Purification of Docosahexaenoic Acid from Tuna Oil by a Two-Step Enzymatic Method: Hydrolysis and Selective Esterification

Yuji Shimada[a,*], Kazuaki Maruyama[b], Akio Sugihara[a], Shigeru Moriyama[b], and Yoshio Tominaga[a]

[a]Osaka Municipal Technical Research Institute, Osaka 536, Japan, and [b]Maruha Corp., Ibaraki 300-42, Japan

ABSTRACT: Purification of docosahexaenoic acid (DHA) was attempted by a two-step enzymatic method that consisted of hydrolysis of tuna oil and selective esterification of the resulting free fatty acids (FFA). When more than 60% of tuna oil was hydrolyzed with *Pseudomonas* sp. lipase (Lipase-AK), the DHA content in the FFA fraction coincided with its content in the original tuna oil. This lipase showed stronger activity on the DHA ester than on the eicosapentaenoic acid ester and was suitable for preparation of FFA rich in DHA. When a mixture of 2.5 g tuna oil, 2.5 g water, and 500 units (U) of Lipase-AK per 1 g of the reaction mixture was stirred at 40°C for 48 h, 83% of DHA in tuna oil was recovered in the FFA fraction at 79% hydrolysis. These fatty acids were named tuna-FFA-*Ps*. Selective esterification was then conducted at 30°C for 20 h by stirring a mixture of 4.0 g of tuna-FFA-*Ps*/lauryl alcohol (1:2, mol/mol), 1.0 g water, and 1,000 U of *Rhizopus delemar* lipase. As a result, the DHA content in the unesterified FFA fraction could be raised from 24 to 72 wt% in an 83% yield. To elevate the DHA content further, the FFA were extracted from the reaction mixture with *n*-hexane and esterified again under the same conditions. The DHA content was raised to 91 wt% in 88% yield by the repeated esterification. Because selective esterification of fatty acids with lauryl alcohol proceeded most efficiently in a mixture that contained 20% water, simultaneous reactions during the esterification were analyzed qualitatively. The fatty acid lauryl esters (L-FA) generated by the esterification were not hydrolyzed. In addition, L-FA were acidolyzed with linoleic acid, but not with DHA. These results suggest that lauryl DHA was generated only by esterification.

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KEY WORDS: Docosahexaenoic acid, hydrolysis, lipase, *Pseudomonas* sp., *Rhizopus delemar*, selective esterification, tuna oil.

The n-3 series of polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (DHA, 22:6n-6), has various physiological functions (1,2), and its medical application has received increasing attention. The purification method of DHA by formation of its complex with silver was recently reported (3), but this method has not been accepted industrially because of residual silver and high cost. Therefore, other suitable methods are desired.

Because PUFA are sensitive to heat and oxidation, enzymatic reactions, which proceed efficiently under nitrogen at ordinary temperature and pressure, are expected to be useful for high-level processing of PUFA-containing oils and their related compounds. Lipases are known to act weakly on PUFA, e.g., γ-linolenic acid (GLA, 18:3n-6), arachidonic acid (20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), and DHA (4–6), and these fatty acids can be enriched by selective hydrolysis (4,7–10) and by selective esterification (11–13). When tuna oil was hydrolyzed with *Candida rugosa* lipase, DHA could be enriched in the glyceride fraction. But the upper limit was approximately 60 wt%, and the yield was not high (7). GLA was purified to 90 wt% as a free fatty acid (FFA) by selective esterification of fatty acids from borage oil with *n*-butanol and *Rhizomucor miehei* lipase (12). However, the system requires a large reactor because the reaction has to be conducted in a large amount of *n*-hexane.

Recently, we noticed that a lipase esterified fatty acids with fatty alcohols but did not hydrolyze the esters, and we developed a new method of selective esterification that did not require any organic solvent. When lauryl alcohol was used as substrate, *Rhizopus delemar* lipase esterified fatty acids other than DHA efficiently. As a result, DHA could be purified from 23 to *ca.* 90 wt% by selective esterification of fatty acids originating from tuna oil (14). However, the fatty acids used as substrates were prepared by hydrolysis of tuna oil in a large amount of ethanol with NaOH as catalyst. This chemical hydrolysis has not been adopted as an industrial method yet.

