Enrichment of γ-Linolenic Acid from Evening Primrose Oil and Borage Oil *via* Lipase-Catalyzed Hydrolysis

M.S.K. Syed Rahmatullah[a,1], **V.K.S. Shukla**[b] and **K.D. Mukherjee**[a,*]

[a]Federal Centre for Cereal, Potato and Lipid Research, Institute for Biochemistry and Technology of Lipids, H.P. Kaufmann-Institute, D-48147 Münster, Germany and [b]International Food Science Centre A/S, DK-8520, Lystrup, Denmark

Lipase-catalyzed selective partial hydrolysis of evening primrose (*Oenothera biennis* L.) seed oil and borage (*Borago officinalis* L.) seed oil led to an increase in the level of γ-linolenic acid (GLA; 18:3n-6) in the unhydrolyzed acylglycerols. Thus, in evening primrose oil, the GLA level could be raised from 9.4% in the starting material to 46.5% in the unhydrolyzed acylglycerols by means of a lipase from *Candida cylindracea*. Selective hydrolysis of borage oil with Pancreatin led to an increase in the GLA content from 20.4% in the oil to 33.5% in the unhydrolyzed acylglycerols. Partial hydrolysis of borage oil with lipase from *C. cylindracea* raised the GLA content of the acylglycerols to 47.8%.

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

Current interest in the production of γ-linolenic acid (GLA; all-*cis* 6,9,12-octadecatrienoic acid) has resulted from its applications in the treatment of a wide range of clinical disorders (1). The occurrence, physical and chemical properties of GLA have been recently reviewed (2).

The most important and commercially available sources of GLA are seed oils of evening primrose (*Oenothera biennis* L.) (3), borage (*Borago officinalis* L.) (1,9) and fungal oils, e.g., from *Mucor* spp. (10) and *Mortierella* spp. (11,12). Methods used for the isolation 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 a separate study (19), we have reported a systematic investigation on the enrichment of GLA-containing fatty acids of borage oil and evening primrose oil *via* lipase-catalyzed selective esterification of these fatty acids with *n*-butanol, leading to enrichment of GLA in the unesterified fatty acids.

In this paper, we report an alternative approach for the enrichment of GLA from borage oil and evening primrose oil, which involves lipase-catalyzed selective hydrolysis of the triacylglycerols, leading to the accumulation of GLA moieties in the unhydrolyzed acylglycerols.

EXPERIMENTAL PROCEDURES

Materials. Crude borage oil and evening primrose oil were provided by the International Food Science Centre, Lystrup, Denmark. Soluble lipase preparations used were Pancreatin (7 U/mg; E. Merck, Darmstadt, Germany), porcine pancreatic lipase (PPL) (50 U/mg; Sigma, Deisenhofen, Germany), *Candida cylindracea* lipase (85 U/mg; Biocatalysts, Pontypridd, Wales) and *C. cylindracea* lipase (850 U/mg; Sigma). Immobilized lipase (Lipozyme) from *Rhizomucor miehei* (25 batch interesterification units/g) was provided by Novo Industrie (Mainz, Germany). All reagents and adsorbents were of analytical grade and were purchased from Merck.

Lipase-catalyzed hydrolysis of the oils. The reactions in the mg-scale were carried out in 10-mL glass tubes sealed with Teflon-lined caps. Glass-stoppered 50-mL Erlenmeyer flasks were used for 10-g scale. The reactions were carried out for various periods at different temperatures.

In mg-scale experiments, the reaction mixture contained borage oil or evening primrose oil (100 mg) and 10 mg lipase powder in 2 mL water or a buffer of a definite molarity and pH, as specified in the tables, together with known concentrations of $CaCl_2$ and an emulsifier, such as sodium cholate. Air in the tube was replaced by nitrogen, and the mixture was stirred magnetically. Aliquots of reaction products were withdrawn at definite intervals, and the lipids were recovered by repeated extractions with diethyl ether.

