Purification of Docosahexaenoic Acid by Selective Esterification of Fatty Acids from Tuna Oil with *Rhizopus delemar* Lipase

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ABSTRACT: To purify docosahexaenoic acid (DHA), we attempted the selective esterification of fatty acids originating from tuna oil with lipases. Tuna oil was hydrolyzed in NaOH–ethanol solution, and the resulting fatty acid mixture [DHA, 23.2%; named tuna–free fatty acid (FFA)] was used as a starting material. *Rhizopus delemar* which acted lightly on DHA, was a suitable catalyst for the selective esterification of tuna–FFA, and lauryl alcohol was the best substrate. The reaction proceeded most effectively when a mixture of 2.4 g lauryl alcohol/tuna–FFA (2:1, mol/mol), 0.6 g water, and 600 U *Rhizopus* lipase was incubated at 30°C for 20 h with stirring at 500 rpm. Under these conditions 72% of tuna-FFA was esterified, and 84% of DHA was recovered in the unesterified fatty acid fraction. The DHA content in the fatty acid fraction rose from 23 to 73% with this reaction. To further elevate the DHA content, the unesterified fatty acids were extracted, and then esterified again under the same conditions. By this repeated esterification, DHA was purified to 89% with a recovery of 71% of its initial content.
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KEY WORDS: Docosahexaenoic acid, fatty alcohol, purification, *Rhizopus delemar* lipase, selective esterification, tuna oil.

The n-3 series of fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have various physiological functions (1,2), and ethyl ester of EPA has been used as a medicine for arteriosclerosis obliterans (3). Oil containing DHA has been used as a health food (4), and medical application of DHA has received increasing attention. The purification method of DHA by formation of its complex with silver was recently reported (5), but this method has not been accepted industrially because of residual silver and high cost. Therefore, other suitable methods are desired.

Lipases are well known to have little reactivity on polyunsaturated fatty acids (e.g., γ-linolenic acid, arachidonic acid, EPA, and DHA) (6,7), and these fatty acids can be enriched by selective hydrolysis (7–11) and by selective esterification (12,13). Glycerides that contained approximately 50% DHA were obtained by selective hydrolysis of tuna oil with *Candida rugosa* lipase (8,14), but higher purity is desirable for medical use. γ-Linolenic acid was purified to 85% as a free fatty acid (FFA) by selective esterification of fatty acids derived from evening primrose oil with *Rhizomucor miehei* lipase (12). However, DHA has not been effectively purified by this reaction system, and furthermore, the reaction required a large amount of *n*-hexane.

In this study, we found that *Rhizopus delemar* lipase effectively esterified fatty acids other than DHA with long-chain fatty alcohols, and that the resulting esters were scarcely hydrolyzed again. Therefore, the esterification extent increased, and DHA was successfully enriched in the FFA fraction. The present paper deals with an effective method by which DHA is purified to a level as high as 90% by selective esterification of fatty acids from tuna oil in the reaction mixture without an organic solvent.

EXPERIMENTAL PROCEDURES

Lipases. Fusarium heterosporum lipase was prepared as reported previously (15). Ammonium sulfate was added to the culture filtrate to give 80% saturation, and the resulting precipitates were dialyzed against water. The lipases from *R. delemar, C. rugosa*, and *Pseudomonas aeruginosa* were gifts from Tanabe Seiyaku Co. (Osaka, Japan), Meito Sangyo Co. (Aichi, Japan), and Toyobo Co. (Osaka, Japan), respectively.

