Two-Step Enzymatic Reaction for the Synthesis of Pure Structured Triacylglycerides

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ABSTRACT: Structured triacylglycerides with medium-chain fatty acids (caprylic acid) in *sn*1- and *sn*3-positions and a long-chain unsaturated fatty acid (oleic or linoleic acid) in the *sn*2-position of glycerol (MLM) were synthesized by lipase catalysis in a two-step process. First, pure 2-monoacylglycerides (2-MG) were synthesized by alcoholysis of triacylglycerides (triolein, trilinolein, or peanut oil) in organic solvents with 1,3-regiospecific lipases (from *Rhizomucor miehei*, *Rhizopus delemar*, and *Rhizopus javanicus*). The 2-MG were purified by crystallization and obtained in up to 71.8% yield. These 2-MG were esterified in a second reaction with caprylic acid in *n*-hexane to form almost pure MLM. For 2-MG obtained from peanut oil, the final product contained more than 90% caprylic acid in the *sn*1- and *sn*3-positions, whereas the *sn*2-position was composed of 98.5% unsaturated long-chain fatty acids. Reaction conditions for both steps were optimized with respect to source and immobilization of lipase, water activity, and solvent.
JAOCS 75, 703–710 (1998).

KEY WORDS: Immobilization, lipase, Lipozyme, 2-monoacylglyceride, peanut oil, structured triacylglyceride.

Fats and oils are complex mixtures of simple and mixed triacylglycerides (TG), which function as energy reserves. TG differ according to the type and composition of fatty acids and their distribution along the glycerol backbone. These characteristics play an important role in the physical, rheological, and nutritional properties of fats and oils. Examples are margarine and cocoa butter that are produced from cheap vegetable oils (1,2). For nutritional purposes, medium-chain TG (MMM) have been used as a concentrated form of calories to meet, for instance, the pancreatic insufficiency of patients (3,4). Structured TG (MLM), which contain medium-chain fatty acids in the *sn*1- and *sn*3-positions of glycerol and an unsaturated long-chain fatty acid in the *sn*2-position, have been reported as versatile TG to substitute for MMM in their nutritional function (5,6), and several studies have dealt with their synthesis.

The first method reported for the production of MMM was chemical synthesis, which involved the fractionation of the medium-chain fatty acids ($C_{8:0}$ and $C_{10:0}$), obtained from the hydrolysis of high-grade vegetable oil, and their subsequent esterification with glycerol and catalysts (7). Recently, other chemical methods, based on the chiral synthesis of mixed TG, have been proposed (8–10). The most important step was the regioselective opening of the epoxide function. Isomerization, which probably occurs either when the epoxide ring is opened or during the various extraction and purification stages, can lead to undesired TG. With such an approach for the production of MLM, it would be difficult to introduce the long-chain fatty acid in the desired position. Alternatively, the enzymatic synthesis of pure MLM was investigated with *sn*1,3-regiospecific immobilized lipases by acidolysis of TG that contained long-chain fatty acids (LLL) with medium-chain fatty acids or by interesterification of LLL with MMM (11–15). However, the recovered products contained a significant amount of long-chain fatty acids in the *sn*1- and/or *sn*3-positions, due to differences in the regiospecificity of the lipases used (16) and acyl migration during synthesis. To overcome these problems, esterification of 2-monoacylglyceride (2-MG) with free medium-chain fatty acids was reported for the esterification of 2-monopalmitin with caprylic acid with lipase from *Rhizopus delemar* in reverse micellar media (17). However, only a small portion of MLM was found, due to isomerization of 2-MG to 1-MG, followed by hydrolysis of the latter.

Unsaturated 2-MG are expensive, thus the synthesis of MLM from these 2-MG as starting materials did not appear economical. Recently, the production of saturated 2-MG by alcoholysis of pure TG was shown to be possible through a detailed study to suppress acyl migration (18,19).

In the present work, we report a two-step lipase-catalyzed synthesis of pure valuable TG of the MLM-type in which, in the first step, 2-MG are produced by alcoholysis of a pure unsaturated TG (triolein, trilinolein) or a natural oil (peanut oil). 2-MG thus obtained are esterified in the second step with fatty acids to produce MLM. Reactions were optimized with respect to lipase source, support for immobilization, organic solvent, and alcohol for the synthesis of 2-MG, as well as variation of the chainlength of medium-chain fatty acids in the second step.