In preparative-scale experiments, typically, a mixture of evening primrose oil (10 g) and potassium phosphate buffer (pH 7.0, 0.08 M, 20 mL) containing *C. cylindracea* lipase preparation (0.1 g, 85,000 units; Sigma) was stirred in a 50-mL Erlenmeyer flask at 20°C for 2 h, and then the reaction mixture was extracted three times with 10 mL diethyl ether. The three extracts were combined and washed with water (2 × 5 mL) containing 20% methanol (vol/vol), and finally the organic solvent was evaporated in a nitrogen stream. To separate unesterified fatty acids from acylglycerols, the products of lipolysis were dissolved in 10 mL hexane and extracted with 40 mL NaOH (1N in 50% aqueous ethanol). The hexane phase and aqueous extracts were separated. The aqueous mixture was extracted two times with 10 mL hexane, and then the hexane extracts were combined and acidified with 10 mL 6N HCl, and the fatty acids were extracted with diethyl ether (3 × 20 mL). The ether extract was washed with water (2 × 10 mL), and the solvents were removed to yield 7 g of fatty acids. Similarly, evaporation of hexane extract containing acylglycerols gave 2 g crude acylglycerol concentrate.

Analytical procedures. The total reaction products, resulting from lipase-catalyzed hydrolysis of borage oil and evening primrose oil, as well as the fractions of fatty acids and acylglycerols recovered from the reaction products in preparative-scale experiments, were fractionated into fatty acids and acylglycerols (tri- + di- + monoacylglycerols) by thin-layer chromatography (TLC) on Silica Gel H (E. Merck) with hexane/diethyl ether/acetic acid (70:30:1, vol/vol/vol) as the developing solvent. The fractions were scraped off from the adsorbent layer and eluted with water-saturated diethyl ether. Known amounts of methyl heptadecanoate, the internal standard, were added to each set of fractions of acylglycerols and fatty acids.

[1]Present address: Department of Chemistry, University of Hong Kong, Pok Fu Lam Road, Hong Kong.

*To whom correspondence should be addressed at Federal Centre for Cereal, Potato and Lipid Research, Institute for Biochemistry and Technology of Lipids, H.P. Kaufmann-Institute, Piusallee 68, D-48147 Münster, Germany.

Both acylglycerols and fatty acids were then converted to methyl esters by adding 50 μL trimethyl sulfonium hydroxide and 200 μL 1,2-dichloroethane (20). 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 methyl esters, containing methyl heptadecanoate as internal standard, was analyzed by gas chromatography as described previously (17).

RESULTS AND DISCUSSION

Several lipases have been shown to discriminate against specific fatty acids or acyl moieties, such as GLA or γ-linolenoyl moieties in lipase-catalyzed hydrolysis (16,17,21), esterification (16–18,21,22) and transesterification (23) reactions. Specifically, the relative inability of lipases to catalyze the hydrolysis of γ-linolenoyl moieties of triacylglycerols, as compared to other fatty acyl moieties, has been used for the enrichment of this acid from fatty acids of GLA-containing oils *via* kinetic resolution during lipase-catalyzed partial hydrolysis; γ-linolenoyl moieties are enriched in the unhydrolyzed acylglycerols, whereas the other fatty acyl moieties are preferentially cleaved (16,21). 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 partial hydrolysis of triacylglycerols of borage oil and evening primrose oil.

Partial hydrolysis of evening primrose oil was carried out with Pancreatin as the lipase preparation in the presence of water and $CaCl_2$ at 40°C for various periods, without buffers and emulsifiers. The results (Table 1) show, in the course of hydrolysis, a minor increase in the level of GLA in the unhydrolyzed acylglycerols, with a concomitant decrease in the GLA content of the fatty acids liberated. After 48 h of reaction, which resulted in 50% hydrolysis, about 1.4-fold enrichment of GLA in the unhydrolyzed acylglycerols was observed.

