Fatty Acid Selectivity of Lipases: γ-Linolenic Acid from Borage Oil[1]

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ABSTRACT: The γ-linolenic acid (*Z,Z,Z*-6,9,12-octadecatrienoic acid, GLA) present in borage oil free fatty acids was concentrated in esterification reactions that were catalyzed by several preparations of the acyl-specific lipase of *Geotrichum candidum*. In this manner, a 95% recovery of the GLA originally present in borage oil (25% GLA) was obtained as a highly enriched fatty acid fraction with a GLA content of >70%. Other fatty acids concentrated in this fraction were the monounsaturated fatty acids with chainlengths of C-20 and longer that were present in the oil. An immobilized preparation of *G. candidum* on silica gel also was used for the enrichment of GLA in borage oil. In this instance, a 75% recovery of GLA was obtained, and the supported lipase was reusable (three cycles) with minimal loss in activity.
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KEY WORDS: Borage oil, fatty acid selectivity, *Geotrichum candidum*, immobilization, γ-linolenic acid, lipase.

There is currently much interest in the lipases of the fungus *Geotrichum candidum* because of the high substrate selectivity expressed by these enzymes for unsaturated fatty acids that contain a *cis*-δ-9-double bond (1–3). It has been demonstrated, however, that not all isoforms of the enzyme expressed by *G. candidum* are uniform in their reactivity to a given fatty acid (4,5). Recently, we compared the relative reactivities of several long-chain fatty acids in esterifications catalyzed by lipases of *G. candidum* (6). In that study, we found that not all *G. candidum* lipase preparations were uniform in their selectivity for fatty acids with δ-9-unsaturation. More importantly, we found that the lipases of *G. candidum* in general discriminate against (i) fatty acids with a chainlength greater than 18 carbons, and/or (ii) fatty acids with unsaturation at the δ-6 and δ-12 position. Recent studies (7,8) have reported that other lipases, including those from rape (*Brassica napus*), *Mucor miehei*, and *Candida cylindracea*, also discriminate against fatty acids with a double bond located near the carboxyl end of the chain (δ-4,-6 and -8). This strong selectivity of a given lipase for, or against, a particular fatty acid can be used for the selective enrichment of a given fatty acid in natural oils. For example, we developed a two-step enzymatic procedure for the isolation of erucic acid (Z-13-docosenoic acid) from rapeseed oil based on chainlength discrimination of the *G. candidum* lipase (9). In recent work, an immobilized lipase preparation of *M. miehei* was used for the enrichment of γ-linolenic acid (GLA; an n-6 fatty acid) in evening primrose and borage oils (10,11). Similarly, the ability of *M. miehei* and *C. cylindracea* lipases to discriminate against the n-3 family of polyunsaturated fatty acids (PUFA) (e.g., eicosapentaenoic acid) was exploited for the selective harvesting of PUFA from fish oils (12,13). This interest in PUFA of the n-6 and n-3 series stems from their clinical effects (14,15). Here we report an evaluation of the selectivity of lipases of *G. candidum* for the enrichment of GLA in borage oil fatty acids. We also evaluated an immobilized preparation of *G. candidum* and compared the results with those obtained with other commercially available supported enzyme preparations.

MATERIALS AND METHODS

Fatty acid methyl esters (FAME) and butyl esters were analyzed by gas–liquid chromatography (GLC) on a Hewlett-Packard (Avondale, PA) Model 5890 chromatograph, equipped with a split capillary injector, electronic pressure control (EPC), a flame-ionization detector and a Hewlett-Packard Model 3396 integrator. FAME and butyl ester separations were made on an SP-2340 column (0.25 mm i.d. × 60 m × 0.25 μm) from Supelco, Inc. (Bellefonte, PA) (9). Free fatty acids and butyl esters were separated on a Hewlett-Packard HP-INNO wax column (0.53 mm × 30 m × 1.0 μm) under EPC with constant flow (9 psi) and a temperature program of 120 to 250°C at 8°C min^{-1}. FAME and free fatty acids (FFA) were identified by comparison with standards, obtained from Nu-Chek-Prep (Elysian, MN), and quantitative analysis was performed as described by Slover and Lanza (16). Butyl esters required for identification by GLC were prepared by reaction of borage oil glycerides (PGE Ind., Saskatoon, Saskatchewan, Canada) with boron trifluoride etherate (Aldrich Chemical Co., Milwaukee, WI) in butanol. Analytical thin-layer chromatography (TLC) was conducted

