Enzymatic Synthesis of High-Purity Structured Lipids with Caprylic Acid at 1,3-Positions and Polyunsaturated Fatty Acid at 2-Position

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ABSTRACT: We attempted to synthesize high-purity structured triacylglycerols (TAG) with caprylic acid (CA) at the 1,3-positions and a polyunsaturated fatty acid (PUFA) at the 2-position by a two-step enzymatic method. The first step was synthesis of TAG of PUFA (TriP), and the second step was acidolysis of TriP with CA. *Candida antarctica* lipase was effective for the first reaction. When a reaction medium of PUFA/glycerol (3:1, mol/mol) and 5% immobilized *Candida* lipase was mixed for 24 h at 40°C and 15 mm Hg, syntheses of TAG of γ-linolenic, arachidonic, eicosapentaenoic, and docosahexaenoic acids reached 89, 89, 88, and 83%, respectively. In these reactions, the lipase could be used for at least 10 cycles without significant loss of activity. In the second step, the resulting trieicosapentaenoin was acidolyzed at 30°C for 48 h with 15 mol parts CA using 7% of immobilized *Rhizopus delemar* lipase. The CA content in the acylglycerol fraction reached 40 mol%. To increase the content further, the acylglycerols were extracted from the reaction mixture with *n*-hexane and were allowed to react again with CA under conditions similar to those of the first acidolysis. After three successive acidolysis reactions, the CA content reached 66 mol%. The content of dicapryloyl-eicosapentaenoyl-glycerol reached 86 wt% of acylglycerols, and the ratio of 1,3-dicapryloyl-2-eicosapentaenoyl-glycerol to 1(3),2-dicapryloyl-3(1)-eicosapentaenoyl-glycerol was 98:2 (w/w). In this reaction, the lipase could be used for at least 20 cycles without significant loss of activity. Repeated acidolysis of the other TriP with CA under similar conditions synthesized 1,3-dicapryloyl-2-γ-linolenoyl-glycerol, 1,3-dicapryloyl-2-arachidonoyl-glycerol, and 1,3-dicapryloyl-2-docosahexaenoyl-glycerol in yields of 58, 87, and 19 wt%, respectively.

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Polyunsaturated fatty acids (PUFA) have various physiological functions. Eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA) play a role in the prevention of a number of human diseases (1–3). The ethyl ester of EPA has been used in the treatment of arteriosclerosis obliterans and hyperlipemia (4), and oil containing 50% DHA has been used as a health food (5). γ-Linolenic acid (18:3n-6; GLA) shows the physiological functions of modulating atopic eczema (6) and rheumatoid arthritis (7), and arachidonic acid (20:4n-6; AA) and DHA are known to be important for preterm infants (8,9).

A structured triacylglycerol (TAG) containing medium-chain fatty acids at the 1,3-positions and a long-chain fatty acid at the 2-position (MLM-type) is absorbed into intestinal mucosa more rapidly than natural oils composed of only long-chain fatty acids (LLL-type) (10,11). These reports have focused a good deal of attention on the physiological activity of MLM-type structured lipids. To evaluate the nutraceutical effects, the ability to synthesize high-purity MLM-type structured lipid on a laboratory scale is necessary. It was recently reported that 1,3-capryloyl-2-eicosapentaenoyl-glycerol (CEC) can be synthesized by interesterification of trieicosapentaenoin (TriE) with ethyl caprylate (EtCA) using immobilized *Rhizomucor miehei* lipase (12). However, 100 mol equiv of EtCA per mol of TriE was used, and the CEC content in the reaction mixture was only 3 wt%. Hence, molecular distillation and/or preparative high-performance liquid chromatography (HPLC) was required for the separation of TAG and fatty acid ethyl esters. Meanwhile, acidolysis of TriE with caprylic acid (8:0, CA) has an advantage that the synthesized CEC and free fatty acids (FFA) can be separated easily by extraction with *n*-hexane.

