Effects of Selected Substrate Forms on the Synthesis of Structured Lipids by Two Immobilized Lipases

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ABSTRACT: Two immobilized lipases, IM 60 from *Rhizomucor miehei* and SP 435 from *Candida antarctica*, were used to synthesize structured lipids (SL). Tricaprin and trilinolein were interesterified to produce SL that contained one linoleic acid per triacylglycerol molecule (SL1) and SL with two linoleic acids (SL2). SL1 and SL2 were separated by silver nitrate thin-layer chromatography according to their unsaturation, and the fatty acid at the *sn*-2 position was determined after pancreatic lipase-catalyzed hydrolysis of SL1 and SL2. With IM 60, 57.7 mol% capric acid and 42.3 mol% linoleic acid were found at the *sn*-2 position of SL1, while 43.3 mol% capric acid and 56.7 mol% linoleic acid were at the *sn*-2 position of SL2. The fatty acid at the *sn*-2 position of SL1 with SP 435 as biocatalyst was 43.6 mol% capric acid and 56.4 mol% linoleic acid, while SL2 contained 56.6 mol% capric acid and 43.4 mol% linoleic acid. Different structural forms of the capric acid-containing substrate (triacylglycerol vs. ethyl ester) and different chainlengths of triacylglycerol were selected to study the substrate selectivity of lipases. Results indicated that SP 435 had some degree of preference for the triacylglycerol form (tricaprin), and IM 60 produced SL more rapidly and reached steady state faster with tricaprin as substrate than with capric acid ethyl ester. For chainlength selectivity, mol% of synthesized SL from tricaprin + trilinolein and tristearin + trilinolein were compared. SP 435 exhibited no apparent preference for either tricaprin or tristearin. However, IM 60 showed a more rapid reaction with tricaprin than with tristearin.

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KEY WORDS: Fatty acid chainlength, hydrolysis, immobilized lipase, interesterification, silver nitrate thin-layer chromatography, *sn*-2 position fatty acid, structured lipids.

Lipases (triacylglycerol acyl hydrolases E.C. 3.1.1.3) are enzymes that preferentially catalyze the hydrolysis and synthesis of esters and triacylglycerols (TAG). Some lipases exhibit substrate selectivity or preference. The interesterification rate of heptadecanoic acid with TAG of ucuhuba seed was about 40% higher than that of the methyl ester (methyl heptadecanoate) after 8 h of reaction with lipase from *Rhizomucor miehei* (1). Kuo and Parkin (2) reported the order of initial rate of acyl exchange of butteroil by pancreatic lipase as glycerol esters > fatty acid methyl esters > fatty acids. Lipase from *Penicillium camembertii* U-150 can hydrolyze mono- and diacylglycerols but not TAG (3). With lipase from *P. caseicolum*, the hydrolysis rate of TAG decreased as the chainlength of fatty acids increased as follows (4): tributyrin > tricaproin > tricaprylin > trilaurin > trimyristin > tripalmitin. Similar results were reported when monoacylglycerols with different chainlengths were hydrolyzed (5). Other lipases show a preference for unsaturated substrates with a double bond at the 9-position (6).

Immobilization of lipases provides some benefits for their industrial application. Immobilization can increase their stability against pH and heat (7). It also allows for easy recovery and reuse. In this study, two immobilized lipases, IM 60 (from *R. miehei*, immobilized on a macroporous anion exchange resin) and SP 435 (from *Candida antarctica*, immobilized on a macroporous acrylic resin), were selected. IM 60 is an *sn*-1,3-specific and SP 435 is a nonspecific lipase, and both can be used for effective synthesis of structured lipids (SL) through esterification (8).

Linoleic acid is an essential fatty acid; it is not synthesized by humans and must be obtained from the diet (9). Medium-chain TAG have been reported to possess several health benefits (10). Thus, SL that contain both linoleic acid and medium-chain fatty acids in the same glycerol molecule are of interest.

