Enzymatic Enrichment of Arachidonic Acid from *Mortierella* Single-Cell Oil

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ABSTRACT: An attempt was made to enrich arachidonic acid (AA) from *Mortierella* single-cell oil, which had an AA content of 25%. The first step involved the hydrolysis of the oil with *Pseudomonas* sp. lipase. A mixture of 2.5 g oil, 2.5 g water, and 4000 units (U) *Pseudomonas* lipase was incubated at 40°C for 40 h with stirring at 500 rpm. The hydrolysis was 90% complete after 40 h, and the resulting free fatty acids (FFA) were extracted with *n*-hexane (AA content, 25%; recovery of AA, 91%). The second step involved the selective esterification of the fatty acids with lauryl alcohol and *Candida rugosa* lipase. A mixture of 3.5 g fatty acids/lauryl alcohol (1:1, mol/mol), 1.5 g water, and 1000 U *Candida* lipase was incubated at 30°C for 16 h with stirring at 500 rpm. Under these conditions, 55% of the fatty acids were esterified, and the AA content in the FFA fraction was raised to 51% with a 92% yield. The long-chain saturated fatty acids in the FFA fraction were eliminated as urea adducts. This procedure raised the AA content to 63%. To further elevate the AA content, the fatty acids were esterified again in the same manner with *Candida* lipase. The repeated esterification raised the AA content to 75% with a recovery of 71% of its initial content.
JAOCS 75, 1213–1217 (1998).

KEY WORDS: Arachidonic acid, enrichment, hydrolysis, lauryl alcohol, *Pseudomonas* sp. lipase, *Rhizopus delemar* lipase, selective esterification, urea adduct.

Arachidonic acid (AA, 20:4n-6) is a rare fatty acid that has potential pharmaceutical value and is a precursor of local hormones, prostaglandins, leukotrienes, and thromboxanes, involved in the AA cascade (1,2). It has also been reported that AA is important for the growth of preterm infants, as is docosahexaenoic acid (DHA, 22:6n-3) (3,4). In addition, arachidonylethanolamide (anandamide) (5) and 2-arachidonylglycerol (6) can be bound to the receptor of cannabinoid, which is a psychotropic agent, which may give them medicinal value.

An AA-containing oil can be produced by a microorganism, and industrial production of the oil with 25 to 40% AA has been achieved (7). In general, the oil with a high concentration of functional fatty acid is effective physiologically even with a small amount of intake. The AA content in glycerides can be raised from 25 to 50% by hydrolyzing AA-containing single-cell oil with *Candida rugosa* lipase (8). However, the AA content could not be raised to more than 60%, even though the hydrolysis was repeated three times. Recently, we have developed a two-step enzymatic method of enriching unstable polyunsaturated fatty acid (PUFA). The first step is the preparation of free fatty acids (FFA) by hydrolysis of PUFA-containing oil with *Pseudomonas* lipase, and the second step is the enrichment of PUFA in the FFA fraction by esterification of the resulting fatty acids with lauryl alcohol and *Rhizopus delemar* lipase. By the enzymatic method, DHA and γ-linolenic acid (GLA, 18:3n-6) were enriched to 89 and 94% from tuna and borage oils, respectively (9–11).

This paper deals with the enrichment of AA by a two-step enzymatic method: The first step is hydrolysis of AA-containing oil (AA content, 25%) with *Pseudomonas* sp. lipase, and the second step is selective esterification of the resulting FFA with lauryl alcohol and *Candida rugosa* lipase. Furthermore, it is shown that the AA content can be raised to 75% by the combination of selective esterification and urea adduct formation for the elimination of saturated fatty acids.

