JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Synthesis of Structured Lipids Containing Medium-Chain and Omega-3 Fatty Acids

FAYEZ HAMAM AND FEREIDOON SHAHIDI*

Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9

The ability of different lipases to incorporate ω3 fatty acids, namely, eicosapentaenoic acid (EPA, C20:5n-3), docosapentaenoic acid (DPA, C22:5n-3), and docosahexaenoic acid (DHA, C22:6n-3), into a high-laurate canola oil, known as Laurical 35, was studied. Lipases from *Mucor miehei* (Lipozyme-IM), *Pseudomonas* sp. (PS-30), and *Candida rugosa* (AY-30) catalyzed optimum incorporation of EPA, DPA, and DHA into Laurical 35, respectively. Other lipases used were *Candida anatrctica* (Novozyme-435) and *Aspergillus niger* (AP-12). Response surface methodology (RSM) was used to obtain a maximum incorporation of EPA, DPA, and DHA into high-laurate canola oil. The process variables studied were the amount of enzyme (2−6%), reaction temperature (35−55 °C), and incubation time (12−36 h). The amount of water added and mole ratio of substrates (oil to n-3 fatty acids) were kept at 2% and 1:3, respectively. The maximum incorporation of EPA (62.2%) into Laurical 35 was predicted at 4.36% of enzyme load and 43.2 °C over 23.9 h. Under optimum conditions (5.41% enzyme; 38.7 °C; 33.5 h), the incorporation of DPA into high-laurate canola oil was 50.8%. The corresponding maximum incorporation of DHA (34.1%) into Laurical 35 was obtained using 5.25% enzyme, at 43.7 °C, over 44.7 h. Thus, the number of double bonds and the chain length of fatty acids had a marked effect on the incorporation ω3 fatty acids into Laurical 35. EPA and DHA were mainly esterified to the sn-1,3 positions of the modified oils, whereas DPA was randomly distributed over the three positions of the triacylglycerol molecules. Meanwhile, lauric acid remained esterified mainly to the sn-1 and sn-3 positions of the modified oils. Enzymatically modified Laurical 35 with EPA, DPA, or DHA had higher conjugated diene (CD) and thiobarbituric acid reactive substance (TBARS) values than their unmodified counterpart. Thus, enzymatically modified oils were more susceptible to oxidation than their unmodified counterparts, when both CD and TBARS values were considered.

KEYWORDS: Acidolysis; eicosapentaenoic acid (EPA); docosapentaenoic acid (DPA); docosahexaenoic acid (DHA); high-laurate canola oil; lipase; medium-chain fatty acids; ω3 fatty acids; positional distribution; response surface methodology; structured lipids; oxidative stability; conjugated dienes; thiobarbituric acid reactive substances (TBARS)

INTRODUCTION

Recognition of the health benefits associated with the consumption of seafoods (ω3 or n-3 fatty acids) is one of the most promising developments in human nutrition and disease prevention research in the past three decades. The n-3 fatty acids have many health benefits related to cardiovascular disease (*1*), inflammation (*2*), cancer (*3*), immune response (*4*), diabetes (*5*), hypertension (*6*), and renal disorders (*7*).

Long-chain n-3 fatty acids present in seafoods and algal sources include eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA). DPA is an elongation metabolite of EPA. DPA has not been studied in much detail because of an availability problem as it is present in much lower concentrations in marine oils as compared with EPA and DHA. It is also difficult to purify it from mixtures containing EPA and DHA, which have similar physicochemical properties (*8*). Whereas most fish oils contain <1% DPA, harp seal oil contains 4−6% of DPA (*8*). About one-third of the long-chain n-3 fatty acids circulating in human blood are attributed to DPA as the effective agent (*9*). DPA may have pharmacological impacts different from those of EPA and DHA. In animals, DHA deficiency is accompanied by increased DPA, which suggests that the ratio of DHA to DPA could be a useful biochemical marker of low n-3 fatty acid status. In contrast to findings in animals, DPA concentration is not a useful measure of low n-3 fatty acid uptake in preschool children, possibly due to physiological differences of n-3 fatty acids deficiency between

* Corresponding author [telephone (709) 737-8552; fax (709) 737-4000; e-mail fshahidi@mun.ca].

10.1021/jf052540r CCC: $33.50
Published on Web 05/20/2006

humans and animals (*10*). It has been demonstrated that angiogenic activity in endothelial cells induced by vascular endothelial growth factor (VEGF) can be suppressed by n-3 PUFA treatment. Among long-chain polyunsaturated fatty acids (LC PUFA), DPA was the most powerful inhibitor of angiogenesis (the growth and proliferation of blood vessels); the inhibitory activity by DPA pretreatment was almost 6 times that of EPA and DHA, pointing out that DPA could be developed as a new drug or supplement against angiogenesis-related diseases (*11*). Angiogenesis plays a significant function in tumor growth and metastasis, and blocking angiogenesis can limit tumor growth. In addition, the stimulative effect of EPA on endothelial cells migration (an important process in the control of wound-healing responses of blood vessels) may be due to DPA. In vitro studies have shown that the activity of DPA to stimulate endothelial cell migration is 10-fold that of EPA (*12*). Hence, it is possible that the antiarteriosclerotic function of seal oil is mainly due to DPA rather than EPA and DHA (*8*). Evidence proposed that DPA is the most significant fatty acid in keeping artery walls soft and plaque-free (*9*). A recent study indicates that DPA can be >10 times as effective as EPA in helping to heal damaged blood vessels (*10*). Moreover, arachidonic acid-stimulated blood platelet aggregation was inhibited by n-3 fatty acids in a dose-dependent manner, among which DPA was the most effective inhibitor (*12*). DPA displays significant activity for interfering with the cyclooxygenase pathways, and accelerating the lipoxygenase pathway, thus inhibiting platelet aggregation most efficiently (13). Eldho et al. (*14*) reported that insufficient supply of DHA or its n-3 precursors during infancy resulted in replacement of DHA by DPA (22:5n-6, an elongation product of arachidonic acid). This replacement is generally nonfatal, but is associated with some loss of brain activity.