SL can be produced by interesterification with lipases in organic solvent, where substrates are soluble. Lipase-catalyzed modifications could provide improvement in the properties of lipids through incorporation of desirable fatty acids into TAG molecules. Thus, modified lipids or SL have prospects for future applications (11). In this study, SL synthesized from tricaprin and trilinolein were separated by silver nitrate–thin-layer chromatography ($AgNO_3$–TLC), and the fatty acid at the *sn*-2 position was determined after pancreatic lipase-catalyzed hydrolysis. Two types of substrates, TAG vs. ethyl ester forms, and TAG of different chainlengths were used to study the substrate selectivities of two immobilized lipases.

MATERIALS AND METHODS

Materials. Tricaprin (1,2,3-tridecanoylglycerol), trilinolein (1,2,3-tri-[(*cis,cis*)-9,12-octadecadienoyl]glycerol), tristearin

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(1,2,3-trioctadecanoylglycerol), capric acid ethyl ester, 1,3 distearoyl-2-oleoyl-glycerol, and porcine pancreatic lipase (Type II, crude) were purchased from Sigma Chemical Co. (St. Louis, MO). Immobilized enzymes SP 435 and IM 60 were provided by Novo Nordisk Biochem North America Inc. (Franklinton, NC). Organic solvents were obtained from Fisher Scientific (Norcross, GA). $AgNO_3$–TLC plates (20% $AgNO_3$/silica gel) were purchased from Alltech Associates, Inc. (Deerfield, IL).

Enzymatic interesterification reactions. To compare the selectivity of lipases for tricaprin and capric acid ethyl ester as substrates to synthesize SL from trilinolein, immobilized enzymes (SP 435 and IM 60, 40% of the total weight of reactants) were added to a 1:1 mole ratio of trilinolein (100 mg) to tricaprin (63 mg) or a 1:3 mole ratio of trilinolein (100 mg) to capric acid ethyl ester (68 mg) in 3 mL hexane in a screw-cap tube, respectively. The reactions were incubated at 55°C in an orbital shaking water bath for 12 h at 200 rpm. Samples (17 µL) were withdrawn from the reaction mixture, mixed with 10 µL of internal standard solution (triolein, 10 mg/mL in hexane), and diluted to 1 mL with an acetone/acetonitrile (A/ACN) (50:50, vol/vol) mixture for high-performance liquid chromatography (HPLC) analysis.

To compare the selectivity for chainlength between tricaprin and tristearin in the synthesis of SL from trilinolein, a 1:1 mole ratio of trilinolein (100 mg) to tristearin (120 mg) or tricaprin (63 mg) was mixed with 40% (total weight of reactants) immobilized enzymes in 3 mL hexane and incubated at 55°C for 12 h at 200 rpm. Samples were collected and prepared for HPLC as described above, except that a mixture of A/chloroform (15:85, vol/vol) was used to dilute and to solubilize tristearin.

HPLC analysis. TAG molecular species were analyzed by HPLC with a Hewlett-Packard 1090 Win liquid chromatographic system (Hewlett-Packard, Avondale, PA), fitted with a reversed phase Ultrasphere® ODS 5 µm spherical 80 Å pore (4.6 mm × 250 mm) column (Beckman Instruments, Inc., Fullerton, CA). The reactants and products were quantitated on-line with an evaporative light-scattering detector (ELSD) (Sedex 45; Richard Scientific, Novato, CA). The ELSD was set to 40°C at a nebulizer gas (N_2) pressure of 2.1 atm and a gain of 10. The injection volume was 20 µL, and the column oven temperature was 40°C. A mobile-phase gradient of ACN and A was used as described in Table 1.

$AgNO_3$–TLC. $AgNO_3$–TLC plates were used to separate TAG according to their unsaturation. A 1:1 mole ratio of tricaprin and trilinolein was interesterified with immobilized enzymes (IM 60 or SP 435, 40% of total weight of reactants) under the conditions described above. After removing the enzyme by passage through an anhydrous sodium sulfate column, the mixture was analyzed by $AgNO_3$–TLC. Each mixture was spotted several times to obtain enough TAG for further analysis. The mobile phase was a mixture of chloroform/benzene (90:10, vol/vol). The bands were visualized, after spraying with 0.2% 2,7-dichlorofluorescein in methanol, under ultraviolet (UV) light. The bands corresponding to TAG, SL1 (containing one linoleic acid) and SL2 (containing two linoleic acids), were scraped and pooled for further analysis. These TAG were extracted twice with diethyl ether and centrifuged (1000 rpm × 1 min).