Fatty acids and fatty alcohols. Tuna oil (100 g), from which gums, FFA, and water were removed by Maruha Co. (Tokyo, Japan), was hydrolyzed at 50°C in 300 mL of 90% ethanol, containing 30 g NaOH, for 30 min, and the fatty acids were extracted with *n*-hexane. The fatty acid composition was not affected by the hydrolysis, and the resulting fatty acid mixture (3.5 mmol/g; DHA content, 23.2 wt%) was named tuna–FFA. Fatty alcohols were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Analyses. Lipase activity was measured by titrating fatty acids liberated from olive oil (Wako Pure Chemical Ind., Osaka, Japan) with 0.05 N KOH, as described previously

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(16). The reaction was carried out at 30°C for 30 min with stirring at 500 rpm. One unit (U) of lipase was defined as the amount of enzyme that liberated 1 μmol of fatty acid per minute.

FFA were esterified at 80°C in 5% HCl–methanol for 3 h. The methyl esters were analyzed with a Hewlett-Packard 5890 gas chromatograph (Avondale, PA), connected to a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) as described previously (8).

Selective esterification of tuna–FFA with lipase. A reaction mixture containing tuna–FFA, alcohol, water, and lipase was incubated at 30°C with stirring at 500 rpm. 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. After the reaction, unesterified fatty acids were extracted with *n*-hexane, and their methyl esters were analyzed by gas chromatography.

RESULTS

Selection of lipase suitable for purification of DHA. Tuna–FFA was esterified with lauryl alcohol by using lipases from *R. delemar, F. heterosporum, C. rugosa,* and *P. aeruginosa*, whose reactivities toward oil and fat were different (Table 1). When *Candida* lipase was used, 98% DHA was recovered in the unesterified fatty acid fraction, and DHA was scarcely esterified with the alcohol. However, the DHA content in the fatty acid fraction was only 42% because of the low esterification extent. The esterification extent by *Pseudomonas* lipase was high, but the DHA content was as low as 51% because the lipase esterified DHA more extensively. *Rhizopus* and *Fusarium* lipases achieved high esterification extents, and the reactivities toward DHA were low. Therefore, DHA was successfully purified as a FFA; DHA was purified from 23 to 65% by *Rhizopus* lipase. From these results, *Rhizopus* lipase was selected as the most effective enzyme.

Effect of water content. The water content in the reaction mixture is generally important when lipase is used as esterification catalyst (17). We first examined the effect of water content on the selective esterification of tuna–FFA (Fig. 1). The DHA content in the fatty acid fraction depended on the esterification extent, and the highest value was obtained at 20% water. Thus, the following esterification was carried out in a reaction mixture that contained 20% water.

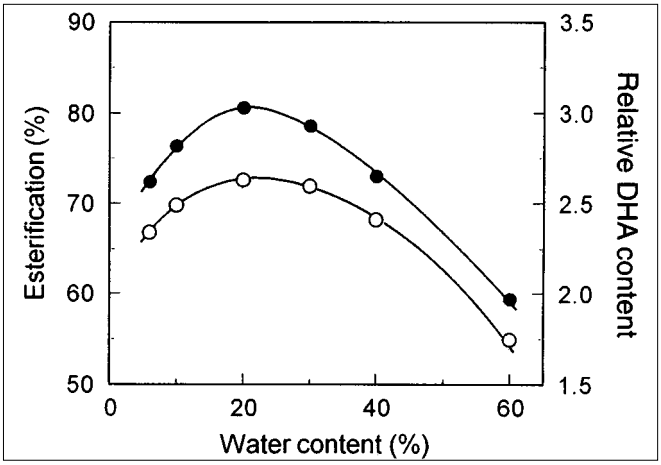

FIG. 1. Effect of water content on the selective esterification of tuna–free fatty acid (FFA) with *Rhizopus delemar* lipase. A reaction mixture (3.0 g) of tuna–FFA/lauryl alcohol (1:2, mol/mol), water, and 600 unit lipase was stirred at 30°C for 16 h. The docosahexaenoic acid (DHA) content in the fatty acid fraction was expressed relative to that in the original tuna–FFA (23.2 wt%). ○, Esterification extent; ●, DHA content in fatty acid fraction.