MATERIALS AND METHODS

Oil and fatty alcohols. AA-containing oil (SUN-TGA25; AA content, 25%) is a commercial product of Suntory Co. (Osaka, Japan). Fatty alcohols were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Lipases. Lipases were gifts from the following companies: *Candida rugosa* lipase (Lipase-OF; Meito Sangyo Co. Ltd., Aichi, Japan), *R. delemar* lipase (Ta-lipase; Tanabe Seiyaku Co. Ltd., Osaka, Japan), *P. aeruginosa* lipase (LPL; Toyobo Co. Ltd., Osaka, Japan), *Chromobacterium viscosum* lipase (Asahi Chemical Industry Co., Ltd., Tokyo, Japan), *Pseudomonas* sp. lipase (Lipase-PS; Amano Pharmaceutical Co., Ltd., Aichi, Japan), *Pseudomonas* sp. lipase (Lipase-AK; Amano), *Pseudomonas* sp. (LIPOSAM; Showa Denko K.K., Tokyo, Japan).

Fractionation of esters and FFA in the 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 the mixture. FFA in the water phase were extracted with 100 mL *n*-hexane after returning to acidic pH (< pH 2) with HCl.

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Urea adduct formation. Urea (25 g) was dissolved at 50°C in 125 mL methanol and 3.5 mL water with stirring, and 25 g FFA were then added gradually. After FFA were completely dissolved, the mixture was gradually cooled to room temperature. The precipitate was removed with filter paper and washed with urea-saturated methanol. The volume of the filtrate was reduced to 50 mL by evaporation, and FFA were then extracted with 150 mL *n*-hexane after adding 100 mL of 0.3 N HCl.

Analysis. Lipase activity was measured by titrating fatty acids liberated from olive oil (Wako Pure Chemicals Ind., Co., Osaka, Japan) with 0.05 N KOH as described previously (12). The reaction was carried out at 30°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/min.

The hydrolysis extent was measured from the acid value of the reaction mixture and the saponification value of the original SUN-TGA25. The esterification extent was calculated on the basis of the amount of fatty acids consumed during the reaction.

Fatty acids in glycerides and lauryl esters were methylated at 75°C for 15 min in methanol with sodium methylate as a methylating reagent. FFA were methylated at 75°C 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 (13).

RESULTS AND DISCUSSION

Hydrolysis of SUN-TGA25. Lipase is effective as a catalyst for hydrolysis of an oil that contains unstable PUFA. Thus, a suitable lipase for hydrolysis of SUN-TGA25 was screened from several commercial lipases with different fatty acid and positional specificities (Table 1). *Candida* and *Rhizopus* lipases act on PUFA weakly, and *Pseudomonas* lipases act on PUFA somewhat more strongly (8,11). It is also well known that *Rhizopus* lipase is a 1- and 3-positional specific enzyme whereas *Candida* and *Pseudomonas* lipases are nonspecific enzymes. *Candida* lipase hydrolyzed the oil strongly, but only 28% of AA was recovered in the FFA fraction because of the weak activity on AA ester. *Rhizopus* lipase hydrolyzed the oil weakly, although it acted on AA ester strongly. On the other hand, *Pseudomonas* lipases acted on the ester of AA as strongly as those of the other constituent fatty acids, and the extent of hydrolysis was also high. Among them, *Pseudomonas* sp. lipase (Lipase-PS) was the most effective; 67% of AA was recovered in the FFA fraction after 20 h hydrolysis. Therefore, Lipase-PS was selected and used for the following experiment.

TABLE 1
Hydrolysis of SUN-TGA25 by Several Lipases[a]

Lipase	Hydrolysis (%)	Fatty acid composition (%)[b] 16:0	18:0	18:1	18:2	20:4
None[c]	—	13.7	6.1	14.3	26.0	24.6
Candida rugosa	64.5	17.6	7.2	18.1	33.1	10.7
Rhizopus delemar	42.0	15.8	6.6	14.0	25.2	23.1
Pseudomonas aeruginosa	60.3	13.1	5.8	14.5	26.8	23.8
P. glumae[d]	65.6	14.7	6.1	13.4	26.5	22.9
Pseudomonas sp.[e]	68.8	13.5	6.5	14.3	26.2	24.3
Pseudomonas sp.[f]	67.6	15.3	6.4	14.8	28.3	18.4
Pseudomonas sp.[g]	62.5	14.3	6.4	14.2	27.7	20.0

