Enzymatic Modification of Triacylglycerols of High Eicosapentaenoic and Docosahexaenoic Acids Content to Produce Structured Lipids

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ABSTRACT: Immobilized lipase, IM60, from *Rhizomucor miehei* was used as a biocatalyst for the incorporation of capric acid (C10:0) into fish oil originally containing 40.9 mol% eicosapentaenoic (20:5n-3) and 33.0 mol% docosahexaenoic (22:6n-3) acid. Acidolysis was performed with and without organic solvent. Pancreatic lipase-catalyzed *sn*-2 positional analysis was performed after enzymatic modification. Tocopherol analysis was performed before and after enzymatic modification. Products were analyzed by gas–liquid chromatography. After a 24-h incubation in hexane, there was an average of 43.0 ± 1.6 mol% incorporation of C10:0 into fish oil, while 20:5 and 22:6 decreased to 27.8 ± 2.2 and 23.5 ± 1.3 mol%, respectively. The solvent-free reaction produced an average of 31.8 ± 8.5 mol% C10:0 incorporation, while 20:5 and 22:6 decreased to 33.2 ± 3.3 and 28.3 ± 3.9 mol%, respectively. The effect of incubation time, substrate molar ratio, enzyme load, and added water were also studied. In general, as the enzyme load, molar ratio, and incubation time increased, mol% C10:0 incorporation also increased. The optimal mol% C10:0 incorporation was 41.2% at 48 h for the reaction in hexane and 46.4% at 72 h for the solvent-free reaction. The highest C10:0 incorporation (65.4 mol%) occurred at a molar ratio of 1:8 (fish oil triacylglycerols/capric acid) in hexane. For the solvent-free reaction, the optimal mol% C10:0 incorporation (56.1 mol%) occurred at a molar ratio of 1:6. An enzyme load of 10% gave the highest mol% C10:0 incorporation (41.4 mol%) in hexane; the highest incorporation (38.3 mol%) for the solvent-free reaction occurred at 15% enzyme load. Mol% incorporation of C10:0 declined with increasing amounts of added water. The optimal mol% C10:0 incorporation occurred at 1% added water (47.9 mol%) for the reaction in hexane, and at zero added water for the solvent-free reaction (21.8 mol%). Fish oil containing capric acid was successfully produced and may be nutritionally more beneficial than unmodified oil.

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KEY WORDS: Acidolysis, capric acid, fish oil, lipase, n-3 polyunsaturated fatty acids, structured lipids, tocopherol content, transesterification.

The n-3 polyunsaturated fatty acids, such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, are found in fish oils and have received much attention in recent years because of the health benefits they offer. Among these health benefits are improved immune function and prevention of heart disease and certain cancers (1). Fish oil contains 14–30% EPA and DHA. Menhaden, sardine, anchovy, herring, and cod liver are the major sources for commercial production of fish oil (2). EPA is an antagonist of the arachidonic acid cascade and competes with arachidonic acid as a substrate for cyclooxygenase and lipoxygenase to produce certain eicosanoids. EPA is a precursor for series-3 prostaglandins and thromboxanes as well as series-5 leukotrienes. Arachidonic acid is a precursor of series-2 prostaglandins and thromboxanes as well as series-4 leukotrienes. Prostanoids are phospholipid-derived inflammatory mediators encompassing prostaglandins and thromboxanes. Dietary lipids play a major role in immune function and platelet aggregation (1). Eicosanoids are only one of many possible mediators through which dietary lipids can influence immune response.

Medium-chain triacylglycerols (MCT) also offer numerous health benefits and have been studied extensively for medical, nutritional, and food applications. MCT have been used to treat fat-absorption abnormalities that occur in premature infants and in patients with cystic fibrosis (3). MCT are burned quickly for energy, and generally are not deposited in adipose tissue (4) except when the medium-chain fatty acid (MCFA) is located at the *sn*-2 position of a structured lipid or when a large amount of MCT is consumed (5). A structured lipid containing both fish oil and MCFA would have the combined effect of providing high energy and other health benefits. In this study, the fatty acid composition of fish oil was modified to contain the MCFA, capric acid (C10:0), by using immobilized lipase (IM60) from *Rhizomucor miehei* as a biocatalyst. The effects of added water, enzyme load, substrate molar ratio, and reaction time were also studied. Pancreatic lipase catalyzed *sn*-2 positional analysis and vitamin E analysis were performed on the fish oils before and after modification.

