Synthesis of Triacylglycerol Containing Conjugated Linoleic Acid by Esterification Using Two Blended Lipases

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ABSTRACT: We have developed an efficient esterification for the synthesis of triacylglycerol (TAG) containing conjugated linoleic acids (CLA) using a blend of two powdered lipases. Two pairs of blended lipases promoted the esterification. *Rhizomucor miehei* lipase plus *Alcaligenes* sp. lipase and *Penicillium camembertii* MAG and DAG lipase plus *Alcaligenes* sp. lipase were used. At the optimal ratio of two lipases, the content of TAG containing CLA (TAG-CLA) in all glycerols reached 82–83% after 47 h using 1 wt% of lipases. With *R. miehei* lipase plus *Alcaligenes* sp. lipase, the reaction time to obtain *ca.* 60% of TAG-CLA was one-third of that needed with *R. miehei* lipase alone. The optimal ratio of two lipases differed between these two pairs. The optimal ratio was 70–80 wt% of *R. miehei* lipase in the last stage of the reaction, whereas it was over a wide range of 10–90 wt% for *P. camembertii* lipase. In the blend of *R. miehei* lipase plus *Alcaligenes* sp. lipase, activity remained very high after 10 cycles of esterification (every 47 h) and could be used in the industrial production of TAG-CLA.

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KEY WORDS: *Alcaligenes* sp., CLA, esterification, *Penicillium camembertii*, powdered lipase, *Rhizomucor miehei*, TAG.

In view of the nutritional and physiological functions of conjugated linoleic acid (CLA), it has attracted great attention in recent years (1–8). Several products containing CLA have been placed on the market as dietary supplements. CLA is generally available as an acid derived from conjugation, and has mainly two isomers consisting of 9-*cis*,11-*trans* and 10-*trans*,12-*cis* linoleic acids. The ester form of CLA, especially the TAG ester, is preferable to its acid form in applications for functional foods (9).

Generally, a synthesis of FA esters by esterification can be conducted with or without a chemical catalyst at high temperatures. When using CLA, some reaction problems may affect the quality of the CLA. For example, deterioration due to excessive heat or isomerization of the CLA may occur (9).

Some methods have been reported for the synthesis of acylglycerols containing CLA by esterification using lipase. For example, Watanabe *et al*. (10) synthesized MAG (MAG-CLA) using powdered lipase, and Arcos *et al*. (11) synthesized TAG (TAG-CLA) using lipase (11). It can be difficult to synthesize TAG by esterification using lipase because some have *sn*-1,3 regioselectivity.

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Previously, we discussed the application of powdered lipase in interesterification reaction systems (12–14). We showed that it was possible to use powdered lipase in such reaction systems and to synthesize TAG consisting of medium- and long-chain FA, phytosterol esters, and structured lipids containing PUFA. Powdered lipases having no support are very useful for blending and easily allow the chemical catalyst to be substituted for an enzymatic one.

When we attempted to synthesize TAG-CLA by the esterification of glycerol with CLA using powdered lipases, we discovered the efficacy of using two lipases having different regioselectivities. Specifically, we selected lipase pairs that could act well in the esterification, one with a high degree of *sn*-1,3 regioselectivity and one with a lower degree of *sn*-1,3 regioselectivity (14). By this method, we could obtain esterified CLA with a high content of TAG (TAG-CLA).

EXPERIMENTAL PROCEDURES

Materials and lipases. The CLA used was a commercially available product (CLA80-HG) manufactured by The Nisshin OilliO Group, Ltd. (Tokyo, Japan). The total CLA content was *ca*. 82 wt% of the FA composition and the two major isomers, 9-*cis*,11-*trans* and 10-*trans*,12-*cis* linoleic acids, were above 90% of the total CLA (10). The molar amount of CLA was calculated as the CLA (M.W. = 280.5). Glycerol was purchased from the Wako Pure Chemical Industry Co. (Osaka, Japan).

Powdered lipases were obtained from the following sources: *Alcaligenes* sp. lipase (lipase QLM) from Meito Sangyo Co. (Aichi, Japan); *Penicillium camembertii* lipase (lipase G "Amano" 50) from Amano Enzyme Inc. (Aichi, Japan); and *Rhizomucor miehei* lipase (a powdered lipase prepared from Palatase 20000 L) from Novozymes A/S (Bagsvaerd, Denmark). *Rhizomucor miehei* lipase powder (lipase RM-P) was prepared by ultrafiltration of a Palatase solution (Novozymes) followed by spray-drying.

