Preparation of Regioisomers of Structured TAG Consisting of One Mole of CLA and Two Moles of Caprylic Acid

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ABSTRACT: TAG (MLM) with medium-chain FA (MCFA) at the 1,3-positions and long-chain FA (LCFA) at the 2-position, and TAG (LMM) with LCFA at the 1(3)-position and MCFA at 2,3(1)-positions are a pair of TAG regioisomers. Large-scale preparation of the two TAG regioisomers was attempted. A commercially available FFA mixture (FFA-CLA) containing 9-*cis*,11-*trans* (9*c*,11*t*)- and 10*t*,12*c*-CLA was selected as LCFA, and caprylic acid (C_8 FA) was selected as MCFA. The MLM isomer was synthesized by acidolysis of acylglycerols (AG) containing two CLA isomers with C_8 FA: A mixture of AG-CLA/C_8 FA (1:10, mol/mol) and 4 wt% immobilized *Rhizomucor miehei* lipase was agitated at 30°C for 72 h. The ratio of MLM to total AG was 51.1 wt%. Meanwhile, LMM isomer was synthesized by acidolysis of tricaprylin with FFA-CLA: A mixture of tricaprylin/FFA-CLA (1:2, mol/mol) and 4 wt% immobilized *R. miehei* lipase was agitated at 30°C for 24 h. The ratio of LMM to total AG was 51.8 wt%. MLM and LMM were purified from 1,968 and 813 g reaction mixtures by stepwise short-path distillation, respectively. Consequently, MLM was purified to 92.3% with 49.1% recovery, and LMM was purified to 93.2% with 52.3% recovery. Regiospecific analyses of MLM and LMM indicated that the 2-positions of MLM and LMM were 95.1 mol% LCFA and 98.3 mol% C_8 FA, respectively. The results showed that a process comprising lipase reaction and short-path distillation is effective for large-scale preparation of high-purity regiospecific TAG isomers.

Paper no. J10924 in *JAOCS 81,* 1013–1020 (November 2004).

KEY WORDS: Acidolysis, *Candida antarctica*, CLA, distillation, immobilized enzyme, lipase, regiospecific analysis, *Rhizomucor miehei*, TAG regioisomer.

Much attention has been focused on the effect of position of FA in TAG on physiological activities. Two TAG regioisomers, which have the same FA composition and carry a functional FA at the 1(3)- and 2-position, are necessary in order to study this subject. The two regioisomers have been synthesized by transesterification of long-chain TAG (LLL) with medium-chain FA (MCFA) using a 1,3-position-specific lipase and by transesterification of medium-chain TAG (MMM) with long-chain FA (LCFA) (1–9). The products can reportedly be purified by HPLC (10,11). The process comprising lipase reaction and HPLC is effective for preparing high-purity TAG regioisomers, and the products have been used for examining the intestinal absorption of TAG (12). However, animal tests involving oral administration require larger amounts of TAG regioisomers, and the purification process by HPLC is not suitable for large-scale preparation. Xu and colleagues (7,8) carried out large-scale preparation of TAG regioisomers by short-path distillation, but the resulting preparations were mixtures of MMM, MLM, and MLL because the distillation removed only FFA. Negishi *et al.* (9) purified MLM from the reaction mixture, but the content of MLM was only 76%. For these reasons, a process for preparing larger amounts of pure TAG regioisomers (purity >90%) is strongly desired.

TAG (MLM) with MCFA at the 1,3-positions and LCFA at the 2-position, and TAG (LMM) with LCFA at the 1(3)-position and MCFA at the 2,3(1)-positions, represent a pair of TAG regioisomers. To evaluate nutritional effects based on the position of FA in TAG (13–15), a FA having significant physiological activities is required. CLA is a group of C_{18} FA containing a pair of conjugated double bonds in either the *cis* or *trans* configuration. A commercially available product contains almost equal amounts of 9-*cis*,11-*trans* (9*c*,11*t*)- and 10*t*,12*c*-CLA. The mixture of the two isomers has various physiological activities, such as reduction of the incidence of cancer (16,17), decrease in body fat content (18–20), beneficial effects on atherosclerosis (21,22), and improvement of immune function (23). Hence, we selected an FFA mixture containing two CLA isomers as a functional LCFA, and caprylic acid (C_8 FA) as a MCFA.

In this paper, MLM is synthesized by acidolysis of TAG containing two CLA isomers with C_8 FA, and LMM is synthesized by acidolysis of tricaprylin with an FFA mixture containing two CLA isomers. It is also shown that the synthesized MLM and LMM can be efficiently purified from the reaction mixtures by stepwise short-path distillation.

