Synthesis of Structured Triglycerides from Peanut Oil with Immobilized Lipase[1]

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ABSTRACT: Structured triglycerides (ST) that contain medium- and long-chain fatty acids were synthesized by lipase-catalyzed interesterification between tricaprylin and peanut oil. To select appropriate enzymes, we investigated nine commercial lipase preparations for their ability to hydrolyze pure triglycerides as well as natural oils. Three microbial lipases from *Rhizomucor miehei* (RML), *Candida* sp. (CSL), and *Chromobacterium viscosum* (CVL) gave good results, and immobilized preparations were used in the interesterification. RML gave the highest yields of ST (73%, 40°C), although its hydrolytic activity toward triolein was low. As the temperature was raised to 50°C, the yield of ST increased to 79%. After 120 h reaction time, remaining activities were high for CSL (71%), moderate for CVL (48%), and low for RML (20%).
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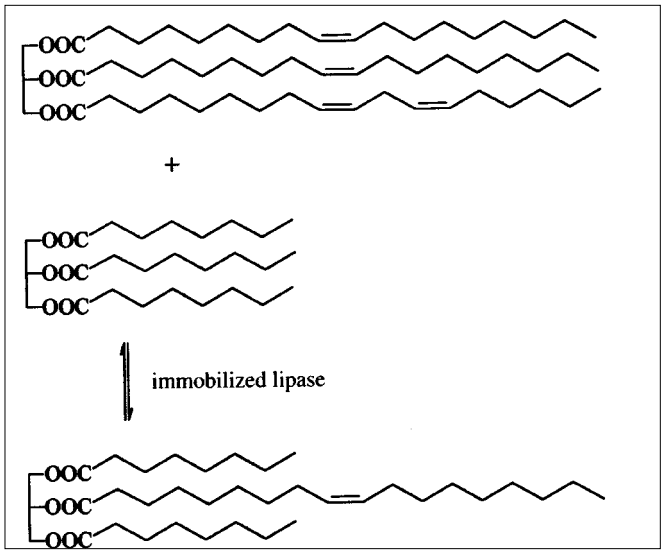
KEY WORDS: Immobilized lipase, interesterification, lipase stability, medium-chain triglycerides, peanut oil, structured triglycerides.

Lipases (triacylglycerol hydrolase, E.C. 3.1.1.3.) are enzymes found in animals, plants and microorganisms, where their function is the hydrolysis of triglycerides. Moreover, they show high activity in organic solvents for synthesis of acylglycerols (1,2), kinetic resolution of chiral compounds (3), and interesterification of lipids (4) to alter the physical and functional properties of fats or oils. One example is the lipase-catalyzed production of cocoa butter—used in the manufacture of chocolate—from low-value oils such as palm oil mid-fractions (5,6).

Medium-chain triglycerides (MMM) are more easily absorbed from the human intestine than long-chain triglycerides. MMM are more soluble in water and can transfer from the bloodstream to cells without forming chylomicrons (7). For this reason, MMM are currently used to feed patients with pancreatic insufficiency and other forms of malabsorption (8–10). A disadvantage of MMM is that they do not contain essential fatty acids, such as linoleic or linolenic acid. This drawback can be overcome by using structured triglycerides of the MLM type. MLM contain medium-chain fatty acids (usually 8:0 and/or 10:0) in the *sn*-1 and *sn*-3 positions of the glycerol backbone and an essential long-chain fatty acid in the *sn*-2 position (Scheme 1). Pancreatic esterases hydrolyze MLM faster than long-chain triglycerides (LLL), and the resulting 2-monoglycerides are absorbed efficiently (11). Nutritional properties, applications/benefits, and enzymatic approaches for the synthesis of these compounds have recently been reviewed, together with examples of already marketed, but chemically synthesized, products (12).

The three enzymatic routes to MLM are: (i) direct esterification of glycerol with long- and medium-chain fatty acids, (ii) ester interchange between long- and medium-chain triglycerides or long (medium)-chain triglycerides with medium (long)-chain fatty acid esters, or (iii) by acidolysis between long (medium)-chain triglycerides with medium (long)-chain fatty acids.



