Synthesis of Docosahexaenoic Acid-Rich Triglyceride with Immobilized *Chromobacterium viscosum* Lipase

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Docosahexaenoic acid (DHA) in the free fatty acid (FFA) derived from enzymically hydrolyzed tuna oil was concentrated by partial titration and precipitation of other FFA as sodium salts with acetone. A triglyceride containing up to 46.2% DHA was synthesized from the DHA-rich glyceride mixture and FFA by use of an immobilized *Chromobacterium viscosum* lipase.

KEY WORDS: ***Candida cylindracea* lipase, *Chromobacterium viscosum* lipase, docosahexaenoic acid (DHA), hydrolysis, immobilized lipase, interesterification, synthesis, tuna oil.**

Since the epidemiological report from Bang *et al.* (1), numerous studies have appeared on the effects of n-3 polyunsaturated fatty acids (PUFA) on human health (2). The composition of lipids consumed is thought to be related to cardiovascular disorders and other diseases. Too much n-6 PUFA, with a shortage of n-3 PUFA can cause problems (3). Docosahexaenoic acid (DHA) is present in great quantities in the phospholipids of the retina (4) and the brain (5). Thus, DHA is presumed to play an important role in the central nervous system.

Since Lawson and Hughes (6) reported that DHA ethyl ester was not as easily incorporated as DHA in triglycerides (TG) into plasma TGs, many researchers have tried to find ways to synthesize DHA-enriched TG. Osada *et al.* (7), Haraldsson *et al.* (8) and Toyoshima *et al.* (9) obtained DHA-enriched TG by interesterification with either *Chromobacterium viscosum* lipase or Lipozyme. Osada *et al.* (10) suggested that, in the case of interesterification of fish oil containing multiple fatty acid species, PUFAs containing many double bonds compete with monoenoic and saturated fatty acids. The result of this competitive reaction is monoenoic and saturated fatty acid incorporation into TG in preference to PUFA. Toyoshima *et al.* (9) reported that a free fatty acid (FFA) fraction containing 70% DHA was more effective than FFA containing 40% DHA for increasing the DHA content in TG by interesterification. Osada *et al.* (7) used pure DHA (99%)-FFA as the substrate for interesterification of fish oil, and obtained DHA-rich TG (about 50%).

These reports indicate two important factors in the incorporation of DHA into TG to obtain DHA-rich TG: (i) the original TG has to be of high DHA content and (ii) the DHA content of FFA used for interesterification has to be high.

The expense of commercial-grade DHA (90–99%) has stimulated the search for an economical means to achieve a product of high purity (9).

Recently, we (11) reported that after 70% hydrolysis of tuna oil with *Candida cylindracea* lipase, followed by FFA removal, the DHA contents in the resulting TG and diglyceride (DG) were 52.4 and 55.3%, respectively (this mixture of TG and DG was referred to as the "glyceride mixture").

Synthesis of DHA-rich TG was carried out with DHA-rich DG in the glyceride mixture as substrates. Conditions are described here for synthesis of DHA-rich TG by interesterifications and esterifications catalyzed by immobilized *C. viscosum* lipase.

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MATERIALS AND METHODS

Lipases and oil. Candida cylindracea lipase and *C. viscosum* lipase were gifts from Meito Sangyo Co., Ltd. (Nagoya, Japan) and Asahi Chemical Industry Co., Ltd. (Tokyo, Japan), respectively. Their hydrolytic activities were measured according to the Japanese industrial standard (JIS) method (12). The specific activities were 92.0 and 12.5 u/mg, respectively. Oleic acid (OA; 18:1 n-9, 99.9% pure), linoleic acid (LA; 18:2 n-6, 99.3% pure), linolenic acid (LN; 18:3 n-3 90.2% pure) and tuna oil were from NOF Corporation (Tokyo, Japan). DHA (92.3% pure) was purchased from Nippon Chemical Feed Co., Ltd. (Hakodate, Japan).

