Purification of γ-Linolenic Acid from Borage Oil by a Two-Step Enzymatic Method

Yuji Shimada[a,*], Akio Sugihara[a], Masahiro Shibahiraki[b], Hiroyuki Fujita[c], Hirofumi Nakano[a], Toshihiro Nagao[a], Tadamasa Terai[b], and Yoshio Tominaga[a]

[a]Osaka Municipal Technical Research Institute, Osaka 536 Japan, [b]Osaka Institute of Technology, Department of Applied Chemistry, Osaka 535 Japan, and [c]The Nippon Synthetic Chemical Industry Co., Ltd., Osaka 565, Japan

ABSTRACT: γ-Linolenic acid (GLA) was purified from borage oil by a two-step enzymatic method. The first step involved hydrolysis of borage oil (GLA content 22.2 wt%) with lipase, *Pseudomonas* sp. enzyme (LIPOSAM). A mixture of 3 g borage oil, 2 g water, and 3000 units (U) LIPOSAM was incubated at 35°C with stirring at 500 rpm. The reaction was 61.5% complete after 2.5 h. The resulting free fatty acids (FFA) were extracted from the reaction mixture with *n*-hexane (GLA content, 22.5 wt%, recovery of GLA, 92.7%). The second step involved selective esterification of borage-FFA with lauryl alcohol by using *Rhizopus delemar* lipase. A mixture containing 4 g borage-FFA/lauryl alcohol (1:2, mol/mol), 1 g water, and 10000 U lipase was incubated at 30°C for 20 h with stirring at 500 rpm. Under these conditions, 74.4% of borage FFA was esterified, and the GLA content in the FFA fraction was enriched from 22.5 to 70.2 wt% with a recovery of 75.1% of the initial content. To further elevate the GLA content, unesterified fatty acids were extracted, and esterified again in the same manner. By this repeated esterification, GLA was purified to 93.7 wt% with a recovery of 65.5% of its initial content.

JAOCS 74, 1465–1470 (1997).

KEY WORDS: Borage oil, hydrolysis, lauryl alcohol, γ-linolenic acid, *Pseudomonas* sp. lipase, purification, *Rhizopus delemar* lipase, selective esterification.

Arachidonic acid (AA), one of n-6 polyunsaturated fatty acids (PUFA), is important as a precursor of local hormones, prostaglandins, leukotrienes, and thromboxanes involved in the AA cascade (1,2), and a single-cell oil containing AA produced by *Mortierella alpina* is expected to be used in an infant formula (3). γ-Linolenic acid (GLA), a precursor of AA, also shows the physiological functions of modulating immune and inflammatory response (4), and borage oil containing GLA is used as the ingredient of cosmetics, food materials, a health food, and an infant formula (5). Thus the highly purified GLA is desired as a medicine and an ingredient of cosmetics.

Because PUFA are very sensitive against heat and oxidation, the enzymatic reactions which proceed effectively under the ordinary temperature and pressure and the nitrogen stream are suitable for the high-level processing of PUFA-containing oils and of their related compounds. Actually, docosahexaenoic acid (DHA) could be enriched in glycerides by selective hydrolysis of tuna oil with a lipase (6–9), and 45 to 50% of DHA-containing oil has been commercialized in Japan since 1994 (10). By using same method, high concentrations of AA- and GLA-containing oils were produced from the single-cell oil from *Mortierella* and borage oil, respectively (11,12). Furthermore, a structured lipid containing oleic acid (OA), linoleic acid (LOA), GLA, α-linolenic acid (ALA), AA, or DHA could be produced by acidolyses of natural oils with medium-chain fatty acid using immobilized 1,3-specific lipase (13–17). It was additionally reported that GLA was purified by selective esterification of fatty acids originating from borage oil with *n*-butanol (18,19). However, since this reaction required large amount of *n*-hexane, the improvement of this system has been desired.

We recently found that selective esterification of fatty acids with lauryl alcohol did not require organic solvent. The reaction proceeded very effectively on using *Rhizopus delemar* lipase as a catalyst, and the DHA content was elevated from 23 to 89% in a good yield when fatty acids from tuna oil were used as substrates (20). In this paper, we describe a method of purifying GLA by a two-step enzymatic method. The first step is hydrolysis of borage oil with *Pseudomonas* sp. lipase, and the second step is selective esterification of fatty acids from borage oil with lauryl alcohol using *Rhizopus* lipase.

