Enzymatic Incorporation of Docosahexaenoic Acid into Borage Oil

S.P.J. Namal Senanayake and Fereidoon Shahidi*

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

ABSTRACT: Lipase-catalyzed acidolysis of acylglycerols of borage (*Borago officinalis* L.) oil with a docosahexaenoic acid (DHA) concentrate, prepared from algal oil, in organic solvents was studied. Seven lipases were used as biocatalysts for the acidolysis reaction. Novozyme 435 from *Candida antarctica,* as compared to lipases from *Mucor miehei* and *Pseudomonas* sp., showed the highest degree of DHA incorporation into borage oil. Other lipases tested, such as those from *Aspergillus niger, C. rugosa, Thermomyces lanuginousus* and *Achromobacter lunatus,* were rather ineffective in the incorporation of DHA into borage oil. Effects of variation of reaction parameters, namely, enzyme load, temperature, time course, and type of solvent, were monitored for *C. antarctica* as the biocatalyst of choice. Incorporation of DHA increased with increasing amount of enzyme, reaching 27.4% at an enzyme concentration of 150 lipase activity units. As incubation time progressed, DHA incorporation also increased. After a reaction time of 24 h, the contents of total n-6 and n-3 polyunsaturated fatty acids in acylglycerols were 44.0 and 27.6%, respectively. The highest degree of DHA incorporation was achieved when hexane was used as the reaction medium. The positional distribution of DHA in modified borage oil was determined using pancreatic lipase hydrolysis. Results showed that DHA was randomly distributed over the *sn*-1, *sn*-2, and *sn*-3 positions of the triacylglycerol. Thus, preparation of modified borage oil acylglycerols containing both DHA (22:6n-3; 27.4%) and γ-linolenic acid (18:3n-6; 17.0%) was successfully achieved and products so obtained may have beneficial effects beyond simple physical mixtures of the two oils. The final oil had a ratio of n-3 to n-6 of 0.42–0.62 which is nutritionally more suitable than the original unaltered borage oil.

Paper no. J8987 in *JAOCS 76,* 1009–1015 (September 1999)

KEY WORDS: Acidolysis, borage oil, *Candida antarctica,* docosahexaenoic acid, γ-linolenic acid, Novozyme 435.

Borage (*Borago officinalis* L.) oil is an important commercially available source of γ-linolenic acid (GLA; 18:3n-6) (1–4). GLA belongs to the n-6 family of polyunsaturated fatty acids (PUFA). In recent years, much research has been directed toward the production of GLA for applications such as curing or treating of certain skin-related diseases as well as diabetes, hypertension, cancer, premenstrual syndrome, inflammatory and cardiovascular disorders (5–7). In humans and in other mammals, GLA is the first metabolite formed during the bioconversion of linoleic acid (18:2n-6) to prostaglandins by the desaturation at the C-6 position *via* the enzyme Δ6-desaturase (1). Individuals who lack Δ6-desaturase suffer from a number of diseases due to an imbalance in GLA production and the formation of successive metabolites that lead to prostaglandins. The physical, chemical, nutritional, and medicinal properties of GLA have also been reviewed (1,5).

The n-3 fatty acids, including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have several health benefits related to cardiovascular disease, immune and renal disorders, inflammation, allergies, diabetes, and cancer (8,9). Apart from having a favorable effect on several disorders, DHA also plays a role in normal brain and retina development in humans and serves as one of the most abundant components of structural lipids of the brain (10–12). Recent studies suggest that a relative deficiency of n-3 fatty acids develops during pregnancy and lactation (13). Some investigators have shown a progressive deterioration of the mother's DHA status during pregnancy, possibly indicating that the mother's capacity to meet the high fetal requirement for DHA is working at its limit and may even be inadequate (12). As a result, researchers have suggested increased intake of n-3 fatty acids, specifically DHA supplementation, during pregnancy. Clinical data on the nutritional importance of n-3 fatty acids, particularly DHA, in infants confirm their critical role in brain and eye development (13). The high concentration of DHA in the retina and cerebral cortex of the fetus suggests its integral involvement in neural and visual function (13).

The beneficial effects of both GLA and DHA are attributed to eicosanoid synthesis. Incorporation of DHA from algal oil into borage oil would provide a unique specialty oil for specific nutritional and clinical needs. This would be possible *via* lipase-catalyzed acidolysis reactions (14). Enzyme-catalyzed reactions have been successfully used for restructuring lipids by interesterification of n-3 fatty acids with either plant or marine oils (15–20). Use of enzymes to produce structured lipids has an advantage over traditional methods (chemical hydrolysis, chemical interesterification, physical blending, etc.) since such methods involve high temperatures which may partially destroy the natural all-*cis* n-3 PUFA by

*To whom correspondence should be addressed.
E-mail: fshahidi@morgan.ucs.mun.ca

oxidation and by *cis-trans* isomerization. Therefore, the mild conditions used in enzymatic reactions provide a promising alternative that could also save energy and increase product selectivity.

In this paper, the ability of different lipases to catalyze the acidolysis of borage oil with DHA was explored. Effects of enzyme load, type of solvent, reaction time, incubation temperature, and the mole ratio of the substrates on the degree of incorporation of DHA in borage oil were also investigated.

MATERIALS AND METHODS

Materials. Borage oil was obtained from Bioriginal Food and Science Corporation (Saskatoon, Canada). Algal oil triacylglycerols with 47.4% DHA were obtained from Martek Biosciences Corporation (Columbia, MD). Seven lipases from *Candida antarctica*, *Mucor miehei*, *Thermomyces lanuginousus*, *Pseudomonas* sp., *C. rugosa*, *Aspergillus niger*, and *Achromobacter lunatus* were acquired from different sources (Table 2). All solvents used in this work were of analytical grade and supplied by Fisher Scientific (Nepeon, Canada).

