Production of MAG of CLA by Esterification with Dehydration at Ordinary Temperature Using *Penicillium camembertii* Lipase

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ABSTRACT: Production of MAG with CLA using *Penicillium camembertii* mono- and diacylglycerol lipase (referred to as lipase) was attempted for the purpose of expanding the application of CLA. The commercial product of CLA (referred to as FFA-CLA) is a FFA mixture containing almost equal amounts of 9*cis*,11*trans* (9*c*,11*t*)-CLA and 10*t*,12*c*-CLA. Esterification of FFA-CLA with glycerol without dehydration achieved 84% esterification but produced almost equal amounts of MAG and DAG. Esterification with dehydration not only achieved a high degree of esterification but also suppressed the formation of DAG. When a mixture of FFA-CLA/glycerol (1:2, mol/mol), 1% water, and 200 units/g-mixture of *P. camembertii* lipase was agitated at 30°C for 72 h with dehydration at 5 mm Hg, the degree of esterification reached 95% and the contents of MAG and DAG were 90 and 6 wt%, respectively. This reaction system may be applied to the industrial production of MAG with unstable CLA.

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KEY WORDS: CLA, dehydration, esterification, lipase, MAG, *Penicillium camembertii*.

CLA refers to a group of C_{18} FA with two conjugated double bonds in either *cis* or *trans* configuration. A commercially available product contains almost equal amounts of 9*cis*,11*trans* (9*c*,11*t*)-CLA and 10*t*,12*c*-CLA. The FFA mixture of the two isomers has various physiological activities, such as reduction of incidence of cancer (1–3), decrease in body fat content (4–6), beneficial effects on atherosclerosis (7,8), and improvement of immune function (9). Much attention has been focused on these useful activities, and CLA has been used as a neutraceutical FA. The application of CLA can be expanded largely by developing MAG esterified with CLA because the new product may be used as an emulsifier that has nutraceutical activities.

Currently, MAG are produced industrially by chemical glycerolysis of oils and fats at high temperatures of 210–240°C (10,11), but the process cannot be applied to the synthesis of MAG of unstable FA, including CLA. On the contrary, because enzymes act effectively under mild conditions, lipase-catalyzed reactions have attracted much attention in the production of MAG and other acylglycerols containing unstable functional FA.

Many research groups have engaged in the synthesis of MAG by enzymatic hydrolysis, esterification, glycerolysis, and ethanolysis, but these reactions are usually conducted in organic solvents (12–16). Organic solvent-free systems are attractive from the viewpoint of industrial production of MAG. In addition, lipase-catalyzed esterification is advantageous for production of MAG-CLA because the FFA mixture containing CLA is the first product in the industrial process. MAG-CLA is produced efficiently in organic solvent-free esterification systems with several lipases including *Penicillium camembertii* MAG and DAG lipase (referred to as lipase) (17–20). These reactions reached 95% esterification, and the yield of MAG-CLA achieved was 90% by weight. However, these reaction systems have the drawbacks of being conducted at 5°C (energy consumption during production) and of requiring a large amount (5 mol) of glycerol. In this paper, we show these drawbacks can be eliminated by esterification with dehydration under reduced pressure.

MATERIALS AND METHODS

Materials. A FFA mixture containing 9*c*,11*t*- and 10*t*,12*c*-CLA was a commercial product (CLA80-HG; water content, 0.09–0.10%) of Nisshin OilliO Group, Ltd. (Tokyo, Japan). The product contained 37.5 wt% 9*c*,11*t*-CLA, 38.5 wt% 10*t*,12*c*-CLA, 1.2 wt% 9*c*,11*c*-CLA, 1.3 wt% 10*c*,12*c*-CLA, and 3.0 wt% other CLA isomers. This FFA mixture was referred to as FFA-CLA. The molar amount of FFA was calculated based on the acid value. Glycerol (water content, 0.21–0.27%) was purchased from Wako Pure Chemical Industry Co. (Osaka, Japan).

Penicillium camembertii lipase (Lipase G) was obtained from Amano Enzyme Inc. (Aichi, Japan). Activity of the lipase was measured by titrating FA liberated from monoolein (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) with 50 mM KOH as described previously (17). One unit (U) of lipase activity was defined as the amount of enzyme that liberated 1 μmol of FA per minute.

Reactions. A standard reaction was conducted at 30°C in a 1-L four-necked round-bottomed flask containing 297 g FFA-CLA/glycerol (1:2, mol/mol) and 3 mL *P. camembertii* lipase

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solution (20,000 U/mL) with agitation at 200 rpm. Dehydration was performed by evaporation at 5 mm Hg using a vacuum pump.