High-laurate canola oil is a genetically engineered oil containing a medium-chain fatty acid (lauric acid, C12:0). This oil was produced by Calgene's Inc. (Davis, CA) to provide an alternate to several palm kernel oil fractions (*15*).

Response surface methodology (RSM) is an optimization procedure that determines optimum process conditions by combining particular experimental designs with modeling using first- or second-order polynomial equations in a sequential testing procedure. RSM examines a number of variables at a time, uses special experimental designs to reduce the number of required determinations, and measures many effects by objective tests (*16*). In this study, the ability of different lipases to catalyze the acidolysis of high-laurate canola oil with EPA, DPA, and DHA was explored. Effects of enzyme amount, incubation time, and reaction temperature on the incorporation of EPA, DPA, and DHA into a lauric acid rich canola oil in using RSM were also investigated. The oxidative stability of the resultant structured lipids (SL) was then assessed.

MATERIALS AND METHODS

Materials. Two lipases from *Candida antarctica* (Novozyme-435) and *Mucor miehei* (Lipozyme 1M) were acquired from Novo Nordisk (Franklinton, NC). Other lipases, namely, *Pseudomonas* sp. (PS-30), *Aspergillus niger* (AP-12), and *Candida rugosa* (AY-30), were obtained from Amano Enzyme (Troy, VA). Fatty acid methyl esters (FAMEs; GLC-461) were purchased from Nu-Check (Elysian, MN). High-laurate canola oil (Laurical 35) was a product from Calgene's Oils Division (Davis, CA). Porcine pancreatic lipase (EC 3.11.3), sodium taurocholate, and silica gel thin-layer chromotographic plates (TLC; 20 × 20 cm; 60 Å mean pore diameter, 2−25 μm mean particle size, 500 μm thickness, with dichlorofluorescein) 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, ON, Canada). EPA samples, >99% pure, were made by Fuso Pharmaceutical Industries Ltd. (Osaka, Japan) and kindly provided by Dr. K. Miyashita. Docosapentaenoic acid was a purified product. Docosahexaenoic acid single-cell oil (DHASCO) containing 40% DHA was obtained from Martek Bioscience Corp. (Columbia, MD), and DHA (>98%) was prepared as described by Wanasundra and Shahidi (*16*).

Methods. *Experimental Design for Response Surface Analysis.* Before RSM was employed, approximate conditions for EPA, DPA, and DHA incorporation, enzyme amount, reaction temperature, and incubation time were determined by changing one factor at a time while keeping the others constant. A proper range for each factor was determined for RSM. A three-factor and three-level face-centered cube design with 17 individual design points was selected in this study (*15*−*18*). To avoid bias, 17 runs were performed in a totally random order. The independent variables or factors investigated were enzyme amount (wt %; X_1), reaction temperature (°C; X_2), and reaction time (h; X_3). Response or dependent variables studied were Y_1 (Laurical 35, DPA%), Y_2 (Laurical 35, DHA%), and Y_3 (Laurical 35, EPA%). Triplicate experiments were conducted at all design points.

The second-order polynomial model used for the optimization of EPA, DPA, and DHA incorporation into oil (Y) was

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum\sum_{i<j=1}^{3} \beta_{ij} X_i X_j$$

where β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively, and X_i and X_j are the independent variables. Data were analyzed using response surface regression (RSREG) procedure (*19*) and fitted to the second-order polynomial equation after logarithmic transformation (*20*). Response surfaces and contour plots were obatained using the fitted model, by keeping the least effective independent variable at a constant value while changing the other two variables. Confirmation experiments were performed using mixtures of variables at different levels (within the experimental range). The independent factors were coded for an experimental design. The center point for each independent variable level was given a code of zero. The highest and lowest levels of concern for each independent factor were coded plus or minus one, respectively, for this three-level design. The major benefit of the design is that it enables one to study one or more parameters at the same time in a single experimental design of a workable size (*21*, *22*).

Acidolysis Reaction. In general, high-laurate canola oil (Laurical 35) (70 mg each) was mixed with EPA, DPA, or DHA at a mole ratio of 1:3 (oil to EPA, DPA, or DHA) in a screw-capped test tube, and then lipase (2−6 wt % of substrates) and water (2 wt % of substrates and enzyme) were added in hexane (3.0 mL). The mixture was incubated for different time periods (12−36 h) in an orbital shaker at 250 rpm at temperatures ranging from 35 to 55 °C.

Separation of Acylglycerols after Acidolysis. After a given time period, a mixture of acetone and ethanol (20 mL; 1:1, v/v) was added to the reaction mixture to stop the reaction. To neutralize free fatty acids, the reaction mixture was titrated against a 0.5 M NaOH solution (using a phenolphathalein 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 (aqueous, 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, the acylglycerol fraction was recovered, and a portion of it (5−10 mg) was transferred to special transmethylation vials for further experimentation.

Preparation of FAMEs. Fatty acid profiles of products were determined following their conversion to methyl esters. The transmethylation reagent (2.0 mL; freshly prepared 6.0 mL of concentrated sulfuric acid made up to 100 mL with methanol and 15 mg of hydroquinone as an antioxidant) was added to the sample vial, followed by vortexing. The mixture was incubated overnight at 60 °C and subsequently cooled to room temperature. Distilled water (1 mL) was

added to the mixture and, after thorough mixing, a few crystals of hydroquinone were added to each vial to prevent oxidation; lipids were extracted three times, each with 1.5 mL of pesticide-grade hexane. The hexane layers were separated, combined, and transferred to a clean test tube and then washed two times, each 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. FAMEs were then dissolved in 1.0 mL of carbon disulfide and used for subsequent gas chromatographic (GC) analysis.

