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Production of monoacylglycerol of conjugated linoleic acid by esterification followed by dehydration at low temperature using *Penicillium camembertii* lipase

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Abstract

Conjugated linoleic acid (CLA) has various physiological activities, and a commercial product is a mixture of free fatty acids (named FFA-CLA) which contains almost equal amounts of 9-*cis*, 11-*trans* (9*c*,11*t*) and 10*t*,12*c* isomers. We attempted to efficiently produce monoacylglycerol (MAG) of CLA by lipase-catalyzed esterification. Study on effect of reaction temperature clarified that synthesis of diacylglycerols (DAGs) from MAGs was repressed at low temperature. When FFA-CLA was esterified at 5 °C with 5 molar equivalents of glycerol using 200 U/g mixture of *Penicillium camembertii* lipase in the presence of 2% water, the degree of esterification reached 89.6% after 45 h and the contents of MAGs and DAGs were 87.0 and 4.5 wt.%, respectively. Triacylglycerols were not synthesized in this *Penicillium* lipase-catalyzed esterification. After the esterification was conducted for 20 h (the degree of esterification, 80.8%), dehydration was started by evaporation at 5 mmHg using a vacuum pump. The degree of esterification increased concomitantly with dehydration and reached 94.5% after 16 h (36 h in total). The contents of MAGs (main components, 1(3)-isomers) and DAGs were 92.7 and 2.9 wt.%, respectively. Fatty acid compositions in MAGs synthesized with and without dehydration were the same as that in FFA-CLA. These results showed that the esterification system with dehydration is effective for producing MAGs in a high yield.

Keywords: Conjugated linoleic acid; Esterification; Lipase; Low temperature; Monoacylglycerol; *Penicillium camembertii*

1. Introduction

Conjugated linoleic acid (CLA) is a group of C_{18} fatty acids containing a pair of conjugated double bonds in either the *cis* or *trans* configuration. A typical commercial product is a mixture of free fatty acids (FFAs) which contains almost equal amounts of 9-*cis*, 11-*trans* (9*c*,11*t*) and 10*t*,12*c* isomers. The mixture containing CLA isomers (named FFA-CLA) has been reported to have various physiological activities, such as reduction of the incidence of cancer [1–3], decrease in body fat content [4–6], beneficial effect on atherosclerosis [7,8], improvement of immune function [9], and suppression of increase in blood pressure [10].

Much attention has been focused on the useful physiological activities of CLA, and CLA has been used as a nutraceutical food. If monoacylglycerols (MAGs) containing CLA were able to be produced, the new products could be used as an emulsifier which has nutraceutical activities. MAGs are currently produced industrially by chemical glycerolysis of oils and fats at high temperatures of 210–240 °C [11,12], but the process can not be applied to synthesize MAGs of unstable fatty acids including CLA. We thus aimed to synthesize MAG-CLA via a lipase-catalyzed reaction.

Many research groups engaged in the synthesis of MAGs by enzymatic hydrolysis, esterification, glycerolysis, and ethanolysis, but these reactions were conducted in organic solvents [13–18]. Meanwhile, organic solvent-free systems are attractive from the viewpoint of industrial production of MAGs. Several organic solvent-free systems have also been reported: (i) glycerolysis of triacylglycerols (TAGs) with *Pseudomonas* lipase [19,20]; (ii) ethanolysis of TAGs

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with immobilized *Candida antarctica* lipase [21,22]; (iii) esterification of FFAs with glycerol using *Penicillium camembertii* mono- and diacylglycerol lipase (referred to as *Penicillium* lipase) [23]. Because the first product of CLA is a mixture of FFAs, we attempted to produce MAGs from FFA-CLA and glycerol using *Penicillium* lipase and developed a two-step in situ reaction system that comprised esterification at 30 °C and glycerolysis at 5 °C [24]. This reaction produced 92 wt.% MAGs based on total content of MAGs and diacylglycerols (DAGs) at 95% esterification, but >2 weeks was necessary for completion of the reaction.

This paper shows that esterification of FFA-CLA with glycerol at low temperature represses the synthesis of DAGs from MAGs, resulting in an efficient production of MAGs.

