Lipase-Catalyzed Enrichment of Long-Chain Polyunsaturated Fatty Acids

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ABSTRACT: Lipase hydrolysis was evaluated as a means of selectively enriching long-chain ω3 fatty acids in fish oil. Several lipases were screened for their ability to enrich total ω-3 acids or selectively enrich either docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA). The effect of enzyme concentration, degree of hydrolysis, and fatty acid composition of the feed oil was studied. Because the materials that were enriched in long-chain ω3 acids were either partial glycerides or free fatty acids, enzymatic reesterification of these materials to triglycerides by lipase catalysis was also investigated. Hydrolysis of fish oil by either *Candida rugosa* or *Geotrichum candidum* lipases resulted in an increase in the content of total ω3 acids from about 30% in the feed oil to 45% in the partial glycerides. The lipase from *C. rugosa* was effective in selectively enriching either DHA or EPA, resulting in a change of either the DHA/EPA ratio or the EPA/DHA ratio from approximately 1:1 to 5:1. Nonselective reesterification of free fatty acids or partial glycerides that contained ω3 fatty acids could be achieved at high efficiency (approximately 95% triglycerides in the product) by using immobilized *Rhizomucor miehei* lipase with continuous removal of water.

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KEY WORDS: *Candida rugosa*, DHA, enrichment, EPA, esterification, ω3, fish oil, lipase, fatty acids, *Rhizomucor miehei*.

Polyunsaturated fatty acids are essential components in human nutrition. Recently, considerable attention has been given to the biological function of individual polyunsaturated fatty acids, such as the ω3 and ω6 families. It is now recognized that the ω3 and ω6 fatty acids have distinct and sometimes opposing roles in human metabolism (1,2). Because ω-3 and ω6 fatty acids are not interconverted in humans, they must both be ingested in the diet. Nutritionists believe that the typical diet of many developed countries contains adequate quantities of the ω6 family, but a deficiency of the ω3 fatty acids. This has led to recommendations from expert committees that the intake of ω3 fatty acids should be increased (3,4), particularly the long-chain fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acids (EPA). Both DHA and EPA are immediate precursors of biologically active molecules such as prostaglandins and thromboxanes, which participate incontrolling a wide range of biological functions. Although DHA and EPA can be synthesized in the body by elongation and desaturation of α-linolenic acid, ingestion of the preformed molecules usually is more effective, especially for the very young or the elderly (5).

Some of the benefits of increasing dietary EPA and DHA include reduction of blood pressure, plasma triglycerides, increased blood coagulation time (6), and control of an overactive immune function, resulting in alleviation of autoimmune diseases, such as arthritis and some types of dermatitis (7). DHA is an important structural component in the membranes of the brain and eye, and of particular importance in the development of infants (8).

Dietary supplementation of long-chain polyunsaturated ω-3 fatty acids is hampered because these acids are present in low concentrations in the commonly available edible oils.

Most fish oils contain only moderate levels of DHA and EPA, and microbial oils that contain high levels of either EPA or DHA can be produced by fermentation, but at a relatively high cost. The main objective of this work was to evaluate selective lipase hydrolysis of commodity fish oils as a means to produce low-cost ω3 fatty acid concentrates. In addition, the feasibility of producing these concentrates in triglyceride (TG) form by lipase-catalysed esterification of the hydrolysis products was investigated.

MATERIALS AND METHODS

Lipases were obtained from the following companies: Meito Sangyo (Tokyo, Japan) (*Candida rugosa*); Amano Pharmaceutical (Nagoya, Japan) (*Geotrichum candidum, Rhizopus niveus*); Novo Nordisk (Bagsvaard, Denmark) (immobilized *Rhizomucor miehei, Humicola lanuginosa*); and Toyo Jozo (Shizuoka, Japan) (*Chromobacterium viscosum*). Chilean 1 and Chilean 2 fish oils were provided by Unilever Research (Sharnbrook, England). Cod liver oil and refined fish oil were purchased in a local foodstore. Enriched fish oil free fatty acid (FFA) (EPA-Chol 750) was purchased from EPA Limited (Mulgrave, Nova Scotia, Canada).

