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Lipase-Catalyzed Incorporation of Different Fatty Acids into Tripalmitin-Enriched Triacylglycerols: Effect of Reaction Parameters

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ABSTRACT: Tripalmitin-enriched triacylglycerols were concentrated from palm stearin by acetone fractionation and as the substrate reacted with a mixture of equimolar quantities of fatty acids (C8:0−C18:3). The incorporation degree and acyl migration level of the fatty acids and acylglycerols composition were investigated, providing helpful information for the production of human milk fat substitutes. Higher incorporation degrees of the fatty acids were obtained with lipase PS IM, Lipozyme TL IM, and Lipozyme RM IM followed by porcine pancreatic lipase and Novozym 435-catalyzed acidolysis. During reactions catalyzed by Lipozyme TL IM, Lipozyme RM IM, and lipase PS IM, incorporation degrees of C12:0, C14:0, C18:1, and C18:2 were higher than those of other fatty acids at operated variables (molar ratio, temperature, and time), and the triacylglycerols content reached the highest (82.09%) via Lipozyme RM IM-catalyzed acidolysis. On the basis of significantly different levels of acyl migration to the sn-2 position, lipases were in the order of lipase PS IM < Lipozyme TL IM < Lipozyme RM IM.

KEYWORDS: *acidolysis, lipase, tripalmitin-enriched triacylglycerols, incorporation degree of fatty acid, human milk fat substitute, structured lipids*

INTRODUCTION

Human milk fat provides >50% of the dietary energy requirements for infants.[1] The chemical structure of human milk fat is rather unique containing 17.30−23.30% of palmitic acid, which is primarily located at the sn-2 position of the triacylglycerol (TAG) (>70%), and unsaturated fatty acids (FAs) are mainly occupied at sn-1,3 positions.[2] Such a structure helps the simultaneous absorption of FAs in the gut lumen of the infant and the loss of calcium through the feces.[3,4] Until now, many studies have been carried out to resemble TAGs of human milk fat to produce human milk fat substitutes by enzymatic catalysis. In contrast to chemical methods, enzyme-modified lipids are currently receiving more attention by virtue of enzyme-catalyzed reactions with desirable acyl moieties or their esters, environmentally friendly processes, mild conditions, and product safety.[5] One of the common routes reported in the literature to the successful synthesis of human milk fat substitutes is based on a simple enzymatic acidolysis between TAGs and FAs, leading to the exchange of acyl groups. Therefore, research on enzymatic processes of acidolysis reaction is indispensable, and it will benefit the effective production of structured lipids.

In the production of modified fats and oils, degrees of reactivity of different FAs are influenced by the composition of substrates, water activity, nature of solvents, and source of lipase. The ability of different lipases to discriminate among FAs can be utilized to facilitate new applications. A number of studies have focused on determining the reactivity of FAs or FA selectivity of various lipases in hydrolysis, esterification, and transesterification reactions. Hamam and Shahidi have reported that all five lipases from *Candida antartica*, *Mucor miehei*, *Pseudomonas* sp., *Candida rugosa*, and *Aspergillus niger* better catalyzed incorporation of oleic acid in acidolysis of tristearin with a combination of equimolar quantities of FAs (oleic, conjugated linoleic, linoleic, α-linolenic, and γ-linoleinic acids), suggesting that the unsaturation degree and location of double bonds of the FA as well as specificity of the enzymes used might lead to variation in the reactivity.[6] In another study, Karabulut et al.[7] have reported that three lipases (Lipozyme TL IM, Lipozyme RM IM, and Novozym 435) showed a bell-shaped distribution in incorporation versus chain length plot with a maximum C12:0−C16:0 in acidolysis of triolein with saturated FAs of C6:0−C22:0. The reason for this result is that the free energy changes of the reactions (ΔG) for medium-chain FAs are less than the short-chain FAs and greater than C18 FAs; thus, lesser ΔG leads to a higher extent of reaction.[7,8] Such helpful information could be drawn from these studies on acidolysis for desirable preparation of structured lipids, such as MLM (L, long-chain FAs; M, medium-chain FAs) type and cocoa butter substitutes. Till now, there are very few studies investigating incorporation degree of different FAs into the substrate of tripalmitin-enriched TAGs (PPP-enriched TAGs) in lipases-catalyzed acidolysis. Karabulut et al.[9] have reported that incorporation of FAs was in the order C18:3 > C18:2 > C18:1 in acidolysis of PPP (purity, 99%) with the equimolar quantities of unsaturated FAs (C18:1, C18:2, and C18:3), catalyzed by Lipozyme TL IM, Lipozyme RM IM, and Novozym 435, separately. Nevertheless, more systematic studies need to be carried out for exploring the incorporation degree of

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different chain-length FAs (from C8:0 to C18:3) into PPP-enriched TAGs. Because human milk fat contains seven FAs in amounts greater than 1%, it is crucial to study the incorporation degree of different FAs (from C8:0 to C18:3) into PPP-enriched TAGs, which might give more information for research on the synthesis of human milk fat substitutes on a molecular basis. Acyl migration was another important factor that affects the quality and yield of human milk fat substutes. Diacylglycerols (DAGs) are the necessary and unavoidable intermediates in lipase-catalyzed transesterification, and its existence is the main reason leading to acyl migration. The mechanism of acyl migration is initiated by the nucleophilic attack of the lone pair of electrons of the free hydroxyl oxygen in DAGs on the ester carbonyl carbon, which forms a five-member ring intermediate.[10] The ring opens resulting in two products, the original DAGs and a migrated one. When the migrated DAG resynthsizes into a TAG, an undesirable TAG product is produced. Many factors could possibly influence acyl migration, such as water content, reaction time, reaction temperature, reaction type, and carries of lipases.[11]

