Synthesis of MAG of CLA with *Penicillium camembertii* Lipase

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ABSTRACT: CLA has various physiological activities, and a FFA mixture containing almost equal amounts of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA (named FFA-CLA) has been commercialized. We attempted to produce MAG of CLA by a two-step successive reaction. The first step was esterification of FFA-CLA with glycerol. A mixture of FFA-CLA/glycerol (1:5, mol/mol), 2 wt% water, and 200 units/g of *Penicillium camembertii* mono- and diacylglycerol lipase was agitated at 30°C to form a homogeneous emulsion. The esterification degree reached 84% after 10 h. To further increase the degree, the reaction was continued with dehydration at 5 mm Hg. The esterification degree reached 95% after 24 h (34 h in total), and the reaction mixture contained 50 wt% MAG and 44 wt% DAG. The second step was glycerolysis of the resulting DAG. The reaction mixture in the first-step esterification was transferred from the reactor to a beaker and was solidified by vigorous agitation on ice. When the solidified mixture was allowed to stand at 5°C for 15 d, glycerolysis of DAG proceeded successfully, and MAG content in the reaction mixture increased to 88.6 wt%. Hydrolysis of the acylglycerols was not observed during the second reaction. FA composition in the synthesized MAG was completely the same as that in the original FFA-CLA, showing that *Penicillium* lipase does not have selectivity toward FA in the FFA-CLA preparation.

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MAG are very good emulsifiers and are widely used as food additives. MAG are produced industrially by chemical alcoholysis of oils and fats with two molar equivalents of glycerol in the presence of metal catalysts at high temperatures of 210–240°C (1,2). But the process cannot be applied to synthesize MAG of unstable FA. Hence, many research groups have engaged in the synthesis of MAG through lipase-catalyzed reactions (hydrolysis, esterification, glycerolysis, and ethanolysis) (3–8). These reactions were, however, conducted in organic solvent systems, which are not suitable for industrial production of MAG esterified with functional FA. Meanwhile, several organic solvent-free systems also have been reported: (i) glycerolysis with *Pseudomonas* lipase (9,10); (ii) ethanolysis of TAG with immobilized *Candida antarctica* lipase (11); and (iii) esterification of FFA with glycerol using *Penicillium camembertii* mono- and diacylglycerol lipase (referred to as *Penicillium* lipase) (12).

CLA is produced by alkali conjugation of safflower or sunflower oil in propylene glycol or ethylene glycol. The first product is a FFA mixture containing almost equal amounts of *cis*-9,*trans*-11 (*c*9,*t*11)-CLA and *trans*-10,*cis*-12 (*t*10,*c*12)-CLA, and the acylglycerols (main component, TAG) esterified with the FFA are also commercially available. The FFA mixture containing CLA isomers has various physiological activities that have been demonstrated in animal models, such as reduction of the incidence of cancer (13–15), decrease in body fat content (16,17), beneficial effects on atherosclerosis (18,19), and improvement of immune function (20). The activities attracted a great deal of attention, and CLA has been used as a functional food. If CLA is efficiently converted to its MAG, the new product can be used as a functional emulsifier for various kinds of foods and can also be added in beverages. Hence, development of a process for producing MAG of CLA has strongly been desired. In light of the instability of CLA, an enzymatic process is suitable for its production. Furthermore, an esterification reaction is advantageous, because the FFA mixture containing CLA is the first product in the industrial process. As reported by Yamaguchi and Mase (12), a reaction system with *Penicillium* lipase efficiently catalyzed esterification of oleic acid with glycerol but produced DAG in addition to MAG. Indeed, when CLA was esterified with glycerol using the lipase, almost equal amounts of MAG and DAG were synthesized. We thus attempted to synthesize MAG of CLA by a two-step successive reaction comprising esterification and glycerolysis. In this paper, we propose an effective procedure for producing MAG of CLA using *Penicillium* lipase.