This paper presents the synthesis of high-purity CEC by acidolysis of TriE (made from glycerol and EPA) with CA. In addition, it shows that MLM-type structured lipid containing AA can be synthesized in good yields.

MATERIALS AND METHODS

Chemicals. EPA and DHA were gifts from Maruha Corp. (Tokyo, Japan) and their purities were 95 and 92%, respectively. AA (purity, 91%) was donated by Suntory Ltd. (Osaka, Japan). GLA was purified by an enzymatic method described previously (13), and the resulting purity was 98%. The molar amount of PUFA was calculated on the basis of its acid value. CA (purity, 98%) and EtCA (purity, 98%) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). The other chemicals were of reagent grade.

Lipases. Immobilized *Candida antarctica* lipase (Novozym 435) and immobilized *R. miehei* lipase (Lipozyme RMIM) were products of Novozymes (Bagsvaerd, Denmark). *Rhizopus delemar* lipase (Ta-lipase; Tanabe Seiyaku Co. Ltd., Osaka, Japan) was immobilized on a weak anion exchange resin, Dowex WBA

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(Dow Chemical Co., Midland, MI), as described previously (14). After 10 g Dowex WBA was suspended in 8 mL of *Rhizopus* lipase solution (125 mg/mL; 6,100 units/mL), immobilized lipase was prepared by drying under reduced pressure. To activate the immobilized lipase preparation, pretreatment was performed as described elsewhere (14,15). A mixture containing substrates, 2% water, and 7% immobilized *Rhizopus* lipase was incubated at 30°C for 48 h with shaking at 130 oscillations per minute. The substrates used here were PUFA-TAG (TriP)/CA or TriP/EtCA. The subsequent reactions were conducted by transferring the immobilized lipase into the same amount of substrate mixture without water, followed by shaking under the same conditions as those for the pretreatment. The immobilized lipase obtained from the above treatment was chosen for transesterification of TriP with CA or EtCA. Immobilized *Candida* and *Rhizomucor* lipases were used without any pretreatment.

Reactions. TriP was synthesized in a 100-mL flask fitted to a rotary evaporator under reduced pressure (15 mm Hg). Standard reaction mixtures were composed of 5 g PUFA/glycerol (3:1, mol/mol) and 250 mg immobilized *Candida* lipase, and the reaction was performed at 40°C for 24 h with complete mixing by rotation.

Transesterification reactions were carried out in 7-mL screw-capped vessels. Acidolysis of TriP with CA was conducted at 30°C for 48 h in a reaction mixture of 4 g TriP/CA (1:15, mol/mol) and 280 mg immobilized *Rhizopus* or *Rhizomucor* lipase. Repeated acidolysis was performed as follows. TAG were extracted from the reaction mixture with 50 mL *n*-hexane under alkaline conditions (16). The resulting hexane layer was washed with 40 mL of 0.5 N KOH (20% ethanol solution) to completely remove the remaining FFA. The extracted TAG were subjected to acidolysis with 15 mol parts CA under conditions similar to those in the first reaction. Interesterification of TriP with EtCA was performed at 30°C for 48 h in a mixture of 4 g TriP/EtCA (1:15 mol/mol) and 280 mg immobilized lipase with shaking.

To obtain a large amount of reactant, esterification and transesterification were repeated by transferring immobilized lipases to fresh substrates.

Purification of TriP. Esterification of PUFA with glycerol was repeated several cycles using the same immobilized *Candida* lipase. Twenty grams of the reaction mixture (*ca.* 90% esterification) was applied to a silica gel 60 column (120 g; 30 × 390 mm; Merck, Darmstadt, Germany), and TriP was eluted with a mixture of *n*-hexane/ethyl acetate (98:2, vol/vol).