MATERIALS AND METHODS

Oil, fatty acids, and alcohol. Borage oil refined by the Nihon Synthetic Chemical Industry Co., Ltd. (Osaka, Japan; GLA, 22.2% w/w) was used. Lauryl alcohol was of reagent grade from Wako Pure Chemicals Ind., Co. (Osaka, Japan).

Lipases. Lipases were gifts from the following companies: *Pseudomonas aeruginosa* lipase (LPL; Toyobo Co., Ltd., Osaka, Japan); *Pseudomonas* sp. (Lipase-AK; Amano Pharmaceutical Co., Ltd., Aichi, Japan); *Pseudomonas* sp. KWI-56 lipase (Kurita Water Ind., Ltd., Tokyo, Japan); *Pseudomonas* sp. lipase (LIPOSAM; Showa Denko K.K., Tokyo, Japan); *Chromobacterium viscosum* lipase (Asahi Chemical Industry Co.,

*To whom correspondence should be addressed at Osaka Municipal Technical Research Institute, 1-6-50 Morinomiya, Joto-ku, Osaka 536, Japan. E-mail: shimaday@comtricity.osaka.jp

Ltd., Tokyo, Japan), *Candida rugosa* lipase (Lipase-OF; Meito Sangyo Co. Ltd., Aichi, Japan); *R. delemar* lipase (Ta-lipase; Tanabe Seiyaku Co. Ltd., Osaka, Japan).

Fractionation of esters and free fatty acids (FFA) in reaction mixture. Esters (glycerides and lauryl esters) and lauryl alcohol were extracted with 100 mL *n*-hexane after adding 70 mL of 0.5 N KOH (30% ethanol solution) into 5 g of reaction mixture. FFA in the water phase were extracted with 100 mL *n*-hexane after acidification with HCl (8).

Analysis. Lipase activity was measured by titrating fatty acids liberated from olive oil (Wako Pure Chemical) with 0.05 N KOH as described previously (21). The reaction was carried out at 35°C for 30 min with stirring at 500 rpm. One unit (U) of lipase activity was defined as the amount of enzyme that liberated 1 μmol of fatty acid per minute.

Fatty acids were methylated at 80°C in 5% HCl-methanol for 3 h, and 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 (20).

Fatty acid specificity of lipase in hydrolysis. Evaluation of fatty acid specificity of lipases in hydrolysis was carried out using the randomly interesterified oil as a substrate (11,17). The randomly interesterified oil was prepared by the conventional interesterification of several triglycerides using Na-methylate as a catalyst according to the previous paper (11,22). A reaction mixture containing 1 g of the interesterified oil, 3 mL of water, and 100 U of lipase was incubated at 35°C with stirring at 500 rpm for 30 min. After the hydrolysis, the FFA fraction was extracted with *n*-hexane, and its fatty acid composition was analyzed. The activity on a fatty acid ester (A_{hyd}) was expressed according to Equation 1,

$$A_{hyd} = F_{fa}/F_{ox} \qquad [1]$$

where F_{fa} and F_{ox} are the content (mol%) of particular fatty acid in the FFA fraction after hydrolysis and in the interesterified oil before hydrolysis, respectively.

Hydrolysis of borage oil and preparation of FFA. Unless otherwise specified, borage oil was hydrolyzed under the following conditions: A mixture of 3 g borage oil, 2 g water, and 5000 U lipase was incubated at 35°C with stirring at 500 rpm for 24 h. The hydrolysis extent was calculated from the acid value of the reaction mixture and the saponification value of the original oil.

After the hydrolysis, glycerides were removed by extracting with *n*-hexane, and then FFA were extracted from the water phase with *n*-hexane after returning it to acidic pH. The resulting FFA were named borage-FFA and used for the substrate of selective esterification.

Selective esterification of borage-FFA. Selective esterification of borage-FFA was carried out according to the previous paper (20). A mixture of 4 g borage-FFA/lauryl alcohol (1:2, mol/mol), 1 g water, and 1000 U *Rhizopus* lipase was incubated at 30°C for 20 h with stirring at 500 rpm. The repeated esterification was conducted under the same conditions using FFA obtained from the single reaction as a substrate. The acid values before and after the reactions were measured by titrating with 1.0 N KOH, and the esterification extent was calculated on the basis of the amount of fatty acid consumed during the reaction.

