Selective Hydrolysis of Borage Oil with *Candida rugosa* Lipase: Two Factors Affecting the Reaction

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ABSTRACT: A 46% γ-linolenic acid (GLA)-containing oil was produced by selective hydrolysis of borage oil (GLA content, 22%) at 35°C for 15 h in a mixture containing 50% water and 20 units (U)/g reaction mixture of *Candida rugosa* lipase. The GLA content was not raised over 46%, even though the hydrolysis extent was increased by extending the reaction time and by using a larger amount of the lipase. However, 49% GLA-containing oil was produced by hydrolysis in a reaction mixture with 90% water. This result suggested that free fatty acids (FFA) that accumulated in the mixture affected the apparent fatty acid specificity of the lipase in the selective hydrolysis and interfered with the increase of the GLA content. To investigate the kinetics of the selective hydrolysis in a mixture without FFA, glycerides containing 22, 35, and 46% GLA were hydrolyzed with *Candida* lipase. The result showed that the hydrolysis rate decreased with increasing GLA content of glycerides, but that the release rate of GLA did not change. Thus, it was found that the apparent fatty acid specificity of the lipase in the selective hydrolysis was also affected by glyceride structure. When 46% GLA-containing oil was hydrolyzed at 35°C for 15 h in a mixture containing 50% water and 20 U/g of the lipase, GLA content in glycerides was raised to 54% at 20% hydrolysis. Furthermore, GLA content in glycerides was raised to 59% when the hydrolysis extent reached 60% using 200 U/g of the lipase. These results showed that repeated hydrolysis was effective to produce the higher concentration of GLA oil. Because film distillation was found to be extremely effective for separating FFA and glycerides, large-scale hydrolysis of borage oil was attempted. As a result, 1.5 kg of 56% GLA-containing oil was obtained from 7 kg borage oil by repeated reaction.

JAOCS 75, 1581–1586 (1998).

KEY WORDS: Borage oil, *Candida rugosa* lipase, γ-linolenic acid, selective hydrolysis.

γ-Linolenic acid (GLA, 18:3n-6) has the physiological functions of modulating immune and inflammatory response (1); borage, blackcurrant seed, and evening primrose oils containing GLA are used as an ingredient of cosmetics, food materials, health foods, and an infant formula (2). Higher GLA concentration is predicted to have the following characteristics: (i) greater physiological effect can be expected from even a small amount of intake; (ii) when it is used as an effective ingredient of various kinds of foods, the processing procedures will be easier; (iii) it can be used as a more effective starting material when high purity of GLA is produced. Thus, a useful method of enriching GLA is strongly desired. Studies in the last decade have proposed several methods for GLA enrichment: urea adduct formation (3), separation on cation-exchanged Y-zeolite columns (4), solvent winterization (5), and enzymatic methods (6–10). Enzymatic methods have been shown to be effective for producing GLA-rich glycerides and high concentrations of free GLA.

In general, lipases act weakly on polyunsaturated fatty acids (PUFA) (11,12). Thus, PUFA can be enriched in the undigested glyceride fraction by selective hydrolysis of PUFA-containing oil with a lipase (6,13–16), and can be enriched in the free fatty acid (FFA) fraction by selective esterification of FFA from the oil with an alcohol and a lipase (7–10,17). When GLA is highly purified, selective esterification is very effective from the viewpoint of large-scale production, cost, and purity. Actually, we succeeded in raising the GLA content to 94% by selective esterification of FFA originating from borage oil with lauryl alcohol and *Rhizopus delemar* lipase (10). The reaction did not require any organic solvent, and the high esterification extent was attained even though the mixture contained 20% water (10,17). On the other hand, the GLA concentrate for food is more desirable as a glyceride than as FFA or ethyl ester, from the viewpoint of taste and absorption. In addition, no chemical compound can be used in the production. Thus, selective hydrolysis with a lipase is the best procedure for the production of GLA-rich oil. Syed Rahmatullah *et al*. (6) reported a method of producing 48% GLA oil by hydrolysis of borage oil in a mixture of 70% water with *Candida rugosa* lipase. However, higher concentrations of GLA-containing oil have not been produced, and the kinetics of selective hydrolysis have not been clarified. In addition, no large-scale production method of GLA-rich oil has been established.

