High-Purity γ-Linolenic Acid from Borage Oil Fatty Acids

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ABSTRACT: High-purity γ-linolenic acid (GLA) was obtained by employing a modified low-temperature solvent crystallization process, followed by a lipase-catalyzed esterification, to borage oil fatty acid. By applying a two-stage solvent crystallization process to the borage oil fatty acid, GLA content was increased from 23.4 to 92.1% with a yield of 89.3%. After the esterification of GLA-rich fatty acid with butanol catalyzed by Lipozyme IM-60, GLA content in the fatty acid was further raised from 92.1 to 99.1%. The overall yield of the combined process was 72.8%. The effects of operation parameters on the Lipozyme IM-60 catalyzed esterification between fatty acid and alcohol were systematically investigated.

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KEY WORDS: Borage oil, esterification, GLA, lipase, Lipozyme IM-60, solvent crystallization.

γ-Linolenic acid (GLA, all-*cis*-6,9,12-octadecatrienoic acid) is a metabolite of linoleic acid (LA, 18:2n-6) and the first intermediate in the conversion of LA to arachidonic acid (AA, 20:4n-6) (1). GLA also possesses important physiological functions, such as modulating immune and inflammatory response (2). *In vitro* and *in vivo* studies have shown that GLA can selectively kill tumor cells without harming normal cells (3). There is also evidence for the therapeutic value of GLA-rich acylglycerols (evening primrose oil) in treatment of atopic eczema, rheumatoid arthritis, diabetic neuropathy, cirrhosis of the liver, psychiatric disorders and premenstrual breast pain, etc. (4). GLA, as a lithium salt, has been reported to prolong the survival of patients with irresectable pancreatic cancer in a dose-dependent fashion (5). The synthesis of GLA concentrate or GLA-rich acylglycerol from natural sources is important for pharmaceutical and dietary purposes.

Various techniques are available for the enrichment of GLA from natural sources. Solvent winterization has been employed to increase the GLA content of fungal oil (6). GLA content in the galactolipids of the biomass of the cyanobacterium *Spirulina platensis* was increased from 39.0 to 90.5% by urea fractionation (7). Guil-Guerrero *et al.* used a four-step method, which included urea fractionation and silica gel chromatography, for the concentration of GLA in seed oil (8). GLA ethyl ester can be concentrated by using Y-Zeolite (9). Enzyme-catalyzed reactions have become popular methods for obtaining concentrated GLA. Lipase-catalyzed esterification was employed for the enrichment of GLA from borage oil fatty acid, and free fatty acids (FFA) with a GLA content of *ca.* 90% were obtained (10,11). By a two-step process, which involved enzyme-catalyzed hydrolysis of borage oil and the esterification of the resulting FFA with lauryl alcohol, a 93.7% GLA content in FFA with a corresponding yield of 67.5% was obtained (12). Shimada *et al.* (13) used a series of enzyme-catalyzed reactions, distillations, and urea fractionation to obtain an FFA that contained 98.6% GLA with a yield of 49.4%.

Low-temperature solvent crystallization was developed for the concentration of polyunsaturated fatty acid decades ago. The basic principle of this method is that the solubility of a fatty acid or its ester in a solvent depends on the chain length and the number of double bonds of the fatty acid. Chen and Ju (14) reported that the GLA content in borage oil fatty acid was increased to 88.9% with a yield of 62.0% by a solvent crystallization process. An improved two-stage solvent crystallization process was employed for the enrichment of GLA in borage oil fatty acid. A GLA content of 93.9% with a corresponding yield of 86.0% was reported (15).

In this work, a process consisting of solvent crystallization and lipase-catalyzed esterification was developed for the enrichment of GLA in borage oil fatty acid. The purpose is to obtain an FFA preparation with very high GLA content and reasonably high yield.

EXPERIMENTAL PROCEDURES

Materials. Borage oil was purchased from Sigma (St. Louis, MO). Except for 20:1, 22:1, and 24:1, which were provided by Nu-Chek-Prep Inc. (Elysian, MN), all other standards were provided by Sigma. Immobilized lipase (Lipozyme IM-60) from *Rhizomucor miehei* was a gift of Novo Nordisk Bioindustry Co. Ltd. (Bagsvaerd, Denmark). All solvents and reagents used were of high-performance liquid chromatography or ACS grade.