2. Materials and methods

2.1. Materials

FFA-CLA was a commercial product (CLA-80; Rinoru Oil Mills Co. Ltd., Tokyo, Japan) produced by alkali conjugation of sunflower oil in propylene glycol. The FFA-CLA was composed of 34.3 wt.% 9*c*,11*t*-CLA, 35.3 wt.% 10*t*,12*c*-CLA, 3.7 wt.% other CLA isomers; 6.4 wt.% palmitic acid, 2.5 wt.% stearic acid, and 16.0 wt.% oleic acid. 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. Glycerol (water content, 0.2%) and olive oil was purchased from Wako Pure Chemical Industry Co. (Osaka, Japan), and monoolein and oleic acid were from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). DAG-rich oil (Econa Cooking Oil, Kao Corp., Tokyo, Japan) was purchased from a local supermarket. Other chemicals were of analytical grade.

2.2. Reactions

A small-scale esterification was conducted in a 50-ml vessel with stirring at 500 rpm. The reaction mixture (5.0 g in total) was composed of FFA-CLA, glycerol, and *Penicillium* lipase solution. Reaction temperature, the ratio of FFA-CLA/glycerol, and the amounts of the lipase and water were changed to study their effects on the esterification. When a reaction was conducted without addition of water, powdered enzyme was added gradually to the reaction mixture with agitation.

A standard large-scale reaction was conducted at 5 °C in a 1-l four-necked round-bottomed flask containing 294 g FFA-CLA/glycerol (1:5, mol/mol) and 6 ml *Penicillium* lipase solution (10,000 U/ml). The mixture was agitated at 200 rpm with an impeller. Dehydration during the reaction was performed at 5 mmHg using a vacuum pump. The degree of esterification was expressed as a ratio (mol%) of the amount of fatty acids esterified to total fatty acids in the reaction mixture. The molecular weights used were 280, 354, and 616 for FFA, MAG, and DAG, respectively.

2.3. Purification of MAGs

The solid reaction mixture was heated at 40 °C, and was then separated into oil and glycerol layers by centrifugation (6500 × *g*, 5 min). The oil layer (ca. 15 g) was diluted with the same volume of *n*-hexane and was applied to a silica gel column (120 g; 30 × 390 mm; Merck, Darmstadt, Germany). After DAGs and FFAs were eluted with 600 ml *n*-hexane/ethyl acetate (80:20, v/v), MAGs were eluted with 1000 ml *n*-hexane/ethyl acetate (50:50, v/v). Organic solvents in the MAG fraction were removed with an evaporator.

2.4. Analyses

About 1 g of the reaction mixture was separated into oil and glycerol layers by centrifugation (6500 × *g*, 5 min). A solid-state mixture was heated at 40 °C before centrifugation. The contents of MAGs, DAGs, and FFAs in the oil layer were measured by a TLC/FID analyzer (Iatroscan MK-5; Iatron Labratories Inc., Tokyo, Japan) after development with a mixture of *n*-hexane/ethyl acetate/acetic acid (90:10:1, v/v/v).

Fatty acid composition was determined by gas chromatography of fatty acid methyl esters. The constituent fatty acids in MAGs were converted to their methyl esters in 3 ml methanol containing 1% Na-methylate by heating at 70 °C for 15 min. FFAs were methylated in 3 ml of 5% HCl–methanol by heating at 70 °C for 10 min. The resulting fatty acid methyl esters were analyzed with a Hewlett-Packard 5890 gas chromatograph (Avondale, CA, USA) connected to a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA, USA) under the condition described previously [25].

1(3)-MAGs and 2-MAGs were detected by thin-layer chromatography (TLC) on boric acid-impregnated silica gel 60 plates (Merck). The TLC plate was prepared as follows: the plate, on which 3% boric acid had been sprayed, was dried overnight at room temperature, and then was baked at 120 °C for 10 min before use. The sample spotted onto the plate was developed in a mixture of chloroform/acetone/acetic acid (96:4:1, v/v/v). Components of the reaction products were visualized by spraying 50% sulfuric acid in methanol, followed by heating at 150 °C.

