Candida rugosa Lipase LIP1-Catalyzed Transesterification To Produce Human Milk Fat Substitute

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Structured lipids (SLs) containing palmitic and oleic acids were synthesized by transesterification of tripalmitin with either oleic acid or methyl oleate as acyl donor. This SL with palmitic acid at the sn-2 position and oleic acid at sn-1,3 positions is similar in structure to human milk fat triacylglycerol. LIP1, an isoform of *Candida rugosa* lipase (CRL), was used as biocatalyst. The effects of reaction temperature, substrate molar ratio, and time on incorporation of oleic acid were investigated. Reaction time and temperature were set at 6, 12, and 24 h, and 35, 45, and 55 °C, respectively. Substrate molar ratio was varied from 1:1 to 1:4. The highest incorporation of oleic acid (37.7%) was at 45 °C with methyl oleate as acyl donor. Oleic acid resulted in slightly lesser (26.3%) incorporation. Generally, higher percentage incorporation of oleic acid was observed with methyl oleate (transesterification) than with oleic acid (acidolysis). In both cases percentage incorporation increased with reaction time. Incorporation decreased with increase in temperature above 45 °C. Initially, oleic acid incorporation increased with increase in substrate molar ratio up to 1:3. LIP1 was also compared with Lipozyme RM IM as biocatalysts. The tested reaction parameters were selected on the basis of maximum incorporation of C18:1 obtained during optimization of LIP1 reaction conditions. Reaction temperature was maintained at 45, 55, and 65 °C. Lipozyme RM IM gave highest oleic acid incorporation (49.4%) at 65 °C with methyl oleate as acyl donor. Statistically significant ($P < 0.05$) differences were observed for both enzymes. SL prepared using Lipozyme RM IM may be more suitable for possible use in human milk fat substitutes.

INTRODUCTION

Structured lipids (SLs) are often referred to as the new generation of fats that can be considered as nutraceuticals: food or parts of food that provide medical or health benefits, including potential for the prevention and/or treatment of diseases (*1*). The SL is produced by chemical or enzymatic modification of triacylglycerols (TAG) (*2*). In the past few years preference for enzymatic modification of TAG over chemical modification has gained attention due to its ability to catalyze reactions under mild conditions and to produce specific composition and functionality of the end product. Therefore, much research has been performed for the production of SLs using lipases as biocatalysts (*3–5*). Typical applications of SLs are margarines, modified fish oil products, cocoa butter, human milk fat substitute, and many other lipid products (*5–7*).

Human milk contains ∼3–5% total lipids, and >90% of milk fat is TAG, in which fatty acids represent 88% of total lipids

(*8*). TAG are the main source of energy in both breast milk and infant formula, providing 50% of dietary energy (*9*). Human milk fats (HMF) mostly contain long-chain fatty acids. The major saturated fatty acid in human milk fat TAG is palmitic acid (16:0), which represents about a fourth of the fatty acids in breast milk. HMF contains ∼70% of its palmitic acid at the *sn*-2 position. Stearic acid (∼5–7%), oleic acid (∼30–35%), and linoleic acid (∼7–14%) are generally esterified to the *sn*-1,3 positions (*6*). This gives a unique structure to HMF, and lard is the only fat that has a similar structure (*8*). The palmitate at the *sn*-2 position is not hydrolyzed by pancreatic lipase, and as 2-monopalmitin it forms a mixed micelle with bile salt, which is efficiently absorbed (*10*, *11*). The palmitic acids in the *sn*-1,3 positions are hydrolyzed by pancreatic lipase, producing free palmitic acids, which form poorly absorbed calcium soap in the intestine, and results in reduced absorption of calcium and fat (*12–14*). Because of the formation of these calcium soaps, stool hardness, constipation, and in some cases bowel obstructions may occur. The presence of palmitic acid at the *sn*-2 position of HMF increases the absorption of 16:0 in the infant and reduces calcium loss in the feces (*15*). Therefore, this unique TAG molecule with palmitic acid esterified at the *sn*-2 position