SCHEME 1

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To our knowledge, only two enzymatic methods for the synthesis of structural triglycerides (ST) have been described (12,13), and an almost quantitative incorporation of caprylic acid in an interesterification with triolein (12) or of capric acid in Trisun 90, an oil containing 90% triolein, (13) was achieved with Lipozyme IM60 as catalyst in *n*-hexane. During these enzymatic, and also chemical, syntheses, a mixture of all triglyceride isomers (MLM, MML, LMM, LML, LLM, and MML) is formed, which we refer to as ST.

In this study, we report on the enzymatic synthesis of ST by using two triglycerides (peanut oil and tricaprylin) as starting materials. The selection of appropiate lipases and the influence of the temperature on the stability of immobilized lipases were also studied, targeting the development of a method that produces MLM as major products and makes a continuous process feasible.

MATERIALS AND METHODS

Lipase. Lipases used in this study were from *Rhizopus* sp. (Solvay Enzymes, Hannover, Germany), *Rhizomucor miehei*, *Humicola lanuginosa*, *Pseudomonas fluorescens*, and *Candida cylindracea* (Biocatalysts Ltd., Pontybridd, England), *C. cylindracea* (lipase OF; Meito Sangyo Co., Ltd., Nagoya, Japan), *Geotrichum candidum* (lipase GC; Amano Pharmaceutical Co., Ltd., Nagoya, Japan), and *Chromobacterium viscosum* (CVL, crude 1 and crude 2; Asahi Chemical Industry Co., Ltd., Tokyo, Japan). Two commercial lipases from *R. miehei* (RML, Lipozyme IM 49) and *Candida* sp. (CSL, SP 382), immobilized on an anion exchange resin, were from Novo (Bagsvaerd, Denmark). All chemicals and solvents used for analysis of the reaction products were reagent-grade and purchased from common commercial suppliers, except peanut oil (Vaselin Fabrik, Bonn, Germany), coconut oil (Sigma, Deisenhofen, Germany), and cocoa butter (Caelo, Hilden, Germany).

Protein determination. The protein content of the commercial lipases was determined by the Bradford method with Bio-Rad reagent (14).

Polyacrylamide gel electrophoresis (PAGE). Samples of lipases were dissolved in buffer (50 mM potassium phosphate, pH 7.0), and insoluble material was removed by centrifugation. The supernatant was used directly for native PAGE or heated at 90°C for denaturing sodium dodecyl sulfate- (SDS-) PAGE. Native and SDS-PAGE were analyzed with a PhastSystem (Pharmacia/LKB, Freiburg, Germany) and gradient polyacrylamide gels (8–25%). As reference proteins, the low-molecular-weight standard mixture from Pharmacia/LKB was used. After electrophoresis for 67 Vh, the gels were stained with $AgNO_3$ in the PhastSystem development unit by the method of Butcher and Tomkins (15).

Activity staining (zymogram). The activity staining was performed as described previously (16): Solution A contained 20 mg of α-naphthyl acetate dissolved in 5 mL acetone, to which 50 mL of 50 mM potassium phosphate buffer, pH 7, was added. Solution B consisted of 50 mg Fast Red salt in 50 mL of the same buffer. The solutions were freshly prepared and mixed 1:1 immediately before incubation of the gels in the reaction on a gel shaker for 5 to 15 min. A red color indicated regions of hydrolase activity.

Hydrolytic activity of lipase. The hydrolytic activity of lipases was tested with 5% (wt/vol) oil emulsion or 5 mM pure triglyceride emulsion (pH 8.0) that contained 2% (wt/vol) gum arabic at 37°C. To 20 mL of the emulsion, 470 µL of $CaCl_2$ solution 22% (wt/vol) and 50 µL of lipase solution (6 mg diluted in 2 mL of 50 mM phosphate buffer at pH 8.0) were mixed, and the liberated fatty acid was titrated automatically with 0.1 N NaOH to maintain a constant pH at 8.0. One unit (U) of lipase activity was defined as the amount of enzyme that liberates 1 µmol fatty acid per minute under assay conditions. The remaining activity was determined after 120 h. The reaction mixture was filtered, and the lipase was washed with chilled acetone, followed by the hydrolytic assay as described above. Remaining activity was expressed as percentage of the activity of fresh lipase.

Immobilization of lipase. Crude lipase solution (2 mg/mL) (3 mL) from *C. viscosum* (crude 2), dissolved in 50 mM potassium phosphate buffer, pH 8.0, was mixed with 1 g of Hyflo Super Cel (Fluka, Buchs, Switzerland, initially heated at 80°C for activation) and gently stirred for 30 min at 5°C. The suspension was dried at room temperature under vacuum for 48 h, assayed as described above, and stored at 5°C until use.