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on 0.25 mm silica gel 60 plates (5 × 20 cm); preparative TLC was done on 1 mm silica gel 60 plates (20 × 20 cm), both obtained from Analtech (Newark, DE). Plates were developed with hexane/ether/formic acid (80:20:2), air-dried and developed by either charring with 50% H_2SO_4 or by exposure to I_2 vapor. Titrations were conducted with a Radiometer AGU Autoburette (Copenhagen, Denmark), and reaction mixtures were agitated with a Lab-Line shaker bath (Wheeling, IL).

Commercial preparations of the acyl-specific lipase from *G. candidum* were obtained from Amano Co. (Troy, VA) and Biocatalysts, Ltd. (Mid Glamorgan, United Kingdom). The strain, NRRL Y-553, was cultured in our laboratory as previously described (17). The stated specific activities of the commercial enzymes (GC-4, 4400 and BL, 4000) in hydrolysis of olive oil were expressed as μmol of FFA released per min per g of powder. The Y-553 lipase contained 19.4% protein and had a specific activity in an olive oil assay (0.05 M Tris buffer, pH 8 with 10 mM calcium chloride) of 69 μmol FFA min^{-1} g^{-1} protein and relative reactivity of oleic to palmitic acid of 20:1 (17). The GC-4 lipase was supported on silica gel (100 mesh, Aldrich Chemical) and had a specific activity of 1150 μmol FFA min^{-1} g^{-1} (P.E. Sonnet, unpublished results). Other immobilized enzymes used included commercial preparations of the 1,3-specific lipase from *M. miehei* (Lipozyme), with an activity on olive oil as substrate of 1613 μmol FFA min^{-1} g^{-1} and the nonspecific lipase of *C. antarctica* (SP 435) with an activity of 1477 μmol FFA min^{-1} g^{-1} obtained from Novo Nordisk (Danbury, CT). Activities of the enzyme preparations are expressed as meq of oleic acid released from olive oil per gram of supported lipase.

Partial hydrolysis of borage oil. The equivalent weight of the oil was determined by titrating borage oil FFA, obtained by saponification with alcoholic KOH, with 0.1 N NaOH to pH 12. The average value (n = 3) was 290.5, which formed the basis for determining percent hydrolysis or esterification in the enzyme-catalyzed reactions. Borage oil (200 mg) and 0.2 mL Tris buffer (0.1 M, pH 8, 10 mM in $CaCl_2$), containing 10 mg of GC-4, were shaken in 5-mL tubes at 25°C for 24 h. The tube contents were acidified with 2N HCl and extracted with hexane (2 × 2 mL). The hexane extracts were washed with water, dried ($MgSO_4$) and pumped free of solvent to constant weight. The residue was dissolved in hexane, transferred to a 5-mL volumetric flask, and brought to volume with hexane. Aliquots of 1 mL were taken for determination of percent hydrolysis. The released FFA and partial glycerides were isolated by preparative TLC, converted to FAME with BF_3/methanol, and analyzed by GLC.

Esterification of borage oil FFA. Esterification reactions were carried out at 25°C in 10-mL Erlenmeyer flasks by mixing 2 mL of 250 mM borage FFA in hexane with 2 mL of 500 mM 1-butanol in hexane. The powdered *G. candidum* enzymes (20–100 mg) were added, together with 100 μL Tris buffer, and the mixtures were shaken for 24–48 h. When one of the supported enzymes (20–60 mg) was used as the catalyst, reactions were quenched by filtering through Celite to remove the catalyst, followed by the addition of 2 mL of 2 N HCl. The hexane layer was separated, and the aqueous phase extracted with 2 mL hexane. The hexane layers were combined and worked up as above. The butyl ester products were separated from unreacted FFA by TLC. The FFA, after conversion to FAME, and the butyl esters were then analyzed by GLC.