Lipase hydrolysis was carried out in a jacketed glass vessel equipped with an overhead stirrer. The vessel was blanketed with nitrogen and kept sealed throughout the reaction.

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Typically, the lipase powder was dissolved in 100 mL of 0.1 M buffer, pH 7, and added to 100 g of fish oil which contained 200 ppm of added tocopherols. The reaction was initiated by vigourous stirring, and the reaction temperature was maintained at 25°C. The course of the reaction was followed by withdrawing samples at appropriate intervals and measuring the FFA content in the oil phase by titration against 0.1 M NaOH. The fatty acid composition in the FFA, TG, diglyceride (DG), and monoglyceride (MG) fractions was determined as follows: FFA, TG, DG, and MG were separated by thin-layer chromatography on Silica gel G plates obtained from Analtech Inc. (Newark, DE). One drop of oil was dissolved in 0.3 mL developing solvent and then applied in a band on a 20 mm × 20 mm × 0.5 mm plate. Plates were developed in 60:40:1 of diethylether/hexane/formic acid, dried, and sprayed with 1% 2,7-dichlorofluorescein in methanol. The bands were visualized under an ultraviolet lamp (254 nm).

The fatty acid composition in each fraction was determined by first scraping the bands from the plates and converting the glycerides and FFA to methyl esters without further extraction. Methylating reagent for FFA was HCl/MeOH (5:95), heated at 70°C for 15 min. The glycerides were converted with 0.5% NaOMe/MeOH at 60°C for 10 min. The methyl esters were extracted by the addition of 2 mL hexane and 2 mL deionized water. Methyl esters were separated by gas chromatography on a 30 m × 0.53 mm column of bonded DB wax (J&W Scientific, Folsom, CA), 1.0 μm film thickness with on-column injection.

The MG and DG content in the samples was determined by separation on silica-phase high-performance liquid chromatography with a light-scattering detector. Quantitation was achieved by calibration by using an internal standard (12-OH octadecanol) with known weights of mono- and diolein.

Esterification reactions were carried out in flat-bottomed vials with magnetic stirring in a hot block. The water generated during the reaction was continuously removed by blowing dry nitrogen over the surface of the reaction mixture. Typically, 5 g of FFA or glyceride mixture was used. For FFA, the stoichiometric amount of glycerol was added, which would result in 100% TG. Enough FFA was added to partial glyceride mixtures to convert the glycerides completely to TG. The reaction was initiated by adding immobilized lipase (*R. miehei* on duolite ion exchange resin, 100,000 LU/g), 1–5% based on the weight of oil. The temperature was kept at 55°C during the reaction. Analysis for FFA, partial glycerides, and fatty acid compositions was identical to that described previously.

RESULTS

Lipase concentration. The effect of lipase concentration on the hydrolysis of Chilean 1 fish oil is shown in Figure 1. At the lowest enzyme concentration evaluated (0.08% based on oil), approximately 50% hydrolysis of the oil could be achieved in 24 h. However, no further increase in FFA content could be achieved at longer reaction times. To increase the degree of hydrolysis, it was necessary to add successively higher amounts of lipase and run the reaction for relatively long periods. A maximum of approximately 90% hydrolysis could be reached in 60 h with 0.875% lipase.

