Structured Lipids: Lipase-Catalyzed Interesterification of Tricaproin and Trilinolein

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ABSTRACT: Structured lipids were synthesized by interesterification of trilinolein and tricaproin with *sn*-1,3-specific (IM 60) and nonspecific (SP 435) lipases. The interesterification reaction was performed by incubating a 1:2 mole ratio of trilinolein and tricaproin in 3 mL hexane at 45°C for the IM 60 lipase from *Rhizomucor miehei*, and at 55°C for the SP 435 lipase from *Candida antarctica*. Reaction products were analyzed by reverse-phase high-performance liquid chromatography with an evaporative light-scattering detector. The fatty acids at the *sn*-2 position were identified after pancreatic lipase hydrolysis and analysis with a gas chromatograph. IM 60 lipase produced 53.5 mol% dicaproyllinolein (total carbon number = C33) and 22.2% monocaproyldilinolein (C45). SP 435 lipase produced 41% C33 and 18% C45. When caproic acid was used in place of tricaproin as the acyl donor, the IM 60 lipase produced 62.9% C33. The effects of variation in mole ratio, temperature, added water, solvent polarity, and time course on the interesterification reaction were also investigated. In the absence of organic solvent, IM 60 lipase produced 52.3% C33.
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KEY WORDS: *Candida antarctica*, interesterification, lipase, organic solvent, *Rhizomucor miehei*, structured lipid.

Triacylglycerol (TAG) lipases are enzymes that hydrolyze TAG to diacylglycerols (DAG), monoacylglycerols (MAG), fatty acids, and glycerol (1). Annual sales of lipases currently account for about 20 million dollars, which corresponds to less than 4% of the worldwide enzyme market that has recently been estimated at 600 million U.S. dollars (2). Lipases can be used in several ways to modify TAG. In aqueous media, hydrolysis is the predominant reaction, but in organic media, transesterification reactions are predominant (3). Different lipases show preferences for both the position of the fatty acid group on the TAG and the nature of the fatty acid (4). Based on specificity of lipases, two main groups of lipases can be identified: random lipases, which catalyze reactions at all three positions on the glycerol molecule (e.g., lipases from *Candida antarctica* and *C. rugosa*) and *sn*-1,3-specific lipases, which act preferentially at the *sn*-1 and *sn*-3 positions of the glycerol molecule (e.g., lipases from *Rhizomucor miehei* and *Aspergillus niger*). Some lipases show specificity toward specific types of fatty acids, e.g., *Geotrichum candidum,* which shows a unique but not absolute specificity for fatty acids that contain *cis*-9 unsaturation, regardless of the position within the TAG molecule (5). The positional specificity of lipases is usually retained when they are placed in organic solvents. Lipases also exhibit novel characteristics in organic solvent, such as altered chemo- and stereoselectivity, enhanced stability, and increased rigidity (3). Chemical catalysts as well as nonspecific lipases randomize fatty acids in TAG mixtures and may prevent the formation of specialty products with desired physicochemical characteristics (6).

The *sn*-1,3-specific lipases can be distinguished from nonspecific lipases based on the nature of the fatty acid at the *sn*-2 position of the TAG (7). Transesterification with *sn*-1,3-specific lipases results in the *sn*-2 fatty acids remaining almost intact in the TAG product. This is significant from a nutritional point of view because the 2-MAG produced by pancreatic lipase digestion are the main carriers of fatty acids through the intestinal wall (8). Fatty acids esterified at the *sn*-2 position are therefore more efficiently absorbed than those at the *sn*-1 and *sn*-3 positions. Structured TAG with an essential fatty acid (EFA) at the *sn-2* position and short- or medium-chain fatty acids at the *sn*-1 and *sn*-3 positions have the advantage of efficiently providing an EFA and at the same time serving as a rapid energy source (9).

In this paper, we report on the interesterification of trilinolein and tricaproin to produce structured lipids (SL) that contain linoleic acid and caproic acid. The effects of different lipases, time courses, temperatures, solvent polarities, mole ratios, and added water were studied, and the fatty acid at the *sn*-2 position, after pancreatic hydrolysis, was also determined.

