Lipase-Assisted Acidolysis of High-Laurate Canola Oil with Eicosapentaenoic Acid

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ABSTRACT: The production of structured lipids via acidolysis of high-laurate canola oil (Laurical 15) with EPA in hexane was carried out using lipase from *Pseudomonas sp*. The optimal reaction conditions used 4% lipase, at a mole ratio of oil to EPA of 1:3 at 45°C over 36 h. The positional distribution of FA on the glycerol backbone of unmodified oil indicated that lauric acid was mainly located at the *sn*-1,3 positions. Stereospecific analysis of the oil modified with EPA showed that lauric acid remained mostly esterified to the *sn*-1,3 positions of the TAG molecules and that EPA was also primarily in the *sn*-1,3 positions of the TAG molecules. Thus, the resultant structured lipids may have optimal value for use in applications where quick energy release and EPA supplementation are required.

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KEY WORDS: Docosahexaenoic acid, eicosapentaenoic acid, high-laurate canola oil, lipase, long-chain triacylglycerols, medium-chain triacylglycerols, positional distribution, structured lipids.

Medium-chain FA (MCFA) comprise 6- to 12-carbon saturated FA and are typically obtained by hydrolysis of tropical plant oils such as those of coconut and palm kernel (1,2). Pure medium-chain TAG (MCT) have a caloric value of 8.3 kcal/g and do not supply EFA (3,4). MCFA have many distinctive features such as high oxidative stability, low viscosity, and low m.p. (5). MCT exhibit unique structural and physiological characteristics; they are different from other fats and oils because they can be absorbed *via* the portal system. MCT do not require chylomicron formation to transfer from the blood stream to cells and have a more rapid β-oxidation to form acetyl CoA end products, which are further oxidized to yield CO_2 in the Krebs cycle (6). Absorption and metabolism of MCT are as quick as for glucose, and MCT have approximately twice the caloric value of proteins and carbohydrates and have little propensity to accumulate as body fat. The higher solubility and smaller molecular size of MCFA make their absorption, transport, and metabolism much easier than for long-chain FA. MCT are hydrolyzed more quickly and completely by pancreatic lipase than long-chain TAG (LCT).

The n-3 FA have many health benefits related to cardiovascular disease (CVD), inflammation, allergies, cancer, immune response, diabetes, hypertension, and renal disorders (7). Epidemiological studies have linked the low incidence of coronary heart disease in Greenland Eskimos with their high dietary intake of n-3 PUFA (8,9). The n-3 FA are essential for normal growth and development throughout the life cycle of humans and therefore should be included in the diet. The n-3 FA have been extensively studied for their influence on CVD. The exact mechanism by which these effects are rendered remains unknown, but research results have shown that these FA in marine oils may prevent CVD by decreasing serum TAG and acting as antiatherogenic and antithrombotic agents (10).

In many foods for medical applications, a mixture of MCT and long-chain TAG (LCT) is used to provide both rapidly and slowly metabolizing fuel as well as EFA. Any abnormality in the many enzymes or processes involved in the digestion of LCT can cause symptoms of fat malabsorption. Thus, patients with certain diseases (such as Crohn's disease, cystic fibrosis, colitis, and enteritis) have shown improvement when MCT are incorporated into their diets (11).

Structured lipids (SL) provide the most efficient means of delivering desired FA for nutritional and/or medical purposes. Strategies for lipid modification include genetic engineering of oilseed crops, production of oils containing high levels of PUFA, and lipase- or chemically-assisted interesterification reactions. Depending on the type of substrate available, chemical or enzymatic reactions can be used for the synthesis of modified lipids, including direct esterification (reaction of FA and glycerol), acidolysis (transfer of acyl group between an acid and ester), and alcoholysis (exchange of alkoxy group between an alcohol and an ester) (6). However, the common methods cited in the literature for production of SL are based on reactions between two TAG molecules (interesterification) or between a TAG and an acid (acidolysis). Chemically catalyzed interesterification, using alkali such as sodium methoxide, is cheap and easy to scale up. However, such reactions lack specificity and offer little or no control over the positional distribution of FA in the final product (6). An alternative to the chemical synthesis of SL is an enzymatic process using a variety of lipases. Lipase-assisted interesterification offers many advantages over the chemical process. It produces fats or oils with a defined structure because it incorporates a specific FA at a specific position of the glycerol moiety. It requires mild experimental conditions without potential for side reactions, with a reduction of energy consumption and with reduced heat damage to reactants, and easy purification of products (1,2).