Immobilized lipases (IML). Chromobacterium viscosum lipase was immobilized onto Celite 535 (Nakarai Tesque, Inc. Kyoto, Japan): 1,000 u (hydrolytic activity) of lipase was dissolved in 10 mL distilled water. Celite 535 was added to the lipase solution, and the mixture was stirred with a magnetic stirrer. The mixture was then dried *in vacuo* at 60°C for 24 h. Measurement of activities was performed according to the following method: 500 mg IML was added to the mixture of 2.2 g dioleyl glycerol (OA-DG), 2.0 g OA and molecular sieves. After being stirred at 50°C for 2 h, the mixture was titrated with 0.1N KOH ethanolic solution. A lipase synthetic unit (u) is defined as the amount of enzyme that consumes 1 μmole FFA/min. The synthetic activity of the IML was 0.3 u/mg.

Analysis. Glycerides [TG, DG and monoglyceride (MG)] and FFA were methylated with boron trifluoride methanol reagent, before analysis of the fatty acid components. A Hewlett-Packard (Palo Alto, CA) 5890 gas chromatograph, equipped with a flame-ionization detector and capillary column (DB-WAX, 30 m × 0.2 mm i.d.; J&W Scientific, Folsom, CA), was used. The column temperature was raised from 150 to 210°C at 5°C/min. The injector and detector temperatures were both 250°C. Fatty acid methyl esters were identified by comparisons of their retention times with those of authentic standards.

The hydrolysis mixture was analyzed by means of thin-layer chromatography (TLC) on a silica gel plate (5721; Merck, Darmstadt, Germany) with $CHCl_3$/acetone (96:4, vol/vol) developing solvent. Relative amounts of TG, DG, MG and FFA were determined by means of a dual-wavelength chromato-scanner (CS-930; Shimadzu, Kyoto, Japan). Water content was measured with a Karl Fischer moisture meter (CA-05; Mitsubishi Chemical Ind. Ltd., Tokyo, Japan).

Hydrolysis of tuna oil. Fifty grams each of tuna oil and distilled water, containing 10,000 u of *C. cylindracea* lipase, were mixed and stirred at 500 rpm and 37°C. The reaction was stopped at about 70% hydrolysis.

Synthesis of TG with lipase solution. Glyceride mixture (10 g) and 2,000 u of *C. viscosum* lipase solution, dissolved in minute amounts of water, were mixed and stirred at 50°C and 500 rpm. The water content was measured by Karl Fischer titration.

Synthesis of TG with IML. Glyceride mixture (10 g), 2,000 u of IML and 40 wt% of molecular sieves were mixed and stirred at 50°C and 500 rpm.

In some experiments, an OA/DHA mixture was added. The ratio of OA to DHA in the mixture varied between 0:100 and 100:0. The ratio of FFA substrate to DG in the glyceride mixture before synthesis was 3.3:1 (mol/mol).

Interesterification. Trioleyl glycerol (OA-TG) and FFA (LA, LN or DHA) were mixed (1:3, mol/mol) with 200 u IML per 1 g of FFA and 40 wt% molecular sieves 3A 1/16 (Wako Pure Chemical Ind. Ltd., Osaka, Japan). The mixture was stirred at 50°C and 500 rpm for 24 h. Tridocosahexaenoyl glycerol (DHA-TG) and FFA (OA, LA or LN) were reacted under similar conditions.

Removal of FFA in acetone. Acetone (500 mL) was added to 50 mL of the tuna oil hydrolysis mixture. Aqueous 0.5N NaOH was then added, and the resulting mixture was stored at 4°C for 30 min. The white precipitate produced was removed by filtration and washed with cold acetone several times before drying. The substrates were separated by TLC and then extracted from silica gel with diethyl ether. The precipitate was neutralized with 1N HCl, and FFA were extracted with hexane. Methyl esters were then prepared for gas chromatographic analysis.

Synthesis of DHA-rich TG. The products treated by the above method (10 g), 2,000 u of IML and 40 wt% of molecular sieves were mixed and stirred at 50°C and 500 rpm.

