# Continuous Production of Structured Lipid Containing γ-Linolenic and Caprylic Acids by Immobilized *Rhizopus delemar* Lipase

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**ABSTRACT:** Production of a structured lipid containing γ-linolenic acid (GLA) achieved by the continuous acidolysis of borage oil with caprylic acid (CA) using 1,3-specific *Rhizopus delemar* lipase as a catalyst. The lipase immobilized on a ceramic carrier was activated by feeding the borage oil/CA (1:2, w/w) mixture saturated with water into a column packed with the enzyme. However, the generation of partial glycerides (20%) in the reaction mixture showed that hydrolysis occurred concomitantly with acidolysis. The concomitant hydrolysis was completely repressed by feeding the oil/CA substrate mixture without adding additional water. When the substrate mixture was fed at 30°C and a flow rate of 4.5 mL/h into a column packed with 8 g of the carrier with immobilized lipase, the content of CA incorporated in glycerides was 50 to 55 mol%. The acidolysis activity scarcely changed even though the substrate mixture was continuously fed for 60 d; then it gradually decreased. The CA content in glycerides was decreased to 73% of the initial value after 100 d, but returned to the initial level when the flow rate was reduced to 3.1 mL/h. Molecular distillation was employed to separate the transesterified oil from the reaction mixture. No glycerides were detected in the distillate, and the transesterified oil was recovered as the residue (acid value, 2.6). Regiospecific analysis of the transesterified oil showed that only fatty acids at the 1- and 3-positions of borage oil were exchanged for CA. It was additionally found by high-performance liquid chromatography analysis that all the triglycerides contained one or two CA, and that the triglyceride with two GLA and one CA was also present, because the lipase acted on GLA very weakly.

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**KEY WORDS:** Acidolysis, borage oil, caprylic acid, continuous reaction, immobilized enzyme, γ-linolenic acid, lipase, packed-bed reactor, *Rhizopus delemar*, structured lipid.

Arachidonic acid, an n-6 polyunsaturated fatty acid, is important as a precursor of local hormones involved in arachidonic acid cascade (1,2). γ-Linolenic acid (GLA), a precursor of arachidonic acid, also shows the physiological functions of modulating immune and inflammatory response (3), and borage and evening primrose oils containing GLA are used as an ingredient in cosmetics, food materials, a health food, and an infant formula (4).

Because structured lipids with medium-chain fatty acids at the 1- and 3-positions are rapidly absorbed into mucosal cells (5,6), a useful method of producing such lipids containing functional fatty acids is strongly desired. We previously reported a production method for structured lipid containing docosahexaenoic acid by exchanging fatty acids at the 1- and 3-positions of tuna oil for caprylic acid (CA) with immobilized *Rhizopus delemar* lipase, which is a 1,3-specific lipase used in food processing (7). By using the same method, structured lipids containing linoleic and α-linolenic acids were also produced effectively from safflower and linseed oils, respectively (8). This method is a batch reaction with the immobilized enzyme, and suffers from the defect that the enzyme is easily destroyed in an industrial agitator. In addition, the long period (48 h) necessary for the reaction is not desirable for the unstable polyunsaturated fatty acid. Therefore, we attempted to accomplish a continuous reaction with a packed-bed reactor from the viewpoint of industrial production.

We reported that fatty acids at the 1- and 3-positions of borage oil were exchanged for CA using immobilized *Rhizopus* lipase as a catalyst (9). The present paper deals with methods for producing structured lipid containing GLA by a continuous reaction with a column of the immobilized lipase and for purifying the lipid from the reaction mixture by molecular distillation.

## MATERIALS AND METHODS

*Oil and fatty acids.* Borage oil was a gift from the Nippon Synthetic Chemical Industry Co., Ltd. (Osaka, Japan; GLA, 22.2 wt%). CA and tricaprylin were commercial products of Yashiro Co., Ltd. (Osaka, Japan; purity, >98%).