Effect of alcohol. Our preliminary experiments showed that *Rhizopus* lipase esterified fatty acids with lauryl or oleyl alcohol, but that the enzyme scarcely hydrolyzed the resulting esters (data not shown). This result showed that the fatty alcohol effectively shifted the equilibrium toward esterification. Therefore, an alcohol suitable for selective esterification was screened from various fatty alcohols, which were in the liquid state at the reaction temperature. As shown in Table 2, long-chain fatty alcohols enhanced esterification, and the DHA con-

TABLE 1
Selective Esterification of Tuna–FFA with Several Lipases[a]

Lipase	Esterification (%)	DHA content[b] (wt%)	Recovery of DHA[c] (%)
None	—	23.2	100
Rhizopus[d]	67.8	65.4	90.8
Fusarium[e]	63.9	58.5	91.0
Candida[f]	45.6	41.9	98.2
Pseudomonas[g]	72.0	50.6	65.9

[a]A reaction mixture of 2.7 g lauryl alcohol/tuna–free fatty acid (FFA) (2:1, mol/mol), 0.3 g water, and 600 units lipase was stirred at 30°C for 16 h.
[b]Docosahexaenoic acid (DHA) content in unesterified fatty acid fraction.
[c]DHA amount recovered in unesterified fatty acid fraction.
[d]*Rhizopus delemar.*
[e]*Fusarium heterosporum.*
[f]*Candida rugosa.*
[g]*Pseudomonas aeruginosa.*

TABLE 2
Effect of Alcohol on Selective Esterification of Tuna–FFA[a]

Alcohol	Esterification (%)	DHA content[b] (wt%)	Recovery of DHA[c] (%)
Methanol	14.1	23.2	83.7
Ethanol	19.1	23.8	80.9
Propanol	26.5	27.9	86.2
Butanol	36.7	32.7	87.0
Pentanol	56.9	49.2	89.1
Hexanol	66.1	62.4	88.9
Octanol	70.0	67.6	85.2
Decanol	72.4	70.3	81.5
Lauryl alcohol	72.7	70.9	81.3
Oleyl alcohol	66.4	60.7	85.7
Glycerol	30.4	23.1	67.6

[a]A reaction mixture of 2.4 g tuna–FFA/alcohol (1:2, mol/mol), 0.6 g water, and 600 U *Rhizopus delemar* lipase was stirred at 30°C for 16 h. See Table 1 for abbreviations.
[b]DHA content in unesterified fatty acid fraction.
[c]DHA amount recovered in unesterified fraction.

tent in the fatty acid fraction depended on the esterification extent. The ability of oleyl alcohol was the same as that of hexanol, although it was a long-chain fatty acid. In addition, when glycerol and short-chain fatty alcohols (methanol, ethanol, and propanol) were used as substrates, the esterification extents were low, and selectivities on fatty acids were not observed. From these results, lauryl alcohol, which was the most effective substrate, was chosen for the selective esterification of tuna–FFA.

The suitable molar ratio of lauryl alcohol to tuna–FFA was next examined, and the result is shown in Table 3. The esterification extent was 45% in the reaction system that contained equimolar amounts of lauryl alcohol and fatty acids, and DHA was purified to only 38%. However, when the ratio of lauryl alcohol to the fatty acids exceeded 2:1, the DHA content in the fatty acid fraction was elevated from 23 to 71% with an esterification extent of 73%. Thus, the ratio of lauryl alcohol to tuna–FFA of 2:1 (mol/mol) was selected.

Determination of other reaction conditions. Figure 2 shows the effect of the amount of lipase on the selective esterification of tuna–FFA. The esterification extent was increased by increasing the amount of the lipase and reached a constant value at 200 U/g of reaction mixture. On the other hand, the DHA content in the unesterified fatty acids showed a maximum value in the range of 200–300 U/g of reaction mixture. In the following reaction, the enzyme amount was set at 200 U/g of reaction mixture.