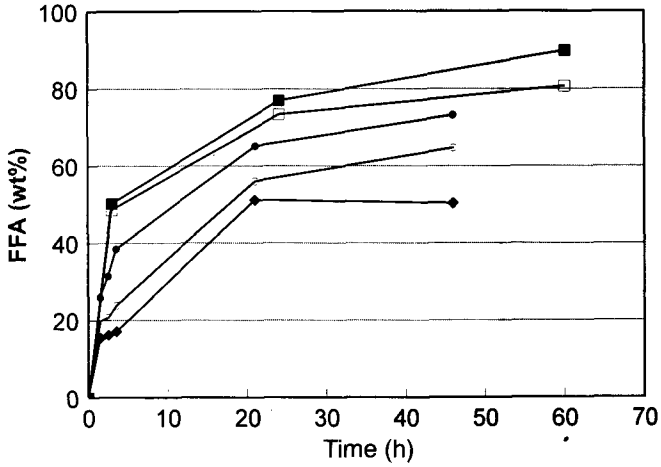

FIG. 1. Effect of *Candida rugosa* lipase concentration on hydrolysis of fish oil. Wt% of lipase based on oil: 0.88% (■), 0.63% (□), 0.5% (●), 0.25% (○), 0.08% (◆). FFA, free fatty acids.

Degree of conversion. The compositions of the major fatty acids in the glyceride and FFA fractions after 60 or 80% hydrolysis of Chilean 1 fish oil with *C. rugosa* lipase are shown in Table 1. The content of MG in the reaction mixture was too low to allow determination of the fatty acid composition in this fraction.

After 60% hydrolysis, the total ω3 fatty acid content of the TG and DG fractions had increased by approximately 1.5-fold, compared to the original fish oil, giving a final concentration of approximately 45%. Most of the enrichment was due to DHA, which increased twofold, whereas the EPA content remained essentially unchanged, compared to the feed oil. This difference in selectivity is reflected in the increased DHA/EPA ratio. The enrichment of ω3 fatty acids was compensated by a reduction in the content of C_{14}–C_{18} saturated and monounsaturated fatty acids, exemplified in Table 1 by a decrease of approximately 1.5-fold in the content of oleic and palmitic acids. The FFA fraction was correspondingly increased in the C_{14}–C_{18} saturated and monounsaturated fatty acids, with a fivefold depletion in DHA compared to the feed oil. This results in a greatly reduced ratio of DHA to EPA.

When the reaction reached 80% hydrolysis, the partial glycerides were further enriched in DHA, but the EPA content was reduced. The overall ω3 content had increased slightly, but the DHA/EPA ratio had now reached approximately four. The composition of most fatty acids in the FFA fraction was similar to that found in the original fish oil, with the exception of DHA, which was reduced by approximately twofold.

Figure 2 shows the change in the DHA and EPA contents of the FFA fraction and the combined glyceride fractions during the course of hydrolysis of Chilean 1 fish oil by *C. rugosa*

TABLE 1
Composition of the Major Fatty Acids in the Glyceride and Free Fatty Acid Fraction After 60 or 80% Hydrolysis of Fish Oil (Chilean 1) by *Candida rugosa* Lipase[a]

		$C_{16:0}$ (wt%)	$C_{18:1}$ (wt%)	$C_{20:5}$ (wt%)	$C_{22:6}$ (wt%)	Total ω3	DHA/EPA ratio[a]
Chilean 1		18.3	13.6	15.9	12.3	30.6	0.8
60% Hydrolysis	TG	11.2	7.4	17.4	22.0	43.2	1.3
	DG	10.0	6.7	15.3	27.3	46.8	1.8
	FFA	24.0	17.1	13.2	2.3	16.4	0.2
80% Hydrolysis	TG	6.6	8.6	10.6	35.1	48.9	3.3
	DG	5.8	7.2	8.6	41.2	43.0	4.8
	FFA	21.7	13.9	16.8	4.9	23.6	0.3

[a]DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; TG, triglyceride; DG, diglyceride; FFA, free fatty acid.