Analyses. About 4 g of the reaction mixture obtained from esterification of FFA-CLA with glycerol was separated into the oil and glycerol layers by centrifugation (6,500 × *g*, 5 min). The contents of MAG, DAG, 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). Analysis of a mixture of FFA/MAG/DAG (1:1:1, by wt) showed the same areas under the peaks. Hence, their contents were determined from the area percentages of their peaks. The degree of esterification was expressed as a ratio (mol%) of the amount of FA esterified to that of total FA in the reaction mixture. The M.W. used were 280, 354, and 616 for FFA, MAG, and DAG, respectively.

1(3)- and 2-MAG were detected by TLC on silica gel 60 plates (Merck, Darmstadt, Germany) impregnated with boric acid (19). The sample spotted onto the plate was developed in a mixture of chloroform/acetone/acetic acid (96:4:1, by vol). Components of the reaction products were visualized by spraying with 50% sulfuric acid in methanol, followed by heating at 150°C. Water content was measured by Karl Fischer titration with a Moisture Meter (Mitsubishi Chemical Corp., Tokyo, Japan).

All analyses were conducted three to five times under the same experimental conditions, and the average values were presented.

RESULTS AND DISCUSSION

The reaction mixture produced by esterification of FFA-CLA with glycerol using *P. camembertii* lipase was composed of FFA, MAG, DAG, and glycerol. Among the m.p. of these components, that of MAG was the highest: 35°C (20). Hence, the viscosity of the reaction mixture would increase as the reaction temperature decreased, and the mixture would solidify below 10°C. The mixture must be sufficiently fluid to allow its recovery from the reactor. The reaction temperature was therefore fixed at 30°C, at which the mixture maintained adequate fluidity during the reaction.

Effect of dehydration. A mixture of 294 g FFA-CLA/glycerol (1:5, mol/mol) and 6 mL *P. camembertii* lipase solution (10,000 U/mL) was agitated at 30°C without dehydration (Fig. 1A). MAG was synthesized in the early stage of the reaction. However, conversion of MAG to DAG started after 2 h, and the reaction reached nearly steady state after 24 h. The degree of esterification at 24 h was 83.9%, the content of MAG in the oil layer was 41.3 wt%, and the content of DAG was 44.6 wt%. Esterification of FFA with glycerol generates water. Hence, the content of water in the reaction mixture increased from 2.2 to 4.1% after 24 h (the content in the glycerol layer increased from 3.3 to 5.9%, and that in the oil layer increased from 0.4 to 1.6%) (Fig. 1C). On the contrary, the reaction with dehydration at 5 mm Hg efficiently synthesized MAG, and little conversion

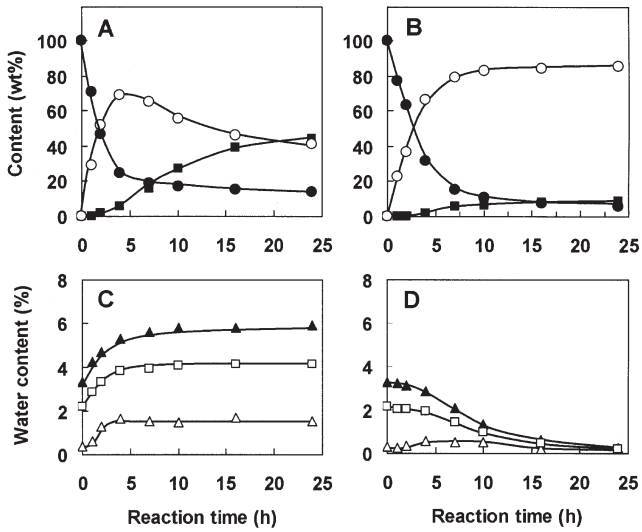

FIG. 1. Effect of dehydration on MAG production from a FFA mixture of CLA (FFA-CLA) and glycerol by *Penicillium camembertii* lipase. A 300-g mixture of FFA-CLA/glycerol (1:5, mol/mol), 2% water, and 200 U/g-mixture of lipase was agitated at 30°C and 200 rpm. (A and C) Reaction conducted without dehydration. (B and D) Reaction was conducted with dehydration at 5 mm Hg. (A and B) Contents of FFA (●), MAG (○), and DAG (■) in the oil layer. (C and D) Contents of water in the reaction mixture (□), glycerol layer (▲), and oil layer (△).