[a]A reaction mixture of 2.5 g SUN-TGA25 (Suntory Co., Osaka, Japan), 2.5 g water, and 2000 units (U) lipase was stirred (500 rpm) at 30°C for 20 h.
[b]Fatty acid composition in the free fatty acid fraction.
[c]Fatty acid composition of the original SUN-TGA25.
[d]Same as *Chromobacterium viscosum.*
[e]Lipase-PS (Amano Pharmaceutical Co., Ltd., Aichi, Japan).
[f]Lipase-AK (Amano Pharmaceutical Co., Ltd.).
[g]LIPOSAM (Showa Denko K.K., Tokyo, Japan).

TABLE 2
Hydrolysis of SUN-TGA25 with *Pseudomonas* sp. Lipase (Lipase-PS)[a]

Reaction time (h)	Hydrolysis (%)	Fatty acid composition (%)[b] 16:0	18:0	18:1	18:2	20:4	Recovery of AA[c] (%)
0[d]	—	13.9	6.2	13.7	24.2	24.6	—
20	79.8	12.6	6.4	14.4	24.0	23.6	76.5
40	89.1	12.8	6.4	14.0	24.1	24.6	89.1
60	88.2	13.7	6.1	13.6	24.1	24.5	87.8

[a]See text for reaction conditions.
[b]Fatty acid composition in the free fatty acid (FFA) fraction.
[c]Recovery of arachidonic acid (AA) in the FFA fraction.
[d]Fatty acid composition of the original SUN-TGA25. See Table 1 for company sources.

The effects of temperature, lipase amount, and water content on the hydrolysis of the oil were examined with 5 g of the reaction system, and the reaction conditions were set as follows: A mixture of 2.5 g oil, 2.5 g water, and 4000 U Lipase-PS was incubated at 40°C with stirring at 500 rpm. Table 2 shows the effect of reaction period on hydrolysis of the oil under the set conditions. The hydrolysis extent reached 89% after 40 h and did not increase, even though the reaction period was extended to 60 h. Thus, FFA originating from the oil were prepared by extracting 40-h hydrolysate with *n*-hexane (TGA25-FFA; AA recovery, 89%). The acid value of TGA25-FFA was 191, i.e., 3.4 mmol FFA/g.

Selective esterification of TGA25-FFA. TGA25-FFA was esterified with lauryl alcohol by using lipases from *C. rugosa*, *R. delemar*, and *P. aeruginosa,* whose reactivities toward oils and fats were different (Table 3). When *Rhizopus* and *Pseudomonas* lipases were used, AA did not remain in the unesterified fatty acid fraction because the esterification proceeded strongly. However, *Candida* lipase acted on AA only

TABLE 3
Selective Esterification of TGA25-FFA with Several Lipases[a]

Lipase	Esterification (%)	AA content[b] (%)	Recovery of AA (%)
None[c]	—	24.8	100
Candida rugosa	54.9	52.5	95.4
Rhizopus delemar	90.6	34.0	12.9
Pseudomonas aeruginosa	86.1	44.9	25.2

[a]A reaction mixture of 4 g TGA25-FFA (2:1, mol/mol), 1 g water, and 1000 U lipase was stirred at 30°C for 20 h.
[b]The content of AA in the FFA fraction.
[c]Fatty acid composition of TGA25-FFA. See Tables 1 and 2 for abbreviations.

