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Structured Lipids via Lipase-Catalyzed Incorporation of Eicosapentaenoic Acid into Borage (*Borago officinalis* L.) and Evening Primrose (*Oenothera biennis* L.) Oils

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Enzymatic acidolysis of borage oil (BO) or evening primrose oil (EPO) with eicosapentaenoic acid (20:5*n*−3; EPA) was studied. Of the six lipases that were tested in the initial screening, nonspecific lipase PS-30 from *Pseudomonas* sp. resulted in the highest incorporation of EPA into both oils. This enzyme was further studied for the influence of enzyme load, temperature, time, type of organic solvent, and mole ratio of substrates. The products from the acidolysis reaction were analyzed by gas chromatography (GC). The highest incorporation of EPA in both oils occurred at 45−55 °C and at 150−250 enzyme activity units. One unit of lipase activity was defined as nanomoles of fatty acids (oleic acid equivalents) produced per minute per gram of enzyme. Time course studies indicated that EPA incorporation was increased up to 26.8 and 25.2% (after 24 h) in BO and EPO, respectively. Among the solvents examined, *n*-hexane served best for the acidolysis of EPA with both oils. The effect of the mole ratio of oil to EPA was studied from 1:1 to 1:3. As the mole ratio of EPA increased, the incorporation increased from 25.2−26.8 to 37.4−39.9% (after 24 h). The highest EPA incorporations of 39.9 and 37.4% in BO and EPO, respectively, occurred at the stoichiometric mole ratio of 1:3 for oil to EPA.

KEYWORDS: Structured lipids; acidolysis; borage oil; evening primrose oil; *Borago officinalis*; *Oenothera biennis*; eicosapentaenoic acid; enzymes; Lipase PS-30; *Pseudomonas* sp.

INTRODUCTION

There has been much interest in borage (*Borago officinalis* L.) and evening primrose (*Oenothera biennis* L.) oils in recent years because they contain appreciable amounts of γ-linolenic acid (18:3*n*−6; GLA). Seeds of borage and evening primrose contain 29−35 and 17−25% lipids, respectively, with corresponding GLA contents of 20−25 and 7−10% in the oil (*1*, *2*). GLA is the Δ6 desaturase product of linoleic acid, and it becomes essential under certain disease conditions (e.g., atopic eczema, multiple sclerosis, premenstrual syndrome, diabetes, cancer). These conditions cause impairment of Δ6 desaturase activity and decrease synthesis of dihomo-γ-linolenic acid (20:3*n*−6; DGLA) and its metabolites such as prostaglandin E_1 (PGE_1) and 15-hydroxydihomo-γ-linolenic acid (15-OH-DGLA) (*3*). It is generally recognized that disease conditions associated with an impaired Δ6 desaturase activity may be alleviated by dietary supplementation with GLA (*4*, *5*).

Similarly, eicosapentaenoic acid (20:5*n*−3; EPA) has been used in the prevention and treatment of atherosclerosis, arthritis, thrombosis, inflammation, diabetes, and cancer (*6*). EPA also exhibits various physiological functions on being incorporated into membrane phospholipids. Fish and marine oils are the predominant sources of *n*−3 polyunsaturated fatty acids (PUFA). Consumption of fish or fish oils that are rich in *n*−3 PUFA has been associated with reduced risk of developing cardiovascular diseases and a variety of other pathological conditions. Recently, several animal studies and clinical trials have shown that supplementation of nutritional formulas with EPA and GLA can reduce inflammation while promoting vasodilation and oxygen delivery following acute lung injury (*7*−*9*). Thus, EPA and/or other *n*−3 fatty acids have been incorporated into borage (*6*, *10*−*14*), evening primrose (*6*, *13*−*15*), and vegetable oils (*16*, *17*).

In recent years, lipase-catalyzed reactions have become the subject of interest for the production of structured lipids (SL). Structured lipids are triacylglycerols (TAG) that have fatty acid profiles and/or stereospecific distribution of fatty acids modified via chemical or enzymatic means to achieve specific health benefits and/or functional properties (*6*, *18*). These products can be efficiently synthesized by exchanging fatty acids at primary positions and/or *sn*-2 position of TAG with desired fatty acids using a variety of lipases. Presently, such TAG are designed for use in selected nutritional and food applications (*6*). This paper deals with the synthesis of SL by acidolysis of borage oil (BO) and evening primrose oil (EPO) with EPA using lipase PS-30 from *Pseudomonas* sp. The effects of enzyme load, reaction temperature, time, type of organic solvent, and mole