MATERIALS AND METHODS

Materials. An FFA mixture containing 9*c*,11*t*- and 10*t*,12*c*-CLA was a commercial product (CLA-80; Nisshin Oillio, Ltd., Tokyo, Japan) obtained by alkali conjugation of safflower oil in propylene glycol. The product contained 33.9

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wt% 9*c*,11*t*-CLA, 34.8 wt% 10*t*,12*c*-CLA, 1.0 wt% 9*c*,11*c*-CLA, 1.0 wt% 10*c*,12*c*-CLA, 2.2 wt% other CLA isomers, 6.2 wt% palmitic acid, 2.3 wt% stearic acid, and 16.7 wt% oleic acid. This FFA mixture is referred to as FFA-CLA. The molar amount of FFA-CLA was calculated based on the acid value. Caprylic acid (C_8 FA; purity, 99.8%) and tricaprylin (purity, 99.3%) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Glycerol was obtained from Wako Pure Chemical Industry Co. (Osaka, Japan). Immobilized *Rhizomucor miehei* lipase (Lipozyme RM IM) and *Candida antarctica* lipase (Novozym 435) were from Novozymes (Bagsvaerd, Denmark). Other chemicals were of analytical grade.

Preparation of acylglycerols (AG) containing CLA. AG (mainly TAG) containing CLA were synthesized by esterification of FFA-CLA with glycerol according to our previous paper (24). The esterification was performed at 50°C for 48 h in a 1-L four-necked round-bottomed flask containing 600 g FFA-CLA/glycerol (3:1, mol/mol) and 30 g immobilized *R. miehei* lipase, with agitation at 200 rpm. To remove water generated by the esterification, the flask was connected with a vacuum pump and the reaction was conducted with evacuation at 670 Pa. The enzyme was recycled five times in total by addition of fresh substrates after removing the reaction mixture from the flask. The degree of esterification reached >88% during five cycles. All the reaction mixtures were combined and dehydrated at 80°C/670 Pa for 30 min. To remove the residual glycerol and FFA as distillates, the resulting mixture (2,899 g) was applied to a distillation apparatus (Wiprene type 2-03; Kobelco Eco-Solutions Co. Ltd., Hyogo, Japan). A two-step distillation at 200°C/2.7 Pa and 220°C/2.7 Pa produced 2,496 g of residue that contained 80.8 wt% TAG, 19.2 wt% DAG, and negligible amounts (<0.5 wt%) of FFA and MAG. The preparation is referred to as AG-CLA.

Acidolyses. A small-scale reaction was conducted at 30°C in a 50-mL screw-capped vessel overlain with nitrogen gas with shaking at 130 oscillations/min. Acidolysis of AG-CLA with C_8 FA was performed in a mixture of 30 g AG-CLA/C_8 FA (mole ratio = 1:3, 1:5, 1:7, 1:10, and 1:15) and 1.2 g immobilized *R. miehei* lipase, and acidolysis of tricaprylin with FFA-CLA was performed in a mixture of 30 g tricaprylin/FFA-CLA (mole ratio = 1:1, 1:2, 1:3, and 1:5) and 1.2 g immobilized *R. miehei* lipase.

A large-scale acidolysis of AG-CLA with C_8 FA was conducted at 30°C for 72 h under nitrogen gas in a 1-L four-necked round-bottomed flask containing 400 g AG-CLA/C_8 FA (1:10, mol/mol) and 16 g immobilized *R. miehei* lipase, with agitation at 200 rpm. A large-scale acidolysis of tricaprylin with FFA-CLA was performed for 24 h in a mixture of 420 g tricaprylin/CLA-FFA (1:2, mol/mol) and 16.8 g immobilized *R. miehei* lipase. The other conditions were the same as those for the acidolysis of AG-CLA with C_8 FA.

Distillation. MLM and LMM in the reaction mixtures were purified by stepwise short-path distillation. The reaction mixture was first dehydrated at 80°C/670 Pa for 30 min to reduce the water content (<100 ppm) and was then applied to a distillation apparatus (Wiprene type 2-03). The first distillation was conducted at 125°C/27 Pa, and the reaction mixture was separated into distillate 1 and residue 1. Residue 1 was next distilled at 180°C/27 Pa and was separated into distillate 2 and residue 2. The subsequent distillations were conducted similarly at 200°C/27 Pa, 230°C/27 Pa, 230°C/2.7 Pa, 240°C/2.7 Pa, and 250°C/2.7 Pa.

Analyses. FA composition was determined by GC of FAME. The constituent FA in AG were converted to their methyl esters in 3 mL methanol containing 1% Na-methylate by heating at 70°C for 5 min. FFA were methylated in 5% HCl/methanol by heating at 70°C for 5 min. The resulting FAME were analyzed with an Agilent 6890 N gas chromatograph (Palo Alto, CA) connected to a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) under the conditions described previously (25).

The contents of FFA, MAG, DAG, and TAG were analyzed by GC. The GC analysis was performed with a DB-1ht capillary column (0.25 mm × 5 m; J&W Scientific). The column temperature was held for 0.5 min at 120°C. It was then increased at 15°C/min from 120 to 280°C and at 10°C/min from 280 to 370°C, and finally held for 1 min at 370°C. The injector and detector (FID) temperatures were set at 370 and 390°C, respectively. MAG, DAG, MMM, LMM, LML, and LLL were detected at almost the same sensitivity, but the FID response for FFA was different from that of AG. For this reason, the contents of FFA and AG were accounted for by using correction factors (FFA, 1.25; AG, 1.0).