Preparation of free fatty acids from algal oil. Preparation of free fatty acids from algal oil was carried out according to Wanasundara and Shahidi (21). Algal oil (25 g, treated with 200 ppm butylated hydroxytoluene) was saponified using a mixture of KOH (5.75 g), water (11 mL) and 95% (vol/vol) aqueous ethanol (66 mL) by refluxing for 1 h at the boiling temperature of the mixture (60°C) under an atmosphere of nitrogen. To the saponified mixture, distilled water (60 mL) was added and the unsaponifiable matter was extracted into hexane (2 × 100 mL) and discarded. The aqueous layer containing saponifiable matter was acidified (pH = 1.0) with 3 N HCl. The mixture was transferred to a separatory funnel, and the liberated fatty acids were extracted into hexane (50 mL). The hexane layer containing free fatty acids was then dried over anhydrous sodium sulfate and the solvent removed in a rotary evaporator at 40°C to recover the free fatty acids which were then stored at −60°C until use.

Preparation of DHA concentrate from algal oil by urea complexation. The separation of DHA from the hydrolyzed fatty acids mixture of algal oil was carried out using urea–fatty acid adduct formation according to Wanasundara and Shahidi (21). Free fatty acids (10 g) were mixed with urea (20%, wt/vol) in 150 mL 95% aqueous ethanol and heated at 60°C with stirring until the whole mixture turned into a clear homogeneous solution. Initially, the urea–fatty acid adduct was allowed to crystallize at room temperature but was later placed in a cold room where the temperature was maintained at 4°C for a period of 24 h for further crystallization. The crystals formed were separated from the liquid (nonurea complexing fraction, NUCF) by suction filtration through a thin layer of glass wool. The filtrate (NUCF) was diluted with an equal volume of water and acidified to pH 4–5 with 6 N HCl; an equal volume of hexane was subsequently added and the mixture stirred thoroughly for 1 h, then transferred to a separatory funnel. The hexane layer containing liberated fatty acids was separated from the aqueous layer containing urea and washed with distilled water to remove any remaining urea and then dried over anhydrous sodium sulfate. The solvent was subsequently removed at 40°C using a rotary evaporator. The NUCF was weighed and percentage recovery calculated. The fatty acid composition of this fraction was determined using a gas chromatographic procedure as described elsewhere (22).

Determination of lipase activity. Lipase activity was measured by assaying fatty acids produced from the hydrolysis of triacylglycerols. All experiments were carried out in screw-capped test tubes in triplicate. Triolein was used as the substrate. It was emulsified at a concentration of 50 mM in 5% (wt/vol) gum arabic for 1 min using a Polytron (model PT-3000; Brinkmann, Littau, Switzerland) at 8000 rpm. The assay mixture contained 1 mL substrate-emulsion and the enzyme (10–100 mg). Reactions were carried out for up to 1 h in a shaking water bath at 35°C. Fatty acids release varied linearly with time for more than 1 h. The released fatty acids were assayed colorimetrically as copper soaps using cupric acetate-pyridine reagent (23,24). The purity of triolein was verified by thin-layer chromatography–flame-ionization detection (TLC–FID); no breakdown products were present.

The enzyme reaction in the emulsion system was stopped by adding 6 N HCl (1 mL) and isooctane (5 mL) followed by mixing for 1 min. Cupric acetate (1 mL, 5% wt/vol, pH 6.1) solution was then added to the mixture and stirred for 90 s with a vortex mixer; the absorbance of the upper isooctane layer was read at 715 nm (25). One unit (U) of lipase activity was defined as nanomoles of fatty acids (oleic acid equivalents) produced per min per g of enzyme.

Acidolysis reaction. In general, borage oil (300 mg) was mixed with DHA concentrate (120 mg) at a mole ratio of 1:1 in a screw-capped test tube, then lipase and water (2% by weight of substrates and enzyme) were added in hexane (3 mL). The mixture was incubated at 37°C for 24 h in a shaking water bath at 250 rpm. The reaction was stopped by the addition of 20 mL of a mixture of acetone/ethanol (1:1, vol/vol). All reactions were performed in triplicate.

Analysis of products. The reaction mixture was titrated against a 0.5 N NaOH solution (using a phenolphthalein indicator) in order to neutralize free fatty acids. The mixture was transferred into a separatory funnel and thoroughly mixed with 25 mL of hexane. The lower aqueous layer was separated and discarded. The upper hexane layer containing acylglycerols was passed through a bed of anhydrous sodium sulfate. The acylglycerol fraction was subsequently recovered following hexane removal at 45°C using a rotary evaporator. The fatty acid compositions of the acylglycerols were analyzed by gas chromatography as described elsewhere (22).

Solvent screening. Seven organic solvents, namely, isooctane, acetone, ethyl acetate, hexane, petroleum ether, benzene, and toluene, were used in this study. Borage oil (300 mg) was mixed with DHA concentrate (120 mg), then *C. antarctica* lipase (150 units) and water (2% by weight of substrates and enzyme) were added to the organic solvent (3 mL).

The reaction mixture was incubated at 37°C for 24 h in an orbital shaking water bath at 250 rpm.