Reactions. Enzymatic esterification of glycerol with CLA (1:3, mol/mol) was carried out by applying vacuum dehydration after stirring at atmospheric pressure for 30 min. The reaction temperature was held at 40°C for the first 3 h and then increased to 60°C at a rate of 5°C an hour (7 h in total). The starting temperature of 40°C was selected to prevent thermal deactivation of the powdered lipases. The pressure was gradually reduced to 20 hPa for the initial 8 h and held constant. The reaction was conducted for 47 h in total.

Two lipases were mixed at different ratios and added to the CLA mixture at 1 wt% under stirring. When used alone, the amount used was also 1 wt%. When lipase G plus lipase QLM were used, 2 wt% of water was added before the addition of the lipases.

The repeated-use operation was conducted by the following process. The reaction mixture obtained by esterification for 47 h was filtered under reduced pressure and the blended lipases were recovered. The content of TAG-CLA was analyzed from the filtrate to provide a value for the first batch. Using the recovered lipases, we carried out subsequent esterifications for 47 h. After the reaction, the recovery procedure was repeated.

Analysis. The content of TAG-CLA was determined by GLC (Shimadzu GC-17A) using a DB-1ht (5 m) column (Agilent Technologies, Palo Alto, CA) under the following conditions: injector temperature 370°C, detector temperature 370°C, initial temperature 50°C, heating rate 15°C/min, and helium carrier gas at a constant pressure. Samples were treated with trimethylsilyl reagent for detection of glycerol, and the content of TAG-CLA was calculated as a percentage of the total peak areas (glycerol, MAG-CLA, DAG-CLA, and TAG-CLA).

RESULTS AND DISCUSSION

Effect of two lipases. Lipase RM-P catalyzed the esterification under vacuum dehydration; the content of TAG-CLA reached 63% in 47 h (Fig. 1).

When using lipase RM-P plus lipase QLM mixed at a weight ratio of 80:20, the synthesis of TAG-CLA reached about 82% in one-third of the reaction time needed to produce *ca*. 60% of TAG-CLA with lipase RMP alone. Lipase QLM alone did not catalyze the esterification, producing only 3% TAG-CLA after 30 h. We speculate that the notable improvement obtained by using a blend of two lipases was due to the reactivity of these lipases (14). We also speculate that lipase QLM converted 1(3)-MAG and 1,3-DAG to 2-MAG and 1,2-DAG by interesterification. As the content of glycerol decreased, the level of TAG-CLA increased from 7 (4 h) to 41% (8 h) (Fig. 2).

With lipase G plus lipase QLM mixed at a weight ratio of 70:30, the synthesis of TAG-CLA reached about 83% (Fig. 1). In this blend, the role of each lipase was more clear. Lipase G alone synthesized almost no TAG-CLA and large amounts of DAG-CLA. For lipase G alone, the synthesis of TAG-CLA was 3% and the synthesis of DAG-CLA was 74% after 30 h. Lipase QLM alone was ineffective in catalyzing the esterification in the presence of glycerol. With both lipase G and lipase QLM, the synthesis of TAG-CLA was 20% after 4 h and 47% after 8 h (Fig. 3).

Ratio of lipases. We investigated the effects of changing the ratios of two lipases. For lipase RM-P plus lipase QLM, the optimal ratio was about 60 wt% of lipase RM-P during the initial stage (8 h) and 70–80 wt% during the last stage (after 30 h) (Fig. 4). Therefore, for an efficient esterification

