Immobilized Lipase-Catalyzed Production of Structured Lipids with Eicosapentaenoic Acid at Specific Positions

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ABSTRACT: Structured lipids (SL) were synthesized by the interesterification reaction between medium-chain triacylglycerols and eicosapentaenoic acid (EPA) ethyl ester. The products were partially purified, and the fatty acid at the *sn*-2 position was determined after pancreatic lipase-catalyzed hydrolysis. The effect of additives (water and glycerol) on the rate of reaction was also investigated. Mol% EPA incorporated into the triacylglycerols was increased by adding water when trilaurin and tricaprylin were the substrates and IM 60 was the biocatalyst. With SP 435, EPA incorporation was always less with additional water than without water. The addition of glycerol (0.005 g or 0.01 g) improved interesterification catalyzed by IM 60 to some degree, but an excess amount (0.02 g) inhibited the reaction. The reaction with glycerol showed no significant difference with SP 435. After scale-up and fractionation by column chromatography, we could recover approximately 0.3–0.4 g of product/g of reaction products. After hydrolysis by pancreatic lipase, we can conclude that IM 60 has a high specificity for *sn*-1,3 positions. With SP 435 lipase, 34.8–39.3 mol% of EPA was found at the *sn*-2 position of the recovered SL.
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KEY WORDS: Additives, column chromatography, eicosapentaenoic acid, hydrolysis, interesterification, medium-chain triacylglycerols, pancreatic lipase.

The ω-3 fatty acids, such as EPA (5,8,11,14,17-eicosapentaenoic acid), DHA (7,10,13,16,19-docosahexaenoic acid), and α-linolenic acid (9,10,15-octadecatrienoic acid), have several health benefits on cardiovascular disease, immune disorders and inflammation, renal disorders, allergies (1,2), diabetes (3), and cancer (4). These fatty acids also may be essential for brain and retina development in humans (5). Metabolically, EPA is an antagonist of the arachidonic acid cascade and competes with arachidonic acid as substrate for cyclooxygenase and lipoxygenase to produce eicosanoids. As a result, EPA can be used for the synthesis of eicosanoids, such as series-3 prostaglandins, which ameliorate immunodisfunction. On the other hand, arachidonic acid may form the series-2 prostaglandins, which may impair the immune function (6). Medium-chain triacylglycerols (MCT) are composed of saturated 8- to 12-carbon fatty acids. MCT are not formed into chylomicrons for transport and are not deposited in the adipose tissue. Rather, unlike long-chain fatty acids, they are metabolized in the liver for quick energy. MCT provide 8.3 cal/g and could lower serum cholesterol. They are nontumor promoting and antitumor fats with promise for several applications and clinical uses (7,8).

Lipases are a group of enzymes that preferentially catalyze the hydrolysis and synthesis of triacylglycerols. These enzymes are active at the oil–water interface (9). Modification with lipase provides a useful way to improve the properties of triacylglycerols. Through enzymatic interesterification, it is possible to incorporate a desired acyl group onto a specific position of the triacylglycerol, whereas chemical interesterification does not possess this regiospecificity due to the random nature of the reaction. In addition, enzymatic reactions occur at mild conditions.

Immobilization of lipase provides improved stability and makes enzyme recovery and reuse for industrial application possible. In this work, two immobilized lipases were used. IM 60 (from *Mucor miehei*, *sn*-1,3 specific; Novo Nordisk Bioindustrial, Danbury, CT) was preimmobilized on a macroporous anion exchange resin, and SP 435 (from *Candida antarctica*, nonspecific; Novo Nordisk) on a macroporous acrylic resin.

Water may have a dual role on the activity of lipases in organic solvent (9). It serves as an essential for reaction by maintaining layers around the molecule. Above the critical amount of water, it promotes hydrolysis, which is the reverse reaction of interesterification. Some scientists have suggested that additives, such as glycerol and other polyols, could increase the rate of interesterification (10,11). We report the synthesis of structured lipids (SL) by the interesterification between MCT and EPA ethyl ester (EPA-EE). The products were partially purified, and the fatty acid at the *sn*-2 position was determined after pancreatic lipase-catalyzed hydrolysis. The effects of additives (water and glycerol) on EPA incorporation were also investigated.