The present paper deals with the purification of DHA by a two-step enzymatic method: The first step is preparation of FFA by hydrolysis of tuna oil with *Pseudomonas* sp. lipase with strong activity on DHA, and the second step is selective esterification of the resulting FFA with lauryl alcohol and *R. delemar* lipase with weak activity on DHA. This paper also shows that lauryl DHA (L-DHA) is generated only by esterification, although fatty acid lauryl esters (L-FA) can be substrates in transesterification (acidolysis).

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

MATERIALS AND METHODS

Oil, fatty acids, and alcohol. Tuna oil used was refined by Maruha Corp. (Tokyo, Japan; DHA, 22.9 wt%). Oleic acid (OA, 18:1n-9) and linoleic acid (LA, 18:2n-6) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan), and DHA was purchased from Nakalai Tesque Inc. (Kyoto, Japan). Lauryl alcohol of reagent grade was from Wako Pure Chemical Industries 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. Ltd., Tokyo, Japan); *Candida rugosa* lipase (Lipase-OF; Meito Sangyo Co. Ltd., Aichi, Japan); and *R. delemar* lipase (Ta-lipase; Tanabe Seiyaku Co. Ltd., Osaka, Japan).

Hydrolysis of tuna oil and preparation of FFA. Unless otherwise specified, hydrolysis of tuna oil was conducted under the following conditions: A mixture of 2.5 g tuna oil, 2.5 g water, and 2,500 units (U) of the Lipase-AK was incubated at 40°C with stirring at 500 rpm for 48 h. The hydrolysis with *Candida* lipase was done under the same conditions except that the temperature was set at 35°C. After hydrolysis, glycerides were removed by extracting with *n*-hexane at alkaline pH (>pH 13), and then FFA were extracted from the water phase with *n*-hexane after returning to acidic pH (<pH 2) with HCl. The resulting FFA prepared by hydrolysis with Lipase-AK and *Candida* lipase were named tuna-FFA-*Ps* and tuna-FFA-*Can*, respectively. The hydrolysis extent was calculated from the acid value of the reaction mixture and the saponification value of the original oil.

Selective esterification of FFA from tuna oil. Selective esterification of fatty acids with lauryl alcohol was carried out according to a previous paper (14). In brief, a mixture of 4 g of fatty acids/lauryl alcohol (1:2, mol/mol), 1 g water, and 1,000 U of *Rhizopus* lipase was incubated at 30°C for 20 h with stirring at 500 rpm. The repeated esterification was conducted under the same conditions with FFA obtained from the first reaction as substrates. 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.

Fractionation of L-FA, FFA, and lauryl alcohol in reaction mixture. The mixture of L-FA and lauryl alcohol, described in the previous section, was extracted with 100 mL *n*-hexane after adding 70 mL of 0.5 N KOH (30% ethanol solution) to 5 g of the reaction mixture. FFA in the water phase were extracted with 100 mL *n*-hexane after returning to acidic pH (<pH 2) with HCl (4). L-FA were purified by applying a mixture of L-FA and lauryl alcohol onto a silica gel 60 (Merck, Darmstadt, Germany) column (30 × 250 mm), and eluting with a mixture of *n*-hexane/ethyl acetate (98:2, vol/vol).

Analysis. Lipase activity was measured by titrating fatty acids liberated from olive oil (Wako Pure Chemical) with 0.05 N KOH as described previously (15). The reaction was carried out at 35°C for 30 min with stirring at 500 rpm. One U of lipase activity was defined as the amount of enzyme that liberated 1 μmol of fatty acid per minute.

Fatty acids in glycerides and L-FA were methylated at 75°C in methanol for 15 min with Na-methylate as methylating reagent, and 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 (7).

RESULTS

Screening for a lipase suitable for hydrolysis of tuna oil. A mixture of 2.5 g tuna oil, 2.5 g water, and 2,500 U of lipase was incubated for 16 h with stirring at 500 rpm. The hydrolyses by *Rhizopus* and *Candida* lipases and by *Pseudomonas* lipases were carried out at 35 and 40°C, respectively. Table 1 shows the hydrolysis extent, the contents of EPA and DHA in the FFA fraction, and the percentage of DHA recovered in the FFA fraction. *Rhizopus* lipase acted moderately on the DHA ester, and the hydrolysis extent was only 39%. DHA could not be recovered efficiently in the FFA fraction by hydrolysis with *Candida* lipase, because the lipase acted moderately on DHA ester although the hydrolysis extent was high. On the other hand, *P. aeruginosa* lipase acted on DHA as strongly as the other fatty acids, and the hydrolysis extent was also high. Therefore, tuna oil was hydrolyzed with several *Pseudomonas* lipases (Table 1). The results showed that all *Pseudomonas* lipases acted strongly on the ester bond of DHA, and that the hydrolysis extents were high. Among these enzymes, Lipase-AK hydrolyzed the DHA ester more strongly than the EPA ester, and DHA was recovered in the FFA fraction in the highest yield. Thus Lipase-AK was chosen for hydrolysis of tuna oil.

