

Purification of Ethyl Docosahexaenoate by Selective Alcoholysis of Fatty Acid Ethyl Esters with Immobilized *Rhizomucor miehei* Lipase

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ABSTRACT: Ethyl docosahexaenoate (E-DHA) is efficiently enriched by the selective alcoholysis of ethyl esters originating from tuna oil with lauryl alcohol using immobilized lipase. Alcoholysis of ethyl esters by immobilized *Rhizopus delemar* lipase raised the E-DHA content in the unreacted ethyl ester fraction from 23 to 49 mol% in 90% yield. However, the content of ethyl eicosapentaenoate (E-EPA) was higher than the initial content. Hence we attempted to screen for a suitable lipase to decrease the E-EPA content, and chose *Rhizomucor miehei* lipase. Several factors affecting the alcoholysis of ethyl esters were investigated, and the reaction conditions were determined. When alcoholysis was performed at 30°C with shaking in a mixture containing ethyl esters/lauryl alcohol (1:3, mol/mol) and 4 wt% of the immobilized *R. miehei* lipase, the E-DHA content in the ethyl ester fraction was increased and the E-EPA content was decreased. By alcoholyzing ethyl esters in which the E-DHA content was 45 mol% (E-tuna-45) for 26 h, the E-DHA content was increased to 74 mol% in 71% yield and the E-EPA content was decreased from 12 to 6.2 mol%. To investigate the stability of the immobilized lipase, batch reactions were carried out continually by replacing the reaction mixture with fresh E-tuna-45/lauryl alcohol (1:3, mol/mol) every 24 h. The decrease in the alcoholysis extent was only 17% even after 100 cycles of reaction. It was found that increasing the proportion of lauryl alcohol increased the conversion of E-EPA to lauryl-EPA. When an ethyl ester mixture in which the E-DHA content was 60 mol% (E-tuna-60) was alcoholized for 24 h with 7 molar equivalents of lauryl alcohol, the E-DHA content was raised to 93 mol% with 74% yield and the E-EPA content was reduced from 8.6 to 2.9 mol%.

JAOCS 75, 1565–1571 (1998).

KEY WORDS: Ethyl docosahexaenoate, immobilized enzyme, lipase, *Rhizomucor miehei*, selective alcoholysis, tuna oil.

It is now well established that n-3 polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (20:5, EPA) and docosahexaenoic acid (22:6, DHA), exhibit beneficial effects

on human health (1–3). The ethyl ester of EPA (E-EPA) has been used in the treatment of arteriosclerosis obliterans and hyperlipemia (4), and fish oil containing DHA has been used as a food material, a component in infant formulas, and a health food (5). DHA has recently attracted great interest because of its special function in the brain (6) and retina (7), and there is a growing demand for the medical application of the ethyl ester of DHA (E-DHA). The purification of E-DHA by the formation of a complex with silver (8), and by high-performance liquid chromatography (9) was recently reported, but these methods have not been accepted because of the cost. Therefore, other suitable purification methods are desired.

PUFA is very labile to heat and oxidation. Thus, enzyme reactions have drawn attention, because they proceed efficiently at ambient temperature and pressure and under a nitrogen stream. Several lipases do not act to an appreciable degree on PUFA, and PUFA can be enriched by taking advantage of this property. For example, when fish oil, borage oil, and arachidonic acid-containing oil from *Mortierella* were hydrolyzed with *Candida rugosa* or *Geotrichum candidum* lipase, DHA, γ -linolenic acid, and arachidonic acid, respectively, were enriched in the glycerides (10–15). DHA and γ -linolenic acid were also enriched in the free fatty acid fraction by selective esterification of fatty acids originating from tuna and borage oils, respectively (16–20). In addition, it was reported that DHA and EPA were enriched in glycerides by alcoholyzing fish oil with ethanol (21,22).