MATERIALS AND METHODS

Materials. Tricaproin, trilinolein, porcine pancreatic lipase, TAG standards, and 1,3-distearoyl-2-oleoyl-glycerol were obtained from Sigma Chemical Company (St. Louis, MO). Immobilized lipases IM 20 and IM 60 from *R. miehei* and SP435

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from *C. antarctica* were obtained from Novo Nordisk Biochem North America Inc. (Franklinton, NC). Nonspecific lipase AK from *Pseudomonas* sp., *sn*-1,3-specific lipase L from *C. lipolytica*, lipase N from *Rhizopus niveus*, lipase PS from *Pseudomonas* sp., and PGE (pregastric esterase) from cow tongue root and salivary glands were kindly provided by Amano International Enzyme Co. (Troy, VA). The 20% silver nitrate plates were purchased from Alltech Associate Inc. (Deerfield, IL). All solvents were of high-performance liquid chromatography (HPLC) grade and were obtained from Fisher Scientific (Norcross, GA).

Interesterification reaction. SL synthesis was performed in screw-capped test tubes. Eight lipases were screened for their ability to catalyze the interesterification reaction. For enzyme screening, a 1:1 mole ratio of trilinolein (50 mg) to tricaproin (22 mg) was incubated at 55°C for 24 h with 7.4 mg of enzyme. Unless otherwise specified, all other interesterification reactions typically contained a mixture of 50 mg trilinolein, 44 mg tricaproin (i.e., 1:2 mole ratio), and 9.4 mg of lipase (i.e., 10% w/w of total substrate) in 3 mL hexane that was previously dried over molecular sieves of 4 Å. The reaction mixture was incubated in an orbital shaking waterbath at 200 rpm, 45 and 55°C, for IM 60- and SP 435-catalyzed reactions, respectively. All reactions were performed in duplicate.

Determination of interesterified products. Reaction products were cooled and filtered through a sodium sulfate column to remove moisture and enzyme particles. Products were analyzed with a Hewlett-Packard 1090 HPLC (Palo Alto, CA) equipped with a Sedex 45 evaporative light-scattering detector (ELSD, Richard Scientific, Novato, CA). The ELSD was set at 40°C, a nebulizer nitrogen gas pressure of 2.1 atm, and a gain of 5 for the nonaqueous reverse-phase system. A Hewlett-Packard 35900 digital A/D analog interface connected the ELSD electronically to the on-line computer. TAG species were separated by nonaqueous reverse-phase HPLC with a Beckman/Altex (San Ramon, CA) Ultrasphere ODS 5 μm (4.6 mm × 25 cm) column. The mobile phase consisted of acetonitrile (A) and acetone (B) with a gradient profile as follows: initial conditions (A/B) 50:50 at a flow rate of 1.8 mL/min held for 4 min, then 5:95 (A/B) at a flow rate of 2.0 mL/min, held for 8.5 min, and then returned to the original conditions. Trilaurin as the internal standard was added to the reaction products prior to HPLC analysis. Product identification was based on polarity, total carbon number (TCN), and use of TAG standards.

Positional analysis of TAG. SL were separated by spotting samples along with standards on 20% silver nitrate thin-layer chromatography (TLC) plates (Alltech Associates, Inc.) and developed in chloroform/benzene (85:15, vol/vol). SL bands were visualized under ultraviolet (UV) radiation after spraying with 0.2% 2,7-dichlorofluorescein in methanol. Bands corresponding to SL were scraped and eluted with diethyl ether. The composition of each band was determined by HPLC. Positional analysis of TAG was accomplished by the pancreatic hydrolysis method of Luddy *et al.* (10). After hydrolysis, the mixture was extracted with ethyl ether, filtered, and dried over anhydrous sodium sulfate. The individual products were isolated by TLC on silica gel G plates, developed with hexane/ethyl ether/acetic acid (50:50:1, vol/vol). The *sn*-2 MAG was visualized under UV after spraying with 0.2% 2,7-dichlorofluorescein in methanol. The MAG band was propylated with 6% HCl in propanol at 75°C for 2 h. The fatty acid propyl esters (FAPE) were extracted with hexane and 0.1 M KCl solution. To establish the accuracy of the pancreatic hydrolysis method for *sn*-2 positional analysis, a TAG standard with known structure, 1,3-distearoyl-2-oleoyl-glycerol was similarly analyzed except that the standard was dissolved in 0.5 mL hexane prior to hydrolysis. This was done because the standard was not soluble in the assay buffer. The fatty acid composition of the MAG band was determined in a Hewlett-Packard 5890 gas chromatograph, equipped with a flame-ionization detector (FID) and operated in a splitless mode. Helium was the carrier gas, and the total gas flow rate was 24 mL/min. The oven temperature was 70°C initially and was held for 4 min, then programmed to 210°C at 10°C/min and held isothermally for 10 min. Heptadecanoic acid was the internal standard. The FAPE were analyzed and integrated by an on-line computer.

