Enrichment of γ-Linolenic Acid from Borage Oil *via* Lipase-Catalyzed Reactions

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ABSTRACT: Three lipase-catalyzed reactions were utilized to enrich γ-linolenic acid in borage oil: (i) selective hydrolysis in isooctane by *Candida rugosa* lipase immobilized on microporous polypropylene, (ii) selective esterification of free fatty acid from saponified borage oil and *n*-butanol by Lipozyme IM-20, and (iii) acidolysis of the products of the previous two reactions, that is, unhydrolyzed acylglycerols and unesterified free fatty acid. In the selective hydrolysis, γ-linolenic acid content could be raised from 23.6 mol% in borage oil to 51.7% in the unhydrolyzed acylglycerols. On the other hand, γ-linolenic acid content in free fatty acid could be increased to 87% after selective esterification. Products with 65% γ-linolenic acid in their acylglycerols were obtained by means of the acidolysis reaction.

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KEY WORDS: Acidolysis, borage oil, immobilization, γ-linolenic acid, selective esterification, selective hydrolysis.

In humans and other mammals, γ-linolenic acid (GLA; all-*cis* 6,9,12-octadecatrienoic acid) is a pre-essential fatty acid. It is also an important intermediate in the normal bioconversion of linoleic acid (18:2*n*-6) to the eicosanoid precursor arachidonate. The bioconversion includes a series of alternating desaturation and elongation steps. The desaturation of linoleic acid is catalyzed by 6-desaturase, the rate-determining step. 6-Desaturase may be impaired by aging, by high levels of cholesterol or alcohol, and in certain cancer cells and virally infected cells (1). Presently, GLA-rich acylglycerides have been applied directly in curing certain diseases, such as atopic eczema (2), multiple sclerosis (3), and rheumatoid arthritis (4). Therefore, many investigators have actively participated in research of GLA concentration from borage oil, evening primrose oil, and fungal oils for pharmaceutical and dietetic purposes. Borage oil has the highest GLA content of the various available sources and is suitable for GLA enrichment. Available methods for GLA enrichment include urea adduct formation (5), separation on Y-zeolite (6), solvent winterization (7), and enzymatic selective hydrolysis or esterification (8–10). Syed Rahmatullah *et al*. (10) reported that *Candida rugosa* lipase is more efficient for GLA concentration in the selective hydrolysis reaction of borage oil and evening primrose oil.

Aqueous systems were employed for the enzymatic selective hydrolysis reactions of borage oil and evening primrose oil (10,11). Oil hydrolysis in an organic two-phase system has received extensive attention. Brink *et al*. (12) and Dordick (13) gave various reasons to justify the use of organic media instead of aqueous solutions, such as increased solubility of nonpolar substrates, enhanced thermostability of the enzyme and elimination of microbial contamination. An immobilized biocatalyst is present as solid phase in the organic–water biphasic system. If the organic phase is continuous, the aqueous phase is nondiscrete, and the biocatalyst is insoluble. The interfacial area available for mass transfer is determined by the specific surface area of the biocatalyst. Consequently, a liquid dispersed in another liquid is unnecessary (14). Lipase immobilized on hydrophobic microporous supports yields higher lipolytic activity than that on hydrophilic supports (15–17). Supports with lower aquaphilicity (Aq) are generally recommended (18). Lipase is generally immobilized on hydrophobic microporous supports by physical adsorption, and lipase leakage may be made negligible (19).

In this study, a systematic investigation was carried out on the enzymatic enrichment of GLA in borage oil. The authors attempted to modify the currently available methods that involve lipase-catalyzed selective hydrolysis, selective esterification, and acidolysis in order to enrich the GLA content in acylglycerols of borage oil.