In general, lipases can recognize long-chain fatty alcohol as a substrate, but not its fatty acid ester (19). Therefore, when long-chain fatty alcohol, especially lauryl alcohol, was used as a substrate, esterification and alcoholysis proceeded efficiently. We recently reported that E-DHA was enriched in the ethyl ester fraction by the selective alcoholysis of fatty acid ethyl esters originating from tuna oil with lauryl alcohol (23). When immobilized *Rhizopus delemar* lipase was used as a catalyst, the E-DHA content was raised to 83 mol%. However, E-EPA was not efficiently converted into the lauryl ester, because *Rhizopus* lipase acted very weakly on EPA as well as on DHA. As a result, the E-EPA content in the ethyl ester fraction was not decreased to less than 7 mol%. To fur-

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ther decrease the E-EPA content, we screened for a lipase which acted somewhat on EPA but very weakly on DHA, and *Rhizomucor miehei* lipase was found to be suitable for this purpose.

This paper deals with a method of purifying E-DHA by selective alcoholysis of fatty acid ethyl esters with lauryl alcohol using immobilized *Rhizomucor* lipase. The selective alcoholysis raised the E-DHA content in the ethyl ester fraction to 93 mol%, and lowered the E-EPA content to 2.9%.

MATERIALS AND METHODS

Fatty acid ethyl esters and alcohol. Fatty acid ethyl esters, of which the E-DHA contents were 23, 45 and 60 mol%, were prepared from tuna oil using the oil processing line of Maruha Corp. (Tokyo, Japan), and were designated as E-tuna-23, E-tuna-45, and E-tuna-60, respectively. Lauryl alcohol was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Lipases. Immobilized *R. miehei* lipase (Lipozyme IM) was purchased from Novo Nordisk (Bagsvaerd, Denmark). *Fusarium heterosporum* lipase was prepared as reported previously (24). Ammonium sulfate was added to the culture filtrate to give 80% saturation, and the resulting precipitates were dialyzed against water. *Rhizopus delemar* lipase (Ta-lipase) was purchased from Tanabe Seiyaku Co. (Osaka, Japan). *Aspergillus niger* lipase (Lipase-AP) was a gift from Amano Pharmaceutical Co. Ltd. (Aichi, Japan). Lipase activity was measured by titrating fatty acids liberated from olive oil (Wako Pure Chemical) with 0.05 N KOH as described previously (25). 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 which liberated 1 μmol of fatty acid per minute.

Preparation of immobilized lipase. Lipases, except *Rhizomucor* lipase, were immobilized on a ceramic carrier, SM-10, a gift from NGK Insulators Ltd. (Aichi, Japan), as described in our previous paper (26). After the ceramic carrier (10 g) was suspended in 40 mL of 10% lipase solution (*Fusarium* lipase, 7200 U/mL; *Aspergillus* lipase, 6500 U/mL; *Rhizopus* lipase, 9800 U/mL), 120 mL of cold acetone (−80°C) was gradually added with stirring, and the precipitate was dried *in vacuo*. Approximately 90% of the lipase was immobilized on the carrier by this procedure.

Reaction. Twelve grams of a mixture consisting of fatty acid ethyl esters, lauryl alcohol, and immobilized lipase was incubated with shaking (140 oscillations/min) at 30°C in a 20-mL screw-capped vessel. The extent of alcoholysis was calculated from the molar ratio of lauryl esters to the sum of the ethyl and lauryl esters, which were determined by gas chromatography as described below. The extent of hydrolysis was measured from the acid value of the reaction mixture and the saponification value of the original ethyl esters.

Analysis. Ethyl and lauryl esters of fatty acids were analyzed with a Hewlett-Packard 5890 Plus gas chromatograph (Avondale, PA) connected to a DB-5 capillary column (0.25 mm × 10 m, J&W Scientific, Folsom, CA) as described previously (23). The column temperature was raised from 150 to

300°C at 10°C/min, and maintained for 10 min at 300°C, respectively. The temperatures of injector and detector were set at 250 and 320°C, respectively. The carrier gas was helium at a flow rate of 25 cm/s. Ethyl and lauryl esters of fatty acids were identified by comparison with standards prepared as described previously (23), and their quantitative analysis was carried out based on the peak area of each fatty acid ester.

