a_w = 0.53). The minimum equilibrium period was 72 hours. The dehydration of solvent (a_w = 0.06) was done by addition of molecular sieve (3Å) directly in the solvent and equilibrated overnight, and the enzyme was pre-

equilibrated with molecular sieve (3Å) in a sealed container overnight to reach the a_w 0.06. The selection of these solvents was based on their hydrophobicities and the solubility of substrates in these solvents. Aliquots were taken at regular time intervals over the course of a 5-h period.

3.2.4.3. Effect of Enzyme Concentration

The effect of enzyme concentration on the bioconversion yield of MLM-type SLs was investigated by varying enzyme concentrations from 5 to 20 mg/mL hexane. The interesterification reaction was carried out for a period of 5 h, using hexane as the reaction medium, with initial a_w of 0.06 at 30°C with Lipozyme IM as the biocatalyst.

3.2.4.4. Effect of Substrate Molar Ratio

The effect of TC to TLN ratios were investigated by increasing the initial concentration of TC in the reaction system (5 to 160 mM) at a defined TLN concentration (20 mM), with the ratios of TC to TLN of 1:4 to 8:1, respectively. Enzymatic reaction were carried out for a 13-h period, using the optimized assay conditions (a_w= 0.06, hexane, 30°C, 10 mg solid enzyme/mL).

3.2.4.5. Effect of Molecular Sieve

In order to favor the enzymatic synthesis of MLM-type SL, the effect of the addition of molecular sieve on the interesterification reaction was investigated. Using Lipozyme IM (10 mg solid enzyme/mL hexane) at 30°C, the enzymatic reaction was investigated in the presence of molecular sieve (Type 3Å; rod) at concentrations ranging from 5 to 20 mg/mL hexane.

3.2.5. Effect of Selected Parameters on the Productivity of CLnC

The productivity of the interesterification reaction was calculated on the basis of weight of CLnC (M_t) and substrate (M_0), reaction time (t), enzyme unit (U) and/or reaction volume (V). The mass productivity (P_M) was calculated by the number of mg of CLnC produced per h divided by the initial substrate mass (Eq. 1). The enzymatic productivity (P_E) was defined as number of mg of CLnC produced per h per unit Lipozyme IM (Eq. 2),

while the volumetric productivity (P_V) was calculated by the number of mg of CLnC synthesized per h per liter of reaction medium (Eq. 3).

$$P_M = \frac{M_t}{t\ (M_0)} \qquad \text{[Eq. 1]}$$

$$P_E = \frac{M_t}{t\,(U)} \qquad \text{[Eq. 2]}$$

$$P_V = \frac{(M_t)}{t\,(V_R)} \qquad \text{[Eq. 3]}$$

3.2.5.1. Effect of Substrate Concentration

The effect of substrate concentration on the enzymatic synthesis of SLs was investigated by varying the concentrations of TLN from 40 to 200 mM. The enzymatic reaction were carried out in 25 mL round-bottom flask, using the optimized assay conditions (a_w = 0.06, hexane, 30°C, and substrate molar ratio of 6:1, TC/TLN), the volume of the reaction mixtures was 1 mL. The enzymatic reactions were initiated by the addition of 40 mg of Lipozyme IM.

3.2.5.2. Effect of Selected Limiting Factors on the Productivity of SLs

In order to maximize the productivity, selected limiting factors, including enzyme denaturation, shifting reaction equilibrium and the formation of a hydrophilic hindrance layer of glycerol surrounding the enzyme support, were investigated.

(a) Denaturation of the Enzyme

To examine the denaturation of the enzyme, the solid enzyme was substituted by the new one after 7 h of the interesterification reaction.

(b) Controlling of Water Activity

The a_w during the enzymatic reaction process was controlled by binary salt as described by Halling (1992), salt pairs with certain a_w, including LiCl/LiCl.1H2O (a_w = 0.023);

$Li_2SO_4/Li_2SO_4.1H_2O$ (a_w = 0.10) and $Ba(OH)_2.1H_2O/Ba(OH)_2.8H_2O$ (a_w = 0.34), were initially added to the reaction medium.

(c) Formation of Glycerol Layer

In order to investigate the limitation of the productivities by the formation of a hydrophilic glycerol hindrance layer, glycerol was added after 7 h of enzymatic reaction to the reaction mixture containing an initial TC to TLN ratio of 6:1.

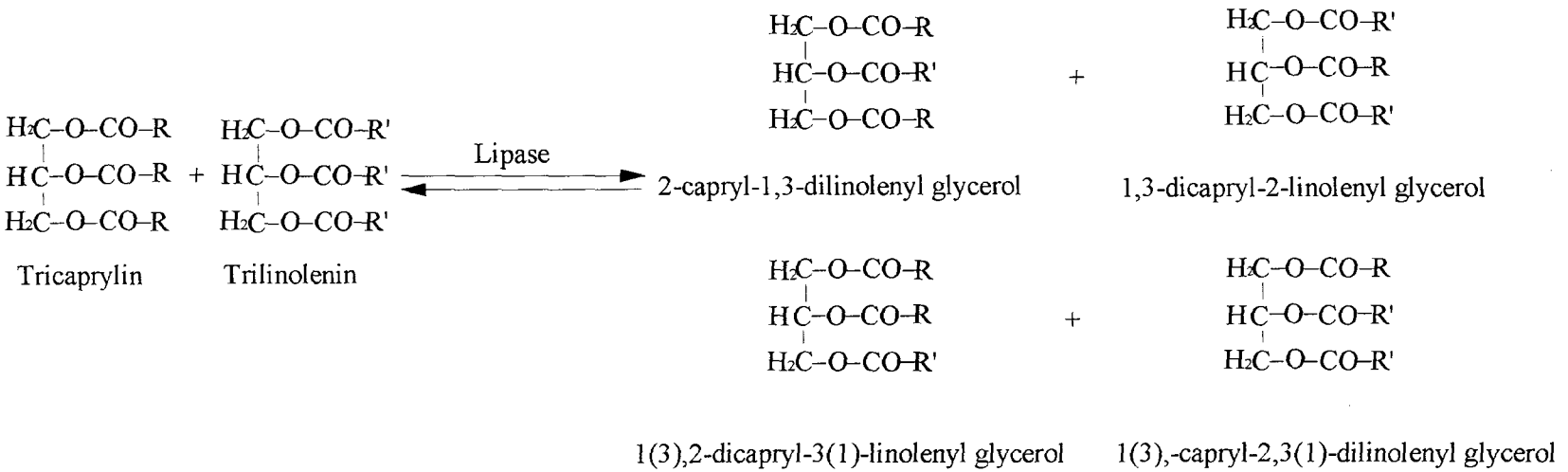
To avoid the limit increase in biosynthesis of SLs, the addition of Silica gel to the reaction mixture, as an adsorbent support for glycerol, was investigated. Different amounts of Silica gel (1.5, 3.0, 4.5 and 6.0 mg) were added to the reaction mixture composed of 0.2×10^3 mM TLN, 1.2×10^3 mM TC and hexane. The experimental trials were carried in a period of 11 h, using the optimized assay conditions (a_w = 0.06, hexane, 30°C, and substrate molar ratio of 6:1 of TC/TLN).

4. RESULTS AND DISCUSSION

4.1. Characterization of End Products of Lipase-Catalyzed Interesterification of Tricaprylin with Trilinolenin

The enzymatic synthesis of medium-long-medium (MLM)-type structured lipids (SLs) by the interesterification reaction of tricaprylin (TC) with trilinolenin (TLN) was carried out using lipase Lipozyme IM (Fig. 9). The reaction components were recovered and separated by silver-ion column equipped with an evaporative light scatter detector (ELSD). Adlof (1997) indicated that the separation of positional isomers by silver-ion column depends on the number of double bonds and the chain-length of triacylglycerols (TAGs); the chain with more double bonds has the longer elution time. Fig. 10 shows the typical chromatogram of HPLC using ELSD. With reference to the standards, TC (peak #1) was eluted first at 6.9 min, while TLN (peak #6) was eluted at 50.6 min (Fig. 10A). Fig. 10B shows the chromatogram of the interesterification reaction mixture of TC and TLN after 3 h of incubation; the elution profiles indicated peaks of substrates as well as four new peaks # 2, 3, 4, and 5 that were characterized as the potential end product SLs.

In order to determine the positional distribution of fatty acids on glycerol backbone, the potential SL-isomers were purified and subjected to hydrolysis by pancreatic lipase, characterized by a high specificity at the *sn*-1 and *sn*-3 positions. The hydrolysis products were separated by thin-layer chromatography (TLC), recovered and methylated before gas-liquid chromatography (GC) analysis. By comparing the elution time with the standards, peaks # 3 and 4, eluted at 17.2 and 35.1 min, were characterized as 1,3-dicapryl-2-linolenyl glycerol (CLnC), and 1(3)-capryl-2,3(1)-dilinolenyl glycerol (CLnLn), respectively. While peaks #2 and #5, as positional isomers of peaks #3 and 4, were identified as 1(3),2-dicapryl-3(1)-linolenyl glycerol (CCLn) and 2-capryl-1,3-dilinolenyl glycerol (LnCLn), respectively.



R = C8:0
R' = C18:3

Figure 9 . The reaction scheme of lipase-catalyzed interesterification reaction of tricaprylin with trilinolenin.