MATERIALS AND METHODS

Materials. Fish oil triacylglycerol (TAG) was provided by Pronova Biocare (Sandefjord, Norway). Immobilized 1,3-specific lipase IM60 (Lipozyme IM) was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). Capric acid was

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obtained from Sigma Chemical Co. (St. Louis, MO). Organic solvents were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA).

Enzymatic modification reaction. Unless otherwise stated, 100 mg of fish oil TAG was mixed with 36.8 mg of C10:0 free fatty acid (FFA) at a molar ratio of 1:2 in 3 mL of hexane for reactions conducted in organic solvent. The same substrate ratio was used for the solvent-free reactions. Immobilized IM60 lipase (10% by weight reactants) was added to the mixture and incubated in an orbital shaking water bath at 55°C for 24 h at 200 rpm. All reactions were performed in duplicate and average values were reported.

Analysis of product. The enzyme was removed by passing the reaction mixture through an anhydrous sodium sulfate column. Fifty microliters of the reaction product was analyzed by thin-layer chromatography (TLC) on silica gel 60 plates and developed with petroleum ether/ethyl ether/acetic acid (80:20:0.5, vol/vol/vol). The bands were visualized under ultraviolet light after being sprayed with 0.2% dichlorofluorescein in methanol. The bands corresponding to TAG were scraped from the TLC plate and methylated with 3 mL 6% HCl in methanol at 70–80°C for 2 h. The fatty acid methyl esters (FAME) were extracted twice with 1 mL of hexane, dried over a sodium sulfate column, and concentrated under nitrogen. The gas chromatograph was an HP 5890 Series II (Hewlett-Packard, Avondale, PA) equipped with a DB-225 fused-silica capillary column (30 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA), a flame-ionization detector, and operated in a splitless mode. The injector and detector temperatures were 250 and 260°C, respectively. The column temperature was held at 120°C for 3 min and programmed to 215°C for 10 min at the rate of 10°C/min. The carrier gas was helium and the total gas flow rate was 23 mL/min. The relative content of FAME as mol% was calculated by computer with 17:0 as the internal standard.

Lipase catalyzed sn-*2 positional analysis*. Fifty microliters of the reaction mixture was spotted onto a silica gel 60 TLC plate and developed as described above. The band corresponding to TAG was scraped from the TLC plate, extracted twice with ethyl ether, and passed through a sodium sulfate column. The ethyl ether was then evaporated under nitrogen. One milliliter of Tris buffer (pH 7.6), 0.25 mL of bile salts (0.05%), 0.1 mL of $CaCl_2$ (2.2%), and 8.0 mg of purified pancreatic lipase were added to the reaction mixture (6). The mixture was then incubated for 3 min at 37–40°C, extracted two times with ethyl ether, evaporated under nitrogen, brought to a final volume of 200 µL, and spotted onto a TLC plate as described above. The band corresponding to *sn*-2 monoacylglycerol (2-monoolein used as a standard) was scraped after the TLC plate was developed in hexane/diethyl ether/acetic acid (50:50:1.0, vol/vol/vol). The *sn*-2 monoacylglycerol was then methylated and analyzed by gas–liquid chromatography (GLC).