In the next set of experiments, hydrolysis of evening primrose oil with PPL and Pancreatin was carried out at 40°C, according to (24), in the presence of Tris-buffer (pH 8.0, 0.08 M), $CaCl_2$ (22% wt/vol) and sodium cholate (0.1% wt/vol). The results given in Table 2 show that with both lipase preparations, the hydrolysis was much faster under these conditions as compared to those used in Table 1. After 1 h of reaction in the presence of Pancreatin, as much as 58% hydrolysis occurred, which resulted in an increase in the GLA content in the unhydrolyzed acylglycerols to 16.8%, as compared to 9.4% in the starting oil; concomitantly, the level of GLA in the liberated fatty acids dropped to 4.6% (Table 2). After 15 min reaction in the presence of PPL, the extent of hydrolysis was 45%, and the level of GLA in the unhydrolyzed acylglycerols was increased to 14.4% (1.5-fold enrichment), whereas the GLA content of the liberated fatty acids dropped to 2.4% (Table 2).

Partial hydrolysis of borage oil with Pancreatin under the reaction conditions specified by Luddy *et al.* (24) yielded the results given in Table 3. The extent of hydrolysis increased with time, reaching 51% hydrolysis after 2 h reaction. Simultaneously, the level of GLA in unhydrolyzed acylglycerols increased up to 33.5% after 2 h reaction, compared to 21.8% GLA in the starting oil; concomitantly, the level of GLA in the liberated fatty acids dropped to 8.7%.

In another set of experiments, hydrolysis of evening primrose oil was carried out with Lipozyme (an immobilized lipase preparation from *R. miehei*) and a small amount of water (20 μL) at 40°C without the addition of emulsifier, $CaCl_2$ and buffer. The results (Table 4) show that despite substantial hydrolysis (26%) after 4 h reaction, the extent of enrichment of GLA in the acylglycerols was rather low (1.3-fold). Increasing the reaction time to 16 h increased the extent of hydrolysis to 44%. However, the enrichment of GLA in the acylglycerols was minimal (1.1-fold).

Partial hydrolysis of evening primrose oil was also carried out with a lipase preparation from *C. cylindracea* (Biocatalysts) in potassium phosphate buffer (pH 7.0, 0.08 M) without the addition of $CaCl_2$ and sodium cholate, at 20°C for various periods. The results (Table 5) show that of all lipases tested so far, the lipase preparation from *C. cylindracea* is the most effective in the partial hydrolysis of evening primrose oil for the enrichment of GLA in the unhydrolyzed acylglycerols. Thus, only after 2–3 h reaction, leading to about 90% hydrolysis of the oil, the level

TABLE 1

Enrichment of GLA in Acylglycerols *via* Partial Hydrolysis of Evening Primrose Oil Catalyzed by Pancreatin in the Presence of Water at 40°C[a]

Time (h)	Component	Amount in total products (wt%)	Fatty acid composition[b] (%)					Enrichment of GLA
			16:0	18:0	18:1	18:2	γ-18:3	
0	Acylglycerols	≈100	7.4	2.1	9.0	71.2	9.4	1.0
2	Fatty acids	15	12.0	3.2	9.9	70.8	4.2	
2	Acylglycerols	85	6.4	1.5	9.5	72.3	10.3	1.1
24	Fatty acids	36	9.5	2.2	9.0	73.9	5.5	
24	Acylglycerols	64	5.2	1.3	9.5	72.6	11.4	1.2
48	Fatty acids	50	9.5	1.6	11.3	71.1	6.5	
48	Acylglycerols	50	6.9	1.1	9.5	69.7	12.8	1.4

[a]Reactions were carried out in mg-scale as described in the 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 in Acylglycerols *via* Partial Hydrolysis of Evening Primrose Oil Catalyzed by Pancreatic Lipases at 40°C in the Presence of $CaCl_2$ (22% wt/vol), Tris Buffer (pH 8.0, 0.08 M) and Sodium Cholate (0.1% wt/vol)[a]

Time (h)	Component	Amount in total products (wt%)	Fatty acid composition[b] (%)					Enrichment of GLA
			16:0	18:0	18:1	18:2	γ-18:3	
0	Acylglycerols	≈100	7.4	2.1	9.0	71.2	9.4	1.0
Pancreatin								
15	Fatty acids	47	11.5	2.0	9.3	74.0	3.2	
15	Acylglycerols	53	5.2	1.0	9.5	69.8	14.5	1.5
60	Fatty acids	58	10.6	2.1	9.3	73.4	4.6	
60	Acylglycerols	42	7.3	0.6	8.7	66.6	16.8	1.8
Porcine pancreatic lipase								
15	Fatty acids	45	10.6	2.2	9.2	75.6	2.4	
15	Acylglycerols	55	5.4	1.3	9.1	69.8	14.4	1.5

[a]As in Table 1.
[b]As in Table 1.