MATERIALS AND METHODS

Lipase. Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) were from *Rhizopus delemar* (RDL), *Rhizopus javanicus*

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(RJL) (both from Amano Pharmaceutical Co. Ltd., Nagoya, Japan), and *Rhizomucor miehei* (RML) (Biocatalysts Ltd., Pontybridd, England). One commercial lipase from RML (Lipozyme IM), immobilized on anion exchange resin, was from Novo (Bagsvaerd, Denmark). All chemicals and solvents used were of reagent grade and purchased from common commercial suppliers, except peanut oil (Vaselin Fabrik, Bonn, Germany), EP 100 (porous polypropylene particles, 200–400 µm; Akzo, Obernburg, Germany), and Celite 545 (Fluka, Buchs, Switzerland).

Hydrolytic activity of lipase. The hydrolytic activity of lipases was measured at 37°C with 5% (wt/vol) olive oil emulsion (pH 8.0) that contained 2% (wt/vol) gum arabic. Twenty mL of the emulsified solution, 470 µL of $CaCl_2$ solution (22% wt/vol), and a known amount of immobilized lipase were mixed, and liberated fatty acids were titrated automatically with 0.01 N NaOH to maintain the pH constant at pH 8.0. One unit (U) of lipase activity was defined as the amount of enzyme that liberates 1 µmol fatty acid/min under assay conditions.

Immobilization of lipase. Celite 545 and EP 100 were used as supports for adsorptive immobilization of commercial lipases. Before immobilization, Celite was washed several times with purified water and ethanol (95% vol/vol) to remove fines and dried at 80°C. The washed Celite was mixed with 5% HNO_3 and stirred at 80°C for 4 h. The acid-washed Celite was carefully washed with purified water until the pH of the water was neutral, and was then dried overnight at 80°C. One gram of lipase powder was dissolved in 20 mL phosphate buffer (pH 7.0, 20 mM). The solution was added to 1.5 g acid-washed Celite, stirred at 5°C for 8 h, and followed by addition of 5 mL chilled acetone (−15°C). The immobilized lipase was collected by filtration, washed three times with phosphate buffer (pH 7.0, 20 mM), dried at room temperature under vacuum for 48 h, and stored at 5°C until use. For EP 100, 1.5 g support was mixed with 5 mL ethanol, before the lipase solution (1 g in 25 mL phosphate buffer, pH 6.0, 20 mM) was added. After stirring overnight at room temperature, the immobilized enzyme was treated as described above.

Alcoholysis of TG. Triolein (99%), trilinolein (99%), or peanut oil (all 0.56 mmol), dissolved in methyl *tert*-butyl ether (MTBE) (2 mL), was equilibrated to a water activity of 0.75 over saturated NaCl in a closed vessel. Ether solvents were chosen because they efficiently suppress acyl migration (18,19). Dry ethanol (dried over molecular sieve 4 Å, which was activated by heating overnight to 250°C) was added to a final concentration of 5.6 mmol. The reaction mixture was incubated at 40°C in a stirred oil bath for 15 min, then the reaction was started by addition of 60 mg immobilized RML, RDL, or RJL, pre-equilibrated at water activity 0.11 over saturated LiCl. The reaction was stopped after 30 h by removal of immobilized lipase, and the composition of the reaction mixture was determined by thin-layer chromatography–flame-ionization detection (TLC–FID) as described previously (20). After evaporation of excess solvent *in vacuo*, the oily residue was dissolved in 20 mL *n*-hexane/MTBE (70:3 vol/vol) and stored at −25°C overnight. After this period, white crystals formed, which were collected by filtration at −25°C. The supernatant, which contained ethyl esters, diacylglycerides (DG), fatty acids and a small amount of TG, was discarded. The 2-MG was recrystallized several times until the TLC plate showed only one band of 2-MG. Yields given in Tables 2 and 4–6 are percentages of the theoretical yield of 33.3% by weight. The purity of 2-MG was confirmed by ^{13}C nuclear magnetic resonance (NMR) spectroscopy (Table 1).

Esterification reaction. The purified 2-MG obtained by alcoholysis (0.12 mmol) and caprylic acid (0.24 to 0.72 mmol) were dissolved in 2 mL *n*-hexane that was equilibrated to a water activity of 0.11. The reaction mixture was incubated at 38°C and stirred magnetically at 800 rpm. The reaction was started by addition of 12% (based on the weight of 2-MG) of immobilized lipase (water activity 0.11) and 0.5 g activated molecular sieve (4 Å). At selected times, an aliquot of the reaction medium was applied to a Kieselgel 60 plate (Merck, Darmstadt, Germany), which was developed in a mixture of *n*-hexane/diethyl ether/acetic acid (70:30:1.5). The components of the reaction mixture were visualized by spraying with 50% (vol/vol) sulfuric acid (dissolved in methanol) and heating at 150°C. For comparison of the reaction systems, interesterification of triolein or peanut oil (0.12 mmol) with caprylic acid (0.6 mmol) in 2 mL *n*-hexane was performed.

