The Incorporation of n-3 Polyunsaturated Fatty Acids into Acylglycerols of Borage Oil *via* Lipase-Catalyzed Reactions

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ABSTRACT: The purpose of this work was to add n-3 polyunsaturated fatty acids (PUFA) into the acylglycerols of borage oil. The acidolysis reaction between borage oil and n-3 PUFA was carried out with lipase (Lipozyme IM-60) in organic solvent. The effects of temperature, solvent, and water content on the reaction product were investigated. For the acidolysis reaction between acylglycerols (product of the selective hydrolysis of borage oil, catalyzed by immobilized *Candida rugosa* lipase) and n-3 PUFA, the total content of n-3 and n-6 PUFA in acylglycerols was 72.8% after a reaction time of 18 h. The contents of γ-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid were 26.5, 19.8, and 18.1%, respectively. By properly controlling the reaction time, acylglycerols with *ca.* 70–72% PUFA and a ratio of n-3 PUFA to n-6 PUFA from 0–1.09 can be obtained.
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KEY WORDS: Acidolysis, borage oil, DHA, EPA, GLA, lipase, menhaden oil, n-3 PUFA, n-6 PUFA.

In humans and other mammals, γ-linolenic acid (GLA, 18:3n-6; all-*cis*-6, 9, 12-octadecatrienoic acid) is a pre-essential fatty acid, and it is also an important intermediate in the normal metabolism of linoleic acid to the eicosanoid precursor arachidonate. Both *cis*-5, 8, 11, 14, 17-eicosapentaenoic acid (EPA, 20:5n-3) and *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA, 22:6n-3) are also important polyunsaturated fatty acids (PUFA). Basically, EPA and DHA are metabolized from the essential fatty acid α-linolenic acid (18:3n-3). The metabolic processes consist of a series of alternating steps of desaturation and elongation, with the desaturation being the rate-limiting step. Because Δ6 desaturase may be impaired by aging, by high levels of alcohol or cholesterol, and in certain cancer and virally infected cells (1), many investigators have actively participated in research to concentrate GLA, EPA, and DHA from borage oil, evening primrose oil, and deep-sea fish oil (2–5) for dietary and pharmaceutical purposes. However, an excess amount of n-6 PUFA can cause vasoconstriction and platelet aggregation (6). Dietary supplementation of n-3 PUFA is necessary to shift the physiological balance in the direction of vasodilation and antiaggregation because n-3 PUFA is a competitor of arachidonic acid and has been shown to prevent cardiovascular diseases (7,8). Recently, EPA and DHA ethyl esters have been incorporated into vegetable oil (9), trilinolein (10), melonseed oil (11), evening primrose oil (12), and borage oil (13) with immobilized lipase IM-60 from *Mucor miehei* and SP 435 from *Candida antarctica* as biocatalysts. The incorporation of n-3 PUFA (from saponification of cod liver oil) into vegetable oil has also been reported (14).

Lipase-catalyzed acidolysis requires low water content to shift the chemical equilibrium in favor of product formation. The polarity or hydrophobicity of solvents can have profound effects on the three-dimensional structure of an enzyme and the retention of enzyme-associated water, necessary for enzyme catalysis. In this work, we examined the incorporation of n-3 PUFA, derived from the saponification of menhaden oil, into borage oil by means of an immobilized lipase IM-60 catalyzed acidolysis reaction in organic solvent. The effects of temperature, water content, and organic solvent on product formation are reported. A two-step reaction is proposed in which borage oil is first partially hydrolyzed with *C. rugosa* lipase, immobilized on microporous polypropylene, and the acidolysis between the partially hydrolyzed borage oil and n-3 PUFA is then carried out with IM-60 lipase.

MATERIALS AND METHODS

Materials. Borage oil (average M.W. 873) and menhaden oil (average M.W. 891) were purchased from Sigma Chemical (St. Louis, MO). Immobilized lipase (Lipozyme IM-60) from *Mucor miehei* was a gift of Novo Nordisk Bioindustry Co. Ltd. (Bagsvaerd, Denmark). All solvents were of either high-performance liquid chromatography or analytical grade and were obtained from commercial sources. Heptadecanoic acid, 18:2n-6, 18:3n-6, 20:5n-3, 22:6n-3 for calibration curves for gas–liquid chromatography (GLC) analysis were purchased from Sigma Chemical.