PPP-enriched TAGs are the most common and important material for the production of human milk fat substitutes. In the present study, PPP-enriched TAGs were concentrated from palm stearin by acetone fractionation and as the substrate reacted with different kinds of FAs (e.g., C8:0, C10:0, C12:0, C14:0, C18:1, C18:2, and C18:3). Five lipases, namely, Novozym 435, Lipozyme RM IM, Lipozyme TL IM, porcine pancreatic lipase (PPL), and lipase PS IM, were screened to catalyze the acidolysis reactions. The effect of operating variables such as molar ratio, temperature, and reaction time on the incorporation degree of different FAs into PPP-enriched TAGs was further investigated. Acyl migration of different FAs to the sn-2 position and acylglycerols composition in acidolysis reaction were also examined. The information obtained in this work would allow scientists and manufacturing to design and/or predict reaction results for incorporating different FAs of interest into TAGs in the production of human milk fat substitutes.

■ MATERIALS AND METHODS

Materials. A nonspecific lipase Novozym 435 (*Candida antarctica* B lipase immobilized on a macroporous acrylic resin), and two sn-1,3 specific immobilized lipases Lipozyme RM IM (*Rhizomucor miehei* lipase immobilized on an ion-exchange resin) and Lipozyme TL IM (*Thermomyces lanuginosus* lipase immobilized on silica gel) were supplied by Novozymes A/S (Bagsvaerd, Denmark). Other lipases (in powder form), namely, lipase PS IM from *Burkholderia cepacia* (immobilized on diatomaceous earth) and PPL (sn-1,3 specific), were purchased from Amano Enzyme (Nagoya, Japan) and Kayon Biological Technology Co. Ltd. (Shanghai, China), separately. Properties of some of the lipases, including FA specificity and regiospecificity, are listed in literature.[11−13] Enzyme activity determination was performed with a coconut oil emulsion substrate according to the published procedure.[14] One unit of lipase (U) is the amount of enzyme that releases 1 μmol of titratable free FAs per minute under the described conditions, and the activities of Lipozyme TL IM, Lipozyme RM IM, Novozym 435, lipase PS IM, and PPL were detected as 11527.69, 812.80, 169.33, 10724.44, and 4741.33 U/g, respectively. Four FAs, namely, caprylic (C8:0, >99%), capric (C10:0, >99%), lauric (C12:0, >99%), and myristic (C14:0, >99%) acids, were purchased from Aladdin Chemistry Co. Ltd. (Shanghai, China). Organic flaxseed oil was provided by Xin-Qi-Dian Biotechnology Co. Ltd. (Hebei, China), and soybean oil was from Yihai Kerry Co. Ltd. (Guangzhou, China). Standards of the 37 FA methyl esters (CAS no. 113 47885-U), dioleoylglycerol (15% of 1,2-dioleoylglycerol, 85% of 1,3-dioleoylglycerol, CAS no. 25637-84-7), and trioleoylglycerol (CAS no. 122-32-7) were sourced from Sigma-Aldrich (China). Palm stearin was from Nian-Nian-Feng Grains & Oils (Group) Co. Ltd. (Shenzhen, China). All other reagents and solvents used for analysis were of chromatographic or analytical grade.

Preparation of Equimolar Quantities of FAs. The preparation of free FAs from soybean oil and flaxseed oil was conducted according to the method descried previously.[15] The prepared FAs from soybean oil (soybean oil FAs) and prepared FAs from flaxseed oil (flaxseed oil FAs) were stored at −25 °C until use.

A mixture of equimolar quantities of different FAs (C8:0, C10:0, C12:0, C14:0, C18:1, C18:2, and C18:3) was prepared by mixing caprylic acid, capric acid, lauric acid, myristic acid, soybean oil FAs, and flaxseed oil FAs at a molar ratio of 0.127:0.126:0.125:0.126:0.255:0.059:0.182. Metlab version 7.0 was used to calculate the molar ratio of seven kinds of FA samples. The water activity of blended FAs was 0.484 (msl Set-aw, Novasina Company, Switzerland).

Preparation and Analysis of PPP-Enriched Fraction. Enrichment of PPP-enriched TAGs from palm stearin was carried out according to the method descried by Son et al.[16] TAG fractions were separated by a serial liquid chromatograph (Waters Tech.) equipped with an XbridgeTM C18 column (250 mm × 4.6 mm, 5 μm particle size, Waters) and an evaporative light scattering detector (ELSD). The sample (10 mg) was dissolved in 1 mL of hexane. Sixty-five percent of the initial elution was acetonitrile, and then, the concentration of acetonitrile was decreased linearly with a gradient from 65 to 60% in the first 10 min and from 60 to 55% over the next 40 min. After completion of the chromatograph elution, the mobile phase was set to its initial concentration within 5 min. The flow rate was 0.8 mL/min, and the injection volume was 5 μL.

The peak identification of a single TAG was carried out via the coupled with mass spectrometry, and mass spectrometry conditions were as follows: nebulizer gas, N2; P, 0.14 MPa; nebulizer gas flow rate, 10 mL/min; atmospheric pressure chemical ionization (APCI) mode, positive; APCI temperature, 350 °C; Q array (quadrupole array), scan; and mass range, 50−1500 *m/z*.