Purification of produced TG. Lipase was removed by centrifugation, the organic phase was dried with anhydrous sodium sulfate, and excess solvent was removed by evaporation *in vacuo*. The reaction mixture was purified by column chromatography. Silica gel (10 g) and aluminum oxide (10 g) were mixed in 50 mL *n*-hexane to make a slurry, which was poured into the column (300 × 30 mm). The reaction mixture (1 g) that contained DG, fatty acids, and TG was applied to the column. The column was eluted with a mixture of *n*-hexane/diethyl ether (95:5 vol/vol). The recovered fractions were analyzed by TLC as described previously (15).

TABLE 1
Chemical Shifts (ppm) of Carbon Atoms in the Acyl Chain and in the Glycerol Moiety of 1-MO (standard) and 2-MO (reaction product)

Monoolein	δCH_2OH	$\delta CHOH$	δCH_2OCOR	$\delta CHOCOR$	δC_1 [a]	δC_2 [b]
1-MO	63.74	70.65	65.51	—	174.75	34.52
2-MO[c]	62.79	—	—	75.35	174.49	34.72

[a]Carbon atom 1 in the acyl chain of monoolein.
[b]Carbon atom 2 in the acyl chain of monoolein.
[c]Recovered from alcoholysis of triolein with ethanol in methyl *tert*-butyl ether (MTBE) after 30 h. MO, monoolein.

High-performance liquid chromatography (HPLC) separation of triacylglycerols. The composition of the triacylglycerols formed during the enzymatic esterification and interesterification was determined by HPLC on a nucleosil C_{18} column (5 µm, 250 × 4 mm, Sykam, Gilching, Germany) with an evaporative light-scattering detector (S.E.D.E.R.E, Vitry/Seine, France) at a column temperature of 38°C and a flow rate of 1.0 mL/min. Elution was performed with a linear gradient elution system of 80% acetonitrile to 90% dichloromethane over 40 min. The extent of reaction was calculated from the molar or weight percentage of produced TG present in the mixture.

Determination of the fatty acid composition. Ten milligrams of TG (peanut oil) or 2-MG, dissolved in 1 mL *n*-heptane, was methylated with 20 µL sodium methoxide solution (0.5 M in methanol) and shaken for 1 min (21). After centrifugation, 1 µL was taken from the supernatant and analyzed by gas–liquid chromatography (GLC; Fisons Instruments Mega series, Mainz, Germany) on a polar column (FFAP, 25 m × 0.53 mm i.d., Machery & Nagel, Düren, Germany). Analysis was carried out with temperature programming from 100 to 210°C at 5°C/min, 200°C as injection and 210°C as detector temperature (FID). Ethyl esters produced by alcoholysis of peanut oil were recovered from TLC plates, dissolved in *n*-heptane, and analyzed for their fatty acid composition.

The regiospecific analysis of produced TG was conducted by partial deacylation with ethyl magnesium chloride (22). Purified TG (10 mg) from column chromatography was dissolved in dry diethyl ether (1 mL), a freshly diluted solution of ethyl magnesium chloride (250 µL) was added, and the mixture was shaken for 1 min before glacial acetic acid (6 µL) in hexane (5 mL) and water (2 mL) were added to stop the reaction. The organic layer was washed twice with water (2 mL) and dried over anhydrous sodium sulfate. After evaporation, the mixture of deacylation products was separated by TLC plates, which were impregnated with boric acid, with *n*-hexane/diethyl ether/acetic acid (70:30:1.5, vol/vol/vol) as developing system. The *sn*-1,2 (2,3)-DG bands were scraped and methylated to determine their fatty acid composition. The molar percentage of fatty acid composition at *sn*1(3)- and *sn*2-positions of the produced TG were calculated according to a published method (23).