Preparation of borage oil fatty acid. FFA obtained by the saponification of borage oil were prepared according to the method described by Haagsma *et al.* (16). Typically, a NaOH solution was prepared by dissolving 48 g NaOH and 0.5 g Na_2EDTA in 160 mL water. To this solution, 160 mL ethanol was added. A mixture containing 100 g oil and 200 mL NaOH solution was heated at 60°C with magnetic stirring at 550 rpm for 1 h. Then added were 40 mL water and 400 mL hexane, and the solution was stirred for 1 h. The upper layer containing unsaponifiable matter was removed and discarded. To the

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lower layer was added 160 mL water, and 12 N hydrochloric acid was then added until the pH equaled 1. The resulting lower layer was removed by using a separating funnel and discarded. The FFA-containing upper layer was dried with anhydrous magnesium sulfate, and solvent was evaporated in a vacuum rotary evaporator at 35°C. FFA obtained from the saponification of borage oil will be referred to as FFA-I.

Solvent crystallization. The enrichment of GLA by solvent crystallization consisted of two stages. In the first stage, 250 mL acetonitrile was added to a vial containing 4 g FFA-I. This mixture was stirred with a magnetic stirrer at 35°C under nitrogen atmosphere until all FFA-I were dissolved. The solution was cooled to room temperature and then stored in an ultra-low temperature freezer (Sanyo MDF-192; Gunma, Japan) chamber at –40°C for 48 h. Solid formed was removed by using a Buchner funnel operated at room temperature. GLA-rich FFA was obtained after solvent in the remaining liquid was removed in a vacuum rotary evaporator operated at 35°C. GLA-rich FFA resulting from the first-stage operation was subjected to the second-stage operation. All procedures were the same as those described in the first-stage solvent crystallization except the following: The solvent was a mixture of 30% acetonitrile and 70% acetone, the storage temperature was –80°C, 1 g FFA and 110 mL solvent were used, and the storage time was 24 h. GLA-rich FFA obtained from the second-stage operation was designated as FFA-II.

Lipase-catalyzed esterification. Esterification between FFA-II and alcohol catalyzed by Lipozyme IM-60 was carried out in organic solvent at 37°C with magnetic stirring at 600 rpm. Typically, both FFA-II and alcohol concentrations were 100 mM with a reaction volume of 5 mL. The amount of Lipozyme IM-60 used was 20 mg.

Analysis of the composition and yield of nonesterified FFA. At various reaction times, 100-μL samples were taken for the analysis of GLA content and yield in the nonesterified FFA. To this sample, 100 μL *n*-hexane containing 20 mM tridecanoic acid was added as internal standard. After adding 400 μL of 1 N KOH solution, FFA and FFA esters were separated by centrifugation (860 × *g*, 3 min). FFA in the water layer was extracted with 300 μL *n*-hexane after returning to acidic pH (<pH 2) with HCl. After removing *n*-hexane by nitrogen, the resulting FFA were transformed into methyl esters and analyzed for the composition and yield of FFA by gas–liquid chromatography. The yield of GLA in nonesterified FFA was expressed as the ratio of the amount of GLA in the sample to the initial amount of GLA before the reaction.

Gas–liquid chromatographic analysis of fatty acid composition. FFA were transformed into the corresponding methyl esters with trimethylsulfonium hydroxide (17). FFA composition was analyzed by gas–liquid chromatography, with flame-ionization detection. The column used was SP-2330 (30 × 0.25 mm i.d.; Supelco, Bellefonte, PA). The temperatures of the injector and the detector were set at 250 and 270°C, respectively. The column was held at 180°C for 10 min and then increased to 235°C at a constant rate of 15°C/min and kept at 235°C for 3 min. One microliter of sample was injected at a split rate of 1:50.