MATERIALS AND METHODS

Materials. A FFA mixture containing CLA (named FFA-CLA) was a commercial product (CLA-80; Rinoru Oil Mills Co. Ltd., Tokyo, Japan) produced by alkali conjugation of safflower oil in propylene glycol. The FFA-CLA was composed of 33.1 wt% *c*9,*t*11-CLA, 33.9 wt% *t*10,*c*12-CLA, 0.9

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wt% *c*9,*c*11-CLA, 1.4 wt% *c*10,*c*12-CLA, and 1.8 wt% other CLA isomers; 6.7 wt% palmitic acid, 2.7 wt% stearic acid, and 17.4 wt% oleic acid. Glycerol (water content, 0.20%) was purchased from Wako Pure Chemical Industry Co. (Osaka, Japan). The molar amount of FFA was calculated based on the acid value. *Penicillium* lipase (Lipase G) was a gift from Amano Enzyme Inc. (Aichi, Japan). The lipase was dissolved in deionized water at a concentration of 10,000 units (U)/mL (200 mg/mL), and the solution or diluted solution was added to a reaction mixture.

Reactions. A small-scale esterification was conducted at 30°C in a 50-mL vessel with stirring at 500 rpm. The standard reaction mixture contained 4.9 g FFA-CLA/glycerol, 200 U/g *Penicillium* lipase, and 0.1 mL of water originating from the enzyme solution. A large-scale esterification was performed at 30°C in a 1-L four-neck round-bottomed flask containing 294 g FFA-CLA/glycerol and 6 mL *Penicillium* lipase solution (10,000 U/mL). The mixture was agitated at 250 rpm with an impeller to form a homogeneous emulsion. Dehydration during the reaction was performed at 5 mm Hg using a vacuum pump. Esterification degree was determined from the amount of FA consumed by the reaction.

Glycerolysis of DAG was performed as follows. A reaction mixture after a large-scale esterification was transferred from the reactor to a beaker, and the mixture was solidified by mixing for 2 min on ice with a homogenizer (Bio-Mixer BM-2; Nihon Seiki Kaisha Ltd., Tokyo, Japan). The resulting reaction mixture was allowed to stand at 5°C.

Purification of acylglycerols. The reaction mixture (15 g) was applied to a silica gel 60 column (120 g; 30 × 390 mm; Merck, Darmstadt, Germany). After washing the column with 200 mL *n*-hexane/ethyl acetate (98:2, vol/vol), DAG of FFA-CLA (DAG-CLA) and FFA were eluted with a mixture of *n*-hexane/ethyl acetate (80:20, vol/vol), and MAG of FFA-CLA (MAG-CLA) were eluted with a mixture of *n*-hexane/ethyl acetate (50:50, vol/vol). FFA in the DAG fraction were removed with *n*-hexane under alkaline conditions as described elsewhere (21).

Analyses. Lipase activity was measured by titrating FA liberated from monoolein (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) with alkali. The reaction was performed as described previously (22). In brief, the assay mixture containing 1.0 mL monoolein, 5.0 mL of Na-acetate buffer (pH 5.6), and 50–200 μL enzyme solution was incubated at 30°C for 30 min with stirring at 500 rpm. The reaction was stopped by the addition of 20 mL ethanol. FA released during the incubation was titrated with 50 mM KOH to pH 10. One unit of lipase activity was defined as the amount of the enzyme that liberated 1 μmol of FA per minute.

About 1 g of the liquid-state reaction mixture obtained from esterification of FFA-CLA with glycerol was separated into oil and glycerol layers by centrifugation (6500 × *g*, 5 min). Meanwhile, about 1 g of the solidified mixture obtained from glycerolysis of DAG was heated at 40°C, and was then separated into oil and glycerol layers by centrifugation. The contents of MAG, DAG, TAG, and FFA in the oil layer were measured by a TLC/FID analyzer (Iatroscan MK-5; Iatron Laboratories Inc., Tokyo, Japan) after development with a mixture of *n*-hexane/ethyl acetate/acetic acid (90:10:1, by vol.).

FA composition was determined by GC of FAME. The constituent FA in acylglycerols were converted to their methyl esters in 3 mL methanol containing 1% Na-methylate by heating at 70°C for 15 min, and FFA were methylated in 3 mL of 5% HCl-methanol by heating at 70°C for 15 min. The resulting FAME were analyzed with a Hewlett-Packard 5890 gas chromatograph (Avondale, CA) connected to a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) under the conditions described previously (23).