TABLE 1
The High-Performance Liquid Chromatography Mobile Phase Gradient

Time (min)	ACN[a] (%)	A[a] (%)	Flow rate (mL/min)
0	50	50	1.8
12.5	5	95	2
13.0	50	50	1.8
18.0	50	50	1.8

[a]ACN, acetonitrile; A, acetone.

Hydrolysis by pancreatic lipase. The hydrolysis condition was slightly modified from the method described by Yoshida and Alexander (12). After evaporating all solvents, 1 mL of 1 M Tris-HCl buffer (pH 7.6), 0.25 mL of bile salt solution, 0.1 mL of 2.2% $CaCl_2$ solution, and 8 mg of pancreatic lipase were mixed and incubated at 37°C for 2 min, followed by vigorous vortex (2 min), centrifugation (1900 rpm, 3 min), extraction with 3 mL of diethyl ether (two times) and elution through an anhydrous sodium sulfate column. Prolonged incubation time may cause acyl migration within the TAG molecule. Extraction of *sn*-2 monoacylglycerol and methylation for gas chromatography (GC) analysis was described in our previous paper (8). Pancreatic lipase hydrolysis method was validated by incubating a solution of 1,3-distearoyl-2-oleoyl-glycerol standard in hexane under the same condition and analyzing the fatty acid at the *sn*-2 position.

GC analysis. For fatty acid composition, a Hewlett-Packard 5890 Series II gas chromatograph equipped with a flame-ionization detector (Hewlett-Packard) was used. A fused-silica capillary column (DB-255, 30 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA) was used. The temperature was programmed (initial temperature: 120°C hold for 3 min, final temperature: 215°C hold for 10 min, rate: 10°C/min). The injector and detector temperatures were 250 and 260°C, respectively.

RESULTS AND DISCUSSION

Figure 1 shows the HPLC reverse-phase separation of the TAG molecular species according to polarity and total carbon number. The identity of each molecular species was established based on our previous report (13). Each peak was collected at predetermined retention time and analyzed by GC after methylation.

Fatty acid composition at the sn*-2 position.* $AgNO_3$–TLC indicated that the more unsaturated TAG (trilinolein) showed the least migration. SL1, with one linoleic acid and two capric acids, has less unsaturation and migrated higher on the $AgNO_3$ plate than SL2, which contains two linoleic acids. The R_f (×100) values were 51.5 for tricaprin, 29.1 for SL with one linoleic acid (SL1), 12.1 for SL with two linoleic acids (SL2), and 3 for trilinolein.