Analysis of FAMEs by GC. The FAMEs 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, ON, 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 flame ionization detector (FID) temperatures were both set at 250 °C. Ultrahigh-purity (UHP) helium was used as a carrier gas at a flow rate of 15 mL/min. The data were treated using a Hewlett-Packard 3365 series II Chem Station software (Agilent). The FAMEs were identified by comparing their retention times with those of an authentic standard mixture (GLC-461; Nu-Check), and the results were presented as weight percentages.

Hydrolysis by Pancreatic Lipase. Hydrolysis of modified oil by pancreatic lipase was performed according to the method described by Christie (23) with a slight modification. Tris-HCl buffer (5.0 mL; 1.0 M, pH 8.0), 0.5 mL of calcium chloride (2.2%, w/v), and 1.25 mL of sodium taurocholate (0.05 w/v) were added to 25 mg of modified oil in a glass test tube. The whole 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 (EC 3.11.3; Sigma). The mixture was subsequently placed in a gyrotory water bath shaker at 250 rpm under nitrogen for 10−15 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 50.0 mL of hexanes, and the upper layer was removed and washed twice with distilled water and then passed through a bed of anhydrous sodium sulfate. The solvent containing hydrolytic products was evaporated under a stream of nitrogen. TLC plates (20 × 20 cm; 60 Å mean pore diameter, 2−25 μm mean particle size, 500 μm thickness, with dichlorofluorescein, Sigma) were evenly sprayed with 5% (w/v) boric acid and dried at 100 °C for 1 h. The hydrolytic products were separated on silica gel TLC plates. The plates were developed using hexane/diethyl ether/acetic acid (70:30:1, v/v/v) 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, v/v). Fatty acid profile of lipids were analyzed by the GC method described earlier.

Oxidative Stability Tests. The oxidative stabilities of the resultant structured lipids as well as the original oil were evaluated under Schaal oven conditions at 60 °C for a period of 72 h. Oils (0.4−0.5 g) were placed in loosely capped test tubes (10 mm diameter and 4.0 cm height) and stored at 60 °C in a forced-air oven (Thelco, model, Prcision Scientific Co., Chicago, IL). Samples were removed at 0, 6, 12, 24, 36, 48, and 72 h from the oven, cooled to room temperature, flushed with nitrogen, capped, and stored at −20 °C until analyzed. The experiments were carried out in triplicate.

Conjugated Dienes (CD). CD in the oils were determined according to IUPAC (*24*) method 20505. Oil samples (0.02−0.04 g) were weighed into 50 mL volumetric flasks, dissolved in iso-octane (2,2,4-trimethylpentane), and made up to the mark with the same solvent; pure iso-octane was used as the blank. The contents were thoroughly mixed, and the absorbance at 234 nm was read using a Hewlett-Packard model 845LA diode array spectrophotometer (Agilent). CD were calculated using the formula CD = $A/(cd)$, where A = absorbance at 234 nm, c = concentration of the solution in g/mL, and d = length of the cell in cm.

Thiobarbituric Acid Reactive Substances (TBARS) Determination. The determination of TBARS was carried out as described by the AOCS (*25*), method Cd 19-90. Oil samples (0.05−0.10 g) were accurately weighed into 25 mL volumetric flasks, dissolved in a small volume of 1-butanol, and made up to the mark with the same solvent. Five milliliters of the mixture (oil and solvent) was transferred into a dry screw-capped test tube, and then 5 mL of freshly prepared TBA reagent (0.5 g of TBA in 250 mL of 1-butanol) was added. The constituents of the mixture were thoroughly mixed and placed in a water bath at 95 °C for 2 h. Heated samples were cooled in an ice bath, and the absorbance of the resulting colored complex was read at 532 nm. The micromole amount of malonaldehyde (MA) equivalents per gram of oil, expressed as TBARS values, was calculated using the formula $C = (0.415A_{532})/w$, where the factor 0.415 is obtained from a standard regression line using 1,1,3,3-tetramethoxypropane as a precursor of MA. In this formula, C is the concentration of MA, A represents the absorbance of the colored complex at 532 nm, and w is the mass of the oil.

Statistical Analysis. All determinations were performed in triplicate. Data are reported as mean ± standard deviation (SD). Normality was tested using Sigma stat. Analysis of variance and Tukey's standardized test were carried out at a level of $p < 0.05$ to assess the significance of differences among mean values.

Table 1. Fatty Acid Composition (Weight Percent) of High-Laurate Canola Oil (Laurical 35) before and after Modification with EPA, DPA, or DHA[a]

fatty acid	unmodified	modified with EPA	modified with DPA	modified with DHA
12:0	37.0 ± 0.20	10.5 ± 2.20	14.3 ± 0.36	18.6 ± 0.16
14:0	3.81 ± 0.03	1.35 ± 0.22	1.62 ± 0.04	2.24 ± 0.04
16:0	3.05 ± 0.05	1.09 ± 0.17	1.48 ± 0.06	2.08 ± 0.06
18:0	12.7 ± 0.29	5.47 ± 0.49	8.02 ± 0.21	9.93 ± 0.45
18:1n-9	33.9 ± 0.30	14.1 ± 1.23	22.1 ± 0.33	28.9 ± 1.39
18:2n-6	3.35 ± 0.04	1.37 ± 0.18	2.22 ± 0.03	0.76 ± 0.22
20:5n-3		64.0 ± 5.30		
22:5n-3			48.7 ± 0.62	
22:6n-3				38.8 ± 3.11

[a] Results are mean values of triplicate determinations ± standard deviation.