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

RESULTS AND DISCUSSION

Screening for a lipase suitable for decreasing E-EPA content. When *Rhizopus* lipase was used in the alcoholysis of ethyl esters originating from tuna oil with lauryl alcohol, E-DHA was efficiently enriched in the ethyl ester fraction, although the E-EPA content was not decreased to less than the initial content (23). The lipases whose enzymatic properties were similar to that of *Rhizopus* lipase were screened for a lipase suitable to decrease the E-EPA content (Table 1). The immobilized *Rhizopus* lipase required that water be present in the reaction (23). Thus the reaction was performed in the mixture of E-tuna-23/lauryl alcohol containing 2 wt% of water. When the reaction was done for 1 d with *Rhizopus* lipase, the alcoholysis extent was 58% and the contents of E-DHA and E-EPA in the ethyl ester fraction were raised from 23 to 49 mol% and from 9 to 15 mol%, respectively. The E-DHA content was raised a little on extending the reaction period to 2 d, but the E-EPA content did not decrease to less than the initial content. When *Fusarium* and *Aspergillus* lipases were used as catalysts, the alcoholysis extents were low and the E-DHA contents were also lower than that obtained with *Rhizopus* li-

TABLE 1
Selective Alcoholysis of Fatty Acid Ethyl Esters with Several Lipases^a

Enzyme	Reaction period (d)	Alcoholysis (%)	Content (mol%) ^b		Recovery of E-DHA ^c (%)
			20:5	22:6	
None	—	—	9.2	22.7	100
<i>Aspergillus</i> ^d	3	10.1	9.5	23.2	91.8
	10	18.8	10.2	25.3	90.5
<i>Fusarium</i> ^d	2	46.5	13.0	38.0	89.5
	5	55.0	12.9	44.7	88.5
<i>Rhizopus</i> ^d	1	58.1	14.9	48.9	90.2
	2	62.7	12.5	52.2	85.8
<i>Rhizomucor</i> ^e	1	66.6	9.0	52.7	77.5
	2	70.6	6.6	50.6	65.5

^aThe reaction was performed at 30°C with shaking in a mixture of 12 g E-tuna-23/lauryl alcohol (1:2, mol/mol), 480 mg immobilized lipase, and 240 μL water.

^bThe content of ethyl eicosapentaenoate (E-EPA) and ethyl docosahexaenoate (E-DHA) in the ethyl ester fraction.

^cRecovery of E-DHA in the ethyl ester fraction.

^dThe lipases from *Fusarium heterosporum*, *Aspergillus niger*, and *Rhizopus delemar* were immobilized on a ceramic carrier as described in the Materials and Methods section.

^eImmobilized *Rhizomucor miehei* lipase, Lipozyme IM; Novo Nordisk, Bagsvaerd, Denmark. E-tuna-23, tuna oil containing 23 mol% ethyl ester of docosahexaenoic acid.

pase. On the other hand, *Rhizomucor* lipase raised the E-DHA content to 51 mol% after 2 d of reaction and lowered the E-EPA content to less than the initial value, although the recovery of E-DHA in the ethyl ester fraction was lower than that obtained with *Rhizopus* enzyme. Because *Rhizomucor* lipase acted on E-EPA most strongly among the lipases tested, the enzyme was used in the following experiment.

Effect of water on the expression of alcoholysis activity. *Rhizopus* lipase immobilized on the ceramic carrier was activated by pretreatment in a reaction mixture containing 2 wt% water (23,26,27). It was reported that *Rhizomucor* lipase did not require any pretreatment in the reaction system containing *n*-hexane (28,29). To confirm that pretreatment is unnecessary in our reaction system, immobilized *Rhizomucor* lipase was shaken in mixtures of E-tuna-23/lauryl alcohol containing 0 to 4 wt% of water. As shown in Table 2, the alcoholysis proceeded efficiently in the mixture without water, and the E-DHA content was raised to 50%. The addition of water decreased the E-DHA content with the acceleration of the hydrolysis. Therefore, the immobilized lipase was used without any pretreatment.

