γ-Linolenic Acid Concentrates from Borage and Evening Primrose Oil Fatty Acids *via* Lipase-Catalyzed Esterification

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γ-Linolenic acid (GLA, all-*cis* 6,9,12-octadecatrienoic acid) has been enriched from fatty acids of borage (*Borago officinalis* L.) seed oil to 93% from the initial concentration of 20% by lipase-catalyzed selective esterification of the fatty acids with *n*-butanol in the presence of *n*-hexane as solvent. The immobilized fungal lipase preparation, Lipozyme, used as biocatalyst, preferentially esterified palmitic, stearic, oleic and linoleic acids and discriminated against GLA, which was thus concentrated in the unesterified fatty acids fraction. In the absence of hexane, concentrate containing about 70% GLA was obtained. When the reaction conditions, optimized for borage oil fatty acids, were applied to fatty acids of evening primrose (*Oenothera biennis* L.) oil, concentrates containing 75% GLA were obtained. From both oils, GLA concentrates were prepared efficiently in short reaction times (1–3 h) at 30–60°C. The process can be applied for the production of GLA concentrates for dietetic purposes.

KEY WORDS: Borage oil, evening primrose oil, γ-linolenic acid, lipase-catalyzed esterification.

In recent years, much research has been directed toward the production of γ-linolenic acid (GLA; all-*cis* 6,9,12-octadecatrienoic acid) for applications in curing certain skin-related, as well as a variety of other diseases (1). In humans and in other mammals, GLA is the first metabolite formed during the bioconversion of linoleic acid (18:2n-6) to prostaglandins by the desaturation at the C-6 position by Δ6-desaturase. People who lack the Δ6-desaturase suffer from a number of diseases due to an imbalance in GLA production and the formation of successive metabolites that lead to prostaglandins. The occurrence, physical, chemical, nutritional and medicinal properties of GLA have been reviewed recently (1,2).

The most important and commercially available sources of GLA are seed oils of evening primrose (*Oenothera biennis* L.) (3), borage (*Borago officinalis* L.) (2,4–8), blackcurrant (*Ribes nigrum* L.) (1,9) and fungal oils, e.g., from *Mucor* spp. (10) and *Mortierella* spp. (11,12). To the best of our knowledge, the highest level of GLA has been found in borage oil, and therefore this oil is expected to be best suited for the concentration of GLA.

Methods used for the concentration of GLA from natural sources include urea adduct formation (13), separation on Y-zeolite (14), solvent winterization (15) and lipase-catalyzed reactions, such as selective hydrolysis of GLA-containing triacylglycerols (16,17) and selective esterification of GLA-containing fatty acid mixtures, derived from oils, with *n*-butanol (16–18).

In the present study, a systematic investigation has been carried out on the enzymatic enrichment of GLA from borage and evening primrose oils. Modifications have been made on the method involving lipase-catalyzed selective esterification reported earlier for evening primrose oil (16,18) and fungal oil (17). The reaction conditions, optimized for the lipase-catalyzed selective esterification of borage oil fatty acids with *n*-butanol, were applied to evening primrose oil fatty acids on a preparative scale.

EXPERIMENTAL PROCEDURES

Materials. Crude borage oil and evening primrose oil were obtained from the International Food Science Centre (Lystrup, Denmark). Immobilized lipase (Lipozyme) from *Rhizomucor miehei* was provided by Novo Industries (Mainz, Germany). All reagents and adsorbents were of analytical grade and purchased from E. Merck (Darmstadt, Germany).

Preparation of fatty acids. Borage oil and evening primrose oil were hydrolyzed chemically for the preparation of fatty acids. Typically, a mixture of the oil (1 g) and a solution of KOH in 90% aqueous ethanol (1N, 6 mL) was heated under nitrogen at 80°C for 1 h. After cooling, the mixture, cold water (6 mL) and aqueous HCl (6N, 2 mL) were added, and the products were extracted with diethyl ether (3 × 10 mL). The ether extracts were combined, washed with water (3 × 5 mL), and then diethyl ether was evaporated at 40°C by blowing a stream of dry nitrogen. The method was scaled up for the preparation of larger lots (10–20 g) of fatty acids.

Lipase-catalyzed esterification of fatty acids with n-butanol. The reactions in mg-scale were carried out in 10-mL glass tubes, sealed with Teflon-lined caps, and 250-mL Erlenmeyer flasks were used for 10-g scale experiments. The reactions were carried out for various periods at different temperatures.