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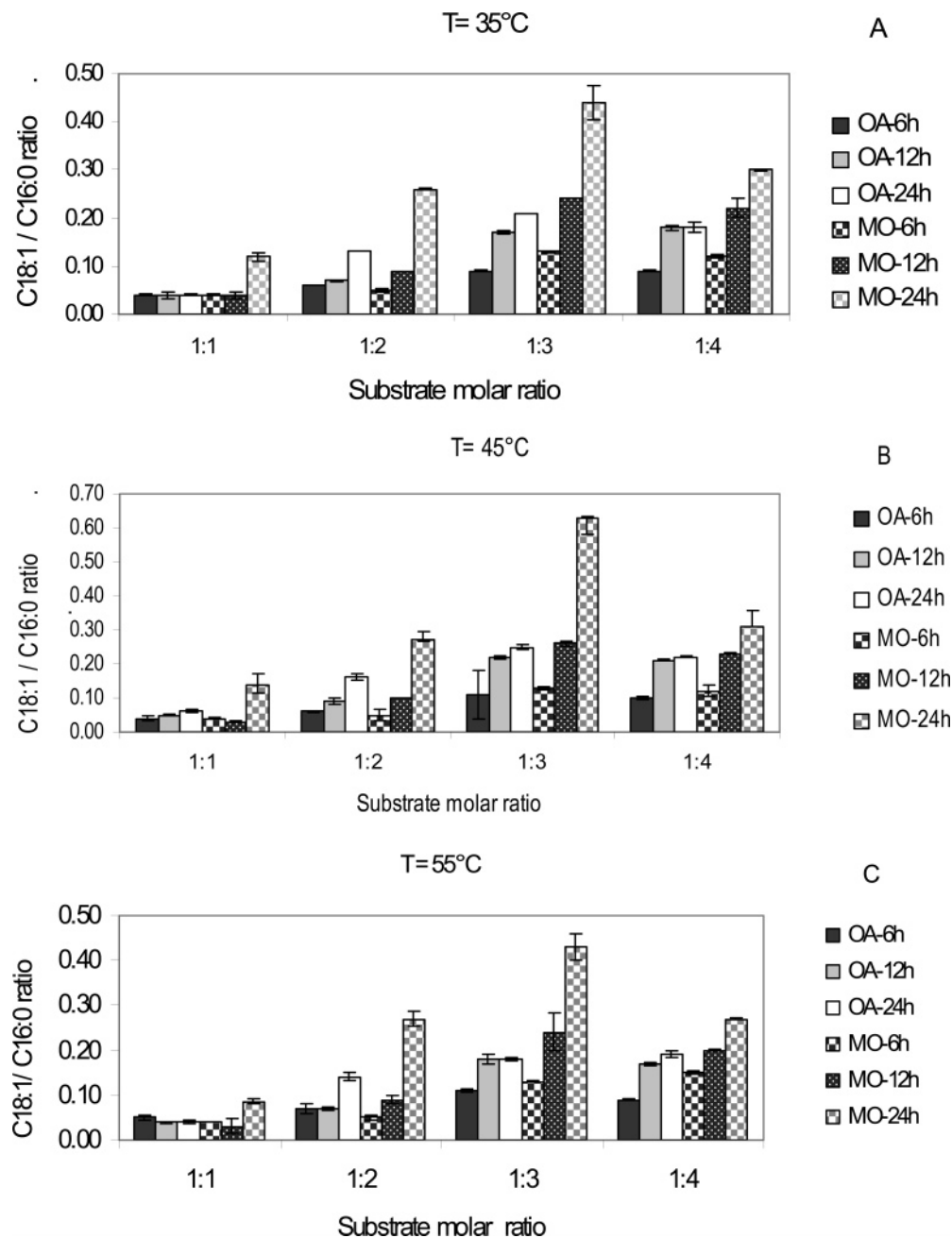† University of Georgia.
§ Academia Sinica.
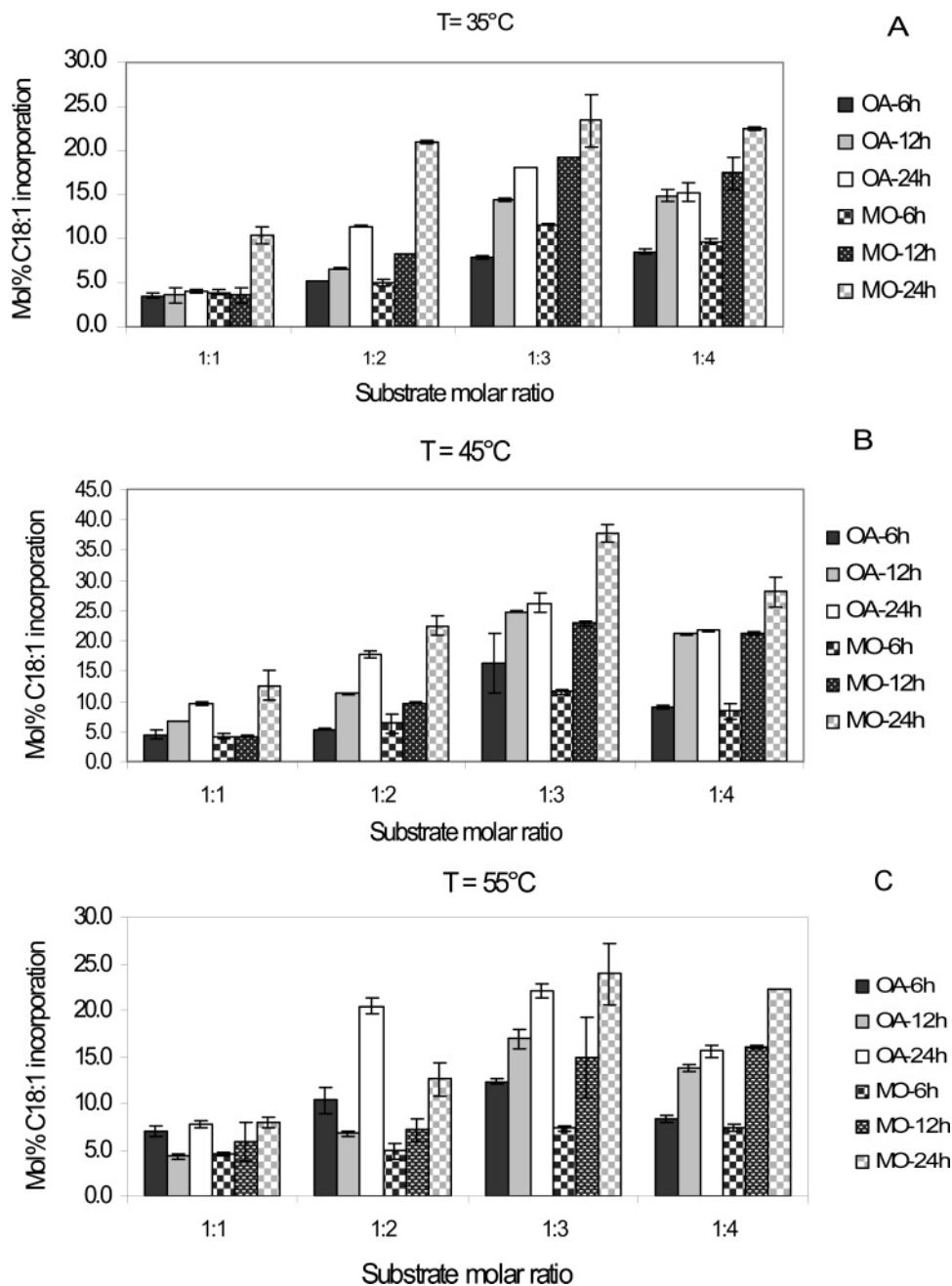
Figure 1. Oleic acid incorporation at substrate molar ratios of 1:1, 1:2, 1:3, and 1:4 (tripalmitin/oleic acid and tripalmitin/methyl oleate). Samples were analyzed at 6, 12, and 24 h. The *X*-axis represents substrate molar ratio. The *Y*-axis represents mole percent of C18:1 incorporation. Enzyme (LIP1) amount was 10 wt % of total substrates. Incubation was at 200 rpm. Reaction was performed in *n*-hexane. Incubations were at (**A**) 35 °C, (**B**) 45 °C, and (**C**) 55 °C. OA, oleic acid; MO, methyl oleate.