MATERIALS AND METHODS

Materials. Candida rugosa lipase (EC 3.1.1.3; triacylglycerol ester hydrolase) and borage oil (av. MW *ca.* 873.4) were purchased from Sigma (St. Louis, MO). Lipozyme IM-20 was acquired from Novo Industrie (Mainz, Germany). Microporous PP (Accurel EP 100, particle size: 200–1000 μm) was donated by Akzo Chemical Inc. (Obernburg, Germany) and used as a carrier for enzyme immobilization. Particle sizes in the range of 300–500 μm were selected by using a Tyler sieve. Isooctane was obtained from J.T. Baker (Phillipsburg, NJ) and was employed as a solvent. Heptadecanoic acid, 16:0, 18:0, 18:1, 18:2, and 18:3*n*-3 for GLC analysis were obtained from Sigma; other standards (20:1, 22:1, and 24:1) were pur-

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chased from Nu-Chek-Prep Inc. (Elysian, MN). Tri-γ-linolenin (GGG) and trilinolein (LLL) for high-performance liquid chromatography (HPLC) analysis were obtained from Nu-Chek-Prep Inc.; 1,2-dilinoleoyl-3-oleyl-*rac*-glycerol (OLL), 1,2-dilinoleoyl-3-palmitoyl-*rac*-glycerol (PLL) were obtained from Sigma. Diolein (85% 1,3- and 15% 1,2-isomer) and 1-monooleoyl-*rac*-glycerol, used for thin-layer chromatography (TLC) analysis, were provided by Sigma. Olive oil emulsion was purchased from Sigma and was utilized as a substrate for lipase assay.

Lipase immobilization. Ten milligrams of *C. rugosa* lipase powder was dissolved in 250 μL of 0.1 M phosphate buffer solution (pH 7.0). After centrifugation, the upper clear enzyme solution was removed. An appropriate amount of the removed enzyme solution was pipetted onto a glass sheet. Polypropylene (100 mg, prewetted with 0.5 mL ethanol) was added to the enzyme solution and flattened by using a small wood bar. This was then immediately placed into a vacuum oven to strip off excess water at room temperature (20).

Lipase assay. Hydrolysis activity of the immobilized lipase was assayed with olive oil emulsion. The immobilized lipase was added to 10 mL of 10% (vol/vol) olive oil emulsion and preincubated at 37°C for 20 min; the emulsion was then stirred at 600 rpm for 20 min with a magnetic stirrer. The reaction was terminated by adding 10 mL of 1:1 acetone/ethanol (vol/vol). The liberated free fatty acid was titrated with 0.1 N NaOH in a Metrohm 686 Titroprocessor (Herisau, Switzerland). A control was performed using the same procedure, except that no immobilized lipase was added to the emulsion. One unit of activity is equivalent to 1 μmol of free fatty acid liberated/min at 37°C.

Preparation of fatty acids. Borage oil (2 g) and 12 mL of solution that contained 1 N KOH in 90% aqueous ethanol was placed in a test tube (15-mm i.d.). The mixture was heated to 80°C under nitrogen and stirred with a magnetic stirrer at 600 rpm for 90 min. After cooling to room temperature, 12 mL of deionized water and 4 mL HCl (6 N) were added to the mixture. Fatty acids were extracted three times with 10 mL diethyl ether. All extracts were collected and dried under vacuum at room temperature. The dried products were dissolved in 10 mL isooctane and stored at −15°C.

Selective hydrolysis reaction. Isooctane (2 mL) containing borage oil (0.05–0.3 M) was placed into a test tube (15 mm), and 1 mL of phosphate buffer was added. This mixture was incubated in a water bath for 20 min at 30°C, and immobilized lipase was added to the test tube. The reaction mixture was shaken for 1 min by vortex, then returned to the water bath, and stirred by magnetic stirrer (600 rpm) as the reaction proceeded.

Selective esterification. Esterification reactions were carried out with various amounts of free fatty acids (FFA) from saponified borage oil and *n*-butanol (molar ratio = 1:1–1:4) and 15 mg Lipozyme IM-20 powder in 3 mL isooctane. The mixture was agitated at 600 rpm with a magnetic stirrer, and 0.2 g of molecular sieve was added during the reaction.

Acidolysis reactions. The reaction mixture contained variable amounts of unhydrolyzed acylglycerols from enzymatic hydrolysis of borage oil, and FFA obtained from selective esterification (molar ratio of acylglycerol to FFA = 1:2–1:5) and 15 mg Lipozyme IM-20 in 3 mL isooctane. The mixture was agitated at 600 rpm with a magnetic stirrer, and 0.2 g of molecular sieve was added during the reaction.

