γ-Linolenic Acid-Rich Triacylglycerols Derived from Borage Oil *via* Lipase-Catalyzed Reactions

Fang-Cheng Huang[a], Yi-Hsu Ju[b,*], and Jen-Chung Chiang[b]

Departments of Chemical Engineering, [a]Cheng Shiu College of Technology and Commerce, Kaohsiun County 833, Taiwan, and [b]National Taiwan University of Science and Technology, Taipei 106-07, Taiwan

ABSTRACT: γ-Linolenic acid (GLA), a precursor of arachidonic acid, possesses physiological functions of modulating immune and inflammatory response. Highly purified GLA is desired both as a medicine and as an ingredient of cosmetics. In this work, urea fractionation and lipase-catalyzed reactions were employed for the enrichment of GLA in borage oil. GLA content in free fatty acids from saponified borage oil can be increased from 23.6 to 94% by the method of urea fractionation. Partial hydrolysis of borage oil catalyzed by immobilized *Candida rugosa* lipase raises GLA content in the unhydrolyzed acylglycerols from 23.6 to 52.1%. The IM-60 catalyzed acidolysis reaction between the GLA-rich free fatty acid and the unhydrolyzed acylglycerols increases the GLA content in the acylglycerols from 52.1 to 75%. The acylglycerols in the reaction product contains *ca.* 90% triacylglycerol. The effects of temperature, water content, substrate weight ratio, and organic solvents on the GLA content in the acylglycerols were examined.

Paper no. J8985 in *JAOCS 76,* 833–837 (July 1999).

KEY WORDS: Acidolysis, borage oil, immobilized lipase, γ-linolenic acid, selective hydrolysis, urea fractionation.

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 the linoleic acid (18:2n-6) to the eicosanoid precursor arachidonate. The etiology of certain diseases may derive from a deficiency of GLA (1). GLA-rich acylglycerides have been applied directly in the treatment of certain skin-related and other diseases, such as atopic eczema (2), multiple sclerosis (3), rheumatoid arthritis (4), and premenstrual syndrome (5). Therefore, many investigators have actively investigated the concentration of GLA from borage oil, evening primrose oil, and fungal oil for pharmaceutical and dietetic purposes. Borage oil is one of the richest natural sources of GLA with a GLA content of up to 25%, and is suitable for the enrichment of GLA. Available methods for the enrichment of GLA include urea adduct formation (6), separation on Y-zeolite (7), solvent winterization (8), and enzymatic selective hydrolysis or esterification (9–11). Syed Rahmatullah *et al.* (11) reported that *Candida rugosa* lipase is more efficient for the concentration of GLA by the selective hydrolysis of borage oil and evening primrose oil. Huang *et al*. (12) obtained a GLA content of 65% in acylglycerols by an acidolysis reaction catalyzed by Lipozyme® IM-20 (*Mucor miehei* lipase immobilized on anion exchange resin). In this work, GLA-rich free fatty acids derived from the urea fractionation of saponified borage oil were used as a substitute for pure GLA. The incorporation of GLA into the unhydrolyzed borage oil in an acidolysis reaction catalyzed by IM-60 in organic solvent was then studied systematically. A small amount of water is required for lipase-catalyzed acidolysis reaction in organic solvent. The polarity or the hydrophobicity of solvent can have profound effects on the three-dimensional structure of enzyme and the retention of enzyme-associated water necessary for its activity. The effects of temperature, water content, substrate weight ratio, and organic solvents on the acidolysis reaction are reported.