Several factors affecting alcoholysis with immobilized *Rhizomucor* lipase. E-tuna-23 was alcoholized with lauryl alcohol using various amounts of the immobilized lipase (Fig. 1). The alcoholysis extent reached 50% when 1 wt% of the immobilized lipase was used, and did not increase considerably even when more enzyme was used. Because the lipase acted well on ethyl palmitate (E-PA) and ethyl oleate (E-OA), their contents in the ethyl ester fraction decreased substantially. The E-DHA content increased with increase of the alcoholysis extent because of the very weak activity of the lipase on E-DHA, and showed a constant value when more than 2 wt% lipase was used. On the other hand, E-EPA was converted somewhat to the lauryl ester. As a result, the E-EPA content was lower than the initial content when more than 4 wt% lipase was used.

To investigate the effect of the amount of lauryl alcohol on the alcoholysis, the reaction was conducted at various ratios of lauryl alcohol to E-tuna-23 with 4 wt% lipase for 16 h at 30°C. The alcoholysis extent depended on the amount of lauryl alcohol, and reached 60% at a molar ratio of 2. The alcoholysis extent gradually increased with increasing amounts of

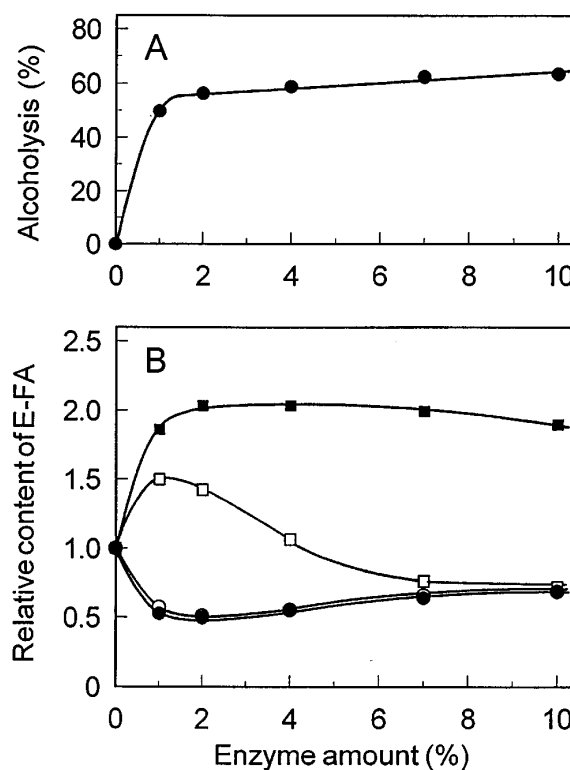


FIG. 1. Effect of amount of immobilized *Rhizomucor miehei* lipase on alcoholysis of E-tuna-23. A mixture of 12 g of E-tuna-23/lauryl alcohol (1:2, mol/mol) was shaken at 30°C for 16 h with various amounts of the immobilized lipase. (A) Alcoholysis extent; (B) the content of fatty acid ethyl ester (E-FA) in the ethyl ester fraction. The E-FA content was expressed relative to the initial content of E-tuna-23. ○, Ethyl palmitate (initial content, 21.1 mol%); ●, ethyl oleate (13.6 mol%); □, ethyl eicosapentaenoate (9.2 mol%); ■, ethyl docosahexaenoate (22.7 mol%). E-tuna-23, tuna oil containing 23 mol% ethyl ester of docosahexaenoic acid.

lauryl alcohol, and reached 78% at a molar ratio of 10. A large amount of lauryl alcohol raised the E-DHA content in the ethyl ester fraction, and efficiently lowered not only the contents of E-PA and E-OA but also that of E-EPA. When 10 molar equivalents of lauryl alcohol was used, the E-DHA content was increased to 72 mol%, and the contents of E-PA, E-OA, and E-EPA were decreased to 5.8, 3.7, and 5.1 mol%, respectively. From the viewpoint of industrial efficiency, the upper limit of the lauryl alcohol amount can be presumed to be two- to threefold that of the ethyl esters, by weight, i.e. three- to fivefold in molar ratio. Thus, the ratio of lauryl alcohol to ethyl esters was fixed at 3:1 (mol/mol) in the following experiment.