has a significant function in the development of infants. Recently, many studies were done to produce SLs with similar configuration for use in infant milk formula or as HMF substitute (*16, 17*). Many of these studies were done using Lipozyme RM IM as biocatalyst for the transesterification reactions (*10, 16*). Lipase from *Candida rugosa* is also one of the most attractive commercially available lipases for the complete hydrolysis of TAG. The main advantage of this enzyme is that it works under mild conditions with optimum reaction temperature at 40 °C (*18*). Commercial preparations of *C. rugosa* lipase (CRL) are mixtures of lipase isomers. This enzyme is nonpositionally specific. There are seven isoforms (LIP1−LIP7) present in crude CRL (*19−23*). Lipase genes for LIP1, LIP2, and LIP3 were successfully identified, and LIP1 and LIP3 were purified from commercial CRL preparations (*24*).

The objectives of the present work were as follows: (1) The reaction conditions required to synthesize SL using lipase isoform 1 (LIP1) from *C. rugosa* as biocatalyst were determined. Tripalmitin, oleic acid, and methyl oleate were used as substrates. The reactions were followed with respect to oleic acid incorporation. Substrate molar ratio, reaction time, and reaction temperature were studied. Products were also analyzed for *sn*-2 positional fatty acids. (2) A comparative study using Lipozyme RM IM, a 1,3-positional preferential lipase from *Rhizomucor meihei*, and a nonpositionally specific lipase (LIP1) from *C. rugosa* was performed.

MATERIALS AND METHODS

Materials. Tripalmitin (glycerol tripalmitate, minimum purity = 99%), oleic acid (∼99%), methyl oleate (∼99%), and porcine pancreatic

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Figure 2. Oleic acid/palmitic acid ratio at substrate molar ratios of 1:1, 1:2, 1:3, and 1:4 (tripalmitin/oleic acid and triplamitin/methyl oleate). Samples were analyzed at 6, 12, and 24 h. The *X*-axis represents substrate molar ratio. The *Y*-axis represents mole percent of C18:1 incorporation. Enzyme (LIP1) amount was 10 wt % of total substrates. Incubation was in *n*-hexane at 200 rpm. Incubations were at (**A**) 35 °C, (**B**) 45 °C, and (**C**) 55 °C. OA, oleic acid; MO, methyl oleate.

lipase (type II, crude) were purchased from Sigma Chemical Co. (St. Louis, MO). LIP1, a nonspecific *C. rugosa* lipase isoform 1, was supplied by the Institute of Botany, Academia Sinica, Taipei, Taiwan. Immobilized 1,3-specific lipase, Lipozyme RM IM, was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). Organic solvents and thin-layer chromatography (TLC) plates were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ) and Fisher Scientific (Fair Lawn, NJ), respectively. All solvents and reagents used in analysis were of chromatographic or analytical grade.