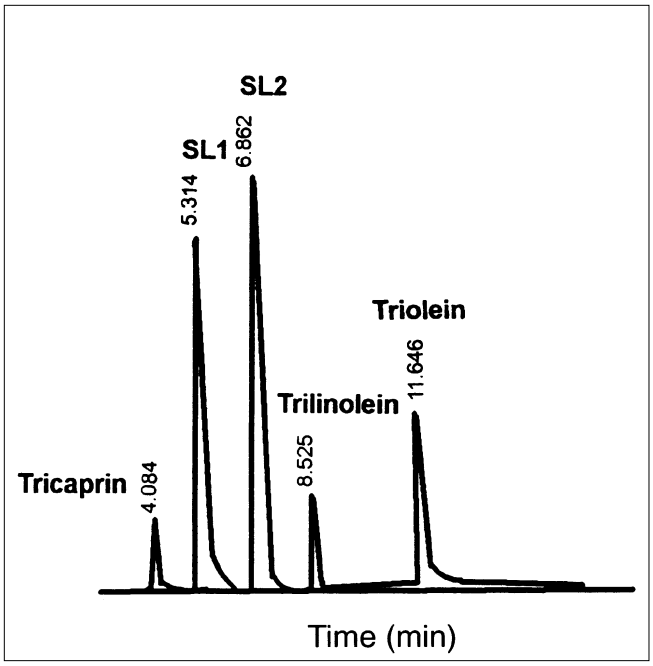
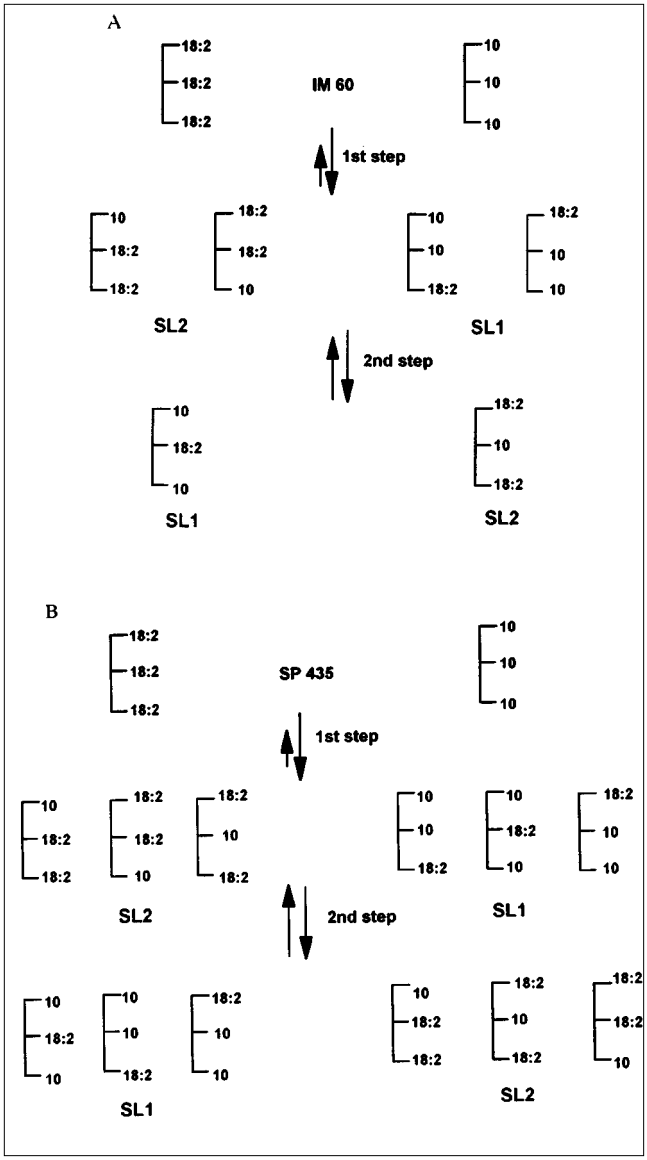


FIG. 1. High-performance liquid chromatography chromatogram showing the molecular species of reactants and structured lipid (SL) products. After 24 h incubation with SP 435, the reaction mixture was analyzed with a reversed-phase column. Conditions are described in the Materials and Methods section. Internal standard was triolein. Reaction with tricaprin and trilinolein as substrates and SP 435 as biocatalyst, where peak #1 = unreacted tricaprin; #2 = SL1; #3 = SL2; #4 = unreacted trilinolein; #5 = internal standard.



SCHEME 1

It has been reported that the fatty acid at the *sn*-2 position is easily absorbed (14). Thus, the study of the fatty acid profile at the *sn*-2 position is useful for further metabolic studies. IM 60 is an *sn*-1,3-selective and SP 435 is a nonspecific lipase. Using TAG substrate forms and IM 60 and SP 435, we can expect SL with linoleic acid at the *sn*-2 position from both the first and second steps [Scheme 1: interesterification between trilinolein and tricaprin (A) with IM 60; (B) with SP 435]. After GC analysis, the fatty acid composition at the *sn*-2 position was determined (Table 2). With IM 60, 57.7 mol% capric acid and 42.3 mol% linoleic acid were obtained at the *sn*-2 position for SL1 and 43.3 mol% capric acid and 56.7 mol% linoleic acid were obtained for SL2. On the other hand, the fatty acids at the *sn*-2 position with SP 435 were 43.6 mol% capric acid and 56.4 mol% linoleic acid for SL1 and 56.6 mol% capric acid and 43.4 mol% linoleic acid for SL2, respectively (Table 2). Confirmation of the hydrolysis method was achieved with a known molecule, 1,3-distearoyl-2-oleoyl-glycerol which gave only oleic acid at the *sn*-2 position. Possible products from a reaction between capric acid ethyl ester and trilinolein are illustrated in Scheme 2 (A: with IM 60; B: with SP 435).