TABLE 3

Enrichment of GLA in Acylglycerols *via* Partial Hydrolysis of Borage Oil Catalyzed by Pancreatin at 40°C in the Presence of $CaCl_2$ (22% wt/vol), Tris Buffer (pH 8.0, 0.08 M) and Sodium Cholate (0.1% wt/vol)[a]

Time (h)	Component	Amount in total products (wt%)	Fatty acid composition[b] (%)						Enrichment of GLA
			16:0	18:0	18:1	18:2	γ-18:3	20:1-24:1	
0	Acylglycerols	≈100	11.9	4.7	19.1	38.2	20.4	5.7	1.0
1	Fatty acids	9	16.9	6.2	26.5	40.2	4.2	6.0	
1	Acylglycerols	91	14.1	3.8	18.7	36.0	21.8	4.0	1.1
15	Fatty acids	15	17.6	5.6	26.5	37.2	6.3	6.8	
15	Acylglycerols	85	13.7	3.6	18.4	35.9	23.0	5.4	1.2
30	Fatty acids	22	18.2	6.0	24.7	37.6	6.2	7.3	
30	Acylglycerols	78	12.9	3.5	18.2	35.8	24.7	4.9	1.2
60	Fatty acids	38	18.7	5.8	23.5	36.6	7.3	8.1	
60	Acylglycerols	62	10.1	2.7	17.3	36.4	29.5	4.0	1.5
120	Fatty acids	51	19.3	6.0	22.8	35.9	8.7	7.3	
120	Acylglycerols	49	10.7	2.0	17.0	33.5	33.5	3.3	1.6

[a]As in Table 1.
[b]As in Table 1.

TABLE 4

Enrichment of GLA in Acylglycerols *via* Partial Hydrolysis of Evening Primrose Oil Catalyzed by Immobilized Lipase from *Rhizomucor miehei* (Lipozyme) in the Presence of Water at 40°C[a]

Time (h)	Component	Amount in total products (wt%)	Fatty acid composition[b] (%)					Enrichment of GLA
			16:0	18:0	18:1	18:2	γ-18:3	
0	Acylglycerols	≈100	7.4	2.1	9.0	71.2	9.4	1.0
2	Fatty acids	13	11.9	2.8	10.0	71.4	3.9	
2	Acylglycerols	87	5.9	1.5	9.2	73.1	10.3	1.1
4	Fatty acids	26	11.6	2.4	9.0	73.6	3.4	
4	Acylglycerols	74	5.3	1.2	9.0	72.4	12.1	1.3
16	Fatty acids	44	7.5	1.3	7.7	77.0	6.4	
16	Acylglycerols	56	8.3	1.6	9.1	70.7	10.3	1.1

[a]Reactions were carried out in mg-scale as described in the Experimental Procedures section with the addition of 20 μL water.
[b]As in Table 1.

TABLE 5

Enrichment of GLA in Acylglycerols *via* Partial Hydrolysis of Evening Primrose Oil Catalyzed by *Candida cylindracea* Lipase at 20°C in Potassium Phosphate Buffer (pH 7.0, 0.08 M)[a]