Acidolysis Reaction. Binary mixtures of PPP-enriched TAGs (100 mg) with equimolar quantities of the selected FAs mixture were used in acidolysis. Reactions were carried out in tightly closed, screw-capped, brown glass vials (20 mL) containing a mixture of PPP-enriched TAGs with an amount of the selected FAs solution dissolved in 3 mL of hexane. The sample vials were incubated in an air bath with a shaker speed of 200 rpm. The effects of substrate molar ratios (PPP-enriched TAGs:a mixture of equimolar quantities of the selected FAs, from 1:1 to 1:4), temperatures (from 40 to 60 °C), and reaction time (from 3 to 24 h) on the incorporation degree of different FAs were studied, respectively. At the end of the reaction, the suspensions were filtered through syringe membrane filter (0.45 μm) to remove the enzyme particles, and filtrated hexane solutions were set for subsequent analysis.

FA Composition Analysis. Fifty microliters of the concentrated sample solution was applied to thin-layer chromatography plates (10 cm × 20 cm) coated with silica gel. The developing solvent was petroleum ether/ethyl ether/acetic acid (80:20:1, by vol). The bands were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under ultraviolet light. The TAGs band was scraped off for the analysis of total and sn-2 positional FA composition.

Small amounts of the scraped TAGs were methylated to FA methyl esters according to the method of ISO 5509:2000(E) and then were analyzed on an Agilent 7890A gas chromatograph (GC) equipped with a capillary column CP-Sil 88 (60 m × 0.25 mm × 0.2 μm; Dikma Technologies, Beijing, China).[17] The incorporation degree of FA (mol %) was calculated as follows:

$$\text{the incorporation degree of FA} = \text{FA content}_{\text{synthesized TAGs}} - \text{FA content}_{\text{PPP-enriched TAGs}}$$

and the total incorporation degree of FAs was the sum of the incorporation degree of each FA.

The sn-2 Positional FA Analysis. The scraped TAGs from thin-layer chromatography plate were applied for sn-2 positional analysis based on the method described by Sahin et al.[18] The 2-monoacyglycerols band was identified and scraped off and then was methylated to FA methyl esters for GC analysis as described above. The relative percentage of FA at sn-2 position in synthesized TAGs was calculated as follows:

$$\text{relative percentage of FA at sn-2 position} = \frac{\text{FA content at sn-2 position}}{3 \times \text{total FA content}} \times 100$$

HPLC Analysis of Acylglycerols in Acidolysis Reaction. The composition of acylglycerols in the acidolysis reaction was analyzed by high-performance liquid chromatography (Waters 1525) and refractive index detector (HPLC-RID). Lipids were separated over a Phenomenex Luna column (Phenomenex Corporation, 250 mm × 4.6 mm i.d., 5 μm particle size) at a column temperature of 35 °C. A mixture of *n*-hexane and isopropyl alcohol (15:1, by vol) with a flow rate of 1.0 mL/min was used as the mobile phase. Samples were diluted with mobile phase and dehydrated by anhydrous Na_2SO_4 prior to HPLC analysis.[19]

Statistics. Experiments were repeated for duplicate, and data reported are means ± standard deviations. SPSS version 13.0 was used to perform statistical calculations. A least significant differences test and analysis of variance procedure ($p < 0.05$) were carried out at a level of $p < 0.05$ to assess the significance of differences among mean values.

RESULTS AND DISCUSSION

Characteristics of PPP-Enriched TAGs. PPP-enriched TAGs are the most common and important substrates used for production of human milk fat substitutes. In this study, PPP-enriched TAGs were concentrated from palm stearin by acetone fractionation, and the solid fraction was targeted as PPP-enriched TAGs. The compositions of palm stearin and PPP-enriched TAGs were analyzed by HPLC-ELSD, and the results are presented in Table 1. PPP-enriched TAGs were found to contain four individual molecular species of TAG, including MPP, POP, PPP, and PPS. The PPP accounted for 88.02% of the solid fraction. As a comparison, the POP content was 53.10% in palm stearin and was decreased to 2.09% in PPP-enriched TAGs. Besides, the FA composition of PPP-enriched TAGs was determined by GC, and the main FAs contents (mol %) were 1.38% of C14:0, 89.83% of C16:0, 5.67% of C18:0, and 2.33% of C18:1, respectively.

Table 1. Identified TAG of Palm Stearin and PPP-Enriched TAGs

samples		
TAG species[a]	palm stearin	PPP-enriched TAGs
PLO	1.19 ± 0.24	ND[b]
PLL	2.26 ± 0.25	ND
MPP	0.59 ± 0.11	1.45 ± 0.10
POO	9.08 ± 0.45	ND
POP	53.09 ± 0.66	2.09 ± 0.19
PPP	31.91 ± 0.23	88.02 ± 0.14
POS	1.16 ± 0.27	ND
PPS	0.72 ± 0.10	8.44 ± 0.23

[a]M, myristic acid; P, palmitic acid; O, oleic acid; L, linoleic acid; S, stearic acid. [b]ND, not detectable.

Screening of Lipases. Five lipases, including, Lipozyme TL IM, Lipozyme RM IM, Novozym 435, lipase PS IM, and PPL, were screened for their ability to incorporate the selected FAs into PPP-enriched TAGs. The reaction conditions were set at the substrate molar ratio of 1:3, temperature of 50 °C, reaction time of 6 h, and enzyme dosage of 10% (by total weight of substrates). Under this enzyme dosage, the hydrolytic activities of the lipases (Lipozyme TL IM, Lipozyme RM IM, lipase PS IM, Novozym 435, and PPL) were 209.80, 14.79, 195.18, 3.08, and 82.29 U per total wieght of subsrtates, respectively. The results are shown in Figure 1. In Figure 1A, three lipases (Lipozyme TL IM, Lipozyme RM IM, and lipase PS IM) had higher incorporation abilities for the selected FAs than the other lipases tested (Novozym 435 and PPL). The total incorporation degree of FAs was 31.18, 30.82, and 30.49% in acidolysis reactions catalyzed with Lipozyme RM IM, Lipozyme TL IM, and lipase PS IM, respectively. Lipozyme TL IM, Lipozyme RMIM, and lipase PS IM better catalyzed the acidolysis of PPP-enriched TAGs, although their hydrolytic activities were different, indicating that enzyme reactivity depends on the specific reastion system.