to DAG occurred (Fig. 1B). The degree of esterification reached 93.1% after 24 h, and the contents of MAG and DAG in the oil layer were 85.3 and 9.1 wt%, respectively. Water originating from the lipase solution and from generation by esterification was removed by dehydration, and the content of water

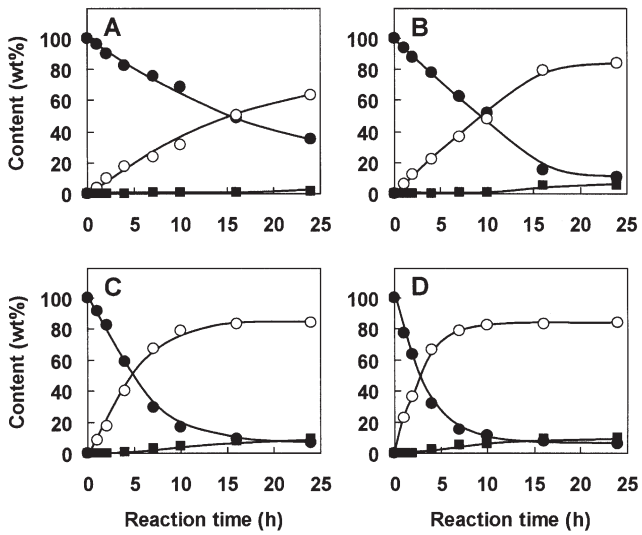

FIG. 2. Effect of glycerol amount on MAG production by *P. camembertii* lipase. A 300-g mixture of FFA-CLA/glycerol, 2% water, and 200 U/g-mixture of lipase was agitated at 30°C and 200 rpm with dehydration at 5 mm Hg. Reaction was conducted with (A) 1 mol glycerol for FFA-CLA; (B) 2 mol glycerol; (C) 3 mol glycerol; (D) 5 mol glycerol. Symbols are the same as those in the legend for Figure 1. See Figure 1 for abbreviations.

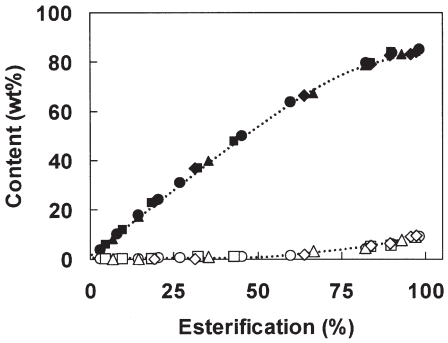

FIG. 3. Correlation among the degree of esterification and the contents of MAG and DAG in the reactions of Figure 2. The reaction with 1 mol glycerol was extended to 110 h. (●) Content of MAG in the reaction with 1 mol glycerol; (■) content of MAG in the reaction with 2 mol glycerol; (▲) content of MAG in the reaction with 3 mol glycerol; (◆) content of MAG in the reaction with 5 mol glycerol; (○) content of DAG in the reaction with 1 mol glycerol; (□) content of DAG in the reaction with 2 mol glycerol; (△) content of DAG in the reaction with 3 mol glycerol; (◇) the content of DAG in the reaction with 5 mol glycerol.

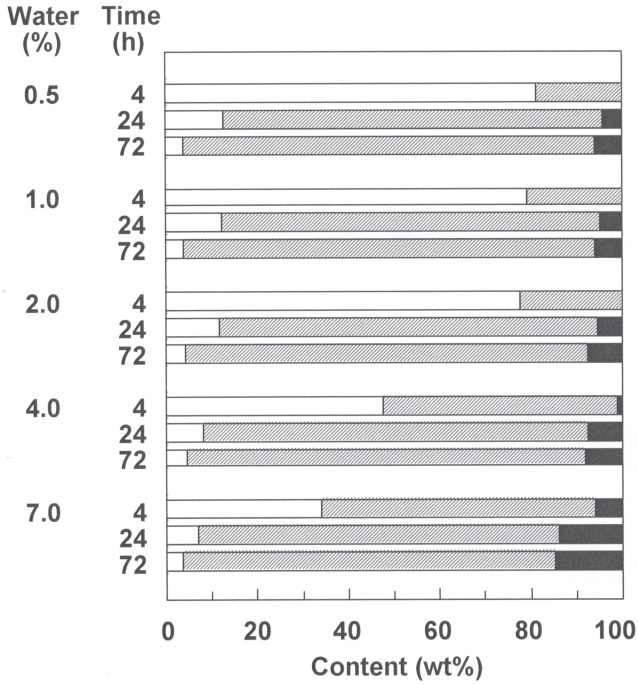

FIG. 4. Effect of initial water content on MAG production with *P. camembertii* lipase. A 300-g mixture of FFA-CLA/glycerol (1:2, mol/mol), 200 U/g-mixture of lipase, and 0.5–7% water was agitated at 30°C and 200 rpm with dehydration at 5 mm Hg. Open bars, content of FFA-CLA; hatched bars, content of MAG; closed bars, content of DAG.