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Table 1. Effect of Different Lipases on EPA Incorporation (Percent) into Borage (BO) and Evening Primrose Oils (EPO)

enzyme	commercial code	enzyme activity (units)	EPA incorporation (%) in BO[a]	EPA incorporation (%) in EPO[a]
Candida antarctica	Novozym-435	554	21.0 ± 0.8	22.9 ± 1.9
Mucor miehei	Lipozyme-IM	13613	23.3 ± 2.1	25.6 ± 0.7
Pseudomonas sp.	PS-30	11936	28.7 ± 0.6	30.7 ± 0.2
Aspergillus niger	AP-12	8142	14.1 ± 0.6	13.0 ± 2.4
Candida rugosa	AY-30	38707	12.0 ± 1.5	10.7 ± 1.1
Thermomyces lanuginosus	Novozym-677BG	7658	6.81 ± 2.1	8.51 ± 2.9

[a] The reaction mixture contained 500 mg of oil, 178 mg of EPA, 500 units of enzyme, and 3 mL of hexane. The reaction mixture was incubated at 37 °C for 24 h in an orbital shaking water bath at 250 rpm. Results are mean of triplicate determinations from different experiments.

ratio of substrates on the percent incorporation of EPA in BO and EPO were studied.

MATERIALS AND METHODS

Materials. Borage oil was obtained from Bioriginal Food and Science Corp. (Saskatoon, SK, Canada), and evening primrose oil was provided by Efamol, Inc. (Kentville, NS, Canada). EPA concentrate (93.1% EPA) was provided through Dr. T. Ohshima (Tokyo, Japan). Lipases, Novozym-435 (*Candida antarctica*), Lipozyme-IM (*Mucor miehei*), and Novozym-677BG (*Thermomyces lanuginosus*) were acquired from Novo Nordisk (Franklinton, NC). Lipases PS-30 (*Pseudomonas* sp.), AP-12 (*Aspergillus niger*), and AY-30 (*Candida rugosa*) were obtained from Amano Enzyme USA Co. Ltd. (Troy, VA). All other chemicals were of American Chemical Society (ACS) grade or better.

Determination of Enzyme Activity of Microbial Lipases. Lipase activity was measured by assaying fatty acids produced from the hydrolysis of triacylglycerols (triolein) (*12*). 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% (w/v) gum arabic for 1 min using a Polytron homogenizer (model PT-3000; Brinkmann, Littau, Switzerland) at 8000 rpm. The assay mixture contained 1 mL of substrate emulsion and the enzyme (10−100 mg). Reactions were carried out for up to 1 h in a shaking water bath at 250 rpm and 35 °C. Fatty acid 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 (*19*, *20*). The purity of triolein, prior to hydrolysis, was verified by thin layer chromatography−flame ionization detection (TLC-FID); no breakdown products (monoacylglycerols, diacylglycerols, or free fatty acids) 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% w/v, pH 6.1) solution was then added to the mixture and stirred for 90 s using a vortex mixer; the absorbance of the upper isooctane layer was read at 715 nm (*21*). One unit of lipase activity was defined as nanomoles of fatty acids (oleic acid equivalents) produced per minute per gram of enzyme. Enzyme activities of microbial lipases tested are shown in **Table 1**.

Acidolysis of Borage and Evening Primrose Oils with EPA. Unless otherwise specified, for general modification of oils, BO (300 mg) or EPO (297 mg) was mixed with EPA (115 mg), in a screw-capped test tube, and then lipase (150 enzyme activity units) and water (2 wt % of substrates plus enzyme) were added in hexane (3 mL). The mixture was stirred in an orbital shaking water bath at 37 °C for 24 h at 250 rpm. All reactions were performed in triplicate and mean values reported.

Enzyme Screening. Six microbial enzymes, namely, lipases from *Candida antarctica*, *Mucor miehei*, *Pseudomonas* sp., *Aspergillus niger*, *Candida rugosa*, and *Thermomyces lanuginosus*, were used in this study. BO or EPO (500 mg) was mixed with EPA (178 mg), and then lipase (500 enzyme activity units) and water (2 wt % of substrates plus enzyme) were added in hexane (3 mL). The reaction mixture was incubated at 37 °C for 24 h in an orbital shaking water bath at 250 rpm.

Analysis of Products. The enzymes were removed by passing the reaction mixture through a bed of anhydrous sodium sulfate. Samples were placed in 250-mL conical flasks, and 20 mL of a mixture of acetone/ethanol (1:1, v/v) was added. The reaction mixture was titrated with 0.5 N NaOH to a phenolphthalein endpoint. The mixture was transferred to 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 acylglycerol fraction was analyzed by TLC-FID. The fatty acid composition of the acylglycerols was analyzed by gas chromatography as described by Senanayake and Shahidi (*22*).