Time (h)	Component	Amount in total products (wt%)	Fatty acid composition[b] (%)					Enrichment of GLA
			16:0	18:0	18:1	18:2	γ-18:3	
0	Acylglycerols	≈100	7.4	2.1	9.0	71.2	9.4	1.0
0.5	Fatty acids	76	7.0	0.8	9.3	81.4	1.6	
0.5	Acylglycerols	24	9.3	1.2	10.7	45.3	33.5	3.6
1	Fatty acids	86	6.9	1.4	9.4	79.0	3.3	
1	Acylglycerols	14	7.8	1.4	6.4	41.6	42.8	4.6
2	Fatty acids	89	6.9	1.4	9.3	75.0	5.3	
2	Acylglycerols	11	6.3	1.0	5.9	40.3	46.5	4.9
3	Fatty acids	90	8.2	1.2	9.5	73.7	7.4	
3	Acylglycerols	10	7.0	1.2	5.3	41.3	45.2	4.8
4	Fatty acids	93	7.7	1.2	8.7	72.1	10.3	
4	Acylglycerols	7	6.6	6.3	11.0	44.3	43.0	4.6
6	Fatty acids	94	8.5	1.2	9.2	72.5	10.3	
6	Acylglycerols	6	4.9	0.0	6.7	50.8	37.6	4.0
16	Fatty acids	97	8.2	1.0	9.0	68.0	13.8	
16	Acylglycerols	3	12.2	2.3	7.9	61.5	16.1	1.7

[a]As in Table 1.
[b]As in Table 1.

TABLE 6

Preparative-Scale Enrichment of GLA in Acylglycerols *via* Partial Hydrolysis of Borage Oil Catalyzed by *Candida cylindracea* Lipase at 20°C in Potassium Phosphate Buffer (pH 7.0, 0.08 M)[a]

Time (min)	Component	Amount in total products (wt%)	Fatty acid composition[b] (%)						Enrichment of GLA
			16:0	18:0	18:1	18:2	γ-18:3	20:1-24:1	
0	Acylglycerols	≈100	11.9	4.7	19.1	38.2	20.4	5.7	1.0
30	Fatty acids	22	12.7	2.7	24.5	51.7	7.4	2.0	
30	Acylglycerols	78	11.7	3.1	19.0	27.6	33.0	5.5	1.5
120	Fatty acids	40	16.1	5.3	25.6	46.4	2.0	4.6	
120	Acylglycerols	60	11.1	5.8	16.5	24.5	33.8	8.3	1.7
240	Fatty acids	65	14.8	4.9	23.7	46.9	4.8	4.9	
240	Acylglycerols	35	8.4	5.6	12.7	20.9	43.3	9.0	2.1
300	Fatty acids	71	14.9	5.1	23.7	45.0	6.5	4.8	
300	Acylglycerols	29	7.6	4.0	12.0	21.8	47.8	6.7	2.3
300[c]	Fatty acids	(84)	13.6	5.1	21.8	40.1	14.3	5.2	
300[c]	Acylglycerols	(16)	6.0	3.7	11.9	24.5	45.6	8.3	2.2

[a]Reactions were carried out in preparative-scale as described in Experimental Procedures section.
[b]As in Table 1.
[c]The reaction product from the foregoing experiment was worked up, as described in the Experimental Procedures section, to yield 6.4 g fatty acids and 1.2 g acylglycerols, which correspond to the weight distribution in the products (given in parentheses).

of GLA in the acylglycerols is increased to as much as 45–47%, corresponding to almost fivefold enrichment of GLA. Further increase in reaction time leads to a decrease in the extent of enrichment of GLA (Table 5). Prolonging the reaction time to 72 h led to complete hydrolysis (99%) of the oil; all fatty acids, including GLA, were found in the fatty acid fraction. It might be noted in this context that selective hydrolysis of fish oils, catalyzed by lipase from *C. cylindracea*, has also been employed for the enrichment of n-3 polyunsaturated fatty acids in the unhydrolyzed acylglycerols (25,26).

In view of the impressive ability of the lipase from *C. cylindracea* (Sigma) to enrich GLA in the acylglycerols *via* kinetic resolution during hydrolysis, a preparative-scale experiment with another commercial lipase preparation from *C. cylindracea* was carried out to enrich GLA from borage oil. The details are outlined in the experimental procedures. The results, given in Table 6, show that a 30-min reaction yields 78% of the products as acylglycerols containing 33% GLA. Progressively increasing the reaction time increases the degree of hydrolysis and the level of GLA in the unhydrolyzed acylglycerols. Increasing the reaction time to 5 h yields 29% of the product as acylglycerols containing approximately 48% GLA. Working up the reaction products *via* alkaline extraction of the fatty acids, as described in the Experimental

Procedures section, yields 1.2 g acylglycerols containing about 46% GLA (Table 6). TLC revealed that the acylglycerol fraction was almost exclusively composed of triacylglycerols.