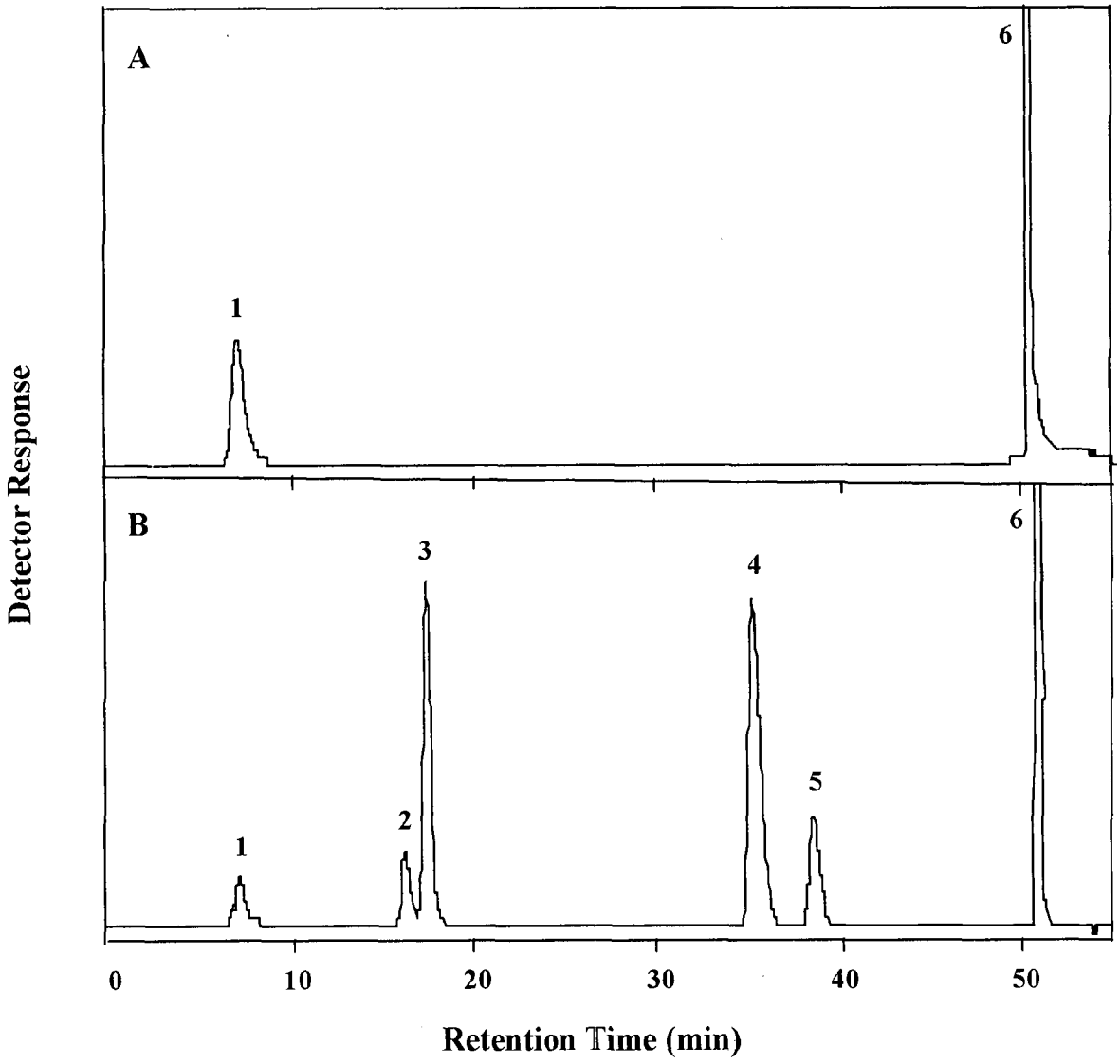


Figure 10. Chromatogram of silver-ion exchange high-performance liquid chromatography (HPLC) analysis of interesterification reaction monitored by evaporative light scattering detector (ELSD), using tricaprylin (TC) and trilinolenin (TLN) as substrates; (A) control; (B) interesterification reaction products after 3 h of incubation. Peak characterized as TC (peak #1), 1(3),2-dicapryl-3(1)-linolenyl glycerol (peak #2), 1,3-dicapryl-2-linolenyl glycerol (peak #3), 1(3),-capryl-2,3(1)-dilinolenyl glycerol (peak #4) and 2-capryl-1,3-dilinolenyl glycerol (peak #5), and TLN (peak #6), respectively.

The use of silver-ion column in the analysis of reaction mixtures of SLs has been reported in recent years. Contrary to the experimental findings, Irimescu *et al.* (2001) separated the mixtures of acidolysis reaction of trieicosapentaenoyl glycerol with caprylic acid in a binary solvent gradient made of acetone and acetone/acetonitrile mixture, and reported the following sequence of CCE, CEC, ECE and EEC (C and E stand for the acyl group of C8:0 and C20:5, respectively). However, another elution sequence of SLs was obtained by Chandler (2001) in the transesterification reaction of decanoic acid with enriched sunflower oil in a solvent gradient of toluene/*n*-hexane to toluene/ethyl acetate, which was DOD, DDO, DOO, ODO (D and O represent the acyl group of C10:0 and C18:1, respectively). These differences in the elution sequence of SLs may be due to the use of different solvents. In addition to the number of double bonds and the chain-length of TAGs, the separation of the SL-isomers on the silver-ion column depends greatly on the nature of the mobile phase.

Overall, the silver-ion HPLC and GC analysis confirmed the formation of MLM-type SLs by lipase-catalyzed interesterification of TC with TLN.

4.2. Optimization of Lipase-Catalyzed Interesterification of Tricaprylin with Trilinolenin

Our preliminary work on the enzymatic interesterification reaction of TC with TLN provided the foundation for the development of a model system for the synthesis of medium-long-medium (MLM)-type SLs. In order to optimize the biosynthesis of MLM-type SLs, selected parameters, including type of biocatalyst, temperature, solvent type, initial water activity (a_w), enzyme concentration, substrate molar ratio and the use of molecular sieve were investigated.

4.2.1. Selection of Appropriate Biocatalyst/Reaction Temperature

Two lipases, Lipozyme IM from *Mucor miehei* and Novozym 435 from *Candida*

antarctica, were selected for the enzymatic synthesis of MLM-type SLs. Lipozyme IM and Novozym 435 do not only differ in their positional selectivity but also in their substrate specificity. Lipozyme IM, which is a 1,3-specific lipase, preferentially rearranges fatty acids at the *sn*-1 and *sn*-3 positions on the glycerol backbone of TAGs, while the *sn*-2 position remains unattached (Gunstone, 1999). Novozym 435, being a non-specific lipase, randomly rearranges fatty acids at the *sn*-1,3 and *sn*-2 positions (Gunstone, 1999; Chandler, 2001). With regard to the substrate specificity, Lipozyme IM showed a higher substrate specificity towards the medium-chain TAGs (Berger and Schneider, 1991). The literature (Lee and Parkin, 2002 and 2003) reported that Novozym 435 had a high specificity towards medium-chain fatty acids (MCFAs) and their diacylglycerols.

It is suggested that the reaction temperature could affect significantly the enzyme activity (Whitaker, 1993) and the acyl migration of MCFAs on *sn*-2 position of glycerol backbone (Mu *et al.*, 2001). Lipase-catalyzed interesterification of TC and TLN was carried out at different temperatures ranging from 20 to 50°C using both selected lipases. The effects of different reaction temperatures with selected lipases on the maximum bioconversion yield (BY) of CLnC are shown in Fig. 11. Using Lipozyme IM as biocatalyst, the maximal BY of CLnC increased slightly from 22.5 to 24.7% when the reaction temperature was raised from 20 to 50°C, whereas using Novozym 435 as biocatalyst, the BY increased 21-fold when the temperature was increased from 30 to 50°C. These results suggested that the BY of CLnC, with Novozym 435 as biocatalyst, was affected more significantly by the change in reaction temperature than that with Lipozyme IM. Similarly, Wei *et al.* (2003) obtained a 2-fold increase in the BY of propyl-glycoside lactate by the Novozym 435-catalzyed esterification reaction of lactic acid and propyl-glycoside when the temperature increased from 30 to 80°C. In contrast, Fomuso and Akoh (1998) reported that the BY of MLM-type SL (dicaproyllinolein) was constant for the Lipozyme IM-catalyzed interesterification of trilinolein and tircapronin, when the reaction

temperature was changed within the range of 25 to 65°C.

The results in Table 4 show a 3.2-fold increase in the initial enzyme activity of Lipozyme IM when the reaction temperature was increased from 20 to 50°C. However, Novozym 435 showed a 17.2-fold increase in the initial enzyme activity when the reaction temperature changed form 30 to 50°C. Similarly, Lue *et al.* (2005) reported a substantial increase of 11-fold in the enzyme activity of Novozym 435-catalzyed esterification reaction of *t*-cinnamic acid with oleyl alcohol, when the reaction temperature was increased from 35 to 55°C. The overall results suggest that the activity of Novozym 435 was greatly affected by the change in temperature. Whitaker (1993) indicated that the higher temperature generally caused more collisions of the substrate molecule, hence increased its interactions between substrate and the active site of the enzyme. The difference in the temperature dependence between Novozym 435 and Lipozyme IM may be due to the difference in their 3-dimensional structures which could require different levels of energy to catalyze the selected reactions (Senanayake and Shahidi, 1999).

The results in Fig. 11 also show the effect of different temperatures on the required time to reach the equilibrium state. For Novozym 435-catalyzed interesterification reaction of TC with TLN (Fig. 11A), the time to reach the equilibrium was decreased from 6 to 4 h when the reaction temperature was increased from 40 to 50°C, whereas for Lipozyme-catalyzed interesterification reaction (Fig. 11B), it was decreased from 2 to 1 h when the reaction temperature was increased from 20 to 50°C. These results suggest that the required time to reach the equilibrium state was also dependent on the temperature of the enzymatic reaction. Similarly, Martinez *et al.* (2005) reported a decrease in the required time to reach the equilibrium from 25 to 8 h for Chirazyme L-9-catalyzed esterification of glycerol with conjugated linoleic acid, when the reaction temperature was increased from 30 to 50°C. A decrease in the BY after reaching its maximum

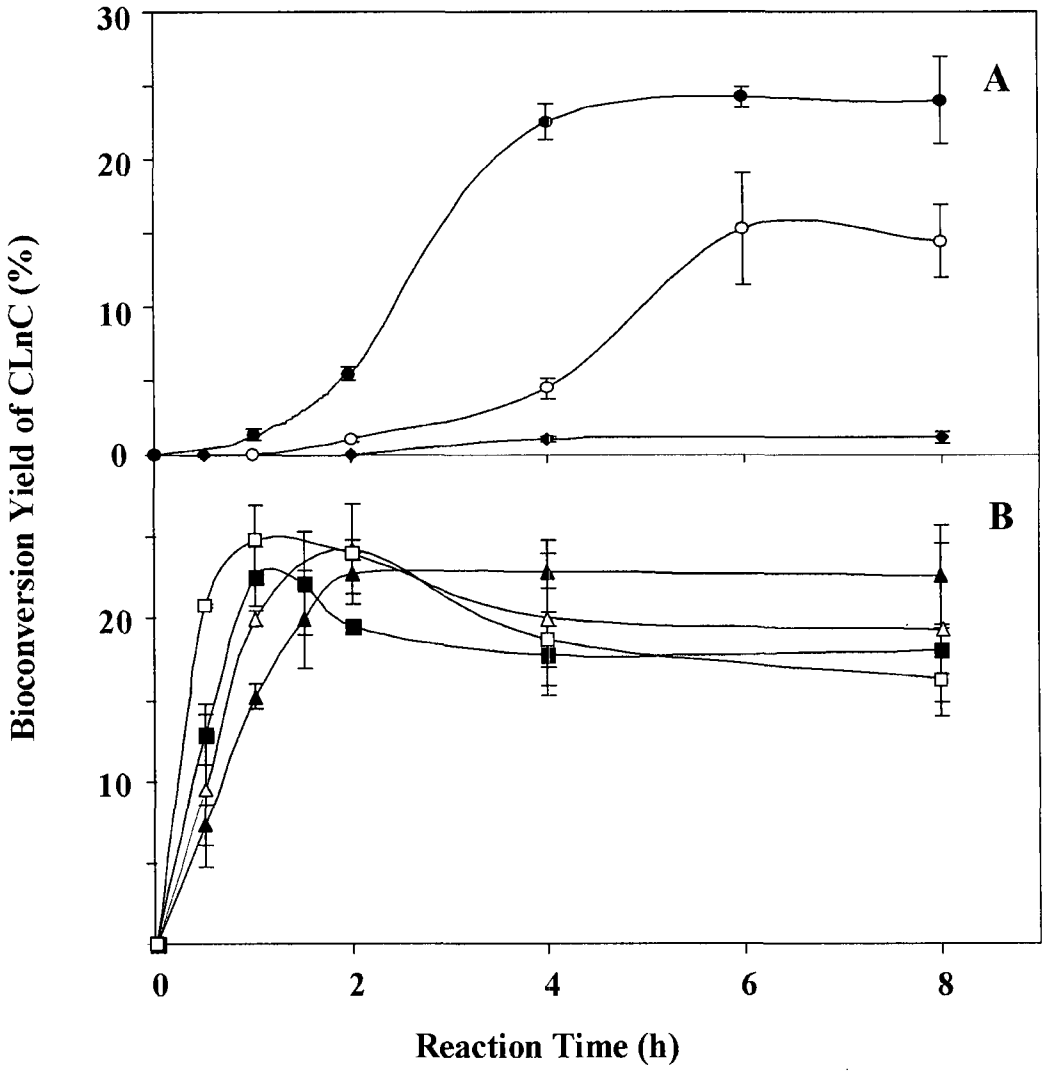


Figure 11. The effect of temperature on bioconversion yield of 1,3-dicapryl-2- linolenyl glycerol (CLnC) in lipase-catalyzed interesterification of tricaprylin with trilinolenin throughout 8 hours; Novozym 435 at 50°C (●), Novozym 435 at 40°C (o), Novozym 435 at 30°C(◆), Lipozyme at 20°C (▲), Lipozyme at 30 °C (△), Lipozyme IM at 40°C(■) and Lipozyme at 50°C(□).