TABLE 4
Effect of Alcohol on Selective Esterification of TGA25-FFA with *Candida rugosa* Lipase[a]

Alcohol	Esterification (%)	AA content[b] (%)	Recovery of AA (%)
None	—	24.8	100
Methanol	22.5	30.0	93.4
Ethanol	14.7	27.7	95.1
Propanol	21.1	30.0	95.2
Butanol	33.5	34.8	93.2
Pentanol	36.9	37.9	96.2
Hexanol	39.7	36.1	87.7
Heptanol	45.0	40.9	90.5
Octanol	48.2	43.1	89.8
Nonanol	48.0	43.8	91.7
Decanol	52.0	46.8	90.4
Lauryl alcohol	55.7	50.8	90.6
Oleyl alcohol	55.7	49.6	88.4

[a]A mixture of 4 g alcohol/TGA25-FFA (2:1, mol/mol), 1 g water, and 1000 U *Candida* lipase was stirred at 30°C for 20 h.
[b]AA content in the FFA fraction. See Tables 1 and 2 for abbreviations.

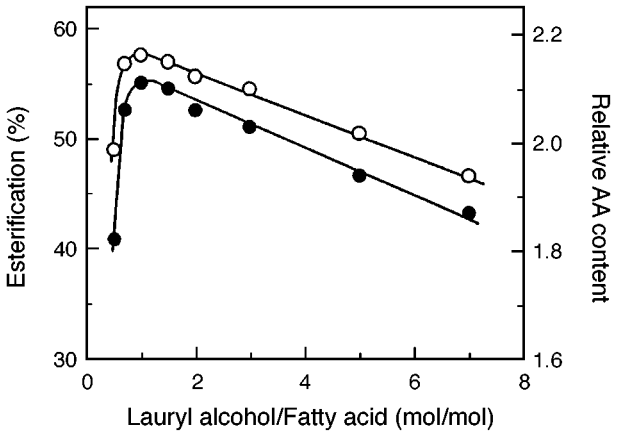

FIG. 1. Effect of lauryl alcohol on selective esterification of free fatty acids (FFA) from SUN-TGA25 oil (TGA25-FFA) with *Candida* lipase. A mixture of 4 g of TGA25-FFA/lauryl alcohol (1:1, mol/mol), 1 g water, and 1000 units (U) of lipase was stirred (500 rpm) at 30°C for 20 h. ○, esterification extent; ●, arachidonic acid (AA) content in the unesterified fatty acid fraction. The content of AA was expressed relative to that in the original TGA25-FFA (24.9%). SUN-TGA25, Suntory Co., Osaka, Japan.