Pancreatic lipase hydrolysis. Hydrolysis of DHA-enriched borage oil by pancreatic lipase was carried out according to the method described by Christie (26). Tris-HCl buffer (5 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%, wt/vol) were added to 25 mg of oil in a glass test tube. The whole mixture was allowed to equilibrate at 40°C in a water bath for 1.0 min and subsequently 5.0 mg of porcine pancreatic lipase (EC 3.1.1.3; Sigma) was added to it. The mixture was then placed in a Gyrotory water bath shaker (model G76; New Brunswick Scientific Co. Inc., New Brunswick, NJ) at 250 rpm under nitrogen for 1 h at 40°C. Ethanol (5 mL) was added to stop the enzymatic hydrolysis, followed by addition of 5.0 mL of 6.0 N HCl. The hydrolytic products were extracted three times with 50 mL of diethyl ether and the ether layer was washed twice with distilled water and dried over anhydrous sodium sulfate. After removal of solvent under nitrogen, the hydrolytic products were separated on silica gel TLC plates (with dichlorofluorescein) impregnated with 5% (wt/vol) boric acid. The plates were developed using hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol). After drying, the bands were located by viewing under short (254 nm)- and long (365 nm)-wavelength lights (Spectroline, Model ENF-240C; Spectronics Co., Westbury, NY). The bands were scraped off, and their lipids were extracted into diethyl ether and subsequently used for fatty acid analysis.

RESULTS AND DISCUSSION

A concentrate of docosahexaenoic acid (DHA) was obtained by urea complexation of fatty acids of algal oil. Urea complexation is a well-established technique for elimination of saturated and monounsaturated fatty acids (27). The fatty acid compositions of the original algal oil and that of the DHA concentrate produced from it are given in Table 1. The major fatty acids present in algal oil were 22:6, 14:0, 16:0, and 18:1. As a result of urea adduct formation, 14:0, 16:0, and 18:1 were eliminated almost completely while 22:6 was enriched from 47.4 to 97.1%.

TABLE 1
Fatty Acid Composition of Algal Oil and Its DHA Concentrate Obtained by Urea Complexation[a]

Fatty acid	Algal oil	DHA concentrate
10:0	0.58 ± 0.06	0.47 ± 0.04
12:0	1.12 ± 0.05	0.51 ± 0.02
14:0	14.92 ± 0.07	0.13 ± 0.02
14:1	0.20 ± 0.03	0.16 ± 0.1
16:0	9.05± 0.12	ND
16:1	2.20 ± 0.08	0.25 ± 0.01
18:0	0.20 ± 0.01	ND
18:1	18.92 ± 0.32	0.22 ± 0.01
18:2	1.01 ± 0.02	0.65 ± 0.03
22:5	0.51 ± 0.05	0.41 ± 0.01
22:6	47.42 ± 0.15	97.1 ± 0.02

[a]Results are averages of triplicate determinations from different experiments. ND, not detected; DHA, docosahexaenoic acid.

Seven commercial enzymes from *C. antarctica*, *M. miehei*, *T. lanuginousus*, *Pseudomonas* sp., *C. rugosa*, *Aspergillus niger*, and *Achromobacter lunatus* were screened for their ability to incorporate DHA into borage oil at 37°C in hexane (Table 2). These lipases catalyzed DHA incorporation into borage oil to various extents. The degree of DHA incorporation attained with the various lipases were in the order of *C. antarctica* > *Pseudomonas* sp. > *M. miehei* > *Aspergillus niger* and *Achromobacter lunatus* > *C. rugosa* > *T. lanuginousus*. The lipases from *Aspergillus niger, Achromobacter lunatus, C. rugosa,* and *Thermomyces lanuginousus* were less effective in this regard. The nonspecific lipase from *C. antarctica* gave the highest degree of incorporation (25.8%, after 24 h). Thus, this lipase was selected for subsequent experiments to determine optimal acidolysis conditions.

The effect of enzyme load on the incorporation of DHA (%) into acylglycerols of borage oil is shown in Figure 1. The extent of DHA incorporation was increased by increasing the amount of enzyme in the mixture, but a significant increase was not observed when the enzyme was present at a level greater than 100 units. Thus, 100 units of enzyme were sufficient to saturate the reaction system in terms of the enzyme load.

Temperature is well known to affect enzyme activity. The rate of enzyme activity increases as the environment heats up,

TABLE 2
Effect of Different Lipases on DHA Incorporation into Borage Oil

Enzyme	Commercial code	Manufacturer	Enzyme activity (U)	DHA incorporation (%)[a]
Candida antarctica	Novozyme-435	Novo	554	25.8 ± 0.1
Mucor miehei	Lipozyme-IM	Novo	13,613	13.1 ± 1.2
Pseudomonas sp.	PS-30	Amano Enzyme	11,936	16.8 ± 0.6
Aspergillus niger	AP-12	Amano Enzyme	8,142	2.7 ± 0.5
C. rugosa	AY-30	Amano Enzyme	38,707	2.0 ± 0.2
Thermomyces lanuginousus	Novozyme-677BG	Novo	7,658	1.7 ± 0.4
Achromobacter lunatus	YL-15	Amano Enzyme	2,475	2.7 ± 0.1

[a]The reaction mixture contained 500 mg borage oil, 194 mg DHA, 500 units of enzyme, and 3 mL hexane. The reaction mixture was incubated at 37°C in an orbital shaking water bath at 250 rpm. Results are averages of triplicate determinations from different experiments. Manufacturer locations: Amano, Troy, VA; Novo Nordisk, Franklinton, NC. For abbreviation see Table 1.