Analyses. Monoacylglycerol (MAG), diacylglycerol (DAG), and TAG were extracted with 100 mL *n*-hexane after adding 70 mL of 0.5 N KOH (20% ethanol solution) to 5–10 g of the reaction mixture (16). FFA contaminating the solvent layer were completely removed by washing with 40 mL 0.5 N KOH (20% ethanol solution). Fatty acids in the acylglycerols were transmethylated in methanol with Na-methylate as a catalyst. The methyl esters were analyzed with a Hewlett-Packard 5890 gas chromatograph (Avondale, PA) with a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) as described previously (16).

MAG, DAG, TAG, and FFA were quantified with a thin-layer chromatograph (TLC)/flame-ionization detector analyzer (Iatroscan MK-5; Iatron Co., Tokyo, Japan) after development with a mixture of benzene/chloroform/acetic acid (50:20:0.7, by vol).

Acylglycerols were analyzed on two octadecyl silica (ODS) columns (4.6 × 150 mm, Cosmosil 5C18-AR; Nakalai Tesque Inc., Kyoto, Japan) connected to a high-performance liquid chromatography (HPLC) system (LC-9A; Shimadzu Co., Kyoto, Japan) with a refractometer as described previously (15). The mobile phase of acetone/acetonitrile (1:1, vol/vol) was used at a flow rate of 0.4 mL/min and 40°C. The positional isomers of dicapryloyl-eicosapentaenoyl-glycerol (diCE) were analyzed on a Chrompack silver ion chromatography column (4.6 × 250 mm; Chrompack, Middelberg, Netherlands) as described by Irimescu *et al*. (12).

Water contents of the reaction mixtures were determined by Karl Fischer titration (Moisture Meter CA-07; Mitsubishi Chemical Corp., Tokyo, Japan).

RESULTS AND DISCUSSION

We attempted to synthesize structured lipids with PUFA at the 2-position and CA at the 1,3-positions by a two-step enzymatic method. The first step was synthesis of TriP by esterification of glycerol with PUFA, and the second step was conversion of TriP to the desired structured lipids by acidolysis of TriP with CA.

Effects of dehydration and temperature on synthesis of TriE. EPA was esterified for 24 h at 30–50°C and 15 mm Hg with 1/3 mol equiv glycerol using 5% immobilized *Candida* lipase or 10% immobilized *Rhizomucor* lipase (Table 1). When immobilized *Candida* lipase was used, TriE synthesis depended slightly on the reaction temperature. The reaction at 40°C produced 89 wt% TriE at 96% esterification, and the TriE content did not increase at 50°C. Esterification with 1,3-positionally specific *Rhizomucor* lipase under reduced pressure was also effective for the synthesis of TriE, and the TriE content reached 86 wt% at 94% esterification at 50°C. *Rhizomucor* lipase produces 1,3-dieicosapentaenoin (1,3-DiE), and the spontaneous acyl migration of 1,3-DiE to 1(3),2-DiE accelerates at a higher temperature (17–19). TriE synthesis can be explained by esterification of 1(3),2-DiE with EPA.

The esterifications of EPA with glycerol at ambient pressure using 5% *Candida* lipase (40°C) and 10% *Rhizomucor* lipase (50°C) proceeded to lower esterification extents, 59 and 77%, respectively. The TriE contents in the reaction mixtures were only 27 and 37%, respectively. When EPA is completely esterified in a mixture of EPA/glycerol (3:1, mol/mol), the water content in the reaction mixture becomes 5.4%. Because the free water content in the 24-h reaction mixture was 1900 ppm, most of the generated water could be bound to immobilized lipase at ambient pressure. The free water content in the reaction at 15 mm Hg was only 250 ppm. Thus, the efficiency of esterification of EPA with glycerol was remarkably increased by removing the generated water under reduced pressure.