RESULTS AND DISCUSSION

Dependence of water content and FFA amount. A reduced water content in the reaction system proved to be an important factor for glyceride synthesis. More than 0.1% water content in the reaction system resulted in hydrolysis of TG and DG in the glyceride mixture and decreasing TG content (Table 1). It is necessary to reduce the water content to as low as possible for conversion of DG into TG. However, enzymes need some water to promote their activity. In this work, synthesis with IML was performed to react with a minute amount of water. This reaction left the TG content almost the same as that of the original glyceride mixture (this is shown in the case of FFA/DG = 0, in Table 1). Therefore, FFA was added to produce a larger proportion of TG. The amount of synthesized TG content depended on the amount of added FFA (Table 1). Kurashige (13) reported that in the case of the conversion of DG to TG with equimolecular concentrations of FFA, the equilibrium moisture was around 150 ppm. During the synthesis of TG, the amount of FFA incorporated into that fraction depended inversely on the water content in the reaction system. So, it was necessary to keep the moisture content below 300 ppm to obtain 90-95% TG.

Interesterification. It is presumed that during synthesis of TG from DG and an excess molecular amount of FFA, interesterification between TG and FFA occurred simultaneously. Previously, we reported (14) the synthesis of OA-TG from OA-DG and OA with IML to be more easily achieved than the synthesis of DHA-TG from didocosahexaenoylglycerol (DHA-DG) and DHA. Osada and Hatano (15) also reported the interesterification activity of *C. viscosum* lipase for palmitic acid as higher than that for DHA. Similarly, we now find that the interesterification ratio between DHA-TG and OA was higher than that between OA-TG and DHA (Fig. 1). The authors presume that (i) saturated and monoenoic acids are incorporated into the TG in preference to DHA or (ii) that DHA in the TG tends to take the place of the saturated and monoenoic acids in the FFA.

TABLE 1

Synthesis with *Chromobacterium viscosum* Lipase (soluble and immobilized) of Triglycerides from the Glyceride Mixture and Free Fatty Acid (FFA)-Derived from Lipase-Treated Tuna Oil[a]

		TG content (%)	
	Lipase form	Before	After
Water content			
<0.1%			71.6
0.25	Solution	70.2	65.2
0.5			56.3
Ratio of FFA/DG (mol/mol)			
1.96			59.6
3.73	Solution	70.2	65.4
4.66		76.1	
0			73.6
3.73	Immobilized	73.4	91.2
9.32			95.3

[a]TG, triglyceride; DG, digylceride.

The glyceride mixture, derived from hydrolysis of tuna oil with *C. cylindracea* lipase, was reacted with excess FFA, which contained the 13.7% DHA derived from hydrolysis of tuna oil (DG/FFA = 1:15, mol/mol). The DHA content in the reacted mixture of TG and DG decreased with increasing TG content (Fig. 2). Assuming that DHA does not react with DG at all in this case, the decreased amount of DHA should have been about 10%. The DHA content actually varied from 55 to 32%. This result indicates that DHA in TG was released into the FFA fraction, and saturated and monoenoic acids were incorporated into TG in place of DHA.

To simplify the reaction system, a glyceride mixture (DHA content 47.8%) was reacted with an OA/DHA mixture. The DHA contents of synthesized TG varied from 59.3 to 22.1% (Fig. 3). Assuming that OA and DHA react

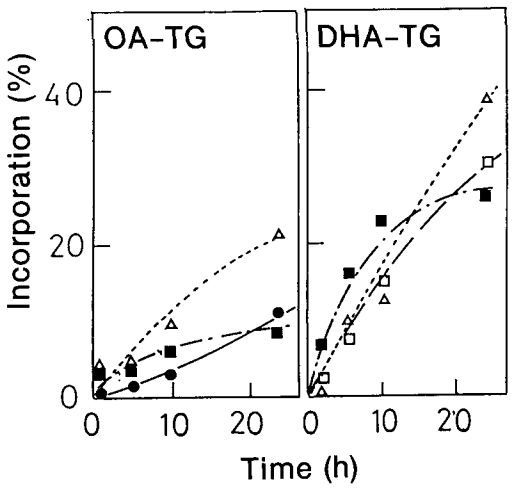

FIG. 1. Degree of incorporation of each free fatty acid (FFA) into trioleylglycerol and tridocosahexaenoylglycerol with immobilized *Chromobacterium viscosum* lipase. The ratio of FFA/triglyceride (TG) was 3:1 (mol/mol). The water content was less than 300 ppm. Docosahexaenoic acid (DHA); □, oleic acid (OA); ■, linoleic acid; and △, linolenic acid.