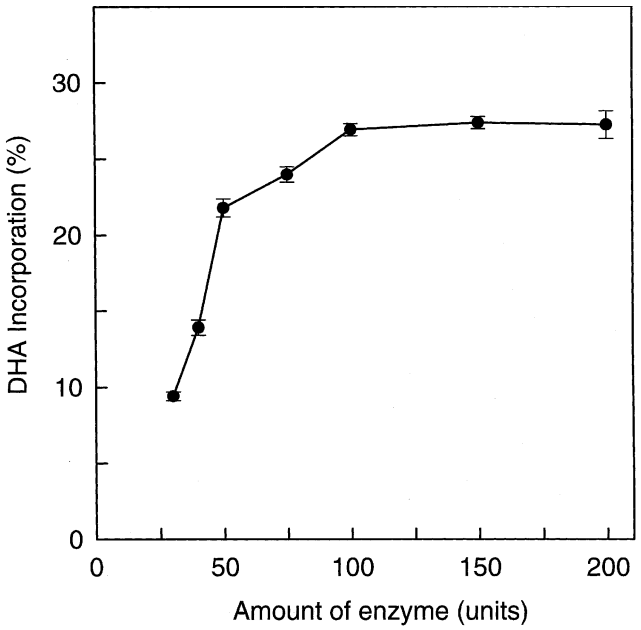

FIG. 1. Effect of enzyme load on the incorporation of docosahexaenoic acid (DHA) into borage oil. The reaction mixture contained 300 mg borage oil, 120 mg DHA, 30–200 units of *Candida antarctica* lipase preparation, and 3 mL hexane. The reaction mixture was incubated at 37°C for 24 h in an orbital shaking water bath at 250 rpm.

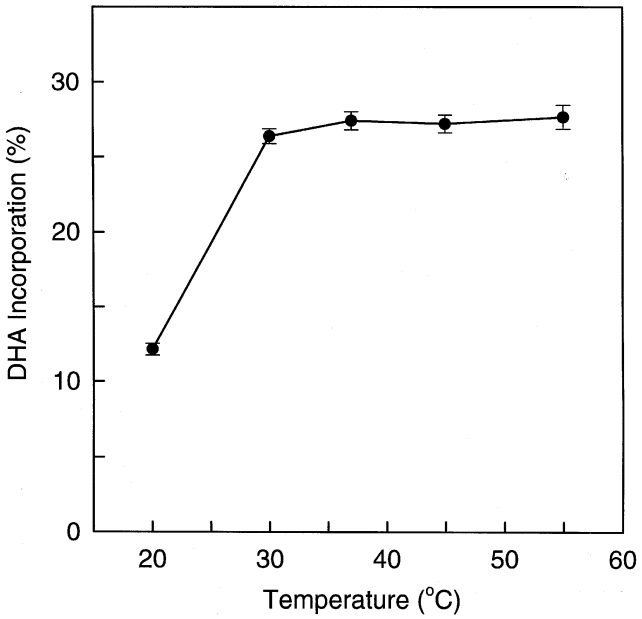

FIG. 2. Effect of temperature on DHA incorporation into borage oil. The reaction mixture contained 300 mg borage oil, 120 mg DHA, 150 units of *Candida antarctica* lipase preparation, and 3 mL hexane. The reaction mixture was incubated at different temperatures (20–55°C) for 24 h in an orbital shaking water bath at 250 rpm. For abbreviations see Figure 1.

until a maximal rate is reached. That rate is the optimal temperature for a given enzyme. When temperature increases further, the reaction rate plummets. The reason is that hydrogen bonds and other weak attractions holding the enzyme in its three-dimensional shape are sensitive to temperature changes in its surroundings. Increased temperature increases the kinetic energy of the enzyme's molecular framework; and molecules in the surroundings also collide more frequently with the enzyme itself. At some point, the disturbances are so great that denaturation occurs. Figure 2 illustrates the effect of temperature on lipase-catalyzed acidolysis by *C. antarctica* lipase. The temperature range tested was 20 to 55°C. DHA incorporation increased as the temperature was increased up to 37°C. When the temperature increased further, the degree of DHA incorporation remained constant. The optimal temperature range for this reaction was 37–55°C. Thus, higher temperatures, up to 55°C, seemed more suitable for better performance of *C. antarctica* lipase. A similar finding has been reported by Akoh and Huang (28).

Table 3 shows the changes in fatty acid composition of borage oil with time. This is useful in monitoring the progress of enzyme-catalyzed reactions. It is also used to determine the shortest time necessary to obtain the highest incorporation of DHA and minimize production costs. DHA was successfully incorporated into borage oil using *C. antarctica* lipase as the biocatalyst. After 24 h of incubation in hexane, 27.4% DHA was incorporated (Table 3). DHA incorporation increased as incubation time increased, up to 24 h. Figure 3 shows the changes in fatty acid composition of borage oil acylglycerols after lipase-catalyzed acidolysis with DHA for up to 48 h. Predominant fatty acids found in borage oil prior to acidolysis reaction were linoleic acid, 18:2n-6 (37.8%) and GLA, 18:3n-6 (23.5%), in agreement with values reported in the literature (1,19,29). As DHA incorporation increased, the proportion of monounsaturated, saturated, and total n-6 fatty acids (linoleic acid and GLA) decreased (Fig. 3). However, the amount of GLA was decreased to a lesser extent (from 23.5 to 17.0% after 24 h incubation). The maximal amount of DHA incorporation was 27.4% (after 24 h). The modified oil had an n-3/n-6 ratio of 0.42–0.62. This oil may prove to be nutritionally more favorable than unmodified borage oil. Similar results from the reaction of borage oil and EPA ethyl ester were obtained with an immobilized *C. antarctica* at a substrate mole ratio of 1:3 (n-3/n-6 of 0.64) (19).