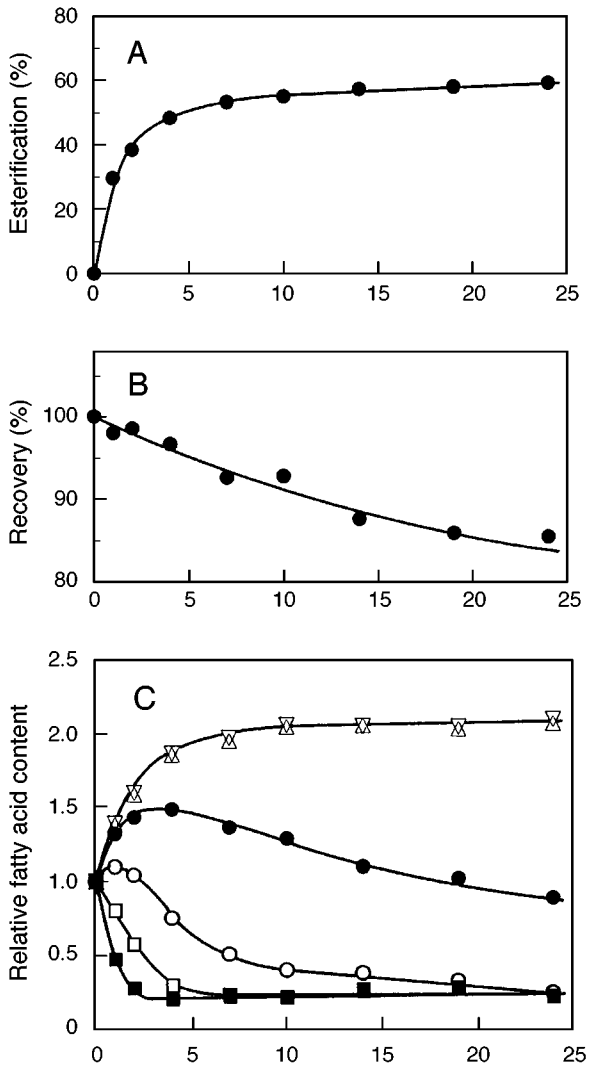

FIG. 2. Time course of selective esterification of TGA25-FFA with *Candida* lipase. A mixture of 3.5 g TGA25-FFA/lauryl alcohol (1:1, mol/mol), 1.5 g water, and 1000 U *Candida* lipase was stirred at 30°C. (A) Esterification extent; (B) recovery of AA in the unesterified fatty acid fraction; (C) fatty acid content in the FFA fraction. The content of each fatty acid was expressed relative to the initial content of the fatty acid in TGA25-FFA. ○, Palmitic acid (the content of TGA25-FFA, 13.7%); ●, stearic acid (6.2%); □, oleic acid (13.7%); ■, linoleic acid (24.2%); △, dihomo-γ-linolenic acid (3.2%); ▽, AA (24.6%). See Figure 1 for abbreviations.

TABLE 5
Enrichment of AA by Selective Esterification and Urea Adduct Formation

Step	Reaction extent (%)	Fatty acid composition (%)[a] 16:0	18:0	18:1	18:2	18:3	20:3	20:4	22:0	24:0	Recovery of AA(%)
Original[b]	—	13.9	6.2	13.7	24.2	2.1	3.2	24.6	2.0	4.1	100
Hydrolysis[c]	90.1	13.7	6.1	13.4	23.6	2.4	3.1	24.9	1.9	3.8	91.2
Esterification[d]	55.3	5.2	6.7	3.5	6.5	4.0	6.1	51.0	3.8	7.5	83.5
Urea adduct[e]	—	4.5	2.9	3.5	6.4	5.1	7.9	63.0	n.d.	n.d.	78.0
Esterification[d]	23.0	1.8	2.8	1.4	2.5	5.4	8.9	74.9	n.d.	n.d.	71.4

[a]Fatty acid composition in the FFA fraction.
[b]Fatty acid composition of SUN-TGA25.
[c]A mixture of SUN-TGA25/water (1:1, wt/wt) and 800 U/g of Lipase-PS was stirred at 40°C for 40 h.
[d]A mixture of FFA/lauryl alcohol (1:1, mol/mol), 30% water, and 200 U/g *Rhizopus* lipase was stirred at 30°C for 16 h.
[e]Urea adduct formation was carried out according to the Materials and Methods section. See Tables 1 and 2 for abbreviations and company sources.

weakly, and the AA content in the FFA fraction was raised from 25 to 53% with a 95% yield. Thus, *Candida* lipase was chosen for the selective esterification of TGA25-FFA.

A suitable fatty alcohol for the esterification with *Candida* lipase was screened from various fatty alcohols that were in the liquid state at the reaction temperature. As shown in Table 4, long-chain fatty alcohols enhanced esterification, and the AA content in the unesterified fatty acid fraction depended on the esterification extent. When lauryl alcohol and oleyl alcohol were used as substrates, the esterification extent reached 56%, and the AA content was raised to 50%. From these results, lauryl alcohol was chosen as a substrate for the selective esterification of TGA25-FFA.