Selectivity between tricaprin and capric acid ethyl ester forms. The results of time course studies of interesterification between trilinolein and tricaprin or capric acid ethyl ester are illustrated in Figure 2. IM 60 showed a more rapid reaction than SP 435. Most of the interesterification occurred within the first 2 h with IM 60 as the biocatalyst (Fig. 2A). With SP 435, the interesterification was slow and continued throughout the 12-h reaction (Fig. 2B). The amount of SL1 produced with capric acid ethyl ester was less than with tricaprin up to at least 12 h with IM 60. After 24 h, the amount of SL1 was 32.1 mol% with capric acid ethyl ester and 31.6 mol% with tricaprin (data not shown). Capric acid ethyl ester as substrate produced more SL2, compared to tricaprin, up to 12 h with IM 60; but after 24 h, less SL2 was produced with capric acid ethyl ester (50.9 mol% with capric acid ethyl ester and 58.9 mol% with tricaprin, data not shown).

Interesterification is initiated by hydrolysis of an ester bond and the formation of an acyl–enzyme intermediate, followed by exchange of the acyl moiety and new ester bond formation (15). If capric acid ethyl esters are used as acyl donor for trilinolein to produce SL, SL2 is first produced and then used for the synthesis of SL1 (Scheme 2). When tricaprin is

TABLE 2
Fatty Acid Positional Analysis of Structured Lipids Synthesized by Interesterification of Tricaprin and Trilinolein Catalyzed by Immobilized Lipases[a]

	Fatty acid at the *sn*-2 position (mol%)			
	SL1[b]		SL2[b]	
Enzyme	Capric acid	Linoleic acid	Capric acid	Linoleic acid
IM 60	57.7	42.3	43.3	56.7
SP435	43.6	56.4	56.6	43.4

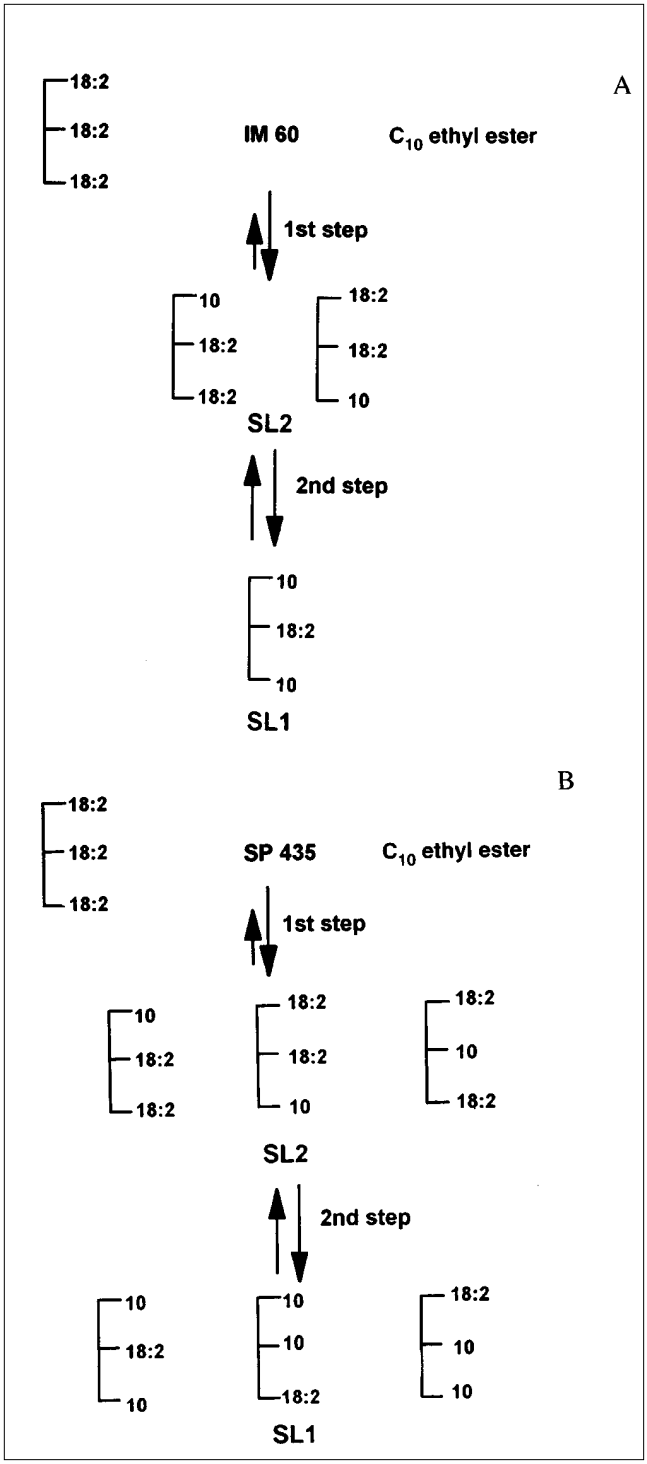
[a]Conditions for reaction are in the Materials and Methods section.
[b]SL1: Structured lipid containing one linoleic acid and two capric acid molecules; SL2: structured lipid containing two linoleic acid and one capric acid molecules.