Table 2. Effect of Enzyme Type on the Incorporation (Percent) of EPA, DPA, and DHA into High-Laurate Canola Oil (Laurical 35)[a]

enzyme source	EPA	DPA	DHA
C. antarctica	52.4 ± 1.35 bac	28.0 ± 1.29 cdb	39.8 ± 0.95 d
M. miehei	52.0 ± 0.91 abc	40.5 ± 0.77 e	29.6 ± 2.20 c
Pseudomonas sp.	62.5 ± 1.58 de	30.5 ± 4.29 dcb	42.5 ± 0.75 e
C. rugosa	64.0 ± 5.30 ed	21.5 ± 5.26 bcda	10.8 ± 0.75b
A. niger	53.9 ± 2.11 cab	17.5 ± 1.82 ab	7.73 ± 0.50 a

[a] Results are mean values of triplicate determinations ± standard deviation. Values in each column with different letters are different ($p < 0.05$).

RESULTS AND DISCUSSION

Fatty Acid Profile of High-Laurate Canola Oil (Laurical 35). Table 1 shows the fatty acid composition of high-laurate canola oil, commercially known as Laurical 35, before and after modification with EPA, DPA, or DHA. The original oil contained 37.0% lauric acid as well as saturated fatty acids such as stearic (12.7%), palmitic (3.05%), and myristic (3.81%) acids. The major unsaturated fatty acids found in Laurical 35 before enzymatic incorporation were oleic (33.9%), and linoleic (3.35%) acids.

Enzyme Screening. Table 2 shows the effect of enzyme type on percent incorporation of EPA, DPA, or DHA into Laurical 35. The incorporation of EPA into Laurical 35 was effectively catalyzed by only two of five lipases tested. However, there was no significant difference ($p > 0.05$) in the EPA incorporation into Laurical 35 when lipases from *C. antarctica*, *A. niger*, and *M. miehei* were employed. *C. rugosa* gave the highest degree of incorporation of EPA into Laurical 35 (64.0%, after 24 h). Lipase from *M. miehei* catalyzed the highest degree of

Table 3. Positional Distribution (sn-2 and sn-1 + sn-3) of Fatty Acids in Modified and Unmodified High-Lauric Acid Canola Oil (Laurical 35)[a]

	unmodified		modified with DPA		modified with DHA	
fatty acid	sn-2	sn-1 + sn-3	sn-2	sn-1 + sn-3	sn-2	sn-1 + sn-3
C12:0	31.2 ± 6.87 (28.1)	49.4 ± 10.7 (71.9)	14.3 ± 0.40 (33.3)	11.9 ± 1.35 (66.7)	16.9 ± 1.52 (30.3)	15.2 ± 3.82 (69.7)
C14:0	4.15 ± 0.15 (36.3)	4.42 ± 0.76 (63.7)	1.70 ± 0.40 (34.9)	1.93 ± 0.11 (65.1)	2.90 ± 0.35 (43.2)	1.96 ± 0.50 (56.8)
C16:0	4.12 ± 0.87 (45.0)	3.30 ± 0.75 (54.5)	1.64 ± 0.13 (36.9)	2.52 ± 0.39 (63.1)	3.61 ± 0.91 (57.9)	1.62 ± 0.06 (42.1)
C18:0	14.5 ± 2.49 (37.8)	8.70 ± 1.09 (62.2)	7.23 ± 0.59 (30.0)	9.64 ± 0.86 (70.0)	12.6 ± 2.37 (42.3)	6.36 ± 0.28 (57.7)
C18:1n-9	41.0 ± 3.45 (40.2)	29.1 ± 9.81 (59.7)	18.8 ± 2.31 (28.4)	22.2 ± 0.55 (71.6)	30.9 ± 0.45 (35.6)	17.8 ± 3.23 (64.4)
C:22 5n-3			48.2 ± 0.89 (32.9)	42.3 ± 0.50 (67.1)		
C:22 6n-3					24.6 ± 4.25 (22.4)	36.5 ± 4.51 (77.6)

[a] Values in parentheses indicate percent fatty acid distribution of total triacylglycerols present at the sn-1 + sn-3 and the sn-2 positions. Values are (% fatty acid at the sn-2 position/% fatty acids in triacylglycerols × 3) × 100; for sn-1 + sn-3 = 100 − sn-2.

Table 4. Positional Distribution (sn-2 and sn-1 + sn-3) of Fatty Acids in Modified and Unmodified High-Lauric Acid Canola Oil (Laurical 35)[a]

	unmodified		modified with EPA	
fatty acid	sn-2	sn-1 + sn-3	sn-2	sn-1 + sn-3
C12:0	31.2 ± 6.87 (28.1)	49.4 ± 10.7 (71.9)	35.3 ± 4.48 (31.8)	2.47 ± 0.93 (68.2)
C14:0	4.15 ± 0.15 (36.3)	4.42 ± 0.76 (63.7)	3.41 ± 0.29 (84.2)	0.31 ± 0.10 (15.8)
C16:0	4.12 ± 0.87 (45.0)	3.30 ± 0.75 (54.5)	2.92 ± 0.12 (89.3)	0.32 ± 0.11 (10.7)
C18:0	14.5 ± 2.49 (37.8)	8.70 ± 1.09 (62.2)	10.3 ± 0.55 (62.8)	1.22 ± 0.39 (37.2)
C18:1n-9	41.0 ± 3.45 (40.2)	29.1 ± 9.81 (59.7)	26.5 ± 0.99 (62.6)	3.12 ± 1.15 (37.4)
C20:5n-3			6.43 ± 2.16 (3.35)	87.9 ± 2.65 (96.7)

[a] Values in parentheses indicate percent fatty acid distribution of total triacylglycerols present at the sn-1 + sn-3, and sn-2 positions. These values are (% fatty acid at the sn-2 position/% fatty acids in triacylglycerols × 3) × 100; for sn-1 + sn-3 = 100 − sn-2.

incorporation of DPA into Laurical 35. This is despite the activity of enzymes tested (*C. antarctica*, 554 units; *M. miehei*, 13613 units; *Pseudomonas* sp., 11936 units; *A. niger*, 8142 units; and *C. rugosa*, 38707 units). The order of incorporation of DHA into Laurical 35 was *Pseudomonas* sp. > *C. antarctica* > *M. miehei* > *C. rugosa* > *A. niger*. *Pseudomonas* sp. gave the highest degree of incorporation of DHA (42.5%, after 24 h) into high-laurate canola oil. Results indicated that lipase from *C. rugosa* was most effective in incorporating EPA into Laurical 35. Lipases from *M. miehei*, *Pseudomonas* sp., and *C. rugosa* catalyzed optimum incorporation of EPA, DPA, and DHA into Laurical 35, respectively. Thus, these three enzymes were chosen for subsequent experiments.