TABLE 1
Hydrolysis of Tuna Oil with Several Lipases

Lipase	Hydrolysis (%)	Fatty acid content (wt%)[a] 20:5	22:6	Recovery of DHA[b] (%)
Rhizopus delemar	38.5	5.1	15.3	25.7
Candida rugosa	64.4	6.8	13.1	36.8
Pseudomonas aeruginosa	64.8	6.6	23.9	67.6
P. glumae[c]	65.4	6.5	23.5	67.1
Pseudomonas sp. KWI-56	58.8	6.7	23.1	59.3
Pseudomonas sp.[d]	68.4	4.3	24.2	71.9
Pseudomonas sp.[e]	78.9	5.2	19.8	68.2

[a]The contents of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-6) in the free fatty acid (FFA) fraction. The contents of EPA and DHA in the original tuna oil were 6.5 and 22.9 wt%, respectively.
[b]The amount of DHA recovered in the FFA fraction.
[c]Same as *Chromobacterium viscosum.*
[d]Lipase-AK (Amano Pharmaceutical Co. Ltd., Aichi, Japan).
[e]LIPOSAM (Showa Denko K.K., Tokyo, Japan).

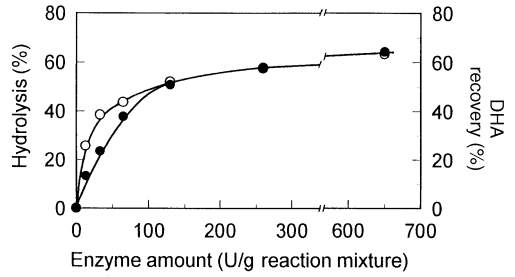

FIG. 1. Effect of lipase amount on hydrolysis of tuna oil with *Pseudomonas* sp. lipase (Lipase-AK; Amano Pharmaceutical Co., Ltd., Aichi, Japan). ○, extent of hydrolysis; ●, recovery of docosahexaenoic acid (DHA) in the free fatty acid fraction.

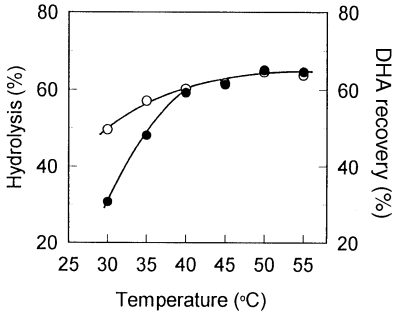

FIG. 3. Effect of temperature on hydrolysis of tuna oil with *Pseudomonas* sp. lipase (Lipase-AK). ○, extent of hydrolysis; ●, recovery of DHA in the free fatty acid fraction. For manufacturer and abbreviation see Figure 1.

Determination of optimal conditions for hydrolysis of tuna oil with Lipase-AK. A reaction mixture of 2.5 g tuna oil, 2.5 g water, and various amounts of Lipase-AK was stirred at 40°C for 16 h, to investigate the effect of enzyme amount on the hydrolysis (Fig. 1). When tuna oil was hydrolyzed with less than 120 U of lipase per 1 g of reaction mixture, the hydrolysis rate of DHA ester was slower than that of the other constituent fatty acids, and the DHA content in the FFA fraction was lower than in tuna oil (23 wt%). However, when the oil was hydrolyzed with more than 120 U/g, the hydrolysis rate of the DHA ester was almost the same as that of the other constituent fatty acid esters. Thus, the recovery of DHA in the FFA fraction agreed with the extent of hydrolysis.

To investigate the effect of water content on hydrolysis, 5 g of a mixture of tuna oil/water was incubated at 40°C with stirring for 16 h with 2,500 U of Lipase-AK. As shown in Figure 2, the amount of DHA recovered in the FFA fraction was associated with the extent of hydrolysis, which did not increase even though the water content was more than 50%.