RESULTS AND DISCUSSION

Lipase screening. Eight commercial lipases were screened for their ability to catalyze the interesterification of trilinolein and tricaproin. The enzymes screened were immobilized lipozyme IM 60, IM 20, and SP 435 (Novo Nordisk Biochem North America Inc.), unimmobilized lipase AK, PS, L, PGE, and LN (Amano Enzyme Co.). For simplicity, the same amount of enzyme was used irrespective of their specific activities. Figure 1 shows that immobilized lipases IM 60, IM 20, SP 435, and

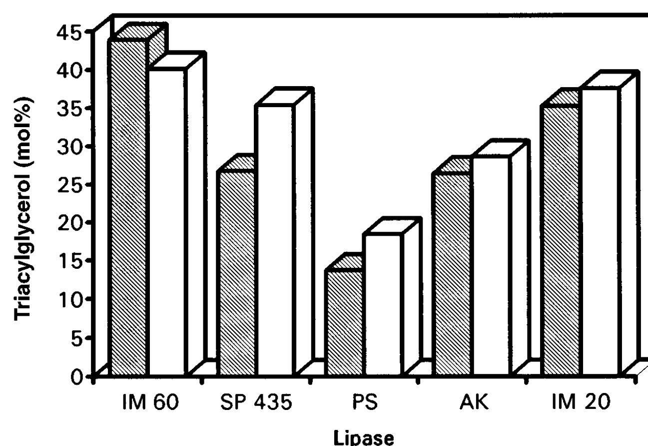

FIG. 1. Lipase screening for the interesterification reaction between trilinolein and tricaproin with 7.4 mg lipase as biocatalyst. Substrates consisted of 50 mg trilinolein and 22 mg tricaproin. The reaction mixture was incubated in 3 mL hexane at 55°C, open bar: C33 = dicaproyllinolein; hatched bar: C45 = monocaproyldilinolein. Number after C indicates total carbon number of the triacylglycerols. See the Materials and Methods section for abbreviations and lipase sources.

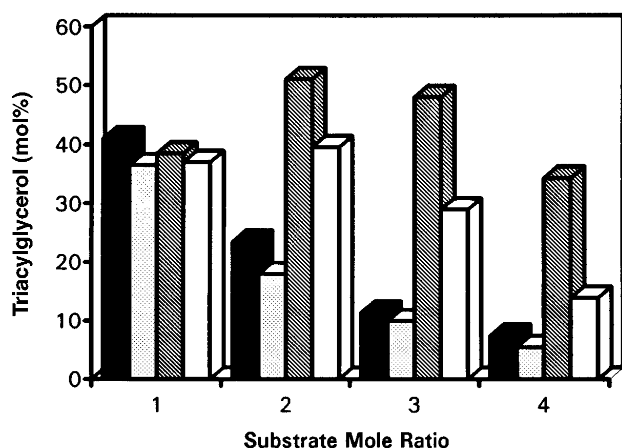

FIG. 2. Effect of mole ratio of substrates on interesterification of trilinolein and tricaproin with 7.4 mg IM 60 or SP 435 lipase as biocatalyst. The mole ratio of trilinolein and tricaproin was varied from 1:1 to 1:4. The reaction mixture was incubated in 3 mL hexane at 55°C. IM = IM 60, SP = SP 435, C33 = dicaproyllinolein, C45 = monocaproyldilinolein. Number after C indicates total carbon number of the triacylglycerols. C45 IM, solid bar; C45 SP, cross-hatched bar; C33 IM, hatched bar; C33 SP, open bar.