RESULTS

Screening of lipase hydrolyzing borage oil. We have shown that lipases are classified into five families of *Staphylococcus*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Candida*, and *Rhizopus* lipases on the basis of their primary structures. Lipases in the same family were produced by the similar production mechanism, and their tertiary structures resembled each other. In addition, they also possess similar properties, e.g., fatty acid and positional specificities, the equilibrium of hydrolysis and esterification, and so on (23,24). Since commercial lipases belong to the three families of *Pseudomonas cepacia*, *Candida*, and *Rhizopus* groups, a lipase was chosen from each family and the fatty acid specificity in hydrolysis was investigated (Fig. 1). It is well known that lipases generally act on PUFA weakly (25,26), as demonstrated by *C. rugosa* and *R. delemar* lipases. On the contrary, since *P. aeruginosa* lipase hydrolyzed the ester bond of GLA strongly, borage oil was hydrolyzed with several *Pseudomonas* lipases. As shown in Table 1, all *Pseudomonas* lipases were found to hydrolyze the GLA ester as strongly as the other fatty acid esters, because the GLA content of the FFA fraction was the same as that of the original oil (22.2%). However, the hydrolysis extent by *Pseudomonas* sp. KWI-56 lipase was only 55%, and that by *Pseudomonas* sp. lipase (LIPOSAM) showed the highest hydrolysis extent (87%) and GLA recovery (89%). Therefore, the lipase, LIPOSAM, was selected and used for the following experiment.

Several factors affecting hydrolysis of borage oil. Because LIPOSAM hydrolyzed the ester bond of GLA as well as those of the other constituent fatty acids, GLA can be effectively recovered in the FFA fraction by higher hydrolysis extent. So, several factors affecting hydrolysis of borage oil were examined to find out the optimum conditions.

Figure 2 shows the effect of enzyme amount on the hydrol-

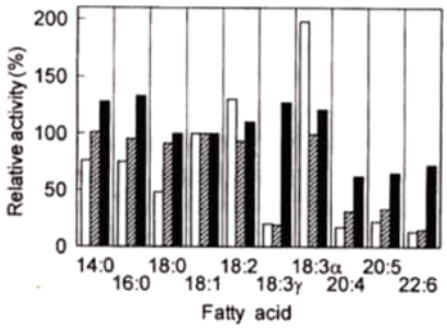

FIG. 1. Fatty acid specificity of several lipases in hydrolysis. The activities on various fatty acid esters were evaluated using randomly interesterified oil as a substrate according to the Materials and Methods section. The activity was expressed relative to that on oleic acid. Open bar, *Candida rugosa* lipase; hatched bar, *Rhizopus delemar* lipase; solid bar, *Pseudomonas aeruginosa* lipase.

TABLE 1
Hydrolysis of Borage Oil by Several *Pseudomonas* Lipases[a]

Enzyme	Hydrolysis (%)	Content of GLA[b] (wt%)	Recovery of GLA (%)
Pseudomonas aeruginosa[c]	80.9	22.9	83.5
Pseudomonas sp. KWI-56[d]	55.4	21.8	54.4
Pseudomonas sp.[e]	79.3	23.0	82.2
Pseudomonas sp.[f]	87.3	22.7	89.3
Chromobacterium viscosum[g]	77.3	22.2	77.3

[a]The mixture containing 3 g of borage oil, 3 g of water, and 6000 U of lipase was stirred (500 rpm) at 35°C for 16 h. The γ-linolenic acid (GLA) content of borage oil was 22.2%.
[b]The content of GLA in the free fatty acid fraction.
[c]LPL; Toyobo Co., Ltd., Osaka, Japan.
[d]Kurita Water Ind., Ltd., Tokyo, Japan.
[e]Lipase-AK (Amano Pharmaceutical Co., Ltd., Aichi, Japan).
[f]LIPOSAM (Showa Denko K.K., Tokyo, Japan).
[g]Same as *P. glumae*.

TABLE 2
Effect of Water Content on Hydrolysis of Borage Oil with *Pseudomonas* sp. Lipase (LIPOSAM)[a]

Water content (%)	Reaction mixture		Hydrolysis (%)	Content of GLA[b] (%)	Recovery of GLA (%)
	Oil (g)	Water (g)			
20	4.0	1.0	71.3	22.2	71.3
30	3.5	1.5	81.2	22.4	81.9
40	3.0	2.0	86.9	22.5	88.1
50	2.5	2.5	85.1	22.6	86.6
70	1.5	3.5	51.0	22.3	51.2
90	0.5	4.5	42.9	22.5	43.5

[a]The reaction was carried out at 35°C with 5000 U of lipase for 16 h. For manufacturer see Table 1.
[b]The content of GLA in the free fatty acid fraction. See Table 1 for abbreviation and company source.

ysis extent of borage oil. When the lipase amount was below 500 U/g of reaction mixture, the hydrolysis extent was elevated with increase in the amount of enzyme. The hydrolysis extent was not so increased even though more amount of enzyme was used, and reached 94.5% with 2000 U/g of reaction mixture.