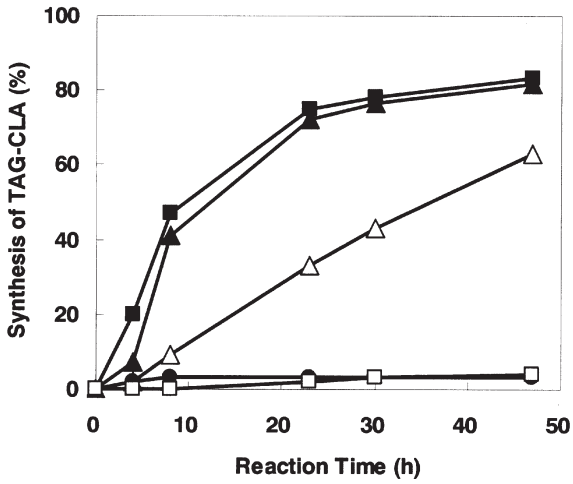

FIG. 1. Synthesis of TAG containing CLA (TAG-CLA) by the esterification of glycerol with CLA (1:3, mol/mol) under dehydration. Lipase RM-P alone (1 wt% of the mixture of glycerol and CLA), △; Lipase RM-P plus lipase QLM (80:20), ▲; Lipase G alone, □; Lipase G plus lipase QLM (70:30), ■; Lipase QLM alone, ●.

to synthesize TAG, a higher percentage of lipase RM-P was needed, and we obtained *ca*. 80% of TAG-CLA in the blend at 70–80 wt% of lipase RM-P after 30 h. Lipase QLM was able to improve the synthesis of TAG-CLA at contents as low as 5 wt%.

With lipase G plus lipase QLM, we observed a phenomenon of great interest (Fig. 5). The optimal ratio of lipase G during the initial stage (8 h) was 10–30 wt%. The synthesis of TAG-CLA was 64%, higher than that obtained with the best ratio of lipase RM-P plus lipase QLM. In contrast, for the result obtained with lipase RM-P plus lipase QLM, the effect was dependent on higher contents of lipase QLM. Thus, we speculate that lipase G efficiently synthesized MAG-CLA

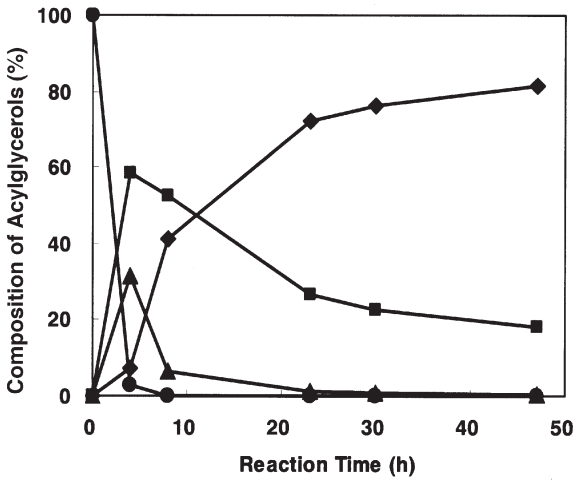

FIG. 2. Time course of esterification for the blend of lipase RM-P plus lipase QLM (80:20). Glycerol, ●; MAG containing CLA (MAG-CLA), ▲; DAG containing CLA (DAG-CLA), ■; TAG-CLA, ◆. For other abbreviation see Figure 1.

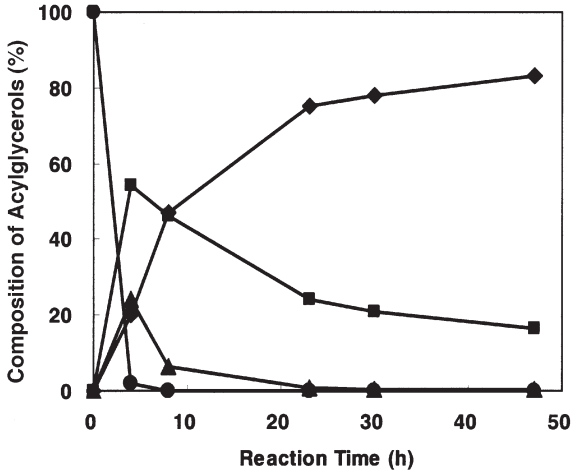

FIG. 3. Time course of esterification for the blend of lipase G plus lipase QLM (70:30). Glycerol, ●; MAG-CLA, ▲; DAG-CLA, ■; TAG-CLA, ◆. For abbreviations see Figures 1 and 2.