unimmobilized lipases PS and AK are capable of synthesis of SL by interesterification. No SL were formed with unimmobilized lipases L, PGE, and LN. We selected IM 60 and SP 435 lipases for the rest of the study because they gave high conversions of substrates to desired products (C33 and C45). Both lipases were chosen to allow a comparison of the interesterification activity of a 1,3-specific lipase and a nonspecific lipase in our model reaction. IM 20 was not studied further because it is, for all intents and purposes, a lower-activity version of IM 60. Recent reports (11,12) indicated comparable yields (73–79%) of SL from tricaprylin and peanut oil when catalyzed by a lipase from *R. miehei.*

Substrate mole ratio. Mole ratio study was performed with SP 435, which contains the lipase from *C. antarctica,* and IM 60, which contains the lipase from *R. miehei*. The mole ratio of trilinolein to tricaproin was varied from 1:1 to 1:4. Trilinolein amount (50 mg) was kept constant while the amount of tricaproin was varied (22–88 mg) to achieve the desired mole ratio. Enzyme amount was kept constant at 7.4 mg, rather than at 10% w/w substrates, so that any effect on yield will be attributed to mole ratio. Figure 2 shows that, with IM 60 lipase, a mole ratio of 1:2 gave optimal incorporation of caproic acid in the products, yielding 50.7% dicaproyllinolein (C33) and 23.6% monocaproyldilinolein (C45). The amounts of unreacted tricaproin (C21) and trilinolein (C57) in the final product were 2.3 and 23.5%, respectively (Table 1). Less than 1% MAG and DAG were formed at this level of assay. All yield calculations were based on the amount of SL formed and the unreacted trilinolein and tricaproin. From the amounts of unreacted trilinolein and tricaproin in the final product, it can be deduced that hydrolysis of tricaproin was more predominant than that of trilinolein at a 1:2 mole ratio (trilinolein/tricaproin). With SP 435 as biocatalyst, a similar TAG profile was observed at a substrate mole ratio of 1:2, to produce optimal incorporation of caproic acid into the SL. However, the product yields with SP 435 were lower than with IM 60, with yields of 41 and 18% for C33 and C45, respectively. The mol% of unreacted trilinolein and tricaproin were 8 and 33%, respectively (data not shown), with SP 435 as the biocatalyst. Previous reports showed an inhibitory effect of short-chain fatty acids on the reactivity of some lipases (13). We found no significant inhibition of both IM 60 and SP 435 lipases by tricaproin, although a small decrease in C33 yield was observed in the SP 435 system.

Temperature effect. This reaction parameter was studied to determine the optimal reaction temperature for IM 60 and SP 435 lipases. Optimal reaction temperature of enzymes can depend on the type of immobilization, chemical modification of enzymes, and pH of reaction mixture (13). The temperature range was varied from 25 to 65°C (Fig. 3), and the substrate mole ratio was kept at 1:2 trilinolein/tricaproin. With IM 60 lipase, the product yield varied slightly over the temperature range studied and showed an optimal yield of C33 (53.5%) and of C45 (22.2%) at 45°C. The variation with SP 435 was not as subtle; at 25°C only 5% C33 and 3% C45 were formed. The product yields increased with an increase in temperature, with a maximal yield at 55°C of 41% C33 and 18% C45. These results show that lipase SP 435 is more active at higher temperatures than at lower temperatures. IM 60 is active at both low and high temperatures. Our current observations are consistent with a previous study on the interesterification of caprylic acid ethyl ester and triolein (14).