Enzymatic Modification Reactions. For transesterification and acidolysis reactions, 100 mg of tripalmitin was mixed with methyl oleate or oleic acid in a screw-cap 30 mL test tube at substrate molar ratios of 1:1, 1:2, 1:3, and 1:4 using 3 mL of hexane as organic solvent. LIP1 in powder form was added to the mixture (10 wt % of total reactants). Shaker water bath was adjusted to 200 rpm, and incubation temperature was set at 35, 45, and 55 °C, whereas incubation times

were set for 6, 12, and 24 h. All reactions were performed in duplicate, and average results were reported. In comparative study incubation temperature was set at 45, 55, and 65 °C, reaction time was set for 24 h, and substrate molar ratio was maintained at 1:3. LIP1 or Lipozyme RM IM at 10 wt % of total reactants was added to the reaction mixture.

Fatty Acid Composition Analysis. The reactions were stopped, and 4 mL of diethyl ether was added and the mixture vortexed. Products were passed through an anhydrous sodium sulfate packed in a Pasteur pipet column to remove the enzyme and moisture. Diethyl ether was removed by nitrogen aeration. The sample was redissolved in chloroform and concentrated to 300 μL using nitrogen aeration. Fifty microliters of the reaction product was applied to TLC plates (20 cm × 20 cm) coated with silica gel G. The developing solvent was petroleum ether/ethyl ether/acetic acid (80:20:0.5, v/v/v). The bands were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under UV light. The band corresponding to TAG was scraped
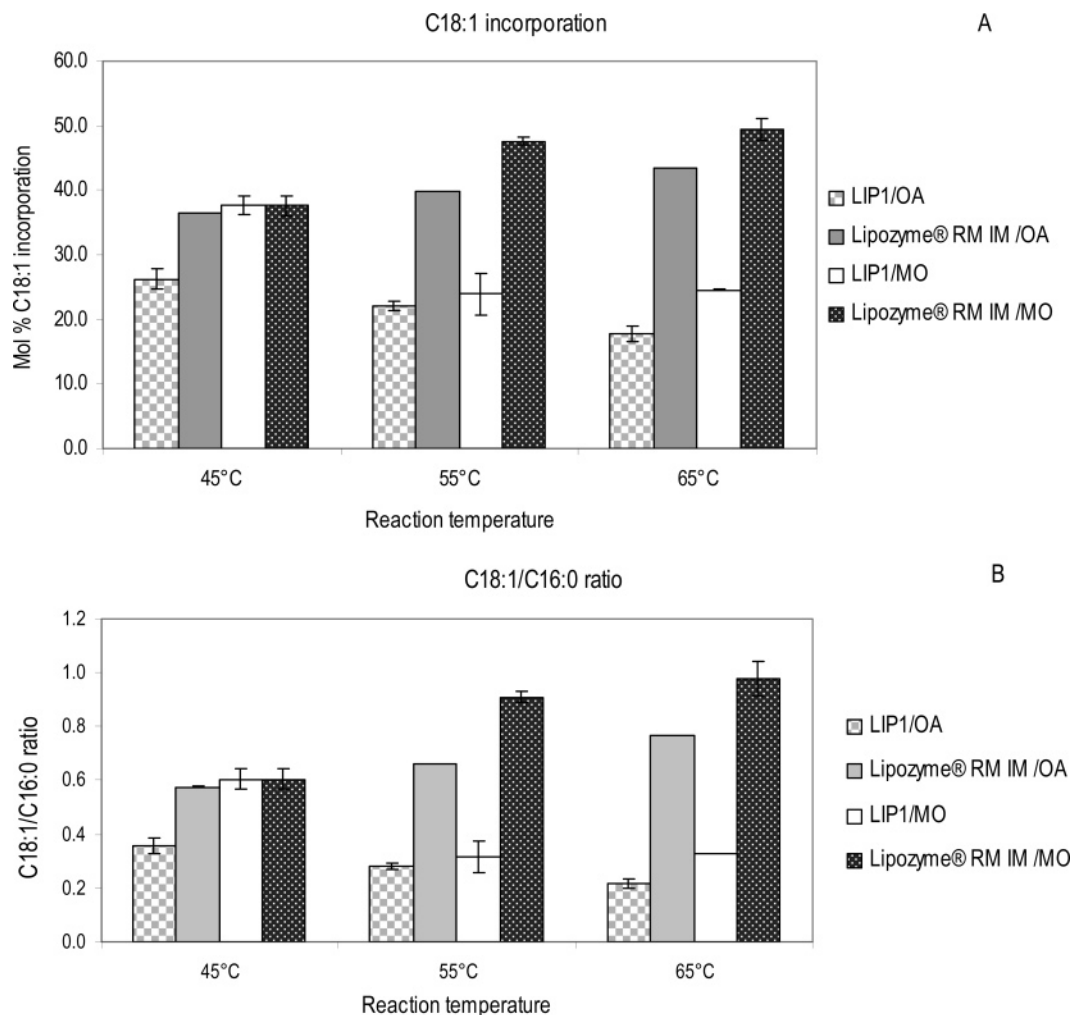
Figure 3. (**A**) Oleic acid incorporation at a substrate molar ratio of 1:3 (tripalmitin/oleic acid and tripalmitin/methyl oleate). The *X*-axis represents substrate molar ratio. The *Y*-axis represents mole percent of C18:1 incorporation. (**B**) Oleic acid/palmitic acid ratio at a substrate molar ratio of 1:3 (tripalmitin/oleic acid and triplamitin/methyl oleate). The *X*-axis represents substrate molar ratio. The *Y*-axis represents C18:1/C16:0 ratios. Samples were analyzed after 24 h. Enzyme (LIP1 or Lipozyme RM IM) amount was 10 wt % of total substrates. Incubation was in *n*-hexane at 200 rpm. Incubations were at 45, 55, and 65 °C. OA, oleic acid; MO, methyl oleate.