Hydrolysis of Enzymatically Modified Oils by Pancreatic Lipase. Removal of constituents other than triacylglycerols (TAG) from modified BO and EPO was carried out using column chromatography (1.25 cm i.d. and 10 cm height) on silicic acid (100−200 mesh size). The column was first washed with hexane, and then 1.0 g of oil was introduced onto it. Hexane (50 mL) was added to the column, which was then eluted with 10% (v/v) diethyl ether in hexane (250 mL). The solvent was removed under vacuum at 40 °C using a rotary evaporator. The recovered oil was then passed through a layer of anhydrous sodium sulfate. To prevent oxidation of purified oils, a few crystals of butylated hydroxytoluene (BHT) were added to the mixture.

Hydrolysis of modified TAG by pancreatic lipase was carried out according to the method described by Christie (*23*). Trishydrochloric buffer (5 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 oil in a glass test tube. The whole mixture was allowed to equilibrate at 40 °C in a water bath for 1 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 200 rpm under nitrogen for 8−10 min 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 the solvent under vacuum at 30 °C, the hydrolytic products were separated on silica gel TLC plates (20 × 20 cm; 60-Å mean pore diameter, 2−25 μm mean particle size, 500-μm thickness, with dichlorofluorescein, Sigma) impregnated with 5% (w/v) boric acid. The plates were developed using hexane/diethyl ether/acetic acid (70:30:1, v/v/v). After drying, the bands were located by viewing under short (254 nm) and long (356 nm) wavelength UV lights (Spectraline, model ENF-240C, Spectronics Co., Westbury, NY). The bands were scraped off and their lipids extracted into chloroform/methanol (1:1, v/v) or diethyl ether and subsequently used for fatty acid analysis by the GC procedure described elsewhere (*22*).

RESULTS AND DISCUSSION

Lipase Screening. Six commercial enzymes from *C. antarctica*, *M. miehei*, *Pseudomonas* sp., *C. rugosa*, *A. niger*, and *T. lanuginosus* were screened for their ability to incorporate EPA into BO and EPO at 37 °C in hexane (**Table 1**). Novozym-435 from *C. antarctica* was immobilized on a macroporous acylic resin. Lipozyme-IM is a 1,3-specific lipase from *M.*

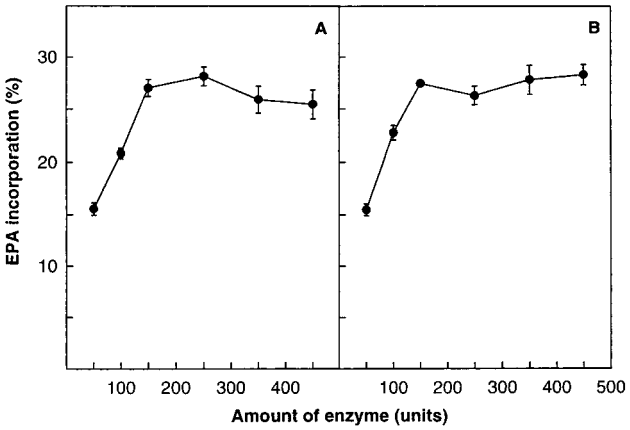

Figure 1. Effect of enzyme load on the incorporation of EPA into borage (A) and evening primrose oils (B). The reaction mixture contained 297–300 mg of oil, 115 mg of EPA, 50–450 units of *Pseudomonas* sp. lipase preparation, and 3 mL of hexane. The reaction mixture was incubated at 37 °C for 24 h in an orbital shaking water bath at 250 rpm.

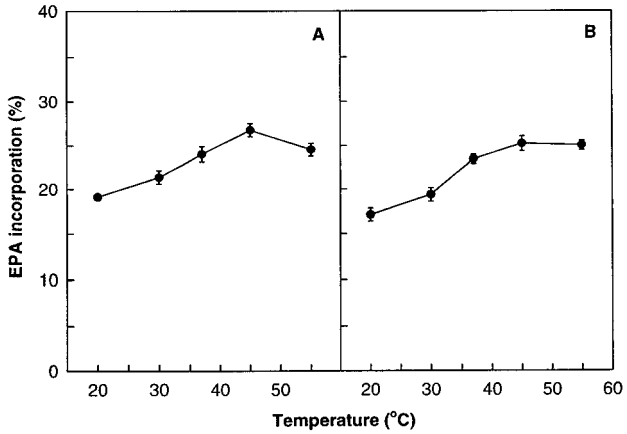

Figure 2. Effect of temperature on EPA incorporation into borage (A) and evening primrose oils (B). The reaction mixture contained 297–300 mg of oil, 115 mg of EPA, 150 units of *Pseudomonas* sp. lipase preparation, and 3 mL of hexane. The reaction mixture was incubated at different temperatures (20–55 °C) for 24 h in an orbital shaking water bath at 250 rpm.