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Another approach to the production of SL is through bioengineering. An early attempt was the production of a high-laurate canola oil containing 40% lauric acid (C12:0) (12). It could be useful to use this oil as a model oil when incorporating long-chain EFA into MCT. Recently, the incorporation of n-3 PUFA, especially EPA and DHA, into melon seed oil (13), vegetable oils (14), borage oil (15–18), evening primrose oil (18,19), and trilinolein (20) has been reported. Synthesis of SL containing MCFA in the *sn*-1,3 positions and an unsaturated LCFA in the *sn*-2 position of the glycerol backbone using 1,3-regiospecific lipases was recently reported by Soumanou *et al*. (21). The final product contained more than 94% caprylic acid in the *sn*-1 and *sn*-3 positions, whereas more than 98% of the unsaturated FA (oleic acid 20.1%, linoleic acid 78.0%) was present in the *sn*-2 position. Shimada *et al.* (22) described an effective method of producing SL that contain linoleic acid and α-linolenic acid by acidolysis of safflower and linseed oils with caprylic acid (CA), respectively, using immobilized *Rhizopus delemar* lipase. Acidolysis of safflower with CA produced only 1,3-capryloyl-2-linoleoylglycerol and 1,3-capryloyl-2-oleoylglycerol, with a ratio of 86:14 (w/w). Additionally, all TAG in linseed oil were converted to 1,3-capryloyl-2-α-linolenoylglycerol, 1,3-capryloyl-2-linoleoylglycerol, and 1,3-capryloyl-2-oleoylglycerol, at a ratio of 60:22:18 (by wt). Kosugi and Azuma (23) described a simple method to prepare and isolate high yields of pure TAG from PUFA using immobilized lipase from *Candida antarctica* and *Rhizomucor miehei*. The esterification of glycerol and FFA or ethyl esters of the FA was performed at 50–60°C over 24 h. More than 95% of PUFA was converted to TAG. Fomuso and Akoh (24) synthesized SL from high-laurate canola oil and stearic acid using lipase from *C. antarctica*. The resulting SL was then used to prepare *trans*-free margarine. Addition of canola oil to the SL improved spreadability at refrigeration temperatures and reduced the hardening effect of lauric acid in the SL.

The aim of this study was (i) to produce SL *via* acidolysis of high-laurate canola oil with a long-chain FA, EPA, (ii) determine the effect of reaction variables such as type of enzyme, enzyme concentration, incubation time, and substrate mole ratio on the extent of incorporation of EPA into high-laurate canola oil, and (iii) determine the positional distribution of FA in the modified and unmodified oils.

MATERIALS AND METHODS

Materials. Lipase preparations from *C. antarctica* (Novozyme-435) and *Rhizomucor miehei* were acquired from Novo Nordisk (Franklinton, NC). Other lipases, namely *Pseudomonas* sp. (PS-30), *Aspergillus niger* (AP-12), and *C. rugosa* (AY-30), were obtained from Amano Enzyme (Troy, VA). All lipases used in this work were obtained in the powder form. Samples of high-laurate canola oil, known as Laurical 15, were products of Calgene's Oils Division (Davis, CA). FAME (GLC-461) were purchased from Nu-Chek-Prep (Elysian, MN). Porcine pancreatic lipase (EC 3.1.1.3), sodium taurocholate, silica gel TLC plates (20 × 20 cm; 60 Å mean pore diameter, 2–25 μm mean particle size, 500 μm thickness, with dichlorofluorescein), and TBHQ were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents used in these experiments were of analytical grade and were purchased from Fisher Scientific (Nepean, Ontario, Canada). EPA samples, >99% pure, were made by Fuso Pharmaceutical Industries Ltd. (Osaka, Japan) and kindly provided by Dr. K. Miyashita.