The effect of temperature on the alcoholysis was finally examined. A mixture of 12 g of E-tuna-23/lauryl alcohol (1:3, mol/mol) and 480 mg of the immobilized lipase was shaken for 16 h at a range of temperatures from 20 to 50°C. The extent of alcoholysis was a little lower below 25°C, but hardly changed at temperatures from 30 to 50°C.

On the basis of the above results, the reaction conditions were set as follows: a mixture of 12 g of ethyl esters/lauryl alcohol (1:3, mol/mol) and 480 mg of immobilized *Rhizomucor* lipase was incubated at 30°C with shaking at 140 oscillations/min.

TABLE 2
Effect of Water Content in the Reaction Mixture on Alcoholysis of E-tuna-23 by *Rhizomucor miehei* Lipase^a

Water content (%)	Alcoholysis (%)	Hydrolysis (%)	E-DHA content ^b (mol%)
0	59.3	1.9	49.5
0.2	60.3	2.2	49.1
0.4	59.9	3.3	49.7
0.8	60.5	3.6	48.4
2.0	49.1	8.2	47.5
4.0	47.2	11.1	41.4

^aA mixture of 12 g of E-tuna-23/lauryl alcohol (1:2, mol/mol), 480 mg of the immobilized lipase, and 0 to 4 wt% of water was shaken at 30°C for 16 h.

^bThe content of ethyl docosahexaenoate (E-DHA) in the ethyl ester fraction. For abbreviation see Table 1.

Time course of alcoholysis of E-tuna-45 with lauryl alcohol.

Because E-tuna-45 can be produced industrially without any significant loss of E-DHA, the alcoholysis of E-tuna-45 with lauryl alcohol was conducted under the set conditions. Figure 2 shows a typical time course. The extent of alcoholysis increased rapidly up to 4 h, and then increased gradually. The content of E-DHA in the ethyl ester fraction increased with the alcoholysis extent, and reached a constant value after 7 h. The contents of ethyl stearate (E-SA) and E-OA decreased rapidly in the early stage of the reaction, but increased gradually after 4 h. The E-EPA content gradually decreased after an initial increase at 2 h, and attained a constant value after 20 h. When the reaction was performed for 26 h, the alcoholysis extent was 60% and the E-DHA and E-EPA contents in the ethyl ester fraction were 74 and 6.2 mol%, respectively.

Figure 2C shows the amount of lauryl ester converted from ethyl ester. The lipase acted strongly on E-SA and E-OA, and more than 80 mol% of these fatty acid ethyl esters were alcoholized to the lauryl esters at 4 h. E-EPA was alcoholized somewhat, and the conversion to lauryl ester ceased at 26 h. On the other hand, E-DHA was the poorest substrate of *Rhizomucor* lipase, and was gradually alcoholized even after the 30-h reaction. These results show that the gradual increases of E-SA and E-OA contents after 4-h reaction (Fig. 2 B) were due to the continued alcoholyses of E-EPA and E-DHA after the cessation of alcoholyses of E-SA and E-OA.

Stability of immobilized lipase. The alcoholysis of E-tuna-45 was continued by replacing the reaction mixture with a fresh ethyl ester/lauryl alcohol mixture every 24 h (Fig. 3). The extent of alcoholysis decreased linearly, and was 83% of the initial value after 100 cycles of reaction. The E-DHA content in the ethyl ester fraction was constant up to 100 cycles of reaction, and the E-OA content decreased gradually. The E-EPA content increased with the decrease of the alcoholysis extent, but was lower than the initial content of E-tuna-23 even after 100 cycles of reaction.