In this paper, we explain that glyceride structure and accumulated FFA affect the apparent fatty acid specificity in selective hydrolysis of borage oil with *Candida* lipase, and that

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the GLA content in glycerides is raised by repeating the hydrolysis after the removal of FFA from the reaction mixture. It is also shown that film distillation is very effective for the large-scale separation of FFA and glycerides.

MATERIALS AND METHODS

Lipase. Candida rugosa lipase (Lipase-OF) was purchased from Meito Sangyo Co. Ltd. (Aichi, Japan). Lipase activity was measured by titrating fatty acids liberated from olive oil (Wako Pure Chemical Industries, Osaka, Japan) with 50 mM KOH as described previously (18). 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 min.

Hydrolysis of borage oil. Borage oil (GLA-22) refined by the Nippon Synthetic Chemical Industry Co. Ltd. (Osaka, Japan; GLA, 22.2 wt%) was used. Standard hydrolysis conditions were as follows: the oil was hydrolyzed at 35°C in a mixture containing 50% water and 20 U/g reaction mixture of *Candida* lipase with stirring at 500 rpm for 15 h. The extent of hydrolysis was calculated from the acid value of the reaction mixture and the saponification value of the substrate oil. Large-scale reaction was carried out using a 2-L reactor (TBR-2-3; Oriental Yeast Co. Ltd., Tokyo, Japan) or a 30-L reactor (Mituwa Co. Ltd., Osaka, Japan). Their agitation speeds were 500 and 200 rpm, respectively.

Fractionation of glycerides and FFA in reaction mixture. Glycerides were extracted with 100 mL *n*-hexane after adding 70 mL of 0.5 N KOH (30% ethanol solution) in 10 g reaction mixture. FFA in the water layer were extracted with 100 mL *n*-hexane after returning to acidic pH (below pH 2) with 4 N HCl. Large-scale separation of FFA and glycerides was performed by film distillation with a molecular distillation apparatus (MS-150; Nippon Sharyo Ltd., Aichi, Japan).

Analysis. Fatty acids in glycerides were methylated at 75°C in methanol for 15 min with Na-methylate as methylating reagent, and FFA were methylated at the same temperature in 5% HCl-methanol for 3 h. 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 (15). The contents of mono-, di-, and triglycerides in the glyceride fraction were analyzed with thin-layer chromatography/flame-ionization detector (Iatroscan MK-5; Iatron Co., Tokyo, Japan) after development with a mixture of benzene/chloroform/acetic acid (50:20:0.7, vol/vol/vol).

RESULTS

Factors affecting selective hydrolysis of GLA-22. GLA-22 was hydrolyzed at 35°C in a reaction mixture of 50% water for 15 h with various amounts of *Candida* lipase (Fig. 1). When the oil was hydrolyzed with less than 12 U/g reaction mixture of the lipase, the hydrolysis extent and the GLA content in glycerides increased rapidly with increasing amount of the lipase. However, when the oil was hydrolyzed with more than 20 U/g of the lipase, the GLA content was not raised above 46%, although the hydrolysis extent increased gradually. Figure 2 shows a time course of hydrolysis of GLA-22 at 35°C in a reaction mixture containing 50% water and 100 U/g of the lipase. The hydrolysis extent after 1 h of reaction reached 48%, and the GLA content in glycerides was raised to 38%. The GLA content reached 46% after 4 h and then remained unchanged, though the hydrolysis extent increased gradually.