As shown in Figure 1B, Novozym 435 acted strongly on C12:0 and C14:0 followed by C10:0 among the FAs tested. For PPL-catalyzed acidolysis, incorporation degrees for C8:0, C10:0, and C12:0 were found markedly higher than those for C14:0, C18:1, C18:2, and C18:3 ($P < 0.05$). For acidolysis reactions catalyzed by Lipozyme TL IM and Lipozyme RM IM, incorporation degrees for C12:0, C14:0, C18:1, and C18:2 were found higher than those for C8:0, C10:0, and C18:3 ($P < 0.05$) (Figure 1B). Lipase PS IM from *B. cepacia* catalyzed weakly on C8:0 and C18:3 in competitive substrate medium of selected FAs (C8:0−C18:3) but showed a significant reactivity in a wide range of FAs from C10:0 to C18:2 ($P < 0.05$).

Among the unsaturated FAs (C18:1, C18:2, and C18:3), no significant differences were observed ($P < 0.05$) in incorporation degree between C18:1 and C18:2, while the incorporation degree of C18:3 was found significantly lower than those of C18:1 and C18:2. A lower incorporation degree of C18:3 might be linked to the formation of a hooked shape of FAs with more double bonds.[20] Therefore, the number of double bonds and their location in the unsaturated FA molecule have influence on the reactivity of FA. Besides, it has been reported that chain-length specificity profiles also depend on the various reactions involving different substrates or cosubstrates.[21]

On the basis of on the above results, three lipases (Lipozyme TL IM, Lipozyme RM IM, and lipase PS IM) with higher incorporation ability for the selected FAs were chosen for further experiments, and the incorporation degree of different FAs, acyl migration of different FAs, and acylglycerols composition in acidolysis reaction were investigated in acidolysis catalyzed by the three lipases.

Effect of Substrate Molar Ratio on Incorporation Degree of Different FAs. The substrate molar ratio is involved in reaction equilibrium; hence, it can affect the incorporation ability of FAs in acidolysis. In the present research, molar ratios (PPP-enriched TAGs:equimolar quantities of the selected FAs) were set as 1:1, 1:2, 1:3, and 1:4, respectively, when the reaction temperature was kept at 50 °C, the enzyme loading was at 10% by weight of the total substrates, and the reaction time was kept at 6 h. Effects of molar ratio on incorporation degree of different FAs are presented in Figure 2. It was found that as the molar ratio increased from 1:1 to 1:4, the total incorporation degree of FAs increased significantly for the reactions catalyzed by Lipozyme TL IM, Lipozyme RM IM, and lipase PS IM, respectively. In most cases, incorporation

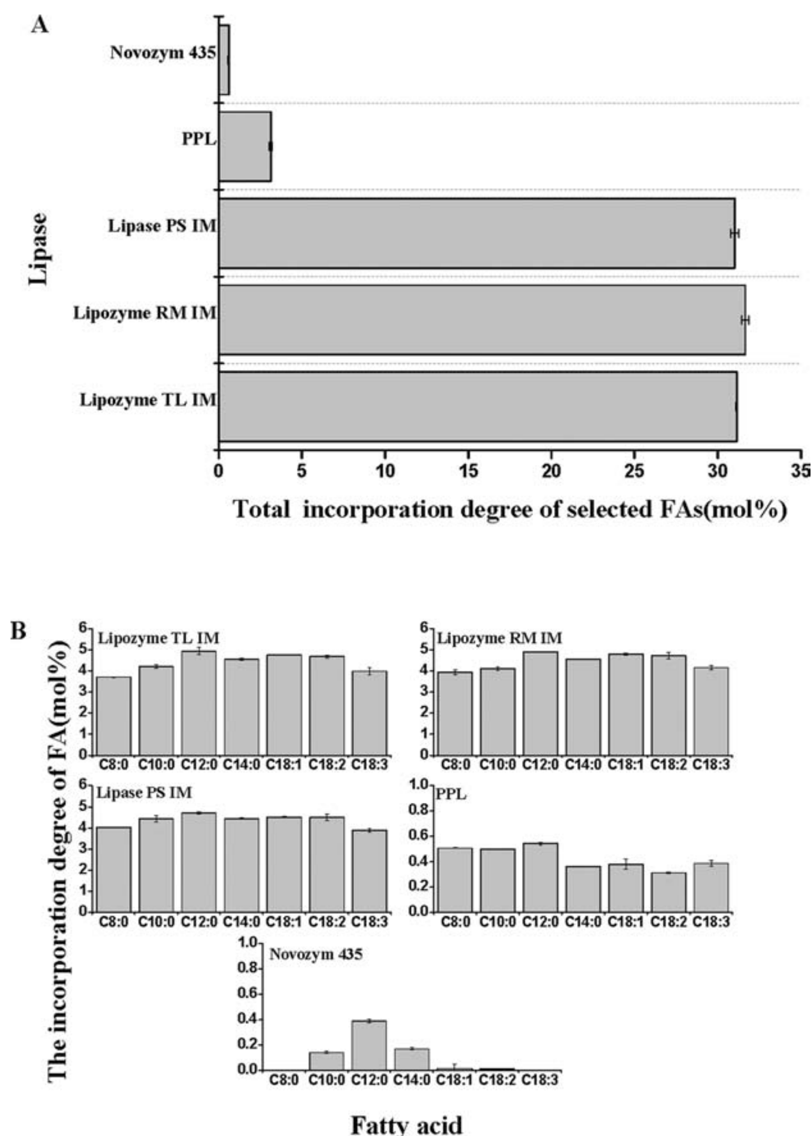

Figure 1. Effect of lipases on total incorporation degree of selected FAs (A) and the incorporation degree of each FA (B) into PPP-enriched TAGs. The reaction conditions are as follows: substrate molar ratio (PPP-enriched TAGs:equimolar quantities of selected FAs), 1:3; temperature, 50 °C; reaction time, 6 h; and enzyme dosage, 10%.

abilities of the three lipases showed no significant difference at each substrate molar ratio (Figure 2A). However, for each enzyme tested at each molar ratio, the incorporation degree for each FA (C8:0, C10:0, C12:0, C14:0, C18:1, C18:2, and C18:3) showed a difference (Figure $2B_{1-3}$).