miehei immobilized on a macroporous anion exchange resin. However, lipases from *Pseudomonas* sp., *A. niger*, *C. rugosa*, and *T. lanuginosus* used were free enzymes. These lipases catalyzed EPA incorporation into BO and EPO to various extents. The degree of EPA incorporation attained with various lipases was in the order of *Pseudomonas* sp. > *M. miehei* > *C. antarctica* > *A. niger* > *C. rugosa* > *T. lanuginosus*. The lipase from *Pseudomonas* sp. gave the highest degree of EPA incorporation into BO (28.7%, after 24 h) and EPO (30.7%, after 24 h). Because the acidolysis of both oils with EPA was best with lipase from *Pseudomonas* sp., this enzyme was chosen for subsequent experiments.

Effect of Enzyme Load. The effect of enzyme load (lipase PS-30 from *Pseudomonas* sp.) on the incorporation of EPA (percent) into BO and EPO is shown in **Figure 1** parts A and B, respectively. There was a gradual increase in EPA incorporation in both oils when the enzyme load was increased up to 150 enzyme activity units. In the case of BO, however, EPA was only marginally increased after more enzymes were added up to 250 enzyme activity units. Above 250 enzyme activity units, there was a slight decrease in EPA incorporation into BO. With an increase in enzyme activity units from 150 to 450, the incorporation of EPA into EPO did not change ($p < 0.05$). The highest EPA incorporation in both oils (28.1% EPA in BO and 27.4% EPA in EPO) was obtained with 150−250 units of enzyme. Moussata and Akoh (*24*) used nonspecific lipase PS-30 from *Pseudomonas* sp. to modify the fatty acid composition of melon seed oil by incorporation of oleic acid (18:1*n*−9). An enzyme load of 5−10% (wt %) was suggested to minimize the cost of the overall process under their assay conditions. However, our results showed that an enzyme load of 3−5% (150−250 enzyme activity units) was sufficient to minimize the cost of the overall process.

Effect of Temperature. The effect of temperature on lipase-mediated acidolysis of oils with fatty acids has been investigated (*11*, *12*, *25*). Temperature effects on lipase-catalyzed ester synthesis were reported to be dependent on the reaction medium, enzyme source, and substrate (*26*). Kosugi and Azuma (*27*) used an immobilized lipase from *C. antarctica* for production of pure TAG from EPA or docosahexaenoic acid (22:6*n*−3; DHA) with glycerol. The rate of TAG formation was reported to be faster at 60 °C even though the TAG yield was the same at 40 and 60 °C.

The effect of temperature on acidolysis of BO and EPO with EPA by lipase PS-30 is shown in **Figure 2**. This reaction parameter was studied to determine the optimum reaction temperature for *Pseudomonas* sp. lipase. Reaction temperatures were varied from 20 to 55 °C. Low EPA incorporation was observed at lower temperatures (20−30 °C). The increase in temperature increased activity without inactivation of enzyme up to 45−55 °C. At this temperature range, the incorporation of EPA reached 25−26 and 25% in BO and EPO, respectively. These results show that *Pseudomonas* sp. lipase is more active at higher temperatures (45−55 °C) than at 20−30 °C. This finding lends further support to those reported by Noureddini and Harmeier (*28*), who used lipase PS from *Pseudomonas* sp. for production of monoacylglycerols from soybean oil with glycerol. The amount of monoacylglycerol formation was higher at 55 °C. Meanwhile, Ju et al. (*11*) studied the effect of temperature on acidolysis reaction (micromoles of *n*−3 PUFA per time) of BO with *n*−3 PUFA. The IM-60 (from *M. miehei*) lipase-catalyzed acidolysis reaction showed a maximum rate at 50 °C.

Time Course. Table 2 shows the changes in fatty acid composition of BO with time during the course of acidolysis with EPA. Using *Pseudomonas* sp. lipase as the biocatalyst, EPA incorporation increased as incubation time increased, up to 24 h. The most abundant fatty acids present in BO prior to the acidolysis reaction were linoleic acid (37.8%) and GLA (23.5%), in agreement with literature values (*2*, *10*, *29*). The maximum amount of EPA incorporation in BO was 26.8% (after 24 h). The modified oil had an *n*−3/*n*−6 ratio of 0.18−0.66 (**Table 2**), which may prove to be nutritionally more favorable than unmodified BO. Similar results for the reaction of BO 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) (*10*).