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MATERIALS AND METHODS

Materials. Tricaprylin (1,2,3-trioctanoylglycerol; T8), tricaprin (1,2,3-tridecanoylglycerol; T10), trilaurin (1,2,3-tridodecanoylglycerol; T12), and porcine pancreatic lipase (Type II, crude) were purchased from Sigma Chemical Co. (St. Louis, MO). EPA-EE (97% purity) was provided by the United States Department of Commerce, National Fisheries Service (Charleston, SC). Silica gel (28–200 mesh) and silicic acid (100 mesh) for column chromatography were purchased from Aldrich Chemical Company (Milwaukee, WI). Organic solvents were obtained from Fisher Scientific (Norcross, GA).

Interesterification. One hundred mg each of T8, T10, and T12 was mixed with EPA-EE at 1:2 molar ratio in 3 mL hexane in a screw-cap tube. Immobilized enzymes, SP 435 and IM 60 (10% of combined weight of substrates), were added. The incubations were carried out in an orbital-shaking water bath at 200 revolutions/min for 24 h at 55°C. For studies on the effect of water and glycerol on the rate of interesterification, 2 μL water or 5, 10, and 20 mg glycerol was added to the reaction mixture. Scale-up (10 times, 2.1–2.7 g total weight of reactants) was conducted with MCT and EPA-EE (1:2 molar ratio) in a 125-mL culture media flask under the same incubation conditions.

Analytical procedures. The products of the interesterification reaction were passed through an anhydrous sodium sulfate column to remove enzymes. Solvent was evaporated under nitrogen, and 50 μL of the reactant was analyzed by thin-layer chromatography (TLC) on a silica gel G plate (Fisher Scientific), developed with petroleum ether/ethyl ether/acetic acid (90:10:1, vol/vol/vol). The bands were visualized under ultraviolet radiation after spraying with 0.2% 2,7-dichlorofluorescein in methanol. Bands corresponding to triacylglycerols were scraped, methylated with 3 mL of 6% HCl in methanol at 75°C for 2 h, extracted with hexane (2 mL) and 0.1 M KCl solution (1 mL), centrifuged (1000 rpm, 3 min), and concentrated under nitrogen.

Fatty acid compositional analysis. A Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA), equipped with a flame-ionization detector and a fused-silica capillary column (DB-225, 30 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA) was used. The column 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 injector and detector temperatures were 250 and 260°C, respectively. The fatty acid methyl esters were identified by comparing retention times with standards and quantitated with heptadecanoic acid as an internal standard.

Column chromatography. After the scale-up reaction with MCT and EPA-EE, the reactants were poured into a round-bottom flask. Solvents were evaporated with a Büchi rotary evaporator (Postfach, Switzerland). Silicic acid and silica gel were dried for 4 h at 110°C. After cooling in a desiccator, 12 and 13 g of silica gel were mixed in 80 mL hexane to make a slurry, which was poured into the column (300 mm × 10.5 mm i.d.), which was equipped with an internal reservoir (250 mL) and a Teflon stopcock. An additional 50 mL hexane was poured, and the flow rate (3 mL/min) was adjusted. The reaction mixture (1 g), containing unreacted substrates (MCT and EPA-EE) and products (SL), was applied to the column. The column was eluted with solvent A (95:5, hexane/diethyl ether, 150 mL) followed by solvent B (90:10, hexane/diethyl ether, 200 mL). The first eluant (fraction I) with solvent A and second eluant (fraction II) with solvent B were collected, evaporated, and stored at –4°C until use. These products were analyzed by TLC as described previously.