off and methylated with 3 mL of 6% HCl in methanol at 70−80 °C for 2 h. The fatty acid methyl ester (FAME) was analyzed by gas−liquid chromatography (GLC). The gas chromatograph was an Agilent Technologies 6890 N equipped with fused silica capillary column (DB-225, 30 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA) and a flame ionization detector (FID) and operated in a splitless mode. The injector and detector temperatures were maintained at 250 and 260 °C, respectively. The column temperature was held at 150 °C for 3 min and programmed to 215 °C for 10 min at the rate of 10 °C/min. The carrier gas was helium, and the total gas flow rate was 23 mL/min. One microliter sample was injected into GLC, and relative content of FAME as mole percent was calculated by computer using C17:0 as internal standard (*25*).

Pancreatic Lipase-Catalyzed *sn*-2 Positional Analysis. The TAG band of SL was scraped off from the TLC plate as described earlier and used for the *sn*-2 analysis. One milliliter of 1 M Tris buffer (adjusted to pH 8.0 with HCl) was first added to the test tubes containing the TAG samples. Then 0.25 mL of 0.05% bile salt solution (sodium cholate), 0.1 mL 2.2% calcium chloride solution, and 20 mg of purified pancreatic lipase (porcine pancreatic lipase, crude type II) were added. The mixture was incubated at 37 °C for 3 min. After 3 min, the tubes were vortexed for 2 min and then 0.5 mL of 6 N HCl solution and 4 mL of anhydrous diethyl ether were added. This mixture was vortexed and centrifuged, and the diethyl ether layer containing tripalmitin, free fatty acid, and SLs was passed through the sodium sulfate column. Four milliliters of diethyl ether was added again, and the process was

repeated to extract the product thoroughly. After extraction, diethyl ether was completely evaporated under nitrogen and redissolved in 2 mL of chloroform. The mixture was transferred to small vials, and chloroform was concentrated to 300 μL. A 200 μL aliquot was spotted onto a silica gel G TLC plate and developed with hexane/diethyl ether/acetic acid (50:50:1, v/v/v). The 2-monoacylglycerol (2-MAG) band was visualized under UV light after being sprayed with 0.2% 2,7-dichlorofluorescein in methanol. The 2-monoolein standard (Sigma) was used for TLC confirmation of 2-MAG of reaction products. The band corresponding to the *sn*-2 MAG was scraped from the plates, methylated and analyzed by GLC as described earlier (*25*).

Statistical Analysis. Statistical analysis was done with the SAS software package (*25*). One-way analysis of variance (ANOVA) was performed to determine the difference among SL samples. When *F* values for the ANOVA were significant, differences in means were determined using Duncan's multiple-range tests as a procedure of mean separation ($P < 0.05$).

RESULTS AND DISCUSSION

Tripalmitin contained predominantly palmitic acid (97.7%) with traces of oleic acid (0.4%). LIP1, a nonpositionally specific lipase, was the biocatalyst for tripalmitin modification. This lipase can catalyze hydrolysis or esterification at the *sn*-1, *sn*-2, and *sn*-3 positions of TAG molecules with preference for

Table 1. Total Fatty Acid Composition (Mole Percent)[a] and Fatty Acid at the sn-2 Position (Mole Percent)[b] of Structured Lipids (SL)

| | | temperature | | | | | |
| | | 45 °C | | 55 °C | | 65 °C | |
SL[c]	FA	total	sn-2	total	sn-2	total	sn-2
1	C16:0	73.8 ± 1.5B	81.5 ± 0.7A	77.9 ± 0.7B	43.5 ± 0.4B	82.2 ± 1.2A	44.0 ± 0.6B
	C18:1	26.3 ± 1.5A	18.5 ± 0.7B	22.1 ± 0.7A	56.5 ± 0.4A	17.8 ± 1.2B	56.0 ± 0.6A
	C18:1/C16:0	0.4 ± 1.5		0.3 ± 0.7		0.2 ± 1.2	
2	C16:0	63.4 ± 0.0A	91.9 ± 0.0A	60.2 ± 0.0B	79.2 ± 0.1C	56.6 ± 0.1B	79.8 ± 0.1B
	C18:1	36.6 ± 0.0A	08.1 ± 0.0C	39.8 ± 1.0A	20.8 ± 0.1A	43.4 ± 0.1A	20.2 ± 0.1B
	C18:1/C16:0	0.6 ± 0.0		0.7 ± 0.0		0.8 ± 0.1	
3	C16:0	62.3 ± 1.5B	53.9 ± 0.2B	76.1 ± 3.3A	56.6 ± 0.2A	75.4 ± 0.0A	48.6 ± 0.4C
	C18:1	37.7 ± 1.5A	46.2 ± 0.2B	23.9 ± 3.3B	43.5 ± 0.2C	24.6 ± 0.0B	51.5 ± 0.4A
	C18:1/C16:0	0.6 ± 1.5		0.3 ± 3.3		0.3 ± 0.0	
4	C16:0	62.4 ± 1.5A	86.6 ± 0.3A	52.4 ± 0.6B	74.3 ± 0.0C	50.6 ± 1.6C	76.7 ± 0.0B
	C18:1	37.6 ± 1.5C	13.4 ± 0.3B	47.6 ± 0.6B	25.8 ± 0.0A	49.4 ± 1.6A	23.3 ± 0.0B
	C18:1/C16:0	0.6 ± 1.5		0.9 ± 0.6		1.0 ± 0.0	