Similarly, EPA was incorporated into EPO using *Pseudomonas* sp. lipase; the changes in fatty acid composition with time are given in **Table 3**. After 24 h of reaction, the amount of EPA into EPO was 25.2%. The modified EPO had an *n*−3/*n*−6 ratio of 0.14−0.40 (**Table 2**). Huang et al. (*30*) incorporated EPA into melon seed oil using two immobilized lipases, IM60 from *M. miehei* and SP435 from *C. antarctica*. The IM60 lipase gave a higher incorporation of EPA (31.2% in 24 h) than the SP435 lipase (24.0% in 24 h). Time courses

Table 2. Fatty Acid Composition of Borage and Evening Primrose Oils before and after Acidolysis with EPA by Lipase from *Pseudomonas* sp.[a]

	borage oil		evening primrose oil	
	0 h	24 h	0 h	24 h
14:0	0.07 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	ND[b]
16:0	9.60 ± 0.50	6.42 ± 0.56	6.17 ± 0.09	4.02 ± 0.50
16:1	0.20 ± 0.05	0.10 ± 0.08	0.04 ± 0.02	ND
17:0	0.10 ± 0.01	ND	0.08 ± 0.01	ND
18:0	3.50 ± 0.03	2.59 ± 0.07	1.75 ± 0.12	1.11 ± 0.08
18:1	15.5 ± 0.70	12.0 ± 0.63	8.65 ± 0.56	5.56 ± 0.41
18:2*n*–6	37.8 ± 1.10	25.3 ± 0.52	72.6 ± 0.91	55.6 ± 0.94
18:3*n*–6	23.5 ± 0.85	15.2 ± 0.84	9.12 ± 0.38	7.12 ± 0.55
18:3*n*–3	0.21 ± 0.05	0.15 ± 0.02	0.16 ± 0.03	ND
20:0	0.22 ± 0.08	0.21 ± 0.05	0.34 ± 0.05	ND
20:1	4.20 ± 0.10	3.00 ± 0.53	0.29 ± 0.07	ND
20:2	0.21 ± 0.05	0.14 ± 0.03	0.05 ± 0.05	ND
20:5*n*–3	ND	26.8 ± 0.96	ND	25.2 ± 1.45
22:0	0.15 ± 0.07	0.15 ± 0.06	0.14 ± 0.05	ND
22:1	2.35 ± 0.12	2.10 ± 0.54	0.12 ± 0.01	ND
24:1	1.50 ± 0.10	1.10 ± 0.07	ND	ND
n–3/*n*–6 ratio	0.003	0.66	0.001	0.40

[a] The reaction mixture contained 297–300 mg of oil, 115 mg of EPA, 150 units of *Pseudomonas* sp., and 3 mL of hexane. The reaction mixture was incubated at 37 °C in an orbital shaking water bath at 250 rpm. Experimental results are means of triplicate determinations. [b] Not detected.

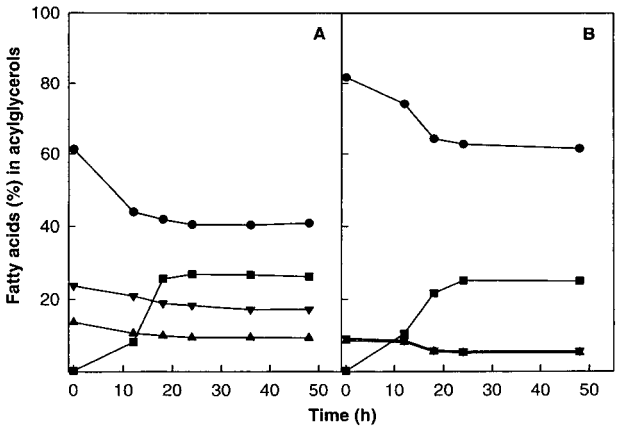

Figure 3. Changes in total contents of *n*–3, *n*–6, saturated, and monounsaturated fatty acids of borage (A) and evening primrose oils (B) during lipase-catalyzed acidolysis with EPA. The reaction mixture contained 297–300 mg of oil, 115 mg of EPA, 150 units of *Pseudomonas* sp. lipase preparation, and 3 mL of hexane. The reaction mixture was incubated at 37 °C in an orbital shaking water bath at 250 rpm. Abbreviations: PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids. Symbols: ●, total *n*–6 PUFA; ■, total *n*–3 PUFA; ▲, total SFA; ▼, total MUFA.