For the reaction catalyzed by lipase PS IM, the incorporation degree of each FA increased smoothly as the molar ratio increased from 1:1 to 1:4 (Figure $2B_3$). The reactions, catalyzed by Lipozyme RM IM and Lipozyme TL IM, were found to deviate from a linear relationship between the incorporation degrees of FAs (C18:2 and C18:3) and the substrate molar ratio at 1:3 (PPP-enriched TAGs to the selected FAs) (Figure $2B_{2,3}$). The possible reason for the decrease in incorporation degrees for C18:2 and C18:3 might be due to inhibition of lipases (Lipozyme TL IM and Lipozyme RM IM) at higher substrate (FAs) concentration. As compared to Lipozyme TL IM and Lipozyme RM IM, lipase PS IM had a higher tolerance in excessive substrate concentration.

For the Lipozyme RM IM-catalyzed acidolysis, the lipase exhibited a better preference for C12:0, C14:0, and C18:1 than the other FAs at each molar ratio, therein the highest incorporation degrees for C12:0, C14:0, and C18:1 reached to 5.53, 5.37, and 5.30% at the substrate molar ratio of 1:4, respectively (Figure $2B_2$). A similar phenomenon was observed for the Lipozyme TL IM-catalyzed acidolysis (Figure $2B_1$). However, for the lipase PS IM-catalyzed acidolysis, no significant differences were observed in the incorporation degree of each FA at substrate molar ratio of 1:1 (Figure $2B_3$); among the selected FAs, the highest incorporation degrees were 4.10% for C18:1 at substrate molar ratios of 1:2 and 5.37 and 5.46% for C12:0 and C18:1 at substrate molar ratios of 1:4, respectively.

Effect of Reaction Temperature on Incorporation Degree of Different FAs. The reaction temperature is a key parameter for production of structured lipids and can affect parameters such as enzyme stability, affinity of enzyme for substrate, preponderance of competing reactions, and the content of byproduct caused by acyl migration. To clarify the effect of temperature on incorporation degree of different FAs, the reaction temperatures were set at 40, 50, and 60 °C, respectively. The substrate molar ratio (PPP-enriched TAGs

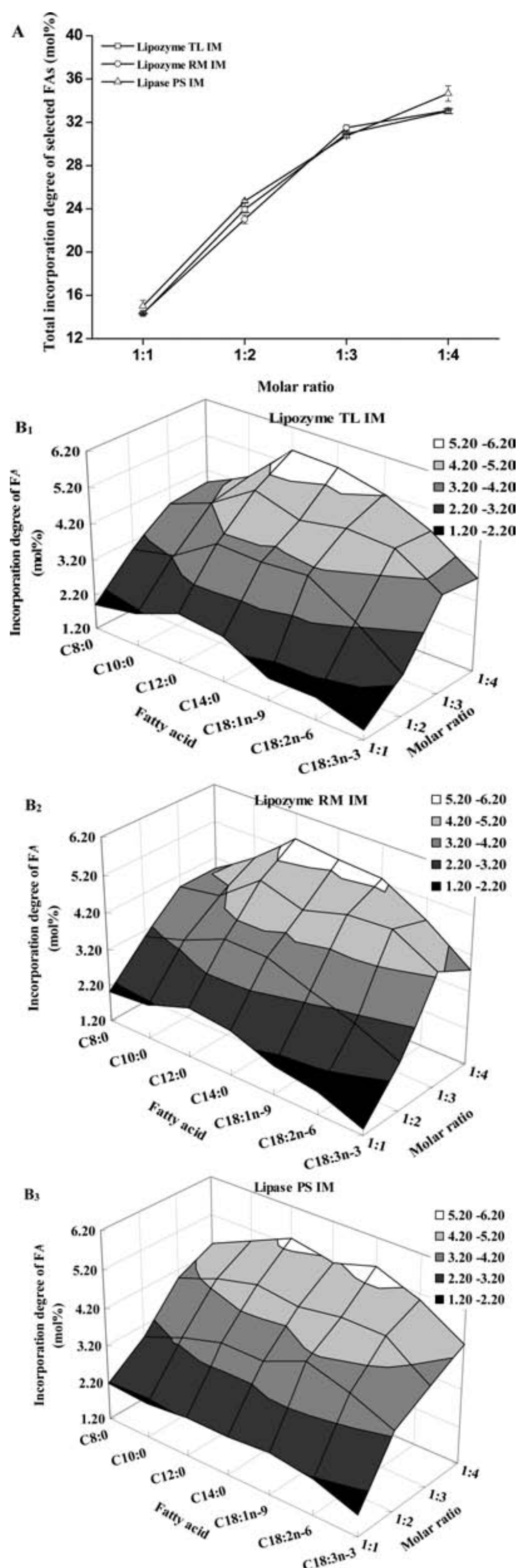

Figure 2. Effect of substrate molar ratio on total incorporation degree of selected FAs (A) and the incorporation degree of each FA (B_{1-3}) into PPP-enriched TAGs. The reaction conditions are as follows: reaction temperature, 50 °C; reaction time, 6 h; and lipase dosage, 10%.