HPLC fractionation of triacylglycerols. HPLC analyses were carried out with a Jasco system module PU-980 HPLC (Tokyo, Japan). The column used was Supelco LC-18 (5 μm, 150 mm × 4.6 mm; Supelco, Bellefonte, PA), housed in a column oven (30°C) for separation and analysis of the highly unsaturated triacylglycerol (TG) in borage oil. A solvent mixture of 1:1 acetone/acetonitrile (vol/vol) was used to elute the TG fraction in the borage oil isocratically. A computer was employed to record the actual reading of eluents detected by a refractive index detector.

TLC analysis for acylglycerols and fatty acids in the hydrolyzed products. The hydrolyzed products were analyzed by TLC on a plate coated by silica gel (Plate 60, Merck; Darmstadt, Germany) with chloroform/acetone (96:4, vol/vol) as the developing solvent (21). Fatty acid compositions in TG, diacylglycerol (DG), and monoacylglycerol (MG) were analyzed.

Gas–liquid chromatography (GLC) analysis of fatty acid components in acylglycerols. Products resulting from the lipase-catalyzed hydrolysis of borage oil were fractionated into a water phase that contained FFA and an organic phase that contained acylglycerols (TG, DG, and MG) by adding 0.1 mL of 1 N NaOH in a 50% ethanol solution. Acylglycerols were collected by evaporating organics in nitrogen atmosphere (22). To convert acylglycerols into fatty acid methyl esters (FAME), 5 mg of acylglycerol, a known amount of internal standard (heptadecanoic acid dissolved in 50 μL of 1,2-dichloroethane), and 50 μL of 0.2 M TMSH (trimethylsulfonium hydroxide)-methanol solution were placed in a test tube; the tube was shaken by vortex for approximately 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, fitted with a flame-ionization detector. The column used was a DB-23 (30 m × 0.53 mm, J&W Scientific, Folsom, CA).

RESULTS AND DISCUSSIONS

In the selective hydrolysis of GLA-rich oils by *C. rugosa* lipase, lipase tends to discriminate against γ-linolenoyl moieties of triacylglycerols; this has been employed for the enrichment of GLA. The GLA content could consequently be enriched in the unhydrolyzed acylglycerols. Similarly, GLA content could be increased in acidolyzed acylglycerols from a lipase-catalyzed acidolysis reaction. On the other hand, GLA is enriched in the unesterified fatty acids in the selective esterification.

Selective hydrolysis. Table 1 shows the results of selective hydrolysis of borage oil with immobilized *C. rugosa* lipase in isooctane at 30°C. After reacting for 4 h, a maximum GLA

TABLE 1
Enrichment of γ-Linolenic Acid (GLA) in Acylglycerols *via* Selective Hydrolysis of Borage Oil Catalyzed by Immobilized Lipase of *Candida rugosa* in Isooctane[a]

Reaction time (h)	Component[b]	Fatty acid composition[c] (mol%)								Degree of hydrolysis (%)[d]	Yield of GLA in acylglycerol (%)
		16:0	18:0	18:1	18:2	γ-18:3	20:1	22:1	24:1		
0	Acylglycerols	11.2	3.5	17.1	38.6	23.6	3.2	2.0	0.5	0	100
0.5	Acylglycerols	8.3	3.9	12.2	25.1	41.9	5.5	2.6	0.8	45	98
0.5	Fatty acids	12.7	2.8	22.4	51.8	7.5	1.8	0.8	0.2		
1	Acylglycerols	7.3	3.4	11.3	24.8	45.0	5.6	2.5	1.0	57	82
1	Fatty acids	13.2	3.5	24.8	46.4	8.0	2.2	1.2	0.7		
2	Acylglycerols	6.9	2.5	11.0	23.9	47.5	5.4	2.2	1.2	64	72
2	Fatty acids	14.0	3.7	24.9	45.0	7.5	2.6	1.5	0.8		
3	Acylglycerols	6.5	2.4	10.6	23.7	48.6	5.2	2.2	0.8	70	62
3	Fatty acids	12.2	3.6	22.7	44.0	13.3	2.4	1.2	0.6		
4	Acylglycerols	6.0	2.1	9.9	23.0	51.7	5.0	1.8	0.5	73	59
4	Fatty acids	12.0	3.6	21.8	44.8	13.5	2.8	0.9	0.7		
5	Acylglycerols	6.2	2.1	10.2	23.6	50.0	5.2	2.2	0.5	75	53
5	Fatty acids	12.3	3.9	21.3	44.0	13.5	2.6	1.5	0.7		
6	Acylglycerols	6.3	2.1	10.2	23.2	50.2	5.2	2.2	0.5	77	49
6	Fatty acids	12.1	3.9	21.4	44.8	13.3	2.7	1.2	0.5		
8	Acylglycerols	6.4	2.2	10.1	23.4	50.1	5.3	2.3	0.2	78	47
8	Fatty acids	12.0	3.8	21.8	44.7	13.2	2.8	0.9	0.8		