The effect of water content on hydrolysis of borage oil was examined using 1000 U of lipase per 1 g of reaction mixture as a catalyst (Table 2). Borage oil was hydrolyzed effectively at the water content of 40 to 50%, and the hydrolysis reaction was 85% complete. Since lipase catalyzes not only hydrolysis but also esterification simultaneously, large amounts of water are necessary to shift the equilibrium to hydrolysis. However, when hydrolysis was conducted in the mixture containing more than 70% of water, hydrolysis was decreased. This phenomenon may be explained by the decrease of hydrolysis rate, which is attributed to the low enzyme concentration in the water phase due to the large amount of water. Actually, when the reaction was extended to 72 h, the hydrolysis reached 91.3%. In addition, when 0.5 g of oil was hydrolyzed in a mixture containing 4.5 g of water and 15,000 U of the lipase, the hydrolysis after 16-h reaction was elevated to 90.5%.

Borage oil was hydrolyzed at a temperature between 30 and 55°C (Fig. 3). The hydrolysis extent after 3 h was somewhat low at 30°C, but did not change at above 35°C. Furthermore, the extent after 16 h was not so affected by the reaction temperature. From these results, the optimal conditions for the hydrolysis of borage oil were determined as follows: A mixture of 3 g borage oil, 2 g water, and 5000 U LIPOSAM was incubated at 35°C with stirring at 500 rpm.

Time course of hydrolysis of borage oil. Figure 4 shows a typical time course of hydrolysis of borage oil with LIPOSAM. Hydrolysis proceeded rapidly until 7 h, and the extent did not increase much after 24 h, being 92% after 50 h (Fig. 4A). The fatty acid contents of the FFA fraction are shown in Figure 4B. The contents of palmitic acid (PA) and stearic acid (SA; data not shown) increased a little at the early stage of the hydrolysis, but the contents of all fatty acids of the FFA fraction approximately agreed with their contents of borage oil after 10 h. These results showed that LIPOSAM hydrolyzed the ester bonds of all the constituent fatty acids at nearly the same rate, although the hydrolysis rates of ester bonds of PA and SA were a little higher than those of the other fatty acid ester bonds. From these results, hydrolysis was carried out for 24 h, and the resulting fatty acid mixture (borage-FFA) was used as a starting material for selective esterification with lauryl alcohol.

Selective esterification of borage-FFA with R. delemar *lipase.* The purification of GLA was attempted by selective es-

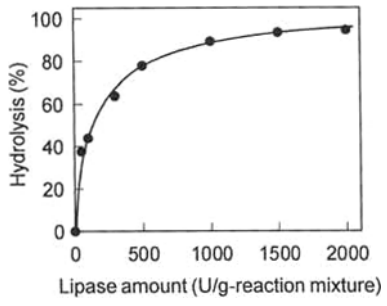

FIG. 2. Effect of amount of *Pseudomonas* sp. lipase (LIPOSAM; Showa Denko K.K., Tokyo, Japan) on hydrolysis of borage oil. A mixture containing 3 g of borage oil, 3 g of water, and various amount of lipase was stirred at 35°C for 16 h.

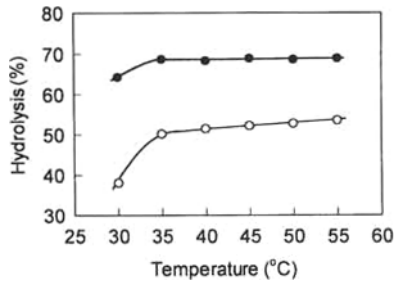

FIG. 3. Effect of temperature on hydrolysis of borage oil with *Pseudomonas* sp. lipase (LIPOSAM). ○, Hydrolysis for 3 h; ●, hydrolysis for 16 h. See Figure 2 for company source.