The use of organic solvents is necessary to carry out bioconversion of lipophilic compounds effectively (30,31). Organic solvents induce various physicochemical effects on enzyme molecules, and these effects differ depending upon the type of organic solvent and enzyme used (32). Several variables are critical to enzyme activity in organic media. The nature of solvent is crucial for maintaining a layer of essential water around the enzyme molecules. The most hydrophobic solvents are best for this purpose, as there is no driving force for the essential water to partition into the solvent; thus it remains on and around the enzyme (33). To select the most suitable solvent for acidolysis by *C. antarctica* lipase, we examined the effect of the presence of various organic solvents in the reaction medium (Fig. 4). Hexane was found to be the best solvent, thus the present results lend further support to the findings of Akoh *et al.* (20,34).

TABLE 3
Fatty Acid Composition of Borage Oil Before and After Lipase-Catalyzed Acidolysis with DHA[a]

Major fatty acids	Duration of acidolysis (h)			
	0	12	18	24
14:0	0.07 ± 0.02	0.07 ± 0.04	0.07 ± 0.02	ND
16:0	9.60 ± 0.50	7.10 ± 0.20	7.07 ± 0.20	6.90 ± 0.33
16:1	0.20 ± 0.05	0.16 ± 0.05	0.16 ± 0.05	0.17 ± 0.23
17:0	0.10 ± 0.01	ND	ND	ND
18:0	3.50 ± 0.03	2.62 ± 0.02	2.62 ± 0.02	2.55 ± 0.50
18:1	15.52 ± 0.70	13.36 ± 0.42	11.53 ± 0.30	11.27 ± 0.21
18:2n-6	37.80 ± 1.10	30.84 ± 0.92	27.60 ± 0.55	27.0 ± 0.82
18:3n-6	23.52 ± 0.85	18.73 ± 0.56	17.10 ± 0.70	17.0 ± 0.50
18:3n-3	0.21 ± 0.05	0.18 ± 0.07	0.17 ± 0.08	0.17 ± 0.06
20:0	0.22 ± 0.08	0.20 ± 0.01	0.17 ± 0.02	0.17 ± 0.02
20:1	4.20 ± 0.10	3.0 ± 0.01	3.0 ± 0.12	3.09 ± 0.01
20:2	0.21 ± 0.05	ND	0.15 ± 0.02	0.15 ± 0.06
22:0	0.15 ± 0.07	ND	ND	0.12 ± 0.05
22:1	2.35 ± 0.12	1.90 ± 0.10	1.86 ± 0.05	1.81 ± 0.22
24:1	1.50 ± 0.10	1.0 ± 0.06	0.53 ± 0.16	ND
22:6n-3	ND	20.70 ± 0.57	26.50 ± 0.20	27.4 ± 0.10
n-3/n-6 Ratio	0.003	0.42	0.59	0.62

[a]The reaction mixture contained 300 mg borage oil, 120 mg DHA, 150 units of *C. antarctica* lipase, and 3 mL hexane. The reaction mixture was incubated at 37°C in an orbital shaking water bath at 250 rpm. Experimental results are averages of triplicate determinations. For abbreviations see Table 1.

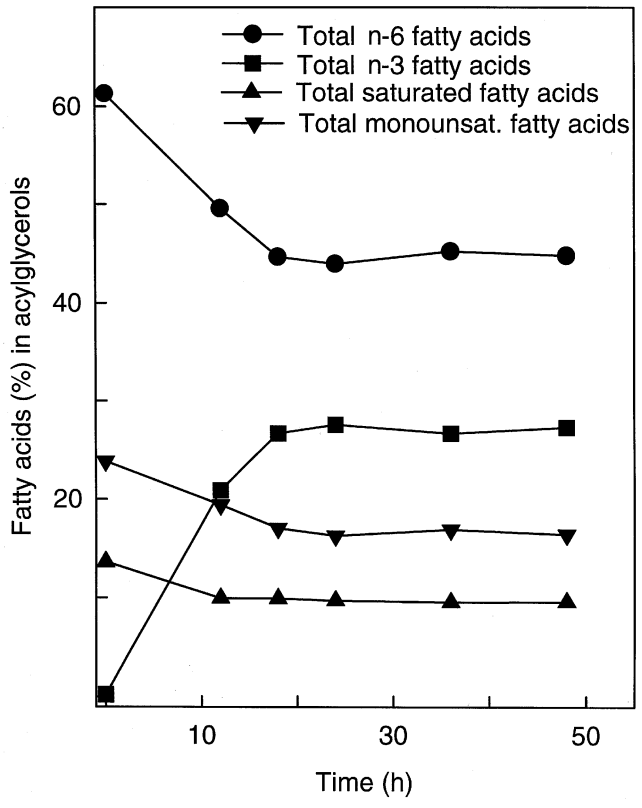

FIG. 3. Changes in total contents of n-3, n-6, saturated, and monounsaturated fatty acids of borage oil during lipase-catalyzed acidolysis with DHA. The reaction mixture contained 300 mg borage oil, 120 mg DHA, 150 units of *C. antarctica* lipase, and 3 mL hexane. The reaction mixture was incubated at 37°C in an orbital shaking water bath at 250 rpm. For abbreviations see Figure 1.