Hydrolysis by pancreatic lipase. Determination of the positional distribution of fatty acids in triacylglycerols from column chromatography was conducted by the method of Luddy *et al.* (12): 1 mg of triacylglycerol was mixed with 1 mL of 1 M Tris–HCl buffer (pH 7.6), 0.25 mL of 0.05% bile salts, 0.1 mL of 2.2% $CaCl_2$, and 1 mg pancreatic lipase. The mixture was incubated in a water bath at 37°C for 3 min, vortexed vigorously (1 min), centrifuged (1900 rpm, 3 min), extracted with 3 mL diethyl ether (two times), and eluted in a sodium sulfate column. TLC analysis was on silica gel G, and the developing solvent system was hexane/diethyl ether/acetic acid (50:50:1, vol/vol/vol). The band corresponding to 2-monoacylglycerol was scraped, methylated, and analyzed by gas chromatography.

Statistics. The Statistical Analysis System (1986; SAS, Cary, NC) was used to perform statistical computations. Stepwise regression and Duncan's multiple range test were performed on means of mol% incorporated EPA. The tested significance level was 5%.

RESULTS AND DISCUSSION

The effect of added water on interesterification between MCT and EPA-EE is depicted in Figure 1. Interesterification was affected by the addition of water. With IM 60 (Fig. 1A), mol% of incorporated EPA into the triacylglycerols was increased by adding water to T12 and T8. Without water addition, the highest overall incorporation was found in T10, even though it was not different from that found with water addition. However, in the presence of additional water, T12 shows the highest incorporation of EPA. With SP 435, the result was somewhat different. EPA incorporation was always less with additional water than without water (Fig. 1B). The highest EPA incorporation was found in T8, followed by T10 and T12, in both the presence and the absence of water. It is safe to assume that addition of water to the reaction catalyzed by SP 435 exceeded the critical amount of water, and thus led to hydrolysis, which is the reverse reaction. But with IM 60, some additional water was required for catalysis (forward reaction).

The best independent variable in each reaction was determined by the stepwise regression method. In the reaction with SP 435, the best independent variable that affected the reaction most was water (×1). With IM 60, that variable was glycerol (×2). After verifying these variables, we studied the kind of relationship (positive or negative) that water or glycerol

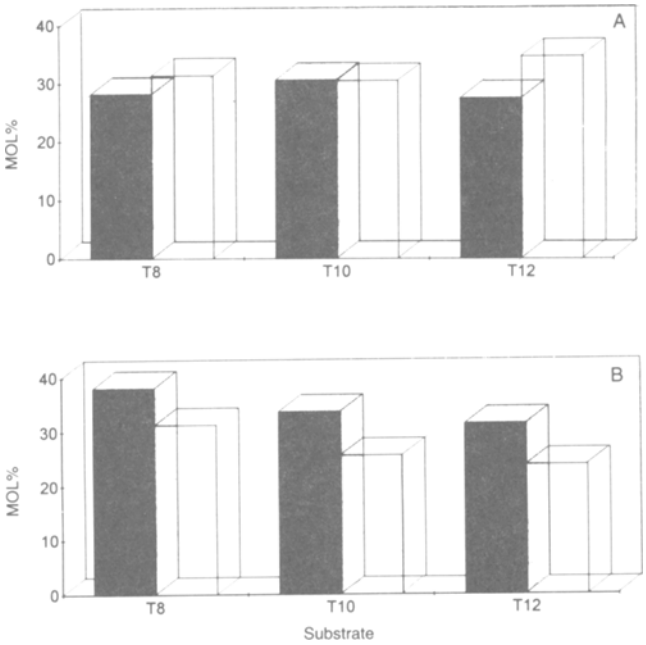

FIG. 1. Mol% of eicosapentaenoic acid incorporated into medium-chain triacylglycerol without glycerol addition. A: IM 60; B: SP 435 (Novo Nordisk, Danbury, CT) ■, no water addition; □, 2 μL water addition. T8, tricaprylin; T10, tricaprin; and T12, trilaurin.