RESULTS AND DISCUSSION

The ability of the lipases of *G. candidum* to discriminate against GLA as substrate was used for the enrichment of GLA in borage oil FFA. Borage oil is an excellent source of GLA, as it comprises 25% of the fatty acids of this oil, compared with other sources, such as evening primrose oil (10% GLA) (18). Another feature of this oil (Table 1) is that approximately 10% of its fatty acids are C-20 or longer. It is gener-

TABLE 1
Esterification of Borage Oil Fatty Acids by *Geotrichum candidum* Lipases[a]

			Fatty acid composition (%)[d]							
Reaction time (h)	Lipase[b]	Conversion[c] (%)	C16:0	C18:0	C18:1	C18:2	C18:3	C20:1	C22:1	C24:1
0	—	—	9.2[e]	3.1	15.8	37.1	24.9	4.0	2.8	1.6
24	GC-4	58E	8.0	2.6	22.1	50.4	10.6	3.3	1.9	1.0
		42A	10.9	3.8	8.5	20.2	45.2	5.0	4.0	2.4
48	GC-4	64E	11.7	3.7	23.8	54.8	2.1	2.9	0.6	0.4
		36A	6.3	2.6	3.3	4.8	65.5	6.8	6.7	4.0
48	Y-553	59E	8.8	2.9	23.8	48.9	8.4	3.2	1.9	2.1
		41A	9.9	3.4	8.9	20.4	47.2	5.2	4.1	0.9
48	BC	67E	10.7	3.3	23.8	55.4	1.8	3.6	0.7	0.9
		33A	4.2	2.7	1.8	2.6	71.8	6.9	7.0	3.0

[a]Borage oil free fatty acids (FFA) reacted with 1-butanol in hexane; see Materials and Methods section for details.

[b]*Geotrichum candidum* lipases: GC-4 (Amano, Troy, VA); BC (Biocatalysts Ltd., Mid Glamorgan, United Kingdom); Y-553 (NRRL Y-553).

[c]Conversion expressed as % butyl esters (E); % unreacted FFA (A) determined by FFA titration.

[d]Values are average of 2–4 determinations as wt% butyl esters, unreacted FFA and/or methyl esters.

[e]Composition (wt%) of unreacted borage oil FFA.

ally accepted that the expression of a lipase's selectivity for or against a given fatty acid is more pronounced in the esterification mode than in the hydrolysis mode (7,9). In this work, we compared the activity and selectivity of two commercial preparations of this lipase (GC-4 and BC) with a laboratory preparation (Y-553) of the enzyme in the esterification of borage oil FFA with butanol. This was done because the fatty acid selectivity of the lipase from *G. candidum* varies with the isoform isolated (4,5). The esterification capacity of the three lipase preparations studied is shown in Table 1. The two commercial lipases (GC-4 and BC) had comparable reactivity in esterifying borage oil FFA in that both preparations gave similar conversion to ester (approximately 65%) after a 48-h reaction. On the other hand, the Y-553 enzyme was somewhat less reactive, with a conversion of only 60% after the same time period. This result was similar to that obtained with the GC-4 enzyme (Table 1) after a 24-h reaction. Because all reactions were conducted with approximately equal activity units, it is not apparent why the Y-553 enzyme had lower reactivity. More importantly, Table 1 shows that the three strains of this enzyme strongly discriminate against GLA (Table 1). For example, in the BC-catalyzed reaction, the unreacted borage fatty acids contained 71% GLA. This amounted to a threefold enrichment in GLA over the starting fatty acids. Similar enrichments of GLA in evening primrose and borage oil FFA with *M. miehei* lipase have been reported (10), although in lower absolute amounts because of the higher reaction conversions required with the latter enzyme. Table 1 also shows that there was a concomitant enrichment of the longer-chain fatty acids present in borage oil FFA into the unreacted FFA fraction so that the longer-chain acids (C20–C24) accounted for almost 20% of this fraction. This further established that the *G. candidum* lipase, regardless of source, strongly discriminates against fatty acids with chainlengths longer than 18 carbons (6), a feature that is not commonly expressed by all lipases.