in the reaction mixture declined from 2.2 to 0.22% after 24 h (the content in the glycerol layer decreased from 3.3 to 0.22%, and that in the oil layer was almost constant, 0.2–0.5%) (Fig. 1D). Dehydration not only achieved a high degree of esterification but also suppressed conversion of MAG to DAG.

Effect of glycerol amount. FFA-CLA was esterified at 30°C with 1–5 mol of glycerol with dehydration at 5 mm Hg (Fig. 2). An increase of glycerol accelerated the esterification. When the reaction with 1 mol glycerol was extended to 120 h, the degree of esterification reached 97.1%, and the contents of MAG and DAG were 84.2 and 9.3 wt%, respectively. Thus, dehydration suppressed the conversion of MAG to DAG, even though the mole ratio of FFA/glycerol was 1:1.

The contents of MAG and DAG in the reactions of Figures 2A–D were plotted as a function of the degree of esterification (Fig. 3). The contents of MAG and DAG depended on the degree of esterification, but not on the amount of glycerol. When the esterification was conducted with 1 mol glycerol per mole of FFA, the reaction velocity was slow; thus, the mole ratio of FFA-CLA/glycerol was fixed at 1:2.

Effect of initial water content. Esterification was conducted at initial water contents of 0.5–7% (Fig. 4). The initial velocity expressed by the reduction of FFA after 4 h was accelerated as the initial content of water was increased, although it was almost the same when the initial content was 2% or less. The content of DAG at the steady state (72 h) was the same when the initial water content was 1% or less, but 2% or more of water made the content of DAG increase. Hence, the initial content of water in subsequent reactions was fixed at 1%.

Time course. FFA-CLA was esterified with 2 mol glycerol at 1% of the initial water content using *P. camembertii* lipase. A typical time course is shown in Figure 5. The FFA was esterified efficiently in the first 24 h, and the content of MAG increased with the decrease of FFA (Fig. 5A). The esterification proceeded gradually even after 24 h, and the contents of MAG and DAG after 72 h were 89.8 and 5.9 wt%, respectively (degree of esterification, 94.7%). In this reaction, the oil and glycerol layers were difficult to separate at or after 16 h of reaction time. Hence, the content of water in the reaction mixture was measured (Fig. 5B). The content of water decreased from 1.1 to 0.16% during the first 24 h and was constant thereafter.

Penicillium camembertii lipase has been shown to be 1,3-positionally specific in the esterification of FFA-CLA with glycerol at 5°C (19). To study the positional specificity of the lipase in this reaction, regioisomers of synthesized MAG were analyzed by TLC. The main regioisomer was 1(3)-MAG throughout the reaction, and only a small amount of 2-MAG was detected after 10 h. Thus, the lipase displayed 1,3-position specificity during esterification with dehydration as well as in esterification at lower temperature.

The reaction system with dehydration succeeded in the production of MAG-CLA in a high yield and eliminated the drawbacks associated with conducting the esterification at low temperature (<5°C) and using large amounts (>5 mol) of glycerol. This reaction may be effective for an industrial production of MAG with unstable FA.

Effect of additional water on conversion of MAG to DAG. To confirm that water in the reaction mixture accelerates the conversion of MAG to DAG, addition of water in the course of the reaction was attempted. Esterification of FFA-CLA with 2 mol glycerol was conducted at 1% of initial water content with dehydration for 24 and 72 h. Water was added to the reaction mixtures at final concentrations of 0, 0.5, 1.0, and 2.0%, and

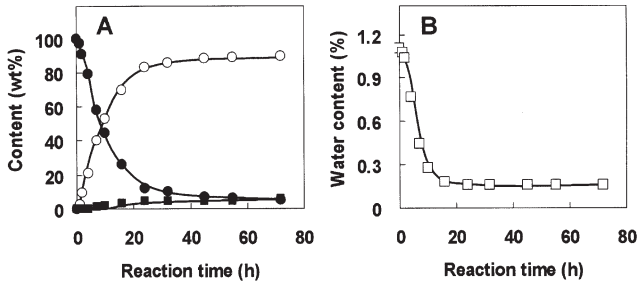

FIG. 5. Time course of MAG production by *P. camembertii* lipase. A mixture of 297 g FFA-CLA/glycerol (1:2, mol/mol) and 3 mL (20,000 U/mL) lipase solution was agitated at 30°C and 200 rpm with dehydration at 5 mm Hg. (A) Contents of FFA-CLA, MAG, and DAG in the oil layer. (B) Water contents in the reaction mixture. Symbols are the same as those in the legend for Figure 1.

each reaction was continued further for 48 h without dehydration (Table 1).