Tocopherol analysis. Tocopherol analysis was performed on the fish oils before and after enzymatic modification. The unmodified oil was diluted to 0.1 g of oil to 25 mL of hexane. Enzymatically modified oil was filtered through a sodium sulfate column to remove the enzyme and diluted (1.0 mL of modified oil reaction mixture to 25 mL hexane). Tocopherol standards were obtained from Sigma. The purity and stability of standards were monitored by specific absorption coefficient ($E^{1\%}_{1cm}$) values measured with a DU-64 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). Specific $E^{1\%}_{1cm}$ and maximum wavelengths (λ_{max}) for tocopherols were 71.0 and 294, respectively, for α-tocopherol; 86.4 and 297, respectively, for β-tocopherol; 92.8 and 298, for γ-tocopherol; and 91.2 and 298 for δ-tocopherol, respectively (7). Twenty-five milligrams of each homolog was accurately weighed and dissolved in 25 mL of hexane containing butylated hydroxytoluene (BHT; 0.01%, wt/vol). Appropriate dilutions were made with the mobile phase to give a stock standard concentration of 18.54, 0.51, 20.70, and 3.30 µg/mL for α-, β-, γ-, and δ-tocopherol, respectively. For a daily working standard, 2 mL of the stock standard solution was diluted into a 50-mL volumetric flask with mobile phase. The working standard concentrations of α-, β-, γ-, and δ-tocopherol were 0.74, 0.02, 0.82, and 0.14 µg/mL, respectively. Concentrations of the tocopherol homologs were calculated from a peak area determined by a Waters 764 integrator (Millipore Corp., Cary, NC). The oil samples were then analyzed on a normal-phase high-performance liquid chromatography (HPLC) system equipped with a Shimadzu LC-6A pump and RF-10A spectrofluorometric detector (Shimadzu Corp., Kyoto, Japan), a Spectra series AS100 autosampler (Termo Separation Products Inc., San Jose, CA), and a 25 cm × 4 mm, 5-µm Lichrosorb Si 60 column (Hibar Fertigsaub RT, Darmstadt, Germany) equipped with a precolumn packed with Perisorb A 30–40 µm (Darmstadt, Germany). The isocratic mobile phase contained 0.6% isopropanol in *n*-hexane (J.T. Baker Chemical Co., Phillipsburg, NJ), and the flow rate was 1.0 mL/min. The mobile phase was filtered with a 0.22-µm nylon filter (MSI Inc., Westboro, MA) and degassed by stirring under vacuum. The wavelengths were set at 290 nm for excitation and 330 nm for emission for the determination of tocopherol homologs (8).

RESULTS AND DISCUSSION

The fish oil used for this study contained 14 major fatty acids, with 20:5 and 22:6 being the predominant ones. After modification, 10:0, 20:5, and 22:6 were the major fatty acids present, as were small amounts of 18:1 and 18:3 (Table 1). IM60, from *R. miehei* was used as a biocatalyst for the incorporation of C10:0 into fish oil because its 1,3-positional specificity would place C10:0 at these positions on the TAG molecule for maximal metabolic benefit. EPA and DHA remained as 2-MAG during digestion and absorption while C10:0 was hydrolyzed and absorbed as FFA to provide quick energy. The effects of incubation time, enzyme load, molar ratio, and added water were studied with and without hexane (Figs. 1–4). Generally, as enzyme load, substrate molar ratio, and incubation time increased, mol% incorporation of C10:0 also increased (Fig. 1–3). This is consistent with other studies involving the enzy-

TABLE 1
Fatty Acids (mol%) in Pronova Fish Oil Before and After Modification With and Without Hexane

Fatty acid	Before modification	After modification	
		Hexane	No solvent
10:0	0.0 ± 0.0	43.0 ± 1.7	31.8 ± 8.5
14:0	0.8 ± 0.4	NDa	ND
16:0	1.6 ± 0.2	ND	ND
16:1n-7	3.8 ± 0.2	ND	ND
18:0	5.8 ± 0.1	ND	ND
18:1n-9	1.7 ± 0.0	5.5 ± 1.6	6.9 ± 1.3
18:2n-6	1.9 ± 0.4	ND	ND
18:3n-3	1.2 ± 0.1	0.1 ± 0.0	ND
20:1n-9	1.9 ± 0.5	ND	ND
20:3n-6	0.8 ± 0.4	ND	ND
20:4n-6	2.3 ± 0.0	ND	ND
20:5n-3	40.9 ± 1.0	27.8 ± 2.2	33.2 ± 3.3
22:0	2.0 ± 0.1	ND	ND
22:6n-3	33.0 ± 0.3	23.5 ± 1.3	28.3 ± 3.9