Regiospecific analysis of MLM and LMM. The MLM preparation obtained by stepwise distillation contained a small amount of DAG, which potentially could alter the regiospecific analysis of TAG. The DAG contaminants were removed by silica gel column chromatography. The MLM preparation (2.0 g) was introduced onto a silica gel column (17 g; 15 × 150 mm; Merck, Darmstadt, Germany), and TAG were eluted with 250 mL *n*-hexane/ethyl acetate (98:2, vol/vol). The purity of the TAG was checked by TLC analysis (development solvent, chloroform/acetone/acetic acid = 96:4:1).

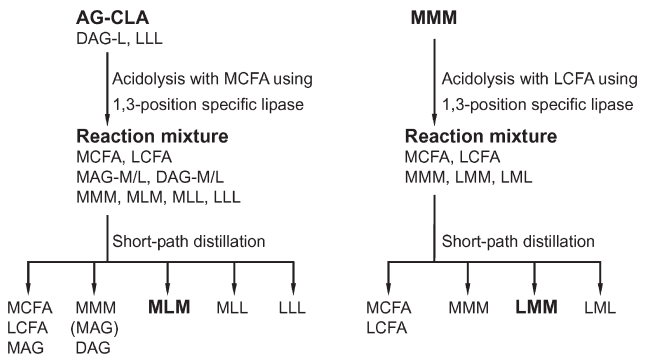
Regiospecific analysis of purified MLM and LMM was conducted basically by ethanolysis of TAG with immobilized *C. antarctica* lipase (26). A mixture of 2.0 g TAG, 6.0 g ethanol, and 0.32 g immobilized *C. antarctica* lipase was shaken at 30°C. Only 2-MAG were detected in the early stage of the reaction, and 1(3)-MAG were not observed. The 2-MAG content reached a maximum value (29–30 mol%) based on the total amount of FA in the reaction mixture after 3 h. The reaction mixture (*ca.* 2 g), which did not contain immobilized lipase, was taken out by the filtration of the reaction mixture, and ethanol in the mixture was evaporated. 2-MAG in the reaction mixture was recovered by chromatography with SEP-PAK Silica Cartridges (Part No. 51900; Waters Corp., Milford, MA). The ethanol-free sample (100 μL) was introduced onto the column, which was equilibrated with 10 mL *n*-hexane/ethyl acetate (80:20, vol/vol). FA ethyl ester and small amounts of DAG and TAG were eluted with 5 mL

n-hexane/ethyl acetate (80:20, vol/vol), and the column was washed further with 5 mL of the same solvent mixture. 2-MAG were then eluted with 5 mL methanol, and the purity of the eluted MAG was checked by TLC analysis as described in the previous report (26). To the MAG/methanol was added Na-methylate at the final concentration of 1%, and the mixture was heated at 70°C for 5 min. After the reaction mixture had cooled to room temperature, 0.5 mL *n*-hexane and 2 mL water were added to the mixture, and the mixture was mixed vigorously for 3 min with a vortex mixer. FAME were recovered in the *n*-hexane layer and were analyzed on a DB-23 capillary column as described in the previous section.

RESULTS AND DISCUSSION

Strategy for preparation of MLM and LMM regioisomers containing CLA in high purity. The strategy is shown in Scheme 1. Because we wished to develop a process for the large-scale preparation of MLM and LMM regioisomers containing CLA, FFA-CLA containing FA other than CLA (27.4 mol%) were used directly for the preparation of each regioisomer. Thus, the LCFA on the resulting regioisomers were a mixture of CLA and other FA. AG-CLA was synthesized by the esterification of FFA-CLA in a manner that could be applied on an industrial scale. The resulting AG-CLA was composed of 74.4 mol% TAG and 25.6 mol% DAG. When this preparation underwent acidolysis with C_8 FA using immobilized *R. miehei* lipase, TAG were converted efficiently to MLM-structured TAG, and DAG were converted to MMM and MLM because acyl migration, esterification of DAG with C_8 FA, and acidolysis of DAG and TAG with C_8 FA occurred simultaneously during the reaction (27,28). Hence, the AG-CLA preparation was used as a starting material without further purification.

The reaction mixture obtained by acidolysis of AG-CLA with C_8 FA contains C_8 FA (M.W., 144), C_{18} FA (M.W., 280), MAG of C_8 FA (M.W., 218), MAG of C_{18} FA (M.W., 354), DAG (main constituent FA were C_8 and C_{18} FA; M.W., 480), MMM (M.W., 470), MLM (M.W., 606), MLL (M.W., 742), and LLL (M.W., 878). Meanwhile, the reaction mixture in the acidolysis of MMM (tricaprylin) with FFA-CLA contained C_8 FA, C_{18} FA, MMM, LMM, and LML. Because the M.W. of these components are different, it should be possible to purify MLM and LMM highly by short-path distillation.