Enzymatic reaction system in organic solvent. Interesterification between peanut oil and tricaprylin was initiated by adding 10% (w/w total triglyceride) immobilized lipase to 3 mL *n*-hexane, which contained 0.6 mmol peanut oil and 0.43 mmol tricaprylin. The temperature was maintained at 40°C, and the mixture was stirred magnetically at 800 rpm. An aliquot of the reaction medium was applied to a kieselgel 60 plate, 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 heated at 150°C.

pH of the reaction medium. A volume of 0.1 mL of the reaction mixture was taken periodically and stirred with 10 mL deionized water. Then the pH of the reaction mixture was measured (17). All samples were measured in triplicate.

Determination of free fatty acid composition. Quantitative analysis of free caprylic (8:0), stearic (16:0), oleic (18:1), and linoleic (18:2) acids generated during the reaction was performed by gas–liquid chromatography (GLC; Fisons Instruments Mega Series, Mainz, Germany). The reaction mixture (100 µL), after evaporation of *n*-hexane (no loss of caprylic acid was observed during this step), was dissolved in *n*-heptane (1 mL) in a closed test tube, and 200 µL methanol containing 20% hydrochloric acid was added. The mixture was shaken in a water bath at 85°C. After 15 min, the tube was removed from the bath and centrifuged (18). One µL was taken from the supernatant for GLC analysis on a polar column (25 m × 0.53 mm i.d.; Macherey & Nagel, Düren, Germany). Under these conditions, only free fatty acids will react; partial glycerides and triglycerides will not be cleaved and con-

verted to methyl esters (18). Analysis was carried out with temperature programming from 150 to 210°C at 5°C/min, 200°C as injection temperature, and 210°C as detector temperature (flame-ionization detector).

High-performance liquid chromatography (HPLC) separation of triacylglycerols. The composition of the triacylglycerols formed during an enzymatic interesterification was characterized by HPLC (19) with a nucleosil C_{18} column (5 µm, 250 × 4 mm; Sykam, Gilching, Germany) and an evaporative light-scattering detector (S.E.D.E.R.E, Vitry/Seine, France) at a column temperature of 50°C and a flow rate of 1.5 mL/min. Elution was performed with a linear gradient elution system of 100% acetonitrile/isooctane (90:10, vol/vol) to 26% acetonitrile/ethanol/isooctane (40:35:25, vol/vol/vol) over 45 min. The extent of reaction was calculated from the weight percentage of produced triglycerides present in the mixture. Because isomers of MLM (namely, MML and LMM) and LML (namely, LLM and MLL) cannot be separated, the data given in Table 4 refer to the total amount of triglycerides of either the MLM or LML type.

RESULTS AND DISCUSSION

Screening of lipases for positional and chainlength specificity. A lipase for the synthesis of ST by interesterification should catalyze ester exchange of both long- and medium-chain fatty acids at comparable rates and should exhibit 1,3-regiospecificity. Reactions were performed with commercially available lipases, without any pretreatment and after immobilization, first with pure triglycerides, to simplify the interpretation of data, and finally on natural vegetable oils as substrates.

We first tested the lipases for their abilities to hydrolyze pure MMM (tricaprylin, tricaprin) and LLL (triolein) (Table 1). The highest activity was observed with lipase (crude 2) from *C. viscosum,* followed by lipase (crude 1) from *C. viscosum*, crude lipases from *P. fluorescens,* and *R. miehei* for all three substrates used. Lipases from *Rhizopus* sp., *H. lanuginosa*, *C. cylindracea*, and lipase OF showed a higher specificity for tricaprylin and tricaprin rather than for triolein. With lipase from *G. candidum*, the hydrolytic activity was low on both pure MMM and triolein.

A similar pattern was found with vegetables oils as substrates (Table 2). Lipase (crude 2) from *C. viscosum* showed again the highest activity on both substrates: medium-chain triglycerides, such as coconut oil, and long-chain triglycerides, such as cocoa butter, olive oil and peanut oil. For crude lipases from *C. viscosum* (crude 1), *Rhizopus* sp., *P. fluorescens*, and *R. miehei*, the hydrolytic activity on fats mentioned above was also relatively high. For most cases, the hydrolytic activity decreased from cocoa butter to peanut oil, except for crude lipases from *C. viscosum*.