In mg-scale experiments, the reaction mixture contained fatty acids of borage oil or evening primrose oil (100 mg, about 0.36 mmol), 53.3 mg or 66 μL *n*-butanol (0.72 mmol) and 15.3 mg lipase powder (about 10% by weight of the total weight of the reaction partners) with or without the addition of 2 mL hexane. Air in the tube was replaced by nitrogen and the mixture was stirred magnetically. Aliquots were withdrawn at definite intervals for analyses. At the end of the reaction period, the lipase preparation was separated from the reaction products by centrifugation.

In preparative-scale experiments, typically, a mixture of 10 g borage crude fatty acids (about 36 mmol), 5.33 g or 6.6 mL *n*-butanol (36 mmol), 1.53 g lipase powder and 180 mL *n*-hexane was stirred in an Erlenmeyer flask (fitted with a reflux condenser) at 60°C for 2 h. The mixture was cooled to room temperature, and the lipase preparation was separated from the reaction products by filtration under gravity. The lipase was washed three times with five mL hexane, filtered, and the filtrates were combined.

For the separation of the unesterified fatty acids from the butyl esters, the solution of the reaction products was transferred to a separatory funnel and extracted with 15

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mL 1N NaOH in 50% aqueous ethanol. The aqueous phase was withdrawn. The hexane phase was reextracted with 5 mL of the above NaOH solution, and the aqueous phase was collected and combined with the previously withdrawn aqueous solution. The combined aqueous solutions were washed with hexane, and the extract was returned to the earlier hexane extracts. The aqueous phase was acidified with 4 mL 6 N HCl and extracted three times with 10 mL diethyl ether. The ether extract was washed with water until it was neutral, dried with anhydrous Na_2S0_4 and evaporated in a rotary evaporator to give 1.2 g of the fatty acid fraction. The hexane phase, containing butyl esters and unreacted *n*-butanol, was washed once with 5 mL water, dried and evaporated in a rotary evaporator under reduced pressure to give 9.3 g butyl ester fraction.

Analytical procedures. The fatty acids obtained from borage oil and evening primrose oil by chemical hydrolysis, the total reaction products resulting from lipase-catalyzed esterification as well as the fractions of fatty acids and butyl esters recovered from the reaction products in preparative-scale experiments, were fractionated into lipid classes by thin-layer chromatography on Silica Gel H with hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol) as the developing solvent. The fractions were identified by co-chromatography with authentic standards, scraped from the thin-layer plate and eluted with water-saturated diethyl ether. Known amounts of methyl heptadecanoate, the internal standard, were added to each set of fractions of butyl esters and fatty acids. The fatty acids were then converted to methyl esters by adding 50 μL trimethyl sulfonium hydroxide and 200 μL 1,2-dichloroethane (19). The mixture was shaken vigorously for 1 min and kept at room temperature for 15 min prior to direct injection onto the gas chromatograph.

Each of the fractions of butyl esters and methyl esters, containing methyl heptadecanoate as internal standard, was analyzed by gas chromatography as described earlier (17).

RESULTS AND DISCUSSION

Several lipases have been shown to discriminate against specific fatty acids or acyl moieties, such as γ-linolenic acid or γ-linolenoyl moieties in lipase-catalyzed hydrolysis (16,17,20), esterification (16–18,20,21) and transesterification (22) reactions. Specifically, the relative inability of lipases to catalyze the esterification of γ-linolenic acid, as compared to other fatty acids, with *n*-butanol has been utilized for the enrichment of this acid from fatty acids of GLA-containing oils *via* kinetic resolution during lipase-catalyzed esterification; GLA is enriched in the unesterified fatty acids, whereas the other fatty acids are selectively converted to butyl esters (16–18). In view of these findings, a systematic study was undertaken to determine the effect of various reaction parameters on the enrichment of GLA *via* lipase-catalyzed esterification of fatty acids of borage oil and evening primrose oil with *n*-butanol.

The results given in Table 1 show the effect of reaction time on the enrichment of GLA from fatty acids of borage oil *via* esterification with *n*-butanol, catalyzed by Lipozyme in the presence of hexane as solvent at 60°C. The maximum enrichment of GLA was obtained after a reaction period of 90 min, which resulted in an unesterified fatty acid fraction in a yield of 15% and containing 91.8% GLA; most of the other fatty acids were converted to butyl esters. When the reaction was continued for 150 min, the GLA content (89.5%) and yield (5%) of the unesterified fatty acid fraction decreased.