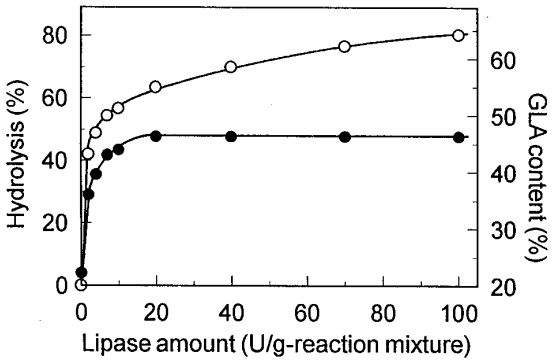

FIG. 1. Effect of lipase amount on hydrolysis of borage oil (GLA-22) with *Candida rugosa* lipase. GLA-22 was hydrolyzed at 35°C for 15 h in a mixture containing 50% water and various amounts of the lipase. ○, Hydrolysis extent; ●, γ-linolenic acid (GLA) content in glycerides. GLA-22, refined by the Nippon Synthetic Chemical Industry Co. Ltd. (Osaka, Japan).

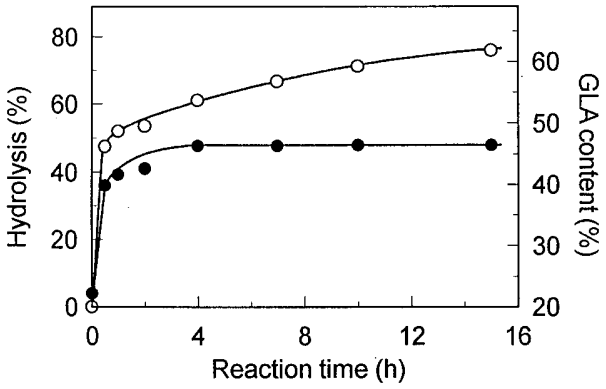

FIG. 2. Effect of reaction time on hydrolysis of GLA-22 with *Candida* lipase. GLA-22 was hydrolyzed at 35°C in a mixture containing 50% water and 100 units (U)/g of the lipase. ○, Hydrolysis extent; ●, GLA content in glycerides. See Figure 1 for abbreviation and for manufacturer.

GLA-22 was hydrolyzed in mixtures of 50 and 90% water to investigate the effect of water content on the hydrolysis. When the lipase reaction was carried out in a mixture containing a large amount of water, the equilibrium shifted to hydrolysis and the rate was fast. Thus, 20 and 100 U/g reaction mixtures of *Candida* lipase were used in the reaction systems containing 90 and 50% water, respectively. The time course of each reaction was investigated, and the relationship between the hydrolysis extent and the GLA content in glycerides is shown in Figure 3. When the hydrolysis extent was less than 60%, the GLA content increased with increasing

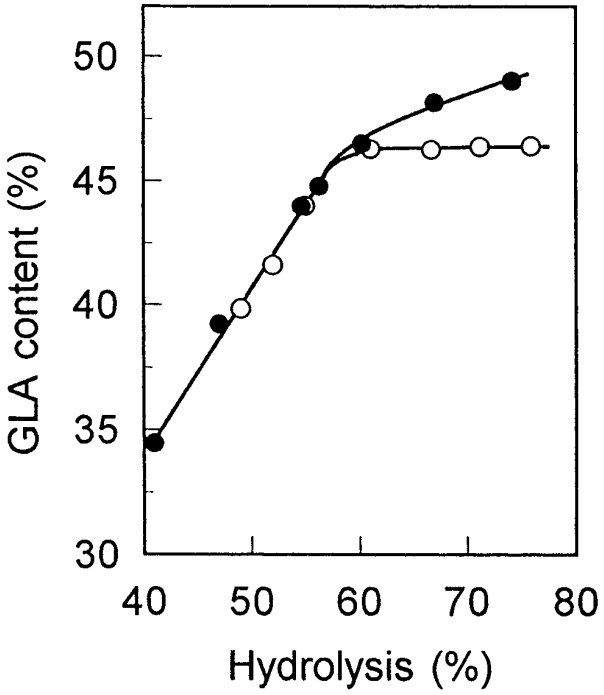

FIG. 3. Correlation between hydrolysis extent of GLA-22 with *Candida* lipase and GLA content in glycerides. GLA-22 was hydrolyzed at 35°C in the mixture containing 90% water and 20 U/g of the lipase (●) and in that of 50% water and 100 U/g of the lipase (○). See Figure 1 for abbreviation and for manufacturer.