*Assay of lipase activity.* Lipase activity was measured by titrating fatty acids liberated from olive oil (Wako Pure Chemical Industries Ltd., Osaka, Japan) with 50 mM KOH, as described previously (10). The reaction was carried out at 30°C for 30 min with stirring at 500 rpm. One unit (U) of li-

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pase activity was defined as the amount of the enzyme that liberated 1 μmol of fatty acid per minute.

*Preparation of immobilized lipase. Rhizopus delemar* lipase (Ta-lipase, 120,000 U/g) was a gift from Tanabe Seiyaku Co. (Osaka, Japan). The lipase was immobilized on a ceramic carrier, SM-10, a gift from NGK Insulators (Aichi, Japan), as described in our previous paper (7). In brief, after the ceramic carrier (50 g) was suspended in 200 mL of 10% lipase solution, cold acetone (–80°C) was gradually added with stirring, and the precipitate was dried *in vacuo*.

*Continuous reaction with packed-bed reactor*. The immobilized lipase (8 g) was suspended in 50 mL of the substrate mixture (borage oil/CA = 1:2, w/w), and then packed into a glass column (bed volume, 1.5 × 6.2 cm). The immobilized lipase was activated at 30°C by feeding 150 mL of the substrate mixture saturated with water (water content, 1.18%) at a flow rate of 4.5 mL/h. Then, 50 mL of the substrate mixture without adding additional water was fed at the same flow rate to repress hydrolysis occurring concomitantly with acidolysis of borage oil. The continuous reaction was performed under the same conditions.

*Batch reaction.* The reaction was carried out in a screw-capped vessel (20 mL) according to a method described elsewhere (8). The immobilized lipase was activated by the following reaction: a reaction mixture containing 12 g of the substrate mixture (borage oil/CA = 1:2, w/w), 0.24 g water, and 0.48 g of the immobilized lipase was incubated at 30°C for 48 h with shaking at 140 oscillations/min. Because the activated enzyme catalyzed acidolysis and hydrolysis simultaneously, the pretreatment of the enzyme was performed in 12 g of a fresh substrate mixture, without adding additional water, in the same manner as the activation process. The concomitant hydrolysis was completely repressed by repeating the pretreatment twice. The batch reaction was conducted under the same conditions as those of the pretreatment.

*Analysis.* Glycerides were extracted with 100 mL *n*-hexane after adding 70 mL of 0.5 N KOH (20% ethanol solution) to 5 g of the reaction mixture. Fatty acids in glycerides were methylated in methanol with sodium methylate as a methylating reagent. These methyl esters were analyzed with a Hewlett-Packard 5890 Plus gas chromatograph (Avondale, PA) connected to a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) as described previously (11). The contents of triglycerides (TG), diglycerides (DG), monoglycerides (MG), and free fatty acids (FFA) were measured with a thin-layer chromatograph/flame-ionization detector analyzer (Iatroscan MK-5; Iatron Co., Tokyo, Japan) after developing with a mixture of benzene/chloroform/acetic acid (50:20:0.7, vol/vol/vol). TG were analyzed on two octadecyl silica (ODS) columns (4.6 × 150 mm, Wakosil-II3C18 HG; Wako Pure Chemical) connected to a high-performance liquid chromatography (HPLC) system (LC-9A; Shimadzu Co., Kyoto, Japan). The sample was eluted with a mixture of acetone/acetonitrile (1:1, vol/vol) at a flow rate of 0.4 mL/min and 40°C, and was detected with a refractometer. Regiospecific analysis of TG was carried out by Grignard degradation with allyl magnesium bromide, followed by isolation and analysis of the 1,3-DG fraction (12).

The water content in the oil/fatty acid(s) mixture was determined by Karl Fischer titration (Moisture Meter CA-07; Mitsubishi Chemical Corp., Tokyo, Japan).