RESULTS AND DISCUSSION

Alcoholysis of TG. The first step in the production of TG of the MLM-type was the alcoholysis of a TG that contained long-chain fatty acids. For this purpose, lipases with *sn*1,3-regiospecificity were investigated with triolein as model compound, and initial rates were determined. During the alcoholysis of triolein with ethanol in MTBE, the enzyme activity (measured as initial rate of the alcoholysis of triolein based on lipase units U) was seven to nine times higher with immobilized RML than with immobilized RDL or RJL (Table 2). In contrast, when the initial rate was based on the weight of enzyme preparation, RML was the worst and RJL the best enzyme. However, for a fairer comparison of different lipase preparations, the activity should be expressed in terms of units, which takes into account the weight of carrier material, as well as impurities and additives, as outlined in previous publications (15,24). Although ethanol was used in a 10-fold excess, the reaction mixture contained free oleic acid in significant amounts of about 25% (Table 3). After 24 h, triolein was completely consumed with RDL, and only a minor amount was left with RJL. With both, small amounts of diolein were left. The best yield of 2-monoolein (2-MO) (based on the isolated product after crystallization) was found with RDL (71.8%), while with RML (40.5%), the lowest yield was obtained, despite the highest initial rate (Table 2). RML has been reported as one of the microbial lipases that shows more preference for *sn*-1 compared to *sn*-3 in TG (16), which might explain the lower yield of 2-MO. A comparison of the ^{13}C NMR spectra of 2-MO with commercial 1-MO confirmed the high purity of the product (Table 1). Reactions were also performed in solvents other

TABLE 2
Synthesis of 2-MO by Alcoholysis of Triolein with Ethanol in MTBE with Lipases Immobilized on Celite (after 24 h)[a]

Lipase from	Initial rate (µmol/h/mg)	Initial rate (µmol/h/U)	Yield (%)
Rhizomucor miehei	0.56 ± 0.01	2.23 ± 0.13	40.5
Rhizopus delemar	0.65 ± 0.03	0.30 ± 0.04	71.8
Rhizopus javanicus	0.82 ± 0.04	0.25 ± 0.02	56.9

[a]For abbreviations see Table 1.

TABLE 3
Composition of the Reaction Medium after 24 h from the Alcoholysis of Triolein with Ethanol and Immobilized Lipases, as Determined by TLC–FID[a]

	Composition of reaction medium (%)				
Lipase from	Oleic acid	Monoolein	Diolein	Triolein	Ethylester
Rhizomucor miehei	25.6	13.6	13.6	10.6	36.0
Rhizopus delemar	24.7	25.0	4.9	0.0	45.4
Rhizopus javanicus	25.3	20.6	8.3	1.6	44.2

[a]TLC–FID, thin-layer chromatography–flame-ionization detection.

TABLE 4
Influence of Organic Solvent on the Yield of 2-MO Obtained by Alcoholysis with Immobilized Lipase from *Rhizopus delemar* (after 24 h)[a]

Organic solvent	Initial rate (μmol/h/U)	Yield (%)
MTBE	0.30 ± 0.04	71.8
Petroleum ether	0.20 ± 0.03	60.4
Acetone	0.10 ± 0.04	34.2

[a]For abbreviations see Table 1.

TABLE 6
Influence of Triacylglyceride on the Synthesis of 2-MG by Alcoholysis with Immobilized Lipase from *Rhizopus delemar* in MTBE and Ethanol (after 24 h)[a]

Triacylglyceride	Initial rate (μmol/h/U)	Yield (%)
Triolein	0.30 ± 0.04	71.8
Trilinolein	0.41 ± 0.03	60.6

[a]2-MG, 2-monoacylglyceride. For other abbreviation see Table 1.

than MTBE (Table 4), but the use of petroleum ether or acetone gave no improvement in 2-MO yields.

An increase in the chainlength of the alcohol decreased the yield of 2-MO (Table 5). By comparing the secondary alcohol 2-propanol with 1-propanol, a higher lipase activity could be observed; however, only trace amounts of 2-MO were detected. This might be due to a lower affinity of a secondary alcohol for the active site (25). Besides triolein, trilinolein was also used as substrate. Although the initial rate with trilinolein was higher than with triolein (Table 6), the yield of 2-monolinolein (2-MLn) was only 60.6%, because the crystallization process needs –35°C for the optimal formation of crystals of 2-MLn, compared to –25°C for 2-MO (26).

The alcoholysis finally used for the production of 2-MG from peanut oil with ethanol was conducted in petroleum ether. As indicated in Table 7, the major fatty acids of 2-MG were oleic acid, $C_{18:1}$ (64.3%), and linoleic acid, $C_{18:2}$ (34.4%), while the major fraction of palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$) were converted to ethyl esters. Longer saturated fatty acids, such as arachidic ($C_{20:0}$) and behenic ($C_{22:0}$) acids, were not cleaved and remained in the peanut oil fraction (Table 7). Thus, the alcoholysis of peanut oil is an efficient method to obtain 2-MG with a high content (98.7%) of unsaturated fatty acids, required for the synthesis of structured TG of the MLM-type.