3. Results and discussion

3.1. Effect of reaction temperature

McNeill et al. [19,20] reported that glycerolysis of TAGs with *Pseudomonas* lipases proceeded efficiently by

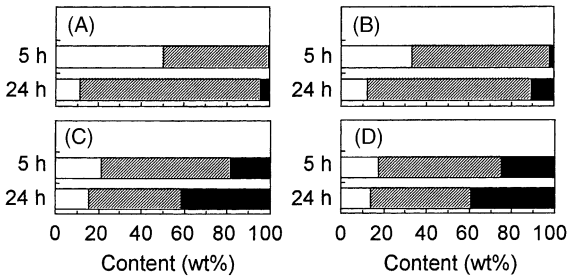

Fig. 1. Effect of temperature on esterification of FFA-CLA with glycerol. A mixture of 4.9 g FFA-CLA/glycerol (1:5, mol/mol) and 0.1 ml (1000 U) *Penicillium* lipase solution was stirred at 5–40 °C. After 5 and 24 h, the reaction mixture was centrifuged, and the contents of FFAs and acylglycerols in the oil layer were determined. The reaction was conducted at (A) 5 °C; (B) 15 °C; (C) 30 °C; (D) 40 °C. Open bars, the content of FFAs in oil layer; hatched bars, MAGs; closed bars, DAGs.

a stepwise decrease in the reaction temperature. Also, we clarified that *Penicillium* lipase catalyzed glycerolysis of DAGs containing CLA when the reaction mixture was kept at 5 °C [23]. It was assumed from these results that an efficient production of MAGs at low temperature is due to the following reason. The melting point of MAGs is the lowest among components in the reaction mixture. Because MAGs solidified at low temperature no longer participated in the reaction, they accumulated in the reaction mixture. In the light of this hypothesis, effect of temperature on esterification of FFA-CLA with glycerol was first studied.

FFA-CLA was esterified at the range of temperature from 5 to 40 °C with 5 molar equivalents of glycerol using 200 U/g mixture of *Penicillium* lipase (Fig. 1). The lipase did not synthesize TAGs, and the products were only MAGs and DAGs. The velocity of esterification increased at higher temperature and reached a maximal value around 40 °C. Almost the same amounts of MAGs and DAGs were synthesized after 24 h in the reaction at 30 and 40 °C. On the other hand, a decrease in the temperature repressed synthesis of DAGs. When the esterification was conducted at 5 °C for 24 h, the content of MAGs reached 95.4 wt.% based on total content of MAGs and DAGs (the degree of esterification, 86.1%). The temperature (5 °C) was the same as that in glycerolysis of DAGs containing CLA using *Penicillium* lipase [24].

3.2. Effect of glycerol amount

FFA-CLA was esterified at 5 °C with different amounts of glycerol using 200 U/g mixture of *Penicillium* lipase (Fig. 2). Larger amounts of glycerol increased the velocity of esterification and attained higher degree of esterification. In addition, large amounts of glycerol repressed synthesis of DAGs and increased the content of MAGs. When the reaction was conducted with 5 molar equivalents of glycerol for 48 h, the content of MAGs was 87.1 wt.% at 89.9% esterification. Based on these results, the amount of glycerol was fixed at 5 molar equivalents against FFA-CLA in the following reactions.

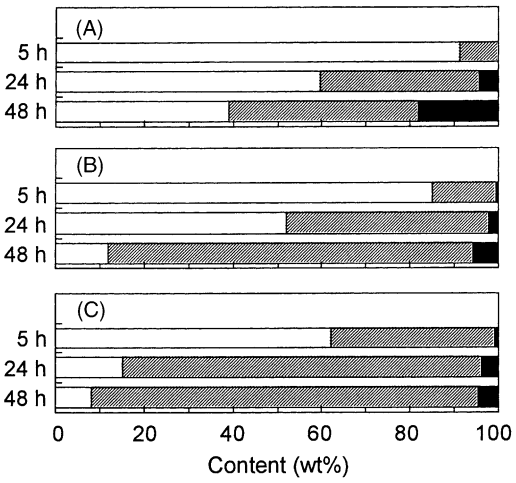

Fig. 2. Effect of glycerol amount on esterification of FFA-CLA with glycerol. A mixture of 4.9 g FFA-CLA/glycerol and 0.1 ml (1000 U) *Penicillium* lipase solution was stirred at 5 °C. The ratio of FFA-CLA/glycerol was (A) 1:1 (mol/mol); (B) 1:3 (mol/mol); (C) 1:5 (mol/mol). Open bars, the content of FFAs in oil layer; hatched bars, MAGs; closed bars, DAGs.

