Fatty Acid Selectivity: The Selectivity of Lipases of *Geotrichum candidum*

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The relative reactivities of several long-chain fatty acids in esterifications with 1-butanol catalyzed by lipases of *Geotrichum candidum* were evaluated. As has been noted previously, these lipases are not uniformly highly selective for *cis*-9 unsaturated fatty acids. However, the lipase preparations examined do uniformly discriminate against fatty acids having a chainlength greater than C-18 such as erucic acid. The reactivities of γ-linolenic and ricinoleic acid were also low compared to that of oleic. An examination of the effect of the alcohol upon the relative reactivities of acids showed that one could enhance fatty acid selectivity by proper choices of alcohol. For example, oleic acid esterifies 2.5 times faster than palmitic acid with 1-butanol catalyzed by Amano GC-4 lipase, but esterifies over 50 times faster with 2-methyl-1-propanol or cyclopentanol.

KEY WORDS: Esterification, fatty acid selectivity, *Geotrichum candidum*, lipases.

The lipase of the fungus *Geotrichum candidum* currently is the subject of intense investigation. The basis for industrial interest has been the characterization of this enzyme as one that shows a strong preference for fatty acids that contain a *cis*-9 double bond (1–3). This preference, however, is not shown uniformly by all available strains of the fungus (4–7), and its selectivity may reside in the structure of one particular isozyme, the presence of which is quite high in NRRL Y-553 (5). Recent research by means of X-ray crystallography in conjunction with molecular modeling aspires to unravel the basis for fatty acid selectivity (8–16).

We recently reported that lipase Y-553 could be employed to obtain erucic acid by catalyzing the esterification of the free fatty acids (FFA) of high-erucic acid rapeseed (HEAR) oil with 1-butanol (17). The HEAR oil contained 47.5% erucic acid; partial esterification of the FFA of this HEAR oil produced material that contained 85.4% erucic acid in the unesterified portion after 51.9% esterification. Additionally, in competitive esterification of 1:1 mixtures of oleic and erucic acid, the relative reaction rate was favoring the oleic acid *ca.* 400:1. The enzyme has been characterized as one that favors a fatty acid bearing an olefinic linkage, suggesting a specific nonbonded interaction involving the protein with the substrate. It is also possible that the selection observed has a basis in geometrical or steric constraints. Another view of the performance of this enzyme, therefore, contemplates its discrimination against saturated C-18 and longer chainlengths. We report here our observations of competitive esterification with a number of fatty acids by two commercial sources of the *G. candidum* lipase and the NRRL Y-553 cultured by us. The two commercial preparations were Amano GC-4 (≡GC-20) (Amano Co., Troy, VA), the isozyme content of which we have examined for selectivity (4), and an offering of Biocatalysts Ltd. (Mid Glamorgan, United Kingdom), which we refer to here as BL. Several of the fatty acids studied are less common specialty fatty acids. The data indicate potential for useful enrichment of some of these acids from the natural milleu, which signifies therefore a profoundly greater industrial value than heretofore anticipated.

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MATERIALS AND METHODS

Nuclear magnetic resonance spectra (^{13}C NMR) were obtained in a JEOLJNM-GX400 FT-NMR spectrometer (Jeol, Peabody, MA) with deuteriochloroform as solvent and tetramethylsilane as internal standard. For ^{13}C NMR, only diagnostic signals are reported, and the signals are given as ppm downfield from tetramethylsilane. Infrared spectra (IR) were obtained in a Perkin-Elmer 1310 Spectrophotometer (Norwalk, CT) with 1% solutions in carbon tetrachloride. Gas–liquid chromatography (GLC) was accomplished with a Hewlett-Packard Model 5895 (Sterling, VA) chromatograph with a split capillary column injector. Helium was the carrier gas, and split ratios were set to 80:1 with flame-ionization detection. The column employed was an SP-2340 column (0.25 mm × 30 m) from Supelco, Inc. (Bellefonte, PA). Elution of fatty acid methyl esters (FAMEs) was carried out with temperature programming from 140 to 155°C at 0.5°C/min; then from 155 to 200° at 2°C/min. FAMEs were identified by comparison with the standard mixture, M-100, from Nu-Chek-Prep (Elysian, MN), and quantitative analysis was performed as described by Slover and Lanza (18). Butyl esters and other esters required for identification by GLC were synthesized by the usual methods. Titrations were conducted with a Radiometer AGU80 Autoburette (Copenhagen, Denmark), and reaction mixtures were agitated with an IKA-Vibrax-VXR obtained from Cole-Palmer (Niles, IL).