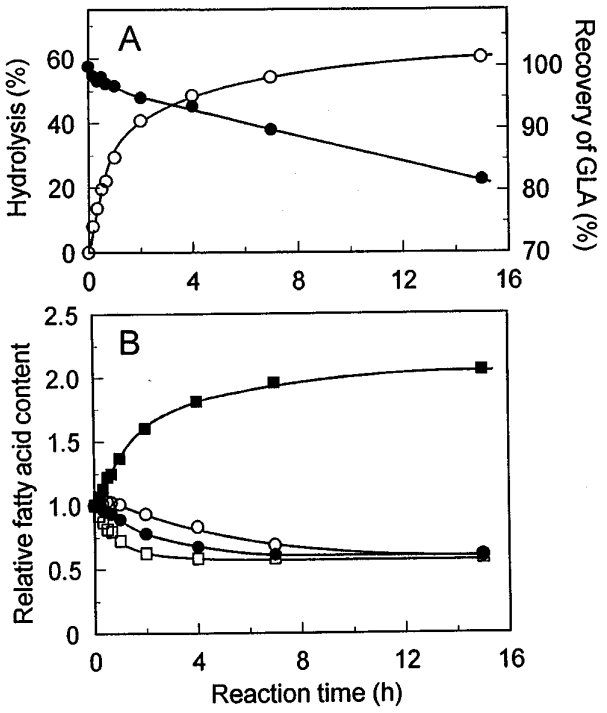

FIG. 4. Time course of selective hydrolysis of GLA-22 with *Candida* lipase. GLA-22 was hydrolyzed at 35°C in a mixture containing 50% water and 20 U/g of the lipase. (A) Hydrolysis extent (○) and recovery of GLA in glycerides (●). The recovery was calculated by assuming that all glycerides were recovered by the extraction with *n*-hexane. (B) Fatty acid content in the glyceride fraction. Each fatty acid content in glycerides was expressed relative to the initial content of the fatty acid in the original oil. ○, Palmitic acid (content of the original oil, 9.7%); ●, oleic acid (17.5%); □, linoleic acid (38.3%); ■, GLA (22.2%). See Figure 1 for abbreviation and for manufacturer.

hydrolysis extent irrespective of the water content in the mixture. When the hydrolysis extent was more than 60%, hydrolysis in a mixture of 90% water raised the GLA content to 49%, but hydrolysis in a mixture of 50% water did not raise it more than 46%. This result showed that the ratio of the release rate of GLA to that of fatty acids other than GLA was 46/54 when the hydrolysis extent exceeded 60% in hydrolysis of GLA-22 in a mixture of 50% water. But the GLA content in glycerides was enriched to 49% in the hydrolysis in a mixture of 90% water, showing that the ratio of the rate of release of GLA to that of the release of fatty acids other than GLA was less than 46/54, even though the hydrolysis extent was more than 60%. Furthermore, it was suggested that accumulated FFA changed the apparent fatty acid specificity of the lipase in the selective hydrolysis and interfered with the increase of the GLA content.

Lipase activity on the constituent fatty acids in oils containing different contents of GLA. When the GLA content in glycerides was raised to 46%, the glyceride structures were changed together with the accumulation of FFA. Thus, the effect of glyceride structure on the fatty acid specificity of the lipase was investigated using glycerides containing different concentrations of GLA.

The glycerides were prepared by hydrolysis of GLA-22 in a mixture containing 50% water and 20 U/g reaction mixture of the lipase. Figure 4 shows a typical time course of the selective hydrolysis. The esters of linoleic, oleic, and palmitic acids were strongly hydrolyzed in the early stage of the reaction, and their contents in glycerides decreased efficiently. However, the GLA content in glycerides increased because the GLA ester was weakly hydrolyzed. The hydrolysis rate was approximately constant, and the recovery of GLA decreased linearly. GLA was enriched from 22 to 35% with 94% recovery after 2 h of reaction, and to 46% with 76% recovery after 15 h of reaction. The glycerides obtained from 2- and 15-h reaction mixtures were named GLA-35 and GLA-46, respectively. The glyceride compositions (triglycerides/diglycerides/monoglycerides) of GLA-35 and -46 were 84:15:1 and 90:9:1, respectively.