[a]Reaction conditions: substrate concentration 0.114 M, enzyme content 4.5 U, magnetic stirrer speed 600 rpm, reaction temperature 30°C
[b]Acylglycerols: triacylglycerol, diacylglycerol, and monoacylglycerol.
[c]Fatty acid /acyl moieties are designated by number of carbon atoms/number of *cis* double bonds.
[d]Fatty acid in acylglycerols/initial fatty acid in acylglycerols.

content of 51.7% in the unhydrolyzed acylglycerols could be obtained with a corresponding yield of 59%. It is clear that linoleic acid is the predominant fatty acid in the FFA, indicating that *C. rugosa* lipase favors linoleoyl moieties in the hydrolysis of acylglycerols. Reaction product evolution is shown in Figure 1. The 1,2-DG content is obviously higher than 1,3-DG content in the unhydrolyzed acylglycerols. The reason is probably most γ-linolenoyl moieties are located on the *sn*-2 position of TG, and lipase is known to discriminate against γ-linolenoyl moieties compared to other fatty acyl moieties in acylglycerol hydrolysis (23). Figure 2 shows the time course of various TG concentrations in the unhydrolyzed acylglycerols in early stages of the reaction. Obviously, the rate of hydrolysis of TG that contain two or three γ-linolenoyl moieties (GGG, LGG, OGG, PGG, and SGG) is markedly lower than for those containing only one γ-linolenoyl moiety (LLG, OLG, and PLG). The LLG content declines rapidly with time, while the OLG and PLG contents remain fairly constant; this agrees with the results of Table 1 that linoleic acid is the predominant FFA in hydrolyzed products. Huang *et al.* (24) obtained similar results, although a different lipase (porcine pancreatic lipase) was employed for the same hydrolysis reaction. Figure 3 shows the compositions of TG, DG, MG, and FFA in the hydrolyzed product after a 4-h reaction. High GLA contents in TG, DG, and MG were observed. The GLA contents in TG, 1,2-DG, 1,3-DG, MG, and FFA are 44.6, 52, 40.8, 54, and 13.5%, respectively. The average GLA content in the unhydrolyzed acylglycerols is 51.7 mol%. In a similar study, Syed Rahmatullah *et al.* (10) reported a GLA content of 47.8% in acylglycerols after 5 h with *C. rugosa* lipase powder in a solvent-free system. The authors were able to obtain a higher GLA content in the unhydrolyzed acylglycerols in a shorter time. Additionally, the immobilized lipase prepared in this work is more stable than the powder lipase (20) used by Syed Rahmatullah *et al.*

Selective esterification. Selective esterification of saponified borage oil and *n*-butanol was carried out with Lipozyme IM-20 in the presence of isooctane. The effect of temperature