In further experiments, the effect of reaction temperature on the enrichment of GLA *via* lipase-catalyzed esterification of fatty acids of borage oil with *n*-butanol was studied. The results presented in Table 2 show that the maximum enrichment of GLA was obtained at 50 and 60°C, which amounted to 93.1 and 92.6% GLA, respectively, in the unesterified fatty acid fraction. Even at 30°C, an unesterified fatty acid fraction containing 85.5% GLA was obtained in a yield of 19%. These

TABLE 1

Enrichment of GLA from Borage Oil Fatty Acids *via* Lipozyme-Catalyzed Esterification with *n*-Butanol at 60°C in Hexane for Various Periods[a]

Reaction time (min)	Component	Amount in total products (wt%)	Composition of fatty acids[b] (wt%)								Enrichment of GLA
			16:0	18:0	18:1	18:2	γ-18:3	20:1	22:1	24:1	
0	Fatty acid	100	11.9	4.7	19.1	38.2	20.4	3.2	1.5	1.0	1.0
30	Fatty acid	34	8.7	3.6	13.3	25.9	44.1	2.5	1.3	0.6	2.2
30	Butyl ester	66	14.8	5.5	24.0	46.7	2.7	3.7	1.6	1.0	
60	Fatty acid	16	3.6	1.5	5.0	10.0	76.2	1.1	0.4	2.2	3.7
60	Butyl ester	84	14.1	5.5	23.2	45.7	4.7	3.8	1.8	1.2	
90	Fatty acid	15	1.1	0.5	1.5	2.8	91.8	0.4	0.2	1.7	4.5
90	Butyl ester	85	14.4	5.4	22.4	44.0	7.3	3.7	1.7	1.1	
120	Fatty acid	10	2.1	0.8	3.4	5.6	85.4	0.6	0.0	2.1	4.2
120	Butyl ester	90	13.3	5.3	21.8	42.7	10.5	3.6	1.7	1.1	
150	Fatty acid	5	1.5	0.5	2.6	3.4	89.5	0.5	0.0	2.0	4.4
150	Butyl ester	95	12.8	5.0	20.7	40.8	14.4	3.6	1.7	1.0	

[a]Reactions were carried out in mg-scale as described in Experimental Procedures section. GLA, γ-linolenic acid.
[b]Fatty acids/acyl moieties are designated by number of C atoms/number of *cis*-double bonds.

TABLE 2

Enrichment of GLA from Borage Oil Fatty Acids *via* Lipozyme-Catalyzed Esterification with *n*-Butanol in Hexane for 90 min at Various Temperatures[a]

Reaction temperature (min)	Component	Amount in total products (wt%)	Composition of fatty acids[b] (wt%)								Enrichment of GLA
			16:0	18:0	18:1	18:2	γ-18:3	20:1	22:1	24:1	
Control	Fatty acid	100	11.9	4.7	19.1	38.2	20.4	3.2	1.5	1.0	1.0
30	Fatty acid	19	2.3	1.0	3.4	6.5	85.5	0.6	0.3	0.4	4.2
30	Butyl ester	81	14.1	5.5	23.1	45.5	4.8	3.9	1.9	1.2	
40	Fatty acid	16	4.0	0.8	3.1	5.3	85.0	0.5	0.3	1.0	4.2
40	Butyl ester	84	14.4	5.4	22.6	44.4	6.6	3.7	1.8	1.1	
50	Fatty acid	14	1.6	0.5	2.3	1.9	93.1	0.0	0.0	0.6	4.6
50	Butyl ester	86	14.9	5.3	22.3	43.8	7.7	3.4	1.6	1.0	
60	Fatty acid	13	1.1	0.5	2.0	3.0	92.6	0.0	0.0	0.8	4.5
60	Butyl ester	87	14.7	5.2	22.1	43.6	8.4	3.4	1.5	1.1	

[a]Same as in Table 1.
[b]Same as in Table 1.

results show that the temperature has a relatively slight effect on the efficiency of enrichment of GLA. However, the rate of esterification is higher at higher temperatures.

Because a lower reaction temperature should ensure the least thermal degradation of GLA, a temperature of 30°C was chosen in one set of experiments. The data given in Table 3 show the effect of reaction time on the enrichment of GLA from borage oil fatty acids *via* selective esterification with *n*-butanol, catalyzed by Lipozyme at 30°C in the presence of hexane as solvent. Highest enrichment of GLA (86.5%) in the unesterified fatty acids was obtained after a reaction period of 150 min.