Table 4. Effect of enzyme type and temperature on the initial enzyme activity and the relative triacylglycerol (TAG) proportion of Lipozyme IM-catalyzed interesterification reaction of tricaprylin with trilinolenin.

Temperature (°C)	Initial enzyme activity (μmol CLnC/g enzyme/min)[a]	Relative TAG proportion (%)[b]			
		CCLn	CLnC	CLnLn	LnCLn
Novozym 20°C	N.D.	N.D.	N.D.	N.D.	N.D.
Novozym 30°C	0.1	7.7 (10.9)[c]	30.8 (12.3)[c]	45.6 (19.9)[c]	16.0 (12.1)[c]
Novozym 40°C	0.4	12.4 (9.5)[c]	47.6 (15.0)[c]	33.3 (13.1)[c]	6.7 (9.9)[c]
Novozym 50°C	1.6	14.1 (7.5)[c]	51.6 (12.5)[c]	34.2 (13.5)[c]	7.8 (11.0)[c]
Lipozyme 20°C	4.6	6.4 (11.3)[c]	27.6 (4.7)[c]	47.8 (7.8)[c]	18.2 (2.8)[c]
Lipozyme 30°C	6.3	8.3 (11.7)[c]	33.2 (2.5)[c]	43.1 (1.0)[c]	15.4 (1.3)[c]
Lipozyme 40°C	7.1	7.3 (10. 0)[c]	29.8 (14.3)[c]	45.6 (5.8)[c]	17.4 (4.8)[c]
Lipozyme 50°C	14.6	7.9 (3.9)[c]	28.8 (8.6)[c]	44.8 (0.6)[c]	18.5 (7.4)[c]

[a] Initial enzyme activity was defined as the concentration of 1,3-dicapryl-2-linolenyl glycerol (CLnC) in μmol per g of solid enzyme per min of reaction.

[b] Relative TAG proportion was calculated on the basis of the concentration of each TAG product divided by the sum of the TAG products at time *t*, multiplied by 100. TAG products include 1(3),2-dicapryl-3(1)-linolenyl glycerol (CCLn), CLnC, 2-capryl-1,3-dilinolenyl glycerol (LnCLn) and 1(3)-capryl-2,3(1)-dilinolenyl glycerol (CLnLn).

[c] Relative percent standard deviation (RSD), calculated as the standard deviation of duplicate samples divided by the mean, multiplied by 100.

was reported for the Lipozyme IM-catalyzed reaction at 30, 40 and 50°C, but not at 20°C; such decrease was more significant at higher temperature (Fig.11). The decrease in the BY may be attributed to a shift in the reaction equilibrium and/or the acyl migration of medium chain of SLs to *sn*-2 position at high temperature (Mu *et al.*, 1998). However, Mu *et al.* (1998) have investigated the effect of reaction temperature on the acyl migration of medium chain of MLM and LML-type SLs and indicated that there was only 1.4% of migration when the reaction temperature was raised from 30 to 70°C. The decrease in BY could also be explained by a shift in the reaction thermodynamic equilibrium which may be due to the increase in the concentration of the polar hydrolytic products, such as glycerol, mono- and diacylglycerols (Sabally *et al.*, 2005). Indeed, the accumulation of glycerol molecules on the enzyme micro-environment may form a hydrophilic hindrance layer which could limit the substrate diffusion (Dossat *et al.*, 1999). Moreover, due to the low boiling point of hexane (69°C), a high temperature should also be avoided to minimize the evaporation of reaction medium.

The effect of the reaction temperature on the relative proportion of each TAG product is shown in Table 4. Although the proportion of each SL was relatively constant over the investigated reaction temperatures (20 to 50°C), for Lipozyme IM there was a slightly higher proportions of CCLn (8.3%) and CLnC (33.2%) at 30°C. For Novozym 435, there were highest proportions of CCLn (14.1%) and CLnC (51.6%) at 50°C, whereas at 30°C they were 7.7 and 30.8%, respectively. These results indicate that with Novozym 435 as biocatalyst, the proportion of MLM-type SL was higher at high temperatures. Similar results were reported by Huang and Akoh (1996) for the Lipozyme IM-catalyzed transesterification of caprylic acid with triolein where the molar proportion of dicaprylolein was increased from 43.3 to 49.5%, when the reaction temperature was increased from 25 to 65°C; these authors also showed that with the use of Novozym 435, the molar proportion of dicaprylolein was increased from 0 to 62.0% when the temperature was increased from 25 to 55°C.

Although the optimal reaction temperature and the enzyme activity (Fig. 11 and Table 4) were different for Lipozyme IM and Novozym 435, both enzymes resulted in a close maximum BY of CLnC of 24.7 and 24.0%, respectively. In addition, the time required to reach the maximum BY of CLnC was lower for Lipozyme IM than that for Novozym 435. The overall experimental results showed that on one hand Lipozyme IM was more appropriate for the biosynthesis of MLM-type SL than Novozym 435, and on the other hand the reaction temperature of 30°C was the most suitable for further investigations.

4.2.2. Effect of Solvent Type/Initial Water Activity on Bioconversion of CLnC

It has been suggested that the water is very critical for maintaining the active enzyme conformation and for controlling the equilibrium between hydrolysis and synthesis reactions (Halling, 1994). On the other hand, the nature of the organic solvent plays a strong effect on the enzymatic activity as a result of the retention of enzyme-associated water, enzyme-solvent interactions and/or substrate solvation (Kvittingen, 1994). The effect of different initial water activity (a_w) of 0.06 to 0.53 on the Lipozyme IM-catalyzed reaction was investigated, using selected organic solvents of different Log P values varied from 3.2 to 4.5; the choice of organic solvents was made on the basis of their capacity for the solubilization of the substrate as well as the absence of their interference with the enzymatic reaction.

The results in Fig. 12 show the time course of the bioconversion yield (BY) of CLnC in the interesterification reaction of TC with TLN in the selected solvents at different initial a_w. At initial a_w of 0.06 (Fig. 12A), the maximal BY of CLnC increased from 27.2 to 34.8% when the Log P value of the solvent was increased from 3.2 (cyclohexane) to 3.5 (hexane); however, there was a decrease in the BY, from 31.0 to 19.7% when the Log P was changed from 4.0 (heptane) to 4.5 (iso-octane), respectively. At initial a_w of 0.11 (Fig. 12B), the BY was increased from 15.9 to 29.7% when the Log P value of solvents

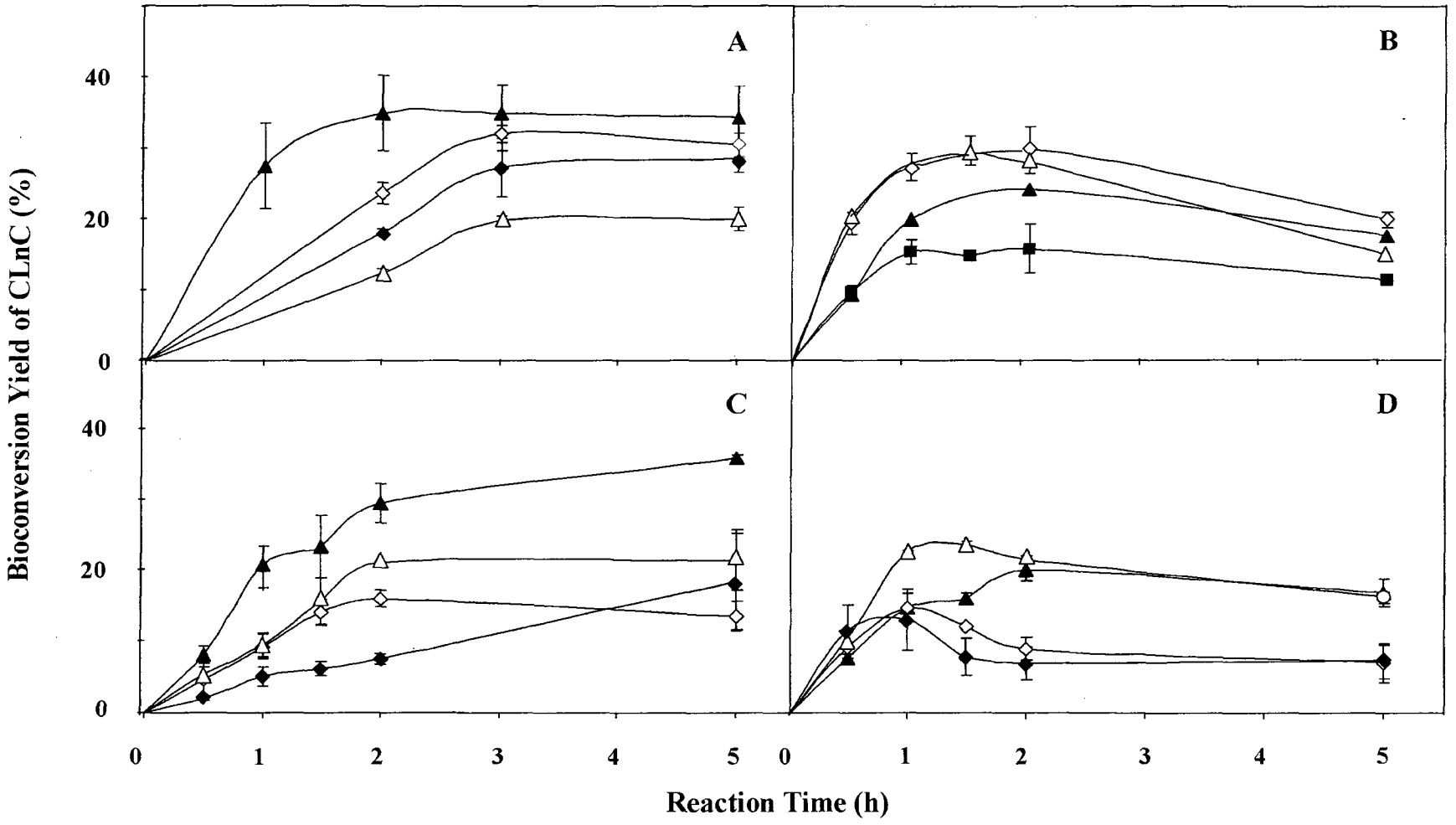


Figure 12. Time course study of the effect of solvent type on the bioconversion yield of 1,3-dicapryl-2-linolenyl glycerol (CLnC) at different initial aw 0.05 (A), 0.11 (B), 0.33 (C) and 0.53 (D); iso-octane (△), heptane (◇), hexane (▲) and cyclohexane (◆), respectively.