The alcoholysis extent and the contents of ethyl esters at 24 h in 100 cycles of reaction coincided with their values at 10 h in the first reaction (Fig. 2A and B). Thus it was estimated that the activity of the immobilized lipase was decreased to 42% (10 h/24 h) by repeating the reaction for 100 cycles. The alcoholysis extent returned to the initial level on extending the reaction time to 48 h.

Alcoholysis of fatty acid ethyl esters with different E-DHA contents. When the relative amount of lauryl alcohol in the reaction mixture was increased, the E-DHA content was raised and the E-EPA content was lowered. Thus E-tuna-23, -45, and -60 were alcoholized with both three- and sevenfold molar excesses of lauryl alcohol. When E-tuna-23 was alcoholized with 3 molar equivalents of lauryl alcohol, the E-EPA content was higher than the initial content. But the content decreased from 9.2 to 7.5 mol% in the alcoholysis with 7 molar equivalents of lauryl alcohol. When ethyl esters with a higher concentration of E-DHA (E-tuna-45 and E-tuna-60) were used as substrates, the E-EPA content in the ethyl ester fraction was also efficiently decreased. The alcoholysis of

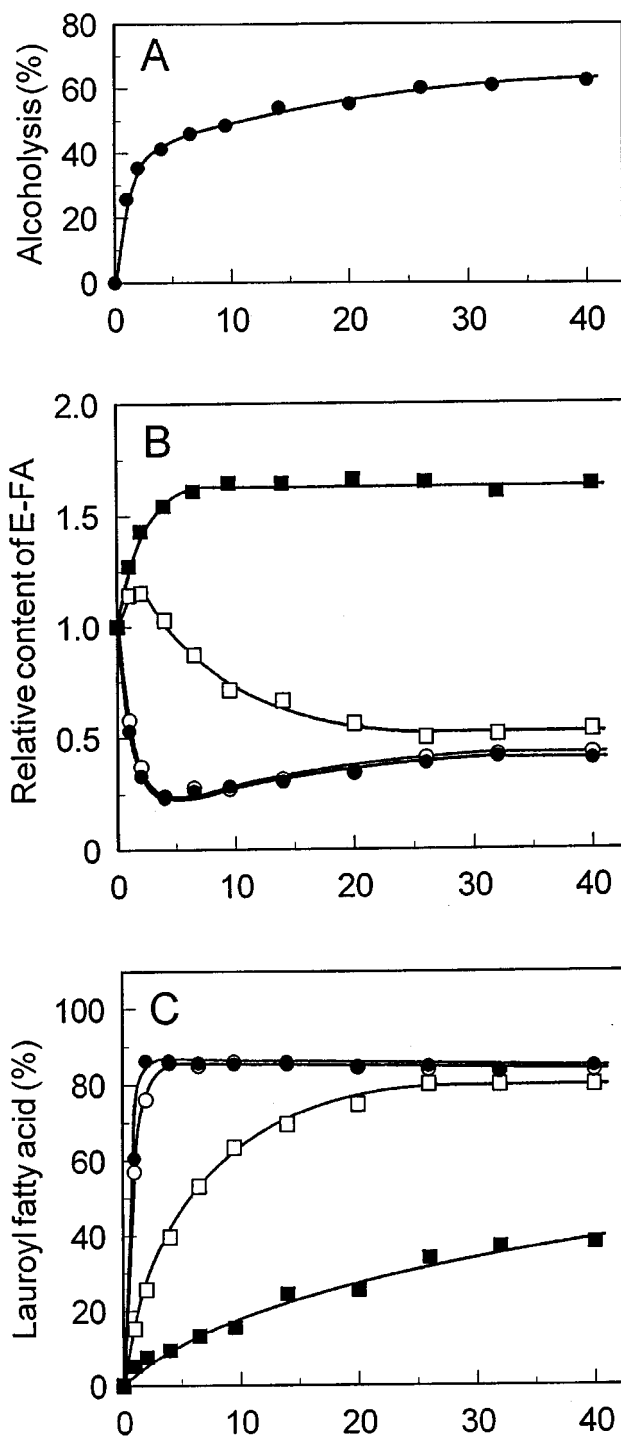


FIG. 2. Time course of the selective alcoholysis of E-tuna-45 with lauryl alcohol by immobilized *Rhizomucor* lipase. The reaction was conducted at 30°C with shaking in the mixture of 12 g E-tuna-45/lauryl alcohol (1:3, mol/mol) and 480 mg immobilized lipase. (A) Extent of alcoholysis. (B) E-FA content in the ethyl ester fraction. The content is expressed relative to the initial content: ethyl stearate, 5.2 mol%; ethyl oleate, 10.8 mol%; ethyl eicosapentaenoate, 12.5 mol%; ethyl docosahexaenoate, 44.9 mol%. (C) Conversion ratio of E-FA to lauryl ester. The amount of lauryl ester is expressed as a percentage of the initial E-FA content. Symbols in Figures B and C: ○, ester of palmitic acid; ●, ester of oleic acid; □, ester of eicosapentaenoic acid; ■, ester of docosahexaenoic acid. For abbreviations, see Figure 1.