MCFA, medium-chain FA; LCFA, long chain FA
MAG-M/L, MAG with MCFA or LCFA
DAG-L, DAG with LCFA; DAG-M/L, DAG with MCFA and/or LCFA
MMM, TAG with MCFA; LLL, TAG with LCFA
MLL, TAG with MCFA at the 1(3)-posion and LCFA at the 2 and 3(1)-positions
MLM, TAG with MCFA at the 1,3-positions and LCFA at the 2-position
LMM, TAG with LCFA at the 1(3)-position and MCFA at the 2 and 3(1)-positions
LML, TAG with LCFA at the 1,3-positions and MCFA at the 2-position

SCHEME 1

Production of the MLM isomer. AG-CLA underwent acidolysis with 3–15 mol C_8 FA using 4 wt% immobilized *R. miehei* lipase. Because the reaction temperature was an important factor for the acyl migrations (2), the reaction was performed at 30°C to reduce the acyl migrations. The contents of AG in the reaction mixtures were identified by GC using a DB-1ht capillary column (Fig. 1). The content of MLM depended on the amount of C_8 FA and reached a constant value (37.7 mol%) at 7 mol C_8 FA after 24 h. When the reaction was extended to 48 h, MLM content reached a constant value (52.3 mol%) at 10 mol C_8 FA. The 72-h reaction with 15 mol C_8 FA produced a little larger amount of MLM than that with 10 mol C_8 FA: MLM contents at 10 and 15 mol C_8 FA were 58.1 and 61.3 mol%, respectively. Large amounts of C_8 FA resulted in a low yield of MLM based on the reaction mixture. Hence, the amount of C_8 FA was fixed at 10 mol.

A 400-g mixture of AG-CLA/C_8 FA (1:10, mol/mol) and 4 wt% immobilized *R. miehei* lipase was agitated at 30°C. A typical time course is shown in Figure 2. When the reaction was scaled up from 30 to 400 g, the acidolysis velocity was not changed significantly. LLL were converted efficiently to MLL and MLM by acidolysis with C_8 FA. The content of MLL attained a maximal value after 24 h and then decreased gradually. DAG content fell, and MMM contents increased. The molar ratio of MLM/(MLM + MLL) reached a constant

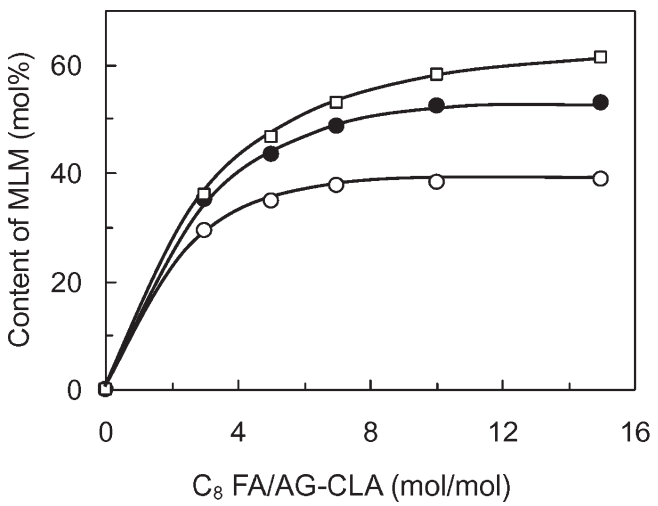


FIG. 1. Effect of caprylic acid (C_8 FA) content on acidolysis of acylglycerols (AG) containing two CLA isomers (AG-CLA). A mixture of 30 g AG-CLA/C_8 FA (1:3–15, mol/mol) and 1.2 g immobilized *Rhizomucor miehei* lipase was shaken at 30°C. The content of MLM (mol%) was expressed relative to that of total AG. ○, MLM content after 24 h; ●, 48 h; □, 72 h. M, medium-chain FA; L, long-chain FA.

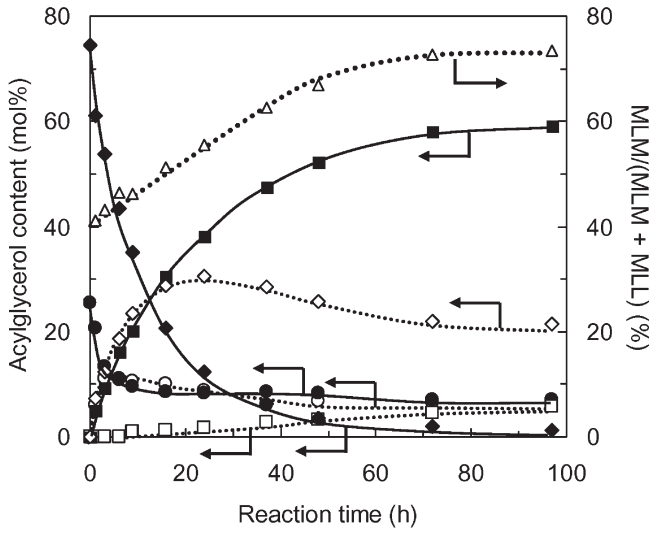


FIG. 2. Time course of acidolysis of AG-CLA with C_8 FA using immobilized *R. miehei* lipase. A mixture of 400 g AG-CLA/C_8 FA (1:10, mol/mol) and 16 g immobilized lipase was agitated at 30°C and 200 rpm. The contents of AG (mol%) were expressed relative to that of total AG. ○, MAG; ●, DAG; □, MMM; ■, MLM; ◇, MLL; ◆, LLL; △, MLM/(MLM + MLL). See Figure 1 for abbreviations.