GLA-22, -35, and -45 were hydrolyzed with *Candida* lipase, and the release rates of all fatty acids and GLA were investigated. The amount of all fatty acids released was expressed by the hydrolysis extent, and the amount of GLA released (R_{GLA}) was expressed relative to that contained in the substrate oil according to the following equation:

$$R_{GLA} = [\{F_{GLA} \times (h/100)\} / G_{GLA}] \times 100 \quad [1]$$

where F_{GLA} and G_{GLA} are the GLA contents (mol%) in the FFA fraction and in the substrate oil at h% hydrolysis, respectively. As shown in Figure 5, the hydrolysis extents of all oils increased linearly in the early stage of the reaction, and the

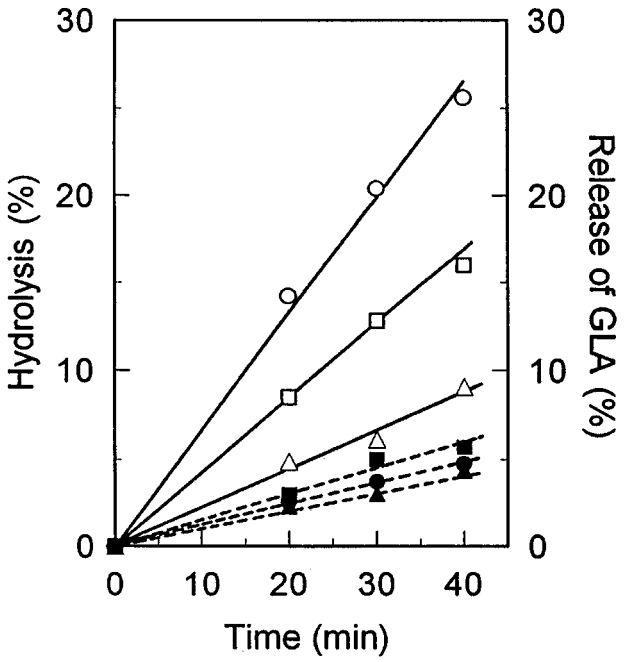

FIG. 5. Hydrolyses of oils containing different GLA contents with *Candida* lipase. GLA-22 (GLA content, 22.2%), GLA-35 (34.8%), and GLA-46 (45.7%) were hydrolyzed at 35°C in a mixture of 50% water and 20 U/g of the lipase. The amount of GLA released was expressed as a percentage of that in the substrate oil. ○, Hydrolysis extent of GLA-22; ●, amount of GLA released from GLA-22; □, hydrolysis extent of GLA-35; ■, amount of GLA released from GLA-35; △, hydrolysis extent of GLA-46; ▲, amount of GLA released from GLA-46. See Figure 1 for abbreviation and for manufacturer.

TABLE 1
Production of γ-Linolenic Acid (GLA)-Rich Oil by Repeated Hydrolysis with *Candida* Lipase

Substrate	Reaction mixture: Water (%)	Reaction mixture: Lipase (U/g)	Hydrolysis (%)	GLA content (%)	GLA recovery[a] (%)
GLA-22	—	—	—	22.2	100
GLA-35[b]	—	—	—	34.8	93.7
	50	7	34.7	47.7	83.9
	50	20	46.2	50.9	73.7
	50	60	54.7	51.5	62.8
	50	200	61.7	51.9	53.5
	90	7	45.8	51.5	75.2
	90	20	63.1	55.4	55.0
	90	60	65.0	56.3	53.1
	90	200	65.4	55.4	51.6
GLA-46[c]	—	—	—	45.7	75.8
	50	7	7.7	47.2	73.5
	50	20	19.8	54.1	73.3
	50	60	50.4	58.2	48.7
	50	200	60.4	59.3	39.6
	90	7	40.2	57.1	57.7
	90	20	46.1	59.0	53.7
	90	60	58.1	58.6	41.5
	90	200	71.6	58.6	28.1