E-tuna-60 with 7 molar equivalents of lauryl alcohol lowered the E-EPA content to 2.9 mol% and raised the E-DHA content to 93 mol% in 74% yield.

Fatty acid specificities of *Rhizopus* and *Rhizomucor* lipases in alcoholysis. The alcoholyses were conducted at 30°C with shaking, using mixtures of E-tuna-23/lauryl alcohol (1:3, mol/mol) with 4 wt% of immobilized *Rhizopus* and *Rhizomucor* lipases. Figure 4 shows the time course of the generation

of lauryl fatty acid. The amount of each lauryl fatty acid increased linearly in the early stage of reaction. The activity of the lipase on a particular fatty acid was calculated on the basis of the initial conversion rate from the ethyl ester to the lauryl ester. There were no significant differences between the activities of the lipases on myristic, palmitic, palmitoleic, stearic, and oleic acids; 82 to 100% relative to oleic acid for *Rhizopus* lipase, and 91 to 100% for *Rhizomucor* lipase. But

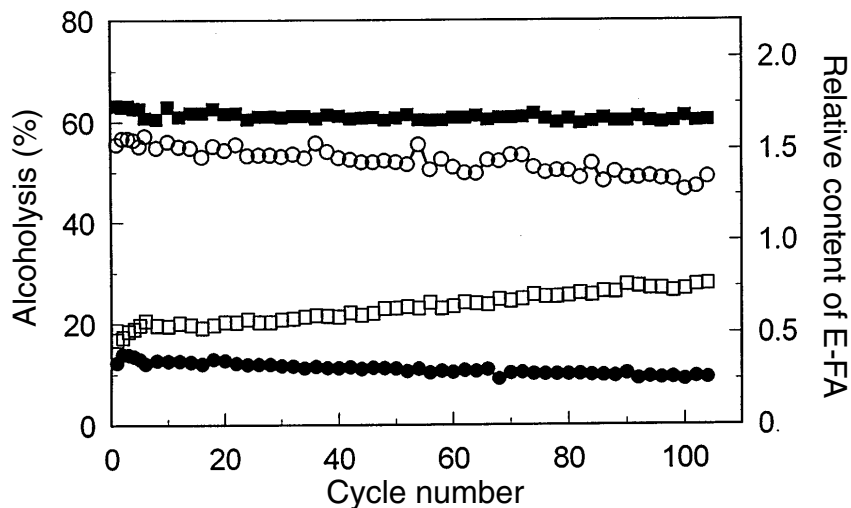


FIG. 3. Stability of immobilized *Rhizomucor* lipase in the alcoholysis of E-tuna-45 with lauryl alcohol. The reaction was conducted at 30°C with shaking in a mixture of 12 g E-tuna-45/lauryl alcohol (1:3, mol/mol) and 480 mg immobilized lipase. The E-FA content is expressed relative to the initial content. ○, Alcoholysis extent; ●, content of ethyl oleate; □, content of ethyl eicosapentaenoate; ■, content of ethyl docosahexaenoate. For abbreviations and the initial E-FA content of E-tuna-45, see Figures 1 and 2.