Positional Distribution. Positional distribution analysis of unmodified Laurical 35 revealed that 71.9% of lauric acid was located at the sn-1 + sn-3 positions of TAG molecules (**Table 3**). The positional distribution of fatty acids in Laurical 35 modified with DPA showed that DPA was randomly distributed over the three positions of the glycerol backbone. However, lauric acid remained mainly attached to the sn-1 and sn-3 positions (**Table 3**). Although DPA or DHA was esterified mainly to the sn-1,3 positions of the TAG molecule of modified Laurical 35, incorporation into the sn-2 position was also observed. The same trend was noted for EPA-modified Laurical 35; lauric acid as well as EPA were esterified mostly to the sn-1,3 positions of the modified oil (**Table 4**).

RSM. RSM is a statistical design that enables one to determine optimal conditions for enzymatically catalyzed reactions by performing a minimum number of experiments. **Table 5** summarizes the experimental data for response variable Y_1 (EPA percent incorporation into Laurical 35), Y_2 (DPA percent incorporation into Laurical 35), and Y_3 (DHA percent incorporation into Laurical 35). Multiple regression coefficients, obtained by employing a least-squares procedure to predict the second-order polynomial model for the EPA, DPA, and DHA incorporation into high-laurate canola oil, are summarized in **Table 6**. In Laurical 35 modified with EPA, all linear, second-order, and interaction terms were insignificant ($p > 0.1$) except for the amount of enzyme (X_1), which is significant ($p < 0.001$). Similarly, interactions of the amount of enzyme (X_1) and reaction temperature (X_2) or reaction time (X_3) were insignificant ($p > 0.1$). In high-laurate canola oil modified with DPA, linear and quadratic terms of amount of enzyme (X_1) were significant ($p \leq 0.01$). Interactions of the amount of enzyme (X_1) and reaction temperature (X_2), reaction time (X_3), and temperature (X_2), as well as the amount of enzyme (X_1) and the reaction time (X_3), were insignificant ($p > 0.1$). In Laurical 35 modified with DHA, all linear, second-order, and interaction terms were insignificant ($p > 0.1$). Similarly, interactions of the amount of enzyme (X_1) and reaction temperature (X_2) or reaction time (X_3) were insignificant ($p > 0.1$).

Table 5. Face-Centered Cube Design Arrangement and Response for the Analysis of High-Laurate Canola Oil (Laurical 35) with EPA, DPA, or DHA[a]

	independent variable[b]			response[c]		
run	enzyme (%)	temp (°C)	time (h)	Y_1	Y_2	Y_3
1	2 (−1)	35 (−1)	12 (−1)	50.4	37.5	29.1
2	2 (−1)	35 (−1)	36 (+1)	53.9	37.2	35.1
3	2 (−1)	45 (0)	24 (0)	55.5	34.9	17.2
4	2 (−1)	55 (+1)	12 (−1)	53.9	33.5	36.6
5	2 (−1)	55 (+1)	36 (+1)	53.6	35.4	32.6
6	4 (0)	35 (−1)	24 (0)	57.8	50.8	35.6
7	4 (0)	45 (0)	12 (−1)	59.5	42.3	33.7
8	4 (0)	45 (0)	24 (0)	64.0	48.5	37.5
9	4 (0)	45 (0)	24 (0)	64.0	49.4	37.5
10	4 (0)	45 (0)	24 (0)	64.0	45.5	37.5
11	4 (0)	45 (0)	36 (+1)	59.1	49.7	23.5
12	4 (0)	55 (+1)	24 (0)	58.9	44.4	35.6
13	6 (+1)	35 (−1)	12 (−1)	57.2	45.9	24.1
14	6 (+1)	35 (−1)	36 (+1)	59.4	48.8	35.1
15	6 (+1)	45 (0)	24 (0)	56.8	50.5	32.4
16	6 (+1)	55 (+1)	12 (−1)	56.7	45.6	40.4
17	6 (+1)	55 (+1)	36 (+1)	50.3	51.9	46.2

[a] Nonrandomized. [b] Uncoded variable levels. Coded variable levels are given in parentheses. [c] Average of triplicate determinations from different experiments. Y_1, % EPA incorporation into Laurical 35; Y_2, % DPA incorporation into Laurical 35; Y_3, % DHA incorporation into Laurical 35.

Table 6. Regression Coefficients of Predicted Quadratic Polynomial Model for Response (Y)

coefficient[a]	Laurical 35, EPA (%) (Y_1)	Laurical 35, DPA (%) (Y_2)	Laurical 35, DHA (%) (Y_3)
β_0	–38.471	39.082	134.43
linear			
β_1	14.077**	8.8692*	2.1454
β_2	2.6007	–0.7528	–5.5640
β_3	1.1566	0.1355	0.6950
quadratic			
β_{11}	–1.1121*	–1.1456*	–1.1144
β_{22}	–0.0225	0.0032	0.0634
β_{33}	–0.0090	–0.0089	–0.0046
interactions			
β_{12}	–0.0800	0.0556	0.1400
β_{13}	–0.0385	0.0411	0.0770
β_{23}	–0.0129	0.0061	–0.0158
β_{123}			
R^2	0.88	0.94	0.57
CV%	3.73	5.09	20.3

[a] Coefficients refer to the general model. R^2, coefficient of determination; CV%, coefficient of variation; *, significant at $p \leq 0.01$;**, significant at $p \leq 0.001$.