To clarify whether these differences in hydrolytic activity are caused by lipase specificity alone or stem from contaminating enzymes, we separated the proteins by PAGE and stained for hydrolytic activity in a zymogram (Table 3). The samples contained <10 wt% protein that was usually a mixture of several proteins. Moreover, hydrolase activity staining showed a single hydrolase for only the lipase from *C. viscosum*. Lipase from *G. candidum* showed no hydrolase activity with this stain, which may explain its low activity shown in Tables 1 and 2.

For the synthesis of ST in organic solvents, we chose lipases from *C. viscosum* (crude 2), *R. miehei,* and *C. cylindracea* because they were the most pure and active lipases. To reduce enzyme deactivation, we immobilized the lipase from *C. viscosum* (CVL, crude 2) on Hyflo Super Cel and used the commercially available immobilized enzymes from *R. miehei* (RML, Lipozyme) and *Candida* sp. (CSL, SP382).

Interesterification of mixed triglycerides by immobilized lipases in n-*hexane.* All three lipases catalyzed efficient interesterification of peanut oil and tricaprylin, and equilibrium was reached usually after about 30 h (Fig. 1). RML (Lipozyme) gave the best yield of ST (31% MLM, 42% LML) at 40°C in spite of its low hydrolytic activity (see section above). CSL and CVL gave similar yields for MLM, but lower amounts of LML. We expected a higher activity of CVL in the interesterification reaction owing to the high activity observed in the hydrolysis reaction of both long- and medium-chain triglycerides. In addition, with olive oil as sub-

TABLE 1
Initial Hydrolytic Activity of Commercial Lipases on Pure Triglycerides (5 mM)

Lipase from	Activity (U/mg)		
	Tricaprylin	Tricaprin	Triolein
Rhizopus sp.	15	13	3
Rhizomucor miehei	17	13	17
Humicola lanuginosa	6	6	1
Pseudomonas fluorescens	31	30	15
Candida cylindracea	7	14	1
C. cylindracea OF	14	6	2
Geotrichum candidum GC	<1	<1	<1
Chromobacterium viscosum (crude 1)	33	35	32
C. viscosum (crude 2)	111	95	106

TABLE 2
Initial Hydrolytic Activity of Commercial Lipases on Vegetable Oils (5% wt/vol)

Lipase from	Activity (U/mg)			
	Coconut oil	Cocoa butter	Olive oil	Peanut oil
Rhizopus sp.	32	37	32	14
Rhizomucor miehei	21	26	16	14
Humicola lanuginosa	9	15	10	6
Pseudomonas fluorescens	32	32	30	17
Candida cylindracea	20	30	11	4
C. cylindracea OF	21	20	14	13
Geotrichum candidum GC	5	13	8	9
Chromobacterium viscosum (crude 1)	65	45	45	46
C. viscosum (crude 2)	247	269	154	179

TABLE 3
Protein Content, Purity and Activity Staining (zymogram) of Commercial Crude Lipase Preparations

Lipase from	Protein content (%)	PAGE SDS[a] (kd)	PAGE Native[b]	Zymogram[c]
Rhizopus sp.	4.8	43,67	10	4
Rhizomucor miehei	3.5	25	5	2
Humicola lanuginosa	2.9	20,30	4	2
Pseudomonas fluorescens	1.2	14,25,40,43	6	3
Candida cylindracea	4.2	20,30,43,67,90	13	2
C. cylindracea OF	6.4	43,67	11	2
Geotrichum candidum	4.3	67	3	0
Chromobacterium viscosum (crude 1)	1.1	17,30,40	3	1
C. viscosum (crude 2)	9.5	17,30,40	2	1

[a]Molecular weight of principal bands; SDS, sodium dodecyl sulfate.
[b]Number of bands on native polyacrylamide gel electrophoresis (PAGE).
[c]Bands from activity staining using α-naphthyl acetate and Fast Red.

strate, immobilized CVL showed even higher activity (73 U) compared to the soluble lipase (46 U) and RML (16 U) or CSL (4 U) (Tables 2 and 4). Hyflo Super Cel, a diatomaceous earth support used for immobilization of CVL, has been reported previously (18) to be a suitable carrier for interesterification reactions. Here, it did not result in a high reaction rate compared to Duolite, which was used for the immobilization of CSL (SP382) and RML (Lipozyme).