*Molecular distillation.* A molecular distillation apparatus (MS-300; Shibata Scientific Technology Ltd., Tokyo, Japan) was used for removing FFA from the reaction mixture obtained by the continuous acidolysis of borage oil with CA.

## RESULTS AND DISCUSSION

*Activation of immobilized lipase. Rhizopus* lipase immobilized on the ceramic carrier did not show any acidolysis activity, but was activated by the contact with the substrate containing water (7). Thus the substrate mixture saturated with water (water content, 1.18%) was fed into the immobilized lipase column. Figure 1 shows the water content in the reaction mixture and the content of CA incorporated in glycerides. The CA content in glycerides rapidly increased, and reached a constant value (50 mol%) after 50-mL feed. The immobilized enzyme gradually adsorbed water in the substrate mixture, and the adsorption was not observed after 100-mL feed. The amount of water adsorbed during this period was calculated to be about 0.5 mL. The glyceride compositions in the reaction mixture on feeding 50, 100, and 150 mL of the substrate were almost the same; TG/DG/MG = 80:17:3, w/w/w. This result showed that hydrolysis occurred concomitantly with acidolysis during the activation of the immobilized lipase.

After feeding 195 mL of the substrate mixture saturated with water, the substrate mixture without additional water (water content, 0.154%) was fed under the same conditions (Fig. 1). The water content in the reaction mixture was rapidly decreased to the level shown by the substrate mixture after

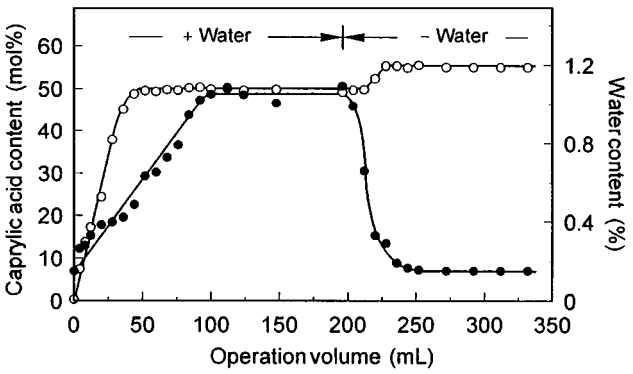


**FIG. 1.** Activation of immobilized *Rhizopus delemar* lipase. The substrate mixture (borage oil/caprylic acid = 1:2, w/w) saturated with water was fed into the immobilized lipase column (8 g lipase; 1.5 × 6.2 cm) at 30°C and a flow rate of 4.5 mL/h. The substrate mixture without additional water was fed under the same conditions after feeding 195 mL of the mixture saturated with water. ○, Content of caprylic acid incorporated in glycerides; ●, water content in the reaction mixture.

50-mL feed (total volume, 245 mL). The CA content in glycerides was increased a little by removing the excess water from the immobilized lipase. Furthermore, the glyceride composition (TG/DG/MG) in the reaction mixture was 98.8:1.0:0.2, suggesting that the concomitant hydrolysis scarcely occurred. From these results, the activation of the immobilized enzyme was performed by feeding 150 mL of the substrate mixture saturated with water into a column packed with 8 g of the enzyme, and the treatment was then done by feeding 50 mL of the substrate mixture without adding additional water.

*Effect of flow rate on acidolysis of borage oil with CA.* The effect of the flow rate of the substrate mixture on the amount of CA incorporated in glycerides was investigated using a column of the activated immobilized lipase (Table 1). The CA content in glycerides increased with reducing the flow rate, and reached a constant value at a flow rate of 6 mL/h.

The batch reaction was conducted by shaking the substrate mixture with 4% of the immobilized lipase. Because 8 g of the immobilized lipase is used in the continuous reaction, the period necessary for feeding 200 g of the substrate mixture in the continuous reaction corresponds to the reaction time in the batch reaction. Thus the feedings at flow rates of 4, 6, 8, 16, and 32 mL/h correspond to 56, 37, 28, 14, and 7 h in the batch reaction, respectively. The CA content in glycerides at each flow rate completely agreed with the time course in the batch reaction reported previously (9). This result showed that the continuous reaction proceeded under the optimal conditions.