Table 5. Effect of initial water activity (a_w) on the initial enzyme activity, maximum bioconversion yield (BY) of 1,3-dicapryl-2-linolenyl glycerol (CLnC) and relative triacylglycerol (TAG) proportion of Lipozyme IM-catalyzed interesterification reaction of tricaprylin with trilinolenin hexane.

Initial a_w	Initial enzyme activity (μmol CLnC/g enzyme /min)[a]	BY (%)[b]	Relative TAG proportion (%)[c]			
			CCLn	CLnC	CLnLn	LnCLn
0.06	8.4	34.8 (15.3)[d]	5.7 (20.2)[d]	43. 6 (15.3)[d]	41.5 (7.5)[d]	9.2 (10.1)[d]
0.11	7.4	24.1 (2.5)[d]	8.3 (11.7)[d]	33.2 (2.5)[d]	43.1 (1.0)[d]	15.4 (1.3)[d]
0.33	7.0	23.2 (3.8)[d]	6.7 (9.7)[d]	29.3 (1.4)[d]	33.5 (13.8)[d]	12.6 (11.8)[d]
0.53	6.7	19.9 (8.1)[d]	9.8 (5.9)[d]	40.3 (0.5)[d]	38.16(6.5)[d]	11.9 (9.9)[d]

[a] Initial enzyme activity was defined as the concentration of 1,3-dicapryl-2-linolenyl glycerol (CLnC) in μmol per g of solid enzyme per min of reaction.

[b] Bioconversion yield is calculated by the concentration of CLnC in mM divided by the concentration of the limiting substrate in mM in the blank reaction at time *t*, multiplied by 100.

[c] Relative TAG proportion was calculated on the basis of the concentration of each TAG product divided by the sum of the TAG products at time *t*, multiplied by 100. TAG products include 1(3),2-dicapryl-3(1)-linolenyl glycerol (CCLn), CLnC, 2-capryl-1,3-dilinolenyl glycerol (LnCLn) and 1(3)-capryl-2,3(1)-dilinolenyl glycerol (CLnLn).

[d] Relative standard deviation (RSD), calculated as the standard deviation of duplicated samples divided by the mean, multiplied by 100.

increased from 3.2 to 4.0. However, when the Log P increased to 4.5, the BY decreased to 28.5%. The results (Fig. 12C) show that at an initial a_w of 0.33, there was a significant increase in the BY from 18.3 to 29.4%, when Log P of solvent increased from 3.2 to 3.5; however, when the Log P values were 4.0 and 4.5, the BY of CLnC was 15.9 and 21.3%, respectively. The results (Fig. 12D) show that at an initial a_w of 0.53, the lowest and the highest BYs were at Log P values of 3.2 and 4.5, respectively. However, the BY of the solvent with the Log P value of 4.0, was lower than that of with a Log P value of 3.5. The experimental findings (Fig. 12) suggest that there was no restrict correlation between the Log P value and the maximum BY of CLnC, at the initial a_w. The increase in BY as Log P value of the solvent increased from 3.2 to 3.5 may indicated that the hydrophobic solvent could favor the partition of monoacylglycerols and diacylglycerols in the polar micro rather than the macro non-polar environment (Halling, 1994); the decrease in the BY at a high Log P value maybe due to the effect of such hydrophobic solvents that have higher solvent-enzyme interactions inside or near the enzyme active site, which might affect the substrate binding (Ross *et al.*, 2000).

The absence of any correlation between the BY and the Log P values of the solvents was also reported by Soumanou and Bornscheuer (2003) in the alcoholysis reaction of sunflower oil with ethanol catalyzed by Lipozyme IM, where the decrease in BY was in concomitant with the nature of the solvents in the sequence of hexane (Log P = 3.5), heptane (Log P = 4.0), iso-octane (Log P = 4.5) and cyclohexane (Log P = 3.2). Similarly, Yesiloglu and Kilic (2004) reported that in the esterification reaction of oleic acid with glycerol catalyzed by lipase from *C. rugosa*, there was an increase in the BY of glycosides in the order of using the solvents of iso-octane (Log P = 4.5), hexane (Log P = 3.5), heptane (Log P = 4.0) and decane (Log P = 5.4). In contrast, Claon and Akoh (1994) indicated that the increase in the Log P value from 3.0 (pentane) to 4.5 (iso-octane) resulted in an 18.2% of the decrease in the BY of citronelly acetate, obtained by the lipase SP 435-catalyzed esterification reaction of citronellol with acetic acid. Fomuso and

Akoh (1998) also reported an increase in the synthesis of dicaproyllinolein when the Log P value of the solvents was increased from 2.0 (benzene) to 4.5 (iso-octane) by Lipozyme IM-catalyzed interesterification of tricaproin with trilinolein.

The results in Figs. 12B and 12D also show significant decreases in the BY of CLnC after reaching its maximum. The decrease in the BY may be due to a shift in the thermodynamic reaction towards the hydrolysis of SLs as a result of the increase in the concentration of the polar hydrolytic products (Sabally *et al.*, 2005) and/or the high water content enhances the acyl migration (Xu *et al.*, 1998), hence decreasing the BY of CLnC. Xu *et al.* (1998) reported that there was a 5.5-times increase in the acyl migration rate of capric acid to *sn*-2 position when the water content was increased from 3.0 to 7.2% by the Lipozyme IM-catalyzed transesterification reaction of capric acid with fish oil. The decrease in the BY of CLnC may also be explained by the enhancement of the acyl migration with the increase in the reaction time (Mu *et al.*, 1998). Mu *et al.* (1998) reported that in Lipozyme IM-catalyzed acidolysis reaction of capric acid with sunflower oil, there was a 10-fold increase in the acyl migration of C10:0 to *sn*-2 position when the reaction time was increased from 1 to 7 h.

The results in Table 5 show that in using hexane as the reaction medium, the BY of CLnC increased from 19.9 to 34.8%, with a concomitant increase in the initial enzyme activity from 6.7 to 8.4 μmol CLnC/g solid enzyme/min, when the initial a_w decreased from 0.53 to 0.06. Table 5 also shows a 1.5-fold increase in the relative TAGs proportion of CLnC, when the initial a_w was decreased from 0.33 to 0.06; however, at an initial a_w of 0.53, this proportion was comparable to that obtained with the initial a_w of 0.06. These results suggest that the initial a_w has a significant effect on the BY and enzyme activity as well as TAGs proportions of end products. The limited increase in the BY and enzyme activity at high initial a_w may be explained by the high water content not only needed to enhance the acyl migration (Xu *et al.*, 1998), but also to favor the hydrolysis reaction (Irimescu *et*

al., 2001). The increase in hydrolysis products, such as mono- and diacylglycerols, in the reaction system may enhance the acyl migration (Mu *et al.*, 1999); moreover, these hydrolysis products might also shift the thermodynamic reaction towards the hydrolysis. Indeed, the accumulation of glycerol molecules on the enzyme micro-environment may form a hydrophilic hindrance layer which would limit the substrate diffusion (Dossat *et al.*, 1999). Similar to the experimental findings, Ma *et al.* (2002) reported that the transesterification of ethyl decanoate with hexanol by lipase from *Rhizopus oryzae* was optimal at low initial a_w of 0.06. Lue *et al.* (2005) pointed out that the highest enzymatic activity of Novozym 435 was obtained at an initial a_w of 0.06 in the esterification reaction of *t*-cinnamic acid with oleyl alcohol. Fomuso and Akoh (1998) reported that the highest TAGs proportion of dicaproyllinolein (44.3%) was obtained with the pre-dry solvents treated with molecular sieve, in Lipozyme IM-catalyzed interesterification reaction of tircapronin and trilinolein.

The overall results indicate that hexane and an initial a_w of 0.06 were the most appropriate conditions since they provided the highest BY of CLnC and consequently were used for further investigations throughout this study.

4.2.3 Effect of Enzyme Concentration on the Biosynthesis of CLnC

In order to maximize the bioconversion yield (BY) of CLnC and to reduce the reaction time, the enzyme-catalyzed interesterification was carried out using a wide range of enzyme concentrations varying from 5 to 20 mg solid Lipozyme IM. The effect of the enzyme concentrations on the maximum BY of CLnC and CLnLn as well as on the initial enzyme activity are depicted in Fig. 13 and Table 6.

The results in Fig. 13A show that the maximum BY of CLnC increased from 20.4 to 33.1% when enzyme concentration was increased from 5 to 10 mg solid enzyme/mL;