of acidolysis reaction of BO and EPO with EPA by lipase PS-30 are illustrated in parts A and B of **Figure 3**, respectively. In both oils, the total content of monounsaturated, saturated, and *n*−6 fatty acids (linoleic acid and GLA) decreased up to 24 h and then reached a plateau (**Figure 3A,B**), whereas EPA incorporation increased up to 24 h. In BO, the amount of GLA decreased from 23.5 to 15.2% in 24 h (**Table 2**). Similarly, for EPO the GLA content decreased from 9.12 to 7.12% in 24 h (**Table 2**). After 24 h of reaction with EPA, the linoleic acid content in BO and EPO decreased by 12.5 and 17.0%, respectively. The EPA incorporation into BO and EPO after 24 h was 26.8 and 25.2%, respectively (**Table 2**). Sridhar and Lakshminarayana (*31*) were able to modify groundnut oil by incorporating EPA and DHA using the *sn*-1,3-specific lipase

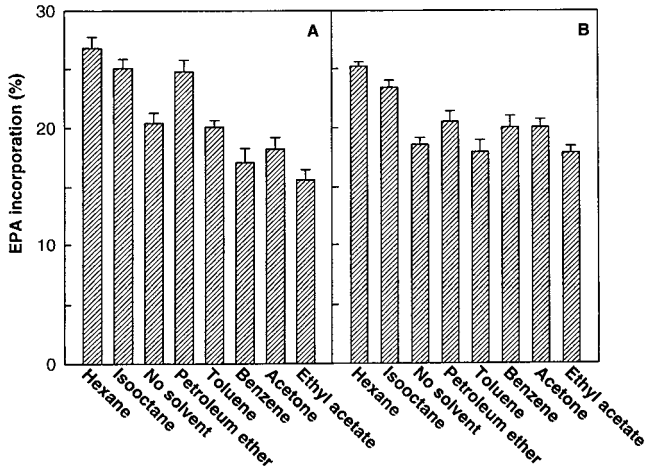

Figure 4. Effect of different organic solvents on the incorporation of EPA into borage (A) and evening primrose oils (B). The reaction mixture contained 297–300 mg of oil, 115 mg of EPA, 150 units of *Pseudomonas* sp. lipase preparation, and 3 mL of hexane. The reaction mixture was incubated at 37 °C for 24 h in an orbital shaking water bath at 250 rpm.

from *M. miehei* as the biocatalyst. The resultant contents of EPA and DHA in the modified oil were 9.5 and 8.0%, respectively. Haraldsson et al. (*32*) prepared EPA-enriched TAG (40% EPA and 25% DHA) and DHA-enriched TAG (48% DHA and 12% EPA) using EPA or DHA concentrates, respectively. *M. miehei* lipase-catalyzed interesterification of cod liver oil with *n*−3 PUFA concentrates was used in the latter study.

Effect of Organic Solvents. The effect of organic solvents on enzyme activity has been demonstrated (*26*, *33*−*35*), but mechanistically the effect of organic solvent on enzyme catalysis is still debated (*35*, *36*). Conformational changes in enzymes, when suspended in organic solvents, are reported to result in alteration of substrate specificity and affinity (*37*). The polarity of an organic solvent is critical to enzyme activity because it is essential to maintain a layer of essential water around the enzyme. It has been proposed that the term log *P* (the logarithm of the partition coefficient between water and octanol) be used as a means of predicting the denaturing effect of a solvent on an enzyme (*36*). Laane et al. (*33*) concluded that, in general, enzyme catalysis in organic solvents is governed by the following rules: (a) solvents with log *P* values of <2 are not suitable for enzyme-catalyzed systems because they distort the essential water from the enzyme, thereby inactivating them; (b) solvents with log *P* values in the range of 2−4 are weak water distorters, and their effect on enzyme activity is unpredictable; and (c) solvents with log *P* values of >4 do not distort the essential water layer, thereby leaving the enzyme in an active state. A certain level of water is necessary for the lipase-catalyzed reactions in organic media (*38*), but when the amount of water exceeds this level, hydrolysis occurs (*37*). Complete depletion of water, however, results in no reaction (*39*).