RESULTS AND DISCUSSION

The two-stage operation. Operation conditions used in the two-stage solvent crystallization that resulted in both high GLA content and yield were selected according to Chen and Ju (14). Table 1 shows the fatty acid compositions of FFA-I (borage oil fatty acid) and FFA-II (fatty acid composition after the two-stage crystallization). As can be seen, most of the saturated and monounsaturated fatty acids in FFA-I were removed by the two-stage solvent crystallization. Both high GLA content (92.1%) and yield (89.3%) were obtained. This result is comparable to the best results available in the literature in terms of GLA content. However, GLA yield obtained here is considerably higher than those reported in the literature (6,9–13).

Esterification of FFA-II and alcohol. Lipase from *R. miehei* is known to favor the esterification of more saturated fatty acids over esterification of polyunsaturated fatty acids such as GLA with alcohol (10,11,18). GLA, being a polyunsaturated fatty acid, mostly remains as nonesterified fatty acid in the reaction. GLA content and yield in the nonesterified fatty acid will be dependent on reaction conditions. Figure 1 shows the effects of alcohol type on GLA content and yield for the esterification of FFA-II and alcohol. In general, the reaction rate decreases with increasing alcohol carbon number except for ethanol, which is known to inhibit lipase activity. Butanol was chosen as the alcohol for further studies since it resulted in highest reaction rate and highest GLA content in the nonesterified FFA. A butanol concentration of 100 mM was chosen in this study since this gave the highest GLA content (results not shown). At a reaction time of 40 min, GLA

TABLE 1
Composition of Borage Oil Fatty Acid Before and After Concentration

FFA	Content (wt%)		
	FFA-I[a]	FFA-II[b]	FFA-III[c]
16:0	10.3	40.07	0.04
18:0	3.61	0.02	0.02
18:1	15.89	1.05	0.09
18:2	37.28	6.71	0.65
GLA[d]	23.35	92.09	99.13
20:1	4.25	0.06	0.06
22:1	3.16	ND[e]	n.d.
24:1	2.12	ND	n.d.
Yield of GLA[f] (%)	—	89.28	81.58
Overall yield of GLA[g] (%)	—	—	72.83

[a]Fatty acid obtained from saponified borage oil.
[b]Fatty acid obtained after the two-stage operation on free fatty acid (FFA-I). The first stage: solvent = acetonitrile, solvent/FFA = 62.5 mL/g, FFA = 4 g, storage temperature = –40°C; the second-stage: solvent = 30% acetonitrile + 70% acetone, solvent/FFA = 110 mL/g, FFA = 1 g, storage temperature = –80°C.
[c]Fatty acid obtained after esterification of FFA-II and butanol. Lipozyme IM-60 Novo Nordisk Bioindustry Co. Ltd. (Bagsvaerd, Denmark) = 20 mg, solvent = hexane, butanol = 100 mM , FFA = 100 mM, reaction temperature = 37°C, reaction volume = 5 mL, magnetic stirrer speed = 600 rpm, reaction time = 40 min.
[d]GLA, all *cis*-6,9,12-octadecatrienoic acid (= γ-linolenic acid).
[e]Not detectable.
[f]Yield of γ-linolenic acid in the two-step operation of FFA-I or in the esterification of FFA-II with butanol.
[g]Combined yield of the two-step operation and the esterification.