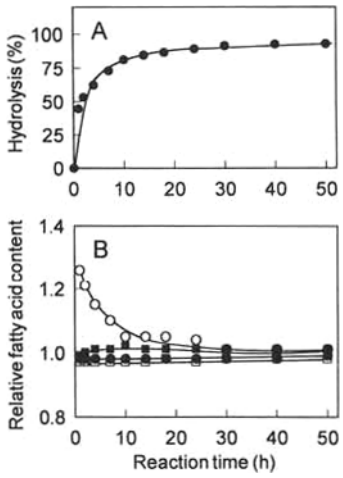

FIG. 4. Time course of hydrolysis of borage oil with *Pseudomonas* sp. lipase (LIPOSAM). A, Hydrolysis extent; B, fatty acid content in free fatty acid fraction. The content of each fatty acid in the free fatty acid fraction was expressed relative to the initial content of the fatty acid in borage oil. ○, Palmitic acid (the content of borage oil, 9.7%); ●, oleic acid (17.5%); □, linoleic acid (38.3%); ■, γ-linolenic acid (22.2%). See Figure 2 for company source.

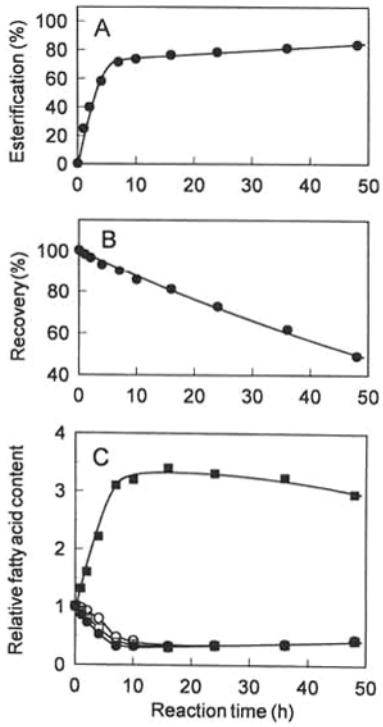

FIG. 5. Time course of selective esterification of fatty acids originating from borage oil (borage-FFA) with lauryl alcohol using *Rhizopus delemar* lipase. A, Esterification extent; B, recovery of γ-linolenic acid (GLA) in the free fatty acid (FFA) fraction; C, fatty acid content in the FFA fraction. The content of each fatty acid in the FFA fraction was expressed relative to the initial content of the fatty acid in borage-FFA. ○, Palmitic acid (the content of borage-FFA, 9.7%); ●, oleic acid (17.2%); □, linoleic acid (37.3%); ■, GLA (22.4%).

terification of borage-FFA with lauryl alcohol. A mixture containing 4 g of borage-FFA/lauryl alcohol (1:2, mol/mol), 1 g of water, and 1000 U of *Rhizopus* lipase was incubated at 30°C with stirring at 500 rpm. The time course is shown in Figure 5. Esterification increased rapidly until 8 h and then increased gradually (Fig. 5A). The contents of PA, OA, and LOA in the FFA fraction decreased with the increase of esterification, and the GLA content increased (Fig. 5C). The GLA content was 76% after 16 h, and 81% of the initial content was recovered in the FFA fraction. The linear decrease of the recovery of GLA in the FFA fraction (Fig. 5B) shows that the amount of lauryl GLA increases linearly in the reaction mixture. If lauryl GLA was generated by transesterification (acidolysis) of lauryl esters with GLA, the exponential increase of lauryl GLA would be observed. Thus the loss of GLA was probably due to the minor esterification of GLA with lauryl alcohol, but not to the acidolysis of lauryl esters with GLA.

To elevate the GLA purity, unesterified fatty acids were extracted with *n*-hexane after the first reaction, and were allowed to react again in the same manner. As shown in Table 3, the GLA content was risen from 22.5 to 70.2% in a 80% yield by the first esterification. The esterification extent of the second reaction was 32%, showing that the reaction efficiency was decreased when the fatty acids that the lipase did not act upon initially were used as a substrate. By the repeated reaction, the GLA content could be elevated to 93.7% in a yield of 73% of the initial content of borage-FFA.

DISCUSSION

We have described a method of highly purifying GLA using two lipases: *Pseudomonas* sp. lipase (LIPOSAM) which acts strongly upon GLA, and *Rhizopus* lipase which acts weakly upon GLA. The first step was hydrolysis of borage oil with LIPOSAM, and the second was selective esterification of the resulting borage-FFA with *Rhizopus* lipase. Because selective esterification with lauryl alcohol does not require any organic solvent, the reaction scale can be made small and the risk of explosion can be avoided. The reaction occurred effectively even in the reaction mixture containing 20% water, so it is not necessary to remove the water generated by esterification. In addition, not only GLA but also DHA can be purified as reported previously (20). From these results, this selective esterification may be effective for industrial-scale purification of PUFA.