The optimal reaction temperature between 25 and 45°C was examined. The reaction mixture was composed of 2.4 g lauryl alcohol/tuna–FFA (2:1, mol/mol), 0.6 g water, and 600 U lipase and was stirred for 16 h. Because the esterification extent and DHA content showed maximum values between 30 and 35°C, the following reaction was carried out at 30°C.

Time course of selective esterification of tuna–FFA. Figure 3 shows the changes of the contents of unesterified fatty acids during esterification of tuna–FFA with lauryl alcohol. The esterification extent did not increase much after 7 h, and DHA content in the fatty acid fraction depended on the esterification extent. The contents of oleic and palmitic acids in the fatty acid fraction rapidly decreased, and that of EPA decreased after the 4-h increase. These results showed that the reactivity of *Rhizopus* lipase toward these fatty acids was in

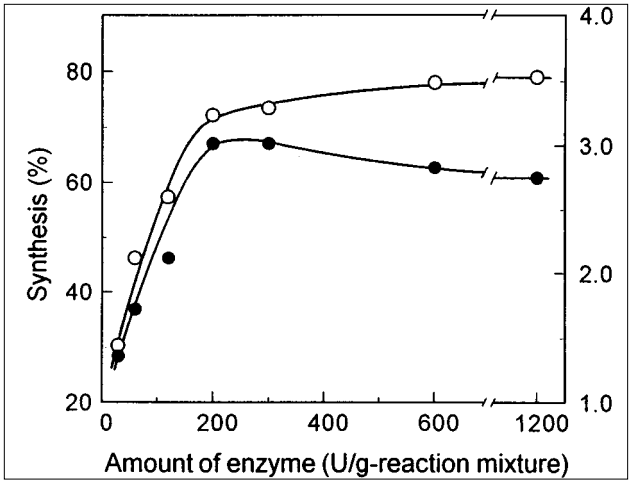

FIG. 2. Effect of enzyme amount on the selective esterification of tuna–FFA with *Rhizopus delemar* lipase. A reaction mixture was composed of 2.4 g lauryl alcohol/tuna–FFA (2:1, mol/mol), 0.6 g water, and various amounts of lipase. The reaction was carried out at 30°C for 16 h with stirring. The DHA content in the fatty acid fraction was expressed as a relative value to that in the original tuna-FFA (23.2 wt%). ○, Esterification extent; ●, DHA content in fatty acid fraction; U, unit. See Figure 1 for other abbreviations.