*To whom correspondence should be addressed.
E-mail: ju@ch.ntust.edu.tw

MATERIALS AND METHODS

Materials. Candida rugosa lipase (EC 3.1.1.3; triacylglycerol ester hydrolase) and borage oil (av. MW 873.4) were purchased from Sigma Chemical (St. Louis, MO). Lipozyme® IM-60 (lipase from *M. miehei* immobilized on anion exchange resin) was a gift of Novo-Nordisk Industrie (Mainz, Germany). Microporous PP (Accurel EP 100, particle size: 200–1000 μm) was a kind gift of Akzo-Nobel (Obernburg, Germany) and used as a carrier for enzyme immobilization. Particle size in the range of 300–500 μm was selected by using a Tyler sieve. All solvents were of either HPLC or analytical grade and were obtained from commercial sources. Heptadecanoic acid, 16:0, 18:0, 18:1, 18:2, and 18:3n-3 for calibration curves in gas–liquid chromatographic analysis were obtained from Sigma Chemical. Other standards (20:1, 22:1, and 24:1) were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Triolein, diolein (85% 1,3- and 15% 1,2-isomer) and 1-monooleoyl-*rac*-glycerol used for thin-layer chromatography (TLC) analysis, were obtained from Sigma Chemical. Urea (99.5% pure) was provided by Acros (Harnstoff, PA). Olive oil emulsion purchased from Sigma Chemical was used as a substrate for *C. rugosa* lipase assay.

Lipase immobilization. Candida rugosa lipase powder (10 mg) was dissolved in 250 μL of 0.1 M phosphate buffer (pH

7.0). Following centrifugation, the upper clear enzyme solution was removed. A proper amount of the enzyme solution was pipetted onto a glass sheet. Polypropylene (100 mg) prewetted with 0.5 mL of ethanol were added to the enzyme solution and flattened by using a small wood bar. This was then placed into a vacuum oven at 25°C to strip off excess water.

Lipase assay. The hydrolytic activity of the immobilized *C. rugosa* lipase was assayed using olive oil emulsion (12). The acidolysis activity of IM-60 was assayed as follows. Isooctane (2 mL) containing equal amounts (on the molar basis) of triolein and palmitic acid were put into a test tube. The test tube was incubated at 40°C, 40 mg of IM-60 was added, and the mixture was agitated by a magnetic stirrer at 600 rpm for 40 min. The reaction was stopped by adding 10 mL of acetone/ethanol (50% vol/vol). The compositions of the reaction product were determined by gas chromatography. One unit of activity is equivalent to 1 μmol of palmitic acid reacted/h at 40°C.

Preparation of fatty acids (11). Two grams of borage oil and 12 mL of alcoholic KOH solution (1 N KOH in 90% aqueous ethanol) were placed in a test tube (15 mm i.d.). The mixture was heated at 80°C under nitrogen atmosphere, and agitated with a magnetic stirrer at 600 rpm for 90 min. After cooling to room temperature, 12 mL of deionized water and 4 mL of 6 N HCl were added to the mixture. The products (fatty acids) were extracted three times, each with 10 mL of diethyl ether. All extracts were collected and then dried under vacuum at room temperature.

Urea fractionation. Urea fractionation described by Traitler *et al.* (6), with slight modification, was employed for the enrichment of GLA in the free fatty acids from saponified borage oil. Typically, 1 g of free fatty acid from saponified borage oil was added into 3 mL of methanol to form a homogeneous solution. Three grams of urea was mixed with 6 mL of methanol in a test tube and incubated in a water bath at 65°C. The free fatty acid containing methanol solution was slowly added into the test tube with slight stirring, until the content of the test tube became clear. The test tube was placed at room temperature for 1 h, then at 4°C for 24 h. The tube was centrifuged at $550 \times g$ for 3 min, and then placed in a refrigerator at −20°C for 1 h. The tube with its contents was centrifuged again and stored overnight at −20°C. The solution in the tube was filtered using a membrane with pore size of 22 μm. Six milliliters of deionized water and 2 mL of 6 N hydrochloride were added to the collected filtrate. Ten milliliters of hexane was added to the above solution to extract the free fatty acid. The organic phase was separated and the water phase was extracted again with hexane. The organic phases from the extraction steps were combined and evaporated in a vacuum oven, yielding GLA-rich free fatty acid.

Selective hydrolysis reaction. Two milliliters of isooctane containing 0.2 g of borage oil was placed into a test tube (15 mm i.d.), and 1 mL of 0.1 M phosphate buffer was added. This mixture was incubated in a water bath for 20 min at 30°C, and 4.5 U of immobilized *C. rugosa* lipase was added into the test tube (1 U is equal to the amount of enzyme required to liberate 1 μmol FFA per min). The mixture was shaken for *ca.*1 min by vortex, then returned to the water bath, and agitated by a magnetic stirrer at 600 rpm as the reaction proceeded.