[a]ND, not detected. Pronova fish oil was supplied by Pronova Biocare (Sandefjord, Norway).

matic modification of TAG performed in our laboratory (9,10). After 24 h of incubation in hexane, there was an average of 43.0 ± 1.7 mol% incorporation of C10:0 into fish oil, and 20:5 and 22:6 declined to 27.8 ± 2.2 and 23.5 ± 1.3 mol%, respectively. The solvent-free reaction gave an average of 31.8 ± 8.5 mol% C10:0 incorporation, while 20:5 and 22:6 decreased to 33.2 ± 3.3 and 28.3 ± 3.9 mol%, respectively. We were able to incorporate capric acid into fish oil as well as retain a reasonable amount of 20:5n-3 and 22:6n-3 in the fish oil.

Time course. The highest mol% C10:0 incorporation (41.2 mol%) occurred at 48 h in hexane and increased up to 46.4 mol% at 72 h for the solvent-free reaction. Previous studies have reported that longer reaction times result in increased acyl migration in a laboratory-scale continuous reactor when MCFA were incorporated into TAG (11).

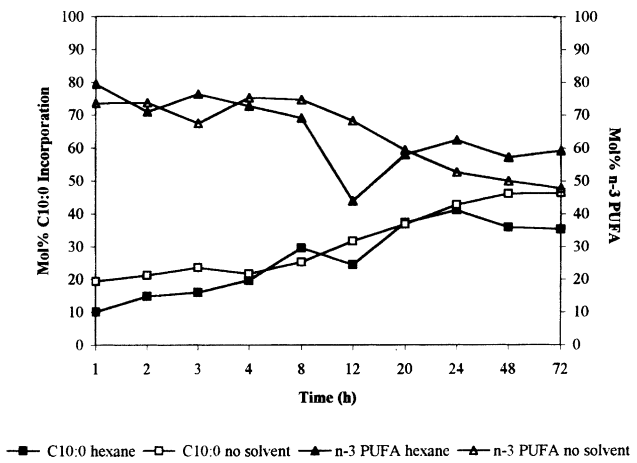

FIG. 1. Time course of IM60 lipase-catalyzed modification of Pronova Biocare (Sandefjord, Norway) fish oil with and without hexane to incorporate capric acid (C10:0) as determined by gas–liquid chromatography. Samples were analyzed at 1, 2, 3, 4, 8, 12, 20, 24, 48, and 72 h (x-axis). All reactions were in duplicate. Enzyme amount was 10% by weight reactants. Incubation was at 55°C and 200 rpm with and without hexane. PUFA, polyunsaturated fatty acid.

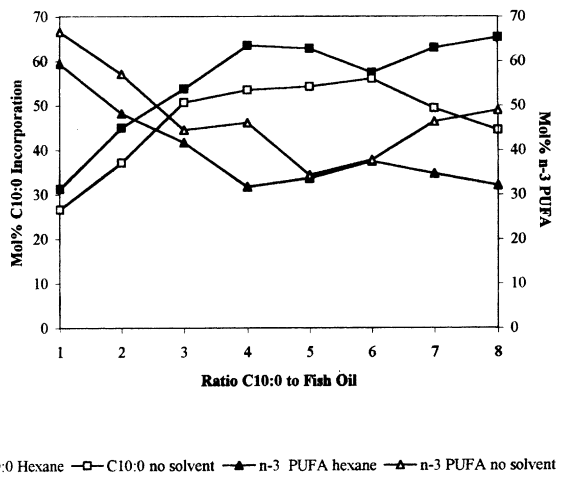

FIG. 2. Effect of molar ratio of substrate (fish oil/C10:0) on C10:0 incorporation with IM60 lipase as a biocatalyst. The numbers 1–8 on the x-axis correspond to molar ratios of 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, and 1:8, respectively. All reactions were in duplicate. Enzyme amount was 10% by weight reactants. Incubation was at 55°C and 200 rpm with and without hexane. See Figure 1 for abbreviations.