[a] Abbreviations: total fatty acid compositions were compared, with mean ± SD, $n = 2$. [b] Fatty acids at sn-2 positions were compared with mean ± SD, $n = 2$; means with the same letter in the same row are not significantly different ($P < 0.05$) for either fatty acid comparison or sn-2. SL 1 was produced from tripalmitin and oleic acid as substrates using LIP1 as biocatalyst; SL 2 was produced from tripalmitin and oleic acid as substrates using Lipozyme RM IM as biocatalyst; SL 3 was produced from tripalmitin and methyl oleate as substrates using LIP1 as biocatalyst; and SL 4 was produced from tripalmitin and methyl oleate as substrates using Lipozyme RM IM as biocatalyst. All reactions were carried out with a substrate molar ratio of 1:3 and incubation for 24 h.

the medium-chain acyl substrates (C_8-C_{10}). Similar activity was shown by commercial CRL, indicating that the major component of commercial CRL is LIP1 (*27*). SL produced in the present study shows that the LIP1 has no positional specificity but an inclination toward sn-1,3 specificity, whereas Lipozyme RM IM is sn-1,3-specific as reported by several researchers (*16, 28*).

Oleic Acid Incorporation Using LIP1 and Oleic Acid as Acyl Donor. Oleic acid incorporation at different temperatures and substrate molar ratios is given in **Figure 1**. Incorporation of oleic acid was affected by all of the parameters. Interaction terms were found to be statistically significant ($P < 0.05$). The highest value of oleic acid incorporation (26.3%) was achieved at 45 °C with a substrate molar ratio of 1:3 and a reaction time of 24 h. Incorporation of oleic acid decreased with increase in temperature above 45 °C. As reported earlier, this enzyme works under mild conditions with optimum temperature of CRL activity at 40 °C (*18*). Lowest incorporation (3.5%) was observed with a substrate molar ratio of 1:1 and a reaction time of 6 h at 35 °C. Initially, percentage incorporation increased with substrate molar ratio up to 1:3. Above this ratio the incorporation was slightly reduced or remained constant. One of the possible reasons for this could be inhibition of enzyme activity due to high substrate concentration or the fact that equilibrium has been reached.

Oleic Acid Incorporation Using LIP1 and Methyl Oleate as Acyl Donor. Oleic acid incorporation at different temperatures and substrate molar ratios is also given in **Figure 1**. Highest incorporation (37.7%) was obtained at 45 °C at a substrate molar ratio of 1:3 and a reaction time of 24 h. Highest incorporation was observed with methyl oleate as acyl donor compared to oleic acid. This indicates that the methyl ester is a better acyl donor than free acid for LIP1 lipase. This could be attributed to better solubility of methyl oleate in hexane than oleic acid and/or easy removal of methoxy group. It was reported that *C. rugosa* lipase does not prefer methyl ester when compared with free acid for the synthesis of canola phytosterol oleate esters (*29*). This was mainly due to the sensitivity of *C. rugosa* lipase to methanol, which was released during the esterification reactions (*29*). We observed an increase in incorporation of oleic acid with an increase in reaction time. Incorporation decreased with further increase in temperature

above 45 °C. There was an increase in oleic acid incorporation with an increase in substrate molar ratio up to 1:3. Thereafter, incorporation was reduced.

Oleic Acid/Palmitic Acid Ratio Using LIP1. The oleic acid/palmitic acid ratio at different temperatures and substrate molar ratios is given in **Figure 2**. The ratio of oleic acid to palmitic acid increased as the incorporation of oleic acid increased and vice versa. The highest ratio (0.6) was observed at 45 °C after 24 h of reaction with methyl oleate as acyl donor. The lowest ratio (0.04) was observed at 35 °C after 6 h of reaction time.