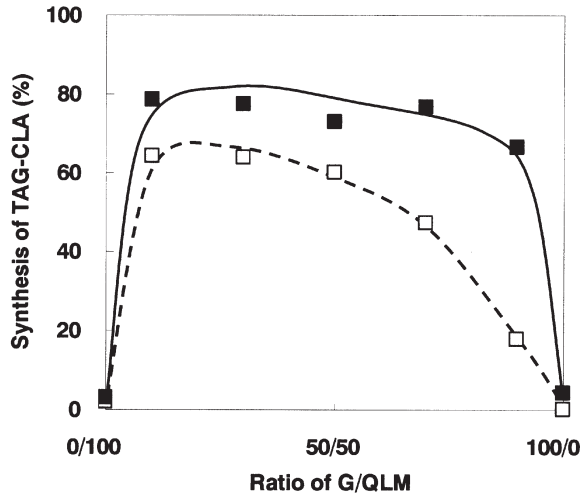

FIG. 5. Effect of the blending ratio. Lipase G/lipase QLM (w/w) at 8 h (□) and at 30 h (■). For abbreviation see Figures 1.

and DAG-CLA by the esterification of glycerol with CLA (10) and that lipase QLM interesterified effectively as the concentration of glycerol decreased. The range of optimal ratios of lipase G during the last stage (after 30 h) was very broad, from 10 to 90 wt%.

Stability in repeated use. In industrial production, the repeated use of lipase as a catalyst for TAG-CLA synthesis is necessary. We examined the stability of lipases in this esterification by conducting several batches without changing catalysts and evaluated the efficiency of the lipases every 47 h. For lipase RM-P plus lipase QLM (80:20), only a slight decrease was detected in the initial stage (8 h), and the yields remained almost unchanged after 10 batches (Fig. 6). In the case of lipase G plus lipase QLM (50:50), the duration of lipase activity was much shorter than that of lipase RM-P plus lipase QLM (80:20). This difference would be due to the different thermal stabilities of lipase RM-P and lipase G at 60°C.

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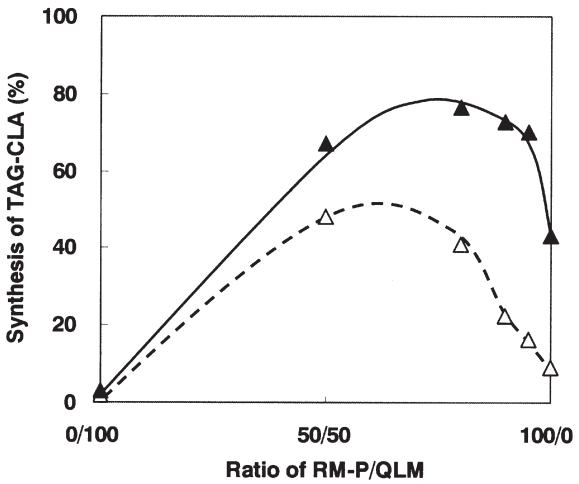

FIG. 4. Effect of the blending ratio. Lipase RM-P/lipase QLM (w/w) at 8 h (△) and at 30 h (▲). For abbreviation see Figures 1.

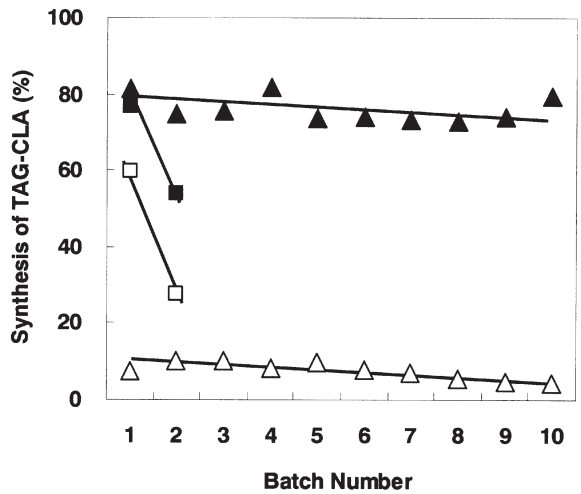

FIG. 6. Yields after repeated use. The reaction was repeated every 47 h. Lipase RM-P plus lipase QLM (80:20) at 8 h (△) and at 47 h (▲). Lipase G plus lipase QLM (50:50) at 8 h (□) and at 47 h (■). For abbreviation see Figures 1.

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