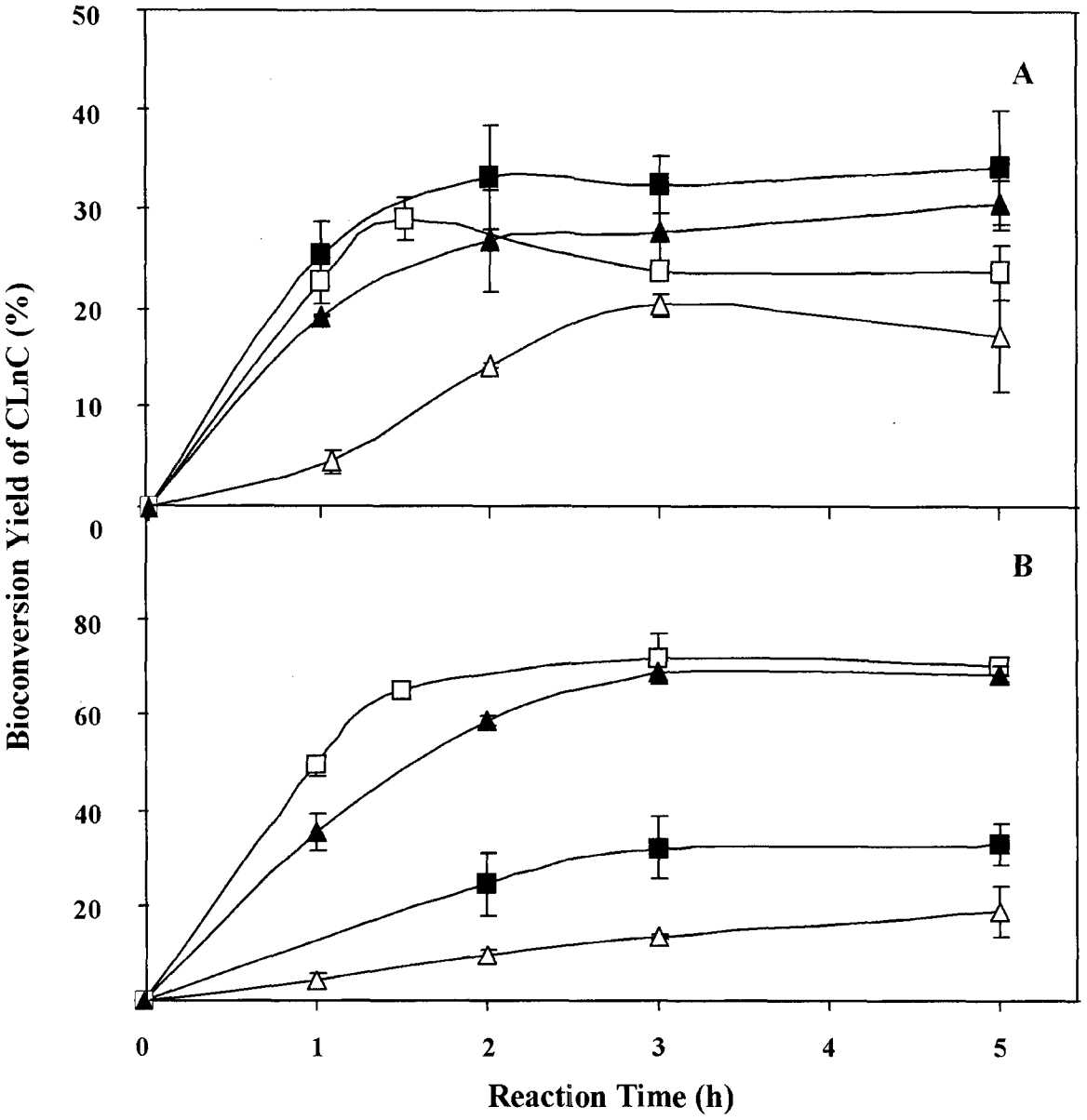


Figure 13. The effect of Lipozyme IM lipase concentration on the bioconversion yield of 1,3-dicapryl-2-linolenyl glycerol (A) and 1(3),-capryl-2,3(1)-dilinolenyl glycerol (B) in lipase-catalyzed interesterificaton reaction of tricaprylin and trilinolenin during 5 hours; 5 mg/mL (△), 10 mg/mL (■), 15 mg/mL (▲) and 20 mg/mL(□).

Table 6. Effect of enzyme concentration on the initial enzyme activity and relative triacylglycerol (TAG) proportion of Lipozyme IM-catalyzed interesterification of tricaprylin with trilinolenin.

Enzyme concentration (mg/mL)	Initial enzyme activity (μmol CLnC/g enzyme/min)[a]	Initial enzyme activity (μmol CLnLn/g enzyme/min)[b]	Relative TAG proportion (%)[c]			
			CCLn	CLnC	CLnLn	LnCLn
5	4.8	2.6	4.3 (5.9)[d]	54.4 (5.8)[d]	36.6 (3.3)[d]	4.7 (5.5)[d]
10	8.4	4.8	5.7 (20.2)[d]	43.6 (15.3)[d]	41.5 (7.5)[d]	9.2 (10.1)[d]
15	6.6	15.8	4.1 (10.5)[d]	25.9 (5.3)[d]	56.7 (2.3)[d]	13.3 (8.0)[d]
20	6.4	17.8	3.3 (10.4)[d]	25.5 (7.3)[d]	57.4 (3.0)[d]	13.8 (2.7)[d]

[a]Initial enzyme activity was defined as the concentration of 1,3-dicapryl-2-linolenyl glycerol (CLnC) in μmol per g of solid enzyme per min of reaction.

[b]Initial enzyme activity was defined as the concentration of 1(3)-capryl-2,3(1)-dilinolenyl glycerol (CLnLn) in μmol per g of solid enzyme per min of reaction.

[c]Relative triacylglycerol (TAG) proportion was calculated on the basis of the concentration of each TAG product divided by the sum of the TAG products at time *t*, multiplied by 100. The TAG products include 1(3),2-dicapryl-3(1)-linolenyl glycerol (CCLn), CLnC, 2-capryl-1,3-dilinolenyl glycerol (LnCLn) and CLnLn.

[d]Relative standard deviation (RSD), calculated as the standard deviation of duplicated samples divided by the mean, multiplied by 100.

however, an additional increase in the enzyme concentration to 20 mg solid enzyme/mL resulted in a decrease in the BY of CLnC to 27.6%. In contrast to the BY of CLnC, the maximum BY of CLnLn (Fig. 13B) increased linearly from 13.7 to 65.2% when the enzyme concentration increased from 5 to 20 mg solid enzyme/mL. Similarly, Table 6 shows a 1.8-time increase in the initial enzyme activity for the biosynthesis of CLnC when its concentration was increased from 5 to 10 mg solid enzyme/mL hexane. Using 15 and 20 mg solid enzyme/mL, the enzyme activity remained unchanged with an average of 6.5 μmol CLnC/g solid enzyme/min. In contrast to CLnC, for the biosynthesis of CLnLn, the initial enzyme activity increased 6.8-times when its concentration was increased from 5 to 20 mg solid enzyme/mL. These results indicated that the enzyme activity and its selectivity were enzyme concentration-dependent. Similar to the present results, changing the substrate selectivity has been reported for the acidolysis of tristearin with lauric and oleic acids (Sellappan and Akoh, 2000); these authors have indicated that Lipozyme IM showed high specificity toward oleic acid rather than for lauric acid when the enzyme concentration was increased by 24%. Carrín and Crapiste (2008) also reported that, using Lipozyme IM as biocatalyst in the acidolysis of sunflower oil with a mixture of palmitic and stearic acid, increasing the enzyme concentration from 8 to 10% resulted in a higher selectivity for palmitic acid over stearic acid.

Regarding the effect of the enzyme concentration on the time required to reach the reaction equilibrium, Fig. 13 indicates a decrease in such time from 3 to 1.5 h for the BY of CLnC when the enzyme concentration was increased from 5 to 15 mg solid enzyme/mL. However, at higher enzyme concentration of 20 mg solid enzyme/mL, there was no change in the required time. For CLnLn, when the enzyme concentration was increased from 5 to 15 mg solid/mL, there was a decrease in the required time from 5 to 3 h and remained unchanged when the enzyme concentration was higher than 20 mg solid enzyme/mL. These results are in agreement with those reported by Martinez *et al.* (2005) where the Chirazyme L-9-catalyzed esterification reaction of conjugated linoleic acid

with glycerol, showed an increase in lipase concentration from 5 to 10% which resulted in reducing the time needed for reaching the equilibrium from 8 to 4 h; however, at higher enzyme concentration there was no significant decrease in such time.

The effect of enzyme concentration on the relative TAGs proportion of CLnC is shown in Table 6. The results indicate that the increase in enzyme concentration from 5 to 15 mg/mL, with a linear decrease in the proportion of CLnC from 54.4 to 35.9%; a further increase in enzyme concentration to 20 mg/mL did not lead to a significant change in CLnC's proportion. In contrast to CLnC, the relative proportion of CLnLn at 15 mg solid enzyme/mL was 1.6-time higher than that at 5 mg solid enzyme/mL; however, with an enzyme concentration of 20 mg solid enzyme/mL, there was little change in this proportion. Similarly, Jennings and Akoh (2001) reported that when the enzyme concentration was higher than 5%, there was no significant difference in the molar percentage of incorporation of capric acid into fish oil. Chopra *et al.* (2008) also reported that the incorporation of stearic acid into rice bran oil by Lipozyme IM-catalyzed acidolysis increased 5-times, when the enzyme concentration increased from 1 to 5%; this enhancement was less significant when the enzyme concentration was further increased to 10%. The decrease in the relative TAGs proportion of CLnC (Table 6) at high enzyme concentration (15 and 20 mg) could be due to the fact that the high protein-support interactions resulting from an excess of enzyme which may have altered its active conformation, affecting hence its catalytic efficiency (Colombie *et al.*, 1998; Yadav and Lathi, 2003) and substrate specificity towards polyunsaturated fatty acids over medium chain fatty acids (Sellappan and Akoh, 2000). The limited increase in the TAGs proportion of CLnLn and initial enzyme activity at 20 mg solid enzyme/mL could be explained by the steric hindrance of the enzyme activity at high enzyme concentration (Hadzir *et al.*, 2001), as well as by the saturation of the enzyme active site with the substrate and/or the external/internal mass transfer limitations (Chen *et al.*, 1995).

Based on the overall findings, 10 mg of solid Lipozyme IM/mL was found to be the most effective biocatalyst concentration for the enzymatic synthesis of CLnC and consequently used for further investigation throughout this study.

4.2.4. Effect of Substrate Molar Ratio on Biosynthesis of CLnC

Substrate molar ratio is seen as an effective mean of shifting the reaction equilibrium toward synthesis direction and hence increasing the bioconversion yield (BY) of SLs (Kim *et al.*, 2001). In order to maximize the BY of CLnC, the effect of substrate molar ratio (4:1 to 1:8 of TLN:TC) was investigated by maintaining the concentration of TLN at 20 mM and varying the TC concentration from 5 to 160 mM (Fig. 14 and Table 7).