Acidolysis reaction. In the acidolysis reaction between the unhydrolyzed acylglycerols of borage oil and GLA-rich free fatty acids, hydrolysis of acylglycerol is followed by reesterification, with the hydrolysis being the rate-limiting step (13). Typically, 20 mg of unhydrolyzed acylglycerols and various amounts of GLA-rich fatty acid are dissolved in 2 mL isooctane, followed by the addition of 1.25% w/w (1.25% combined weight of substrate) 0.1 M phosphate buffer (pH 7.0) and 15% w/w IM-60 (15% combined weight of substrates). The reaction was carried out under nitrogen atmosphere and agitated with a magnetic stirrer at 600 rpm. Product concentrations measured in the first 3 min of incubation were used in the calculation of initial rates.

TLC analysis for the acidolysis reaction products. The acidolysis reaction products were analyzed by TLC on a silica gel plate (60; Merck, Darmastadt, Germany) with chloroform/acetone (96:4 vol/vol) as the developing solvent (14). GLA content in the acidolysis reaction product was analyzed as described in the following.

Fatty acid compositions in acylglycerols by gas chromatography (GC) analysis (15). GC was employed for the determination of fatty acid compositions of the acidolysis reaction. 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 TMSH (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 concentrations of fatty acid esters were determined from the calibration curves by the measured peak area ratio.

RESULTS AND DISCUSSIONS

Enrichment of GLA by urea fractionation. Urea normally forms tetragonal crystals, but in the presence of certain aliphatic compounds it forms hexagonal prisms encompassing some of the aliphatic material. Fatty acids are separated by urea fractionation according to their degree of unsaturation. Table 1 shows that GLA content in the free fatty acid derived from borage oil can be increased from 23.6 to 94% by urea fractionation. The linoleic and oleic acid contents in the free fatty acid decrease from 38.9 to 2.8% and 16.6 to 1.1%, respectively. Thus the saturated, mono-, and diunsaturated fatty acids were separated from the polyunsaturated fatty acids by the urea fractionation. An alternative way to obtain GLA-rich free fatty acids is by the lipase-catalyzed selective esterification between free fatty acids and *n*-butanol (10,12) which yields free fatty acids that contain 87–92% GLA.

TABLE 1
Composition of Saponified Borage Oil Fatty Acids Before and After Urea Fractionation

Fatty acids	Before fractionation (%)	After fractionation (%)
16:0	9.8	1.1
18:0	3.6	1
18:1	16.6	1.1
18:2	38.9	2.8
18:3γ	23.6	94
10:1	3.0	N.D.[a]
22:1	3.4	N.D.
24:1	1.1	N.D.

[a]N.D., not detected.

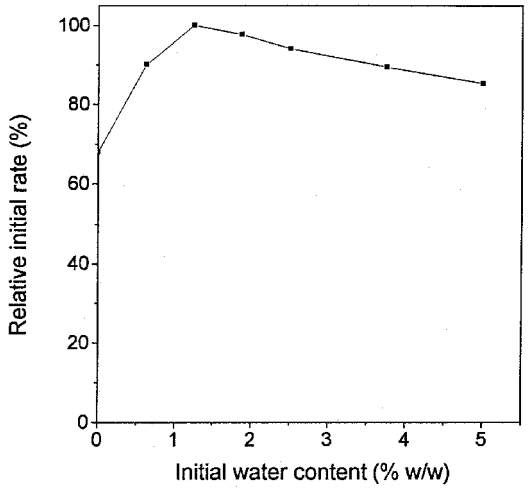

FIG. 1. Effect of initial water content on the initial rate of the acidolysis reaction. Reaction conditions: 20 mg unhydrolyzed borage oil, 40 mg γ-linolenic acid (GLA)-rich free fatty acids in 2 mL isooctane, enzyme loading 15% (w/w), magnetic stirrer speed 600 rpm, reaction temperature 50°C. Initial rate at a water content of 1.25 % (w/w) is taken as 100%.

Selective hydrolysis. Results of the selective enzymatic hydrolysis of borage oil was carried out are listed in Table 2. After a reaction time of 4 h, GLA content in the unhydrolyzed acylglycerols reached a maximum of 52.1% with a yield of 66%. At a reaction time of 0.5 h, acylglycerols with a GLA content of 42% and a yield of 98% can be obtained. Acylglycerols with a GLA content of 42% were chosen as substrate in the acidolysis reaction.