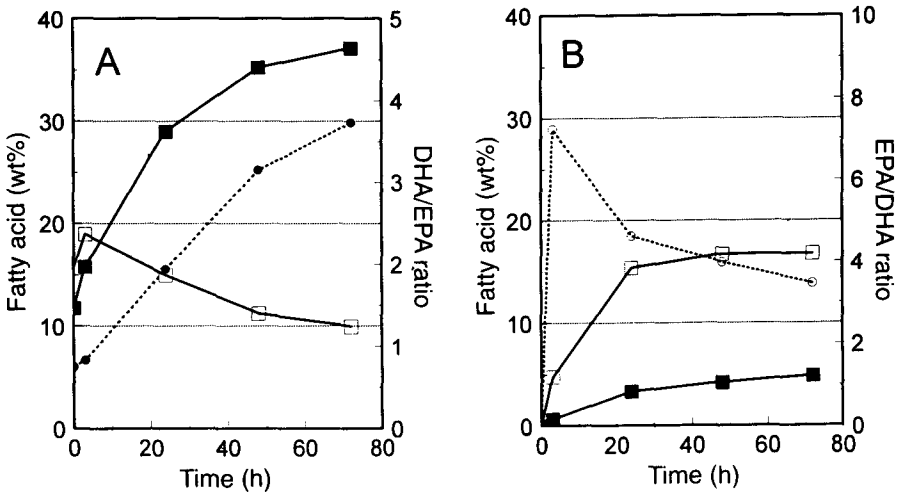

FIG. 2. Change in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in the partial glyceride and free fatty acid (FFA) fractions during the course of hydrolysis of fish oil by *Candida rugosa* lipase. A = partial glyceride fraction, B = FFA fraction. DHA (■), EPA (□), ratio of DHA/EPA (●) and ratio of EPA/DHA (●).

lipase. DHA was consistently enriched in the glycerides throughout the reaction, but EPA was only enriched in the first six hours, followed by a steady decline throughout the rest of the reaction. This indicates strong discrimination by the lipase against DHA, but only moderate discrimination against EPA. The FFA fraction shows a slow increase in the DHA content, but the increase in EPA content was rapid and complete within 20 h.

Effect of varied fish oil fatty acid composition. In an attempt to influence the content of ω3 fatty acids and the ratio of DHA/EPA in the concentrates, fish oils with differing fatty acid compositions were hydrolyzed with lipase from *C. rugosa*. The compositions of the major fatty acids in the TG fraction after 80% hydrolysis are shown in Table 2. In general, the total content of ω3 fatty acids was increased by approximately 1.8-fold, regardless of the composition in the original fish oil. Similarly, the DHA content was increased by approximately threefold in all samples, with a small decrease in the EPA content. The fish oil with the highest initial DHA content resulted in a concentrate with both the highest total ω3 content and the highest DHA/EPA ratio.

Comparison of lipases. The ability of several other lipases to enrich EPA and DHA in the glyceride fraction during hydrolysis of fish oils was evaluated. Table 3 shows that *H. lanuginosa* and *C. viscosum* lipases both enriched DHA in the TG fraction and that *C. viscosum* also enriched EPA. However, these lipases are not as effective as *C. rugosa*. The lipase from *G. candidum* resulted in partial glycerides that were enriched by about 1.5-fold in total ω3 fatty acids, similar to the enrichment obtained with *C. rugosa*. A high DHA/EPA ratio could not be obtained with *G. candidum* lipase.

Reesterification. Esterification of enriched fish oil FFA (EPA-Chol 750, see Materials and Methods section) with glycerol was carried out with either 2 or 5% of immobilized *R. miehei* lipase, based on the weight of oil. The enriched FFA contained 33% EPA and 22% DHA. The disappearance of FFA and appearance of TG during the course of the reaction are shown in Figure 3. Only the highest level of lipase (5% on weight of oil) was effective in TG synthesis. After 150-h reaction, the level of TG in the glyceride fraction was greater than 90%. The content of DHA in the TG fraction at the early stages of the reaction was lower than that in the feed FFA