Next, the optimal temperature was examined by stirring a mixture of 2.5 g tuna oil, 2.5 g water, and 2,500 U of Lipase-AK in a range of 30 to 55°C for 16 h (Fig. 3). The hydrolysis extent depended slightly on the reaction temperature but showed a constant value above 40°C. The recovery of DHA in the FFA fraction was lower than the hydrolysis extent below 40°C (the DHA content in the FFA fraction was less than 23 wt%) but was the same as the extent of hydrolysis above 40°C (the DHA content was approximately 23 wt%).

On the basis of these results, hydrolysis was performed under the following conditions: A mixture of 2.5 g tuna oil, 2.5 g water, and 500 U/g of Lipase-AK was incubated at 40°C with stirring at 500 rpm.

Time course of hydrolysis of tuna oil with Lipase-AK. Figure 4 shows a typical time course under optimal conditions. Tuna oil was hydrolyzed rapidly for several hours after the beginning of the reaction, and then hydrolyzed more gradually. The contents of palmitic acid (PA, 16:0) and OA in the FFA fraction were high in the early stage of the reaction and came close to those of the original tuna oil after 16 h. The ester bond of DHA was hydrolyzed moderately in the early

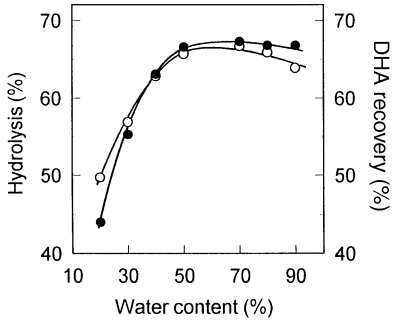

FIG. 2. Effect of water content on hydrolysis of tuna oil with *Pseudomonas* sp. lipase (Lipase-AK). ○, extent of hydrolysis; ●, recovery of DHA in the free fatty acid fraction. For manufacturer and abbreviation see Figure 1.

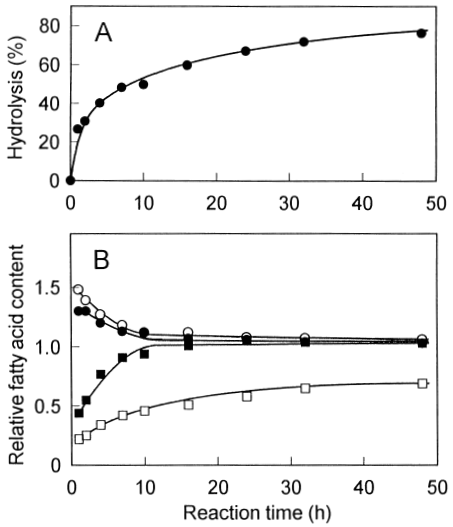

FIG. 4. Time course of hydrolysis of tuna oil with *Pseudomonas* sp. lipase (Lipase-AK) (A) and the contents of main constituent fatty acids in the free fatty acid (FFA) fraction (B). The fatty acid contents in the FFA fraction are expressed relative to those in the original tuna oil. The fatty acid contents in the original oil: palmitic acid (○), 18.6 wt%; oleic acid (●), 20.7 wt%; eicosapentaenoic acid (□), 6.5 wt%; docosahexaenoic acid (■), 22.9 wt%.

stage of the reaction, but its content in the FFA fraction became almost the same as that of the tuna oil after 16 h. On the contrary, the EPA ester was hydrolyzed weakly, and its content in the FFA fraction was lower than that of the original tuna oil, even after 48 h of reaction. These facts showed that the reactivity of Lipase-AK toward the constituent fatty acids was in the order: PA, OA > DHA > EPA.

On the basis of these results, the fatty acids (tuna-FFA-*Ps*) were prepared by extraction with *n*-hexane from the reaction mixture after 48 h of hydrolysis. The hydrolysis extent was 79%, and the recovery of DHA in the FFA fraction was 83%.

Purification of DHA by selective esterification of fatty acids from tuna oil with Rhizopus *lipase.* DHA can be efficiently enriched in the FFA fraction by selective esterification of the fatty acids with lauryl alcohol by using *Rhizopus* lipase (14). As shown in Table 2, when tuna-FFA-*Ps* were esterified with lauryl alcohol according to the Materials and Methods section, the DHA content was raised from 24 to 72 wt% in a 83% yield. To further elevate the DHA content, the unreacted fatty acids were extracted from the reaction mixture with *n*-hexane and were esterified again under the same conditions. As a result, the DHA content could be raised to 91 wt% in an 88% yield. The hydrolysis extent of tuna oil with Lipase-AK was 79%, and 17% of DHA remained in the glyceride fraction. However, the recovery of DHA in the FFA fraction was 60% of the initial DHA content in tuna oil, even after hydrolysis with Lipase-AK and repeated selective esterification with *Rhizopus* lipase.