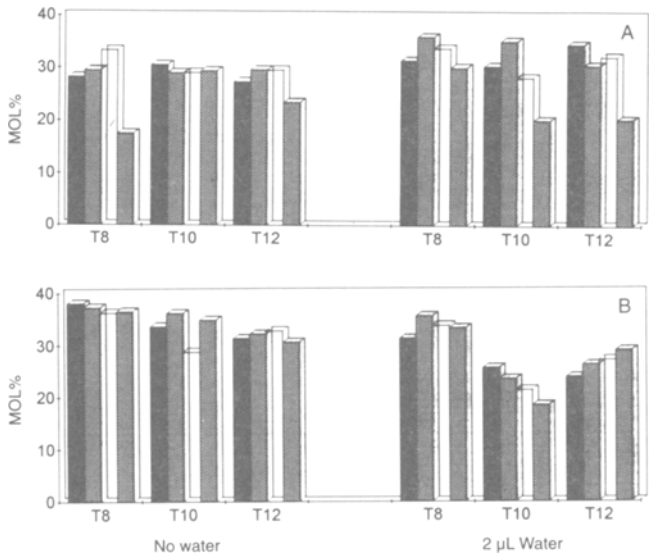

FIG. 2. Mol% of eicosapentaenoic acid incorporated into medium-chain triacylglycerol. Duncan grouping: A: IM 60; no water, T8: A, A, A, B., T10: A, A, A, A, T12: A (B), A, A, B. 2 μL water, T8: A, A, A, A., T10: A, A, A, B., T12: A, A, A, B. B: SP 435; no water, T8: A, A, A, A., T10: A, A, (B), B, T12: A, A, A, A. 2 μL water, T8: A, A, A, A., T10: A, A, A, A., T12: A, A, A, A. Order of letters is from 0 g glycerol (left) to 0.02 g glycerol (right) addition in each reaction. The same letters are not significantly different. (Black bar, 0 g of glycerol addition; right-hatched bar, 0.005 g of glycerol addition; white bar, 0.01 g of glycerol addition; left-hatched bar, 0.02 g of glycerol addition). Abbreviations and company source as in Figure 1.

has on the interesterification reaction. Correlations between water or glycerol and mol% of incorporated EPA (y) were obtained (Table 1). With SP 435, ×1 showed a negative correlation to EPA incorporation in all cases (T8, T10, and T12), and the most negative correlation was found for T10 ($r = -0.856$). Two variables were taken for T10, which means that both water and glycerol addition gave a negative relation to the reaction. With IM 60, ×2 showed a negative correlation with EPA incorporation, and the most negative correlation ($r = -0.74$) was found for T12. For T8, ×1 (positive relation) and ×2 (negative relation) were taken. Thus, we can assume that additional water retarded the reaction with SP 435, and added glycerol (0.005–0.02 g) retarded the reaction with IM 60. Figure 2 shows the effect of glycerol amounts on EPA incorporation. With IM 60 (Fig 2A), the best incorporation was detected at 0.005 and 0.01 g of glycerol addition to T12, 0 g to T10, and 0.01 g to T8, in the absence of water. In the presence of additional water, the best EPA incorporation was at 0 g glycerol to T12, 0.005 g to T10 and to T8. With IM 60, the addition of 0.02 g glycerol showed the lowest EPA incorporation and the most significant difference according to the Duncan test. Therefore, we could assume that addition of a certain amount of glycerol (0.005 or 0.01 g) improved the interesterification (MCT and EPA-EE) to some degree, but an excess amount (0.02 g) inhibited the reaction. Glycerol is not very soluble in organic solvents, and we have observed aggregation of IM 60 when 0.02 g of glycerol was added. This aggregation of immobilized enzymes may be part of the reason for the decreased catalysis. With SP 435 [Fig. 2B], the best incorporation was detected at 0.01 g of glycerol addition to T12, 0.005 g to T10, and 0 g to T8 in the absence of water, and 0.02 g to T12, 0 g to T10, and 0.005 g to T8 in the presence of water.