These studies clearly demonstrate the supremacy of the lipase from *C. cylindracea* in the enrichment of GLA by selective hydrolysis.

REFERENCES

1. Horrobin, D.G., in *Antioxidants, Free Radicals and Polyunsaturated Fatty Acids in Biology and Medicine*, edited by A.T. Diplock, J.M.C. Gutteridge and V.K.S. Shukla, International Food Science Centre A/S, Lystrup, 1993, pp. 181–198.
2. Gunstone, F.D., *Prog. Lipid Res. 31*:145 (1992).
3. Hudson, B.J.F., *J. Am. Oil Chem. Soc. 61*:540 (1984).
4. Kleiman, R., F.R. Earle and I.A. Wolff, *Ibid. 41*:459 (1964).
5. Wolff, R.B., R. Kleiman and R.E. England, *Ibid. 60*:1858 (1983).
6. Whipkey, A., J.E. Simon and J. Janick, *Ibid. 65*:979 (1988).
7. Uzzan, E., *Rev. Franc. Corps Gras 35*:501 (1988).
8. Ucciani, E., G. Mallet and J. Gamisans, *Ibid. 39*:135 (1992).
9. Traitler, H., H. Winter, U. Richli and Y. Ingenbleck, *Lipids 19*:923 (1984).
10. Fukuda, H., and H. Morikawa, *Appl. Microbiol. Biotechnol. 27*:15 (1987).
11. Hansson, L., and M. Dostálek, *Ibid. 28*:240 (1988).
12. Kamisaka, Y., T. Yokochi, T. Nakahara and O. Suzuki, *Lipids 25*:54 (1990).
13. Traitler, H., H.J. Wille and A. Studer, *J. Am. Oil Chem. Soc. 65*:755 (1988).
14. Arai, M., H. Fukuda and H. Morikawa, *J. Ferment. Technol. 65*:569 (1987).
15. Yokochi, T., M.T. Usita, Y. Kamisaka, T. Nakahara and O. Suzuki, *J. Am. Oil Chem. Soc. 67*:846 (1990).
16. Hills, M.J., I. Kiewitt and K.D. Mukherjee, *Biotechnol. Lett. 2*:629 (1989).
17. Mukherjee, K.D., and I. Kiewitt, *Appl. Microbiol. Biotechnol. 35*:579 (1991).
18. Hills, M.J., I. Kiewitt and K.D. Mukherjee, *J. Am. Oil Chem. Soc. 67*:561 (1990).
19. Syed Rahmatullah, M.S.K., V.K.S. Shukla and K.D. Mukherjee, *Ibid. 71*:563 (1994).
20. Schulte, E., in *CRC Handbook of Chromatography: Analysis of Lipids*, edited by K.D. Mukherjee, and N. Weber, CRC Press Inc., Boca Raton, 1993, pp. 139–148.
21. Hills, M.J., I. Kiewitt and K.D. Mukherjee, *Biochim. Biophys. Acta 1042*:237 (1990).
22. Rangheard, M.-S., G. Langrand, C. Triantaphylides and J. Baratti, *Ibid. 1004*:20 (1989).
23. Osterberg, E., A.-C. Blomstrom and K. Holmberg, *J. Am. Oil Chem. Soc. 66*:1330 (1989).
24. Luddy, F.E., R.A. Barford, S.F. Herb, P. Magidman and R.W. Riemenschneider, *Ibid. 41*:693 (1964).
25. Hoshino, T., T. Yamane and S. Shimizu, *Agric. Biol. Chem. 54*:1459 (1990).
26. Tanaka, Y., J. Hirano and T. Funada, *J. Am. Oil Chem. Soc. 69*:1210 (1992).

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