Using the substrate molar ratio of 1:4 to 6:1, the maximum BY of CLnC increased linearly from 4.8 to 73.3%, respectively; however, when the molar ratio was further increased to 8:1, the BY decreased to 65.4%. The results (Fig. 14) also show that the initial enzyme activity increased slightly from 7.4 to 8.4 μmol CLnC/g solid enzyme/min, when the substrate molar ratio was increased from 1:4 to 1:1. However, at the high molar ratio of 8:1, a dramatical decrease in enzyme activity to 2.9 μmol CLnC/g solid enzyme/min was obtained. Similarly, Lue *et al.* (2005) reported an increase in the BY from 38.4 to 100.0% as the substrate molar ratio was increased from 1:0.5 to 1:6 for the esterification reaction of *t*-cinnamic acid with oleyl alcohol. In contrast, Yadav and Lathi (2003) reported a decrease in the BY of *n*-octyl acetate when the substrate molar ratio of *n*-octanol to vinyl acetate was increased from 1:1 to 1:2 in the transesterification reaction catalyzed by Lipozyme IM. The linear increase in BY of CLnC in the presence of excess TC could be explained by the effects of viscosity and polarity of the reaction medium on the substrate availability for the enzyme. The excess of tricaprylin may also favor the partition of tricaprylin towards the enzyme micro-environment and could lead thereby to a higher BY of CLnC (Wei *et al.*, 2003; Karboune *et al.*, 2005). At molar ratio of 8:1, the

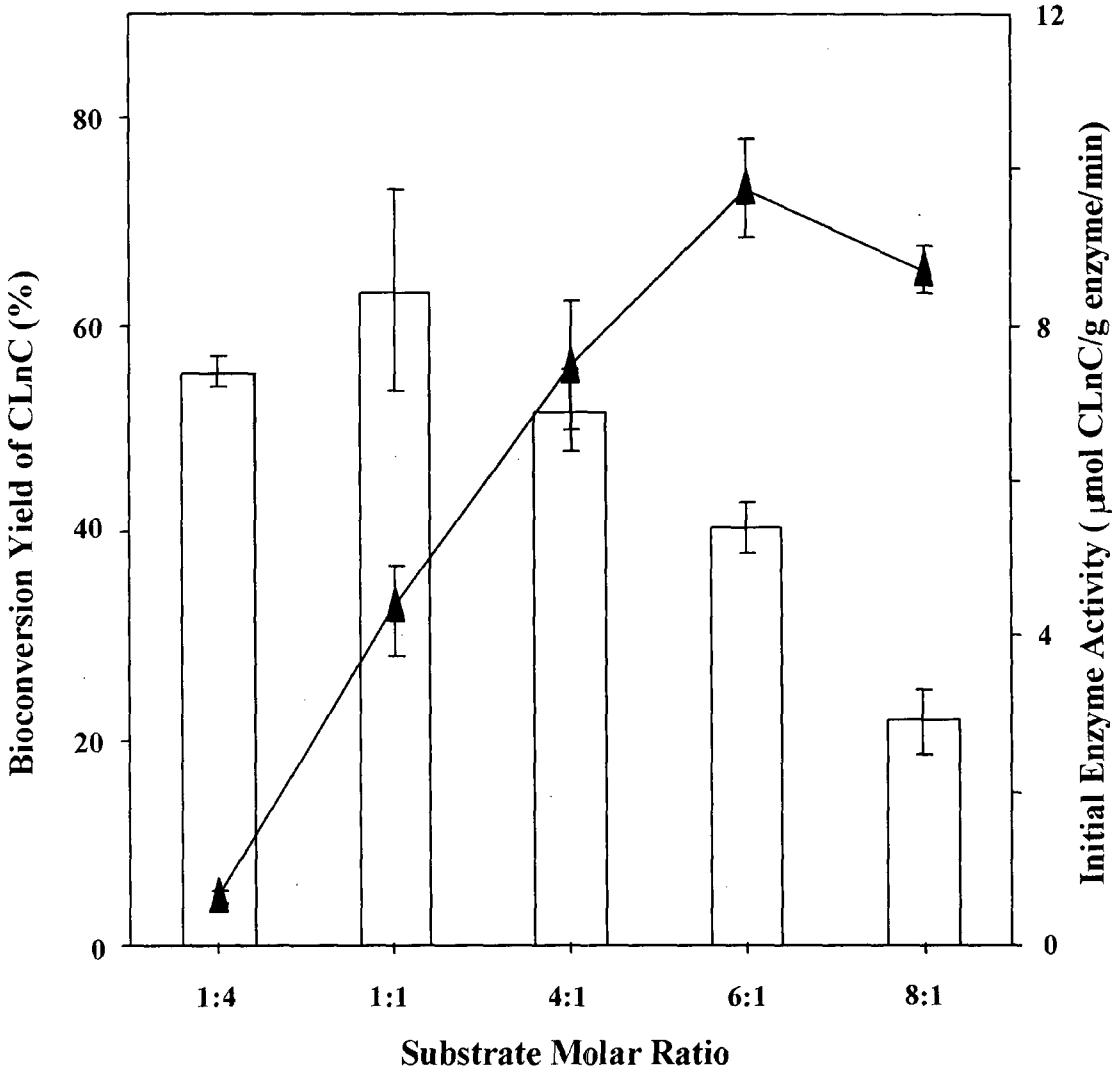


Figure 14. The effect of substrate molar ratio (tricaprylin: trilinolenin) on the biosynthesis of 1,3-dicapryl-2-linolenyl glycerol (CLnC) throughout a 13-h lipase-catalyzed interesterification reaction of TC and trilinolenin (TLN); CLnC (▲), and initial enzyme activity (□).

Table 7. Effect of molar substrate ratio on the enzyme activity and the relative triacylglycerol (TAG) proportion of Lipozyme IM-catalyzed interesterification reaction of tricaprylin with trilinolenin.[a]

Substrate molar ratio	Relative TAG proportion (%)[b]			
	CCLn	CLnC	CLnLn	LnCLn
1:4[c]	5.6 (22.6)[d]	14.4 (16.0)[d]	41.9 (2.1)[d]	38.1 (2.9)[d]
1:1[e]	5.2 (6.6)[d]	44.0 (15.3)[d]	42.9 (20.1)[d]	7.9 (4.5)[d]
4:1[f]	2.9 (13.7)[d]	74.1 (19.0)[d]	22.5 (6.5)[d]	0.5 (21.6)[d]
6:1[g]	3.4 (5.0)[d]	79.8 (10.9)[d]	16.4 (10.7)[d]	0.4 (5.2)[d]
8:1[g]	1.6 (12.8)[d]	84.3 (3.0)[d]	14.0 (11.5)[d]	0.1 (27.2)[d]

[a] Interesterification reaction was carried out in hexane at different tricaprylin to trilinolenin molar ratios using enzyme concentration 10 mg/mL reaction media by fixing the concentration of trilinolenin at 20 mM.

[b] Relative TAG proportion was calculated on the basis of the concentration of each triacylglycerol (TAG) product in mM divided by the sum of the concentration of the TAG products in mM at time *t*, multiplied by 100. TAG products include 1(3),2-dicapryl-3(1)-linolenyl glycerol (CCLn), 1,3-dicapryl-2-linolenyl glycerol (CLnC), 2-capryl-1,3-dilinolenyl glycerol (LnCLn) and 1(3)-capryl-2,3(1)-dilinolenyl glycerol(CLnLn).

[c] Maximum bioconversion yield was reached after 3-h reaction period.

[d] Relative standard deviation (RSD) was calculated from the standard deviation of triplicate samples divided by their mean multiplied by 100.

[e] Maximum bioconversion yield was reached after 2-h reaction period.

[f] Maximum bioconversion yield was reached after 5-h reaction period.

[g] Maximum bioconversion yield was reached after 9-h reaction period.

decrease in BY of CLnC and in the initial enzyme activity may be attributed to the substrate mass transfer limitations (Chen *et al.*, 1995), which resulted in a shift in the thermodynamic equilibrium reaction by an increase in the concentration of free fatty acids and/or to the diffusion hindrance effect of glycerol (Dossat *et al.*, 1999). Moreover, the excess amount of free fatty acids could also have change the pH in the micro-environment of enzyme, and resulted in the enzyme denaturation (Jennings and Akoh, 2001; Can and Özçelik, 2005).

The results in Table 7 show that there was a steady increase in the TAGs proportions of CLnC from 14.4 to 84.3% and decrease in CLnLn from 42.9 to 14.0%, respectively, when the molar ratio of TC to TLN was changed from 1:4 to 8:1. These results suggest that the change in the molar ratio of substrates affected significantly the relative TAGs proportion of end products. These results also indicate that the high amount of acyl donor (TC) shifted the reaction equilibrium to the synthesis of CLnC and decreased the acyl migration (Mu *et al.*, 1999; Kim and Akoh, 2005). Similar to the experimental results (Table 7), the increase in molar proportion of dicaprylolein and the decrease in that of monocaprylolein were also reported by Huang and Akoh (1996), when the substrate molar ratio of caprylic ethyl ester to triolein was changed from 1:1 to 8:1.

The overall results indicate that under the experimental conditions, a substrate molar ratio of 6:1 of tricaprylin to trilinolenin was found to be the most appropriate one for the enzymatic synthesis of CLnC, and consequently it was used in the further investigations throughout this study.

4.2.5. Effect of Molecular Sieve on the Biosynthesis of CLnC

In order to maximize the bioconversion yield (BY) of CLnC by shifting the thermodynamic reaction towards synthesis rather than hydrolysis, a reduction in water content in the reaction mixture was attempted by the addition of molecular sieve acting as

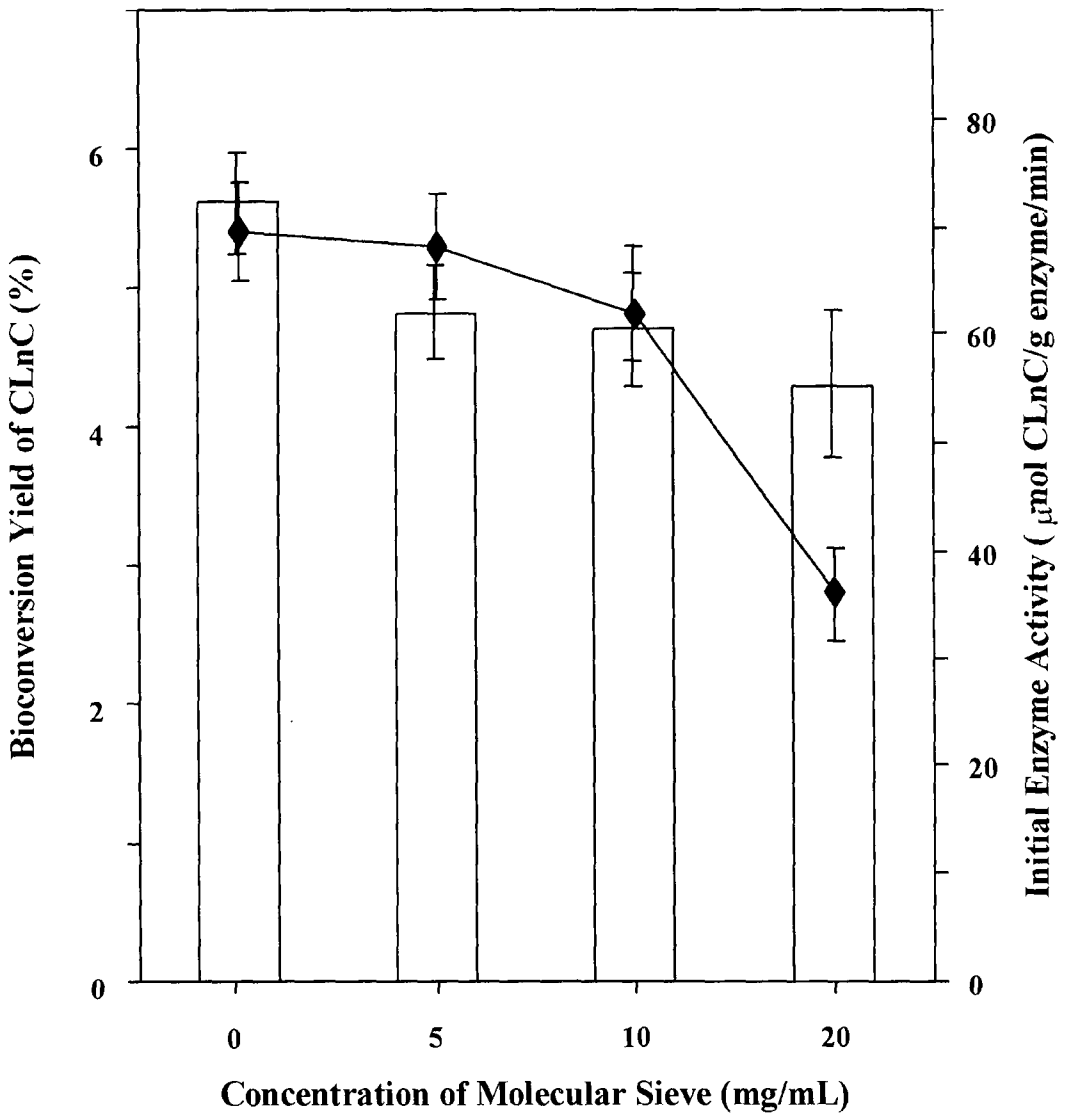


Figure 15. The effect of addition of molecular sieve on the bioconversion yield of 1,3-dicapryl-2-linolenyl glycerol (CLnC) and initial enzyme activity throughout a 14-h lipase-catalyzed reaction; initial enzyme activity (♦) and bioconversion yield of CLnC (□), respectively.

dehydrating agents. The lipase-catalyzed interesterification of TC and TLN was carried out in the presence of various concentrations of molecular sieve from 5 to 20 mg/mL.