*Stability of immobilized enzyme.* The continuous reaction was performed according to the method described in the Materials and Methods section. While water was not added to the substrate mixture, the water content was 0.03 to 0.16% because of the adsorption of water from the atmosphere. Figure 2 shows the CA content in glycerides obtained by the extraction with *n*-hexane. The acidolysis activity scarcely changed for 60 d, and then gradually decreased. The CA content in glycerides was decreased to 73% of the initial value after 100 d, but returned to the initial level after the flow rate was reduced to 3.1 mL/h.

*Removal of FFA from reaction mixture.* The reaction mixture that flowed from the above column after 30–50 d was collected, and 1 kg of the mixture was used for the molecular distillation. The distillation was performed by measuring the acid value of the residue as a guide (Table 2). When the distillation was performed at 130°C under 0.2 mm Hg, 644 g was separated as the first distillate, and the acid value was 369. Because the acid value of the residue was 54, the distillation temperature was increased to 170°C. As a result, 52 g was separated as the second distillate (acid value, 202), but the acid value of the residue was still 26. Further distillation at 195°C brought the third residue to acid value 2.6 with 24.8% of the recovery.

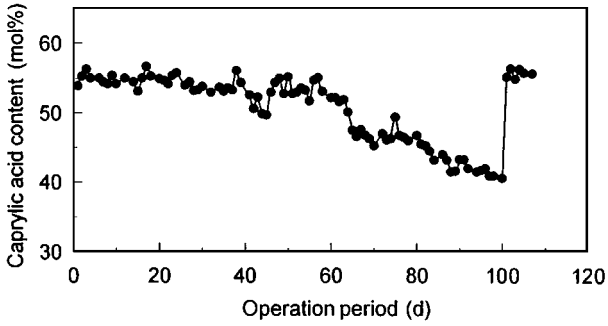


**FIG. 2.** Stability of immobilized lipase in continuous flow reaction. The continuous flow reaction was performed according to the method described in the Materials and Methods section. The flow rate was 4.5 mL/h for 100 d, and was then reduced to 3.1 mL/h.

Only FFA were present in the first, second, and third distillates, and TG, DG, and MG were not found. The third residue contained 98.7% TG, 0.5% DG, and 0.8% FFA. From these results, it was found that molecular distillation very effectively removed FFA from the reaction mixture.

*Composition of transesterified oil purified by molecular distillation.* Table 3 shows fatty acid compositions of the 1(3)- and 2-positions in the original borage oil and the transesterified oil. The fatty acid compositions of the 2-positions in the oils before and after the reactions were the same; CA was not incorporated into the 2-position. This result showed that only fatty acids at the 1(3)-position in borage oil were exchanged for CA by the continuous reaction with immobilized *Rhizo-*

**TABLE 1**
**Effect of Flow Rate on Acidolysis of Borage Oil with Caprylic Acid[a]**

| | Fatty acid composition (mol%)[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Flow rate (mL/h) | 8:0 | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | 20:1 | 22:1 | 22:4 |
| 4 | 54.8 | 1.5 | 0.6 | 6.7 | 16.9 | 18.4 | 0.6 | 0.3 | n.d.[d] |
| 6 | 54.4 | 1.5 | 0.6 | 6.8 | 17.1 | 18.6 | 0.7 | 0.3 | n.d. |
| 8 | 47.3 | 1.8 | 0.7 | 7.4 | 18.7 | 22.8 | 0.8 | 0.4 | n.d. |
| 16 | 44.6 | 2.4 | 1.0 | 9.1 | 22.2 | 22.9 | 1.3 | 0.8 | 0.3 |
| 32 | 36.0 | 3.9 | 1.7 | 9.6 | 22.7 | 22.7 | 1.8 | 1.1 | 0.5 |
| Original[c] | — | 10.6 | 3.9 | 17.5 | 38.4 | 22.4 | 3.9 | 2.1 | 1.2 |

[a]The substrate mixture was fed into an immobilized lipase column (8 g lipase; 1.5 × 6.2 cm) at 30°C and various flow rates.
[b]Fatty acid composition of glycerides in the reaction mixture flowing from the column.
[c]Fatty acid composition of the original borage oil.
[d]Not detectable.