Preparation of n-3 PUFA. The preparation of n-3 PUFA-enriched free fatty acids (FFA) from menhaden oil was car-

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TABLE 1
Fatty Acid Composition (wt%) of Saponified Menhaden Oil Before and After Urea-Adduct Concentration

Fatty acid	Menhaden (%)	Concentrate (%)
14:0	9.1	4.6
16:0	20.9	2
16:1	11.2	1.5
18:0	4.5	—
18:1	12.2	2
18:2n-6	1.3	1.3
18:4n-3	3.5	6.1
20:4n-3	1.3	2.9
20:5n-3	14.8	29
22:4		
22:6n-3	15.6	42.3
Others	4.1	5.1
EPA + DHA[a]	30.4	71.3
Total n-3	35.1	80.3

[a]EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

ried out by the urea inclusion method (15). The method includes saponification of menhaden oil, extraction of FFA, formation of urea inclusion complex, and extraction of n-3 PUFA-enriched FFA. Table 1 shows the composition of concentrated FFA prepared in this work. The composition of concentrated FFA was determined according to the methods described by Shantha and Napolitano (16). Total EPA and DHA content of the n-3 PUFA-enriched FFA is 71.3 wt%.

Acidolysis reaction. In the acidolysis between triacylglycerol of borage oil and n-3 PUFA-enriched FFA, hydrolysis of oil is followed by re-esterification reactions, with the hydrolysis being the rate-limiting step (17). Typically, 40 mg of borage oil and 120 mg of n-3 PUFA were dissolved in 2 mL isooctane, followed by the addition of 0.01 mL 0.05% (wt/vol) phosphate buffer (pH 7.0) and 10 % (w/w) immobilized lipase IM-60 (10% based on the substrates' weight). The mixture was agitated with a magnetic stirrer at 600 rpm, and 0.2 g molecular sieve (4 A, Merck, Darmstadt, Germany) was added. The reaction was carried out under nitrogen atmosphere.

Fatty acid compositions in acylglycerols by GLC analysis (18). Products of the lipase-catalyzed acidolysis were analyzed to determine the fatty acid compositions by GLC. To convert acylglycerols into fatty acid methyl esters (FAME), 0.05 mL of acylglycerols, a known amount of internal standard (heptadecanoic acid, dissolved in 0.05 mL of 1,2-dichloroethane), and 0.05 mL of 0.2 M trimethylsulfonium hydroxide methanol solution were placed in a test tube; the tube was shaken by vortex for *ca.* 20 s and then put aside for 15 min. The FAME mixture was analyzed by a China Chromatography model 8700F (Taipei, Taiwan) gas–liquid chromatograph, equipped with a flame-ionization detector. The column used was DB-23 (30 m × 0.53 mm i.d.; J&W Scientific, Folsom, CA). The temperatures of column, injector, and detector were set at 220, 270, and 250°C, respectively. The concentration of fatty acid esters was determined from the calibration curves by the measured peak area ratio.

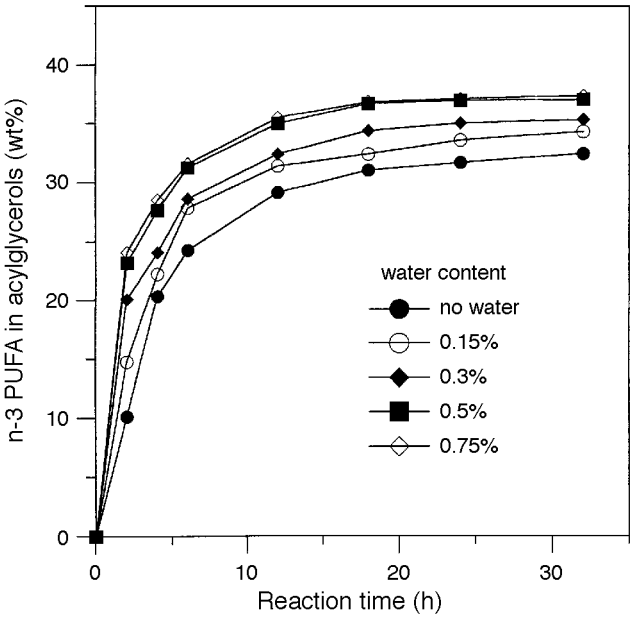

FIG. 1. Effect of added water on the n-3 PUFA content in acylglycerols. Substrate composition: 40 mg borage oil, 120 mg n-3 PUFA in isooctane. Magnetic stirrer speed 600 rpm. PUFA, polyunsaturated fatty acid.