In the literature, almost quantitative incorporation of a short-chain fatty acid into a triglyceride was reported (12,13). The lower amounts of ST formed in the interesterification reaction between peanut oil and tricaprylin, reported in this paper, may be attributed to (i) the use of a natural oil that consisted of several other types of fatty acids, which may lead to lower reaction rates, e.g., by inhibition of the lipase, and (ii) the different type of reaction (interesterification between two triglycerides vs. esterification between a triglyceride and a free fatty acid). The interesterification proceeds *via* several steps, e.g., fatty acids from peanut oil as well as tricaprylin must be released before subsequent esterification to ST can occur. Thus, we also determined the formation of free fatty acids as described in the following section.

pH of the reaction media. During the course of interesterification, released fatty acids may lower the pH in the microenvironment of the lipase, thus affecting its activity. Because the reaction was performed in *n*-hexane, the pH of the reaction mixture was measured indirectly by mixing samples of the reaction mixture with deionized water as described in

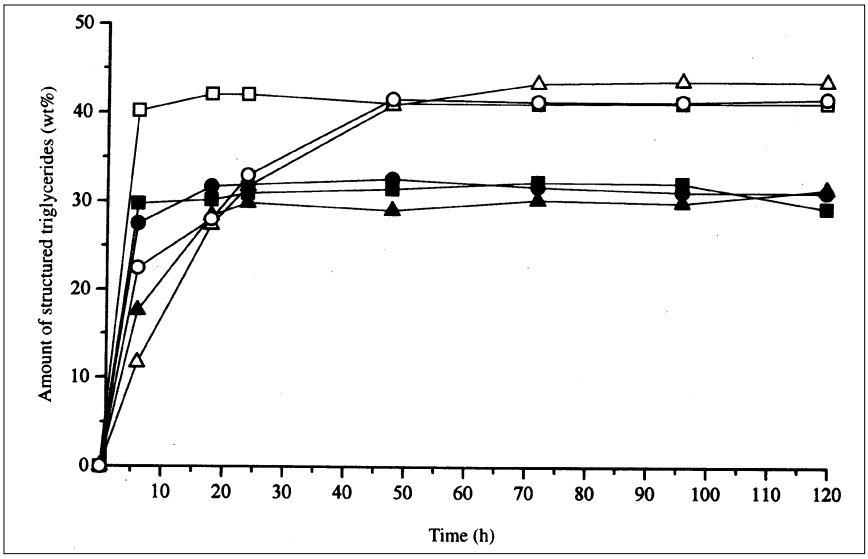

FIG. 1. Time course of the interesterification between tricaprylin (MMM) and peanut oil (LLL) triglycerides (1:1.4, mol/mol) with immobilized RML (Lipozyme) (■, □), CSL (*Candida* sp.) (SP 382) (▲, △) and CVL (*Chromobacterium viscosum*) (crude 2), (●, ○). Solid symbols refer to the total amount of MLM, MML, and LMM; open symbols refer to the total amount of LML, LLM, and MLL where M = medium-chain fatty acids and L = long-chain fatty acids.

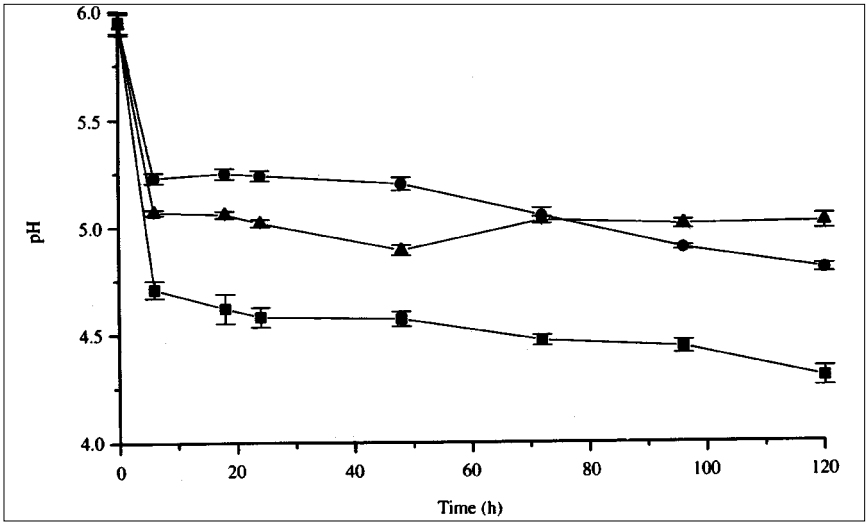

FIG. 2. Changes in pH of reaction medium of interesterified peanut oil and tricaprylin by immobilized *Chromobacterium viscosum* lipase (▲), *Rhizomucor miehei* lipase (■), and *Candida* sp. lipase (●).