The results (Fig. 15) show that the BY of CLnC decreased from 72.1 to 55.3%, with a concomitant decrease in the enzyme activity from 5.6 to 2.8 μmol CLnC/g solid enzyme/min, when the molecular sieve concentration increased from 5 to 20 mg/mL in reaction mixture, respectively. In contrast to the initial enzyme activity, the relative TAGs proportions of CCLn, CLnC, CLnLn and LnCLn remained fairly constant (data not shown). These results suggest that the molecular sieve may strip off the water layer surrounding the enzyme molecule, hence distorting the interaction between the water and the enzyme (Halling, 2002), which could limit the formation of SLs. Similarly, Cerdán *et al.* (1998) have reported a 21.8% decrease in the BY of TAGs in Novozym 435-catalyzed esterification reaction of polyunsaturated fatty acids with glycerol by increasing the molecular sieve concentration from 0.5 to 2.0 g/mL reaction medium. Karboune *et al.* (2005) also reported a 35% decrease in the maximum BY upon the addition of 10 mg/mL of molecular sieves to the lipase-catalyzed biosynthesis of cinnamoylated lipids. The literature (Ergan *et al.*, 1990; Robles-Medina *et al.*, 1999; Martinez, *et al.*, 2005) proposes at which time the molecular sieve should be added. Ergan *et al.* (1990) suggested the addition of the molecular sieve at a stage close to equilibrium, whereas Robles-Medina *et al.* (1999) and Martinez *et al.* (2005) reported the beginning of the reaction to attain high rates of esterification.

The overall experiment results suggested that there was little effect of molecular sieve on the Lipozyme IM-catalyzed interesterification reaction of tricaprylin with trilinolenin.

4.3. Effect of Selected Parameters on the Productivity of CLnC

The productivity of CLnC was applied to assess the reaction efficiency over an incubation period of the Lipozyme IM-catalyzed interesterification reaction. The mass

productivity (P_M) was defined as the weight (mg) of produced CLnC per g of initial substrates per h; the enzymatic productivity (P_E) was defined as the weight (mg) of produced CLnC per unit of Lipozyme IM per h, whereas the volumetric productivity (P_V) was calculated by the weight (g) of produced CLnC in g per liter of reaction mixture per h.

4.3.1. Effect of Substrate Concentration on Productivity

To assess the efficiency of the enzyme-catalyzed interesterification of TC with TLN over a well defined period of incubation time, the effect of substrate concentration (40 to 200 mM) on productivity, including P_M, P_E and P_V, was investigated. The enzymatic reaction was carried out using the optimized assay as described previously.

The results (Table 8) show that when TLN concentration was increased from 40 to 80 mM, the P_M increased from 12.7 to 15.5 mg CLnC/h/g TC and TLN; thereafter, as the concentration was further increased to 200 mM, the P_M decreased linearly to 8.2 mg CLnC/h/g. In contrast, the P_E increased 3 times when TLN concentration was increased from 40 to 100 mM. Further increase in the concentration of TLN to 200 mM did not lead to an increase in P_E. With regard to P_V, increasing the TLN concentration from 40 to 140 mM resulted in an increase from 1.9 to 6.0 g CLnC/h/L reaction medium. The P_V remained fairly stable at a value of 6.1 g CLnC/h/L reaction medium, when the concentration further increased to 200 mM. The relative TAG proportion of CLnC increased slightly from 74.3 to 81.3% when the TLN concentration was increased from 40 to 80 mM, respectively. Thereafter, the TAG proportion of CLnC decreased gradually from 76.9% to 65.4% as the concentration of TLN further increased from 100 to 200 mM. Similar to our results, Dossat *et al.* (2002) reported a 6-fold increase in the P_V in solvent-free system with Lipozyme IM-catalyzed transesterification reaction of sunflower oil with butanol.

Table 8. Effect of trilinolenin concentration on the productivities and relative triacylglycerol (TAG) proportions of Lipozyme IM-catalyzed interesterification reaction of tricaprylin with trilinolenin.[a]

Trilinolenin (mM)	Mass productivity (mg CLnC/g of substrates/h)[b]	Enzymatic productivity (mg CLnC/enzyme unit/h)[c]	Volumetric productivity (g CLnC/mL/h)[d]	Relative TAG proportion (%)[e]
40	12.7 (11.2)[f]	0.02 (2.5)[f]	1.9 (2.5)[f]	74.3 (2.5)[f]
80	15.5 (4.7)[f]	0.05 (6.9)[f]	4.6 (6.9)[f]	81.3 (4.6)[f]
100	14.5 (1.8)[f]	0.06 (3.4)[f]	5.6 (3.4)[f]	76.9 (1.8)[f]
140	10.8 (6.6)[f]	0.07 (7.1)[f]	6.0 (7.1)[f]	71.0 (0.3)[f]
200	8.2 (8.8)[f]	0.07 (7.9)[f]	6.1 (7.9)[f]	65.4 (4.0)[f]

[a] Only shows the productivities of 1,3-dicapryl-2-linolenyl glycerol (CLnC) and its relative TAG proportion at 11 h.

[b] Mass productivity was calculated on the basis of weight of CLnC in mg per g of tricaprylin and trilinolenin per h of reaction.

[c] Enzymatic productivity was calculated on the basis of weight of CLnC in mg per enzyme unit per h of reaction. One enzyme unit was defined as the amount of enzyme which sets free 1 μmol stearic acid per minute at pH 8.0 and 70°C.

[d] Volumetric productivity was calculated on the basis of weight of CLnC in g per mL reaction mixture per h.

[e] Relative triacylglycerol (TAG) proportion was calculated on the basis of the concentration in mM of CLnC divided by the sum of the concentration in mM of TAG products at time *t*, multiplied by 100. TAG products include 1(3),2-dicapryl-3(1)-linolenyl glycerol (CCLn), CLnC, 2-capryl-1,3-dilinolenyl glycerol (LnCLn) and 1(3)-capryl-2,3(1)-dilinolenyl glycerol (CLnLn).

[f] Relative standard deviation (RSD), calculated as the standard deviation of duplicated samples divided by the mean, multiplied by 100.

The linear increase in P_E and P_V values maybe due to an increase in the substrate availability as TLN concentration increased, which may favor the thermodynamic equilibrium towards the synthesis of CLnC. The limited increase in P_E and P_V at high TLN concentration (200 mM) may be due to the denaturation of the enzyme caused by a dead-end inhibition complex formation (Yadav and Lathi, 2003), of both/either reactants and/or products inhibition (Garcia *et al.*, 1999). Such limited increase in the productivity also might be explained by the formation of a hydrophilic hindrance layer of glycerol in the microenvironment of the enzyme, which can also increase internal and external substrate diffusion limitations (Dossat *et al.*, 1999, Karboune *et al.*, 2005; Wei *et al.*, 2008).

4.3.2. Effect of Selected Limiting Factors on Productivity

In order to maximize the productivity (P_M, P_E and P_V), selected limiting factors, including enzyme denaturation, the formation of a hydrophilic hindrance layer of glycerol surrounding the enzyme support and the effect of water activity (a_w) during reaction process were investigated, using 200 mM of TLN, substrate molar of TC to TLN at 6:1, reaction temperature of 30°C and enzyme concentration of 40 mg/mL. The effects of these selected limiting factors on the selected productivity as well as the bioconversion yield (BY) of CLnC are shown in Table 9.

4.3.2.1. Denaturation of Enzyme

Lipozyme IM denaturation was investigated by the replacement of the solid enzyme in the reaction mixture by a new one after 7 h of interesterification reaction. This substitution resulted in a 14.4% increase in P_M and 16.0% increase in both P_E and P_V, after an additional 4-h period of reaction. The BY of CLnC was also increased from 55.0 to 64.1% after the substitution of the enzyme. Similarly, Karboune *et al.* (2005) reported an increase in the BY of cinnamoylated lipid by the substitution of the enzyme with a new one. These results suggest that the limited increase in the productivity may be

partially due to the denaturation of the enzyme.

4.3.2.2. Effect of Water Activity

The water activity (a_w) may have an effect not only on the enzyme activity, but also on the productivity by modulating the reaction equilibrium (Halling, 1994). To investigate the effect of a_w during the reaction process on the productivity, the a_w was controlled by the addition of binary salts to the reaction mixture. The binary salt hydrate pair acts as a buffer by releasing or removing water molecules as required, keeping the a_w during the reaction at a characteristic a_w value at a given temperature, as long as some of both salt hydrate pairs remain in the reaction medium (Halling, 1992). At a_w of 0.34, the productivity was only 15.5% of that of uncontrolled a_w-reaction. However, at a_w of 0.02 the productivity decreased dramatically to 6.5% of uncontrolled a_w-reaction.. Controlling the a_w was also led to at least 12-fold decrease in the BY of CLnC. These results suggest that both substrates, TLN and TC, may have been adsorbed on the binary salts, which may have affected their availability to the enzyme. The mass diffusion limitation, which is due to the high viscosity of the reaction mixture at high salt content, could low productivity (Kim *et al.*, 1998). The experimental findings may also indicate that the enzyme could not maintain its active conformation in the reaction system at extremely low a_w (0.02), which may limit the formation of CLnC.