Methods. (i) Acidolysis reaction. High-laurate canola oil (100 mg) was mixed with EPA at different mole ratios of oil to EPA ranging from 1 to 3, in screw-capped test tubes, and then hexane (3.0 mL) was added to a mixture of lipase (2–10% by weight of substrates) and water (2% by weight of substrates and enzyme). Samples were flushed with nitrogen and the containers capped and incubated for different periods (12 to 48 h) in an orbital shaker at 250 rpm at 25–55°C.

(ii) Separation of acylglycerols after acidolysis. A mixture of acetone and ethanol (20 mL; 1:1, vol/vol) was added to stop the reaction. To neutralize FFA, the reaction mixture was titrated against a 0.5 M NaOH solution (using a phenolphthalein indicator) until the color of the solution turned pink. Hexane (25 mL) was added to the mixture to extract the acylglycerols. The mixture was transferred into a separatory funnel and thoroughly mixed. The two layers (water, hexane) were allowed to separate, and the lower aqueous layer was discarded. The hexane layer was passed through a bed of anhydrous sodium sulfate to remove any residual water. The hexane was evaporated using a rotary evaporator at 45°C, and the acylglycerol fraction was recovered and a portion of it (5–10 mg) transferred to transmethylation vials.

FA composition of products. (i) Preparation of FAME. FA profiles of products were determined following their conversion to methyl esters. The transmethylation reagent (2.0 mL, freshly prepared solution of 6.0 mL of concentrated sulfuric acid made up to 100 mL with methanol and 15 mg of TBHQ as an antioxidant) was added to the sample vial. The mixture was vortexed and then incubated at 60°C overnight; it was subsequently cooled to room temperature. Distilled water (1 mL) was added to the mixture and after thorough mixing, a few crystals of TBHQ were added to each vial to prevent oxidation; lipids were extracted 3× with 1.5 mL of pesticide-grade hexane. The hexane layers were separated, combined, and transferred to a clean test tube and then washed twice with 1.5 mL of distilled water. The hexane layer (the upper layer) was separated from the aqueous layer and evaporated under a stream of nitrogen. FAME were then dissolved in 1.0 mL of carbon disulfide and used for subsequent GC analysis.

(ii) Analysis of FAME by GC. The FAME were analyzed using a Hewlett-Packard 5890 Series II gas chromatograph (Agilent, Palo Alto, CA) equipped with a SUPELCOWAX-10 column (30 m length, 0.25 mm diameter, 0.25 μm film thickness; Supelco Canada Ltd., Oakville, Ontario, Canada). The oven temperature was first set at 220°C for 10.25 min and then raised to 240°C at 30°C/min and held there for 15 min. The injector and the FID temperatures were both set at 250°C.

TABLE 1
FA Composition of Modified and Unmodified Laurical 15 Oils[a]

FA	Weight (%)	
	Unmodified	Modified
12:0	37.6 ± 1.33	9.81 ± 0.08
14:0	3.82 ± 0.07	1.16 ± 0.01
16:0	3.21 ± 0.08	1.14 ± 0.01
18:0	36.0 ± 1.04	15.2 ± 0.33
18:1n-9	14.7 ± 0.71	5.94 ± 0.03
20:5n-3	—	64.9 ± 0.26
Others	4.56	1.85

[a]Values are mean values ± SD (n = 3).

Ultra-high purity helium was used as a carrier gas at a flow rate of 15 mL/min. Data were treated using a Hewlett-Packard 3365 Series II Chem Station Software (Agilent). The FAME were identified by comparing their retention times with those of authentic standard mixtures (GLC-461; Nu-Chek-Prep) and the results were presented as weight percentage of total FAME.