the Materials and Methods section. The pH decreased rapidly from pH 5.9 (at t = 0) to pH 5.0 (CVL), pH 4.8 (CSL), and pH 4.3 (RML) after 120 h (Fig. 2). This observation was supported by the determination of the free fatty acid content for 8:0, 16:0, 18:0 and 18:2, determined from samples of the reaction mixture (Fig. 3A–C). Usually, significantly higher amounts of caprylic acid (black columns) were released compared to long-chain fatty acids. For RML (Fig. 3A), the difference between the concentration of caprylic acid and long-chain fatty acids was the most balanced. After reaction times above 48 h, no significant change in the free fatty acid concentration is observed, which also correlates with the equilibrium observed in the formation of ST (Fig. 1).

Influence of temperature on the stability of lipase. The extent of reaction was examined at two temperatures (40 and 50°C) for the three immobilized lipases. As shown in Table 4, the amount of synthesized ST increased with all lipases as the temperature was raised to 50°C. The remaining activity after 120 h reaction time was higher for lipase CSL at 50°C (81%) compared to the reaction at 40°C (71%) (Fig. 4). One may speculate that this is related to its high heat stability and activity above 50°C, as indicated in the data sheet supplied by the manufacturer. For CVL, the remaining activity was 48% at 40°C; however, it dropped to 21% at 50°C. Although RML gave the highest amount of ST, the remaining activity was low at 40°C (20%) and 50°C (17%).

TABLE 4
Enzymatic Interesterification of a Mixture of Tricaprylin and Peanut Oil (1:1.4, mol/mol)[a]

	IHA[b]	ST[c] at 40°C		ST[c] at 50°C	
Lipase	(U/mg)	MLM[d]	LML[e]	MLM[d]	LML[e]
RML	16	31	42	35	44
CVL	73	32	33	32	39
CSL	4	28	27	31	37

[a]In *n*-hexane after 24 h with immobilized RML (Lipozyme; *Rhizomucor miehei*), CVL (*Chromobacterium viscosum*), and CSL (SP 382; *Candida* sp.).
[b]Initial hydrolytic activity in olive oil pH-stat assay.
[c]ST: structured triglyceride.
[d]Includes MLL and LMM.
[e]Includes MLL and LLM where M = medium-chain fatty acid and L = long-chain fatty acid.