[a]Recovery of the initial GLA content of the original borage oil (GLA-22). The recovery was calculated by assuming that all glycerides were recovered by the extraction with *n*-hexane.
[b]Obtained by hydrolysis of GLA-22 for 2 h in a mixture containing 50% water and 20 U/g of the lipase. GLA-35 was hydrolyzed at 35°C for 15 h in reaction mixture indicated.
[c]Obtained by hydrolysis of GLA-22 for 15 h in a mixture containing 50% water and 20 U/g lipase. GLA-46 was hydrolyzed at 35°C for 15 h in reaction mixture indicated.

rates became slow with increasing GLA content in the substrate oil. However, the relative rates of GLA releases were approximately the same and did not depend on the GLA contents in the oils. These results show that glyceride structure affected the apparent fatty acid specificity of the lipase and interfered with the increase of the GLA content in glycerides.

Production of GLA-rich oil by repeated hydrolysis. The accumulated FFA and the glyceride structure interfered with the increase of the GLA content in glycerides. To investigate the effect of the removal of FFA on the increase of the GLA content, GLA-35 and -46 were hydrolyzed at 35°C for 15 h in mixtures of 50 and 90% water with various amounts of lipase (Table 1). The GLA content was enriched to more than 46% in the hydrolyses of GLA-35 and -46. It was confirmed from the result that the accumulated FFA interfered with greater than 46% enrichment of the GLA content in a single reaction. When GLA-35 was hydrolyzed in the mixture of 50% water, the GLA content was not raised over 52%, even though the hydrolysis extent was increased by using a large amount of the lipase. However, the GLA content was enriched to 56% when GLA-35 was hydrolyzed in the mixture of 90% water. On the other hand, when GLA-46 was hydrolyzed in the mixtures of 50 and 90% water, the GLA content was raised to 59% irrespective of the water content in the reaction mixture. These results show that an oil containing higher GLA concentration was produced, with good yield of GLA, by using GLA-46 as a substrate instead of GLA-35. When GLA-46 was hydrolyzed at 35°C for 15 h in a mixture containing 50% water and 20 U/g of the lipase, the GLA content was raised to 54% in a 73% yield of the initial content of GLA-22. In addition, when GLA-46 was hydrolyzed in a mixture containing 50% water and 200 U/g of the lipase and in that containing 90% water and 20 U/g of the lipase, the GLA content was raised to 59% in 40 and 54% yields, respectively.

Large-scale production of higher concentrations of GLA oil by repeated hydrolysis. It was found that repeated hydrolysis was effective for the production of higher concentrations of GLA-containing oil. When large-scale repeated hydrolysis is performed, FFA have to be removed from the reaction mixture. But solvent extraction is not suitable because of time, cost, and risk of explosion. Film distillation may be effective for the following reasons: (i) the reaction mixture consists of FFA and glycerides (the content of triglycerides, *ca.* 90%), and the average molecular weights of FFA and triglycerides are 280 and 878, respectively; (ii) if the hydrolyses of GLA-46 and GLA-22 are performed at the same time, both reaction mixtures can be successively applied to film distillation; (iii) in general, distillation can recover a desired compound in a high yield. Therefore, we attempted the large-scale production of GLA-rich oil from 7 kg GLA-22 by a combination of repeated hydrolysis and distillation. Table 2 shows a material balance in each production step.