TABLE 1
Correlation (r) Between y and ×1 or ×2[a]

	Substrate	Correlation (r)	Variables
SP	T8	−0.62	×1
435	T10	−0.856/−0.21	×1/×2
	T12	−0.754	×1
IM	T8	0.51/−0.45	×1/×2
60	T10	−0.56	×2
	T12	−0.74	×2

[a]The best independent variables were taken by the stepwise regression method. ×1: water; ×2: glycerol; and y: mol% of incorporated eicosapentaenoic acid (EPA) with each substrate [tricaprylin (T8), tricaprin (T10), and trilaurin (T12)]. Interesterification was between each medium-chain triacylglycerol and EPA-ethyl ester in the absence or presence of water with 0, 0.005, 0.01, or 0.02 g of glycerol. SP 435 and IM 60 from Novo Nordisk (Danbury, CT).

Unlike IM 60, the reaction with 0.02 g glycerol, catalyzed by SP 435, showed no significant difference. It seems that the nature of the support for each immobilized enzyme may contribute to this difference. Also, it confirms that SP 435 requires less water than IM 60 for catalysis. We have previously shown (13) that this is the situation.

The result of the scale-up process is shown in Table 2. The incorporation of EPA was always higher after scale-up than

TABLE 2
Mol% EPA Incorporated into Medium-Chain Triacylglycerols Before and After Scale-Up[a]

	Substrate	Before scale-up	After scale-up (unit: mol%)
IM 60	T8	27.1	33.7
	T10	30.5	32.2
	T12	28.2	36.2
SP 435	T8	31.3	38.2
	T10	33.6	41.6
	T12	38.0	40.5

[a]Company source and abbreviations as in Table 1.

before scale-up. One reason might be the shape of the reactor, which provided better distribution of the immobilized enzyme and more contact with substrates than a test tube reactor. After column chromatography, we collected two fractions (I and II). We could not find bands that corresponded to the triacylglycerol standard in fraction I. But after further elution, the triacylglycerol bands were detected in fraction II. Thus, fraction II contained the SL as well as unreacted MCT used as substrates. After evaporation of the solvent in fraction II, we could recover approximately 0.3–0.4 g of products from each reaction (1 g MCT and EPA-EE with SP 435 or IM 60). These products were subjected to hydrolysis by pancreatic lipase, which is specific for the hydrolysis of fatty acids at the 1- and 3-positions of triacylglycerol molecules and leaves an *sn*-2 monoacylglycerol. After pancreatic lipase-catalyzed hydrolysis, the products were applied to TLC. The R_f (×100) for monoacylglycerol after development was about 3.4. It has been reported that triacylglycerol that contains polyunsaturated fatty acid at the *sn*-2 position and medium-chain fatty acids at the *sn*-1,3 positions are easily absorbed, and that the fatty acid at the *sn*-2 position is efficiently absorbed (14,15). Thus, incorporation of a desirable fatty acid, which has nutritional and/or pharmaceutical properties, at the *sn*-2 position of modified lipids is important.

The mol% EPA at the *sn*-2 position of the structured lipids after hydrolysis is shown in Table 3. Even though the products contain SL, as well as unreacted MCT, we can conclude that these values directly represent the incorporated EPA at the *sn*-2 position of structured lipids because unreacted MCT should not contain EPA. These values show that IM 60 has a high specificity for *sn*-1,3 positions because we could not detect EPA peaks on gas chromatography after pancreatic hydrolysis. Apparently, IM 60 incorporated EPA at the *sn*-1,3 positions, leaving medium-chain fatty acids at the *sn*-2 position. If IM 60 has the ability to esterify at the *sn*-2 position under our experimental conditions, then peaks for EPA must show on gas chromatography. On the other hand, with SP 435 lipase, 34.8–39.3 mol% of EPA was found at the *sn*-2 position from the recovered triacylglycerols (SL + unreacted MCT). This means that SP 435 can incorporate EPA at the *sn*-2 position of MCT. These position-specific SL could be used for further nutritional studies.

TABLE 3
Mol% EPA Incorporated at the *sn*-2 Position of Structured Lipids After Pancreatic Lipase-Catalyzed Hydrolysis[a]

	Substrate	Mol% EPA at *sn*-2 position
IM60	T8	—
	T10	—
	T12	—
SP 435	T8	34.8
	T10	39.3
	T12	35.6

[a]Company source and abbreviations as in Table 1.

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