3.3. Effect of lipase amount

FFA-CLA was esterified at 5 °C with 5 molar equivalents of glycerol using 20–400 U/g mixture of *Penicillium* lipase (Fig. 3). Reaction velocity depended on the amount of enzyme. When >200 U/g mixture of lipase was used, the degrees of esterification after 24 h reached >80%. The amount was, therefore, fixed at 200 U/g mixture.

3.4. Effect of water content

Water content affects generally the degree of esterification at the equilibrium state; thus, effect of initial water content was studied. FFA-CLA was esterified at 5 °C with addition of different amounts of water (Fig. 4). Addition of water accelerated the velocity of esterification. But addition of 10% water deduced the velocity of esterification, and addition of 5 and >5% water decreased the degree of

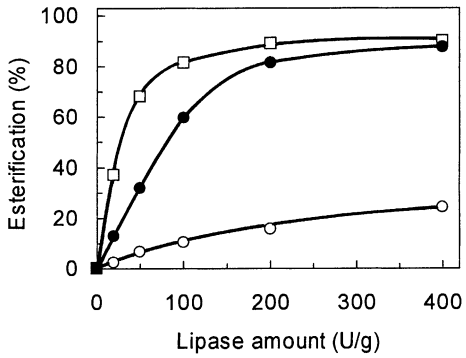

Fig. 3. Effect of lipase amount on esterification of FFA-CLA with glycerol. A mixture of 4.9 g FFA-CLA/glycerol (1:5, mol/mol) and 0.1 ml (100–2000 U) *Penicillium* lipase solution was stirred at 5 °C. Open circles, 3-h reaction; closed circles, 24-h reaction; open squares, 48-h reaction.

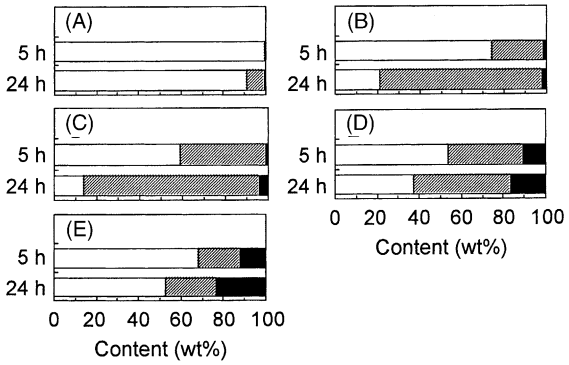

Fig. 4. Effect of water content on esterification of FFA-CLA with glycerol. A 5-g mixture of FFA-CLA/glycerol (1:5, mol/mol) and *Penicillium* lipase solution (1000 u) was stirred at 5 °C. In the reaction without addition of water, powdered enzyme was added gradually to the reaction mixture with agitation. (A) Without addition of water; (B) initial water content was 1.0%; (C) water content, 2.0%; (D) water content, 5.0%; (E) water content, 10.0%. Open bars, the content of FFAs in oil layer; hatched bars, MAGs; closed bars, DAGs.

esterification after 24 h. The inefficient esterification may be attributable to hydrolysis of synthesized products.

When the esterification was conducted with addition of >1.0% water, the reaction mixture changed from liquid to creamy after 2–4 h and its hardness increased gradually. Addition of larger amounts of water decreased the hardness of reaction mixture. This phenomenon suggested that large amounts of water disturb the solidification of MAG; leading to production of DAGs from MAGs (Fig. 4D and E).

The reaction without addition of water showed the low degree of esterification after 24 h (Fig. 4A). Also, the degree of esterification after 24 h with addition of 1.0% water was lower than that with addition of 2.0% water (Fig. 4B and C). A smaller amount of water increases generally the degree of esterification at the equilibrium state; thus, the reaction without addition of water was extended (Fig. 5). It was consequently found that esterification proceeded efficiently after 24-h lag. The degree of esterification reached 92.0% after 72 h, and the contents of MAGs and DAGs were 90.6 and 2.9 wt.%, respectively. This result showed that *Penicillium* lipase requires a small amount of water for full expression of the activity. When water was not added to the reaction mixture, the velocity of esterification was low. However, the lipase was activated fully even by a small amount of water generated by esterification.