The results given in Table 4 show the effect of temperature on the enrichment of GLA from borage oil fatty acids *via* lipase-catalyzed esterification with *n*-butanol without hexane as solvent. These results reveal that, even in the absence of hexane, reaction temperatures between 30 and 60°C have only a slight effect on the enrichment of GLA. In general, the extent of enrichment of GLA is lower in the absence of hexane (Table 4) as compared to experiments in which hexane was used as solvent (Tables 1–3).

The next set of experiments was carried out on the preparative-scale enrichment of GLA from borage oil fatty acids *via* esterification with *n*-butanol and Lipozyme as biocatalyst. In these experiments, esterification was carried out at 60°C in 50, 10 and 5% (wt/vol) hexane solutions of the substrates. The results, summarized in Table 5, show that the highest enrichment of GLA was about 80% in the unesterified fatty acids fraction. The solvent concentration had some effect on the extent of enrichment of GLA. Somewhat higher levels of GLA in the unesterified fatty acids were found at lower substrate concentration.

Finally, as an extension of the work carried out with borage oil fatty acids, preparative-scale esterification of fatty acids of evening primrose oil with *n*-butanol, catalyzed by Lipozyme, was carried out to enrich GLA in the unesterified fatty acid fraction. The reactions were carried out for various periods at 60°C with hexane as solvent at a substrate concentration of 5% (wt/vol). The data, given in Table 6, show that the optimum reaction time under the conditions employed is 2 h.

In the last set of experiments, the effect of subambient temperatures was studied on the enrichment of GLA *via* selective esterification of fatty acids of evening primrose oil with *n*-butanol and Lipozyme as biocatalyst. The data

TABLE 3

Enrichment of GLA from Borage Oil Fatty Acids *via* Lipozyme-Catalyzed Esterification with *n*-Butanol at 30°C for Various Periods[a]

Reaction time (min)	Component	Amount in total products (wt%)	Composition of fatty acids[b] (wt%)								Enrichment of GLA
			16:0	18:0	18:1	18:2	γ-18:3	20:1	22:1	24:1	
0	Fatty acid	100	11.9	4.7	19.1	38.2	20.4	3.2	1.5	1.0	1.0
60	Fatty acid	22	7.3	3.3	10.7	20.5	54.3	2.2	1.2	0.5	2.7
60	Butyl ester	78	17.2	5.2	22.6	45.2	4.3	3.2	1.4	0.9	
90	Fatty acid	16	4.0	0.9	5.7	11.0	74.4	1.3	0.8	0.9	3.6
90	Butyl ester	84	16.5	5.3	23.5	46.3	2.8	3.3	1.4	1.1	
120	Fatty acid	12	1.9	1.1	3.2	5.6	85.3	0.7	0.0	2.2	4.2
120	Butyl ester	88	15.1	5.4	24.0	47.3	3.2	3.6	1.6	1.0	
150	Fatty acid	8	1.9	0.9	3.0	5.1	86.5	0.8	0.7	1.1	4.2
150	Butyl ester	92	13.8	5.3	22.9	43.8	7.5	3.8	1.8	1.1	

[a]Same as in Table 1.
[b]Same as in Table 1.

TABLE 4

Enrichment of GLA from Borage Oil Fatty Acids *via* Lipozyme-Catalyzed Esterification with *n*-Butanol without Hexane for 90 min at Various Temperatures[a]

Reaction temperature (min)	Component	Amount in total products (wt%)	Composition of fatty acids[b] (wt%)								Enrichment of GLA
			16:0	18:0	18:1	18:2	γ-18:3	20:1	22:1	24:1	
Control	Fatty acid	100	11.9	4.7	19.1	38.2	20.4	3.2	1.5	1.0	1.0
30	Fatty acid	23	4.2	2.0	7.5	14.5	68.9	1.5	0.7	0.5	3.4
30	Butyl ester	77	17.7	5.1	22.4	44.6	5.9	2.7	1.0	0.6	
40	Fatty acid	20	4.8	1.9	7.8	14.1	68.0	1.5	1.6	0.3	3.3
40	Butyl ester	80	14.1	5.5	22.6	44.5	6.8	3.7	1.7	1.1	
50	Fatty acid	21	4.4	1.8	8.0	15.3	68.0	1.6	0.6	0.3	3.3
50	Butyl ester	79	15.5	5.2	22.1	44.9	6.9	3.2	1.4	0.8	
60	Fatty acid	19	4.4	1.9	7.5	14.9	68.6	1.6	0.6	0.5	3.4
60	Butyl ester	81	18.0	5.1	22.8	45.1	5.1	2.6	0.9	0.4	

[a]Same as in Table 1.
[b]Same as in Table 1.