The coefficient of determination for Y_1 (Laurical 35, EPA%) ($R^2 = 0.88$) indicates that the fitted model could explain 88% of the variations, whereas the coefficient of variation (CV) was <5%, thus indicating that this model is reproducible. The coefficient of determination for Y_2 (Laurical 35, DPA%) ($R^2 = 0.94$) indicates that the fitted model could explain 94% of the variations. The CV was 5%, thus indicating that this model is reproducible only at 5%. The coefficient of determination for Y_3 (Laurical 35, DHA %) was 0.57, which indicates that the fitted model could explain 57% of the variations. The CV was 20%, thus indicating that this model is reproducible only at 20%.

Canonical analysis is a mathematical method used to locate the stationary point of the response surface and to determine whether it represents a maximum, minimum, or saddle point (*21*). Therefore, to characterize the nature of the stationary points, canonical analysis was conducted on the second-order polynomial models to examine the overall shape of the response surface curves. The canonical forms of the equations for Y_1 (Laurical 35, EPA%), Y_2 (Laurical 35, DPA %), and Y_3 (Laurical 35, DHA %) are

$$Y_1 = 62.2 - 0.86W_1^2 - 2.29W_2^2 - 4.84W_3^2$$

$$Y_2 = 50.8 + 0.48W_1^2 - 1.32W_2^2 - 4.70W_3^2$$

$$Y_3 = 34.1 + 6.61W_1^2 - 0.94W_2^2 - 4.89W_3^2$$

where W_1, W_2, and W_3 are the axes of the response surface for the oil examined. All eigenvalues were negative for Y_3, indicating that the stationary point was a maximum for the Y_1 (Laurical 35, EPA %) model. The eigenvalue was positive for DPA or DHA incorporation into high-laurate canola oil model, pointing out that the stationary points were saddle.

Table 7 shows critical values for the three factors (enzyme load, reaction time, and temperature) examined. The maximum incorporation of EPA (62.2%) into Laurical 35 was predicted at 4.36% of enzyme load at 43.2 °C over 23.9 h. The stationary point for the degree of DPA incorporation (percent) into high-laurate canola oil reached a maximum of 50.8% at 5.41% enzyme concentration, at 38.7 °C in 33.5 h. Similarly, the maximum incorporation of DHA (34.1%) into high-laurate canola oil was obtained when enzyme amounts, reaction temperature, and time were 5.25%, 43.7 °C, and 44.7 h, respectively.

Table 7. Canonical Analysis of Response Surface for Acidolysis of High-Laurate Canola Oil with EPA or DPA or DHA

factor	Laurical 35, EPA (%)	Laurical 35, DPA (%)	Laurical 35, DHA (%)
amount of enzyme (wt %, X_1)	4.36	5.41	5.25
reaction temp (°C, X_2)	43.2	38.7	43.7
reaction time (h, X_3)	23.9	33.5	44.7
stationary point	maximum	saddle	saddle
predicted value[a]	62.2	50.8	34.2
observed value[b]	61.1 ± 0.95	48.7 ± 0.62	38.8 ± 3.11

[a] Predicted using the polynomial model. [b] Mean of triplicate determinations from different experiments ± standard deviation.

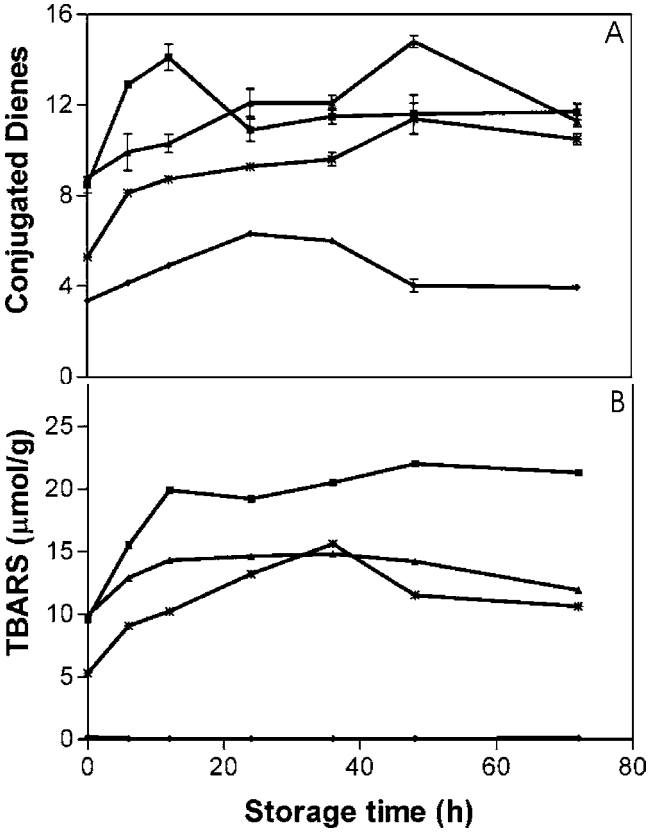

Figure 1. (**A**) Conjugated diene values and (**B**) TBARS values (μmol/g) of (■) Laurical 35 modified with DPA, (▲) Laurical modified with DHA, (∗) Laurical modified EPA, and (◆) the control unmodified oil stored under Schaal oven conditions at 60 °C.