Esterification. The second step in the synthesis of MLM-TG is the esterification of 2-MG with a fatty acid. Reactions were initially performed with 2-MO and optimized with respect to type of fatty acid, molar ratio between fatty acid and 2-MG, and choice and immobilization of lipase, as outlined and discussed in the following sections.

Effect of molar ratio of caprylic acid and 2-MO. The effect of the molar ratio between caprylic acid and 2-MO on the initial rate in the synthesis of MLM was studied with Lipozyme, and the results are shown in Figure 1 for molar ratios between 2 and 6. The optimal molar ratio was 3:1. At higher ratios, the rate of esterification decreased, probably owing to the accumulation of caprylic acid at the interface.

Effect of origin of lipase and support for immobilization. RML and RDL were immobilized on different carriers, and the initial rate in the esterification was investigated in *n*-hexane at a low water activity of 0.11 to suppress acyl migration. In addition, reactions were performed in the presence or absence of molecular sieve. With the exception of reactions with the commercial RML preparation Lipozyme (Resin) and RDL immobilized on EP100, the use of molecular sieves did not favor the synthesis of MLM (Table 8). In comparing RML and RDL immobilized on EP100, conversion of MO to MLM in the presence of molecular sieve took place with RDL, while it did not occur with RML. Highest initial rates and final concentrations of MLM were found with Lipozyme (89.1%) and RDL immobilized on EP 100 (91.2%). When comparing reactions with RML or RDL immobilized on Celite with or without molecular sieve, the synthesis of MLM was much faster in the absence of molecular sieve, and 62.1 (RML) or 69.4% (RDL) MLM was formed. However, the values obtained with Lipozyme (RML) or RDL (EP 100) were not exceeded (Table 8).

In the esterification of 2-MG with medium-chain fatty acids, the water content and/or activity must be low to avoid acyl migration and to convert all 2-MG to MLM. This strongly depends on the origin of lipase, the type of support, and whether molecular sieve is present or not. However, some water is absolutely essential for enzymatic catalysis because the chemical and physical properties of enzymes strongly de-

TABLE 5
Influence of Alcohol on the Synthesis of 2-MO by Alcoholysis with Immobilized Lipase from *Rhizopus delemar* in MTBE (after 24 h)[a]

Alcohol	Initial rate (μmol/h/U)	Yield (%)
Ethanol	0.30 ± 0.04	71.8
1-Propanol	0.15 ± 0.01	32.3
2-Propanol	0.26 ± 0.01	<1.0
1-Butanol	0.26 ± 0.01	29.2

[a]For abbreviations see Table 1.

TABLE 7
Fatty Acid Composition of Peanut Oil, Ethyl Esters, and 2-MG Obtained by Alcoholysis of Peanut Oil with Immobilized Lipase from *Rhizopus delemar* in MTBE (after 24 h)

Fatty acid	Fatty acid composition (mol%)[a]		
	Peanut oil	Ethyl esters	2-MG
$C_{16:0}$	10.4	19.9	1.4
$C_{18:0}$	3.8	5.1	n.d.
$C_{18:1}$	49.4	53.9	64.3
$C_{18:2}$	31.2	21.0	34.4
$C_{18:3}$	0.4	n.d.	n.d.
$C_{20:0}$	1.6	n.d.	n.d.
$C_{22:0}$	3.2	n.d.	n.d.

[a]Determined by gas chromatography; n.d., not detectable. For other abbreviations see Tables 1 and 6.