(iii) Hydrolysis by pancreatic lipase. Hydrolysis of modified oil by pancreatic lipase was performed according to the method described by Christie (25) with a slight modification. Tris-HCl buffer (5.0 mL; 1.0 M, pH 8.0), 0.5 mL of calcium chloride (2.2 %, wt/vol), and 1.25 mL of sodium taurocholate (0.05) were added to 25 mg of modified oil in a glass test tube. The mixture was allowed to stand at 40°C in a water bath for 1.0 min, followed by the addition of 5.0 mg of porcine pancreatic lipase. The mixture was subsequently placed in a gyrotory water bath shaker at 250 rpm under nitrogen for 15 min at 40° C. Ethanol (5.0 mL) was added to the mixture to stop the enzymatic reaction, followed by the addition of 5.0 mL of 6.0 M HCl. The hydrolytic products were extracted three times with 25.0 mL of hexane (1:1, vol/vol), and the upper layer was removed and washed twice with distilled water and passed through a bed of anhydrous sodium sulfate. The solvent, containing hydrolytic products, was evaporated under a stream of nitrogen. TLC plates were evenly sprayed with 5% (wt/vol) boric acid and dried at 100°C for 1 h. The hydrolytic products were separated on these silica gel TLC plates by development using hexane/diethyl ether/acetic acid (70:30:1, by vol) for 40–50 min and then allowed to dry in air. The bands were located by viewing under a short (254 nm)- and a long (365 nm)-wavelength light (Spectroline, Co., Westbury, NY). The bands were scraped off and their lipids extracted into methanol/chloroform (1:1, vol/vol). The FA of the lipids were then analyzed by GC, as described earlier.

Statistical analysis. All experiments were performed in triplicate. Data are reported as mean ± SD. ANOVA and Tukey's test were carried out at a level of $P < 0.05$ to assess the significance of differences between mean values.

RESULTS AND DISCUSSION

FA composition of high-laurate canola oil (Laurical 15) before and after enzymatic modification. The lauric acid content of Laurical 15 was 37.6% (Table 1). This oil also contained other saturated FA, namely, stearic (36.0%), palmitic (3.21%), and myristic (3.82%) acids, and its oleic acid content was almost 15%.

Enzyme screening. Five lipases from *C. antarctica, R. miehei, Pseudomonas* sp., *C. rugosa,* and *A. niger* were screened for their ability to incorporate EPA into Laurical 15. The order of incorporation of EPA into Laurical 15 was *Pseudomonas* sp. (64.6%) > *C. rugosa* (58.1%) > *A. niger* (56.1%) > *C. antarctica* (55.5%) > *R. miehei* (50.8%). It was found that lipase from *Pseudomonas* sp. was most effective in its ability to incorporate EPA into high-laurate canola oil (Laurical 15). This is despite the activity of enzymes tested (*C. antarctica,* 554 U; *R. miehei,* 13613 U; *Pseudomonas* sp., 11936 U; *A. niger,* 8142 U; and *C. rugosa,* 38707 U). Because acidolysis of Laurical 15 with EPA was better obtained using *Pseudomonas* sp., this enzyme was selected for subsequent experiments.

Positional distribution. The positional distribution of FA in modified and unmodified high-laurate canola oil (Laurical 15) was determined (Table 2). In unmodified Laurical 15, lauric acid was located primarily in the *sn*-1 + *sn*-3 positions.

TABLE 2
Positional Distribution[a] (wt%, *sn*-2 and *sn*-1 + *sn*-3) of FA in Modified and Unmodified High-Laurate Canola Oil (Laurical 15)

FA	Unmodified		Modified	
	sn-2	*sn*-1 + *sn*-3	*sn*-2	*sn*-1 + *sn*-3
12:0	32.3 ± 13.7 (28.5)	41.7 ± 5.39 (71.5)	9.87 ± 3.39 (8.72)	19.9 ± 7.55 (91.3)
14:0	3.49 ± 0.86 (30.5)	3.98 ± 0.44 (69.5)	2.03 ± 0.51 (17.7)	2.11 ± 0.92 (82.3)
16:0	3.88 ± 0.97 (40.3)	3.09 ± 0.34 (59.7)	6.49 ± 4.82 (67.4)	3.73 ± 3.43 (32.6)
18:0	34.8 ± 6.50 (32.2)	27.5 ± 9.13 (67.8)	16.9 ± 3.59 (15.7)	15.2 ± 4.83 (84.3)
18:1n-9	15.3 ± 1.29 (34.7)	13.9 ± 0.94 (65.3)	10.0 ± 7.98 (22.7)	8.42 ± 3.46 (77.3)
20:5n-3	—	—	37.2 ± 19.4 (19.2)	44.8 ± 22.8 (80.8)