CD. The CD values of the Laurical 35 modified with EPA, DPA, and DHA and the original oil are shown in **Figure 1**. As the storage time increased from 0 to 24 h, the CD values of the original oil increased steadily and reached its maximum value (6.33) at 24 h. After 24 h of storage, the CD values decreased to 4.04, possibly due to the breakdown of unstable hydroperoxides. The oxidative stability of the unmodified oil reached a plateau over the storage period of 48−72 h. As the storage time was extended to 48 h, the CD value of Laurical 35 modified with EPA increased steadily and reached its maximum (11.4) at 48 h. After 48 h of storage, the CD value decreased to 10.5. As the storage time was increased from 0 to 48 h, the CD values of Laurical 35 modified with DPA increased accordingly and peaked (14.8) at 48 h, hence reflecting a longer induction period for Laurical 35 modified with DPA. The sharp increase in the

CD might be accounted for by the formation of more and more hydroperoxides as primary products of oxidation. After 48 h of storage, the CD values decreased to 11.3, due to the breakdown of unstable hydroperoxides. As the storage time was extended to 12 h, the CD values of high-laurate canola oil modified with DHA increased sharply and peaked (14.1) at 12 h, hence reflecting a shortened induction period. After 24 h of storage, the CD values decreased to 10.9. In general, the CD values of both Laurical 35 modified with either DPA or DHA were higher than those of EPA-modified oil. The values of CD were higher in Laurical modified with DHA, DPA, or EPA than the original oil over the entire storage period. The present results indicate that modification of Laurical 35 led to products with lower oxidative stability, possibly due to the incorporation of highly unsaturated fatty acids, EPA, DPA, or DHA, into the molecules and possibly removal of endogenous antioxidants (*26*).

TBARS. TBARS are secondary oxidation products resulting from the degradation of hydroperoxides. Hence, their appearance depends on the rate of breakdown of the hydroperoxides involved.

Figure 1 shows TBARS values of Laurical 35 modified with EPA, DPA, or DHA as well as the control unmodified oils. The TBARS values of unmodified oil did not change during the entire storage time, indicating good stability of the unmodified oil. TBARS values of Laurical 35 enzymatically modified with EPA increased gradually and peaked (15.6 μmol/g) at 36 h as the accelerated storage period was extended to 36 h. After 36 h of storage, the TBARS values decreased to 10.6 μmol/g. As the storage time was altered from 0 to 12 h, the TBARS values of Laurical 35 modified with DPA increased sharply. As storage time was extended to 36 h, the TBARS values increased accordingly and reached its maximum value (20.5 μmol/g) at 48 h. The TBARS values of high-laurate canola oil modified with DHA increased steadily as storage time increased from 0 to 12 h. After 12 h of storage, the TBARS values reached a plateau over the period 12−36 h. After 36 h of storage, the TBARS values decreased to 11.9 μmol/g. There was a significant difference ($p < 0.05$) between the modified and unmodified oils, in agreement with expectation. Furthermore, the TBARS values of Laurical 35 modified with DPA were significantly higher than those of Laurical 35 modified with EPA or DHA over the entire storage time.

Finally, we examined each lipase and its action on the incorporation of EPA, DPA, and DHA into Laurical 35. *C. rugosa*, which was found best for EPA incorporation (58.2 ± 2.31%) under optimized conditions, afforded 2.97 ± 0.57 and 10.1 ± 1.44% incorporation of DPA and DHA, respectively, into Laurical 35 under these conditions. Meanwhile, *Pseudomonas* sp., which proved to be the best enzyme for DHA incorporation (31.8 ± 0.22%), afforded 61.9 ± 1.68 and 25.0 ± 6.79% of EPA and DPA incorporation, respectively. For DPA, *M. miehei* afforded 29.6 ± 0.31% into Laurical 35 when tested, whereas EPA and DHA were incorporated at 53.2 ± 1.07 and 33.3 ± 0.81%, respectively. In this last set, incorporation of DPA was less than that found in our optimization work. However, the enzyme batch used in this experiment was different. Using three batches of the same enzyme, we found results were always nearly the same for EPA and DHA, whereas variations were observed for DPA incorporation. Thus, the stability of the enzyme and DPA might have affected the results, and caution must be exercised in this regard.

Conclusions. Lipases from *C. rugosa*, *M. miehei*, and *Pseudomonas* sp. and were most effective for incorporating EPA, DPA, and DHA into Laurical 35, respectively. RSM showed that the maximum incorporation of EPA (62.2%) into Laurical 35 was predicted at 4.36% of enzyme load and 43.2 °C over 23.9 h. In Laurical 35-based SL, the maximum incorporation of DHA (34.3%) was obtained at 5.25% enzyme, at 43.7 °C, over 44.7 h. The maximum incorporation of DPA into Laurical 35 (50.8%) was obtained when the enzyme amount, reaction temperature, and time were 5.41%, 38.7 °C, and 33.5 h, respectively. Incorporation of n-3 fatty acids into Laurical 35 was in the order EPA > DPA > DHA, possibly due to the structural differences (chain length and number of double bonds) between these molecules. A study of the positional distribution of fatty acids on the glycerol backbone of modified Laurical 35 showed that whereas EPA and DHA were mainly esterified to the sn-1,3 positions, DPA was randomly distributed over the three positions of the triacylglycerol molecules. Meanwhile, lauric acid remained esterified mainly to the sn-1 and sn-3 positions of the modified oil. Laurical 35 enzymatically modified with EPA, DPA, and DHA had a higher CD value than its unmodified counterpart. Laurical 35 modified with DPA is less stable than Laurical 35 modified with DHA or EPA as reflected in both TBARS and CD values.

ABBREVIATIONS USED

DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; MCFA, medium-chain fatty acids; MCT, medium-chain triacylgylcerol; PUFA, polyunsaturated fatty acids; RSM, response surface methodology; TLC, thin-layer chromatography; TAG, triacylglycerols.

ACKNOWLEDGMENT

Samples of enzymes were generously donated by the Amano and Novo Nordisk Companies. Samples of high-laurate canola oils were kindly provided by Dr. James Daun, to whom we are very grateful.