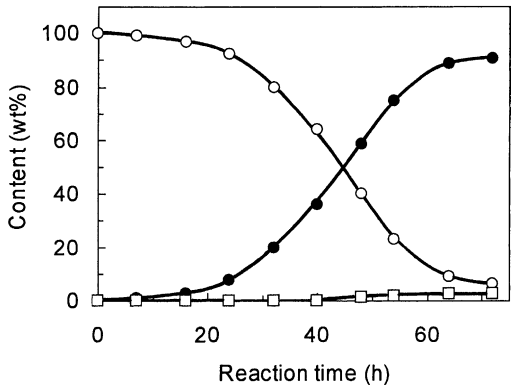

Fig. 5. Time course of esterification of FFA-CLA with glycerol without addition of water. Powdered *Penicillium* lipase (1.2 g; 60,000 U) was added gradually to a 300 g mixture of CLA-FFA/glycerol (1:5, mol/mol) with agitation at 200 rpm. The reaction was conducted at 5 °C and at the same agitation speed. Open circles, the content of FFAs in oil layer; closed circles, MAGs; open squares, DAGs.

3.5. Effect of dehydration

The esterification without addition of water achieved 92.0% esterification and 90.6 wt.% MAGs after 72 h (Fig. 5). Meanwhile, when esterification of FFA-CLA was conducted in a mixture with addition of 2.0% water, the degree of esterification and the content of MAGs after 48 h were 89.3% and 86.5 wt.%, respectively. These reactions generate 2.1% water at 90% esterification; thus, dehydration may be effective to achieve higher degree of esterification and higher yield of MAGs.

Esterification was conducted at 5 °C by agitating a 300-g mixture of FFA-CLA/glycerol (1:5, mol/mol), 200 U/g mixture of *Penicillium* lipase, and 2.0% water. Fig. 6A shows a typical time course of the reaction without dehydration. Esterification proceeded gradually even after 24 h, and the degree of esterification reached 89.6% after 45 h. The contents of MAGs and DAGs were 87.0 and 4.5 wt.%, respectively.

To evaluate the effect of dehydration, esterification of FFA-CLA with glycerol was conducted under the same conditions as those stated above, and dehydration was started after 20 h by evaporation at 5 mmHg using a vacuum pump (Fig. 6B). The degree of esterification increased concomitantly with dehydration and reached 94.5% after 16 h (36 h in total). The contents of MAGs and DAGs were 92.7 and 2.9 wt.%, respectively. These results showed that dehydration is effective for a decrease in reaction time and for a little increase in MAG yield.

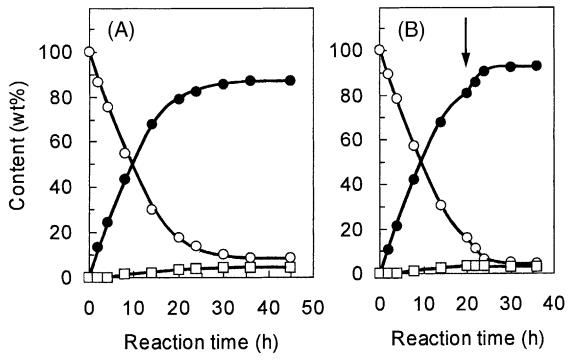

Fig. 6. Effect of dehydration on esterification of FFA-CLA with glycerol. A mixture of 294 g FFA-CLA/glycerol (1:5, mol/mol) and 6 ml (60,000 U) *Penicillium* lipase solution was agitated at 5 °C and 200 rpm. (A) The reaction was conducted without dehydration. (B) The reaction was conducted with dehydration: the reactor was connected to a vacuum pump at 20 h (indicated with arrow), and the reaction was continued with dehydration at 5 mmHg. Open circles, the content of FFAs in oil layer; closed circles, MAGs; open squares, DAGs.