DHA-enriched borage oil was subjected to TLC. The relative content of triacylglycerols (TAG; 86.3%) was much higher than that of diacylglycerols (DAG; 10.7%) and

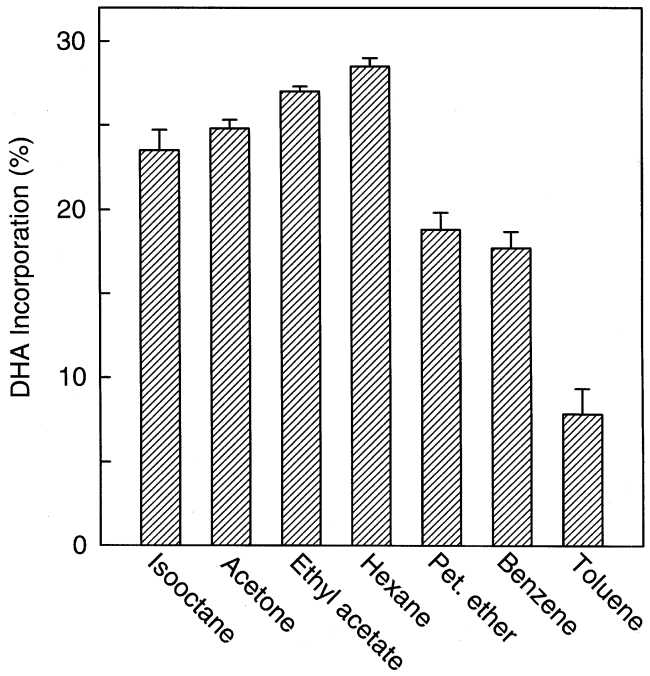

FIG. 4. Effect of different organic solvents on the incorporation of DHA into borage oil. The reaction mixture contained 300 mg borage oil, 120 mg DHA, 150 units of *C. antarctica* lipase preparation, and 3 mL of organic solvent. The reaction mixture was incubated at 37°C for 24 h in an orbital shaking water bath at 250 rpm. For abbreviations see Figure 1.

TABLE 4
Fatty Acid Composition of Acylglycerol Components Separated After Acidolysis by *Candida antarctica* Lipase[a]

	Lipid component (%)		
Fatty acids	TAG (86.3)	DAG (10.7)	MAG (2.9)
14:0	0.10 ± 0.05	0.12 ± 0.01	1.17 ± 0.12
16:0	7.33 ± 0.04	8.47 ± 0.02	9.69 ± 0.06
16:1	0.20 ± 0.02	0.23 ± 0.06	2.13 ± 0.09
18:0	3.10 ± 0.20	3.86 ± 0.21	6.34 ± 0.07
18:1	12.32 ± 0.52	14.83 ± 0.50	16.81 ± 0.67
18:2n-6	26.22 ± 1.20	28.82 ± 0.89	19.70 ± 0.55
18:3n-6	16.10 ± 0.97	15.02 ± 0.20	8.33 ± 0.92
18:3n-3	0.20 ± 0.05	0.20 ± 0.06	0.99 ± 0.10
20:1	2.92 ± 0.20	3.71 ± 0.06	3.53 ± 0.24
20:2	0.13 ± 0.07	0.22 ± 0.05	1.02 ± 0.39
22:0	0.12 ± 0.04	0.21 ± 0.08	0.22 ± 0.10
22:1	1.84 ± 0.10	2.45 ± 0.06	4.35 ± 0.59
22:6-n3	28.31 ± 1.24	25.95 ± 0.78	18.66 ± 0.75

[a]Substrate composition: 300 mg borage oil, 120 mg DHA, 150 units of *C. antarctica* lipase, and 3 mL hexane. Reactions were carried out at 37°C for 24 h in an orbital shaking water bath at 250 rpm. TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols; for other abbreviation see Table 1.

monoacylglycerols (MAG; 2.9%). No free fatty acids were found since these were removed by the NaOH after acidolysis reaction. The fatty acid composition of the isolated bands was analyzed by gas chromatography (Table 4). The content of DHA in the TAG, DAG, and MAG fractions was 28.3, 25.9, and 18.6%, respectively. The content of GLA in TAG, DAG, and MAG fractions was 16.1, 15.0, and 8.3%, respectively. Linoleic acid was mainly found in theTAG (26.2%) and DAG fractions (28.8%).

The effects of borage oil/DHA mole ratio on DHA incorporation by *C. antarctica* are shown in Table 5. As the number of moles of DHA increased, incorporation also increased. DHA incorporation increased by 1.4 times at a 1:2 mole ratio. The degree of DHA incorporation remained constant between borage oil/DHA mole ratios of 1:2 and 1:3. This experiment was designed to go up to a stoichiometric mole ratio of 1:3. As the mole ratio increased, the amount of GLA decreased to a lesser extent (Table 5). Successful incorporation of oleic acid into melon seed oil has been reported by Moussata and

TABLE 5
Effect of Mole Ratio of Substrates on DHA Incorporation into Borage Oil[a]

	Mole ratio		
Major fatty acids	1:1	1:2	1:3
16:0	7.2 ± 0.1	5.8 ± 0.2	5.4 ± 0.1
18:0	3.0 ± 0.1	2.4 ± 0.1	2.2 ± 0.1
18:1	12.3 ± 0.2	10.3 ± 0.3	10.0 ± 0.1
18:2n-6	26.4 ± 0.2	22.4 ± 0.6	21.8 ± 0.2
18:3n-6	16.3 ± 0.2	14.0 ± 0.1	13.4 ± 0.3
22:6n-3	27.0 ± 0.3	37.4 ± 0.8	39.7 ± 1.2

[a]Mole ratios of borage oil to DHA were varied from 1:1 to 1:3. Reactions were carried out at 37°C for 24 h in an orbital shaking water bath at 250 rpm. For abbreviation see Table 1.