The degree of esterification at 24 h was 82.9%, and the contents of MAG and DAG were 80.8 and 5.1 wt%, respectively. The reaction was continued without addition of water and dehydration. During the reaction, esterification proceeded and generated water: The content of water increased from 0.16 to 0.71% after 48 h in total and to 0.74% after 72 h. This generated water led to conversion of MAG to DAG, and the content of DAG increased from 5.1 to 17.3 wt% after 48 h and to 23.5 wt% after 72 h. Addition of larger amounts of water accelerated the conversion, and addition of 2% water resulted in almost equal amounts of MAG and DAG after 48 h in total.

The degree of esterification at 72 h was 96.0%, and the contents of MAG and DAG were 91.2 and 5.6 wt%, respectively. When the reaction was continued without addition of water and dehydration, the degree of esterification increased from 96.0 to 97.6% after 120 h in total. Although esterification increased the content of water in the reaction mixture only slightly (from 0.15 to 0.22%), the content of DAG increased from 5.6 to 10.2 wt%. Of course, the conversion of MAG to DAG was accelerated by addition of larger amounts of water, and the contents of MAG and DAG became almost the same when the reaction was continued for 48 h (120 h in total) after addition of 2% water.

Role of water in conversion of MAG to DAG. We previously observed that conversion of MAG to DAG is repressed moderately by starting dehydration after 10 h of esterification of FFA-CLA with 5 mol glycerol using *P. camembertii* lipase (17). In this study, the conversion depended on the content of water in the reaction mixture (Table 1), agreeing with the previous result. In addition, when esterification of FFA-CLA with glycerol was continued for 48 h without dehydration after 72 h of reaction with dehydration, MAG was converted to DAG although little water was generated (Table 1). Conversion of MAG to DAG in the presence of only a very small amount of water may be explained by heterogeneous distribution of water molecules in the reaction mixture; i.e., more water molecules bond to the enzyme molecule. Under dehydration with a vacuum pump, water molecules generated by the esterification may be released from the enzyme molecule and homogeneously distributed in the reaction mixture. On the contrary, when the esterification is

TABLE 1
Effect of Additional Water on Conversion of MAG to DAG

Reaction mixture[a]	Water added (%)	Reaction time[b] (h)	Content (wt%)[c] FFA	MAG	DAG	Water content[d] (%)
24-h Mixture	—	24	14.1	80.8	5.1	0.16
	0	48	2.9	79.8	17.3	0.71
		72	2.2	74.3	23.5	0.74
	0.5	48	3.5	74.1	22.4	1.18
		72	3.2	62.3	34.5	1.19
	1.0	48	5.7	59.2	35.2	1.56
		72	5.6	48.8	45.6	1.56
	2.0	48	8.7	46.0	45.3	2.41
		72	8.6	44.2	47.2	2.42
72-h Mixture	—	72	3.2	91.2	5.6	0.15
	0	96	2.2	91.2	6.6	0.21
		120	2.0	87.8	10.2	0.22
	0.5	96	3.1	82.3	14.6	0.67
		120	2.7	72.8	24.5	0.68
	1.0	96	4.8	72.3	22.9	1.08
		120	4.8	61.0	34.2	1.08
	2.0	96	7.2	59.0	33.8	1.97
		120	7.1	47.7	45.2	1.98

[a]A 300-g mixture of FFA-CLA/glycerol (1:2, mol/mol), 1% water, and 200 U/g-mixture of *Penicillium camembertii* lipase was agitated at 30°C and 200 rpm with dehydration at 5 mm Hg for 24 and 72 h. To each reaction mixture (50 g) was added 0, 0.5, 1.0, and 2.0% water, and the mixture was stirred at 30°C without dehydration for a further 48 h.
[b]Total reaction time.
[c]The contents of FFA, MAG, and DAG in the oil layer.
[d]The content of water in the reaction mixture.

conducted without dehydration, the enzyme molecule may keep the water generated (perhaps near the catalytic site). The putative "bound" water would contribute to conversion of MAG to DAG more greatly than free water would.

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