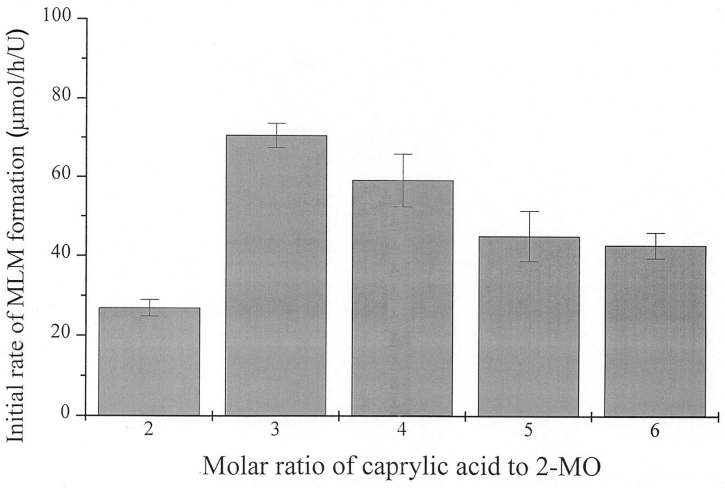

FIG. 1. Influence of the molar ratio of caprylic acid to 2-monoolein (2-MO) on the initial rate of MLM formation with Lipozyme. MLM, structured triacylglyceride with medium-chain fatty acids in the *sn*1- and *sn*3-positions of glycerol and an unsaturated long-chain fatty acid in the *sn*2-position.

TABLE 8
Synthesis of Structured Triacylglyceride (TG) by Esterification of 2-MO with Caprylic Acid (1:3 molar ratio) in *n*-Hexane with Immobilized Lipases

		Initial rate (μmol/h/U)		Concentration of TG[a] (wt%)	
Lipase	Support	MLM[b]	MLL[b]	MLM	MLL
RML[b]	Resin[c]	70.6	3.7	89.1	8.8
	Celite[c]	<0.01	<0.01	0.7	2.1
	Celite	3.5	<0.1	62.1	15.4
	EP100[c]	<0.01	<0.01	0.4	0.2
	EP 100	1.3	<0.1	64.6	14.2
RDL[b]	Celite[c]	<0.01	<0.01	0.5	7.3
	Celite	0.9	<0.01	69.4	7.8
	EP 100[c]	3.1	<0.1	91.2	8.0

[a]Concentration of TG after 15 h.
[b]MLM, structured TG with medium-chain fatty acids in the *sn*1- and *sn*3-positions of glycerol and an unsaturated long-chain fatty acid in the *sn*2-position; MLL, similar to MLM, but medium-chain fatty acid in *sn*1-position and unsaturated long-chain fatty acids in *sn*2- and *sn*3-positions; RML, *Rhizomucor miehei*; RDL, *Rhizopus delamar*; for other abbreviations see Table 1.
[c]In the presence of molecular sieve.

pend on the direct role of water in all noncovalent interactions, which maintain the conformation of active sites (27). The resin used for RML immobilization in the production of Lipozyme and EP 100 for RDL may protect the enzymes against complete removal of water by molecular sieves, and this might explain the high conversion of 2-MO into TG.

Effect of fatty acid chainlength on esterification rate. Various medium-chain fatty acids with carbon chainlengths between 6 and 14 were used as substrates for the esterification of 2-MO [molar ratio (fatty acid/2-MO), 3:1] in *n*-hexane with molecular sieves and Lipozyme (RML). The relative activity was calculated as the percentage of the initial rate of conversion for each fatty acid (the rate for caprylic acid was set to 100). RML clearly showed highest activity for caprylic acid (Fig. 2), and relative activities for capric acid ($C_{6:0}$) or longer fatty acids ($>C_{10:0}$) were much lower. The same range of fatty acids was investigated in the conversion of monoerucin into TG with lipase from *Geotrichum candidum*, where again the highest activity was found for caprylic acid (28).

Comparison of reaction systems. Figure 3 shows the time course of the Lipozyme (RML)-catalyzed esterification of 2-MO with caprylic acid with molecular sieves in *n*-hexane at 38°C. As expected, at the early stage of the reaction (up to 2 h), DG composed of caprylic acid and oleic acid (CyO) is formed, followed by its conversion to CyOCy (Fig. 3). After only 3 h, the reaction plateaued, and the TG composition was 88.5% CyOCy and 9% CyOO. This reaction (boxed), together with possible side reactions occurring during this step, are shown in Figure 4. The formation of CyOO was due to acyl migration to enable the formation of diolein, which is fi-

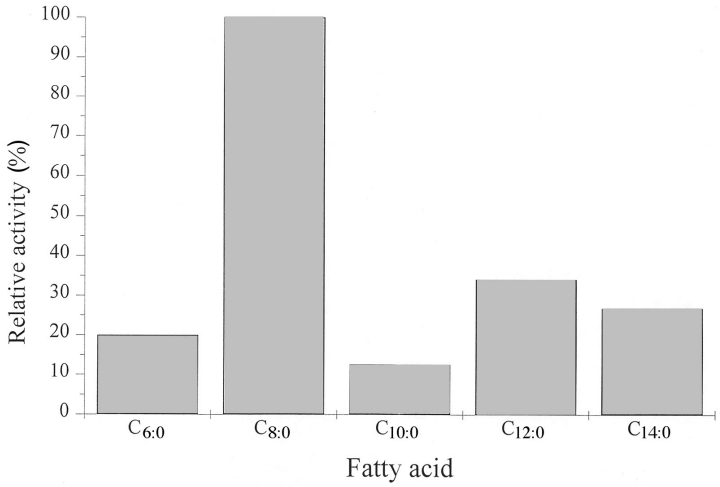

FIG. 2. Influence of fatty acid chainlength in the synthesis of structured triacylglycerides (MOM) with Lipozyme.