Effect of EPA concentration on synthesis of TriE. TriE synthesis was conducted for 24 h at 40°C and 15 mm Hg in a mix-

TABLE 1
Effects of Dehydration Condition and Temperature on Synthesis of Trieicosapentaenoin with Immobilized *Candida antarctica* or *Rhizomucor miehei* Lipases

Lipase	Reaction conditions	Composition[a] (wt%)				
		TriE	1,3-DiE	1(3),2-DiE	MonoE	EPA
Candida[b]	30°C, 15 mm Hg	79.3	6.2	2.3	2.9	9.3
	40°C, 15 mm Hg	88.9	3.8	1.7	1.3	4.3
	40°C, 780 mm Hg	26.9	8.1	5.9	17.6	41.5
	50°C, 15 mm Hg	90.0	3.0	1.2	1.0	4.8
Rhizomucor[c]	30°C, 15 mm Hg	61.3	18.0	3.3	2.1	15.3
	40°C, 15 mm Hg	74.3	11.9	2.1	1.1	10.6
	50°C, 15 mm Hg	85.7	5.8	1.9	1.0	5.6
	50°C, 780 mm Hg	37.4	16.5	6.9	5.8	33.4

[a]TriE, trieicosapentaenoin; DiE, dieicosapentaenoin; MonoE, monoeicosapentaenoin.; EPA, eicosapentaenoic acid.
[b]Reaction mixture: 5 g EPA/glycerol (3:1, mol/mol) and 0.25 g immobilized *Candida* lipase.
[c]Reaction mixture: 5 g EPA/glycerol (3:1, mol/mol) and 0.5 g immobilized *Rhizomucor* lipase.

ture of glycerol, 3 to 4 mol parts EPA, and 5% immobilized *Candida* lipase (Table 2). In the reaction with 3 mol parts EPA, 95% of EPA was esterified, and the TriE content in the acylglycerol fraction reached 93 wt%. The TriE content did not increase when the esterification was conducted with more than 3 mol parts EPA. The EPA amount was thus fixed at 3 mol equiv over glycerol.

Time course of TriE synthesis. TriE was synthesized at 40°C and 15 mm Hg in a mixture of 5 g EPA/glycerol (3:1, mol/mol) and 250 mg immobilized *Candida* lipase (Fig. 1). The content of 1,3-DiE increased concomitant with the decrease of EPA in the early stage of the reaction. The 1,3-DiE content reached a maximum level, 54 wt%, after 4 h, and gradually decreased thereafter. The TriE content increased after a 2-h lag, and 1(3),2-DiE and monoeicosapentaenoin (MonoE) were present in only small amounts throughout the reaction. The reaction reached steady state after 24 h, and the TriE content attained 90 wt% at 95% esterification.

The product sheet from the supplier indicates that the positional specificity of immobilized *Candida* lipase depends on the reaction. In the esterification of EPA with glycerol, 1,3-DiE accumulated in the reaction mixture, but 1(3),2-DiE was present in only a small amount. TriE synthesis with *Candida* lipase proceeded efficiently at lower temperature than that with 1,3-positionally specific *Rhizomucor* lipase. These findings suggest that the lipase prefers the 1,3-positions under our reaction conditions.

Stability of immobilized Candida *lipase*. To investigate the stability of the immobilized enzyme preparation, esterification of EPA with glycerol was conducted under the conditions determined. The reaction was repeated by transferring the immobilized lipase into a fresh substrate mixture every 24 h. In the first reaction cycle, the TriE contents in 6- and 24-h reaction mixtures were 42 and 91 wt%, respectively. Even after the reaction had been repeated 10 cycles, the TriE contents did not decrease; that is, 43 wt% (6-h reaction), 90 wt% (24-h reaction). The immobilized *Candida* lipase preparation was stable under the reaction conditions used in this study.

Syntheses of several TriP. TAG of GLA, AA, and DHA are abbreviated as TriG, TriA, and TriD, respectively. We attempted syntheses of TriP using GLA, AA, and DHA as substrates instead of EPA under similar conditions (Table 3). Esterification rates of AA and EPA by *Candida* lipase were almost identical. The rate of TriG synthesis was slightly higher, and that of TriD synthesis was slightly lower. The TriG, TriA, and TriE contents attained nearly 90 wt% after 24 h at 95% esterification, and the TriD content was 83% at 92% esterification. *Candida* lipase acted strongly on these PUFA.