An oil with 45–50 wt% DHA has been produced industrially by selective hydrolysis of tuna oil with *C. rugosa* lipase (7,16), and the FFA generated have been treated as a waste material. Because the DHA content in this FFA fraction was 13 to 15 wt%, we also attempted selective esterification of the FFA (Table 2). Here, the FFA (tuna-FFA-*Can*) were prepared by hydrolysis of tuna oil with *Candida* lipase according to the Materials and Methods section. The hydrolysis extent was 64%, and the DHA content in tuna-FFA-*Can* was 13 wt%. When selective esterification of tuna-FFA-*Can* was carried out two times, the DHA content could be raised to 81 wt% with a recovery of 70% of the initial content in the FFA. This fact shows that the waste material can be used as a starting material for the purification of DHA.

TABLE 2
Purification of DHA by Two-Step Enzymatic Method

	Fatty acid content (wt%)[a]						Recovery of DHA[b]
Step	16:0	16:1	18:0	18:1	20:5	22:6	(%)
Tuna oil	18.6	4.6	4.5	20.7	6.5	22.9	100
Hydrolysis							
Tuna-FFA-*Ps*[c]	19.4	4.8	4.5	21.1	4.3	24.2	83.0
Esterification							
First	5.2	1.2	1.1	5.7	4.0	71.6	68.5
Second	0.8	n.d.	n.d.	0.9	1.7	90.6	60.3
Hydrolysis							
Tuna-FFA-*Can*[d]	20.6	5.8	4.7	24.4	7.3	13.1	36.8
Esterification							
First	8.0	2.2	1.8	9.4	6.9	58.3	30.0
Second	1.9	0.4	0.5	2.0	5.1	81.0	25.6

[a]The contents of main fatty acids in the free fatty acid (FFA) fraction. See Table 1 for other abbreviation.
[b]Recovery of DHA based on the initial content of tuna oil.
[c]The FFA obtained by hydrolysis of tuna oil with *Pseudomonas* sp. lipase (Lipase-AK; Amano Pharmaceutical Co. Ltd., Aichi, Japan).
[d]The FFA obtained by hydrolysis of tuna oil with *Candida rugosa* lipase (Lipase-OF; Meito Sangyo Co. Ltd., Aichi, Japan).

When selective esterification was conducted two times with tuna-FFA-*Ps* and tuna-FFA-*Can* as substrates, the recoveries of DHA in the FFA fractions were 74 and 70% of initial contents in the FFA, respectively. This result suggested that the recovery of DHA was not so different, even though the DHA content in the FFA was changed. On the contrary, when the FFA rich in DHA were used as substrates for selective esterification, the DHA content in the FFA fraction could be slightly increased. In addition, when the FFA that were poor in EPA were used as substrates, the EPA content in the FFA fraction could be efficiently decreased.

Simultaneous reactions on selective esterification. Selective esterification of fatty acids with lauryl alcohol proceeded most efficiently when 20% water was present in the reaction mixture (14). Because lipase catalyzes hydrolysis, esterification and transesterification, all these reactions may occur during selective esterification. Therefore, we attempted to examine the reactions associated with esterification.

A mixture of 1.0 g OA, LA, or DHA, 1.4 g lauryl alcohol, 0.6 g water, and 600 U of *Rhizopus* lipase was incubated at 30°C for 20 h with stirring at 500 rpm. The esterification extent of DHA was only 5.2%, although those of OA and LA were 90 and 92%, respectively. This result showed that DHA was esterified only weakly in this reaction system.

To investigate whether L-FA can be substrates of *Rhizopus* lipase, L-FA generated by esterification of tuna-FFA-*Ps* were purified by silica gel column chromatography. When a mixture of 0.8 g L-FA, 0.2 g water, and 200 U of *Rhizopus* lipase was incubated at 30°C for 20 h, the hydrolysis extent was only 4.5%. This result shows that simultaneous hydrolysis of L-FA is negligible.