TABLE 1
Effect of Added Water on the Lipase-Catalyzed Interesterification of Trilinolein and Tricaproin with IM 60 as Biocatalyst

Added water	Mol%			
(% w/w enzyme)	C57	C45	C33	C21
0	6.3 ± 0.5	44.7 ± 1.1	44.3 ± 0.01	3.7 ± 2.7
15	7.5 ± 1.5	44.7 ± 2.1	43.9 ± 0.5	3.9 ± 0.1
30	7.1 ± 1.4	42.7 ± 2.2	45.9 ± 0.5	4.3 ± 0.3
60	6.3 ± 0.9	44.0 ± 4.2	45.7 ± 5.2	4.0 ± 0
90	9.7 ± 2.8	41.4 ± 2.9	43.9 ± 1.3	5.0 ± 1.4

[a]Amount of water varied from 0–90% w/w of enzyme. The reaction mixture was incubated at 45°C for 24 h. The mole ratio of trilinolein to tricaproin was 1:2. C57 = unreacted trilinolein, C33 = dicaproyllinolein, C45 = monocaproyldilinolein, and C21 = tricaproin. Number after C indicates total carbon number of the triacylglycerols.

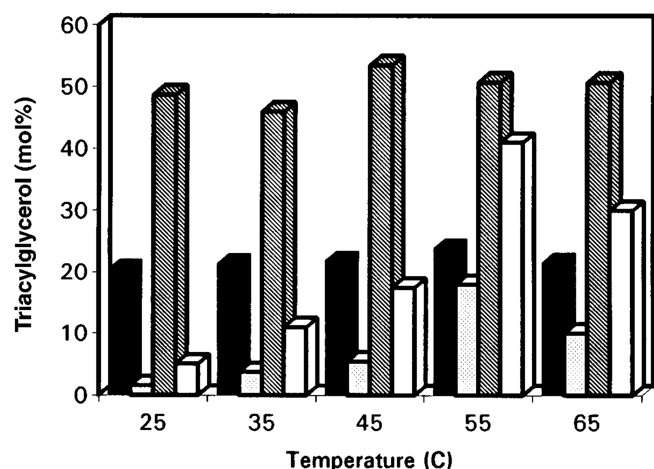

FIG. 3. Effect of temperature on the lipase-catalyzed interesterification of trilinolein and tricaproin (1:2 mole ratio) with IM 60 or SP 435 lipase. The reaction mixture was incubated at 25–65°C in an orbital shaking water bath for 24 h. See Figure 2 for abbreviations and bar symbols.

Added water. It is generally accepted that water is essential for enzymatic catalysis. This could be attributed to the role water plays in all noncovalent reactions. Water is responsible for maintaining the active conformation of proteins, for facilitating reagent diffusion, and also for maintaining enzyme dynamics (15). Studies by Hirata *et al.* (16) led to the conclusion that there is an optimal water content where enzymes show their maximal activity, and this value is affected by hydration of the enzyme and the substrate, coagulation of the enzyme, the nature of the solvent, and the water equilibrium between the enzyme and the substrate solution. Generally, a low-water content favors synthesis over hydrolysis. Table 1 shows the results obtained with IM 60 lipase only. Without water, a higher percentage of C33 was formed (50.7%), this amount dropped to 44.3% with the addition of 10% water. A corresponding increase in C45 from 23.6 to 44.7% was also observed. Added water beyond what was essential for synthesis led to the hydrolysis of the formed product of *C. antarctica* lipase-catalyzed reactions (14). Adding water at 15 wt% of enzyme to SP 435 lipase resulted in a reduction of SL formation from 18% C45 and 41% C33 to 1.5% C45 and 6.2% C33 (data not shown). At water content of 30 wt% enzyme, there was no formation of C33 and C45 by SP 435 lipase. It can be assumed that addition of water to the reaction catalyzed by SP 435 exceeded the critical amount of water necessary for synthesis. We can safely say that the absolute amount of water required for catalysis varies from enzyme preparation to enzyme preparation.

Effect of solvent polarity. Interaction between organic solvents and enzyme-bound water controls the activity of enzymes. The partition coefficient of a solvent between octanol/water is a quantitative measure of solvent polarity (17). In general, catalytic activity is low in solvents with log $P < 2$ and high in apolar solvents with log $P > 2$ (17). Nonpolar solvents, such as hexane, are incapable of containing large amounts of water and are therefore unable to strip away substantial amounts of water from enzymes (18). The influence of organic solvents on enzyme activity has been the subject of several studies (14,18–20). Claon and Akoh (20) found that increasing log P values did not necessarily sustain higher activity of SP 435 lipase. In the current study, both IM 60 and SP 435 lipases showed an apparent increase in SL synthesis (C33) with increase in log P value (Table 2). Interestingly, SP 435 lipase produced SL in acetonitrile (log $P = -0.33$) and none in benzene (log $P = 2.0$). This result appears to confirm conclusions by Gorman and Dordick (18) that desorption of water from enzymes is both solvent- and enzyme-dependent. Hexane and isooctane supported the synthesis of SL in good yields. In the absence of organic solvents, good yields of C33 (52.3%) and C45 (15.6%) were obtained with IM 60 lipase as the biocatalyst.