RESULTS AND DISCUSSION

Effects of water content on the acidolysis. To keep the enzyme active and promote the acidolysis reaction rate, 0.1 M phosphate buffer (pH 7.0) was added to the reaction mixture at the

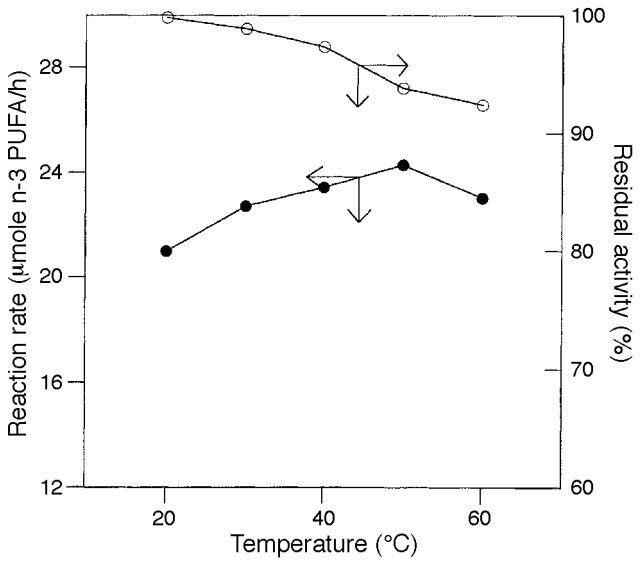

FIG. 2. Effect of temperature on the reaction rate (●) and the stability (○) of IM-60 lipase (Novo Nordisk, Bagsvaerd, Denmark). Substrate composition: borage oil 40 mg, n-3 PUFA 120 mg, and 0.5% (wt/vol) phosphate buffer (pH 7.0) in 2 mL isooctane. Enzyme content 10% (w/w). Reaction conditions: magnetic stirrer speed 600 rpm, reaction time 2 h. Reaction rate of nonincubated IM-60 lipase is taken as 100%. For abbreviation see Figure 1.

beginning of the reaction. As shown in Figure 1, n-3 PUFA (EPA plus DHA) content in acylglycerols increases with the amount of added water up to 0.5% (wt/vol). This amount of water is sufficient for the lipase IM-60 to show its maximal activity because additional amounts of water had no apparent effects on the increase of n-3 PUFA in acylglycerols. Hence, 0.5% (wt/vol) added water was chosen in later experiments.

Effect of temperature on reaction rate and thermostability of the lipase. The effect of temperature on reaction rate and thermostability was examined, and results are shown in Figure 2. The IM-60 lipase-catalyzed acidolysis reaction shows a maximal reaction rate at 50°C. In the thermostability study, the lipase was incubated in 2 mL isooctane for 12 h at the desired temperature. An optimal operating temperature of 50°C was chosen from the results of Figure 2. Figure 3 shows the effect of reaction time on the incorporation of EPA and DHA in the acidolysis reaction. Both EPA and DHA contents in acylglycerols increased with time. GLA content decreased slowly with time, whereas the linoleic acid content in acylglycerols decreased rapidly at the early stage of reaction and remained fairly constant as the reaction proceeded. The time course of the ratio of n-3 PUFA (EPA plus DHA) to n-6 PUFA (linoleic acid plus GLA) in acylglycerols is presented in Figure 4. Similar results from the reaction of borage oil and EPA ethyl ester were obtained with an immobilized nonspecific lipase (SP435 from *C. antarctica*) by Akoh and Sista (13). Their results indicated that, at a substrate molar ratio of 1:3, a PUFA (n-3 plus n-6) content of 74.8% in the acylglycerols could be obtained with linoleic acid, GLA and EPA contents in the acylglycerols of 29.3, 16.2, and 28.1%, respectively.