to equimolar quantities of the selected FAs) was kept at 1:3, the enzyme dosage was 10% (by total weight of substrates), and the reaction time was kept at 6 h. The results are shown in Figure 3. From 40 to 60 °C, the total incorporation degree of selected FAs was found to increase linearly ($R^2 = 0.997$) from 28.68 to 32.64% for the Lipozyme TL IM-catalyzed acidolysis (Figure 3A). Among the three lipases, the total incorporation degree of the selected FAs (32.64%) was highest at 60 °C for the Lipozyme TL IM-catalyzed acidolysis. The total incorporation degree of the selected FAs was at the same level ($P > 0.05$) at temperatures of 50 and 60 °C, with Lipozyme RM IM-catalyzed acidolysis. For the lipase PS IM-catalyzed acidolysis, the total incorporation degree of selected FAs was 30.66% at 50 °C and 30.31% at 60 °C, respectively.

FAs of C12:0, C14:0, C18:1, and C18:2 were found to be more easily incorporated into the PPP-enriched TAGs than the other selected FAs at all temperatures (Figure $3B_{1-3}$). The incorporation degree of C12:0 was the highest (4.93%) among selected FAs at 40 °C for the Lipozyme TL IM-catalyzed acidolysis. Among the selected FAs, the highest incorporation degree was 4.89% for C18:1 at 40 °C for the lipase PS IM-catalyzed acidolysis (Figure $3B_3$). The highest incorporation degree of C12:0 was 4.90% at 60 °C for the Lipozyme RM IM-catalyzed acidolysis. When the temperature rose to 60 °C, differences in incorporation degrees between different FAs were weakened for the acidolysis catalyzed by Lipozyme TL IM, Lipozyme RM IM, and lipase PS IM, respectively. These results showed that the selectivity of lipases for different FAs was much more sensitive at low temperatures. Besides, it has been reported that lower temperatures showed less influence on acyl migration, which is a serious problem for the production of structured lipids.[10] Therefore, a lower temperature benefits the preparation of structured lipids, such as human milk fat substitutes with desirable FAs content and high quality.

Effect of Reaction Time on Incorporation Degree of Different FAs. The reaction time was set at 3, 6, 9, 12, and 24 h, respectively, so as to investigate the effect of reaction time on the incorporation degree of different FAs. The other reaction conditions were as follows: substrate molar ratio (PPP-enriched TAGs to equimolar quantities of the selected FAs), 1:3; reaction temperature, 50 °C; and enzyme dosage, 10% (by total weight of substrates); and the results are shown in Figure 4. The total incorporation degree of the selected FAs was found to increase as the acidolysis time increased (Figure 4A). The total incorporation degree of the selected FAs was lowest (27.03%) at 3 h of acidolysis catalyzed by lipase PS IM. As compared to Lipozyme TL IM and lipase PS IM, Lipozyme RM IM exhibited the maximum total incorporation degree for the selected FAs (31.67%) at 6 h of acidolysis, while the highest total incorporation degree for the selected FAs was 32.98% at 9 h during Lipozyme TL IM-catalyzed acidolysis, as compared to the other lipases-catalyzed acidolysis. The incorporation abilities of the three lipases showed no significant difference at 12 and 24 h, respectively (Figure 4A).

Three lipases showed higher preference for C12:0, C14:0, C18:1, and C18:2 at each given reaction time (Figure $4B_{1-3}$). Higher incorporation degrees for C12:0 and C18:1 at 3 h were 4.48 and 4.71% during Lipozyme TL IM-catalyzed acidolysis and were 4.57 and 4.55% with Lipozyme RM IM-catalyzed acidolysis, respectively, as compared to lipase PS IM at 3 h. During Lipozyme RM IM-catalyzed acidolysis, the incorporation degree of C12:0 (5.59%) was significantly higher than other FAs at 12 h, and incorporation degrees for C12:0, C14:0, and C18:2 were detected at the same level at 24 h (Figure $4B_2$).