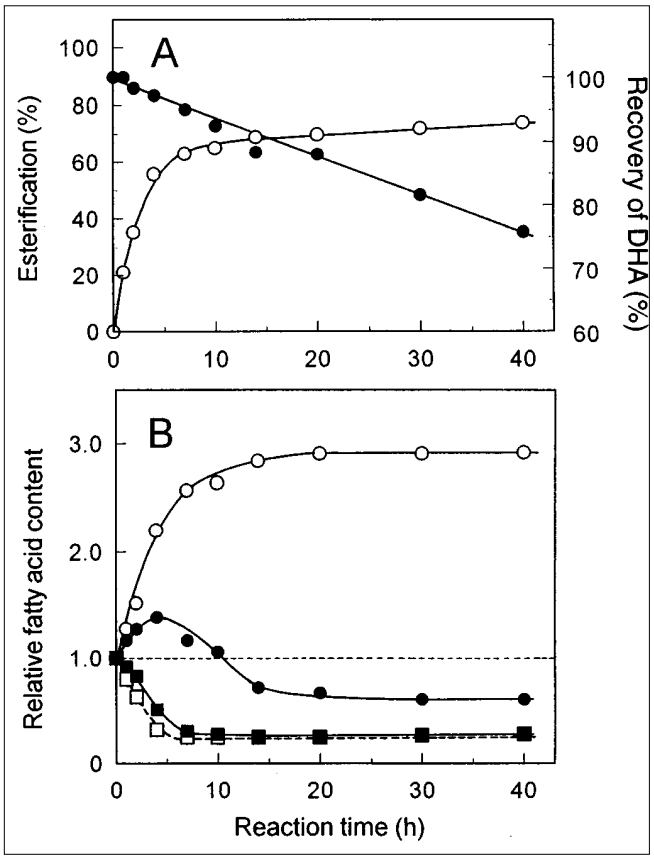

FIG. 3. Time course of the selective esterification of tuna–FFA with *Rhizopus delemar* lipase. The reaction was carried out at 30°C in the mixture of 2.4 g lauryl alcohol/tuna–FFA (2:1, mol/mol), 0.6 g water, and 600 U lipase. A, The esterification extent (○) and DHA recovery (●); B, the contents of unesterified fatty acids. The contents of fatty acids were expressed as relative values to their initial ones. ●, DHA; ●, EPA; □, oleic acid; ■, palmitic acid. See Figures 1 and 2 for abbreviations.

TABLE 3
Effect of Amount of Lauryl Alcohol on Selective Esterification of Tuna–FFA[a]

Alcohol/tuna–FFA	Esterification (%)	DHA content[b] (wt%)	Recovery of DHA[c] (%)
1:1	45.0	38.4	91.0
2:1	72.5	70.6	83.7
3:1	72.7	70.9	83.4
4:1	72.8	70.6	82.8
6:1	73.5	709.2	80.2

[a]A reaction mixture of 2.4 g lauryl alcohol/tuna–FFA (1:1 to 6:1, mol/mol), 0.6 g water, and 600 U *Rhizopus delemar* lipase was stirred at 30°C for 16 h. See Table 1 for abbreviations.
[b]DHA content in unesterified fatty acid fraction.
[c]DHA amount recovered in unesterified fraction.

TABLE 4
The Contents of Unesterified Fatty Acids Obtained by Repeated Selective Esterification[a]

Treatment	Esterification (%)	Fatty acid content (wt%)[b] 14:0	16:0	16:1	18:0	18:1	20:4	20:5	22:6	Recovery of DHA[c] (%)
None	—	2.6	17.9	4.9	3.9	23.0	2.1	5.8	23.2	100
Rhi.[d]	72.4	0.7	4.4	1.2	1.0	6.0	1.7	4.1	73.0	83.6
Rhi. + *Rhi.*[e]	29.7	n.d.[g]	1.0	n.d.	n.d.	1.2	0.8	1.7	88.6	71.3
Rhi. + *Can*[f]	14.6	n.d.	1.3	n.d.	0.8	1.1	1.8	3.5	82.9	81.2

[a]The first reaction was carried out at 30°C for 20 h in a reaction mixture of 7.2 g lauryl alcohol/tuna–FFA (2:1, mol/mol), 1.8 g water, and 1,800 U *Rhizopus delemar* lipase. After the reaction, unesterified fatty acids were extracted with *n*-hexane and were used for the second esterification. The second reaction was carried oiut at 30°C for 20 h in a reaction mixture of 2.4 g lauryl alcohol/fatty acids, 0.6 g water, and 600 U *Rhizopus* or *Candida rugosa* lipase. See Table 1 for abbreviations.
[b]Content of unesterified fatty acid.
[c]DHA amount recovered in unesterified fraction relative to initial DHA content.
[d]The first reaction with *Rhizopus* lipase.
[e]The second reaction with *Rhizopus* lipase after the reaction with *Rhizopus* lipase.
[f]The second reaction with *Candida* lipase after the reaction with *Rhizopus* lipase.
[g]n.d., Not detected.

the order: oleic acid, palmitic acid > EPA > DHA. The contents of unesterified fatty acids reached approximately a constant value after 20 h, although the recovery of DHA decreased with reaction time. The reaction period was therefore fixed to 20 h in the following reactions.