Molar ratio. The highest overall mol% incorporation of C10:0 (65.4%), occurred at a 1:8 molar ratio (fish oil to C10:0) in hexane (Fig. 2). For the solvent-free reaction, the optimal mol% C10:0 incorporation (56.1 mol%) occurred at a molar ratio of 1:6. However, it seemed that the reaction reached equilibrium at 1:4 with hexane, and 1:3 without hexane. There is no economic advantage in using high-substrate molar ratios. Others have shown that a high molar ratio required a shorter reaction time, improved reaction rate, and resulted in less acyl migration in a laboratory-scale continuous reactor when MCFA were incorporated into TAG (11). Depending on the level of C10:0 incorporation desired in the final product, the substrate molar ratio can be manipulated to achieve it. High

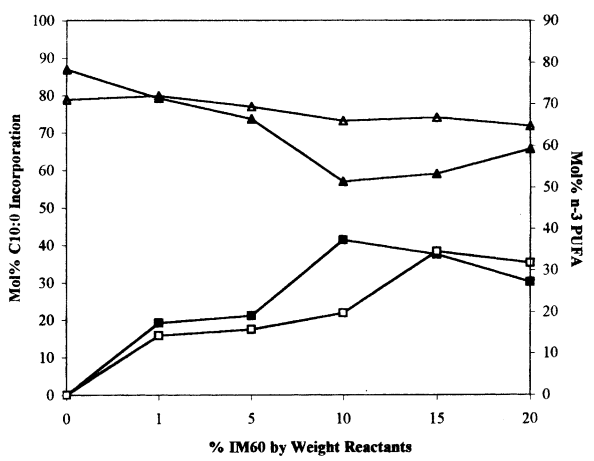

FIG. 3. Effect of enzyme load on C10:0 incorporation into Pronova fish oil with IM60 as a biocatalyst. Amount of enzyme was based on weight of reactants (0, 5, 10, 15, and 20%, respectively). All reactions were in duplicate. Incubation was at 55°C and 200 rpm with and without hexane. See Figure 1 for abbreviations.

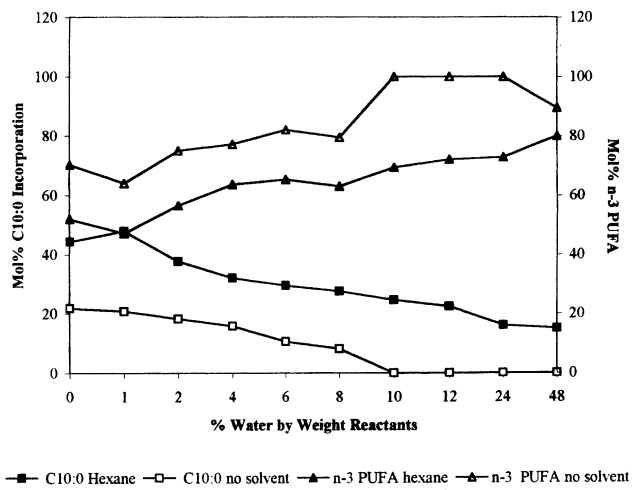

FIG. 4. Effect of added water on C10:0 incorporation into Pronova fish oil with IM60 lipase as a biocatalyst. Amount of water added was based on the weight of the reactants (0, 1, 2, 4, 6, 8, 10, 12, 24, and 48%, respectively). All reactions were in duplicate. Incubation was at 55°C and 200 rpm with and without hexane. See Figure 1 for abbreviations.

C10:0 concentration in the reaction may indeed lead to substrate inhibition.

Enzyme load. Mol% incorporation of C10:0 reached its maximal level (41.4) with hexane at an enzyme load of 10%. The highest mol% incorporation (38.3) occurred at an enzyme load of 15% for the solvent-free reaction (Fig. 3). Other researchers have reported increased incorporation of C10:0 into rapeseed oil with increasing enzyme load, although it is sometimes followed by increased acyl migration (12,13). Increased reaction time can also lead to acyl migration.