LIPOSAM hydrolyzed nonspecifically the esters of the constituent fatty acids of borage oil, and the fatty acid composition of borage-FFA was almost the same as that of the original borage oil. Nonspecific hydrolysis of borage oil can be conducted by incubating in a large amount of ethanol using NaOH as a catalyst. But this chemical hydrolysis has not been used as an industrial method because there is a risk of the isomerization of GLA. We could not find a lipase acting on the ester of GLA more strongly than those of the other constituent fatty acids, but GLA may be purified more efficiently by using such lipase as a catalyst of the first-step hydrolysis.

TABLE 3
Purification of GLA by Repeated Selective Esterification of Fatty Acids from Borage Oil (borage-FFA) with Lauryl Alcohol Using *Rhizopus delemar* Lipase

Purification step	Reaction extent (%)	Fatty acid composition (wt%)[a]								Recovery of GLA[b] (%)
		16:0	18:0	18:1	18:2	18:3	20:1	22:1	24:1	
Original[c]	—	9.7	3.9	17.5	38.3	22.2	4.3	2.6	1.6	100.0
Hydrolysis[d]	91.5	9.8	4.1	17.5	37.8	22.5	4.3	2.5	1.5	92.7
Esterification										
First	74.4	4.7	2.0	6.3	14.3	70.2	1.3	0.8	n.d.	74.1
Second	31.7	0.8	0.4	1.0	2.8	93.7	0.2	n.d.	n.d.	67.5

[a]Fatty acid composition of the free fatty acid (FFA) fraction.
[b]Recovery of GLA based on its initial content of borage-FFA.
[c]The fatty acid content of the original borage oil.
[d]Hydrolysis of borage oil with *Pseudomonas* sp. lipase (LIPOSAM); fatty acid content of borage-FFA. See Table 1 for other abbreviation and company source.

When natural oil was hydrolyzed and acidolyzed by a lipase, the reactions proceeded according to its fatty acid specificity (9,11,15,17). Thus it is very important to know the correct specificity for the screening of lipase suitable for the reaction and for the prediction of the reaction. However, it is well known that lipase catalyzes hydrolysis, esterification, and transesterification in almost all reaction systems reported previously (9,27,28). Therefore, it has been very difficult to evaluate the fatty acid specificity of a lipase in each reaction. Recently, we proposed a new method of investigating fatty acid specificity of lipase in hydrolysis and acidolysis by using randomly interesterified oil (11,17,22), and showed that the fatty acid specificity of *Rhizopus* lipase in acidolysis is stricter than that in hydrolysis (17). In the reaction system described in this study, fatty acids were esterified effectively with lauryl alcohol, but lauryl esters generated were not hydrolyzed (data not shown). It has not been clarified yet whether interesterification between lauryl esters and acidolysis of lauryl esters with fatty acid occurs. However, it was found that each fatty acid was esterified at a constant initial rate (Fig. 5). It was, therefore suggested that fatty acid specificity of *Rhizopus* lipase in esterification could be evaluated by analyzing the early stage of the reaction.

The fatty acid specificity of *Rhizopus* lipase in esterification was tentatively investigated on the basis of the amount of each fatty acid lauryl ester generated after 1-h selective esterification as shown in Figure 5. The esterification activity (A_{est}) of each fatty was calculated according to Equation 2:

$$A_{est} = \{F_{ori} - (1 - E/100) \times F_{ffa}\}/F_{ori} \quad [2]$$

where E is the esterification extent (24.5%) after 1-h reaction, and F_{ori} and F_{ffa} are the content (mol%) of a particular fatty acid in borage-FFA and in the FFA fraction, respectively. Therefore, $\{F_{ori} - (1 - E/100) \times F_{ffa}\}$ shows the amount of a particular fatty acid (mol%) in the lauryl ester fraction. The activity of each fatty acid was expressed as a relative value to that on OA, and compared with those of the fatty acid in hydrolysis (Fig. 1) and in acidolysis as reported previously (17). As shown in Table 4, the fatty acid specificities in all the reactions were similar, but the activities on PA and SA in esterification were a little lower than those in hydrolysis and acidolysis. The activity on GLA decreased in the following order: hydrolysis > esterification > acidolysis. Actually, GLA could not be enriched to more than 28 wt% in glycerides when borage oil was hydrolyzed with *Rhizopus* lipase (data not shown), and GLA of the 1,3-position of the oil was scarcely acidolyzed with caprylic acid at the early stage of the reaction (17). In addition, GLA was esterified somewhat with lauryl alcohol (Fig. 5B). While the difference of the activity on GLA in these reactions is very small, the precision processing of oils may be enabled by taking advantage of this difference of fatty acid specificities in the reactions.