used as substrate, both SL1 and SL2 can be produced in the first step, then used as substrates for further conversions before reaching equilibrium (Scheme 1). Overall production of SL (total mol% of synthesized SL1 and SL2) with IM 60 was greater with tricaprin than with capric acid ethyl ester during 12 h of incubation. Tricaprin was a better interesterification substrate with trilinolein than capric acid ethyl ester for the production of SL1 and SL2 with SP 435 (Fig. 2b). We were able to produce more SL with tricaprin with both enzymes (IM 60 and SP 435). Additionally, from Table 3, we can assume that tricaprin was more rapidly used for synthesis of SL1 and SL2 than trilinolein with IM 60 and SP 435. Because most reaction occurred during the first 2 h with IM 60, the values did not change much after 2 h (steady state). But during the first 2 h, IM 60 showed preference toward tricaprin, resulting in more rapid consumption than trilinolein. SP 435 showed an apparent preference toward tricaprin, compared with trilinolein, during the reaction.

Purified TAG are more expensive than ethyl esters. Thus, from an economic standpoint, the manufacturer has to decide which type of substrate (tricaprin or capric acid ethyl ester) to use for producing SL that contain capric acids.

Selectivity with regard to substrate chainlength. Figures 3A and 3B show the selectivity of IM 60 and SP 435 with regard to substrate chainlength. IM 60 showed a more rapid reaction with tricaprin than with tristearin, because structured lipids (SL1 and SL2) were more rapidly synthesized and reached steady state in 4 h (Fig. 3A). After 2 h, the amount of SL1 and SL2 synthesized with tricaprin were 1.5 and 4.6 times, respectively, compared with tristearin. Most reaction with tricaprin occurred within 2 h. But with tristearin, the reaction was slower, reaching equilibrium in 6 h. Total mol% of synthesized SL were also greater with tricaprin than with tristearin before 4 h, indicating that short-chain fatty acids were rapidly reacted to produce SL. After 12 h of reaction, the amount of SL1 and SL2 with tricaprin was 38.8 and 52.8 mol%, respectively. With tristearin, 44.4 and 46.1 mol% of SL1 and SL2 were obtained, respectively. Tristearin did not fully dissolve in hexane at room temperature, but at the reaction temperature, 55°C, it was fully dissolved.