Water content was measured by Karl Fischer titration with a Moisture Meter CA-07 (Mitsubishi Chemical Corp., Tokyo, Japan).

RESULTS AND DISCUSSION

Penicillium lipase is known to be effective for esterification of FFA with glycerol (12). We thus selected the lipase for production of MAG-CLA by a two-step successive reaction composed of esterification and glycerolysis.

Effect of enzyme amount on the first-step esterification. A mixture of FFA-CLA/glycerol (1:5, mol/mol) and water originating from the lipase solution was stirred at 30°C with various amounts of the enzyme. As the lipase solution prepared was 10,000 U/mL, the amount of water added in the reaction with 400 U/g *Penicillium* lipase became 4 wt%. Hence, the amount of water added in all of the reactions was fixed at 4 wt%. The result is shown in Table 1. Esterification degree after 5 h was proportional to enzyme amount, and the degree after 24 h reached >80% using >100 U of the lipase per 1 g reaction mixture. Conversion of MAG-CLA to DAG-CLA seemed to be accelerated as the esterification degree exceeded 60%, but the DAG were not converted to TAG. It was also found that the amounts of MAG- and DAG-CLA in the reaction mixture were almost the same weight in the equilibrium state.

TABLE 1
Effect of Amount of Enzyme on Esterification of FFA Containing CLA Isomers (FFA-CLA) with Glycerol[a]

Amount of enzyme (U/g-mixture)	Reaction time (h)	Composition (wt%)[b]		
		FFA-CLA	MAG-CLA	DAG-CLA
20	5	64	34	2
	24	37	54	9
50	5	42	55	3
	24	22	53	25
100	5	33	59	8
	24	19	44	37
200	5	25	53	22
	24	18	42	40
400	5	19	50	31
	24	17	43	40

[a]The reaction was conducted at 30°C in a mixture of 4.8 g FFA-CLA/glycerol (1:5, mol/mol) and 200 μL *Penicillium camembertii* lipase solution (500, 1,250, 2,500, 5,000, and 10,000 U/mL) with stirring at 500 rpm. The added amount of water was 4 wt%.
[b]MAG-CLA, MAG of FFA-CLA; DAG-CLA, DAG of FFA-CLA.

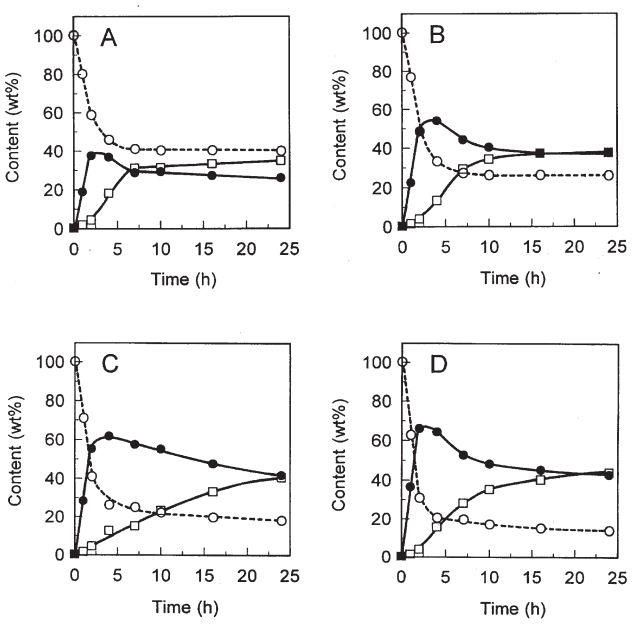

FIG. 1. Effect of glycerol concentration on esterification of FFA containing CLA isomers (FFA-CLA) with glycerol. The reaction was conducted at 30°C in a mixture of 4.9 g FFA-CLA/glycerol and 100 µL *Penicillium camembertii* lipase (10,000 U/mL) with stirring. The added amount of water was 2 wt%. The ratio of FFA-CLA/glycerol was (A) 1:1 (mol/mol); (B) 1:2; (C) 1:3; (D) 1:5. ○, FFA-CLA; ●, MAG of FFA-CLA (MAG-CLA); □, DAG of FFA-CLA (DAG-CLA).