Purification of DHA by repeated esterification. When DHA and arachidonic acid were concentrated in glycerides by selective hydrolyses, repeated reactions were effective, compared with increase of enzyme amount and with extension of reaction time (7,8). Therefore, unesterified fatty acids were recovered from the reaction mixture of the first selective esterification of tuna–FFA and then esterified again with lauryl alcohol by using *Rhizopus* and *Candida* lipases. The results are shown in Table 4. The esterification extent of the first reaction was 72%, and DHA was purified to 73% with a recovery of 84%. When the repeated esterification was carried out with *Candida* lipase, which showed lower esterification activity on DHA than *Rhizopus* lipase (Table 1), DHA was purified to 83% with a recovery of 81% of its initial content, although the esterification extent was only 15%. On the other hand, the esterification extent of the repeated reaction with *Rhizopus* lipase was 30%, and DHA was purified to 89% with a recovery of 71% of its initial content. These results showed that the repeated reaction with *Rhizopus* lipase was effective for purification of DHA from tuna–FFA.

DISCUSSION

We have described a procedure of purifying DHA to about 90% by the repeated esterification of tuna–FFA with lauryl alcohol and *R. delemar* lipase, which effectively esterified fatty acids other than DHA and moderately esterified DHA. This effective method requires no organic solvents, and the resulting purified DHA may be used not only for medicine but also for health foods.

Effect of water content on the selective esterification of tuna–FFA. Because esterification by a lipase is the reverse reaction of hydrolysis, the reaction proceeds effectively with a small amount of water (17). In the reaction system of this study, the lipase dissolved in water exists as a reverse micelle in the substrate mixture of lauryl alcohol and tuna–FFA. Therefore, a small amount of water reduces the contact of the enzyme with the substrates and results in low reactivity (Fig. 1). Because immobilized enzyme is useful for enhancing contact of the enzyme with substrates in the reaction mixture without water, we carried out the selective esterification of tuna–FFA in the reaction mixture without water by using lipase immobilized on Celite 545 or a ceramic support. However, the immobilized enzyme was aggregated by the water produced during the reaction and did not disperse well in the reaction mixture. Therefore, the reaction did not proceed effectively (data not shown). A reaction system from which the water can be removed is being developed for enhancing the efficiency of the selective esterification of tuna-FFA.

Generation of lauryl DHA. Several *Pseudomonas* lipases transesterified fatty acids in triglycerides with DHA (18,19), and *Geotrichum candidum* lipase also catalyzed the transesterification of fatty acids in partial glycerides with DHA (9). It is an interesting problem whether the generation of lauryl DHA during selective esterification of tuna–FFA can be attributed to the esterification of DHA with lauryl alcohol or to the transesterification between DHA and the other fatty acids of lauryl esters. If lauryl DHA was generated by transesterification, the DHA ester would appear after accumulation of lauryl esters of the other fatty acids. However, the DHA ester was detected from the beginning of the reaction (Fig. 3), just like the other fatty acids: palmitic acid, oleic acid, and EPA (calculated from the data of Fig. 3). In addition, when oleyl oleate and DHA were made to react by the lipase, DHA was not detected at all in the ester fraction after the reaction (data not shown). From these results, we conclude that the DHA ester was generated by esterification but not by transesterification.

Fatty acid specificity. The fatty acid specificities of lipase on hydrolysis, esterification, and transesterification were

slightly different: The ester of DHA in triglyceride was lightly hydrolyzed (7), but DHA in glyceride was scarcely transesterified with caprylic acid (20). In this study, DHA was esterified somewhat with lauryl alcohol as substrate, and it was esterified like the other fatty acids with glycerol, methanol, and ethanol as substrates (Table 2). This result showed that the apparent fatty acid specificity on esterification was markedly influenced by the alcohol used as substrate. This difference of the fatty acid specificity may be attributed to the properties of the products; lauryl esters, glycerides, methyl esters, and ethyl esters. Lauryl esters are not substrates of lipase, but the other esters are good ones. Therefore, when glycerol, methanol, and ethanol were used as substrates, the apparent fatty acid specificity might be lost because simultaneous transesterification occurs with esterification.

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