SP 435 showed somewhat different results (Fig. 3B). This enzyme did not show a preference for either substrate (tricaprin and trilinolein) at the early stages. After 12 h, the mol% of SL1 and SL2 from tristearin were approximately 41.3 and 38.9 %, respectively, and 45.8 % for SL2 and 32.1% for SL1 with tricaprin. In addition, tristearin was more rapidly used than trilinolein within the first 2 h, even though trilinolein was used more for synthesis of SL after that period (Table 4). This is surprising to us because some lipases tend



SCHEME 2

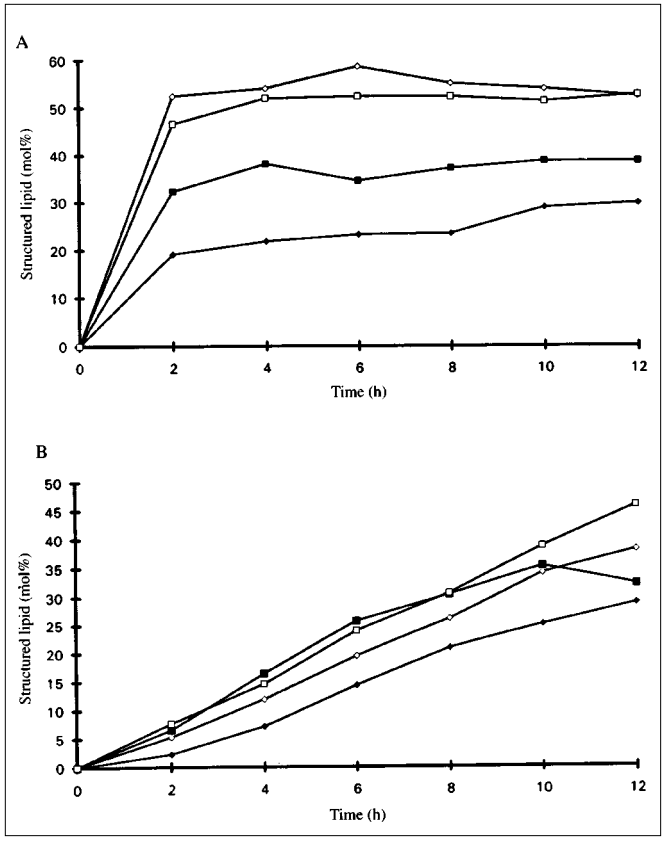


FIG. 2. Mol% of synthesized SL1 and SL2 after the interesterification of tricaprin or capric acid ethyl ester with trilinolein. A: IM 60; and B: SP 435. SL1 with capric acid ethyl ester (–◆–) SL: with one linoleic acid in the reaction between trilinolein and capric acid ethyl ester; SL2 with capric acid ethyl ester (–◇–) SL: with two linoleic acids in the reaction between trilinolein and capric acid ethyl ester; SL1 with tricaprin (–■–) SL: with one linoleic acid in the reaction between tricaprin and trilinolein; SL2 with tricaprin (–□–) SL: with two linoleic acids in the reaction between tricaprin and trilinolein. See Figure 1 for abbreviation.

to prefer unsaturated substrates (9). At this point, we cannot fully explain this unexpected result. Overall, IM 60 showed more rapid interesterification than SP 435 (Fig. 3). From the manufacturer's manual, IM 60 has an interesterification activity of 5–6 BAUN/g (Batch Acidolysis Units Novo) and SP 435 has 7000 PLU/g (Propyl Laurate Units); however, it is not known how these activities compare to one another.

Wang (5) suggested that the increase in chainlength of a substrate could decrease substrate accessibility to the active site of a lipase. It was stated that the lipolysis rate of bile salt-activated lipases decreased with increased acyl chainlength in the order of $C_8 > C_{10} > C_{12} > C_{14} > C_{16}$ (5). It was reported that lipases from *R. miehei* showed a bell-shaped distribution for acyl chainlength in ester synthesis reactions with a maximum around C_4–C_6 (16). Substrates with too short acyl chainlengths and too few double bonds cannot release enough energy, which may be used for conformational changes of the lipase to form an efficient structure, so that substrates can be properly oriented to the active site of the lipase (5). From this study, it seems that SP 435 has a preference to some degree

TABLE 3
Mol% of Unreacted Substrates During Interesterification Reaction Between Tricaprin and Trilinolein with IM 60 and SP 435 Lipases[a]

Enzymes	Substrate	2 h	4 h	6 h	8 h	10 h	12 h
IM 60	Tricaprin	11.2	7.8	7.4	7.7	8.3	8.1
	Trilinolein	20.3	9.0	12.1	9.6	9.0	7.6
SP 435	Tricaprin	29.6	23.4	20.0	14.3	12.1	11.1
	Trilinolein	60.2	52.4	40.2	33.1	22.7	19.5