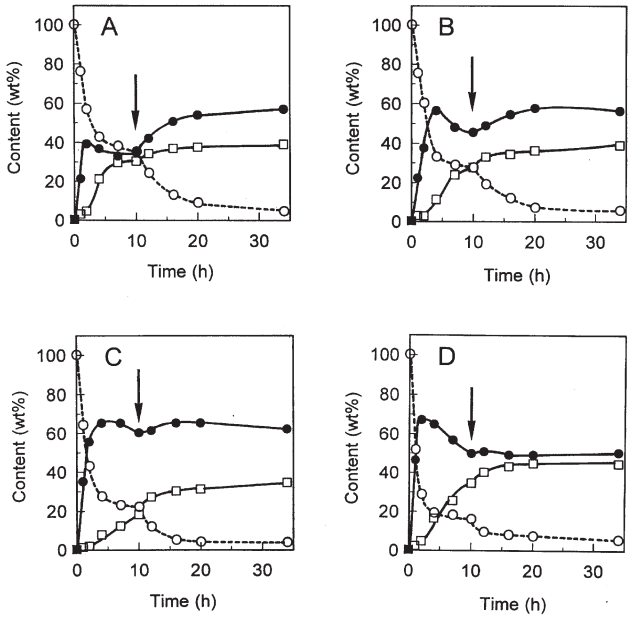

FIG. 2. Effect of dehydration on esterification of FFA-CLA with different amounts of glycerol. The reaction was conducted at 30°C in a mixture of 294 g FFA-CLA/glycerol and 6 mL *Penicillium* lipase (10,000 U/mL) with vigorous agitation. At 10 h, as indicated with arrows, the reactor was connected with a vacuum pump, and the reaction was continued with dehydration at 5 mm Hg. The ratio of FFA-CLA/glycerol was (A) 1:1 (mol/mol); (B) 1:2; (C) 1:3; (D) 1:5. ○, FFA-CLA; ●, MAG-CLA; □, DAG-CLA.

Effect of initial water content on the first-step esterification. To prepare a reaction mixture without added water, the lipase powder was added to the reaction mixture at a concentration of 200 U/g. However, the esterification did not proceed, because the lipase did not disperse in the mixture. Hence, it was necessary first to dissolve the lipase in deionized water, and then add the solution to the reaction mixture. When 5-g mixtures of FFA-CLA/glycerol (1:5, mol/mol); 200 U/g *Penicillium* lipase; and 2, 5, and 10 wt% of water were incubated at 30°C for 24 h, the esterification degrees were 86, 78, and 52%, respectively. The result showed that a smaller amount of water increased esterification degree. Hence, the added amount of water was fixed at 2 wt% for subsequent experiments, which was the minimum content in a system with 200 U/mL of the lipase.

Effect of glycerol concentration on the first-step esterification. A 5-g mixture of FFA-CLA/glycerol, 2 wt% water, and 200 U/g *Penicillium* lipase was stirred at 30°C. Figure 1 shows the time courses in the reactions with 1 to 5 molar equivalents of glycerol against FFA-CLA. A fundamental feature of these experiments was that the time courses were the same even though the glycerol contents were varied: MAG-CLA was at first synthesized by esterification of FFA-CLA with glycerol, and DAG-CLA appeared after MAG-CLA had accumulated in the reaction mixture. Esterification velocity was accelerated by an increase in the amount of glycerol. The esterification degree in the equilibrium state also correlated with the glycerol amounts, which were 60, 74, 82, and 86% in the reactions with 1, 2, 3, and 5 molar equivalents of glycerol, respectively. The conversion of MAG to DAG became slow when glycerol was increased from 1 to 3 molar equivalents (Figs. 1A–C), indicating that the conversion velocity was repressed by excess amounts of glycerol. However, comparison of time courses in the reactions with 3 and 5 molar equivalents of glycerol showed that the velocities (decrease of FFA and increases of MAG- and DAG-CLA) of the reaction with 5-molar glycerol were only 1.5 times faster than those with 3-molar glycerol (Figs. 1C, 1D). It was therefore found that the conversion of MAG-CLA to DAG-CLA was not repressed even though >3 molar equivalents of glycerol was employed. In addition, these time courses indicated that the amounts of MAG- and DAG-CLA in the reaction mixture were almost equal weight in the equilibrium state.