Synthesis of TriP was repeated several cycles using the same immobilized *Candida* lipase. The reaction mixtures

TABLE 2
Effect of Eicosapentaenoic Acid Concentration on Synthesis of Trieicosapentaenoin with Immobilized *Candida* Lipase[a]

EPA/glycerol (mol/mol)	EPA Remaining (wt%)	Acylglycerol composition (wt%)			
		TriE	1,3-DiE	1(3),2-DiE	MonoE
3:1	4.6	92.5	4.8	1.6	1.1
3.5:1	18.6	91.5	5.9	1.4	1.2
4:1	28.9	92.3	4.9	1.2	1.6

[a]A mixture of EPA/glycerol and 5% immobilized lipase was shaken at 40°C and 15 mm Hg for 24 h. See Table 1 for abbreviations.

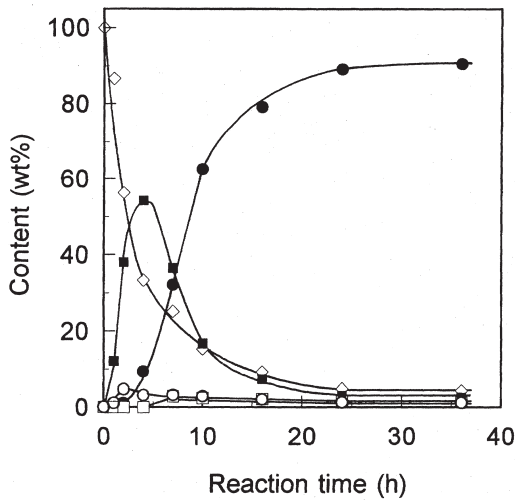

FIG. 1. Time course of trieicosapentaenoin synthesis with immobilized *Candida antarctica* lipase. A mixture of 5 g eicosapentaenoic acid/glycerol (3:1, mol/mol) and 5% immobilized lipase was shaken at 40°C and 15 mm Hg. An aliquot of the reaction mixture (*ca.* 50 mg) was periodically withdrawn to analyze the contents of acylglycerols and free fatty acid. ●, Trieicosapentaenoin; ■, 1,3-dieicosapentaenoin; □, 1(3),2-dieicosapentaenoin; ○, monoeicosapentaenoin; ◇, eicosapentaenoic acid.

TABLE 3
Syntheses of Triacylglycerols of Several Polyunsaturated Fatty Acids (PUFA)[a]

PUFA[b]	Reaction time (h)	Composition (wt%)				
		TAG	1,3-DAG	1(3),2-DAG	MAG	FFA
GLA	4	19.3	44.4	0.8	4.3	31.2
	10	64.8	16.2	2.3	2.8	13.9
	24	89.1	4.0	1.5	1.1	4.3
AA	4	9.6	52.5	ND	4.1	33.8
	10	61.9	17.6	2.4	3.8	14.3
	24	88.8	3.4	1.3	1.9	4.6
EPA	4	9.9	57.0	ND	2.3	30.8
	10	65.7	17.7	2.5	2.0	12.1
	24	88.4	3.8	1.8	1.4	4.6
DHA	4	5.6	54.0	ND	5.1	35.3
	10	44.0	27.6	2.0	4.0	22.4
	24	82.8	5.8	1.0	2.9	7.5

[a]A mixture of PUFA/glycerol (3:1, mol/mol) and 5% immobilized *Candida* lipase was shaken at 40°C and 15 mm Hg.
[b]TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; FFA, free fatty acid; GLA, γ-linolenic acid; AA, arachidonic acid; ND, not detected; DHA, docosahexaenoic acid. See Table 1 for other abbreviation.