LITERATURE CITED

(1) Conquer, J. A.; Holub, B. J. Dietary docosahexaenoic acid as a source of eicosapentaenoic acid in vegetarian and omnivores. *Lipids* **1997**, *32*, 341−345.

(2) Mori, T. A.; Beilin, L. J. Omega-3 fatty acids and inflammation. *Curr. Atheroscler. Rep.* **2004**, *6*, 461−467.

(3) Cave, W. T. n-3 fatty acid diet effects on tumorgenesis in experimental animals. In *Health Effects of ω3 Polyunsaturated fatty Acids in Seafoods*; Simopoulos, A. P., Kifer, R. R., Martin, R. E., Barlow, S. M., Eds.; World Review of Nutrition and Dietetics, Karger: Basel, Switzerland, 1991; Vol. 66, pp 446−456.

(4) Puertollano, M. A. E.; Puertollano, E.; Ruiz-Bravo, A.; Jimenez-Valera, M.; De-Pablo, M. A.; Cienfuegos, G. A. Changes in the immune functions and susceptibility to *Listeria monocytogenes* infection in mice fed dietary lipids. *Immunol. Cell Biol.* **2004**, *82*, 370−376.

(5) Stene, L. C.; Joner, G. Use of cod liver oil during the first year of life is associated with lower risk of childhood onset type 1 diabetes: a large, population-based, case-control study. *Am. J. Clin. Nutr.* **2003**, *78*, 1128−1134.

(6) Aguilera, A. A.; Diaz, G. H.; Barcelata, M. L.; Guerrero, O. A.; Ros, R. M. Effects of fish oil on hypertension, plasma lipids, and tumor necrosis factor-alpha in rats with sucrose-induced metabolic syndrome. *J. Nutr. Biochem.* **2004**, *15*, 350−357.

(7) Plotnick, A. N. The role of omega-3 fatty acids in renal disorders. *J. Am. Vet. Med. Assoc.* **1996**, *209*, 906−910.

(8) Yazawa, K. Recent development of health foods enriched with DHA, EPA and DPA in Japan. *World Rev. Nutr. Diet.* **2001**, *88*, 249−252.

(9) Ho, C. *Omega 3: The Seal Connection*; Flanker Press: St. John's, NF, Canada, 2003.
(10) Innis, S. M.; Vaghri, Z.; King, D. J. n-6 docosapentaenoic acid is not a predictor of low docosahexaenoic acid status in Canadian preschool children. *Am. J. Clin. Nutr.* **2004**, *80*, 768−773.
(11) Tsuji, M.; Murota, S.; Morita, I. Docosapentaenoic acid (22:5, n-3) suppressed tube-forming activity in endothelial cells induced by vascular endothelial growth factor. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **2003**, *68*, 337−342.
(12) Kanayasu-Toyoda, T.; Morita, I.; Murota, S. Docosapentaenoic acid (22:5, n-3), an elongation metabolite of eicosapentaenoic acid (20:5, n-3), is a potent stimulator of endothelial cell migration on pretreatment in vitro. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **1996**, *54*, 319−325.
(13) Akiba, S.; Murata, T.; Kitatani, K.; Sato, T. Involvement of lipoxygenase pathway in docosapentaenoic acid-induced inhibition of platelet aggregation. *Biol. Pharm. Bull.* **2000**, *23*, 1293−1297.
(14) Del Vecchio, A. J. High-laurate canola oil: how Calgene's program began, where it's headed. *INFORM* **1996**, *7*, 230−240.
(15) Mason, R. L.; Gunst, R. F.; Hess, J. L. Response-surface designs. In *Statistical Design and Analysis of Experiments*; Wiley: New York, 1989; pp 204−233.
(16) Wanasundara, U. N.; Shahidi, F. Concentration of omega-3 polyunsaturated fatty acids of seal blubber oil by urea complexation: optimization of reaction conditions. *Food Chem.* **1999**, *65*, 41−49.
(17) Gao, L.; Mazza, G. Extraction of anthocyanin pigments from purple sunflower. *J. Food Sci.* **1996**, *61*, 600−603.
(18) Hamam, F.; Shahidi, F. Lipase-catalyzed acidolysis of algal oils with capric acid: optimization of reaction conditions using response surface methodology. *J. Food Lipids* **2004**, *11*, 147−163.
(19) SAS Institute, Inc. *Statistical Analytical Systems User's Guide*; SAS Institute: Cary, NC, 1990; Vol. 2.
(20) Thompson, D. Response surface experimentation. *J. Food Process. Preserv.* **1982**, *6*, 155−188.
(21) Montgomery, D. C. Response surface methods and designs. In *Design and Analysis of Experiments*; Wiley: New York, 1991; pp 521−568.
(22) Senanayake, S. P. J. N.; Shahidi, F. Lipase-catalyzed incorporation of docosahexaenoic acid (DHA) into borage oil: optimization using response surface methodology. *Food Chem.* **2002**, *77*, 115−123.
(23) Christie, W. W. Structural analysis of lipids by means of enzymatic hydrolysis. In *Lipid Analysis*; Christie, W. W., Ed.; Pergamon Press: New York, 1982; pp 155−166.
(24) IUPAC. *Standard Methods for the Analysis of Oils and Fats and Derivatives*, 7th ed.; Blackwell Scientific Publications: Oxford, U.K., 1987.
(25) AOCS. *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th ed.; AOCS Press: Champaign, IL, 1990.
(26) Senanayake, S. P. J. N.; Shahidi, F. Chemical and stability characteristics of structured lipids from borage (*Borago officialis* L.) and evening primrose (*Oenothera biennis* L.) oils. *Food Chem. Toxicol.* **2002**, *67*, 2038−2045.

Received for review October 13, 2005. Revised manuscript received April 7, 2006. Accepted April 20, 2006. This work was financially supported by the Advanced Foods and Materials Network (AFMnet).

JF052540R