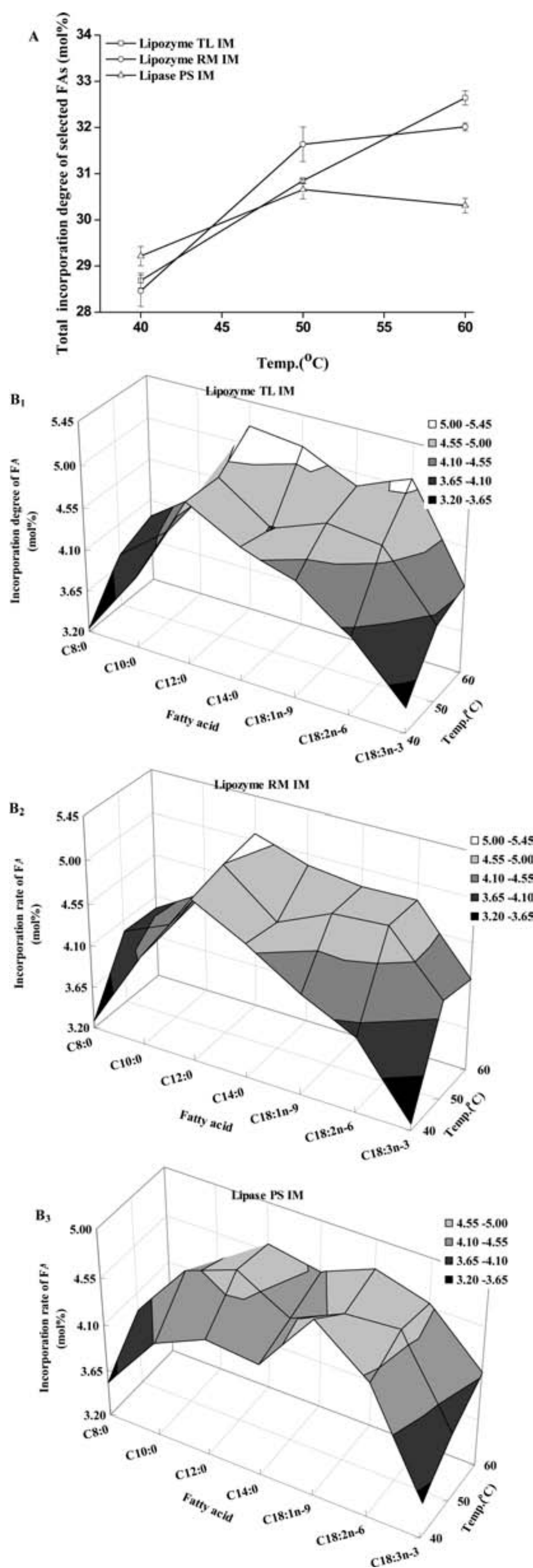

Figure 3. Effect of reaction temperature on total incorporation degree of selected FAs (A) and the incorporation degree of each FA (B_{1-3}) into PPP-enriched TAGs. The reaction conditions are as follows: substrate molar ratio, 1:3 (PPP-enriched TAGs:equimolar quantities of selected FAs); reaction time, 6 h; and lipase dosage, 10%.

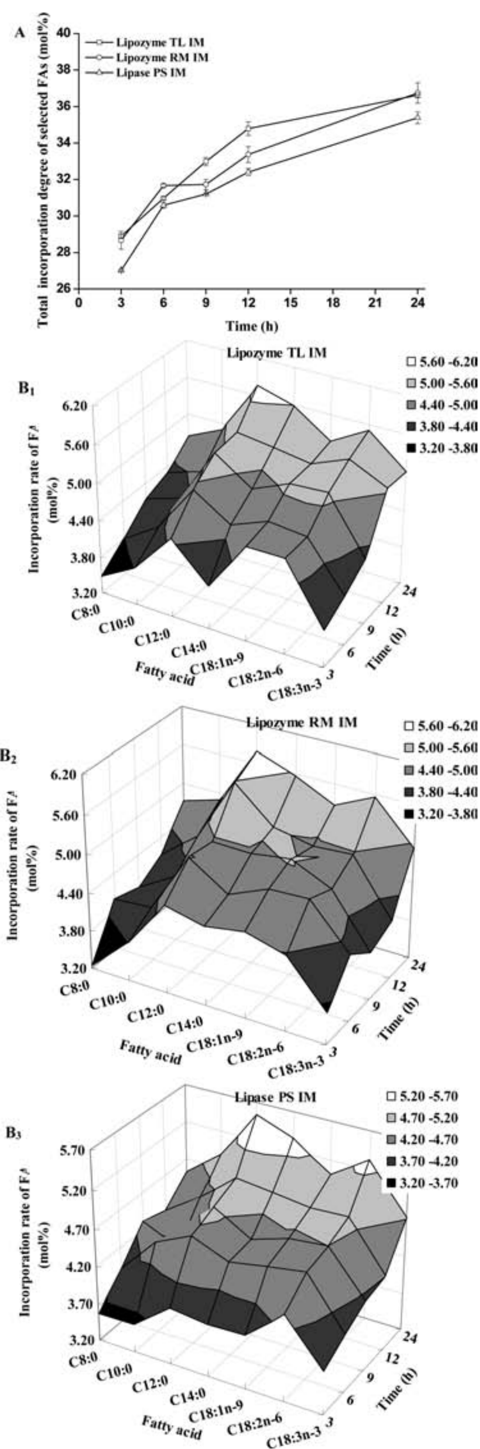

Figure 4. Effect of reaction time on total incorporation degree of selected FAs (A) and the incorporation degree of each FA (B_{1-3}) into PPP-enriched TAGs. The reaction conditions are as follows: substrate molar ratio, 1:3 (PPP-enriched TAGs:equimolar quantities of selected FAs); reaction temperature, 50 °C; and lipase dosage, 10%.

Acyl Migration of Different FAs. Acyl migration unavoidably occurs during lipase-catalyzed acidolysis for production of structured lipids. The level of acyl migration could affect the quality of structured lipids. The migration of different FAs (from sn-1,3 to sn-2 positions) was investigated, and the reaction was carried out under the conditions: a substrate molar ratio of 1:3, temperature of 50 °C, reaction time of 6 h, and enzyme dosage of 10%. The results are shown in Figure 5. Taking into account the difference in the total FA profiles of TAGs during the three lipases-catalyzed acidolysis, the index (relative percentage of FA at sn-2 position) was

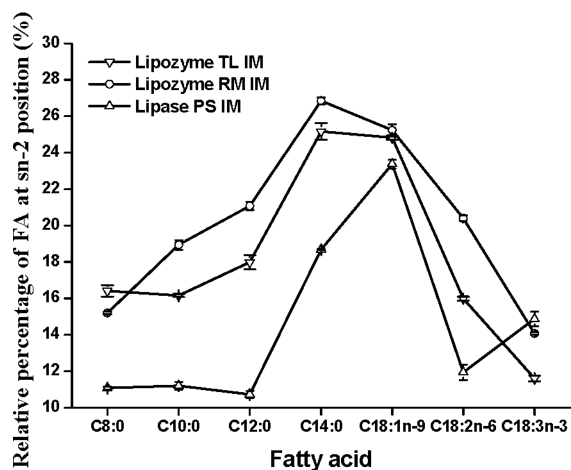

Figure 5. Effect of lipases on acyl migration of different FAs in acidolysis of PPP-enriched TAGs with equimolar quantities of selected FAs. The reaction conditions were as follows: substrate molar ratio, 1:3 (PPP-enriched TAGs:equimolar quantities of selected FAs); temperature, 50 °C; reaction time, 6 h; and enzyme dosage, 10%.