Time course. The time course of IM 60 lipase-catalyzed interesterification of trilinolein and tricaproin was performed at a 1:2 substrate mole ratio. Reaction products were analyzed at 3, 6, 9, 12, 18, 24, 30, 36, 42, and 48 h. Figure 4 shows that the highest incorporation of caproic acid (C33) was achieved at 24 h after which there was no significant increase in yield. The yield of C45 increased initially between 6 to 9 h of incubation and thereafter decreased as more C33 was being formed. We chose 24 h for all other studies because the reaction reached equilibrium at this time.

Effect of free fatty acid as acyl donor. A mole ratio study

TABLE 2
Effect of Selected Organic Solvents on SP 435- and IM 60-Catalyzed Synthesis of Structured Lipids (SL)

Solvent[a]	Water content (wt%)	Log P value[b]	Mol% C33		Mol% C45	
			IM 60	SP 435	IM 60	SP 435
No solvent			52.3 ± 0.2	32.5 ± 0.7	15.6 ± 0.1	6.2 ± 0.1
Isooctane	0.004	4.51	57.6 ± 0.1	44.3 ± 5.0	16.9 ± 0.9	8.6 ± 0.2
n-Hexane	0.004	3.50	54.8 ± 2.5	39.3 ± 0.5	21.4 ± 1.6	14.5 ± 5.0
Benzene	0.016	2.00	34.8 ± 3.0	N/A[c]	4.7 ± 6.0	N/A
Tetrahydrofuran	0.140	0.49	N/A	N/A	N/A	N/A
Acetonitrile	0.053	−0.33	N/A	20.2 ±1.1	N/A	10.3 ± 1.2

[a]Solvents were dried over molecular sieve 4 Å. The water contents of the solvents were measured with a 684 KF coulometer, equipped with a 649 stirrer (Brinkman Instruments, Inc., Westbury, NY). IM = IM 60, SP = SP 435, C33 = dicaproyllinolein, and C45 = monocaproyldilinolein. Number after C indicates total carbon number of the triacylglycerols.
[b]Source: Reference 17; and isooctane, Reference 21.
[c]N/A indicates no SL formation.

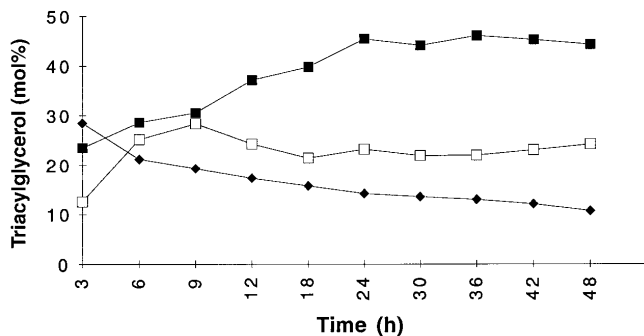

FIG. 4. Time course of IM 60 lipase-catalyzed interesterification of trilinolein and tricaproin. Samples were analyzed at times indicated and in duplicate. The mole ratio of trilinolein to tricaproin was 1:2. C33, ■; C45, □; C57, ◆?