TABLE 6
Positional Distribution of Fatty Acids in DHA-Enriched Borage Oil[a]

		Position	
PUFA (%)	TAG	*sn*-2	*sn*-1 + *sn*-3
18:2n-6	26.22 ± 0.90	23.63 ± 1.20	29.26 ± 0.83
18:3n-6	16.07 ± 0.82	19.84 ± 0.90	16.83 ± 0.56
22:6n-3	28.31 ± 1.50	28.51 ± 0.50	30.31 ± 0.80

[a]PUFA, polyunsaturated fatty acid; for other abbreviations see Tables 1 and 4.

Akoh (35) who showed that the use of nonspecific lipase PS30 from *Pseudomonas* sp. resulted in a 53.4% incorporation at a mole ratio of 1:5.

The fatty acid composition of the *sn*-2 and *sn*-1 + *sn*-3 positions of the DHA-enriched borage oil, performed by pancreatic lipase hydrolysis, is shown in Table 6. Pancreatic lipase is used to hydrolyze quite specifically the fatty acids esterified to the primary positions of TAG yielding 2-MAG, the fatty acid composition of which accurately reflects that of position 2 in the original TAG. Our results showed that DHA was randomly distributed over the three positions, confirming the nonspecificity of the *C. antarctica* enzyme. Previously, the stereospecific distribution of the fatty acids in native borage oil has been determined (36). The GLA was distributed asymmetrically and was preferentially esterified at the *sn*-2 and *sn*-3 positions (3,36). Our results showed that in DHA-enriched borage oil, GLA was mainly associated with the *sn*-2 position. Intestinal absorption of fatty acids has been reported to be dependent on their arrangement in the TAG molecules. During digestion, fatty acids in the *sn*-1 and *sn*-3 positions of the TAG are liberated by a positional specific enzyme such as pancreatic lipase, but the fatty acids attached to the *sn*-2 position of the TAG are absorbed and distributed in the body in the chylomicron form. However, clinical studies need to be carried out to verify this latter assumption. The DHA-enriched oils prepared in this study would be potentially useful as they contain desirable functional fatty acids in the same molecule.

ACKNOWLEDGMENTS

We are grateful to the Natural Sciences and Engineering Research Council (NSERC) of Canada and Memorial University of Newfoundland for partial financial support. The supply of the algal oil from Martek Biosciences Corporation (Columbia, MD) and borage oil from Bioriginal Food and Science Corporation (Saskatoon, Canada) is acknowledged.

REFERENCES

1. Gunstone, F.D., Gamma-Linolenic Acid—Occurrence and Physical and Chemical Properties, *Prog. Lipid Res. 31*:145–163 (1992).
2. Rahmatullah, M.S.K.S., V.K.S. Shukla, and K.D. Mukherjee, γ-Linolenic Acid Concentrates from Borage and Evening Primrose Oil Fatty Acids *via* Lipase-Catalyzed Esterification, *J. Am. Oil Chem. Soc. 71*:563–569 (1994).
3. Redden, R.P., X. Lin, J. Fahey, and F. Horrobin, Stereospecific Analysis of the Major Triacylglycerol Species Containing γ-