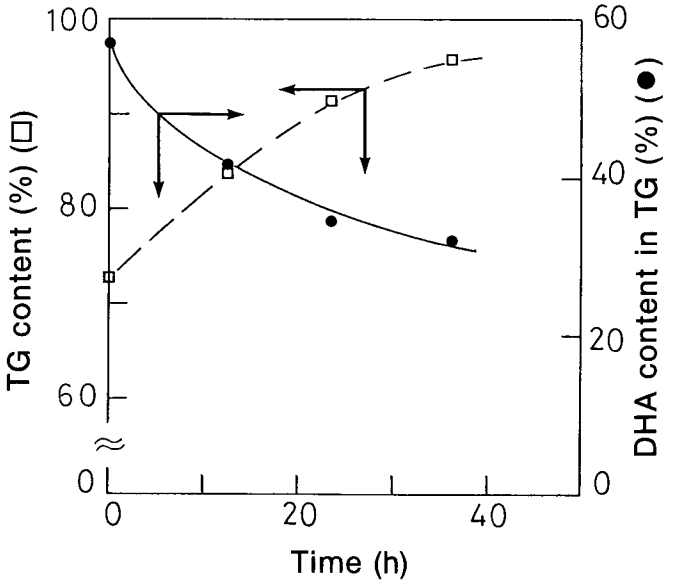

FIG. 2. The time course of the immobilized lipase-catalyzed synthetic reaction with the glyceride mixture and excess free fatty acid. The docosahexaenoic acid (DHA) content (●) in the glyceride mixture decreased with an increase of the triglyceride (TG) content (□) in it.

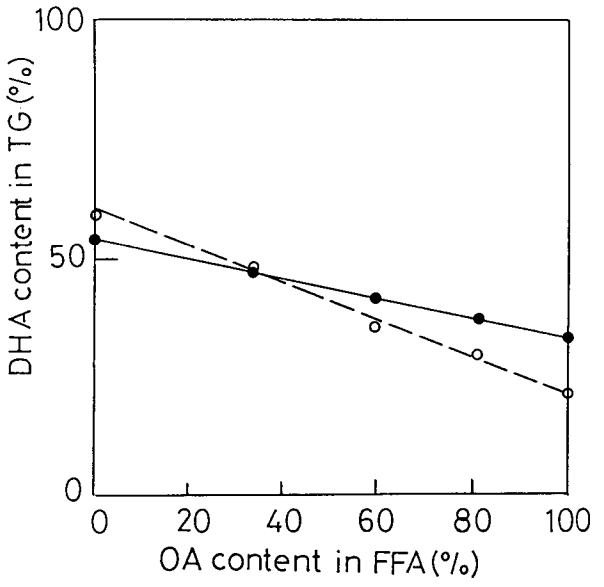

FIG. 3. Relationship between the oleic acid (OA) content in the additional free fatty acid (FFA) fraction and the docosahexaenoic acid (DHA) content in the synthesized triglyceride (TG). The ratio of the additional FFA to the glyceride mixture (mol/mol) was 3.3:1. ○ Indicates DHA and OA calculated or reacting with diglycerides in the same fashion; ● indicates actual result.

in the same fashion and that interesterification does not occur, the DHA content should vary from 53 to 43% (Fig. 3). The authors believe that the OA reacted with DG in preference to DHA, and that excess OA was incorporated into TG by interesterification with DHA in TG.

With 95% pure DHA as the FFA substrate, other fatty acids (e.g., myristic, palmitic and oleic acids) were observed in the FFA fraction, and DHA was incorporated into TG in their place. This result agrees with the report by Osada *et al.* (8). Figure 3 indicates that, to keep the DHA content of synthesized TG the same as that of the original glyceride mixture, it is necessary to have more than 65% DHA in the FFA fraction. Although pure DHA is effective for increasing the DHA content in synthesized TG, this approach is not econonically feasible because of the large amounts of DHA required.

Enhancement of DHA in FFA fraction. FFA derived from hydrolysis of tuna oil with *C. cylindracea* lipase was chosen as an inexpensive source of DHA. To enhance the DHA level in the FFA, the partially hydrolyzed mixture of glycerides and FFA was dissolved in acetone and titrated with NaOH solution. The molar amount of NaOH added was less than the amount of FFA in the hydrolysis mixture. A white precipitate of fatty acid sodium salts formed and was removed by filtration. The components of the untitrated FFA were analyzed after removal of the solvent. The ratio of the amount of untitrated FFA/total fatty acid × 100 is referred to as the fatty acid residual rate (FRR). The DHA content increased with an increase in FRR (Fig. 4).