We also examined the use of the GC-4 lipase for the concentration of GLA into borage oil glycerides. Concentration of GLA in glycerides by other lipases, e.g., *M. miehei*, pancreatin, and PPL, also has been reported (11). These reactions, however, were based upon the positional selectivity of the enzyme or a fortuitously structured triglyceride. Most of these enzymes, however, are not especially acyl or positionally selective, and reactions with them are often complicated by acyl migration, causing loss of the desired acid residue. Other lipases, e.g., *C. cylindracea*, share with the less familiar *G. candidum* lipase a lack of positional selectivity, coupled with fatty acid chainlength/unsaturation selectivity. That selectivity is most pronounced for the *G. candidum* lipase used in this study. A comparison of the data obtained with this enzyme for the esterification of borage oil FFA and hydrolysis of its triglycerides is given in Table 2. In the hydrolysis of borage oil by *G. candidum*, only 64% of the GLA was concentrated into the partial glyceride fraction (compare entries 1 and 2, Table 2). Although the *G. candidum* lipase was less discriminatory toward GLA in hydrolysis reactions, GLA glycerides

TABLE 2
Fractionation of Borage Oil Fatty Acids with Lipases

Lipase[a]	γ-C18:3 (%)[b]	Conversion (%)[c]	Yield (%)[d]
Geotrichum candidum (E)	17.8	33 (A)	95
		67 (E)	
G. candidum (H)	45.5	65 (A)	64
		35 (G)	
G. candidum (E)[e]	53.6	35 (A)	75
(Silica)		65 (E)	
Mucor miehei (E)	55.5	35 (A)	78
(Lipozyme)		65 (E)	
Candida antarctica	53	40 (A)	60
(SP-435)		60 (E)	

[a]*Geotrichum candidum* lipase from Biocatalysts, Ltd. (Mid Glamorgan, United Kingdom); Lipozyme and SP-435 from Novo Nordisk (Danbury, CT). E, esterification of borage oil free fatty acids; H, hydrolysis of borage oil; see Materials and Methods section for details.

[b]wt% γ-Linolenic acid in fraction (average of 2–4 determinations).

[c]Conversion after 48 h reaction as determined by FFA titration: A = % fatty acid fraction; G = % glyceride fraction; E = % butyl ester fraction.

[d]Total recovery of γ-linolenic acid originally present in borage oil (24.9%).

[e]*Geotrichum candidum* lipase supported on silica gel 60.

are considered more desirable because they are apparently more completely absorbed than FFA (19). In addition, the lower GLA content, found in the glyceride fraction, confirmed that enzymes in general are more selective or discriminatory toward a given fatty acid in esterification reactions.

In the harvesting of targeted fatty acids, lipases are often immobilized for practical considerations, because recovery and reuse of the enzyme are important, and immobilization improves product isolation. The immobilization of *G. candidum* lipase onto inorganic supports (e.g., Celite) gave a supported enzyme that proved useful for the interesterification of animal and vegetable triglycerides (20). Similarly, it has been shown that *G. candidum* lipase can be placed onto Celite, provided water is added to the catalyst (21). More recently, the lipases from *G. candidum* have been supported on silica gel (22), and these supported enzymes retained their essential features of fatty acid selectivity. In the present work, we compared the activity of one silica gel-supported *G. candidum* lipase with that of two other supported lipases. One preparation was a lipase from *M. miehei* (Lipozyme), a 1,3-positionally selective lipase, and the second was a nonspecific lipase of *C. antarctica* (SP-435). It was reported previously that the *M. miehei* lipase discriminated against GLA in esterification of borage and evening primrose oils (10). As shown in Table 2, the selectivities of both the *G. candidum* and *M. miehei* lipases were equally effective in concentrating the GLA of borage oil FFA in esterification reactions. For both supported enzymes, GLA was recovered to the extent of about 75% in the FFA fraction. On the other hand, the nonspecific lipase from *C. antarctica* was less biased against GLA as compared with the former supported enzymes. Total recovery in this instance was only about 60%. The lower recovery of GLA from bor-