**TABLE 2**
**Purification of Transesterified Oil by Molecular Distillation**

| Step | Acid value | Recovery (%) |
|---|---|---|
| Reaction mixture[a] | 263 | 100 |
| First distillate[b] | 369 | 64.4 |
| Second distillate[c] | 202 | 5.2 |
| Third distillate[d] | 181 | 3.9 |
| Third residue[d] | 2.6 | 24.8 |

[a]Oil/fatty acids mixture flowing from the immobilized lipase column.
[b]Distilled at 130°C under 0.2 mm Hg.
[c]Distilled at 170°C under 0.2 mm Hg.
[d]Distilled at 195°C under 0.2 mm Hg.

*pus* lipase. The acidolysis extent was calculated to be 79%, because 52.7% CA was incorporated into glycerides.

The TG compositions of the original oil and the transesterified oil were analyzed by HPLC (Fig. 3). All the TG obtained by acidolysis of borage oil were new components, which eluted at retention times much shorter than those of TG in the original oil. Because tricaprylin eluted at 11.7 min, it was shown that all transesterified oils contained one or two CA at the 1(3)-position. To identify the structures of TG fractionated by HPLC, the components numbered in Figure 3 were collected, and their fatty acid compositions were analyzed. On the basis of the molar ratio of fatty acids, the structures of peaks I to IV were estimated as follows: peak I, 1,3-capryloyl-2-γ-linolenoyl-glycerol; peak II, 1,3-capryloyl-2-linoleoyl-glycerol; peak III, 1,3-capryloyl-2-oleoyl-glycerol; peak IV, TG with one molecule of CA and two molecules of GLA.

Partial glycerides and tricaprylin were not detected in the first, second, and third distillates, and tricaprylin was not found in the third residue (purified transesterified oil). In addition, CA was not detected in the 2-position of the transesterified oil. Therefore, the continuous reaction with immobilized *Rhizopus* lipase proceeded 1,3-specifically, and it was confirmed that hydrolysis, esterification, and intramolecular acyl migration did not occur concomitantly.

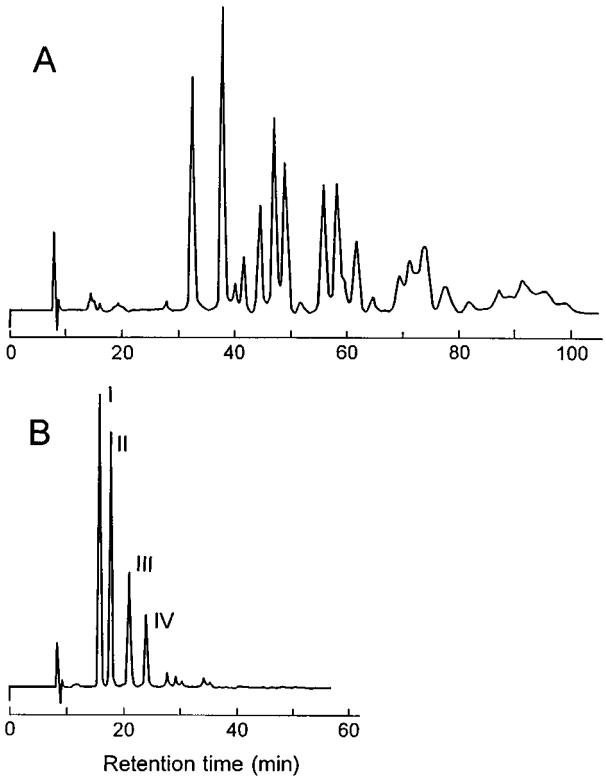


**FIG. 3.** Triglyceride components of the original borage oil (A) and the oil obtained by continuous acidolysis (B). High-performance liquid chromatography analysis was carried out as described in the Materials and Methods section. The peaks numbered in B are described in the text.