[a]Values are mean values ± SD (n = 3). The reaction mixture contained 100 mg Laurical 15, 123 mg EPA, 4% (w/w) enzyme for *Pseudomonas* sp., and 3.0 mL hexane. The reaction mixture was incubated at 45°C for 24 h in an orbital shaker at 250 rpm. Values in parentheses indicate percentage of the subject FA located at the *sn*-1 + *sn*-3 and the *sn*-2 positions. Values in parentheses are, for *sn*-2 (% FA at the *sn*-2 position/% FA in TAG × 3) × 100; for (*sn*-1 + *sn*-3) = 100 – *sn*-2. Sample calculation for EPA; EPA from column 4 of this table and EPA in the unmodified oil as such from Table 1. FA at the *sn*-2 position = (37.2/64.9 × 3) × 100 = 19.2; for (*sn*-1 + *sn*-3) = 100 – 19.2 = 80.8.

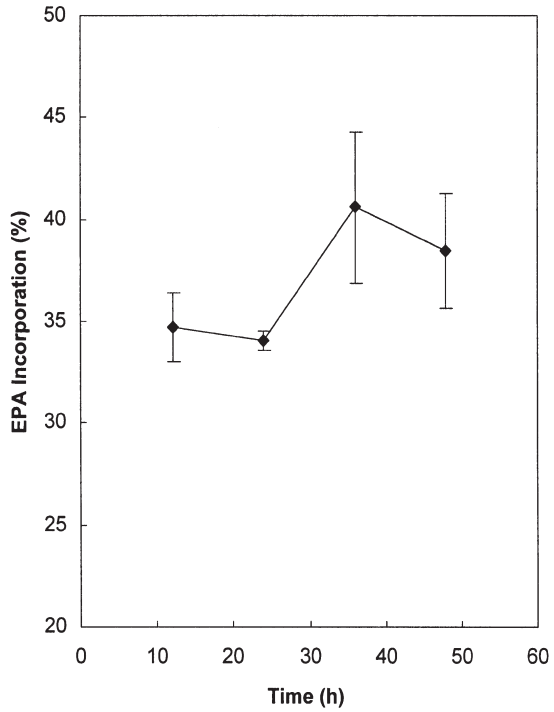

FIG. 1. The effect of time on the incorporation of EPA into Laurical 15. The mixture was incubated at 45°C. The reaction mixture contained 100 mg Laurical 15, 123 mg EPA, 4% (w/w) *Pseudomonas* sp. lipase, and 3.0 mL hexane. The reaction mixture was incubated at 45°C for 12 to 48 h in an orbital shaker at 250 rpm.