TABLE 4
Fatty Acid Specificity of *Rhizopus delemar* Lipase in Hydrolysis, Acidolysis, and Esterification[a]

Fatty acid	Relative activity (%)		
	Hydrolysis[b]	Acidolysis[c]	Esterification[d]
16:0	95	97	77
18:0	91	88	67
18:1	100	100	100
18:2	91	89	90
18:3n-6	19	0	4

[a]The activity was expressed relative to that on oleic acid.
[b]Compiled from Figure 1.
[c]Compiled from Reference 17.
[d]Calculated according to Equation 2.

REFERENCES

1. Borgeat, P., M. Nadeau, H. Salari, P. Poubelle, and B.F. deLaclos, Leukotrienes: Biosynthesis, Metabolism, and Analysis, in *Advances in Lipid Research*, Vol. 21, edited by R. Paoletti and D. Kritchevsky, Academic Press, New York, 1985, pp. 47–77.
2. Minno, G.D., A.M. Cerbone, and A. Postiglione, Lipids in Platelet Function: Platelet and Vascular Prostaglandins in Thromboembolic Disease, *Ibid.*, Vol. 22, 1987, pp. 63–82.
3. Shimizu, S., H. Kawashima, K. Akimoto, Y. Shinmen, and H. Yamada, Production of Odd-Chain Polyunsaturated Fatty Acids by *Mortierella* Fungi, *J. Am. Oil Chem. Soc. 68*:254–258 (1991).
4. Wu, D., and S.N. Meydani, γ-Linolenic Acid and Immune Function, in *γ-Linolenic Acid: Metabolism and Its Roles in Nutrition*