*Advantage of continuous flow reaction.* The ceramic carrier was easily destroyed in an agitator with impeller, although it was stable for shaking in a screw-capped vessel. Thus the immobilized lipase can not be used as a catalyst in the batch reaction with an industrial agitator. On the other hand, the enzyme-immobilized carrier was able to be used for

**TABLE 3**
**Fatty Acid Compositions at 1(3)- and 2-Positions of Borage Oil and Transesterified Oil[a]**

| | Fatty acid composition (mol%) | | | | | |
|---|---|---|---|---|---|---|
| | Borage oil | | | Transesterified oil | | |
| Fatty acid | Total | 1(3) | 2 | Total | 1(3) | 2 |
| 8:0 | — | — | — | 52.7 | 52.8 | n.d.[b] |
| 16:0 | 10.6 | 10.6 | n.d. | 1.6 | 1.5 | 0.1 |
| 18:0 | 3.9 | 3.9 | n.d. | 0.6 | 0.6 | n.d. |
| 18:1 | 17.5 | 12.2 | 5.3 | 6.9 | 1.7 | 5.2 |
| 18:2 | 38.4 | 24.4 | 14.0 | 17.4 | 3.5 | 13.9 |
| 18:3 | 22.4 | 6.0 | 16.4 | 19.6 | 3.2 | 16.4 |
| 20:1 | 3.9 | 3.8 | 0.1 | 0.7 | 0.7 | n.d. |
| 22:1 | 2.1 | 2.2 | n.d. | 0.3 | 0.4 | n.d. |
| 24:1 | 1.2 | 1.1 | 0.1 | 0.1 | n.d. | 0.1 |

[a]The fatty acids at the 1(3)-position of triglyceride were analyzed using 1,3-diglycerides obtained by Grignard degradation. The fatty acids at the 2-position were calculated from the fatty acid compositions of triglyceride and 1,3-diglyceride. Total amount of fatty acids at the 1(3)- and 2-positions is expressed as 100%.
[b]Not detectable.

more than 100 d without being destroyed in the packed-bed reactor, showing that the reaction system was effective for the industrial production of the structured lipid. In addition, 1.3 h (the retention time in the column) was enough for the continuous reaction, although the long period (48 h) was required for the batch reaction (9). The shorter reaction time is advantageous to the processing of an oil containing unstable polyunsaturated fatty acid because of the reduction of oxidation and isomerization.

When CA was allowed to stand in an open vessel for 1 mon, the water content increased from 0.035 to 0.27% by absorbing water from the atmosphere. In this study, the continuous reaction was performed using a substrate mixture with a water content of 0.03 to 0.16%, but reactions other than acidolysis scarcely occurred. When substrate mixtures containing 0.27, 0.51, and 1.02% of water were fed into the column of the immobilized lipase, the CA contents in glycerides were 53.1, 53.7, and 49.5 mol%, respectively, and the contents of partial glycerides were 0.8, 3.7, and 15.3 wt%, respectively. These results showed that concomitant hydrolysis was negligible on feeding the substrate mixture containing less than 0.27% water. Therefore, it is not necessary to control the water content in the substrate mixture precisely.

Acidolysis reported previously was generally conducted in a mixture containing *n*-hexane (13–15). In contrast, the system described here did not require any organic solvents for the reaction, or for the purification of the products. Thus a small reactor can be used, and the risk of explosion can be avoided.

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