Lipases of *G. candidum* were gifts of Amano Co. and Biocatalysts, Ltd. The strain, NRRL Y-553, was cultured as described previously (5). The specific activities of these enzymes in hydrolysis of olive oil, expressed as μmol FFA released/min/g of powder, were: GC-4, 4400 (manufacturer) and BL, 4000 (manufacturer). The Y-553 lyophilized powder was 19.4% protein and had a specific activity in an olive oil assay (0.05 M Tris buffered at pH 8.2 with 11 mmol calcium chloride) of 69 μmol FFA/min/mg protein and relative activity in hydrolysis of umbelliferate esters of oleic and palmitic acids of ≈20:1 (6). These materials were employed without further purification.

Organic solvents were reagent-grade or better. Dimethylsulfoxide (DMSO) and hexamethylphosphoramide (HMPT) were dried over 13Å molecular sieves (Aldrich Chemical Co., Milwaukee, WI), while tetrahydrofuran (THF) was distilled from lithium aluminum hydride before use. Reagent chemicals were purchased from Aldrich Chemical Co. and used directly; fatty acids were obtained

either from Aldrich Chemical Co. or Sigma Chemical Co. (St. Louis, MO) and were ≥99% pure.

Competitive esterifications of fatty acids with 1-butanol. Equimolar solutions of oleic and a second fatty acid were prepared in chloroform at 50% wt/vol. Reaction mixtures contained 100 μL of acid solution (≈25 mg of each acid), 25 μL of 1-butanol and a total of 400 μL of the buffer described above. The lipase powders were dissolved in buffer at 0.10 g/mL and added to the reaction tubes (25, 50, 100, 200, 400 μL). The NRRL Y-553 culture broth was thawed and used directly in the same increments. Mixtures were agitated at 200 rpm for 16–24 h at 34°C. The mixtures were worked up by adding 2 mL of 2N HCl and 2–4 mL ether, vortexing and centrifuging. The ether layer was removed, and a second ether extraction was done. The combined ethereal phase was then washed twice with water, passed through a disposable pipet containing solid sodium sulfate, and concentrated under a stream of nitrogen. Diazomethane was added to produce a mixture that contained a pair of methyl esters that represented the unreacted fatty acids and a pair of butyl esters that constituted the product. GLC area ratios of methyl esters and butyl esters were employed to calculate percentage conversion. This value, in conjunction with the "excess" of one methyl ester over the other, can be employed in an equation derived by Sih and Wu (19) for biocatalytic resolutions to obtain a measure of relative activity.

Competitive esterification of 1-butanol with oleic and palmitic acids catalyzed by Y-553 serves as a specific example: Areas of esters, normalized to unity for a particular sample, were: methyl palmitate, 0.637 and methyl oleate, 0.363 (0.637 − 0.363 = 0.274 = mole fraction excess of palmitic acid in the starting material = SM_{XS}); butyl palmitate, 0.048 and butyl oleate 0.952 (0.952 − 0.048 = 0.904 = mole fraction excess of oleate ester in the product = P_{XS}); C (fraction converted) = $SM_{XS}/SM_{XS} + P_{XS}$ = 0.233 (≡23.3% conv.); S_R (substrate ratio, or relative reactivities of the two acids) = $\ln(1 - C)(1 - SM_{XS})/\ln(1 - C)(1 + SM_{XS})$ = 25. Because oleic acid reacted 25 times as fast as palmitic acid, the rate of palmitic relative to oleic acid, with oleic set to 100, was 4 (Table 1). Each pair of acids was allowed to react to several different degrees of conversion, and the calculated relative reaction velocities (S_r) were averaged. These equations have become commonplace in assessing the potential for resolving the enantiomers of a racemate and are appropriate for application in relative reactivity of other competing reactants. Its application does imply certain provisions (19) but is expected to serve as a useful qualitative guide to the relative reactivities of fatty acids and derivatives reacting competitively, as do relative reactivities based upon initial reaction velocities obtained by independent measurements. The advantage of competitive measurement is that it approximates a real situation more closely. Difficulties were encountered in measuring GLC areas of ricinoleic acid esters, and relative reactivity was evaluated by independent reactions that were monitored by titrations (20) to determine the extent of esterification.