value after 72 h. The 72-h reaction mixture was composed of 6.5 mol% MAG, 7.0 mol% DAG, 4.5 mol% MMM, 58.0 mol% MLM, 22.0 mol% MLL, and 2.0 mol% LLL.

Purification of MLM. Acidolysis of AG-CLA with C_8 FA was performed five times at a 400-g scale using the same lipase. All of the reaction mixtures were combined, and MLM was purified from the mixture by short-path distillation (Table 1).

Because the reaction mixture (1,968 g) contained 4,030 ppm water, it was dehydrated at 80°C/670 Pa for 30 min to reduce the water content to less than 100 ppm. In this step, a small amount of C_8 FA was removed. C_8 FA was then distilled at 125°C/27 Pa (distillation 1), and most of the C_{18} FA was distilled at 180°C/27 Pa (distillation 2). To remove the remaining C_{18} FA, residue 2 was distilled at 200°C/27 Pa (distillation 3). Residue 3 contained 3.2 wt% C_{18} FA, 2.3 wt% MMM, 0.5 wt% MAG, and 5.2 wt% DAG, of which the M.W. were smaller than that of MLM. Hence, these components were removed by distillation at 230°C/27 Pa (distillation 4). The contents of FA, MMM, MAG, and DAG in residue 4 fell to 0.4, 0.3, <0.1, and 2.9 wt%, respectively, although a certain amount of MLM was distilled (MLM content in distillate 4, 44.7 wt%). The desired product, MLM, was then recovered by distillation at 230°C/2.7 Pa (distillation 5) and 240°C/2.7 Pa (distillation 6). Distillate 5 (80.8 g) was composed of 1.0 wt% MMM, 87.1 wt% MLM, 2.0 wt% MLL, and 9.9 wt% DAG, and distillate 6 (54.8 g) was composed of 0.5 wt% MMM, 91.8 wt% MLM, 3.6 wt% MLL, and 4.0 wt% DAG. Because residue 6 still contained 36.2 wt% MLM, it was finally distilled at 250°C/2.7 Pa (distilla-

TABLE 1
Purification of MLM from the Reaction Mixture by Short-Path Distillation

		Weight (g)							
		FFA							
Step	Total	C_8	Others[a]	MAG	DAG	MMM[b]	MLM[b]	MLL	LLL
Reaction mixture	1968.0	1146.0	293.6	24.2	30.4	20.3	270.0	166.5	17.0
Dehydration[c]	1941.0	1130.0	290.0	24.0	30.0	20.0	266.0	164.0	17.0
Distillate 1[d]	1101.6	1101.6	ND[e]	ND	ND	ND	ND	ND	ND
Distillate 2[f]	240.8	16.0	207.2	14.4	ND	3.2	ND	ND	ND
Distillate 3[g]	83.4	6.4	61.6	6.2	3.2	4.0	1.6	ND	ND
Distillate 4[h]	73.3	ND	14.4	2.3	13.2	10.2	32.8	ND	ND
Distillate 5[i]	80.8	ND	ND	ND	8.0	0.8	70.4	1.6	ND
Distillate 6[j]	54.8	ND	ND	ND	2.2	0.3	50.3	2.0	ND
Distillate 7[k]	62.4	ND	ND	ND	0.8	ND	54.4	7.2	ND
Residue 7[l]	202.8	ND	ND	ND	ND	ND	40.8	146.4	15.6
Redistillation of distillate 5[i]									
Distillate 5-2	12.9	ND	ND	ND	3.8	0.7	8.4	ND	ND
Residue 5-2	66.0	ND	ND	ND	4.0	ND	60.5	1.5	ND
Redistillation of distillate 7[k]									
Distillate 7-2	22.9	ND	ND	ND	0.5	ND	22.0	0.4	ND
Residue 7-2	38.6	ND	ND	ND	0.3	ND	31.7	6.6	ND

[a]FFA originating from acylglycerols containing two CLA isomers, which are mainly C_{18} FA.
[b]*M, medium-chain FA; L, long-chain FA*
[c]At 80°C/670 Pa for 30 min.
[d]At 125°C/27 Pa.
[e]ND, not detected (<0.5 wt%).
[f]At 180°C/27 Pa.
[g]At 200°C/27 Pa.
[h]At 230°C/27 Pa.
[i]At 230°C/2.7 Pa.
[j]At 240°C/2.7 Pa.
[k]At 250°C/2.7 Pa.

tion 7). The composition of distillate 7 (62.4 g) was 87.2 wt% MLM, 11.5 wt% MLL, and 1.3 wt% DAG, and the MLM content in residue 7 (202.9 g) was 20.1 wt%.