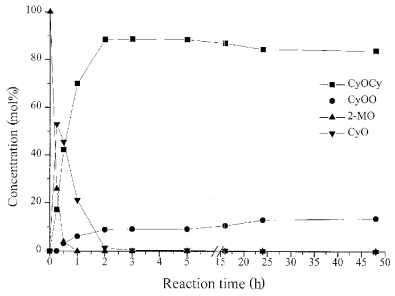

FIG. 3. Time course of the formation of structured triacylglycerides from 2-MO and caprylic acid in *n*-hexane with Lipozyme. Cy, caprylic acid; O, oleic acid. For other abbreviation see Figure 1.

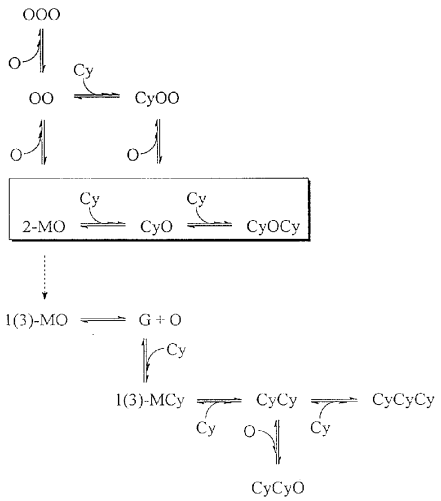

FIG. 4. Possible reactions involved in the esterification between 2-MO and caprylic acid with Lipozyme (*Rhizomucor miehei*). G, glycerol; MCy, monocaprolein; see Figures 1 and 3 for other abbreviations.

nally converted to CyOO. Other products shown in Figure 4 could not be detected.

We have also performed the synthesis of CyOCy by direct interesterification between triolein and caprylic acid with the same enzyme, and the time course of this reaction is shown in Figure 5. It is obvious that the concentration of CyOCy is much lower after direct interesterification, compared to the newly developed two-step synthesis (Fig. 3). In addition, high concentrations of CyOCy (40%) could only be obtained in the interesterification after long reaction times (>20 h), and the residual concentrations of CyOO (22%) and triolein (7%) were also high. Figure 6 shows chromatograms of the purified products obtained after direct interesterification (A, 15 h reaction time) and after the two-step process (B, 6 h reaction time). The TG (CyOCy) synthesized in the esterification between 2-MO and caprylic acid is almost pure (>91%) and can be used as standard.

The same reaction system was also used for the esterification of 2-MG (obtained by alcoholysis of peanut oil) with caprylic acid, and the time course is shown in Figure 7. Again, a rapid formation of MLM occurred, and after 3 h, the TG mixture contained 80% MLM. Besides *n*-hexane, three other organic solvents were used, and the compositions of the reaction products at different reaction times are given in Table

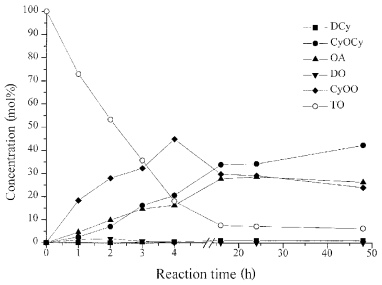

FIG. 5. Time course of the formation of structured triacylglycerides by direct interesterification between triolein and caprylic acid in *n*-hexane with Lipozyme. D, di; T, tri; see Figures 1 and 3 for other abbreviations.

TABLE 9
Effect of Organic Solvents on the Synthesis of Structured Triglycerides by Esterification of 2-MG (obtained from peanut oil by alcoholysis) with Caprylic Acid and RML (Lipozyme).