Competitive esterification of oleic and palmitic acids with other alcohols. The reaction mixtures were prepared as described above with 25 μL of the candidate hydrophobic alcohol and solutions of Amano GC-4. The conduct and analysis of mixtures was as described above.

TABLE 1

Relative Reactivity of Fatty Acids in Esterification with 1-Butanol Catalyzed by Lipases of *Geotrichum candidum*

Acid	Designation	Activity relative to oleic acid[a]		
		GC-4	BL	Y-553
Palmitic	16:0	40	5	4
Stearic	18:0	10		2
Petroselenic	18:1(n-11)	10		<1
Oleic	18:1(n-8)	100	100	100
Elaidic	18:1(*trans*,n-8)	4.3		<0.5
Linoleic	18:2(n-5)	100	59	<83
Linolenic	18:3(n-2)	100	69	77
γ-Linolenic	18:3(n-5)	0		≈0
Ricinoleic	18:1(n-8,12-OH)	1.5		0.9
9-Docosenoic[b]	22:1(n-12)	0.5		<0.5
9-Docosynoic[b]	22(9-yne)	0		≈0
Erucic	22:1(n-8)	0.6	<0.1	0.3

[a]Values are the average of 2–4 determinations. The lipase of Y-553 and that of Biocatalysts, Ltd. (Mid Glamorgan, United Kingdom) were similar enough that the entire set of acids was not evaluated. All double bonds are *cis* unless otherwise noted.
[b]Synthesized for this study (see Materials and Methods).

Synthesis of 9-decynoic acid. A solution of 8-bromooctanoic acid (8.9 g, 40 mmol) in 100 mL DMSO was stirred under nitrogen and cooled in an ice bath while 16 mL of 2.5M butyllithium in hexane was slowly added. To the resulting lithium carboxylate was added lithium acetylide/ethylenediamine (7.4 g, 40 mmol) and sodium iodide (6.0 g, 40 mmol). The reaction was stirred overnight at 45–50°C. The crude product was obtained by acidification and extraction with ether. Distillation (short path) provided 9-decynoic acid: b.p. 101–116°C (0.2 Torr), 4.1 g (31%); m.p. 28–29°C (recrystallized from hexane); IR 1710 cm^{-1}; ^1H NMR δ 2.33 (*t*, *J* = 7.5 Hz, CH_2CO_2H, 2H), 2.11 (*t*, *J* = 6.4 Hz, $CH_2C{\equiv}C$, 4H), 1.61 (*m*, $CH_2CH_2CO_2H$, 2H), 1.44 (*m*, $CH_2CH_2C{\equiv}C$, 4H), 1.2–1.4 (*m*, CH_2 env.), 0.85 (*t*, *J* = 6.8 Hz, CH_3, 3H) ppm; ^{13}C NMR δ 179.7 (CO_2H), 80.4 (C≡C) ppm. GLC retention time for the methyl ester: 11.6 min. (SPB-1/250°C).

Synthesis of Z-9-docosenoic acid. 9-Decynoate (4.77 g, 28 mmol) was dissolved in 50 mL dry THF and cooled to −70°C under nitrogen. Butyllithium (23 mL of 2.5M in hexane) was injected, followed by 20 mL HMPT. The mixture was stirred for 0.25 h, and then 1-bromododecane (6.7 mL, 28 mmol) was injected. The resulting mixture was stirred at room temperature overnight and then worked up by acidifying and extracting with ether. The crude 9-docosynoic acid was passed through a column of alumina with hexane, yielding 4.1 g, 44%. A portion of this acid was converted to the methyl ester with boron trifluoride–methanol (GLC: SPB-1 column/250°C, retention time 11.4 min). The ester (0.85 g, 2.4 mmol) was hydrogenated in hexane (15 mL) over Lindlar catalyst (0.1 g) to which 10 μL of quinoline was added. Hydrogenation was monitored both by hydrogen uptake and GLC (retention time of the *cis*-alkenoate with the conditions noted above was 11.1 min). The reduction product was recovered from the hexane and saponified with 6N KOH in 50% aqueous methanol under reflux for 4 h. The usual workup provided the *Z*-9-dodecenoic acid that was recrystallized from hexane; 0.35 g (41%); mp 30–31°C; IR

1710 cm^{-1}; ^1H NMR δ 5.32 (*m*, CH=CH, 2H), 2.32 (*t*, J = 7.5 Hz, CH_2CO_2H, 2H), 1.98 (*m*, CH_2CH=CH, 4H) 1.62 (*m*, $CH_2CH_2CO_2H$, 2H), 1.2–1.4 (*m*, CH_2 env.), 0.86 (*t*, J = 6.9 Hz, CH_3, 3H) ppm.