Oleic Acid Incorporation Using Lipozyme RM IM and LIP1. Oleic acid incorporation at different temperatures using Lipozyme RM IM and LIP1 is given in **Figure 3A**. Reactions were performed using a substrate molar ratio of 1:3 (tripalmitin/acyl donor) for 24 h. Reaction temperature was maintained at 45, 55, or 65 °C. The statistical significance of difference was determined by a one-way ANOVA with Duncan's multiple-range test of comparison for oleic acid incorporation. Statistically significant ($P < 0.05$) differences were observed for the two enzymes and incorporation of oleic acid. The interaction term temperature × enzyme was also a useful variable in the incorporation of oleic acid. Duncan's multiple-range tests showed highest incorporation of oleic acid (49.4%) with Lipozyme RM IM at 65 °C and methyl oleate as acyl donor. The lowest incorporation (17.8%) was observed with LIP1 at 65 °C and oleic acid as acyl donor. Statistically significant differences ($P < 0.05$) were observed under all temperature conditions (45, 55, and 65 °C) with LIP1 and Lipozyme RM IM when oleic acid was used as acyl donor. However, when methyl oleate was used as acyl donor, there were no significant differences ($P < 0.05$) observed at 55 and 65 °C with Lipozyme RM IM. Similar observations ($P < 0.05$) were obtained for LIP1 at 55 and 65 °C. The best incorporation was at 45 °C for LIP1 enzyme, indicating lack of stability at higher temperatures.

Oleic Acid/Palmitic Acid Ratio. Oleic acid incorporation at different temperatures and substrate molar ratios is given in **Figure 3B.** The overall ratio of oleic acid to palmitic acid (**Table 1**) was low (0.6) when LIP1 was used as biocatalyst and higher (1.0) when Lipozyme RM IM was used as biocatalyst, indicating that Lipozyme RM IM gave higher oleic acid incorporation than LIP1. Reaction products with oleic acid/palmitic acid ratio of

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1.2, similar to that of HMF, were considered to meet the desired ratio for HMF or infant formula (*30*, *31*).

Pancreatic Lipase *sn*-2 Positional Analysis: The fatty acid composition and fatty acids at *sn*-2 positions of SLs are given in **Table 1**. Structured lipid obtained after 24 h, with a substrate molar ratio of 1:3 and reaction temperature of 45, 55, or 65 °C, were used for *sn*-2 analysis. Duncan's multiple-range test of comparison shows that no statistically significant difference ($P < 0.05$) was observed between the two acyl donors (methyl oleate and oleic acid) and incorporation of C18:1 at the *sn*-2 position. Oleic acid contents at the sn-2 position were from 18.5 to 56.5% (**Table 1**) with LIP1 and from 8.1 to 25.8% (**Table 1**) with Lipozyme RM IM. The random incorporation of oleic acid at the *sn*-2 position indicates nonpositional specificity of LIP1 and possibly acyl migration at temperatures above 45 °C. High palmitic acid (91.9%) was observed at the *sn*-2 position due to the *sn*-1,3 specificity of Lipozyme RM IM. Despite the specificity of Lipozyme RM IM at *sn*-1,3 positions, incorporation of fatty acid into TAG at the *sn*-2 position occurred due to acyl migration during reaction (*32*). At a low temperature of 45 °C, acyl migration was only 8.1 for the Lipozyme RM IM reaction.

On the basis of our study, SL produced using Lipozyme RM IM may be more suitable for possible use in infant formulas or human milk fat substitutes when compared with SL produced using LIP1. This is due to the *sn*-1,3 specificity of Lipozyme RM IM, which resulted in high palmitic acid content at the *sn*-2 position. Earlier studies provide convincing information regarding the relationship between calcium absorption and higher saturated fatty acid at the *sn*-2 position and unsaturated fatty acids at *sn*-1,3 positions (*33*, *34*). LIP1 gave variable results at the *sn*-2 position for palmitic and lower incorporation of C18:1 in SL.

LITERATURE CITED

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

(2) Willis, W. M.; Marangoni, A. G. Assessment of lipase- and chemically catalyzed lipid modification strategies for the production of structured lipids. *J. Am. Oil Chem. Soc.* **1999**, *76*, 443–450.

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

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

(5) Hamam, F.; Shahidi, F. Enzymatic incorporation of capric acid into a single cell oil rich in docosahexaenoic acid and docosapentaenoic acid and oxidative stability of the resultant structured lipid. *Food Chem.* **2005**, *91*, 583–591.

(6) Nielsen, N. S.; Yang, T.; Xu, X.; Jacobsen, C. Production and oxidative stability of a human milk fat substitute produced from lard by enzyme technology in a pilot packed-bed reactor. *Food Chem.* **2006**, *94*, 53–60.

(7) Jensen, R. G. Infant formulas. In *Structured and Modified Lipids*; Gunstone, F. D., Ed.; Dekker: New York, 2001; pp 455–464.

(8) Chang, M. K.; Abraham, G.; John, V. T. Production of cocoa butter-like fat from interesterification of vegetable oils. *J. Am. Oil Chem. Soc.* **1990**, *67*, 832–834.

(9) Kennedy, K.; Fewtrell, M. S.; Morley, R.; Abbott, R.; Quinlan, P. T.; Wells, J. C. K.; Bindels, J. G.; Lucas, A. Double-blind, randomized trial of a synthetic triacylglycerol in formula-fed term infants: effects on stool biochemistry, stool characteristics, and bone mineralization. *Am. J. Clin. Nutr.* **1999**, *70*, 920–927.