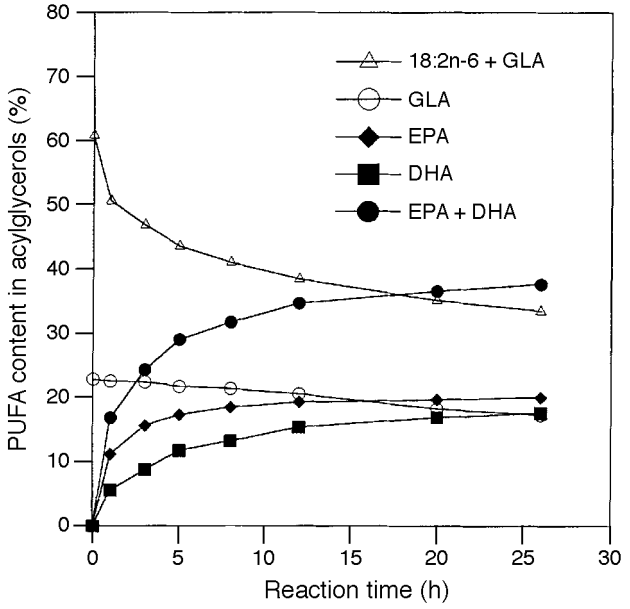

FIG. 3. PUFA contents in product as function of time for acidolysis. Substrate composition: 40 mg borage oil, 120 mg n-3 PUFA, 0.5% (wt/vol) phosphate buffer (pH 7.0) in 2 mL isooctane. Reactions were carried out at 50°C with magnetic stirrer speed 600 rpm; enzyme content 10 % (w/w). GLA, γ-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. For other abbreviation see Figure 1.

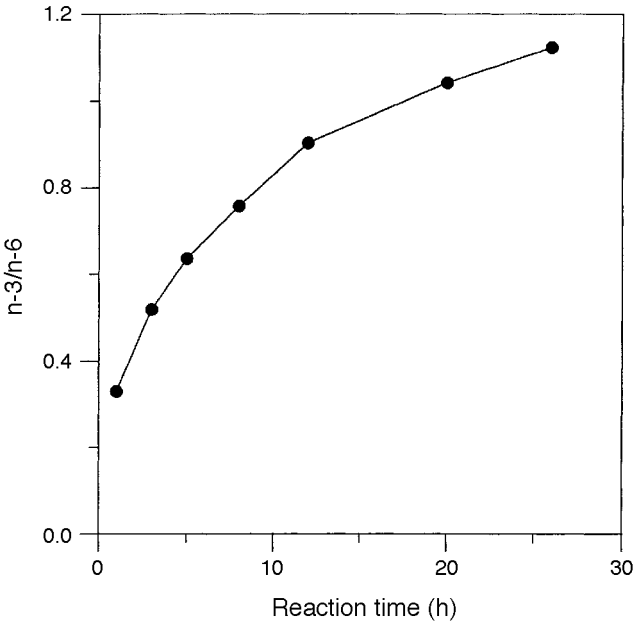

FIG. 4. The ratio of n-3 PUFA to n-6 PUFA in products as a function of time for acidolysis. Reaction conditions are the same as those in Figure 3. For abbreviation see Figure 1.

The corresponding ratio of n-3 PUFA to n-6 PUFA was 0.62. In their work, 97% pure EPA ethyl ester was used as a substrate. If free fatty acids with 45% EPA were used as a sub-

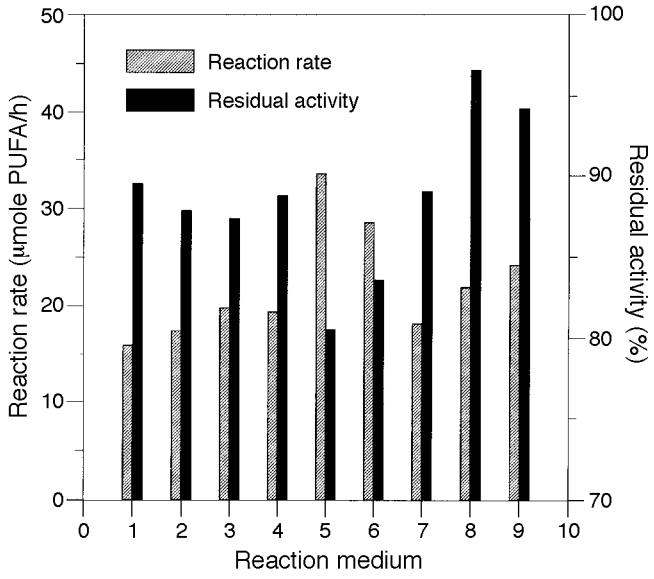

FIG. 5. Effect of organic solvents on the reaction rate and stability of IM-60 lipase. In the stability study, the lipase was incubated in the desired solvent for 12 h at 50°C. Substrate composition and reaction conditions are the same as those in Figure 2 except for the organic solvent. Numbers in the abscissa represent : 1 = acetonitrile (log P = –0.33), 2 = methyl acetate (log P = 0.16), 3 = triethylamine (log P = 1.6), 4 = chloroform (log P = 2.0), 5 = benzene (log P = 2.0), 6 = toluene (log P = 2.5), 7 = xylene (log P = 3.1), 8 = *n*-hexane (log P =3.5), 9 = isooctane (log P = 4.5). P, partition coefficient. For other abbreviation see Figure 1. For manufacturer of lipase see Figure 2.