Effects of water content on the acidolysis reaction. To keep the enzyme active and promote the acidolysis reaction rate, a certain amount of 0.1 M phosphate buffer (pH 7.0) was added into a mixture of GLA-rich free fatty acid and unhydrolyzed acylglycerols at the beginning of the reaction. This added water is referred to as the initial water content (% w/w). As shown in Figure 1, GLA content in the acylglycerols reaches maximum at a water content of 1.25% w/w. Apparently, 1.25% w/w is the proper amount of water for the IM-60 to show its maximum activity, and is chosen as the water content in later experiments.

Effect of temperature on the reaction rate and the stability of IM60. The effects of temperature on the reaction rate and the stability of IM-60 were examined and the results are shown in Figure 2. The IM-60 catalyzed acidolysis reaction shows maximal reaction rate at 50°C. In the thermostability studies, the IM-60 was incubated in isooctane for 24 h at the

TABLE 2
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 acids composition[c] (mol%) 16:0	18:0	18:1	18:2	γ-18:3	20:1	22:1	24:1	Hydrolysis (%)[d]	Yield of GLA in acylglycerols (%)
0	Acylglycerols	9.8	3.6	16.6	38.9	23.6	3	3.4	1.1	0	100
0.5	Acylglycerols	7.5	3.7	13	26.2	42.0	3.2	3.4	1	45	98
0.5	Fatty acids	12.6	3.5	21.0	54.4	1.1	2.8	3.4	1.2		
1	Acylglycerols	7.5	3.5	10.2	24.4	45.7	4.2	3.5	1	54	89
1	Fatty acids	11.8	3.7	22.1	51.3	4.8	2.0	3.3	1.2		
2	Acylglycerols	7.1	3.4	9.2	23.4	46.6	5.2	3.6	1.5	57	85
2	Fatty acids	11.8	3.8	22.2	50.6	6.2	1.3	3.2	0.8		
3	Acylglycerols	5.8	2.5	8.1	23.2	49.1	5.6	3.9	1.8	64	75
3	Fatty acids	12.1	4.2	21.4	47.7	9.3	1.5	3.1	0.7		
4	Acylglycerols	4.2	2.2	7.5	22.6	52.1	5.7	3.9	1.8	70	66
4	Fatty acids	12.2	4.2	20.5	45.9	11.4	1.8	3.2	0.8		
5	Acylglycerols	4.5	2.6	7.9	23.2	51.2	5.5	3.6	1.5	74	56
5	Fatty acids	11.7	4.0	19.7	44.4	13.9	2.1	3.3	1.0		
6	Acylglycerols	4.5	2.7	8.0	23.5	50.7	5.4	3.7	1.5	78	47
6	Fatty acids	11.3	3.9	19.0	43.2	16.0	2.3	3.3	1.0		
8	Acylglycerols	5.4	2.7	8.1	23.9	50.2	5.2	3.3	1.2	80	43
8	Fatty acids	10.9	3.8	18.7	42.7	17.0	2.5	3.4	1.1		

[a]Reaction conditions: 200 mg borage oil in 2 mL isooctane; enzyme content 4.5 U, where 1 U is the amount of enzyme required to liberate 1 μmol free fatty acid per min; magnetic stirrer speed 600 rpm; reaction temperature 30°C.
[b]Acylglycerols (contain tri-, di-, and monoacylglycerols).
[c]Fatty acid/acyl moieties are designated by number of C atoms/number of *cis*-double bonds.
[d]Fatty acid in acylglycerols/initial fatty acid in acylglycerols.

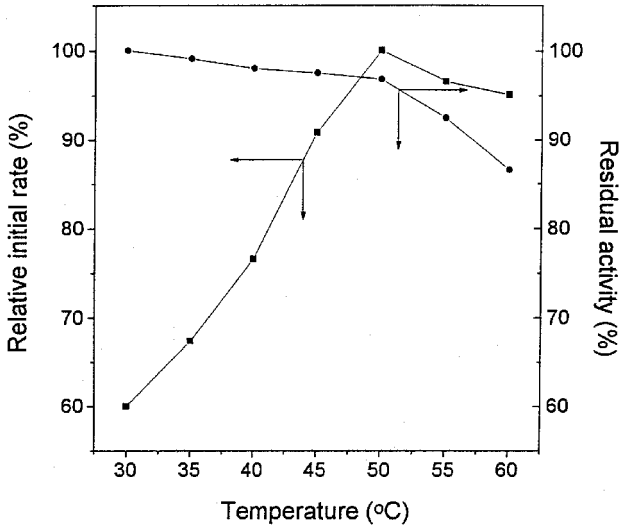

FIG. 2. Effects of temperature on the activity and the stability of IM-60. Reaction conditions are the same as those in Figure 1, except that the water content is 1.25% (w/w). Residual activities at 50 and 30°C are taken as 100% for the activity and stability studies, respectively.