Organic solvent	Time (h)	Composition (%)[a] MLM	MLL	DG	MG	FFA[b]
n-Hexane	0.5	14.5	1.0	79.6	4.9	0
	1	53.8	7.6	38.6	0	0
	4	75.3	13.8	6.9	0	3.9
Cyclohexane	0.5	15.8	0	70.4	13.8	0
	1	30.0	0	70.0	0	0
	4	77.4	4.7	17.9	0	0
Isooctane	0.5	19.4	0	62.2	18.3	0
	1	38.7	1.0	60.3	0	0
	4	79.3	7.8	12.9	0	0
Petroleum ether	0.5	26.7	6.9	66.3	0	0
	1	38.9	9.6	51.5	0	0
	4	54.6	13.6	16.2	0	15.6

[a]Determined by HPLC.
[b]Fatty acids released from 2-MG.

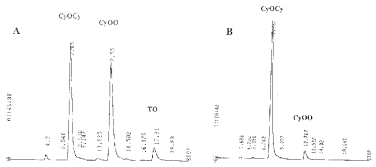

FIG. 6. Chromatograms indicate the purity of structured triacylglycerides. A: one-step process, 15 h reaction time; B: two-step process, 6 h reaction time. See Figures 3 and 5 for abbreviations.

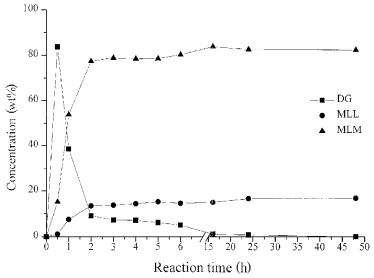

FIG. 7. Time course of the formation of structured triacylglycerides from 2-monoacylglyceride (obtained from peanut oil) and caprylic acid in *n*-hexane with Lipozyme. DG, diglyceride; MLL, structured triacylglyceride with medium-chain fatty acid in the *sn*1-position, and unsaturated long-chain fatty acids in the *sn*2- and *sn*3-positions; for other abbreviation see Figure 1.

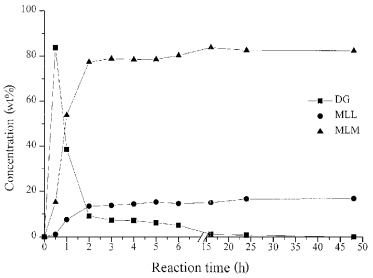

FIG. 8. Time course of the formation of structured triacylglycerides by direct interesterification between peanut oil and caprylic acid in *n*-hexane with Lipozyme. For abbreviations see Figures 1 and 7.

9. Reactions in *n*-hexane, cyclohexane, and isooctane gave similar concentrations of MLM between 75.3 and 79.3% after 4 h reaction time. Petroleum ether was less suitable because only 54.6% MLM was formed, and free fatty acid was produced in significant amounts (15.6%).

A comparison with the direct interesterification between peanut oil and caprylic acid (Fig. 8) gives the same pattern as described above for triolein, but a two-step process allows a faster reaction and gives considerably higher final concentrations of the desired MLM TG, as listed in Table 10. High amounts of caprylic acid (60.5%) were incorporated with high selectivity (>90%) in the *sn*1- and *sn*3-positions, and

TABLE 10
Composition and Positional Distribution of Fatty Acids of Structured Triacylglycerides Formed in the Two-Step Reaction from Peanut Oil and Caprylic Acid with RML (Lipozyme) after 30 h Reaction Time[a]

Fatty acid	Composition (mol%)	Positional distribution	
		1(3)-position (mol%)	2-position (mol%)
$C_{8:0}$	60.5	90.9	<0.1
$C_{16:0}$	1.0	n.d.	0.6
$C_{18:1}$	25.4	8.5	59.3
$C_{18:2}$	13.1	0.6	39.2

[a]For abbreviation see Table 8.

only a small amount of long-chain unsaturated fatty acid (9.1%) was found. As desired, in the *sn*2-position, 98.5% unsaturated fatty acid (oleic acid 59.3%, linoleic acid 39.2%) was bound.

These data, obtained by regiospecific analysis of TG produced in the two-step reaction, clearly show that this process is superior to chemical methods and can also be applied to unsaturated fatty acids (or fats and oils). Moreover, our method for the production of MLM TG makes further studies of nutritional properties of MLM much more versatile.

ACKNOWLEDGMENTS

Financial support to M.M. Soumanou through a grant from the German Academic Exchange Service (DAAD, Bonn, Germany) is gratefully acknowledged. We also thank Novo Nordisk Co., Denmark; Amano Pharmaceuticals, Japan; and Henkel, Germany, for the generous gifts of enzymes and medium-chain fatty acids.

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[Received June 9, 1997; accepted December 11, 1997]