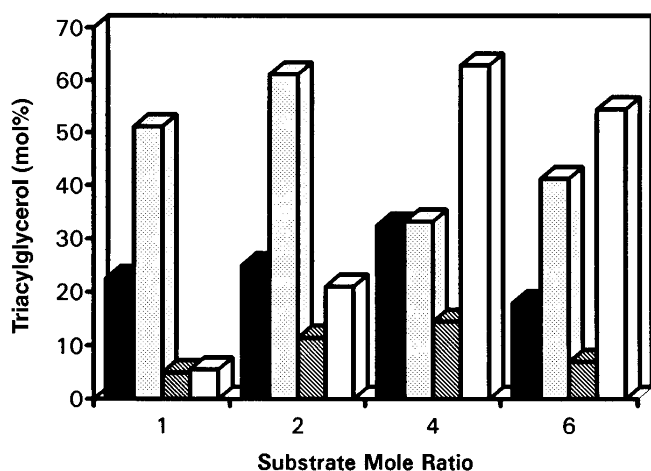

FIG. 5. Effect of free fatty acid as acyl donor. The mole ratio of trilinolein to caproic acid was varied from 1:1 to 1:6. The reaction mixture was incubated for 24 h in 3 mL hexane at 45°C for IM 60 lipase and 55°C for SP 435 lipase. See Figure 2 for abbreviations. C45 SP, solid bar; C45 IM, cross-hatched bar; C33 SP, hatched bar; C33 IM, open bar.

was carried out with caproic acid as the acyl donor instead of tricaproin. The mole ratio of trilinolein to caproic acid was varied from 1:1 to 1:6 (trilinolein/caproic acid), and 5.7 mg of enzyme (10% w/w of total substrate at 1:1 mole ratio) was added in all mole ratio incubations. At a mole ratio of 1:4, maximal incorporation of caproic acid was observed with both enzymes (Fig. 5). IM 60 lipase produced more C33 than SP 435 lipase, which indicates a preference for free acids. At a substrate mole ratio of 1:4, SP 435 lipase produced 32.5% C45 and 14.5% C33, while IM 60 lipase produced 33.3% C45 and 62.9% C33. When caproic acid is compared with tricaproin as the donor, a higher yield of C33 was obtained with caproic acid (82.8% increase) and IM 60 as the biocatalyst. With SP 435 lipase, a lower yield of C33 was obtained with free acid as the acyl donor. In this reaction, SP 435 lipase was more sensitive to the presence of free acid. Above a mole ratio of 1:4, caproic acid inhibited SP 435 activity as reflected by a big decrease in mol% SL. No significant decrease in SL product yield was observed when IM 60 lipase was used.

Pancreatic lipase study. A pancreatic lipase study was carried out to determine the fatty acid composition at the *sn*-2 position of the SL. This study is significant because reports have suggested improved absorption of fatty acids at the *sn*-2 positions of TAG (9). SL were separated based on degree of unsaturation by argentation silver nitrate TLC, and pancreatic lipase analysis was performed on the C33 and C45 SL species. The result is given in Table 3. When IM 60 lipase was used, 47.9% of C33 consisted of LCC and CCL, and 52.1% consisted of CLC, where L = linoleic acid and C = caproic acid. We found more caproic acid (57.6%) at the *sn*-2 position of C33 synthesized with SP 435 lipase than with IM 60 lipase (47.9%). For C45 species, SP 435 produced more C18:2 (73.9%) at the *sn*-2 position than IM 60 lipase (44.9%). IM 60 lipase catalyzes the exchange of esters from the *sn*-1 and *sn*-3 positions of the TAG while leaving the *sn*-2 position intact. With SP 435 lipase, the ester exchange takes place at all three positions of the TAG.

Under the conditions of our assay, IM 60 lipase exhibited a wider degree of temperature stability and higher efficiency in incorporating large amounts of caproic acid into the SL products than SP 435 lipase. However, both lipases are potentially useful in producing SL by interesterification of TAG.

TABLE 3
***Sn*-2 Fatty Acid Analysis After Pancreatic Lipase Hydrolysis**

sn-2 Fatty acid (%)	C33 (IM 60)	C33 (SP 435)	C45 (IM 60)	C45 (SP 435)
C6	47.9 ± 10	57.6 ± 8	55.1 ± 11	26.1 ± 12
C18:2	52.1 ± 10	42.4 ± 8	44.9 ± 12	73.9 ± 12

[a]Pancreatic lipase hydrolysis was done by the method of Luddy *et al.* (10). C33 = dicaproyllinolein and C45 = monocaproyldilinolein. Number after C indicates total carbon number of the triacylglycerols.

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