To select a suitable solvent for acidolysis of BO with EPA by lipase PS-30, the effect of several organic solvents (isooctane, log *P* = 4.5; hexane, log *P* = 3.5; toluene, log *P* = 2.5; benzene, log *P* = 2.0; acetone, log *P* = −0.23; ethyl acetate, log *P* = 0.68; petroleum ether, no log *P* value reported) was examined (**Figure 4**A). *n*-Hexane (log *P* = 3.5) was found to be best for EPA incorporation into BO (26.8%) (**Figure 4A**), which lends further support to the findings of Akoh et al. (*15*, *40*), who reported that *n*-hexane solvent was effective when incorporating EPA and DHA into EPO and trilinolein. Organic solvents such as *n*-hexane have several functions, including increasing the

Table 3. Fatty Acid Profile (Weight Percent) of Modified Borage Oil, Catalyzed by Lipase from *Pseudomonas* sp. at Different Substrate Mole Ratios[a]

	mole ratio		
major fatty acid	1:1	1:2	1:3
16:0	7.22 ± 0.17	5.84 ± 0.28	5.42 ± 0.15
18:0	3.00 ± 0.15	2.40 ± 0.16	2.23 ± 0.18
18:1	12.3 ± 0.24	10.3 ± 0.34	10.0 ± 0.14
18:2*n*–6	26.4 ± 0.28	22.4 ± 0.66	21.8 ± 0.25
18:3*n*–6	16.3 ± 0.29	14.0 ± 0.19	13.4 ± 0.33
20:5*n*–3	26.8 ± 0.30	37.4 ± 0.87	39.9 ± 1.20

[a] Mole ratios of borage oil to EPA were varied from 1:1 to 1:3. Reactions were carried out in hexane with lipase from *Pseudomonas* sp. at 37 °C for 24 h in an orbital shaking water bath at 250 rpm.

Table 4. Fatty Acid Profile (Weight Percent) of Modified Evening Primrose Oil, Catalyzed by Lipase from *Pseudomonas* sp. at Different Substrate Mole Ratios[a]

	mole ratio		
major fatty acid	1:1	1:2	1:3
16:0	4.52 ± 0.50	3.72 ± 0.17	2.80 ± 0.23
18:0	0.22 ± 0.07	0.23 ± 0.05	0.20 ± 0.03
18:1	4.81 ± 0.50	4.3 ± 0.24	3.83 ± 0.52
18:2*n*–6	53.3 ± 1.26	50.6 ± 1.60	47.3 ± 1.34
18:3*n*–6	7.61 ± 0.45	7.44 ± 0.21	6.21 ± 0.25
20:5*n*–3	25.2 ± 0.10	35.1 ± 1.80	37.4 ± 0.82

[a] Mole ratios of evening primrose oil to EPA were varied from 1:1 to 1:3. Reactions were carried out in hexane with lipase from *Pseudomonas* sp. at 37 °C for 24 h in an orbital shaking water bath at 250 rpm.

solubility of nonpolar substrates and shifting the reaction toward synthesis rather than hydrolysis (*34*). However, satisfactory incorporation of EPA (20.4%) into BO was achieved in a solvent-free system. For food applications, the solvent-free reaction may be the method of choice. Claon and Akoh (*41*) have demonstrated that lipase SP-30 worked well in the solvent-free synthesis of primary terpene acetates.

The same solvents were also used as reaction media incorporating EPA into EPO using lipase PS-30 (**Figure 4B**). The highest incorporation was achieved in *n*-hexane (25.2% in EPA), with isooctane producing the second highest EPA incorporation (23.4%). The solvent-free reaction gave acceptable incorporation of EPA (18.6%) into EPO, and from these results it was concluded that *n*-hexane served best for the acidolysis reaction of both oils with EPA.

Effect of Mole Ratio of Substrates. The mole ratio of oil substrate to fatty acid affected EPA (percent) incorporation into BO and EPO (**Tables 3** and **4**, respectively). This study was performed by varying the mole ratio of oils to EPA from 1:1 to 1:3. The amount of BO (300 mg) or EPO (297 mg) was kept constant while the amount of EPA was varied (115−345 mg) to achieve the desired mole ratio. EPA incorporation increased as its mole ratio in the reaction medium increased. **Tables 3** and **4** show that, with lipase from *Pseudomonas* sp., a mole ratio of 1:1 gave EPA incorporation of 26.8 and 25.2% in BO and EPO, respectively. At a 1:2 mole ratio of reactants, 37.4 and 35.1% EPA was incorporated into BO and EPO, respectively. EPA incorporation reached a maximum at the stoichiometric oil/EPA ratio of 1:3. At the mole ratio of 1:3, incorporation of EPA into BO and EPO was 39.9 and 37.4%, respectively (**Tables 3** and **4**, respectively). Previously, EPA has been incorporated into melon seed oil by Huang et al. (*30*), who showed that the mole ratio of substrates to fatty acids affected EPA incorporation (percent) into the oil. There is no economic advantage in using high substrate mole ratios, especially EPA. Depending on the level of EPA incorporation desired in the final product, the substrate mole ratio can be manipulated to achieve it. High EPA concentrations in the medium may indeed lead to substrate inhibition. Such an observation has been reported in a reaction involving lipase synthesis of SL when high mole ratios of caprylic acid to triolein were used (*42*). Kuo and Parkin (*43*) also reported a concentration-dependent substrate preference in lipase-mediated acidolysis reactions, which they attributed to acidification of the microaqueous environment of the lipase and desorption of water by the fatty acid substrate (*43*).