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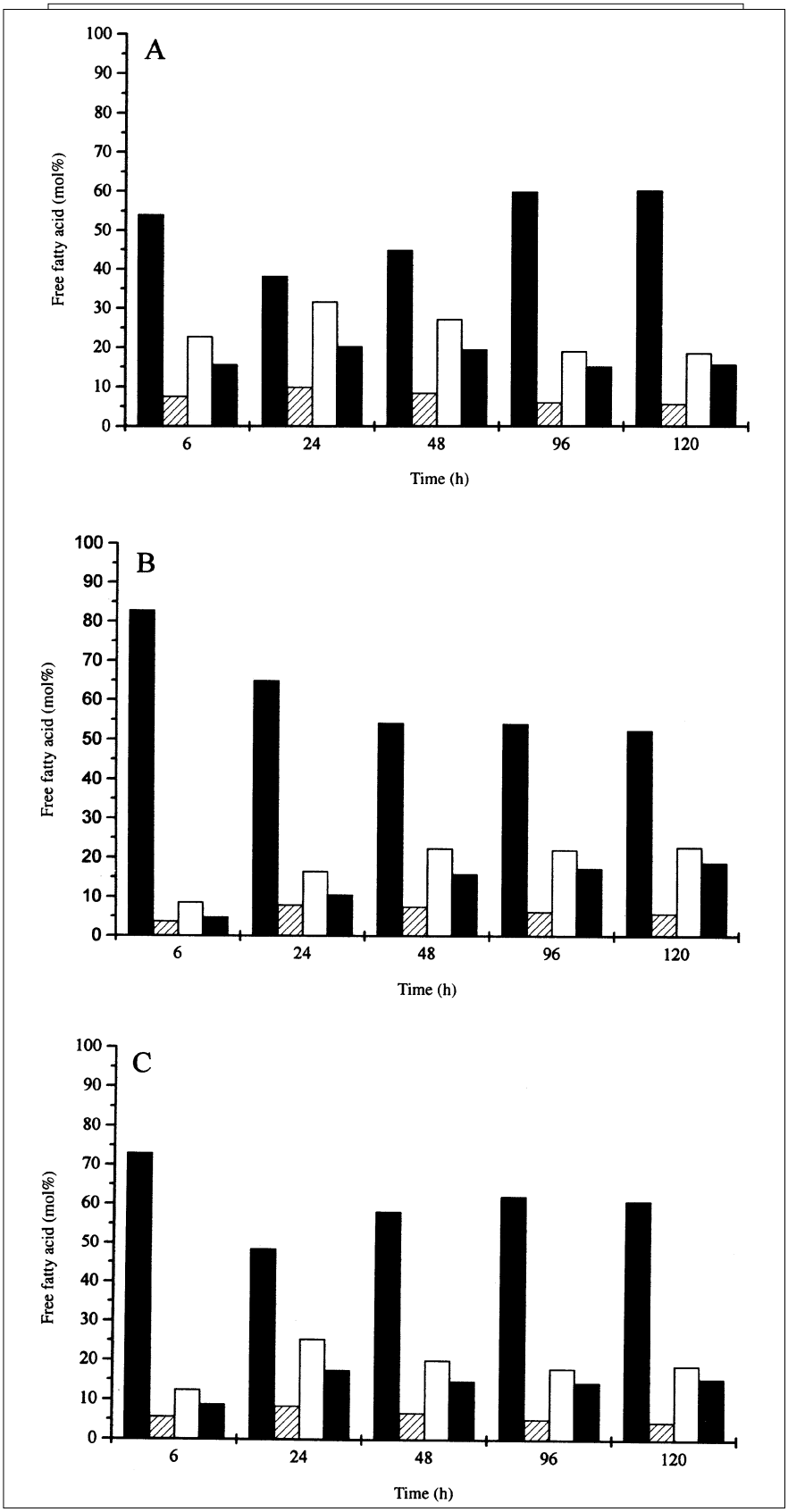

FIG. 3. Changes in the concentration of free fatty acids during the lipase-catalyzed interesterification between peanut oil and tricaprylin in *n*-hexane as determined by gas chromatography. A: *Rhizomucor miehei* lipase, B: *Candida* sp. lipase, C: *Chromobacterium viscosum* lipase, 8:0, solid columns; 16:0, hatched columns; 18:1, open columns; 18:2, dotted columns.

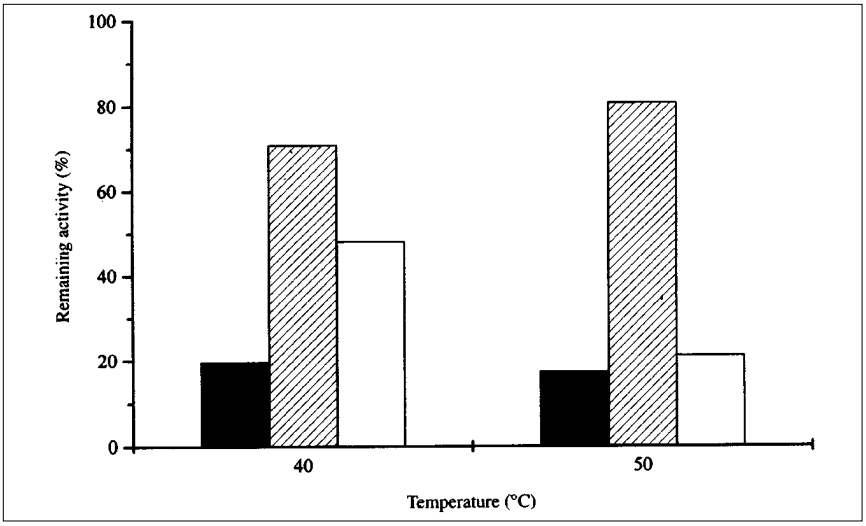

FIG. 4. Remaining activity of immobilized lipases at 40 and 50°C after 120 h reaction time; *Rhizomucor miehei* lipase, solid columns; *Candida* sp. lipase, hatched columns; *Chromobacterium viscosum* lipase, open columns.

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