TABLE 2
Large-Scale Production of High Concentration of GLA-Containing Oil

Procedure	Weight (kg)	Acid value	Amount of glycerides[a] (kg)	GLA content[b] (%)	GLA recovery[b] (%)
GLA-22	7.00	n.d.	7.00	22.2	100
Hydrolysis	6.29	122	2.44	45.7	71.8
Film distillation					
Distillate 1-1[c]	3.26	197	3.26	n.t.	—
Distillate 1-2[d]	0.67	147	0.67	n.t.	—
Residue 1-2[d]	2.28	10	2.17	46.4	64.8
Hydrolysis (2)[e]	2.12	49	1.60	55.3	56.9
Film distillation					
Distillate 2-1[f]	0.52	176	0.06	n.t.	—
Residue 2-1[f]	1.54	4	1.50	55.7	53.8

[a]Calculated by assuming that the acid value of free fatty acids originating from borage oil is 200.
[b]The content and the recovery of GLA in the glyceride fraction.
[c]Distillation was performed at 150°C and 0.05 mm Hg.
[d]Distillation was performed at 160°C under 0.05 mm Hg.
[e]The oil was hydrolyzed at 35°C for 15 h in a mixture containing 50% water and 20 U/g of *Candida* lipase with stirring, and the reaction mixture was then dehydrated.
[f]Distillation was performed at 155°C under 0.04 mm Hg; n.d., not detected; n.t., not tested; see Table 1 for abbreviation and for manufacturer.

When the first hydrolysis was conducted in a mixture containing 50% water and 20 U/g of the lipase, the GLA content in glycerides was raised to 46% at 61% hydrolysis. Film distillation of the reaction mixture was performed at 150°C under 0.05 mm Hg, but the acid value of the residue, 40, was still high. Hence the second cycle of distillation was carried out after increasing the temperature to 160°C, and 2.3 kg of the residue (acid value 10) was obtained. The resulting glycerides were hydrolyzed again under the same conditions as those of the first hydrolysis. As a result, the GLA content in glycerides was raised to 55% at 20% hydrolysis. Film distillation of the reaction mixture at 155°C under 0.04 mm Hg gave 0.5 kg of distillate (acid value 176) and 1.5 kg of residue (acid value 4; GLA content, 56%). The distillations of the reaction mixtures of the first and the second hydrolyses recovered 89 and 94% of glycerides, respectively. This result shows that the distillation was very effective for the removal of FFA in the large-scale production of GLA-rich oil.

DISCUSSION

We have shown that FFA and glyceride structure interfered with the increase of the GLA content in selective hydrolysis of GLA-22 with *Candida* lipase, and that the GLA content was enriched from 22 to 54% by repeating the hydrolysis after the removal of accumulated FFA. The GLA recovery in glycerides was more than 70% of the initial content when the recovery of glycerides by the extraction with *n*-hexane was assumed to be 100%. The repeated hydrolysis of 7 kg GLA-22 actually produced 1.5 kg of glycerides containing 56% GLA by performing film distillation for the removal of FFA in the reaction mixture.

Triglycerides of which the constituent fatty acids are good substrates for a lipase are hydrolyzed more strongly by that lipase than triglycerides of which the constituent fatty acids are poor substrates. It was, for example, reported that triglycerides containing greater amounts of docosahexaenoic acid were more weakly hydrolyzed by lipases (16,21); this property is called triglyceride specificity. GLA, like docosahexaenoic acid, was a poor substrate for *Candida* lipase, and high concentration of GLA oil was weakly hydrolyzed (Fig. 5). However, the release rate of GLA from triglycerides did not change, even though the triglycerides contained a high concentration of GLA. It was therefore shown that triglyceride structure affected the apparent fatty acid specificity of the lipase.

When GLA-22 was hydrolyzed in a mixture of 50% water, the GLA content in glycerides was not raised above 46% (Figs. 1, 2, and 3). This phenomenon was attributed to the change in apparent fatty acid specificity of the lipase not only by triglyceride structure but also by accumulated FFA. When the GLA content in the glycerides was raised to 46% in a single hydrolysis, the amount of FFA was over 60% of total fatty acids. Most of the FFA were palmitic, oleic, and linoleic acids on which the lipase acted strongly. It is well known that lipases catalyze esterification and transesterification simultaneously with hydrolysis (16,19,20). In addition, palmitic, oleic, and linoleic acids were esterified more strongly than GLA (10). Therefore, it is suggested that the simultaneous esterification of accumulated FFA changed the apparent fatty acid specificity of the lipase in the selective hydrolysis.

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[Received November 21, 1997; accepted June 8, 1988]