The suitable molar ratio of lauryl alcohol to TGA25-FFA was examined, and the result is shown in Figure 1. The esterification extent showed the highest value at equal mole amounts of lauryl alcohol and TGA25-FFA, and the excess amount of lauryl alcohol inhibited the esterification. Because the AA content depended on the esterification extent, the ratio of lauryl alcohol to TGA25-FFA was fixed at 1:1 (mol/mol).

The other factors affecting the selective esterification of TGA25-FFA with lauryl alcohol were examined with 5 g of the reaction system. In consequence, water content, lipase amount, and temperature were determined to be 30%, 200 U/g-reaction mixture, and 30°C, respectively. Figure 2 shows a typical time course under the set conditions. The esterification extent increased rapidly during the first 4 h. Oleic acid (18:1n-9) and linoleic acid (18:2n-6) were most preferably esterified, and their contents in the FFA fraction decreased rapidly. The contents of palmitic acid (16:0) and stearic acid (18:0) increased in the early stage of the reaction and decreased gradually after 1 and 4 h, respectively. On the other hand, *Candida* lipase acted on AA and dihomo-γ-linolenic acid (20:3n-6) only weakly, and the contents of these fatty acids were raised by increasing the esterification extent.

Enrichment of AA by repeated selective esterification and urea adduct formation. Enrichment of AA was attempted with 100 g of SUN-TGA25 as a starting material (Table 5). TGA25-FFA was prepared from the hydrolysate of the oil with Lipase-PS. The hydrolysis extent was 90%, and the recovery of AA was 91%. The selective esterification of TGA25-FFA with lauryl alcohol and *Candida* lipase raised the AA content in the FFA fraction from 25 to 51% with a recovery of 92%. Because *Candida* lipase acted on GLA, dihomo-γ-linolenic acid, behenic acid (22:0), and lignoceric acid (24:0) as weakly as on AA, these fatty acids were also enriched in the unesterified fatty acid fraction. The lipase activity on stearic acid was somewhat weak, and the content was not decreased. The conventional urea adduct formation was conducted next to eliminate these saturated fatty acids. As a result, behenic and lignoceric acids were completely eliminated, and a part of palmitic and stearic acids were also removed with an AA recovery of 93%. To further elevate the AA content, the selective esterification was done again. The esterification extent was 23%, and the AA content was raised to 75% with a recovery of 71% of the initial content.