The purities of MLM in distillates 5 and 7 were 87.1 and 87.2%, respectively. To further increase the purity, each fraction was redistilled. Redistillation of distillate 5 at 230°C/27 Pa (distillation 5-2) recovered 66.0 g of residue 5-2, of which the contents of MMM, MLM, MLL, and DAG were 0.3, 91.4, 2.3, and 6.1 wt%, respectively. In addition, redistillation of distillate 7 at 250°C/2.7 Pa (distillation 7-2) recovered 22.9 g of distillate 7-2, of which the contents of MLM, MLL, and DAG were 96.1, 1.7, and 2.2 wt%, respectively.

Distillate 6, residue 5-2, and distillate 7-2 were combined and used as a purified MLM preparation, the composition of which was 4.7 wt% DAG, 0.3 wt% MMM, 92.3 wt% MLM, and 2.7 wt% MLL. A series of distillations purified MLM from the reaction mixture in a yield of 49.1%, showing that stepwise distillation was effective for producing high-purity MLM.

Production of the LMM isomer. Tricaprylin (MMM) underwent acidolysis at 30°C with 1–5 mol FFA-CLA using 4 wt% immobilized *R. miehei* lipase (Fig. 3). The content of LMM in the 24-h reaction reached a maximal value (54.6 mol%) at 3 mol FFA-CLA. The contents of LMM in 48- and 72-h reactions reached maximal values at 2 mol FFA-CLA (58.2 and 56.1 mol%, respectively). Based on these results, the amount of FFA-CLA was fixed at 2 mol for MMM amount.

A mixture of MMM/FFA-CLA (1:2, mol/mol) and 4 wt% immobilized *R. miehei* lipase was agitated at 30°C. A typical time course is shown in Figure 4. MMM was converted efficiently to LMM by its acidolysis with FFA-CLA, and LMM was converted to LML. The content of LMM reached a constant value after 37 h, and the content of LML increased almost linearly. The molar ratio of LMM/(LMM + LML) decreased gradually after 6 h (Fig. 4). Because separation of MMM and MLM is somewhat easier than separation of MLM and MLL (Table 1), effective purification of LMM will be achieved by a low LML content in the reaction mixture. For these reasons, the reaction period was fixed at 24 h. The contents of MMM, LMM, and LML in the mixture of a 24-h reaction were 39.6, 52.6, and 7.8 mol%, respectively, and the content of DAG was less than 0.3 mol%.

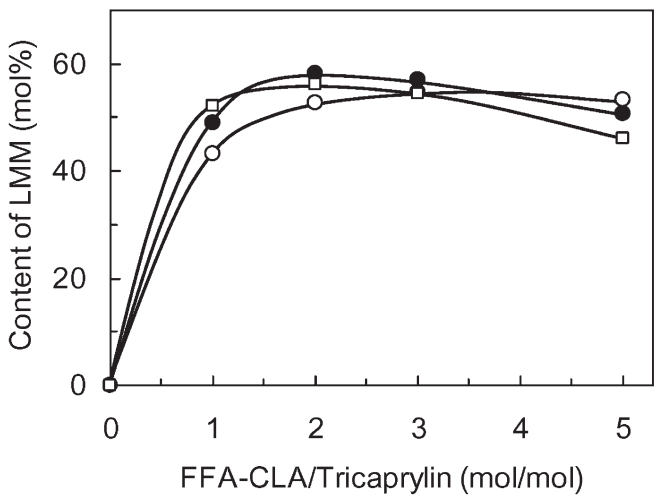


FIG. 3. Effect of FFA mixture containing two CLA isomers (FFA-CLA) on acidolysis of tricaprylin using immobilized *R. miehei* lipase. A mixture of 30 g tricaprylin/FFA-CLA (1:1–5, mol/mol) and 1.2 g immobilized lipase was shaken at 30°C. The content of LMM (mol%) was expressed relative to that of total AG. ○, LMM content after 24 h; ●, 48 h; □, 72 h. For abbreviation see Figure 1.