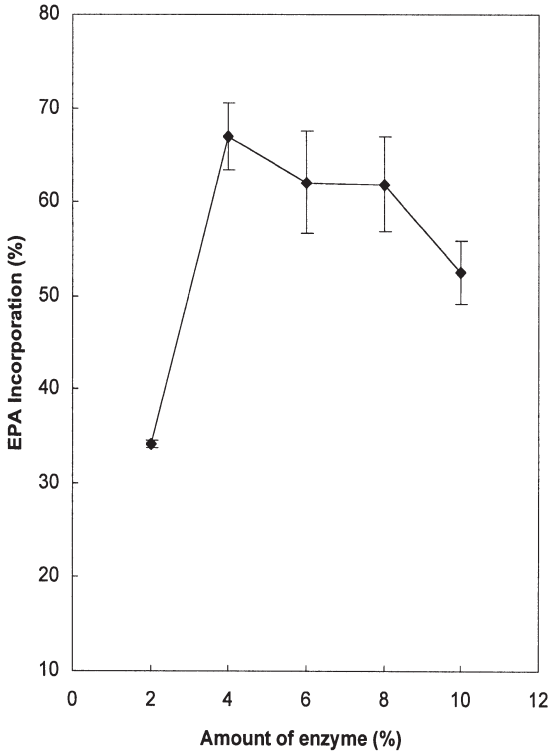

FIG. 3. The effect of enzyme load on the incorporation of EPA into Laurical 15. The reaction mixture contained 100 mg Laurical 15, 123 mg EPA, *Pseudomonas* sp. (2–10%, w/w) enzyme, and 3.0 mL hexane. The reaction mixture was incubated for 36 h at 45°C in an orbital shaker at 250 rpm.

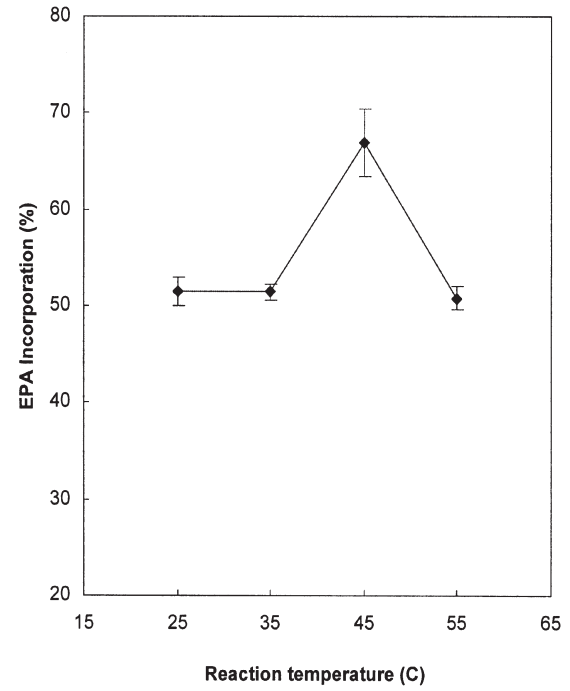

FIG. 2. The effect of reaction temperature on the incorporation of EPA Laurical 15. The reaction mixture contained 100 mg Laurical 15, 123 mg EPA, 4% (w/w) *Pseudomonas* sp., and 3.0 mL hexane. The reaction mixture was incubated for 36 h at 25–55°C in an orbital shaker at 250 rpm.

Stereospecific analysis of the enzyme-modified Laurical 15 showed that lauric acid remained mostly in the *sn*-1,3 positions of the TAG molecules (Table 2).

Mole ratio effect. When the mole ratio of Laurical 15 to EPA was varied (1:1, 1:2, and 1:3), incorporation of EPA into Laurical 15 was 34.1 ± 0.46, 53.4 ± 2.36, and 66.9 ± 3.54, respectively. When the mole ratio of substrates increased from 1:1 to 1:3, incorporation of EPA increased accordingly (data not shown).

Time course. The time course of lipase-assisted acidolysis of EPA into Laurical 15 using PS-30 from *Pseudomonas* sp. is shown in Figure 1. As time progressed from 24 to 36 h, incorporation of EPA into Laurical 15 increased significantly, reaching a maximum at 36 h, followed by a decrease to 38.5% at 48 h, possibly due to the occurrence of a reverse hydrolysis reaction.

Reaction temperature effect. The stimulatory effect of temperature on the reaction rate of enzyme-assisted reactions, below the denaturation temperature, is a well-established phenomenon. Thus, to determine the optimal temperature for incorporation of EPA into Laurical 15, the reaction was carried out at temperatures from 25 to 55°C as shown in Figure 2. Reaction rates increased from 35 to 45°C. The optimal incorporation of EPA into Laurical 15 (66.9%) was obtained at 45°C.

Enzyme load effect. As the enzyme load (%, w/w of enzymes to substrates) increased from 2 to 4%, incorporation of

EPA into Laurical 15 increased gradually, reaching a maximum (66.9%) at 4% (Fig. 3). Above 10% enzyme load, a slight decrease was noted.

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