Effect of dehydration on the first-step esterification. An attempt was made to increase esterification of FFA-CLA with glycerol by removing the water that originated from the enzyme solution and that was generated by the esterification (Fig. 2). A 300-g mixture of FFA-CLA, 1 to 5 molar equivalents of glycerol, 2 wt% water, and 200 U/g lipase was agitated at 30°C to form a complete emulsion. The time courses before dehydration were the same as those in the small-scale reactions (Fig. 1). After the esterification degree had reached a constant value (10 h), dehydration was started by evacuation at 5 mm Hg using a vacuum pump. The esterification degree was increased concomitantly with the dehydration and reached *ca*. 95% after 24 h (34 h in total) in all reactions with

TABLE 2
Effect of Glycerol Concentration on Conversion of DAG- to MAG-CLA Through Glycerolysis at Low Temperature[a]

FFA-CLA/glycerol (mol/mol)	Temperature (°C)	Period (d)	Composition (wt%)		
			FFA-CLA	MAG-CLA	DAG-CLA
1:1	—	0	4.5	56.7	38.8
	30	15	4.1	53.8	42.1
	5	5	3.9	63.5	32.6
		10	3.6	69.0	27.4
		15	3.7	74.6	21.7
1:2	—	0	5.6	55.9	38.5
	30	15	5.2	54.1	40.7
	5	5	5.1	64.0	30.9
		10	4.9	71.5	23.6
		15	3.2	77.7	19.1
1:3	—	0	3.8	61.9	34.3
	30	15	3.5	58.1	38.4
	5	5	3.3	70.0	26.7
		10	4.0	77.2	18.8
		15	3.0	81.9	15.1
1:5	—	0	5.4	50.2	44.4
	30	15	4.8	49.3	45.9
	5	5	4.7	68.4	26.9
		10	4.5	80.1	15.4
		15	4.1	88.6	7.3

[a]Five grams of reaction mixture, obtained from the reaction presented in Figure 2, was allowed to stand at 30°C. When the mixture had separated into oil and glycerol layers, it was mixed with stirring to form a homogeneous emulsion. The rest of the mixture (*ca.* 280 g) was recovered in a beaker, and was homogeneously solidified by vigorous mixing on ice. The solidified mixture was allowed to stand at 5°C.

different amounts of glycerol (Fig. 2). When FFA-CLA were esterified with <3 molar equivalents of glycerol without dehydration, conversion of MAG-CLA to DAG-CLA continued even after esterification degree reached a constant value (Fig. 1). The conversion was, however, depressed somewhat under the dehydration conditions (Fig. 2).

Second step: glycerolysis of DAG. It is well known that the yield of MAG in enzymatic reactions is increased at low temperatures, because MAG with low m.p. are excluded from the reaction system (3). In particular, *Pseudomonas* lipases efficiently catalyze conversion of TAG to MAG owing to a stepwise decrease in reaction temperature to 5°C in an organic solvent-free system (9). On the other hand, it was also reported that *Penicillium* lipase did not catalyze glycerolysis of TAG but did when used together with *Rhizomucor miehei* lipase (10). As described previously, the reaction products obtained by esterification of FFA-CLA with glycerol did not contain TAG, and the reaction mixture was mainly composed of MAG-CLA, DAG-CLA, and glycerol. *Penicillium* lipase is an enzyme that recognizes only MAG and DAG. The lipase may therefore catalyze glycerolysis of DAG-CLA at low temperature.

The four reaction mixtures (*ca.* 270 g) represented in Figure 2 were transferred into beakers and were then solidified by vigorous agitation on ice. The resulting preparations were allowed to stand at 5°C (Table 2). As a control, 5-g reaction mixtures were kept at 30°C with occasional stirring. *Penicillium* lipase did not catalyzed glycerolysis of DAG-CLA at 30°C but did at 5°C. The reaction proceeded efficiently with an increase in the amount of glycerol. When the reaction mixture with 5 molar equivalents of glycerol was allowed to stand at 5°C for 15 d, the MAG-CLA content increased from 50.2 to 88.6 wt% and the DAG content decreased from 44.4 to 7.3 wt%. Hydrolysis of acylglycerols was not observed, because only a slight amount of water was present in the reaction mixture.