and Medicine, edited by Y.-S. Huang and D.E. Mills, AOCS Press, Champaign, 1996, pp. 106–117.
5. Crozier, G.L., and M.-C. Secretin, γ-Linolenic Acid in Infant Formula, *Ibid.*, pp. 246–251.
6. Hoshino, T., T. Yamane, and S. Shimizu, Selective Hydrolysis of Fish Oil by Lipase to Concentrate n-3 Polyunsaturated Fatty Acids, *Agric. Biol. Chem. 54*:1459–1467 (1990).
7. Tanaka, Y., J. Hirano, and T. Funada, Concentration of Docosahexaenoic Acid in Glyceride by Hydrolysis of Fish Oil with *Candida cylindracea* Lipase, *J. Am. Oil Chem. Soc. 69*:1210–1214 (1992).
8. Shimada, Y., K. Maruyama, S. Okazaki, M. Nakamura, A. Sugihara, and Y. Tominaga, Enrichment of Polyunsaturated Fatty Acids with *Geotrichum candidum* Lipase, *Ibid. 71*:951–954 (1994).
9. Shimada, Y., K. Maruyama, M. Nakamura, S. Nakayama, A. Sugihara, and Y. Tominaga, Selective Hydrolysis of Polyunsaturated Fatty Acid-Containing Oil with *Geotrichum candidum* Lipase, *Ibid. 72*:1577–1581 (1995).
10. Maruyama, K., and M. Nishikawa, Physiological Function of a Fish Oil Component and Its Application to Foods, *Food Chemicals 1995(4) (in Japanese)*:31–37 (1995).
11. Shimada, Y., A. Sugihara, K. Maruyama, T. Nagao, S. Nakayama, H. Nakano, and Y. Tominaga, Enrichment of Arachidonic Acid: Selective Hydrolysis of a Single-Cell Oil from *Mortierella* with *Candida cylindracea* Lipase, *J. Am. Oil Chem. Soc. 72*:1323–1327 (1995).
12. Rahmatullah, M.S.K.S., V.K.S. Shukla, and K.D. Mukherjee, Enrichment of γ-Linolenic Acid from Evening Primrose Oil and Borage Oil *via* Lipase-Catalyzed Hydrolysis, *Ibid. 71*:569–573 (1994).
13. Shieh, C.-J., C.C. Akoh, and P.E. Koehler, Four-Factor Response Surface Optimization of the Enzymatic Modification of Triolein to Structured Lipids, *Ibid. 72*:619–623 (1995).
14. Huang, K.-H., and C.C. Akoh, Enzymatic Synthesis of Structured Lipids: Transesterification of Triolein and Caprylic Acid Ethyl Ester, *Ibid. 73*:245–250 (1996).
15. Shimada, Y., A. Sugihara, K. Maruyama, T. Nagao, S. Nakayama, H. Nakano, and Y. Tominaga, Production of Structured Lipid Containing Docosahexaenoic and Caprylic Acids Using Immobilized *Rhizopus delemar* Lipase, *J. Ferment. Bioeng. 81*:299–303 (1996).
16. Shimada, Y., A. Sugihara, H. Nakano, T. Yokota, T. Nagao, S. Komemushi, and Y. Tominaga, Production of Structured Lipids Containing Essential Fatty Acids by Immobilized *Rhizopus delemar* Lipase, *Ibid. 73*:1415–1420 (1996).
17. Shimada, Y., A. Sugihara, H. Nakano, T. Nagao, M. Suenaga, S. Nakai, and Y. Tominaga, Fatty Acid Specificity of *Rhizopus delemar* Lipase in Acidolysis, *Ibid. 83*:321–327 (1997).
18. Hill, M.J., I. Kiewitt, and K.D. Mukherjee, Enzymatic Fractionation of Fatty Acids: Enrichment of γ-Linolenic Acid and Docosahexaenoic Acid by Selective Esterification by Lipases, *J. Am. Oil Chem. Soc. 67*:561–564 (1990).
19. Foglia, T.A., and P.E. Sonnet, Fatty Acid Selectivity of Lipases: γ-Linolenic Acid from Borage Oil, *Ibid. 72*:417–420 (1995).
20. Shimada, Y., A. Sugihara, H. Nakano, T. Kuramoto, T. Nagao, M. Gemba, and Y. Tominaga, Purification of Docosahexaenoic Acid by Selective Esterification of Fatty Acids from Tuna Oil with *Rhizopus delemar* Lipase, *Ibid. 74*:97–101 (1997).
21. Sugihara, A., T. Tani, and Y. Tominaga, Purification and Characterization of a Novel Thermostable Lipase from *Bacillus* sp., *J. Biochem. 109*:211–216 (1991).
22. Shimada, Y., A. Sugihara, T. Nagao, S. Kitai, M. Nakamura, M. Gemba, and Y. Tominaga, Evaluation of Fatty Acid Specificity of Lipase Using Interesterified Oil, *Kagaku to Kogyo* (in Japanese) *69*:95–99 (1995).
23. Shimada, Y., A. Sugihara, and Y. Tominaga, Microbial Lipase: Structure and Production, in *Recombinant Microbes for Industrial and Agricultural Applications*, edited by Y. Murooka, and T. Imanaka, Marcel Dekker, New York, 1994, pp. 359–371.
24. Shimada, Y., A. Sugihara, and Y. Tominaga, Production, Structure and Reaction of Lipase from *Geotrichum candidum*, *Kagaku to Kogyo* (in Japanese) *70*:312–319 (1996).
25. Bottino, N.R., G.A. Vandenburg, and R. Reiser, Resistance of Certain Long-Chain Polyunsaturated Fatty Acids of Marine Oil to Pancreatic Lipase Hydrolysis, *Lipids 2*:489–493 (1967).
26. Yang, L.-Y., A. Kuksis, and J.J. Myher, Lipolysis of Menhaden Oil Triacylglycerols and the Corresponding Fatty Acid Alkyl Esters by Pancreatic Lipase *in vitro*: A Reexamination, *J. Lipid Res. 31*:137–148 (1990).
27. Okumura, S., M. Iwai, and Y. Tsujisaka, Synthesis Estolides During Hydrolysis of Castor Oil by *Geotrichum candidum* Lipase, *J. Jpn. Oil Chem. Soc. 32*:27–29 (1983).
28. Okumura, S., M. Iwai, and Y. Tsujisaka, The Effect of Reverse-Action Triglyceride Hydrolysis by Lipase, *Agric. Biol. Chem. 45*:185–189 (1981).

[Received February 20, 1997; accepted June 22, 1997]