Synthesis of DHA-rich TG. Four samples with FRR values of (i) 0%, (ii) 5.6%, (iii) 15.1% and (iv) 28.6% FFA/DG (mol/mol) were reacted to convert DG to TG. The respective molar ratios FFA/DG in these samples were 0, 1.39, 3.73 and 7.09. With samples (iii) and (iv), more than 90% of DG was converted to TG, but with samples (i) and (ii), no conversion was observed at all. The fatty acid components of FFA before reaction and of the synthesized TG are shown in Tables 2 and 3. The results in Figure 2 indicate that it is necessary to have >65% DHA content in FFA to keep the DHA content in the synthesized TG

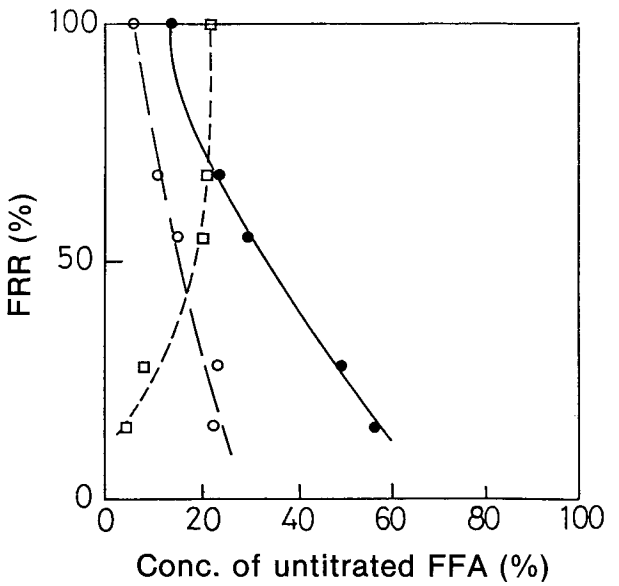

FIG. 4. Relationship between three fatty acid concentrations in the untitrated fatty acid fraction and the ratio of the untitrated fatty acid amount to the total fatty acid amount (FRR). FFA, free fatty acids; ●, docosahexaenoic acid; ○, eicosapentaenoic acid; and □, oleic acid.

TABLE 2

Major Fatty Acid in Untitrated Free Fatty Acids (FFA) Remaining with the Glyceride Mixture

	14:0	16:0	18:0	18:1	EPA	DHA
Original FFA[a]	4.8	25.9	6.2	21.0	6.0	13.7
FFA (iii)	0.6	1.0	0.1	3.2	22.9	57.0
FFA (iv)	0.6	2.6	0.1	4.9	24.0	50.1

[a]Original FFA was derived from tuna oil hydrolysis with *Candida cylindracea* lipase. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

TABLE 3

Major Fatty Acid Components of Docosahexaenoic Acid-Rich Oils Synthesized from the Glyceride Mixture and Untitrated FFA with Immobilized *Chromobacterium viscosum* Lipase[a]

	14:0	16:0	18:0	18:1	EPA	DHA
Tuna oil	4.8	21.6	4.8	14.1	5.6	25.1
M.G.T.[b]	1.7	10.4	3.2	9.5	4.1	53.1
TG (iii)	2.8	12.6	3.3	12.2	4.2	46.2
TG (iv)	3.1	11.8	2.7	14.9	4.3	39.2

[a]Abbreviations as in Table 2.
[b]M.G.T. was the glyceride mixture derived from tuna oil by hydrolysis with *C. viscosum* lipase. Triglycerides (TG) (iii) and (iv) were synthesized from the glyceride mixture and untitrated FFA (iii) and (iv), respectively, of Table 2.

the same as that of the original glyceride mixture. Because the DHA contents of samples (iii) and (iv) were below 65%, the DHA contents of the synthesized TGs became less than the original. These results correspond with those shown in Figure 2. It would be economically feasible to add only a small amount of pure DHA to reaction system (iii) to increase the DHA content in the FFA component to more than 65%.

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