4.3.2.3. Formation of Glycerol Layer

In order to investigate the hindrance effect of glycerol during the interesterification, 150 mM glycerol was added after 7 h of the reaction; the addition of glycerol resulted in 35.3, 32.6 and 32.6% decrease in the P_M, P_E and P_V, respectively. Moreover, the BY of CLnC after the addition of glycerol decreased dramatically from 55.0% to 27.6%. These results may suggest the inhibitory effect of glycerol on the enzyme activity. The agglomeration of the enzyme

Table 9. Effect of selected limiting factors on the productivities and bioconversion yield (BY) of Lipozyme IM catalyzed interesterification reaction of tricaprylin with trilinolenin.[a]

Limiting Factors	Mass productivity (mg CLnC/g of substrates/h)[b]	Enzymatic productivity (mg CLnC/enzyme unit/h)[c]	Volumetric productivity (g CLnC/mL/h)[d]	BY (%)[e]
No adding[f]	8.2 (8.8)[g]	0.07 (7.9)[g]	6.1 (7.9)[g]	55.0 (7.9)[g]
Denature of enzyme[h]	9.4 (11.5)[g]	0.08 (10.3)[g]	7.1(10.3)[g]	64.1 (10.3)[g]
Formation of glycerol layer[i]	5.3 (5.0)[g]	0.05 (5.0)[g]	4.1 (5.0)[g]	27.6 (10.1)[g]
Controlling of water activity[j]	1.3 (10.3)[g]	0.01 (9.1)[g]	0.1 (9.1)[g]	1.5 (14.1)[g]
Addition of Silica gel[k]	12.5 (5.5)[g]	0.10 (7.6)[g]	8.4 (7.6)[g]	84.0 (6.6)[g]
Addition of Silica gel and replace of old enzyme[l]	4.9 (0.6)[g]	0.04 (3.6)[g]	3.7 (3.6)[g]	33.7 (5.4)[g]

[a] Only shows the productivities of 1,3-dicapryl-2-linolenyl glycerol (CLnC) and its bioconversion yield at 11 h.

[b] Mass productivity was calculated on the basis of weight of CLnC in mg per g of tricaprylin and trilinolenin per h of reaction.

[c] Enzymatic productivity was calculated on the basis of weight of CLnC in mg per enzyme unit per h of reaction. One enzyme unit was defined as the amount of enzyme which sets free 1 μmol stearic acid per minute at pH 8.0 and 70°C.

[d] Volumetric productivity was calculated on the basis of weight of CLnC in g per mL reaction mixture per h.

[e] The BY was calculated on the basis of the concentration of CLnC divided by the concentration of the limiting substrate in the blank reaction at time *t*, multiplied by 100.

[f] The experiment was carried out using optimized assay in previous part, using trilinolenin concentration of 200 mM.

[g] Relative standard deviation (RSD), calculated as the standard deviation of duplicated samples divided by the mean, multiplied by 100.

[h] The experiment was done by replace of the old enzyme by unused one after 7-h of interesterification reaction carried out during an 11-h period.

[i] The experiment was done by addition of 0.15 mM glycerol after 7-h of interesterification reaction carried out during an 11-h period.

[j] Only shows the results of water activity at 0.34 during an 11-h reaction process by addition of $Ba(OH)_2 \cdot 1H_2O/Ba(OH)_2 \cdot 8H_2O$ at the beginning of the reaction.

[k] Only shows the results of addition of 3 mg Silica gel at the beginning of the reaction during an 11-h reaction process.

[l] The experiment was done addition of 3 mg Silica gel at the beginning of the reaction and replace of old enzyme by unused one after 7-h of interesterification reaction carried out during an 11-h period.

particles appeared after 4 h upon the addition of glycerol. This phenomenon was also reported by Karboune *et al.* (2005) for the enzymatic synthesis of phenolic lipids. The constant TAGs proportion (data not shown) and the decrease in the productivity suggest that the formation of a glycerol layer surrounding the enzyme was one of the reasons that caused the limited increase in the productivity at high substrate concentrations.

Silica gel, an amorphous form of silicon dioxide, was added to the reaction mixture as an adsorbent of glycerol. Using a 6:1 molar substrate ratio of TC and TLN in hexane, the enzymatic synthesis of CLnC was investigated in the presence of Silica gel at concentrations of 1.5, 3.0, 4.5 and 6.0 mg/mL. Figure 16 summarizes the experimental findings for the productivity of CLnC in the presence of different concentrations of Silica gel. The results show that an increase in the concentration of Silica gel from 1.5 to 3.0 mg/mL resulted in a concomitant increase in P_M, P_E and P_V by 1.6, 1.3 and 1.2-time, respectively; however, further increase in the Silica gel content to 6.0 mg/mL resulted in a 52.8, 36.7 and 36.7% decrease in P_M, P_E and P_V, respectively. Similarly, Wei *et al.* (2003) reported a 50.5% increase in the BY of propyl-glycoside lactate and 6-fold increase in the reaction velocity in the presence of Silica gel (0.2 g/mL). The experimental results (Fig. 16) are in agreement with those reported by Karboune *et al.* (2005) who indicated that there was a 17% increase in the BY of cinnamoylated lipids upon the addition of 2.2 mg Silica gel/mL; however, increasing the Silica gel to 4.4 mg/mL resulted by a 26.0% of decrease in the BY. The overall results suggest that the formation of glycerol during the interesterification reaction increased the mass diffusion limitation and caused the denaturation of the enzyme. Dossat *et al.* (1999) indicated that addition of appropriate amount of Silica gel prevented the adsorption of glycerol on the support of immobilized enzyme, and any excess amount of it may cause its dehydration as this layer surrounding the enzyme, which may result in its inactivation.

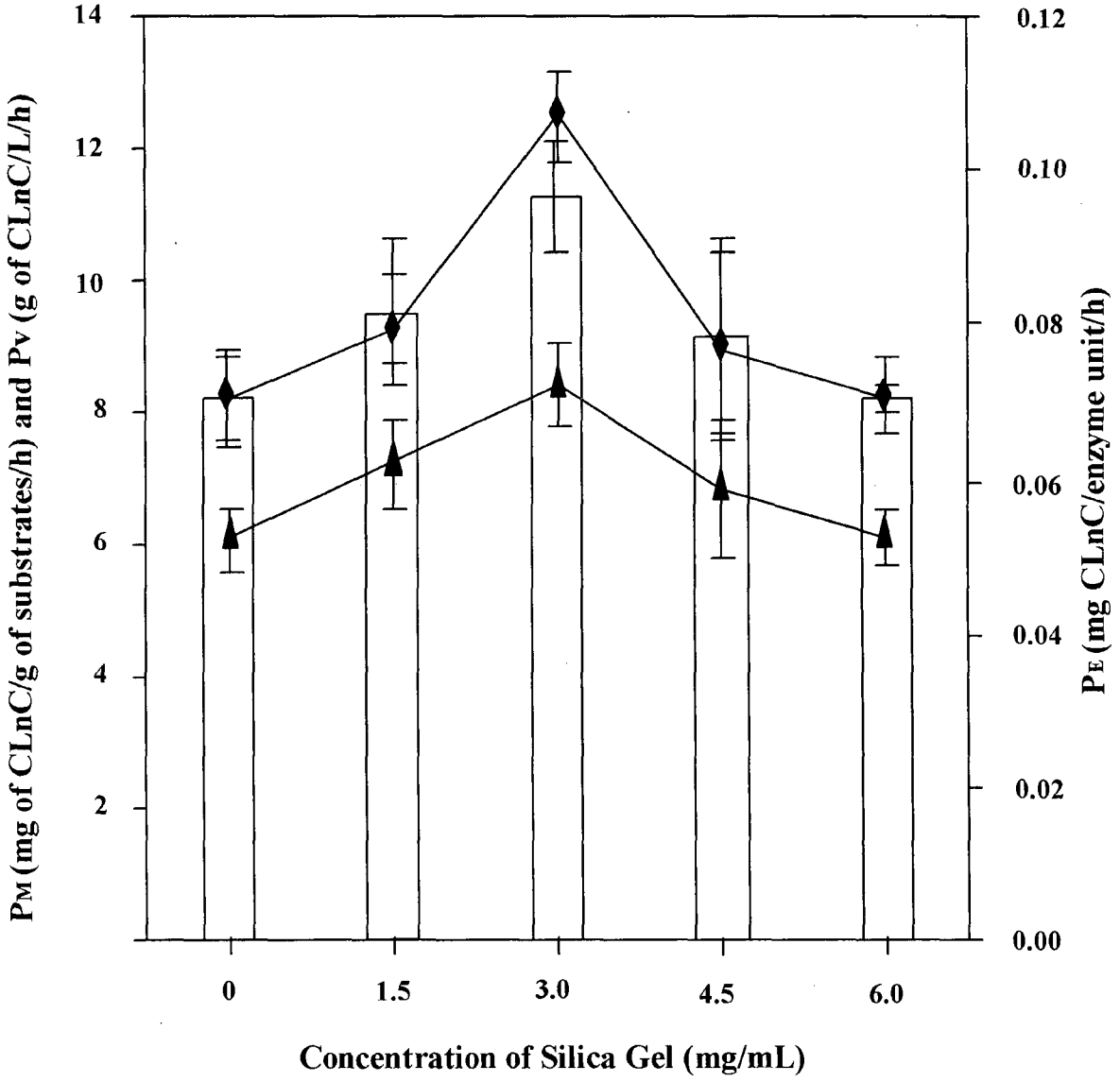


Figure 16. The effect of addition of Silica gel on the mass productivity (P_M), enzymatic productivity (P_E) and volumetric productivity (Pv) 1,3-dicapryl-2-linolenyl glycerol (CLnC) throughout a 11-hour lipase-catalyzed reaction; P_M (◆), P_E (□) and Pv(▲), respectively.

On the basis of the experimental findings, 3 mg of Silica gel should be added in the interesterification reaction of tricaprylin with trilinolenin.

5. CONCLUSION

A model for the biosynthesis of medium-long-medium (MLM)-type structured lipids in organic solvent media, Lipozyme IM from *Mucor miehei* as catalyst, tricaprylin (TC) and trilinolenin (TLN) as substrates was established and optimized.

The experimental results showed that the Lipozyme IM was more efficient than that of the Novozym 435 for the interesterification reaction of TC and TLN. The results also indicated that hexane at a selected initial a_w favored the biosynthesis of the CLnC. The experimental data showed an increase in the bioconversion yield (BY) with the increase in the enzyme concentration; however, high enzyme concentration led to a decrease of CLnC but an increase in CLnLn. Similarly, an increase in the concentration of TC resulted in a concomitant increase in the BY of CLnC; however, any excess in TC resulted in an inhibitory effect on the maximum BY. Moreover, the addition of molecular sieve showed decreases in the initial enzyme activity and in the BY of CLnC.

Using the optimized assay, the reaction efficiency over a defined incubation time was assessed by selected productivity. The results showed a concomitant increase in the productivity with the increase in the TLN concentration. The results also indicated that the formation of a glycerol hindrance layer surrounding the enzyme was one of the factors that could limit the increase in the productivity. Moreover, the addition of an appropriate amount of Silica gel could enhance the productivity.

In conclusion, the optimization of an enzymatic system model and its subsequent application for the production of MLM-type SLs were demonstrated. As such, the development of the model system has laid the groundwork for the future investigation of the biosynthesis of MLM-type SLs, using a wide range of medium and long-chain triacylglycerols.

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