TABLE 2
Incorporation of EPA and DHA into Acylglycerols of Borage Oil *via* a Two-Step Acidolysis Reaction[a]

Reaction time (h)	Component	Fatty acid composition									Yield of acylglycerols (wt%)	Total PUFA in acylglycerols (wt%)	Ratio of n-3 to n-6 PUFA[b]
		16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:5	22:6			
0	Acylglycerols	7.8	6.7	4.0	11.5	21.2	48.1	0.8	—	—	100	69.3	0
3	Acylglycerols	2.3	5.2	2.8	5.5	8.7	33.5	1.9	17.1	11.9	97.5	71.2	0.687
3	FFA	1.8	4.5	2.3	4.6	6.1	4.3	1.4	23.9	36.7			
5	Acylglycerols	2.0	5.1	2.4	5.1	7.9	32.5	2.0	18.9	12.0	97	71.3	0.782
5	FFA	1.7	5.0	3.1	4.2	6.1	4.9	3.0	23.5	35.8			
8	Acylglycerols	1.9	4.8	2.0	5.0	7.3	29.6	1.6	19.1	14.5	97	70.5	0.911
8	FFA	2.1	5.2	2.1	5.1	5.9	5.7	3.6	21.5	33.5			
12	Acylglycerols	1.8	4.6	1.9	4.4	7.6	28.2	1.1	19.5	16.8	95.3	72.1	1.014
12	FFA	2.2	5.5	2.4	4.9	7.6	7.4	3.4	20.9	31.4			
18	Acylglycerols	1.7	4.2	1.8	4.2	8.4	26.5	1.2	19.8	18.1	96	72.8	1.086
18	FFA	2.0	6.3	2.8	4.5	7.7	8.9	3.8	19.2	30.1			

[a]Reaction conditions: 40 mg of unhydrolyzed borage oil and 120 mg of n-3 PUFA are dissolved in 2 mL isooctane. Water content 0.5% wt/vol, enzyme content 10% w/w, reaction temperature 50°C, magnetic stirrer speed 600 rpm.
[b]n-3: EPA and DHA; n-6: linoleic acid and γ-linolenic acid. PUFA, polyunsaturated fatty acid; FFA, free fatty acid. For other abbreviations see Table 1.

strate, the incorporation of EPA into borage oil would be poorer. In this work, EPA- and DHA-rich free fatty acid derived from menhaden oil was used as a substrate, and at a substrate weight ratio of 1:3, we obtained a PUFA content of 71.4%. The contents of linoleic acid, GLA, EPA, and DHA in the acylglycerols were 16.2, 17.3, 20.3, and 17.6%, respectively. The ratio of n-3 PUFA to n-6 PUFA was 1.13.

Effects of organic solvents. The polarity of an organic solvent affects lipase-catalyzed reactions (19) . The effect of log *P* (the logarithm of the partition coefficient, a measure of polarity) value of different organic solvents on the acidolysis reaction was examined. Figure 5 indicates that, in terms of reaction rate, benzene was the best among the nine solvents tested. However, when lipase IM-60 was incubated in various solvents for 12 h, hexane and isooctane showed higher residual activity than benzene. In this study, isooctane was chosen as solvent for the acidolysis reaction.

Two-step acidolysis. In a previous study (20), borage oil was selectively hydrolyzed with *C. rugosa* lipase, immobilized on hydrophobic microporous polypropylene. After 4 h, the GLA content in acylglycerols increased to *ca.* 48–51 %. The unhydrolyzed acylglycerol and n-3 PUFA from the saponification of menhaden oil were employed as substrates in the acidolysis reaction. The relationships between fatty acid compositions in acylglycerols and reaction time are shown in Table 2. Total content of n-3 plus n-6 PUFA in acylglycerols remained fairly constant during the reaction. The ratio of n-3 PUFA to n-6 PUFA in acylglycerols increased rapidly at the early stage of reaction and reaching an asymptotic value of *ca.* 1.09. It is apparent from Table 2 that, by controlling the reaction time, acylglycerols with *ca.* 70–72 wt% PUFA and a ratio of n-3 PUFA to n-6 PUFA from 0–1.09 can be obtained *via* the proposed two-step acidolysis reaction.

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