TABLE 5

Preparative-Scale Enrichment of GLA from Borage Oil Fatty Acids *via* Lipozyme-Catalyzed Esterification with *n*-Butanol Using Different Substrate Concentrations in Hexane at 60°C for Various Periods[a]

Substrate concentration (% wt/vol)	Reaction time (min)	Component	Amount in total products (wt%)/yield (%)	Composition of fatty acids[b] (wt%)								Enrichment of GLA
				16:0	18:0	18:1	18:2	γ-18:3	20:1	22:1	24:1	
Control	0	Fatty acid	100	11.9	4.7	19.1	38.2	20.4	3.2	1.5	1.0	1.0
50	180	Fatty acid	16/13	4.5	1.8	7.2	13.0	71.1	0.5	1.0	0.8	3.5
50	180	Butyl ester	84/75	16.7	4.9	21.8	43.3	8.7	3.5	1.4	1.0	
10	120	Fatty acid	17/13	3.7	1.6	5.9	9.3	76.5	0.5	0.7	1.8	3.8
10	120	Butyl ester	83/78	14.2	5.2	21.7	44.6	8.4	3.4	1.6	1.0	
5	120	Fatty acid	15/13	3.1	1.6	5.5	7.4	79.6	1.0	1.2	0.7	3.9
5	120	Butyl ester	85/79	13.4	5.2	21.8	44.1	9.4	3.6	1.7	1.2	

[a]Reactions were carried out in preparative-scale as described in Experimental Procedures. GLA, γ-linolenic acid.
[b]Fatty acids/acyl moieties are designated by number of C atoms/number of *cis*-double bonds.

TABLE 6

Preparative-Scale Enrichment of GLA from Evening Primrose Oil Fatty Acids *via* Lipozyme-Catalyzed Esterification with *n*-Butanol Using 5% wt/vol Substrate Concentrations in Hexane at 60°C for Various Periods[a]

Reaction time (min)	Component	Amount in total products (wt%)	Composition of fatty acids[b] (wt%)					Enrichment of GLA
			16:0	18:0	18:1	18:2	γ-18:3	
0	Fatty acids	100.0	7.4	2.1	9.0	71.2	9.4	1.0
90	Fatty acids	13.0	5.2	0.0	8.4	31.4	55.0	5.9
90	Butyl esters	87.0	7.4	1.6	11.0	78.6	1.4	
120	Fatty acids	9.0	2.3	0.0	4.6	18.2	74.9	8.0
120	Butyl esters	8.1	8.1	1.6	9.6	77.5	3.2	
240	Fatty acids	6.0	3.4	0.0	6.3	15.5	74.8	8.0
240	Butyl esters	94.0	7.1	1.6	9.7	77.9	3.7	
360	Fatty acids	5.0	6.3	8.3	6.0	15.6	63.8	7.0
360	Butyl esters	95.0	8.4	1.3	9.1	75.0	6.2	

[a]Same as in Table 5.
[b]Same as in Table 5.

TABLE 7

Enrichment of GLA from Evening Primrose Oil Fatty Acids *via* Lipozyme-Catalyzed Esterification with *n*-Butanol in Hexane (substrate concentration 10% wt/vol) for 24 h at various Low Temperatures[a]

Reaction temperature (°C)	Component	Amount in total products (wt%)	Composition of fatty acids[b] (wt%)					Enrichment of GLA
			16:0	18:0	18:1	18:2	γ-18:3	
Control	Fatty acid	100	7.4	2.1	9.0	71.2	9.4	1.0
−20	Fatty acid	26	6.9	1.3	7.4	51.6	32.8	3.5
−20	Butyl ester	74	5.5	0.8	9.4	83.3	1.0	
4	Fatty acid	16	3.6	1.4	6.3	42.2	46.5	4.9
4	Butyl ester	4	7.4	1.2	9.4	79.7	2.3	
20	Fatty acid	12	4.1	0.0	5.8	38.9	51.2	5.4
20	Butyl ester	8	7.5	1.5	9.4	77.1	4.5	

[a]Same as in Table 1.
[b]Same as in Table 1.

presented in Table 7 show that extensive esterification and concomitant enrichment of GLA in the unesterified fatty acids occurred even at 4 and −20°C.

The data presented here show that GLA can be conveniently concentrated to high levels *via* lipase-catalyzed selective esterification of fatty acids of borage oil or evening primrose oil with *n*-butanol.

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