Linolenic Acid in Evening Primrose Oil and Borage Oil, *J. Chromatogr. 704*:99–111 (1995).
4. Rahmatullah, M.S.K.S., V.K.S. Shukla, and K.D. Mukherjee, Enrichment of γ-Linolenic Acid from Evening Primrose Oil and Borage Oil *via* Lipase-Catalyzed Hydrolysis, *J. Am. Oil Chem. Soc. 71*:569–573 (1994).
5. Horrobin, D.F., Clinical Applications of n-6 Essential Fatty Acids: Atopic Eczema and Inflammation, Diabetic Neuropathy and Retinopathy, Breast Pain and Viral Infections, in *Essential Fatty Acids and Eicosanoids*, edited by A. Sinclair and R. Gibson, American Oil Chemists' Society, Champaign, 1992, pp. 367–372.
6. Carter, J.P., Gamma-Linolenic Acid as a Nutrient, *Food Technol. 6*:72–82 (1988).
7. Horrobin, D.F., Unsaturated Lipids: A New Approach to the Treatment of Cancer, in *Effects of Fatty Acids and Lipids in Health and Disease*, edited by C. Galli, A.P. Simopoulos, and E. Tremoli, World Rev. Nutr. Diet, Karger, Basel, 1994, pp. 77–80.
8. Illingworth, D.R., and D. Ullmann, Effects of Omega-3 Fatty Acids on Risk Factors for Cardiovascular Disease, in *Omega-3 Fatty Acids in Health and Disease*, edited by R.S. Lees and M. Karel, Marcel Dekker, Inc., New York, 1990, pp. 39–69.
9. Haumann, B.F., Nutritional Aspects of n-3 Fatty Acids, *INFORM 8*:428–447 (1997).
10. Carlson, S.E., The Role of PUFA in Infant Nutrition, *Ibid. 6*:940–946 (1995).
11. Horrobin, D.F., Medical Roles of Metabolites of Precursor EFA, *Ibid. 6*:428–435 (1995).
12. Salem, N., H. Kim, and J.A. Yergey, Docosahexaenoic Acid: Membrane Function and Metabolism, in *Health Effects of Polyunsaturated Fatty Acids in Seafoods*, edited by A.P. Simopoulos, R.R. Kifer, and R.E. Martin, Academic Press, Inc., New York, 1986, pp. 263–317.
13. Simopoulos, A.P., Omega-3 Fatty Acids in Growth and Development, in *Omega-3 Fatty Acids in Health and Disease*, edited by R.S. Lees and M. Karel, Marcel Dekker, Inc., New York, 1990, pp. 115–156.
14. Akoh, C.C., Structured Lipids—Enzymatic Approach, *INFORM 6*:1055–1061 (1995).
15. Haraldsson, G.G., P.A. Höskuldsson, and S.T. Sigurdsson, The Preparation of Triglycerides Highly Enriched with ω-3 Polyunsaturated Fatty Acids *via* Lipase Catalysed Interesterification, *Tetrahedron Lett. 30*:1671–1674 (1989).
16. Sridhar, R., and G. Lakshminarayana, Incorporation of Eicosapentaenoic and Docosahexaenoic Acids into Groundnut Oil by Lipase-Catalyzed Ester Interchange, *J. Am. Oil Chem. Soc. 69*:1041–1042 (1992).
17. Huang, K.-H., and C.C. Akoh, Lipase Catalyzed Incorporation of n-3 Polyunsaturated Fatty Acids into Vegetable Oils, *Ibid. 71*:1277–1280 (1994).
18. Huang, K.-H., C.C. Akoh, and M.C. Erickson, Enzymatic Modification of Melon Seed Oil: Incorporation of Eicosapentaenoic Acid, *J. Agric. Food Chem. 42*:2646–2648 (1994).
19. Akoh, C.C., and R.V. Sista, Enzymatic Modification of Borage Oil: Incorporation of Eicosapentaenoic Acid, *J. Food Lipids 2*:231–238 (1995).
20. Akoh., C.C., B.H. Jennings, and D.A. Lillard, Enzymatic Modification of Evening Primrose Oil: Incorporation of n-3 Polyunsaturated Fatty Acids, *J. Am. Oil Chem. Soc. 73*:1059–1062 (1996).
21. Wanasundara, U.N., and F. Shahidi, Concentration of Omega-3 Polyunsaturated Fatty Acids of Seal Blubber Oil by Urea Complexation: Optimization of Reaction Conditions, *Food Chem. 65*:41–49 (1999).
22. Wanasundara, U.N., and F. Shahidi, Storage Stability of Microencapsulated Seal Blubber Oil, *J. Food Lipids 2*:73–86 (1995).
23. Lowry, R.R., and I.J. Tinsley, Rapid Colorimetric Determination of Free Fatty Acids, *J. Am. Oil Chem. Soc. 53*:470–472 (1976).
24. Kwon, D.Y., and J.S. Rhee, A Simple and Rapid Colorimetric Method for Determination of Free Fatty Acids for Lipase Assay, *Ibid. 63*:89–92 (1986).
25. Arribére, M.C., N.S. Priolo, and N.O. Caffini, Characterization of the Lipolytic System of Germinated Sunflower Seeds, *Acta Aliment. 23*:313–324 (1994).
26. Christie, W.W., Structural Analysis of Lipids by Means of Enzymatic Hydrolysis, in *Lipid Analysis*, edited by W.W. Christie, Pergamon Press, New York, 1982, pp. 155–166.
27. Ratnayake, W.M.N., B. Olsson, D. Matthews, and R.G. Ackman, Preparation of Omega-3 Concentrates from Fish Oils *via* Urea Complexation, *Fat Sci. Technol. 90*:381–386 (1988).
28. Akoh, C.C., and K. Huang, Enzymatic Synthesis of Structured Lipids: Transesterification of Triolein and Caprylic Acid, *J. Food Lipids 2*:219–230 (1995).
29. Horrobin, D.F., Nutritional and Medical Importance of Gamma-Linolenic Acid, *Prog. Lipid Res. 31*:163–194 (1992).
30. Kang, S.T., and J.S. Rhee, Effect of Solvents on Hydrolysis of Olive Oil by Immobilized Lipase in Reverse Phase System, *Biotechnol. Lett. 11*:37–42 (1989).
31. He, Y., and F. Shahidi, Enzymatic Esterification of ω-3 Fatty Acid Concentrates from Seal Blubber Oil with Glycerol, *J. Am. Oil Chem. Soc. 74*:1133–1136 (1997).
32. Li, Z. and O.P. Ward, Lipase-Catalyzed Esterification of Glycerol and n-3 Polyunsaturated Fatty Acid Concentrate in Organic Solvent, *Ibid. 70*:745–748 (1993).
33. Laane, C.S., K.V. Boeren, and C. Veeger, Rules for Optimization of Biocatalysts in Organic Solvents, *Biotechnol. Bioeng. 30*:81–87 (1987).
34. Akoh., C.C., B.H. Jennings, and D.A. Lillard, Enzymatic Modification of Trilinolein: Incorporation of n-3 Polyunsaturated Fatty Acids, *J. Am. Oil Chem. Soc. 72*:1317–1321 (1995).
35. Moussata, C.O., and C.C. Akoh, Lipase-Catalyzed Incorporation of Oleic Acid into Melon Seed Oil, *J. Am. Oil Chem. Soc. 74*:177–179 (1997).
36. Lawson, L.D., and B.G. Hughes, Triacylglycerol Structure of Plant and Fungal Oils Containing γ-Linolenic Acid, *Lipids 23*:313–317 (1988).

[Received August 21, 1998; accepted April 2, 1999]