Table 5. Positional Distribution of Fatty Acids in Enzymatically Synthesized Borage Oil[a]

	positional distribution	
major fatty acid (wt %)	*sn*-2	*sn*-1,3
16:0	5.82 ± 0.18	6.37 ± 0.84
18:0	1.55 ± 0.43	1.39 ± 0.51
18:1*n*–9	10.8 ± 0.35	11.5 ± 0.74
18:2*n*–6	21.2 ± 0.42	23.6 ± 0.83
18:3*n*–6	18.6 ± 0.63	13.3 ± 0.52
20:1*n*–9	3.80 ± 0.04	2.95 ± 0.37
20:5*n*–3	32.5 ± 0.79	33.4 ± 1.08
22:1*n*–11	1.20 ± 0.28	1.58 ± 0.44
24:1	0.58 ± 0.24	1.77 ± 0.45

[a] Substrate composition: 300 mg of borage oil, 115 mg of EPA, 150 units of *Pseudomonas* sp. lipase preparation, and 3 mL of hexane. The reaction was carried out at 37 °C for 24 h at 250 rpm.

Table 6. Positional Distribution of Fatty Acids in Enzymatically Synthesized Evening Primrose Oil[a]

	positional distribution	
major fatty acid (wt %)	*sn*-2	*sn*-1,3
16:0	2.94 ± 0.15	4.98 ± 0.42
18:0	3.63 ± 0.27	1.00 ± 0.18
18:1*n*–9	8.54 ± 0.44	5.48 ± 0.39
18:2*n*–6	48.4 ± 0.61	40.3 ± 0.57
18:3*n*–6	7.16 ± 0.37	5.39 ± 0.26
20:5*n*–3	27.2 ± 0.58	39.5 ± 1.00

[a] Substrate composition: 297 mg of evening primrose oil, 115 mg of EPA, 150 units of *Pseudomonas* sp. lipase preparation, and 3 mL of hexane. The reaction was carried out at 37 °C for 24 h at 250 rpm.

Separation of Acylglycerols of EPA-Enriched Oils. EPA-enriched oils were subjected to TLC-FID. The results showed that the relative content of TAG (88.7−94.8%) was much higher than that of diacylglycerols (DAG; 4.3−8.1%) and monoacylglycerols (MAG; 0.9−3.2%). No free fatty acids (FFA) were detected in EPA-enriched oils because these were removed by the NaOH after the acidolysis reaction.

Positional Analysis of Structured Lipids. The positional distribution of fatty acids in EPA-enriched oils was determined (**Tables 5** and **6**). For EPA-enriched BO, EPA seemed to be randomly distributed (**Table 5**), whereas for EPA-enriched EPO, EPA was mainly found at the *sn*-1,3-positions (39.5% at *sn*-1 + *sn*-3) followed by the *sn*-2 position (27.2%) (**Table 6**). Accordingly, it is assumed that lipase from *Pseudomonas* sp. shows no regiospecificity and may incorporate EPA at the *sn*-2 and *sn*-1,3-positions of TAG of the oils. In both oils, GLA was esterified predominantly at the *sn*-2 position (18.6 and 7.2% in EPA-enriched BO and EPO, respectively). It has been shown that fatty acids esterified to the *sn*-2 position of TAG are more

efficiently absorbed regardless of fatty acid type (*44*). Christensen et al. (*45*) demonstrated that defined TAG with capric acid (10:0) esterified in the *sn*-1,3 positions and *n*-3 fatty acids in the *sn*-2 position were more readily absorbed compared to randomized TAG with a similar fatty acid composition. In another study, Ikeda et al. (*46*) described the lymphatic absorption of linoleic acid (18:2*n*−6), caprylic acid (8:0), and capric acid from various defined TAG in rats. They found that linoleic acid was better absorbed from a defined TAG with linoleic acid in the *sn*-2 position.

ABBREVIATIONS USED

ACS, American Chemical Society; BO, borage oil; DGLA, dihomo-γ-linoleic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EPO, evening primrose oil; GC, gas chromatography; GLA, γ-linoleic acid; 15-OH-DGLA, 15-hydroxydihomo-γ-linoleic acid; PGE_1, prostaglandin E_1; PUFA, polyunsaturated fatty acids; SL, structured lipids; TLC, thin layer chromatography; TLC-FID, thin layer chromatography−flame ionization detection; TAG, triacylglycerols.

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