(10) Thomson, A. B.; Keelan, M.; Garg, M. L.; Clandinin, M. T. Intestinal aspects of lipid absorption: a review. *Can. J. Physiol. Pharmacol.* **1989**, *67*, 179–191.

(11) Filer, L. J.; Mattson, F.; Fomon, S. J. Triglyceride configuration and fat absorption by the human infant. *J. Nutr.* **1969**, *99*, 293–298.

(12) Lien, E. L. The role of fatty acid composition and positional distribution in fat absorption in infants. *J. Pediatr.* **1994**, *125*, 562–568.

(13) Lien, E. L.; Boyle, F. G.; Yuhas, R.; Tomarelli, R. M.; Quinlan, P. The effect of triglyceride positional distribution on fatty acid absorption in rats. *J. Pediatr. Gastr. Nutr.* **1997**, *25*, 167–174.

(14) Lucas, A.; Quinlan, P.; Abrams, S.; Ryan, S.; Meah, S.; Lucas, P. J. Randomized controlled trial of a synthetic triglyceride milk formula for preterm infants. *Arch. Dis. Child.* **1997**, *77*, 178–184.

(15) Chappell, J. E.; Clandinin, M. T.; Kearny-Volpe, C.; Reichman, B.; Swyer, P. W. Fatty acid balance studies in premature infants fed human milk or formula: effect of calcium supplementation. *J. Pediatr.* **1986**, *108*, 439–447.

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

(17) Mukherjee, K. D.; Kiewitt, I. Structured triacylglycerols resembling human milk fat by transesterification catalyzed by papaya (*Carcia papaya*) latex. *Biotechnol. Lett.* **1998**, *20*, 613–616.

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

(19) Hube, B.; Stehr, F.; Bossenz, M.; Mazur, A.; Kretschmar, M.; Schafer, W. Secreted lipases of *Candida albicans*: cloning, characterization and expression analysis of a new gene family with at least ten members. *Arch. Microbiol.* **2000**, *174*, 362–374.

(20) Ferrer, P.; Montesinos, J. L.; Valero, F.; Sola, C. Production of native and recombinant lipases by *Candida rugosa*: a review. *Appl. Biochem. Biotechnol.* **2001**, *95*, 221–255.

(21) Grochulski, P.; Li, Y.; Schrag, J. D.; Bouthillier, F.; Smith, P.; Harrison, D.; Rubin, B.; Cygler, M. Insights into interfacial activation from an open structure of *Candida rugosa* lipase. *J. Biol. Chem.* **1993**, *268*, 12843–12847.

(22) Kaiser, R.; Erman, M.; Duax, W. L.; Ghosh, D.; Jornvall, H. Monomeric and dimeric forms of cholestrol esterase from *Candida cylindracea*. Primary structure identity in peptide patterns, and additional microheterogeneity. *FEBS Lett.* **1997**, *337*, 123–127.

(23) Diczfalusy, M. A.; Hellman, U.; Alexson, S. E. Isolation of carboxylester lipase (cel) isoenzymes from *Candida rugosa* and identification of corresponding genes. *Arch. Biochem. Biophys.* **1997**, *348*, 1–8.

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

(25) Jennings, B. H.; Akoh, C. C. Enzymatic modification of triacylglycerol of high eicosapentaenoic and docosahexaenoic acids content to produce structured lipids. *J. Am. Oil Chem. Soc.* **1999**, *76*, 1133–1137.

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

(27) Brocca, S.; Schmidt-Dannert, C.; Lotti, M.; Alberghina, L.; Schmid, R. D. Design, total synthesis, and functional overexpression of the *Candida rugosa* Lip1 gene coding for a major industrial lipase. *Protein Sci.* **1998**, *7*, 1415–1522.

(28) Virto, M. D.; Agud, I.; Montero, S.; Blanco, A. A.; Solozabal, R.; Lascary, J. M.; Llama, M. J.; Serra, J. L.; Landeta, L. C.; de Renbales, M. Hydrolysis of animal fats immobilized *Candida rugosa* lipase. *Enzyme Microb. Technol.* **1994**, *16*, 61–65.

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

(30) Mattson, F. H.; Volpenhein, R. A. Rearrangement of glyceride fatty acids during digestion and absorption. *J. Biol. Chem.* **1962**, *237*, 53−55.

(31) Xu, X. Production of specific-structured triacylglycerols by lipase-catalyzed reactions: a review. *Eur. J. Lipid Sci. Technol.* **2000**, *102*, 73−81.

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

(33) Innis, S. M.; Dyer, R.; Quinlan, P.; Diersen-Schade, D. Palmitic acid is absorbed as sn-2 monopalmitin from milk and formula with rearranged triacylglycerols and results in increased plasma triacylglycerides sn-2 and cholestryl ester palmitate in piglets. *J. Nutr.* **1995**, *125*, 73−81.

(34) López-López, A.; Castellote-Bargalló, A. I.; Company-Folgoso, C.; Rivero-Urget, M.; Tormo-Carnicé, R.; Infante-Pina, D.; López-Sabater, M. C. The influence of dietary palmitic acid triacylglycerol position on the fatty acid, calcium and magnesium contents of at term newborn faeces. *Early Hum. Dev.* **2001**, *65*, 83−94.