Next, a mixture of 0.4 g L-FA, 0.4 g LA or DHA, and 200 U of *Rhizopus* lipase was incubated at 30°C for 20 h to examine transesterification (acidolysis) of the esters with fatty acid. After the reaction, L-FA were extracted with *n*-hexane, and the fatty acid composition was analyzed (Table 3). Fatty acids in L-FA, mainly PA and OA, were exchanged for LA, but not for DHA at all. This shows that acidolysis of L-FA occurred simultaneously on esterification with LA but not with DHA.

DISCUSSION

We have described a procedure of purifying DHA by a two-step enzymatic method that consists of hydrolysis of tuna oil and two selective esterifications of the resulting FFA. Lipase-AK hydrolyzed the ester of DHA as strongly as those of the other constituent fatty acids and was suitable for the first-step hydrolysis. *Rhizopus* lipase was suitable for the second-step

TABLE 3
Acidolysis of Fatty Acid Lauryl Esters (L-FA) with Linoleic Acid (LA) or DHA by *Rhizopus delemar* Lipase

Reaction	Fatty acid composition (mol%)[a]						
	16:0	16:1	18:0	18:1	18:2	20:5	22:6
None (L-FA[b])	26.2	7.0	5.5	25.6	1.5	4.0	3.1
L-FA + LA[c]	21.1	5.1	4.6	19.9	18.0	3.7	2.9
L-FA + DHA[d]	26.3	7.0	5.6	25.6	1.6	4.0	3.1

[a]Fatty acid composition in the L-FA.
[b]L-FA used as substrate.
[c]Acidolysis of L-FA with linoleic acid.
[d]Acidolysis of L-FA with DHA. For other abbreviation, see Table 1.

esterification because it acted only weakly on DHA. As shown in Table 2, the hydrolysis extent of tuna oil with Lipase-AK was 79%, and 17% of DHA remained in the glyceride fraction. Thus, the DHA amount recovered in the FFA fraction was 60% after repeated selective esterification of tuna-FFA-*Ps*. However, the undigested glycerides can be used as materials for the next hydrolysis. In addition, DHA could be purified to 91 wt% by selective esterification of tuna-FFA-*Ps,* and to 81 wt% even by that of tuna-FFA-*Can*.

Generally, when esterification is conducted with lipase as a catalyst, even a small amount of water interferes with the reaction efficiency. But, when lauryl alcohol was used as the substrate, esterification proceeded most effectively in a reaction mixture that contained 20% water (14). Therefore, not only the water in the substrates but also the water generated by the esterification does not have to be removed. Furthermore, the selective esterification reported previously was carried out in a mixture with *n*-hexane (11–13), but the esterification system described here did not require any organic solvent. Thus, a small reactor can be used without the risk of explosion. In addition, the moderate reaction conditions with the lipase can avoid degradation of DHA, especially its isomerization. Taking these into consideration, the two-step enzymatic method may be suitable for industrial purification of DHA from tuna oil.

As reported previously, when fatty acids from tuna oil were esterified with short-chain alcohols (methanol, ethanol, and propanol) and glycerol in a reaction mixture that contained 20% water, esterification extent was low, less than 30% (14). But when lauryl alcohol was used as substrate, the esterification extent of fatty acids from tuna oil was more than 70%, and those of OA and LA were more than 90%. That only 4.5% of L-FA was hydrolyzed in the reaction mixture that contained 20% water suggests that the reaction was shifted to esterification.

L-DHA was generated by esterification of DHA with lauryl alcohol, although the esterification extent was only 5.2%. L-FA were acidolyzed with LA, on which *Rhizopus* lipase acted strongly, but not with DHA (Table 3). In addition, the amount of L-DHA in the reaction mixture increased linearly (14). These results suggest that L-DHA is generated by esterification.

L-FA were not hydrolyzed by *Rhizopus* lipase, but 17% of the fatty acids in L-FA were acidolyzed with LA (Table 3). The transesterified LA corresponds to only 10% of LA in the reaction mixture. However, 92% of LA was changed to L-LA by the esterification. These facts show that esterification occurs preferentially in the reaction of fatty acids with lauryl alcohol. Transesterification catalyzed by lipase involves alcoholysis and interesterification in addition to acidolysis. Therefore, alcoholysis and interesterification may also occur simultaneously during selective esterification, but these reactions do not affect the amount of L-DHA in the reaction mixture. The kinetic studies of selective esterification are now in progress to clarify the reaction mechanism.

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