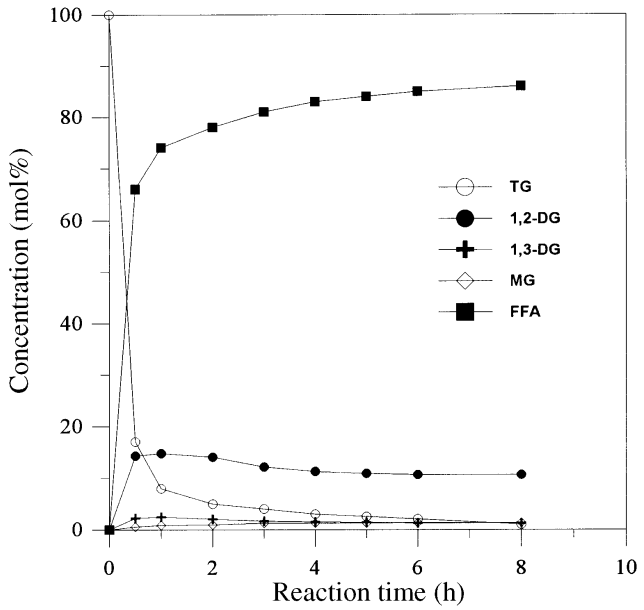

FIG. 1. Relationship between hydrolysis product concentrations and reaction time. Reaction conditions are the same as those in Table 1.

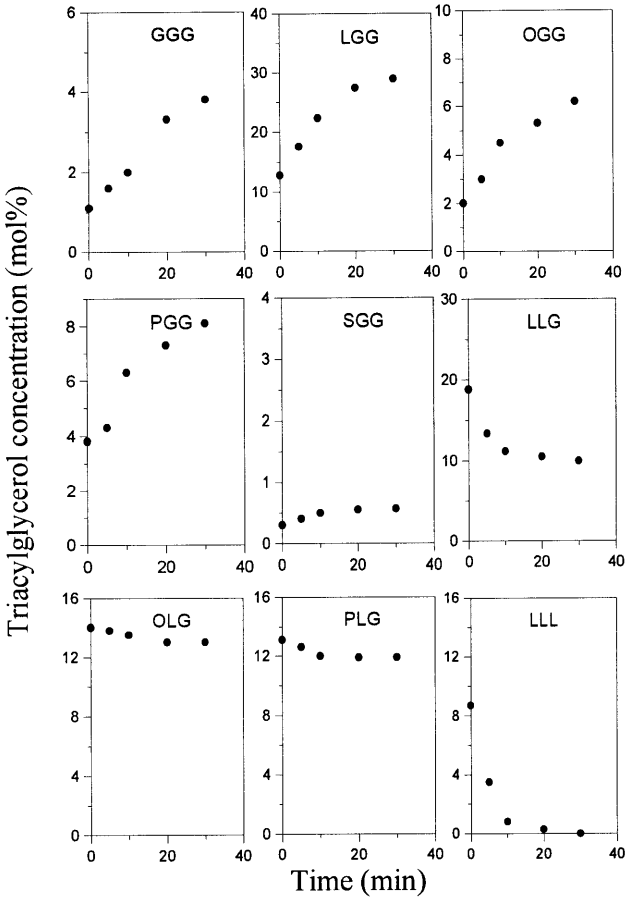

FIG. 2. Time course of triacylglycerol concentrations in the hydrolysis of borage oil. Reaction conditions are the same as those in Table 1. Abbreviations: P, palmitic acid (16:0); S, stearic acid (18:0); O, oleic acid (18:1); L, linoleic acid (18:2); and G, γ-linolenic acid (18:3).

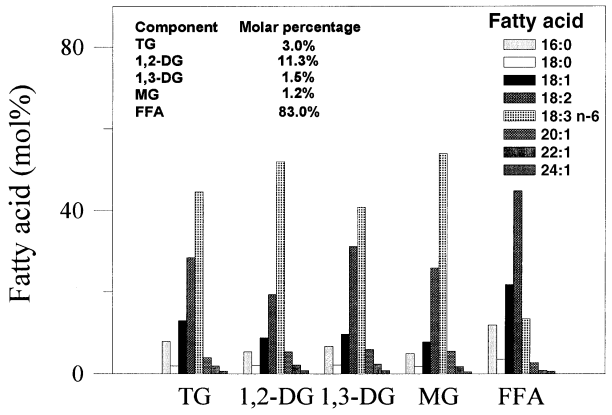

FIG. 3. Fatty acid compositions of the hydrolytic products of borage oil. Reaction time = 4 h. Reaction conditions are the same as those in Table 1. Abbreviations: TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol; FFA, free fatty acids.