The solidified reaction mixture after the glycerolysis was heated at 40°C, and was separated into oil and glycerol layers by centrifugation. The oil layer was allowed to stand at 30°C for 1 wk, but conversion of MAG to DAG was not observed. This result may be explained by the fact that the conversion proceeded poorly under dehydrated conditions (Fig. 2).

Composition of the reaction products. The products obtained from the first- and second-step reactions with 5 molar equivalents of glycerol were applied to a silica gel column, and MAG- and DAG-CLA were purified. FA compositions of the purified preparations are shown in Table 3. The compositions in MAG- and DAG-CLA after the esterification and glycerolysis completely agreed with that in the original FFA-CLA. These results show that *Penicillium* lipase did not act selectively toward FA in the FFA-CLA preparation.

A proposed process for synthesis of MAG-CLA. *Penicillium* lipase solution is added to a substrate mixture of FFA-CLA/glycerol (1:5, mol/mol). The amount of lipase is 200 U/g-mixture, and the added water content is set at <2 wt%. The first-step esterification is conducted at 30°C while agitating the mixture to form a homogeneous emulsion. Under these conditions, the esterification degree achieved is *ca.* 85%, and conversion of MAG-CLA to DAG-CLA starts

TABLE 3
FA Composition of MAG- and DAG-CLA Obtained from First- and Second-Step Reactions

	FA composition (wt%)							
				CLA[a]				
Sample[b]	16:0	18:0	18:1	*c*9,*t*11	*t*10,*c*12	*c*9,*c*11	*c*10,*c*12	Others
Original FFA	6.7	2.7	17.4	33.1	33.9	0.9	1.4	1.8
First-step								
MAG	6.4	2.6	17.9	33.3	34.5	1.0	1.3	1.9
DAG	6.6	2.9	17.2	32.7	34.0	0.9	1.4	2.0
Second-step								
MAG	6.7	2.3	18.3	33.2	33.6	1.1	1.5	1.9
DAG	7.0	2.8	16.9	33.5	33.7	0.8	1.4	1.8

[a]*c, cis; t, trans.*
[b]The products obtained from the reaction with 5 molar equivalents of glycerol against FFA-CLA were purified by silica gel column chromatography. The first-step products were purified from the 34-h reaction mixture depicted in Figure 2D, and the second-step products were purified from the 15-d reaction mixture in Table 2.

when the esterification degree has exceeded 60%. At an esterification degree of 60%, dehydration is initiated with a vacuum pump at 3–5 mm Hg to achieve the esterification degree of nearly 95%. The ratio of MAG-CLA/DAG-CLA in the steady state is approximately 1:1 (wt/wt).

The second step is glycerolysis of DAG-CLA. One procedure is to decrease the reaction temperature from 30 to 5°C. Agitation is not necessary after the reaction mixture is solidified. Alternatively, the reaction mixture after the esterification at 30°C can be recovered from the reactor. When the reaction mixture is taken out from the reactor, the viscosity can be decreased by heating. In this procedure, inactivation of the lipase can be ignored at <40°C, because the lipase in the reaction medium is stable at 50°C for at least 10 h. Vigorous agitation is necessary during solidification of the reaction mixture at low temperature (0–5°C), because the glycerolysis proceeds more efficiently upon making smaller particles of glycerol in the oil layer. The solidified reaction mixture is allowed to stand at 5°C. The two procedures achieve >95% esterification of FFA-CLA, and the content of MAG-CLA increases to nearly 90 wt%.

Molecular distillation is generally adopted for the industrial purification of MAG. FFA and MAG cannot completely be separated by this procedure, although MAG and DAG can be separated. A high esterification degree and high MAG yield are therefore required for production of a MAG product contamined with only a small amount of FFA. Because the two-step reaction achieves a high esterification degree and high MAG yield, the process may be applied to the industrial production of MAG-CLA.

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