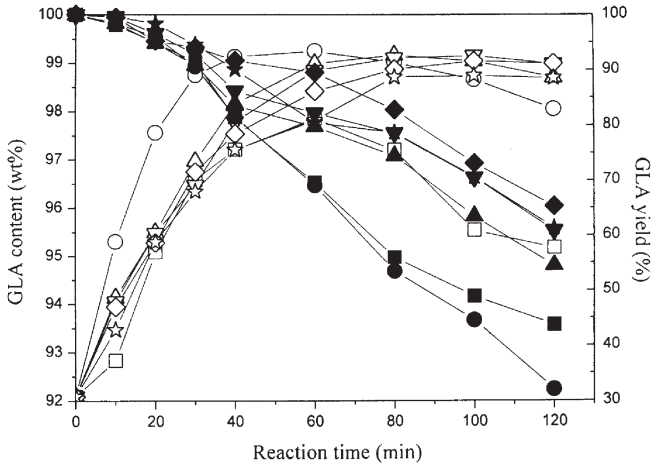

FIG. 1. The effects of alcohol type on the γ-linolenic acid (GLA) content and yield in the esterification of free fatty acid (FFA-II) and alcohol. Reaction conditions: Lipozyme IM-60 = 20 mg, reaction volume = 5 mL, solvent = hexane, FFA-II concentration = alcohol concentration = 100 mM, reaction temperature = 37°C, magnetic stirrer speed = 600 rpm. Both hexane and alcohol were treated with molecular sieve before use. Open symbols = GLA content in remaining FFA, solid symbols = GLA yield. Ethanol (□,■), butanol (○,●), hexanol (△,▲), octanol (▽,▼), decanol (◇,◆), dodecanol (☆,★).

content in the nonesterified fatty acid reached 99.1% with a corresponding yield of 81.6%.

The effect of reaction temperature on maximal GLA content attainable is shown in Table 2. A reaction temperature of 37°C was chosen for the esterification of FFA and butanol because maximal GLA content was reached in a relatively short reaction time.

In general, lipases possess higher activity in solvents with higher log *P* values (19) (*P* is the partition coefficient of the solvent between octanol and water). Table 3 shows the effect of solvent log *P* on maximal GLA content attainable. With hexane as the solvent and at a reaction time of 40 min, GLA content reached 99.1% with a reasonably high GLA yield of 81.6%. The results shown in Figure 1, Tables 2 and 3 generally share the same trend in terms of optimal reaction time. At higher reaction times, the recovery of GLA decreases because it serves more favorably as an acyl donor. At smaller reaction times, many of the non-Δ6 acyl groups have not been esterified; hence, the purity of GLA among the nonesterified FFA is low.

TABLE 2
The Effect of Temperature on the GLA Content of the FFA Fraction Following Esterification by *Rhizomucor miehei* Lipase

Temperature (°C)	GLA content (wt%)[a]	GLA yield (%)[b]	Reaction time (min)[c]
25	99.18	77.37	80
37	99.13	81.58	40
50	99.12	67.86	60

[a]Maximal GLA content attainable at the reaction temperature. See Table 1 for abbreviations.
[b]The corresponding GLA yield at maximal GLA content.
[c]Time required to attain maximal GLA content.

TABLE 3
The Effect of Solvent Log *P* on Maximal GLA Content Attainable

Solvent (log *P*)	GLA content (wt%)[a]	GLA yield (%)[b]	Reaction time (min)[c]
Isooctane (4.5)	99.13	60.07	60
Hexane (3.5)	99.13	81.58	40
Xylene (3.1)	98.61	89.22	120
Toluene (2.5)	98.82	87.33	120
Benzene (2.0)	98.22	89.03	120

[a]Maximal GLA content attainable at the reaction temperature. See Table 1 for abbreviations.
[b]The corresponding GLA yield at maximal GLA content.
[c]Time required to attain maximal GLA content.

Nonesterified fatty acid after the esterification of FFA-II with butanol under optimal reaction conditions was designated as FFA-III. The composition of FFA-III is shown in Table 1. Starting with borage oil fatty acid (FFA-I) that contains 23.4% GLA, we have developed a simple process to obtain FFA that contains 99.1% GLA with an overall GLA yield of 72.8%. This compares favorably with best results available in the literatures. For example, Shimada *et al.* (13) obtained FFA that contains 98.6% GLA with a yield of 49.4% from borage oil fatty acid. The inclusion of the two-stage solvent crystallization is crucial for achieving very high (>99%) GLA content with reasonably high (>70%) GLA yield. If it is omitted, the GLA content attainable is *ca.* 90% with GLA yield of *ca.* 65% (10,11).

A process that combines a low-temperature solvent crystallization and an enzyme-catalyzed esterification was developed for the enrichment of GLA in FFA derived from saponified borage oil. Both high GLA content (99.1%) and yield (72.8%) were obtained. Preliminary studies show that the process developed in this work is also very effective for the enrichment of docosahexanoeic acid (C22:6n-3) from DHASCO (Martek Biosciences Corporation, Columbia, MD), a single-cell oil. It is moderately effective for the enrichment of AA (C20:4n-6) from ARASCO (Martek Biosciences Corporation), also a single-cell oil.

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