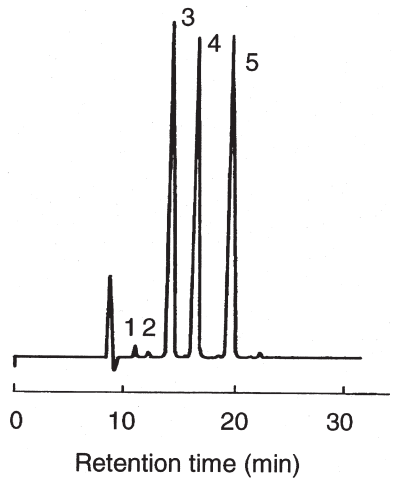

FIG. 2. High-performance liquid chromatogram of acylglycerols obtained by acidolysis of trieicosapentaenoin with caprylic acid. The acidolysis was conducted as follows: a mixture of 5 g trieicosapentaenoin/caprylic acid (1:15, mol/mol) and 350 mg immobilized *Rhizopus delemar* lipase was shaken at 30°C for 48 h. Peak 1, diacylglycerols; peak 2, tricaprylin; peak 3, dicapryloyl-eicosapentaenoyl-glycerol; peak 4, capryloyl-dieicosapentaenoyl-glycerol; peak 5, trieicosapentaenoin.

from several batches were combined, and TriP was purified by silica gel column chromatography described in the Materials and Methods section (TriP recovery, >90%).

Analysis of products obtained by transesterification of TriE with CA or EtCA. Figure 2 shows a typical chromatogram of the acylglycerols extracted from a 48-h reaction mixture of acidolysis of TriE with CA using immobilized *Rhizopus* lipase. Peak 3 (14.3 min), peak 4 (16.7 min), and peak 5 (19.8 min) were identified to be diCE, capryloyl-dieicosapentaenoyl-glycerol (CdiE) and TriE, respectively, from the fatty acid compositions. The retention time of peak 2 (12.3 min) coincided with that of tricaprylin (TriC). Peak 1 (11.3 min) was shown to be DAG from TLC analysis, and the fatty acid composition suggested that the peak was composed of dicaprylin, dieicosapentaenoin, and capryloyl-eicosapentaenoyl-glycerol. The reaction mixture after interesterification of TriE with EtCA was also analyzed by HPLC, but because DAG co-eluted with the ethyl ester of EPA, the contents of DAG were not determined. However, the other components eluted at the retention times indicated in Figure 2. The acylglycerol contents were determined from their peak areas.

Suitable lipase for synthesis of CEC. Fatty acids at 1,3-positions of TAG can be efficiently exchanged by acidolysis with a desired fatty acid or by interesterification with a fatty acid ethyl ester, using an immobilized 1,3-positionally specific lipase (20–22). In our previous studies (15,23), acidolysis of several natural oils with CA proceeded efficiently in a mixture of TAG/CA (1:15, mol/mol). The ratio of TriE to CA or EtCA was therefore fixed at 1:15 (mol/mol) in the present study.

Transesterification of TriE with CA or EtCA was conducted at 30°C with shaking using 7% of immobilized *Rhizopus* or *Rhizomucor* lipase. Table 4 shows the acylglycerol composition as determined by HPLC on an ODS column. If the DAG content in the interesterification reaction mixture was negligible, transesterification of TriE would be more effective in interesterification than in acidolysis. However, the diCE content in the single interesterification with immobilized *Rhizomucor* lipase reached only 53 wt%. Irimescu *et al.* (12) reported recently that the diCE content in the acylglycerol fraction reached nearly 90% in interesterification of TriE with 100 mol equiv of EtCA. Although the interesterification extent was high, the diCE content in the reaction mixture was only 3 wt%. Hence, it is rather difficult to purify diCE from that reaction mixture, because molecular distillation and/or HPLC is required for the separation of TAG and ethyl esters. On the other hand, acidolysis reaction mixtures contain TAG and FFA, which are easily separated by *n*-hexane extraction. When the acidolysis with *Rhizopus* lipase was conducted for 48 h, the diCE content reached 33 wt%. Because the content was higher than that obtained by acidolysis with *Rhizomucor* lipase, we selected immobilized *Rhizopus* lipase for the synthesis of diCE by acidolysis.