Effect of added water. The optimal mol% C10:0 incorporation (47.9) occurred at 1% added water for the reaction in hexane and at 0% added water reaching 21.8 mol% for the solvent-free reaction (Fig. 4). Too much added water shifts the reaction toward hydrolysis rather than synthesis. The n-3 PUFA contents increased beyond 8% added water, which indicated hydrolysis of other fatty acids and concentration of n-3 PUFA. For most reactions, a water content of less than 1% is required, but the optimum water content varies between 0.04 and 11% wt/vol for different lipases (14,15).

Solvent vs. solvent-free reactions. Organic solvents such as hexane have several functions, including increasing the solubility of nonpolar substrates and shifting the reaction toward synthesis rather than hydrolysis (16). Concerns for hexane toxicity, flammability, cost, and the additional time associated with the purification process have led to lipase-catalyzed modification of TAG being performed without organic solvent. Mol% incorporation of C10:0 was comparable for time-course and enzyme-load experiments. Most of the mol% incorporation values were also close for the molar ratio experiment. For food applications, the solvent-free reaction should be the method of choice. Mol% incorporation was lower for the effect of the water experiment for the solvent-free reaction than for the hexane reaction.

Pancreatic lipase-catalyzed sn-*2 positional analysis*. Pancreatic lipase-catalyzed *sn*-2 positional analysis of the fish oil after modification in hexane for 24 h showed the following fatty acid composition at the *sn*-2-position of the structured lipid: C10:0, 11.5 mol%; 18:1n-9, 8.1 mol%; 18:2n-6, 3.4 mol%; 20:5n-3, 24.4 mol%; and 22:6n-3, 52.6 mol%. The molar ratio of fish oil to C10:0 was 1:2. The presence of C10:0 at the 2-position, even though a 1,3 specific lipase was used, demonstrates some acyl migration, possibly occurring during pancreatic lipase hydrolysis or during lipase-catalyzed acidolysis. Others have reported that in addition to reaction time, water content, lipase load, temperature, acyl donor type, and lipase type also influence acyl migration (13). The predominant fatty acids at the *sn*-2-position were EPA and DHA. It should be noted that a high amount of DHA (52.6 mol%) remained at the *sn*-2 position where it is expected to be conserved during digestion. DHA is important for brain development and retinal function. Fatty acids esterified at the 2-position are easily absorbed, regardless of the type of fatty acid esterified at that position (17,18).

Tocopherol analysis. Tocopherols function as antioxidants and are found naturally in oils. Table 2 shows the effect of enzymatic modification on tocopherol content of fish oil. Unmodified fish oil contains predominantly less stable α-tocopherol with total tocopherols of 2.8 mg/g. Menhaden fish oil contains approximately 0.066 mg/g tocopherol (2). Pronova Biocare indicated that tocopherol was added as an antioxidant. This addition would explain elevated levels of tocopherols of the oil tested in this study (Table 2). The tocopherol content of modified fish oil remained close to values obtained for unmodified fish oil for δ-tocopherol, but values declined substantially for the α-, β-, and γ-tocopherols. The α-, β-, and γ-tocopherols are the most biologically active and least stable tocopherols, and were possibly lost during the acidolysis reaction and processing. Therefore, tocopherols and other antioxidants must be added back to the enzymatically modified oils if considerable loss occurs, to protect the highly unsaturated n-3 and n-6 PUFA.

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TABLE 2
Tocopherol Content of Pronova Fish Oil Before and After Enzymatic Modification[a]

	Tocopherol content (mg/g)				
Fish oil	α	β	γ	δ	Total
Before modification	1.70	0.017	0.754	0.308	2.80
After modification	0.021	0.006	0.216	0.273	0.516

[a]Molar ratio of fish oil/C10:0 was 1:2; enzymatic modification was performed in hexane for 24 h with lipase at 10% by weight reactants and no added water; product contained 44.2% capric acid and 48.9% n-3 polyunsaturated fatty acids.

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