adopted to investigate the level of migration of different FAs from sn-1,3 to sn-2 positions. The higher the relative percentage of FA at the sn-2 position is, the more migration of the FA from sn-1,3 to sn-2 positions. There are significant differences between Lipozyme TL IM, Lipozyme RM IM, and lipase PS IM in migration level for C8:0, C10:0, C12:0, C14:0, and C18:2, while no significant differences in the migration level for C18:1 were observed ($P < 0.05$) during Lipozyme TL IM- and Lipozyme RM IM-catalyzed acidolysis. Among the three lipases, the lowest migration (11.60%) of C18:3 occurred with Lipozyme TL IM-catalyzed acidolysis. Besides, the migration level of C14:0 was found higher than those of other FAs during Lipozyme TL IM- and Lipozyme RM IM-catalyzed acidolysis, and the migration level of C18:1 was found greater than those of other FAs during lipase PS IM-catalyzed acidolysis. The enzymes exhibited the order as Lipozyme PS IM < Lipozyme TL IM < Lipozyme RM IM based on their causing the acyl migration level for different FAs from sn-1,3 to sn-2 positions.

Composition of Acylglycerols in Acidolysis Reaction. Partial acylglycerols such DAGs and monoacylglycerols are unavoidable intermediates during a lipase-catalyzed acidolysis for the production of structured lipids.[22] DAGs exist in acidolysis, resulting in low yield and purity of TAGs. The composition of acylglycerols was investigated in the acidolysis reactions catalyzed by Lipozyme TL IM, Lipozyme RM IM, and lipase PS IM, respectively. The reaction conditions were as follows: substrate molar ratio, 1:3; temperature, 50 °C; reaction time, 6 h; and enzyme dosage, 10%; and the results are shown in Table 2. For Lipozyme RM IM-catalyzed acidolysis, acylglycerols in acidolysis reaction consisted of 82.09% of TAGs, 12.18% of 1,3-DAGs, and 5.73% of 1,2-DAGs. The TAGs content (82.09%) is significantly higher ($P < 0.05$) than those in Lipozyme TL IM and lipase PS IM-catalyzed acidolysis. The ratios of 1,3-DAGs to 1,2-DAGs were 2.57, 2.36, and 2.12 in Lipozyme PS IM-, Lipozyme TL IM-, and lipase RM IM-catalyzed acidolysis, respectively. Besides, the amount of partial acylglycerols is also affected by various conditions, such as reaction temperature, enzyme loading, substrate ratio, and water content.[19] In this study, DAGs content (17.91%) was found to be least influenced during Lipozyme RM IM-catalyzed acidolysis. It is possible to increase the level of TAGs in acidolysis by further optimization of reaction conditions.

Table 2. Effect of Lipases on Acylglycerols Composition in Acidolysis Reaction[a]

lipase	content[b] (wt %) TAGs	1,3-DAGs	1,2-DAGs
Lipozyme TL IM	78.99[c] ± 0.06	14.76[c] ± 0.30	6.24[c] ± 0.23
Lipozyme RM IM	82.09[d] ± 0.23	12.18[d] ± 0.31 a	5.73 ± 0.08
Lipase PS IM	79.37 ± 0.23	14.86 ± 0.12	5.78 ± 0.11

[a]The reaction conditions were as follows: substrate molar ratio, 1:3 (PPP-enriched TAGs:equimolar quantities of selected FAs); temperature, 50 °C; reaction time, 6 h; and enzyme dosage, 10%. [b]The content was presented as the mean ± SD; 1,3-DAGs, 1,3-diacylglyecerols; and 1,2-DAGs, 1,2- diacylglyecerols. [c]Denotes significant differences ($P < 0.05$) between the Lipozyme TL IM and the Lipozyme RM IM group. [d]Denotes significant differences ($P < 0.05$) between the Lipozyme RM IM and the Lipase PS IM group.

In conclusion, it is important to understand the chemical process of FA during enzyme-catalyzed synthesis of structured lipids. This work reports the incorporation degree of different FAs into PPP-enriched TAGs in acidolysis catalyzed by lipase PS IM, Lipozyme TL IM, Lipozyme RM IM, Novozym 435, and PPL. Among the selected FAs, Novozym 435 acted strongly on C12:0 and C14:0. Incorporation degrees for C8:0, C10:0, and C12:0 are markedly higher in acidolysis catalyzed by PPL. Lipozyme TL IM, Lipozyme RM IM, and lipase PS IM, the most effective lipases, preferably incorporated C12:0, C14:0, C18:1, and C18:2 at all of the experimental conditions tested. The lipases, based on the acyl migration level for different FAs, were ranked as the order of Lipozyme PS IM < Lipozyme TL IM < Lipozyme RM IM. As compared to Lipozyme TL IM and lipase PS IM, the content of TAGs was the highest (82.09%) in the acidolysis reaction catalyzed by Lipozyme RM IM. Our findings, including the incorporation degree of different FAs, acyl migration of different FAs to sn-2 position, and acylglycerols composition in acidolysis reaction, not only provide helpful information for production of human milk fat substitutes that are required to have highly similarity degree to human milk fat but also will be expected to expand the existing knowledge, both basic and applied, in the area of lipid biotechnology.

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Notes

The authors declare no competing financial interest.

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