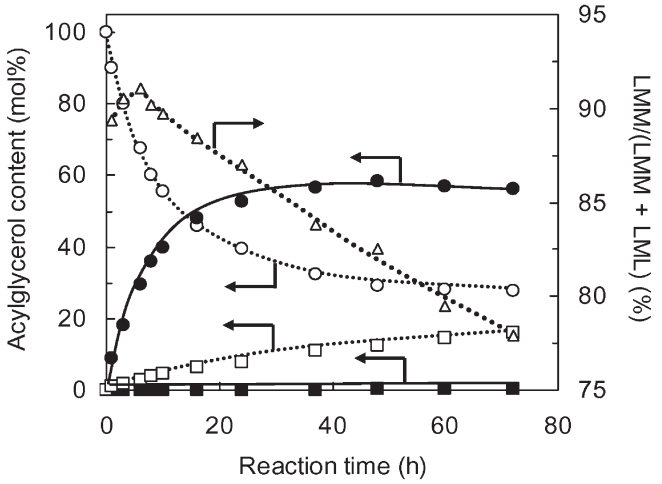


FIG. 4. Time course of acidolysis of tricaprylin with FFA-CLA using immobilized *R. miehei* lipase. A mixture of 420 g tricaprylin/FFA-CLA (1:2, mol/mol) and 16.8 g immobilized lipase was agitated at 30°C and 200 rpm. The content of each TAG (mol%) was expressed relative to that of total TAG. ○, MMM (tricaprylin); ●, LMM; □, LML; ■, DAG; △, LMM/(LMM + LML). See Figure 1 for abbreviations.

Purification of the LMM isomer. Acidolysis of MMM with FFA-CLA was performed twice at a 420-g scale using the same immobilized lipase. The reaction mixtures were combined, and LMM was purified by short-path distillation, the conditions for which were the same as those in Table 1 (Table 2).

The reaction mixture (813 g; water content 590 ppm) was dehydrated for 30 min at 80°C/670 Pa to reduce the water content to less than 100 ppm. After most of the C_8 FA was distilled at 125°C/27 Pa (distillation 1), C_{18} FA were distilled at 180°C/27 Pa (distillation 2) and 200°C/27 Pa (distillation 3). Residue 3 was composed of <0.2 wt% C_{18} FA, 7.9 wt% MMM, 75.0 wt% LMM, and 17.1 wt% LML. To remove MMM, residue 3 was distilled at 230°C/27 Pa (distillation 4). Whereas distillate 4 (38.4 g) contained 57.0 wt% LMM, the content of MMM in residue 4 fell to 2.1 wt%. Hence, LMM was recovered by distillations at 230°C/2.7 Pa (distillation 5) and 240°C/2.7 Pa (distillation 6). Distillate 5 (77.1 g) was composed of 6.6 wt% MMM, 92.1 wt% LMM, and 1.2 wt% LML; distillate 6 (49.7 g) was composed of 1.2 wt% MMM, 94.8 wt% LMM, and 4.0 wt% LML. Residue 6 was finally distilled at 250°C/2.7 Pa (distillation 7). Although 28.1 g of distillate 7 was recovered, the content of LMM was 88.6 wt%.

Distillates 5 and 6 were combined and used as a purified preparation of LMM; the combination contained 4.5 wt%

TABLE 2
Purification of LMM from the Reaction Mixture by Short-Path Distillation

	Weight (g)					
		FFA				
Step	Total	C_8	Others[a]	MMM	LMM	LML
Reaction mixture	812.9	106.8	270.2	159.7	226.0	50.2
Dehydration[b]	796.3	104.1	264.2	157.3	222.2	48.5
Distillate 1[c]	83.0	81.9	1.1	ND[d]	ND	ND
Distillate 2[e]	179.2	19.7	139.2	20.3	ND	ND
Distillate 3[f]	240.9	ND	120.9	111.0	9.0	ND
Distillate 4[g]	38.4	ND	ND	16.0	21.9	0.2
Distillate 5[h]	77.1	ND	ND	5.1	71.0	0.9
Distillate 6[i]	49.7	ND	ND	0.6	47.1	2.0
Distillate 7[j]	28.1	ND	ND	ND	24.9	3.2
Residue 7[j]	78.9	ND	ND	ND	38.0	40.9

[a]FFA originating from a FFA mixture containing two CLA isomers (FFA-CLA), which are mainly C_{18} FA.
[b]At 80°C/670 Pa for 30 min.
[c]At 125°C/27 Pa.
[d]For abbreviations see Table 1.
[e]At 180°C/27 Pa.
[f]At 200°C/27 Pa.
[g]At 230°C/27 Pa.
[h]At 230°C/2.7 Pa.
[i]At 240°C/2.7 Pa.
[j]At 250°C/2.7 Pa.

TABLE 3
FA Composition at the 2-Position of Purified MLM and LMM

	FA composition (mol%)				
		MLM		LMM	
FA	FFA-CLA	Total	2-Position	Total	2-Position
8:0	0	65.0	4.9	67.9	98.3
16:0	6.7	2.1	5.9	2.4	ND[a]
18:0	2.2	0.8	1.8	0.7	ND
18:1	16.5	5.9	15.9	5.6	0.3
CLA					
9*c*,11*t*	33.7	11.6	32.8	10.2	0.7
10*t*,12*c*	34.7	12.0	31.7	11.5	0.7
Others	4.2	1.8	5.0	1.1	ND
Others	2.0	0.8	2.0	0.6	ND

[a]ND, not detected (<0.2 wt%). For other abbreviations see Tables 1 and 2.