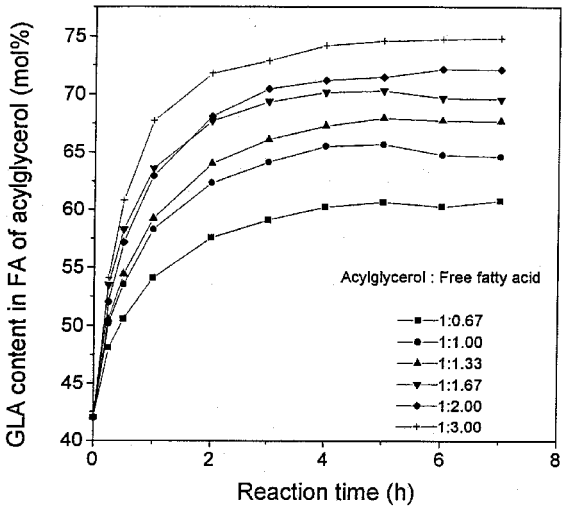

FIG. 3. Effects of substrate weight ratio on the GLA content in acylglycerols. Reaction conditions are the same those in Figure 1, except that the substrate weight ratio is 1:2. FA, fatty acids. See Figure 1 for abbreviation.

desired temperature, and then assayed for its residual activity. By referring to Figure 2, an operating temperature of 50°C was chosen from considerations of both initial rate and thermal stability of IM-60.

Effects of organic solvents. The polarity of organic solvent affects lipase catalyzed reactions (16). The effect of log *P* (the logarithm of partition coefficient, a measure of polarity) of several organic solvents on the acidolysis reaction was examined. Table 3 indicates that isooctane was the best solvent in terms of reaction rate. When IM-60 was incubated in various solvents for 24 h, it shows higher residual activity in hexane and isooctane. In this work, isooctane was chosen as the solvent for the acidolysis reaction.

Effect of weight ratio of substrates on the acidolysis reaction. Figure 3 shows the effect of weight ratio (unhydrolyzed acylglycerols to GLA-rich fatty acid) on the GLA content in the acidolysis reaction product. GLA content in acylglycerols increases with substrate weight ratio and reaction time. At a substrate weight ratio of 1:3 and a reaction time of 7 h, the reaction product was analyzed by TLC and the results show that the acylglycerols contain 90.6% TG. The corresponding GLA content in TG is 74.3%.

TABLE 3
Effects of Solvents on the Initial Rate and the Solution Stability of IM-60[a]

Solvents	Isooctane	Hexane	Xylene	Toluene	Benzene
Log *P*	4.5	3.5	3.1	2.5	2.0
Relative initial rate (%)	100	92.9	60.7	71.4	82.1
Residual activity (%)	96.8	96	85.3	80.1	75.6

[a]Reaction conditions are the same as those in Figure 1, except the solvents and 1.25% w/w water content. Residual activities without incubation (in isooctane) were taken as 100% in the stability study. *P*, partition coefficient.

An alternative approach for the enrichment of GLA from borage oil *via* lipase-catalyzed reactions in isooctane was described. This includes selective hydrolysis of borage oil by immobilized *C. rugosa* lipase, urea fractionation of fatty acids from saponified borage oil, and acidolysis reaction between GLA-rich fatty acid and unhydrolyzed acylglycerols. Triacylglycerol content in the acylglycerols of the acidolysis reaction product can reach 90.6% with a corresponding GLA content in triacylglycerols of 74.3%.

ACKNOWLEDGMENT

This work was supported by the National Science Council of Taiwan through a grant NSC87-2214-E-230-001.

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[Received August 19, 1998; accepted February 8, 1999]