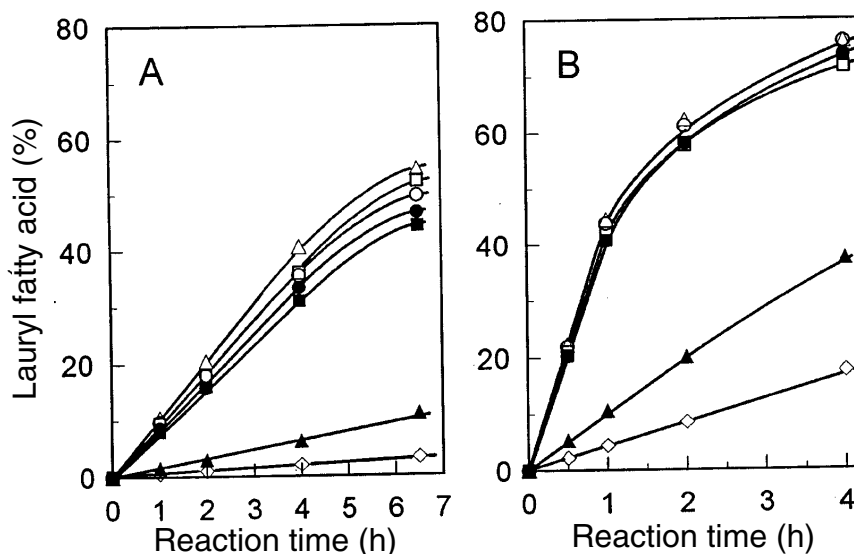


FIG. 4. Time course of the amount of lauryl ester generated in the alcoholysis of E-tuna-23 with immobilized *Rhizopus delamar* (A) or *Rhizomucor miehei* (B) lipase. The alcoholysis was conducted at 30°C with 4 wt% of the immobilized lipase. The amount of lauryl ester was expressed as a percentage of the initial content of the ethyl ester, 5.1 mol%); ●, lauryl palmitate (21.1 mol%); □, lauryl palmitoleate (5.8 mol%); ■, lauryl stearate (3.9 mol%); △, lauryl oleate (13.6 mol%); ▲, lauryl eicosapentaenoate (9.2 mol%); ◇, lauryl docosahexaenoate (22.7 mol%). For abbreviation, see Figure 1.

the activities of *Rhizomucor* lipase on EPA and DHA (24 and 11% relative to oleic acid) were twice those of *Rhizopus* lipase (13 and 5%). *Rhizomucor* lipase converted E-EPA to the lauryl ester at a higher rate than did *Rhizopus* lipase. Furthermore, when *Rhizopus* and *Rhizomucor* lipases were used, the recoveries of E-DHA in the ethyl ester fraction were 90 and 78%, respectively. These phenomena were shown to be due to the difference between the fatty acid specificities of the lipases.

Characteristics of the alcoholysis system described here. In general, it has been reported that transesterification with immobilized lipases requires precise control of water content in the reaction mixture (30,31). Because the continual batch reaction described here was performed using E-tuna-45/lauryl alcohol without controlling the water content, the water content changed from 300 to 1500 ppm. However, alcoholysis proceeded efficiently and the concomitant hydrolysis was less than 0.5%. This result shows that the alcoholysis system does not require control of the water content in the substrate. In addition, many transesterification reactions reported were performed in organic solvents, mainly in *n*-hexane (28–30), but no organic solvent was necessary in our reaction system. Thus a smaller-scale reactor can be used, and the risk of explosion can be excluded.

The mixture after the reaction contains lauryl esters, lauryl alcohol, and ethyl esters. Ethyl esters in the mixture could be easily separated by urea adduct formation and/or short-path distillation. Therefore, we believe that selective alcoholysis of ethyl esters with lauryl alcohol is very effective to purify E-DHA from ethyl esters originating from tuna oil.

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[Received May 19, 1998; accepted August 18, 1998]