MMM, 93.2 wt% LMM, and 2.3 wt% LML. A series of distillations purified LMM from a reaction mixture in a yield of 52.3%.

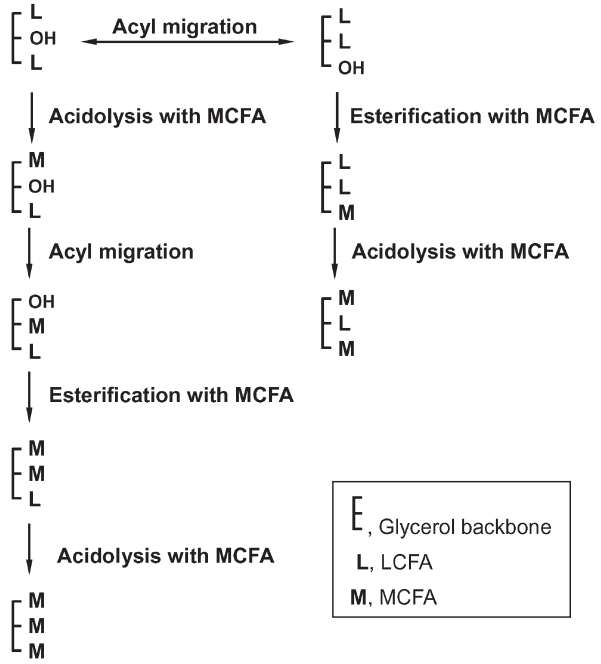
Analysis of purified preparations. The FA composition at the 2-position of TAG has been analyzed by Grignard degradation (29) or by hydrolysis with a 1,3-position-specific lipase (30). Tandem MS provides information on the FA distribution between the 2- and 1(3)-positions of TAG (31). Recently, we reported a new method for the analysis of FA composition at the 2-position (26). When oils underwent ethanolysis with the immobilized *C. antarctica* lipase in the presence of a large amount of EtOH, FA at the 1- and 3-positions were converted regiospecifically to their ethyl esters (32,33). Thus, regiospecific analysis of purified MLM and LMM was conducted according to our method.

The MLM preparation obtained by short-path distillation contained 4.7 wt% DAG, which might disturb regiospecific analysis. The preparation was therefore introduced onto a silica gel column (recovery of TAG, 85%), and the resulting TAG fraction was subjected to regiospecific analysis. Because the LMM fraction obtained by short-path distillation contained a negligible amount of DAG, the fraction was subjected to regiospecific analysis without further purification.

The FA composition of MLM coincided almost completely with that of LMM (Table 3). The contents of LCFA at the 2-position in MLM were 95.1 mol%, and the content of C_8 FA at the 2-position in LMM was 98.3 mol% (Table 3). These results indicated that the two preparations were high-purity regioisomers containing 2 mol C_8 FA and 1 mol LCFA containing CLA. FFA-CLA was the mixture of CLA and other FA. The ratio of CLA and other FA was not significantly changed by our purification process.

Acyl migrations occur during lipase-catalyzed acidolysis and distillation steps (2,7,8), and the acyl migrations are increased by high reaction temperature (>40°C) and long-term distillation. To reduce the acyl migrations, our reactions were performed at 30°C, and short-path distillations were used for the purification. For the quantification of acyl migrations, regiospecific analysis of purified MLM and LMM preparations was performed, and the results showed that the purities of the desired TAG regioisomers were 95.1 and 98.3 mol%, respectively (Table 3). These results showed that the regioisomers of purified MLM and LMM preparations were only 4.9 and 1.7 mol%, respectively.

These purities seemed to be very high, but the results included a small difference. The higher acyl migrations of MLM preparations than those of LMM can be explained by the long reaction times (72 h) and higher molar ratio of FA (AG-CLA/C_8 FA = 1:10) (2), and by the DAG contaminant (25.6 mol%). The DAG are forecasted to be converted to MLM and MMM through a main pathway shown in Scheme 2. 1(3),2-DAG are converted to LLM by their esterification with C_8 FA, and LLM are then converted to MLM by their acidolysis with C_8 FA. Meanwhile, 1,3-DAG first undergo acidolysis with C_8 FA. After C_8 FA at the 1(3)-position has migrated to the 2-position, MML are synthesized by esterification of the 2,3(1)-DAG with C_8 FA. Finally, MML are converted to MMM by their acidolysis with C_8 FA. This reaction produces MLM and MMM as end products, and LLM and MML as TAG intermediates. TAG products, MMM and LLM, were removed by distillation (MMM content in the MLM preparation, 0.5 mol%; LLM content, 2.4 mol%) (Table 1). Even though the MLM preparation contained MLL, FA at the 2-position would be LCFA. On the other hand, a by-product, MML, cannot be separated from the desired products, MLM, by distillation. FA at the 2-position in MML is C_8 FA. Hence, the difference in purities of TAG regioisomers in LMM and MLM preparations may be due to contamination of the MLM preparation with MML.



SCHEME 2

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[Received August 18, 2004; accepted November 19, 2004]