[a]After the reaction, mol% of unreacted substrates were obtained along with mol% of synthesized structured lipids. Conditions for reaction are in the Materials and Methods section.

for the TAG form. IM 60 reacted better with tricaprin than with capric acid ethyl ester for the overall synthesis of SL. Thus, more SL were synthesized with the TAG substrate form than with the ethyl ester form of capric acid. SP 435 exhibited no apparent preference for either tricaprin or tristearin because total mol% of synthesized SL1 and SL2 were similar. But with IM 60 and tricaprin as substrate, SL1 and SL2 were produced more rapidly and reached steady state faster than with tristearin as substrate. Additionally, tricaprin was

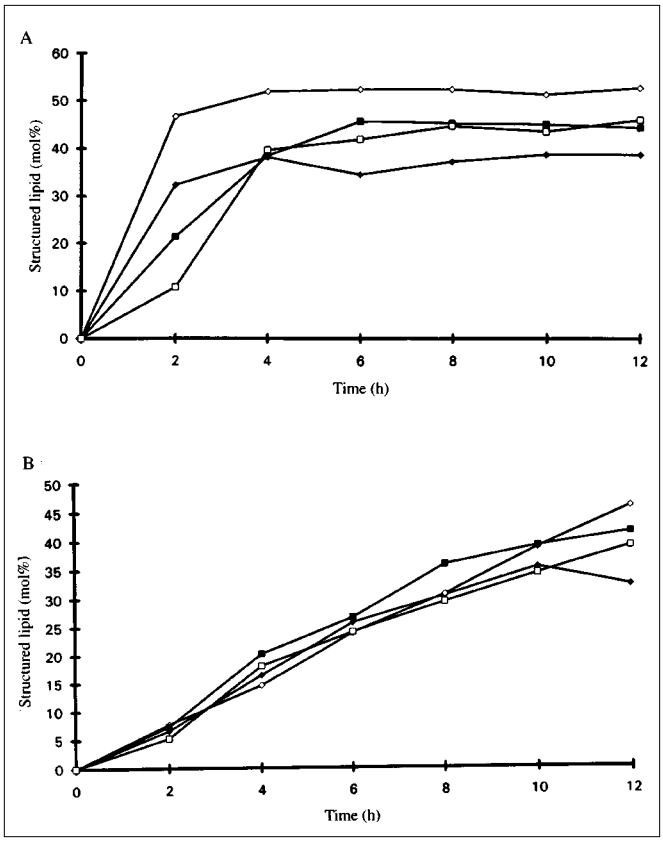


FIG. 3. Mol% of synthesized SL1 and SL2 after the interesterification of tricaprin or tristearin with trilinolein. A: IM 60; and B: SP 435. SL1 with tricaprin (–◆–): SL with one linoleic acid in the reaction between trilinolein and tricaprin; SL2 with tricaprin (–◇–): with two linoleic acids in the reaction between trilinolein and tricaprin; SL1 with tristearin (–■–): SL one linoleic acid in the reaction between tristearin and trilinolein; SL2 with tristearin (–□–) SL: with two linoleic acids in the reaction between tristearin and trilinolein. See Figure 1 for abbreviation.

TABLE 4
Mol% of Unreacted Substrates During Interesterification Reaction Between Tristearin and Trilinolein with IM 60 and SP 435 Lipases[a]

Enzymes	Substrate	2 h	4 h	6 h	8 h	10 h	12 h
IM 60	Tristearin	27.5	20.5	18.7	19.4	13.0	12.5
	Trilinolein	49.9	17.3	10.0	8.1	8.5	8.2
SP435	Tristearin	36.7	42.0	37.2	28.0	21.3	19.1
	Trilinolein	55.3	35.8	31.0	25.1	21.2	16.0

[a]After the reaction, mol% of unreacted substrates were obtained along with mol% of synthesized structured lipids. Conditions for reaction are in the Materials and Methods section.

more rapidly consumed than trilinolein with both enzymes (IM 60 and SP 435).

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