TABLE 2
Effect of Fish Oil Composition on DHA and EPA Enrichment in TG After 80% Hydrolysis by *Candida rugosa* Lipase[a]

	$C_{16:0}$ (wt%)	$C_{18:1}$ (wt%)	$C_{20:5}$ (wt%)	$C_{22:6}$ (wt%)	Total ω3	DHA/EPA ratio
CLO	10.8	20.6	9.2	10.4	20.9	1.1
Enriched CLO TG	7.3	12.1	7.1	27.4	36.5	3.9
RFO	15.4	13.5	19.3	9.7	31.5	0.5
Enriched RFO TG	7.6	8.5	16.1	27.8	48.9	1.7
Chilean 1	18.3	13.6	15.9	12.3	30.6	0.8
Enriched Chilean 1 TG	6.6	8.6	10.6	35.1	48.9	3.3
Chilean 2	16.8	19.2	10.1	14.2	27.3	1.9
Enriched Chilean 2 TG	7.4	13.6	7.3	41.1	51.6	5.6

[a]See Table 1 for abbreviations. CLO, Cod liver oil, RFO, refined fish oil.

TABLE 3
Comparison of Lipases for Enrichment of DHA and EPA in TG During Hydrolysis (750 units/g oil)[a]

	FFA (wt%)	$C_{16:0}$ (wt%)	$C_{18:1}$ (wt%)	$C_{20:5}$ (wt%)	$C_{22:6}$ (wt%)	Total ω3	DHA/EPA ratio[a]
Chilean 2 oil		16.8	19.2	10.1	14.2	26	1.4
Rhizomucor miehei (TG fraction)	14	16.0	18.8	10.7	15.2	28	1.4
Humicola lanuginosa (TG fraction)	51	11.2	20.2	10.2	18.7	32	1.8
Rhizopus niveus (TG fraction)	52	16.0	18.8	9.4	15.6	28	1.8
Chromobacterium viscosum (TG fraction)	74	9.4	18.5	14.1	17.0	35	1.2
Chilean 1 oil		18.3	13.6	15.9	12.3	31	0.8
Geotrichum candidum (TG fraction)	34	9.8	7.9	23.3	18.4	46	0.8

[a]See Table 1 for abbreviations.

(data not shown). However, as the TG content approached 90%, the DHA content in the TG increased to the level in the feed FFA.

Esterification of partial glycerides obtained from the hydrolysis of Chilean 1 fish oil by *C. rugosa* lipase with FFA from either fish oil or sunflower oil was carried out with 5% immobilized *R. miehei* lipase. As shown in Figure 4, the rate of incorporation of FFA was similar for both sunflower and fish oil FFA. The content of TG in the glyceride fraction reached approximately 95% within 100 h for both.

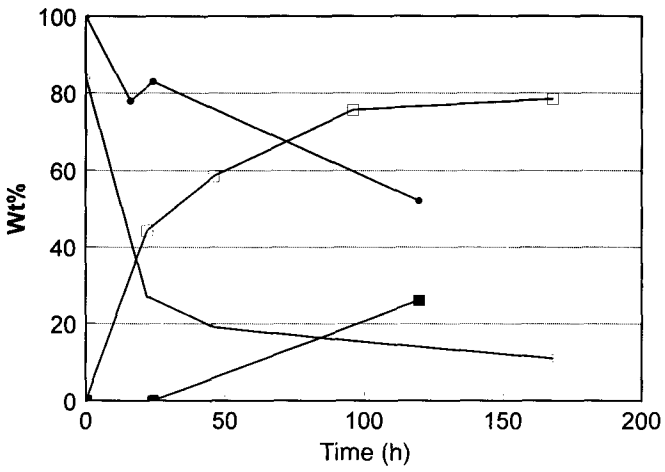

FIG. 3. Esterification of FFA, enriched in DHA and EPA, with glycerol by using immobilized *Rhizomucor miehei* lipase. Effect of lipase concentration on time course. Lipase concentration = 2% (on weight of oil): triglyceride (TG) (■) and FFA (●). Lipase concentration = 5% (on weight of oil): TG (□) and FFA (○). See Figure 2 for abbreviations.