Synthesis of diCE by repeated acidolyses. In acidolysis of TriE with CA using immobilized *Rhizopus* lipase, the content of diCE did not increase to more than 50 wt% even when the reaction time was extended from 2 to 4 d, or the amount of immobilized lipase was increased from 7 to 14%. To increase the yield of diCE, acylglycerols were extracted from the 48-h reaction mixture with *n*-hexane, and the resulting acylglycerols were then allowed to react again with 15 mol equiv of CA for total acylglycerols under similar conditions using the same lipase repeatedly. Table 5 shows the fatty acid composition of acylglycerols obtained by repeated acidolyses and the acylglycerol composition. After the third acidolysis reaction, the CA content of the acylglycerol fraction increased to 66 mol%. The DAG (a mixture of dieicosapentaenoin, capryloyl-eicosapentaenoyl-glycerol, and dicaprylin) and TriC contents after the first reaction were 3.1 and 1.0 wt%, respectively. The diCE content increased to 86 wt% after three repetitions, but the contents of DAG and TriC increased only slightly.

The positional isomers of the diCE obtained by repeated acidolyses were analyzed by silver ion HPLC. The ratio of

TABLE 4
Acidolysis of TriE with Caprylic Acid (CA) and Interesterification of TriE with Ethyl Caprylate (EtCA) Using Immobilized *Rhizopus delemar* or *Rhizomucor miehei* Lipases[a]

Reaction	Source of lipase	Reaction time (h)	Composition[b] (wt%)				
			DAG	TriC	diCE	CdiE	TriE
Acidolysis	*Rhizopus*	24	3.9	ND	16.0	25.9	54.2
		48	4.5	1.9	33.0	31.4	29.2
	Rhizomucor	24	4.3	1.2	6.7	22.6	65.2
		48	5.1	2.3	11.8	36.6	44.2
Interesterification	*Rhizopus*	24	—[c]	ND	27.1	32.6	40.3
		48	—	1.6	48.1	33.4	16.9
	Rhizomucor	24	—	2.9	30.6	47.2	19.3
		48	—	6.2	52.9	35.3	5.6

[a]A mixture of TriE/CA or TriE/EtCA was shaken at 30°C with 7% immobilized lipase.
[b]TriC, tricaprylin; diCE, dicapryloyl-eicosapentaenoyl-glycerol; CdiE, capryloyl-dieicosapentaenoyl-glycerol. See Tables 1 and 3 for other abbreviations.
[c]Not determined because DAG eluted with ethyl ester of EPA.

CEC to 1(3),2-dicapryloyl-3(1)-eicosapentaenoyl-glycerol (CCE) was 98:2 (wt/wt). This ratio, in combination with the fact that DAG and TriC were present in only very small amounts even after three acidolysis reactions (Table 5), indicated that spontaneous acyl migration was negligible under the reaction conditions presented in this study.

Stability of immobilized Rhizopus *lipase*. A mixture of 4 g TriE/CA (1:15, mol/mol) and 280 mg of immobilized lipase was shaken at 30°C for 48 h. The immobilized lipase was then transferred into fresh substrate mixture and incubated under the same conditions, a process repeated for 20 cycles. The CA contents in the acylglycerol fractions in the 1st-, 10th-, and 20th-cycle reactions were 37, 34, and 35 mol%, respectively. In addition, the CEC contents in their reactions were 35, 32, and 34 wt%, respectively. These results showed that immobilized *Rhizopus* lipase preparation can be used more than 20 times without significant loss of activity.