age oil FFA with the silica gel-supported *G. candidum* lipase when compared to the unsupported enzyme (entries 1 and 3, Table 2) is attributed to the lower reactivity of the supported enzyme. The data shown are after a 48-h reaction. If the reaction is allowed to continue for 72 h, the recovery of GLA with the supported lipase approaches that of the unsupported lipase. On the other hand, with the *M. miehei* supported lipase, the recovery of GLA decreased after 72 h, presumably because of the less discriminating selectivity of this enzyme compared to the *G. candidum* lipase. Larger-scale reactions (25 g borage FFA) also were run with the supported lipase of *G. candidum*. In these reactions, although conditions were not optimized, the total recovery of GLA approached that obtained for the smaller-scale reactions. What is more important, the supported *G. candidum* lipase can be recycled so that the recovery of GLA was about 75% after two additional reuses (data not shown), and product isolation required only column chromatographic separation of the ester and acid fractions. From the foregoing, it can be concluded that either a fatty acid or glyceride fraction highly concentrated in GLA (72%) can be obtained from borage oil with lipases from *G. candidum*. Enrichment of GLA in either fraction is specifically a result of the strong discrimination of selectivity of this particular enzyme against certain fatty acids. Moreover, the selectivity of the enzyme against GLA was retained after immobilization.

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REFERENCES

1. Jensen, R.J., D. Rubano Galluzo and V.J. Bush, *Biocatalysis 3*:307 (1990).
2. Baillargeon, M.W., and P.E. Sonnet, *Biotech. Lett. 13*:871 (1991).
3. Rangheard, M.S., G. Langrand, C. Triantaphylides and J. Barratti, *Biochem. Biophys. Acta 1004*:20 (1989).
4. Charton, E., and A.R. Macrae, *Biochim. Biophys. Acta. 1123*:59 (1992).
5. Baillargeon, M.W., *Lipids 25*:841 (1990).
6. Sonnet, P.E., T.A. Foglia and W.W. Baillargeon, *J. Am. Oil Chem. Soc. 70*:1043 (1993).
7. Mukherjee, K.D., I. Kiewitt and M.J. Hills, *Appl. Micro. Biotech. 40*:489 (1993).
8. Hills, M.J., I. Kiewitt and K.D. Mukherjee, *Biochem. Biophys. Acta 1042*:237 (1990).
9. Sonnet, P.E., T.A. Foglia and S.H. Feairheller, *J. Am. Oil Chem. Soc. 70*:387 (1993).
10. Syed Rahmatullah, M.S.K., V.K.S. Shukla and K.D. Mukherjee, *Ibid. 71*:563 (1994).
11. Syed Rahmatullah, M.S.K., V.K.S. Shukla and K.D. Mukherjee, *Ibid. 71*:569 (1994).
12. Tanaka, Y., J. Hirano and T. Funada, *Ibid. 69*:1210 (1992).
13. Hills, M.J., I. Kiewitt and K.D. Mukherjee, *Ibid. 67*:561 (1993).
14. Svennerholm, L.J., *J. Lipid Res. 9*:570 (1968).
15. Dyerberg, J., *Nutr. Rev. 44*:125 (1986).
16. Slover, H.T., and E. Lanza, *J. Am. Oil Chem. Soc. 56*:933 (1979).
17. Baillargeon, M.W., and S. McCarthy, *Lipids 26*:831 (1991).
18. Gunstone, F.D., *Prog. Lipid Res. 31*:145 (1992).
19. Lawson, L.D., and B.G. Hughs, *Biochem. Biophys. 52*:328 (1988).
20. Foglia, T.A., K.A. Petruso and S.H. Feairheller, *J. Am. Oil Chem. Soc. 70*:281 (1993).
21. Macrae, A.R., and A.R. Slabas, *Eur. J. Biochem. 202*:485 (1991).
22. Sonnet, P.E., G.P. McNeill and W. Jun, *J. Am. Oil Chem. Soc. 71*:1421 (1994).