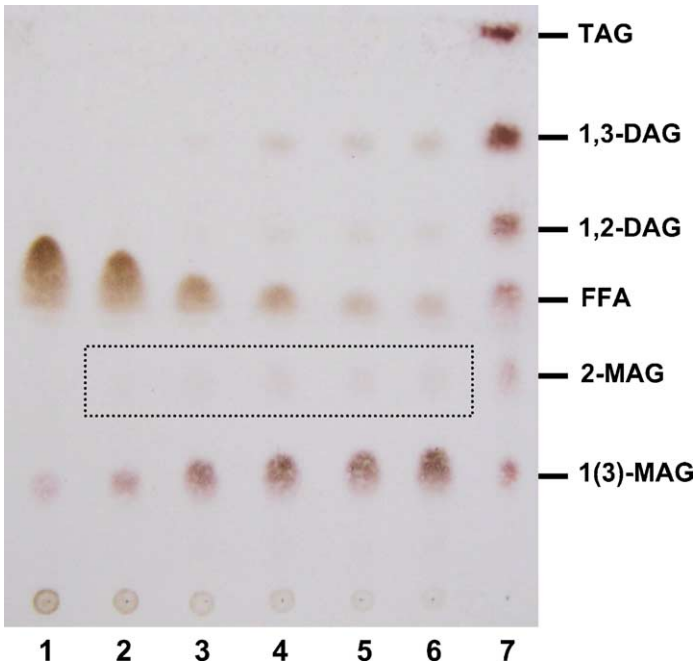

Fig. 7. TLC of reaction mixtures withdrawn from the reaction in Fig. 6B. Small amounts of 2-MAG were detected in the box shown with dotted line. Lane 1, sample after 2-h reaction; lane 2, 4 h; lane 3, 8 h; lane 4, 14 h; lane 5, 20 h; lane 6, 36 h; lane 7, a mixture of olive oil, DAG-rich oil, oleic acid, and monoolein.

Table 1
Fatty acid composition in MAGs obtained by esterification of FFA-CLA with *Penicillium* lipase

Reaction	Fatty acid composition (wt.%)					
	16:0	18:0	18:1	9*c*,11*t*[a]	10*t*,12*c*[a]	Others[a]
None (FFA-CLA)[b]	6.4	2.5	16.0	34.3	35.3	3.7
−Dehydration[c]	5.5	2.1	16.9	34.0	35.9	4.1
+Dehydration[d]	5.9	2.2	16.5	34.1	35.7	4.0

MAGs were purified from reaction mixtures in Fig. 6 by silica gel column chromatography.
[a] CLA, conjugated linoleic acid.
[b] Fatty acid composition in FFA-CLA used as a substrate.
[c] Fatty acid composition in MAGs purified from 45-h reaction mixture in Fig. 6A.
[d] Fatty acid composition in MAGs purified from 36-h reaction mixture in Fig. 6B.

3.6. Regioisomer of synthesized MAG

A portion of reaction mixture in Fig. 6B was periodically withdrawn, and regioisomers of synthesized MAGs were analyzed by TLC (Fig. 7). Main regioisomer was 1(3)-MAG through the reaction, and only a small amount 2-MAG was detected after 4 h. This result suggested that *Penicillium* lipase was 1,3-position-specific, and that acyl migration occurred very weakly because the reaction was conducted at low temperature.

3.7. Fatty acid composition of synthesized MAG

MAGs were purified from the reaction mixtures in Fig. 6A and B by silica gel column chromatography. Fatty acid compositions in the resulting MAGs are shown in Table 1. The compositions in MAGs were almost the same as that in FFA-CLA. It was, therefore, clarified that MAGs, in which fatty acid composition is the same as that in FFA-CLA, can be produced by esterification system with *Penicillium* lipase.

4. Conclusion

We previously reported that MAGs containing CLA can be produced by a two-step in situ reaction which comprises esterification of FFA-CLA with glycerol at 30 °C and glycerolysis of synthesized DAGs at 5 °C [24]. The reaction system, however, included a drawback that the reaction time is too long (ca. 2 weeks). This study clarified that esterification at 5 °C could reduce the reaction time to 36–45 h, and that the combination with dehydration could shorten further to 30–36 h. In addition, fatty acid composition of synthesized MAGs was the same as that in a substrate, FFA-CLA.

Because this esterification system achieves a high degree of esterification and high yield of MAGs, the reaction may be applied to the industrial production of MAG-CLA.

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