Syntheses of high-purity structured lipids with PUFA at 2-position and CA at 1,3-positions by repeated acidolyses. Purified TriG, TriA, and TriD were used as substrates for acidolysis with CA under conditions similar to those in the acidolysis of TriE (Table 6). The repeated acidolysis of TriA incorporated 64 mol% CA into acylglycerols, and the content of dicapryloyl-arachidonoyl-glycerol (diCA) was 87 wt%. These contents were almost the same as those after the repeated acidolysis of TriE. When TriG and TriD were used as substrates and their acidolyses were repeated three times, the CA contents in the acylglycerol fractions reached 53 and 31 mol%, respectively. The contents of dicapryloyl-γ-linolenoyl-glycerol (diCG) and dicapryloyl-docosahexaenoyl-glycerol (diCD) were 58 and 19 wt%, respectively. These results show that *Rhizopus* lipase acts on EPA and AA moderately, on GLA weakly, and on DHA very weakly. These fatty acid specificities agree with previous results obtained with a randomly interesterified oil as a substrate (24).

We have presented a method for synthesizing structured lipids containing CA at the 1,3-positions and PUFA at the 2-position. High-purity CEC and 1,3-dicapryloyl-2-arachidonoyl-glycerol (CAC) were synthesized in good yields. 1,3-Dicapryloyl-2-γ-linolenoyl-glycerol (CGC) was obtained in a moderate yield, and 1,3-dicapryloyl-2-docosahexaenoyl-glycerol (CDC) were synthesized in a poor yield. The procedure presented in this study may be effective for synthesizing high-purity structured lipids, especially CEC and CAC, on a laboratory scale.

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TABLE 5
Synthesis of CdiE by Repeated Acidolyses Using Immobilized *Rhizopus* Lipase[a]

Reaction	FA composition (mol%)[b]		Acylglycerol composition (wt%)				
	CA[c]	EPA	DAG	TriC	diCE	CdiE	TriE
First	39.7	57.3	3.1	1.0	33.5	32.4	30.0
Second	59.3	39.1	2.7	1.8	67.3	22.0	6.2
Third	65.8	32.8	3.2	2.5	86.4	7.9	ND

[a]The first reaction was performed at 30°C for 48 h in a mixture of TriE/CA (1:15, mol/mol) using 7% immobilized *Rhizopus* lipase. Repeated reactions were conducted by incubating the acylglycerols obtained from the first and second reactions with 15 mol parts CA to total acylglycerols using the same lipase.
[b]Fatty acid composition in acylglycerol fraction.
[c]Caprylic acid. See Tables 1 and 4 for other abbreviations.

TABLE 6
Repeated Acidolyses of PUFA-TAG with CA Using Immobilized *Rhizopus* Lipase[a]

		FA composition (mol%)		Acylglycerol composition (wt%)				
Substrate	Reaction	CA	PUFA	DAG	TriC	diCP[b]	CdiP[c]	TriP[d]
TriG[e]	First	35.3	63.4	2.2	0.5	26.2	33.2	37.9
	Second	45.9	53.1	2.8	0.9	43.7	36.5	16.1
	Third	52.6	46.5	2.4	1.3	57.7	29.1	9.5
TriA[f]	First	44.6	51.0	2.9	0.9	41.2	33.5	21.5
	Second	55.7	40.8	2.8	1.5	64.7	22.9	8.1
	Third	63.8	33.3	2.7	1.9	86.5	8.9	ND
TriE	First	41.9	55.2	2.9	1.1	34.0	32.8	29.2
	Second	57.2	40.9	3.3	1.9	68.3	20.3	6.2
	Third	66.0	32.3	3.5	2.7	85.7	8.1	ND
TriD[g]	First	16.5	76.6	0.6	ND	6.5	31.6	61.3
	Second	24.4	67.0	0.7	ND	13.0	35.2	51.1
	Third	31.1	62.6	1.1	0.5	19.0	35.2	44.2

[a]Repeated reactions were performed under conditions similar to those described in Table 5.
[b]TAG with 2 molecules of CA and 1 molecule of PUFA.
[c]TAG with 1 molecule of CA and 2 molecules of PUFA.
[d]TAG of PUFA.
[e]TAG of GLA.
[f]TAG of AA.
[g]TAG of DHA. See Tables 3 and 4 for other abbreviations.

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