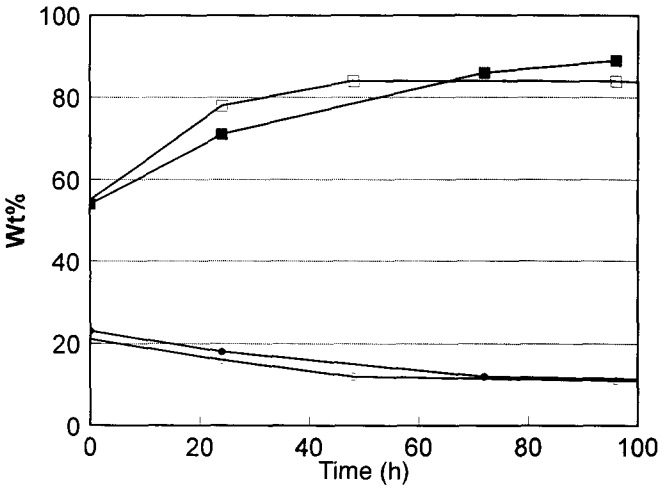

FIG. 4. Esterification of enriched fish oil partial glycerides (60% hydrolysis of Chilean 1 fish oil with *Candida rugosa* lipase) with FFA by using 5% immobilized *Rhizumucor miehei* lipase. Effect of FFA composition on time course. Sunflower FFA: TG (■) and FFA (●). Fish oil FFA: TG (□) and FFA (○). See Figures 2 and 3 for abbreviations.

DISCUSSION

In recent years, a number of workers have demonstrated that lipases can be used to enrich the long-chain ω3 fatty acids in fish oil, either by selective hydrolysis of the TG (9–11), selective esterification of fish oil FFA (12), or selective transesterification with low-molecular weight alcohols (13). Lipase from *C. rugosa* has been shown to be particularly effective (9,10). This could be partly due to its recently discovered fatty acid chainlength selectivity, showing higher activity with C_{18} or shorter fatty acids than with C_{20} or C_{22} acids (14,15). In the present work, optimization of both enrichment of total long-chain ω3 fatty acids and the alteration of the DHA/EPA ratio have been investigated. For enrichment of total long-chain ω3 fatty acids, approximately 50% hydrolysis with a moderate level of lipase is adequate over a 24-h reaction time. About 80% hydrolysis is required to obtain a high DHA/EPA ratio. This necessitates the use of relatively high levels of lipase in combination with long reaction times. Lower yields are obtained as the DHA is concentrated in the unhydrolyzed fraction. Comparing fish oils of differing compositions, the degree of enrichment under the same reaction conditions is essentially the same for all oils examined. However, the actual ω3 content and DHA/EPA ratio depend strongly on the fish oil composition. Lipase from *G. candidum* behaved similarly to *C. rugosa* lipase for enrichment of DHA and EPA. This is not surprising, considering the recent finding that these lipases share a high degree of homology (16).

Esterification and interesterification of pure EPA and DHA (17,18) and of enriched fish oil fatty acids (19,20) using lipases has been attempted previously. In this publication, it was shown that more than 95% TG can be synthesized from either long-chain ω3 FFA or long-chain ω3 partial glycerides by using immobilized *R. miehei* lipase. In the reaction system described here, relatively long reaction times are required (approximately 100 h) with a minimum of 5% immobilized lipase. Continuous removal of water is also essential, as noted by previous investigators (19,20). In contrast to the results of Lie and Molin (19), TG could be synthesized by *R. miehei* lipase that contained the same level of DHA as the feed acid.

The data shown here have been used as the basis for pilot-scale production of various fish oil TG concentrates, and the data will be reported in a subsequent article.

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