RESULTS AND DISCUSSION

Esterification reactions were selected to compare these lipase variants of *G. candidum* because enhanced selectivity may be expected in esterifications as opposed to hydrolysis (17,19). One could hope for a better spread of values to help assess the utility of a particular lipase-catalyzed transformation. It is apparent from Table 1 that there is a greater discriminating power of Y-553 (and BL) compared to GC-4 in the oleic *vs.* palmitic acid competition, as has been noted previously (5). This acid pair can be regarded as a critical pair separation, because the majority of the isozymes of *G. candidum*, save the most discriminating one(s), show roughly equal reactivity with the two acids. Additionally, reactions, such as hydrolysis of soy oils for polyunsaturated fatty acids and of tallow to obtain oleic acid that are targeted to obtaining samples enriched in *cis*-9-unsaturated fatty acids, will generally require high discrimination against palmitic acid. Parenthetically, one expects that the stearic acid content of tallow would not be as much of a problem, given that both lipases discriminate against it. As expected, the reactivities of linoleic (δ-9,12) and linolenic (δ-9,12,15) acids were roughly similar to oleic, whereas petroselenic (δ-6) and γ-linolenic (δ-6,9,12) acids, which possess unsaturation closer to the carbonyl, were much slower to undergo esterification. In these cases, it did appear that the enzyme was indeed selecting for *cis*-9 unsaturation in the chain, and that the least effective catalyst was GC-4. However, neither ricinoleic (δ-9, 12-OH) nor *cis*-9-docosenoic acids showed much reactivity with either the GC-4 or Y-553 lipases. 9-Docosynoic acid was quite unreactive, and erucic acid (C-22, δ-13) was similarly slow to react with any of the three lipases. These results indicate that *cis*-9 unsaturation may not be a sufficient criterion for acceptance by any of the isozymes of these enzyme formulations and that both chainlength and hydrophilic substitution on the chain can suppress reaction. Intriguingly, the three sources of *G. candidum* behaved similarly with the longer-chain fatty acids, and one may not require a special "brand" of material to effect a useful enrichment of a specialty fatty acid. On the other hand, the data reiterate that, if the target is a separation that ultimately requires an ability to discriminate between palmitic and oleic acids, one may indeed require an enzyme rich in a particular isozyme.

The effect of alcohol structure on the esterification selectivity of the GC-4 lipase preparation was studied with the previously mentioned critical pair, oleic *vs.* palmitic acid, as substrates. The calculated values for relative reactivities tended to decline during the course of the reaction. This is probably due to the heterogeneous nature of GC-4, which is a composite of several isozymes. The phenomenon is discussed in a review of biocatalytic resolutions by Sih and Wu (19). The data of Table 2, therefore, show relative reactivities at roughly equivalent low degrees of conversion (5–18%), which is relevant to a comparison of relative reactivities as a function of the alcohol used for the esterification. At 5% conversion, the preference of the GC-4 lipase for oleic acid over palmitic acid in esterification with 1-butanol was equal to the selectivity (2.5–4.0) that had been noted at higher conversions. In contrast, when 2-methyl-1-propanol and cyclopentanol are employed, a twentyfold increase in selectivity was measured. The selectivities observed with cyclohexanol, benzyl alcohol and 3-methyl-1-butanol were about 10 times greater than with 1-butanol. These observations suggest that the selectivity in reactions of an acylated lipase is related to steric access to the alcohol group, with the more hindered alcohols providing greater selectivity. In any case, one may improve selectivities in reactions of fatty acids in esterifications of alcohols by proper choice of an alcohol. Applied to the situation of an enzyme that is only modestly discriminating between oleic and palmitic acids, one may be able to augment selectivity to the point of useful resolutions.

TABLE 2

Effect of Alcohols on Relative Reactivity of Oleic *vs.* Palmitic Acids in Esterifications Catalyzed by Lipase Amano GC-4

Alcohol	Conversion (%)	Relative reactivity[a]
1-Butanol	<5	3.0
2-Methyl-1-propanol	15.4	53
3-Methyl-1-butanol	12.4	16
Benzyl alcohol	12	15
Cyclopentanol	18	52
Cyclohexanol	6.0	17

[a]Reactions were carried out to varying percentages of conversion, and relative reactivities at roughly equivalent conversions are compared.

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