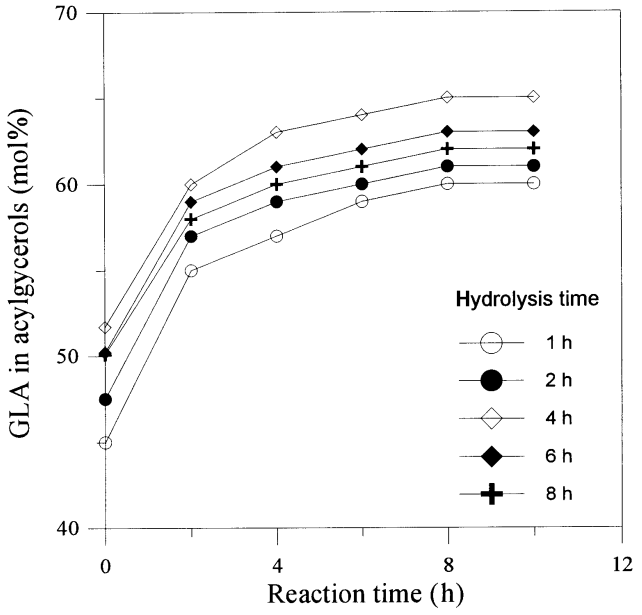

FIG. 4. Effect of reaction time of hydrolysis of borage oil on the γ-linolenic acid (GLA) content in acidolysis reaction. Reaction mixture contains unhydrolyzed acylglycerols from the selective hydrolysis of 200 mg borage oil; 0.5 mmol of GLA-rich (87%) FFA; 3 mL isooctane; 15 mg Lipozyme IM-20, and 0.2 g molecular sieve. Reaction temperature = 50°C; magnetic stirrer speed = 600 rpm. The reaction was conducted under nitrogen atmosphere. See Figure 3 for abbreviation.

on the GLA content in FFA was studied, and the optimal reaction temperature was determined to be 60°C. A reaction temperature of 30°C was chosen to minimize thermal degradation of GLA. A substrate molar ratio of 2:1 (*n*-butanol/FFA) gave the best results in terms of GLA content. Lipase-catalyzed esterification can be described with a ping-pong mechanism that has substrate (alcohol) inhibition; the inhibition increases with the concentration of alcohol (25). A maximal GLA content of 85 mol% in the remaining FFA was obtained with a reaction time of 2 h. Syed Rahmatullah *et al.* (9) reported a GLA content of 92% in the remaining FFA in a similar study. The authors were able to obtain a slightly lower GLA content in the remaining FFA by using a much lower reaction temperature, minimizing the thermal degradation of GLA.

Acidolysis reaction. GLA-rich FFA from selective esterification and unhydrolyzed acylglycerol from the hydrolysis of borage oil were used as substrates in the acidolysis reaction. The reaction was carried out with Lipozyme IM-20 in isooctane at 50°C. The GLA-content effects of hydrolysis time in preparing the unhydrolyzed borage oil acylglycerols of the reaction product are shown in Figure 4. An optimal hydrolysis time of 4 h results in a maximal GLA content in acylglycerols of 65%. As stated previously, a maximal GLA content of approximately 50% in acylglycerols can be obtained from the selective hydrolysis of borage oil. Acidolysis is clearly a better approach to enrich GLA in borage oil acylglycerols.

Lipase-catalyzed reactions for the enrichment of GLA content in borage oil have been described in this work. These reactions include selective hydrolysis of borage oil by immobilized *C. rugosa* lipase, selective esterification of fatty acids

from saponified borage oil with *n*-butanol, and Lipozyme IM-20, and acidolysis of GLA-rich fatty acids from selective esterification and unhydrolyzed acylglycerols from selective hydrolysis. A GLA content of 65% in acylglycerols can be obtained by the acidolysis reaction described in this work.

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