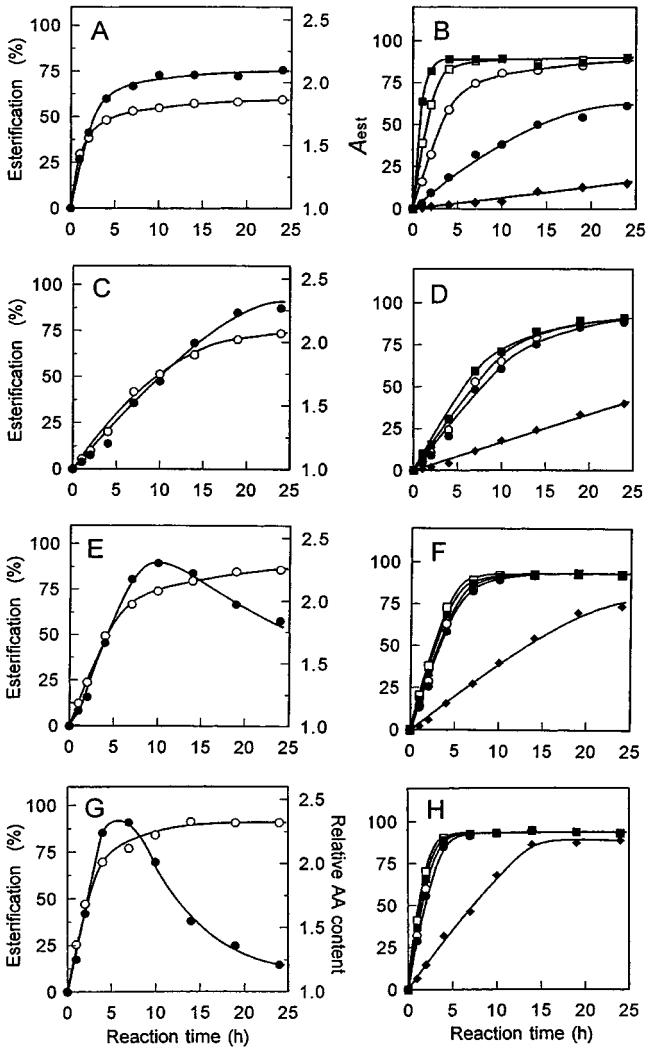

FIG. 3. Activities of *Candida* and *Rhizopus* lipases on fatty acids in TGA25-FFA. TGA25-FFA was esterified at 30°C with the same molar concentration of lauryl alcohol in a mixture containing 30% water. (A, B) Esterification with 200 U/g-reaction mixture of *Candida* lipase; (C, D) 50 U/g-reaction mixture of *Rhizopus* lipase; (E, F) 100 U/g-reaction mixture of *Rhizopus* lipase; (G, H) 200 U/g-reaction mixture of *Rhizopus* lipase. A, C, E, and G show esterification extent (○) and the AA content in the FFA fraction (●); B, D, F, and H show amounts of palmitic acid (○), stearic acid (●), oleic acid (□), linoleic acid (■), and AA (◆) esterified with lauryl alcohol. The amount of each fatty acid esterified (A_{est}) was calculated by Equation 1. See Figure 1 for abbreviations.

Selective esterification of TGA25-FFA with Rhizopus *lipase. Rhizopus* lipase acted weakly on AA as on DHA and GLA (8,14). However, AA was not enriched by the selective esterification of TGA25-FFA with the lipase (Table 3), although DHA and GLA were enriched by the selective esterification of fatty acids that originated from tuna and borage oils, respectively (9–11). Thus, the time course of the selective esterification of TGA25-FFA was investigated with a different amount of *Rhizopus* lipase. The amount of fatty acid esterified (A_{est}) was expressed as a percentage of the original content according to the following equation:

TABLE 6
Fatty Acid Specificities of *Candida rugosa* and *Rhizopus delemar* Lipases[a]

Fatty acid	*Candida*	*Rhizopus*
16:0	41	69
18:0	12	58
18:1	100	100
18:2	164	102
20:4	3	23

[a]Activity on each fatty acid was calculated from the initial velocity in the reactions of Figures 3B and D according to Equation 1 and expressed relative to that of oleic acid.

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

where F_{est} and F_{ffa} are the content (mol%) of a particular fatty acid in the lauryl ester fraction and in TGA25-FFA, respectively.

When the selective esterification of TGA25-FFA was conducted for 24 h with 200 U/g *Candida* lipase, the esterification extent was only 57%, and the AA content was not raised over 52% (Fig. 3A). Even though 700 U/g of the lipase was used, the esterification extent after 24 h of reaction was 59% (data not shown). On the other hand, *Rhizopus* lipase catalyzed the esterification more strongly than *Candida* lipase, and the esterification extent reached 91% after 24 h with 200 U/g lipase (Fig. 3G). The AA content in the FFA fraction showed the highest value (57%) at 75% extent of esterification (Figs. 3E and 3G). These phenomena observed in the esterification with *Rhizopus* lipase can be explained by the following results: (1) *Rhizopus* lipase acted on AA more weakly than the other fatty acids, but acted on AA more strongly than *Candida* lipase did (Table 6); (2) AA was esterified with lauryl alcohol even after cessation of the esterification of fatty acids other than AA (Figs. 3F and 3H). From the viewpoints of the control of the reaction and the recovery of AA, *Candida* lipase seems more suitable for the selective esterification of TGA25-FFA with lauryl alcohol than *Rhizopus* lipase.

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[Received January 16, 1998; accepted April 28, 1998]