Fatty Acid Selectivity of Lipases: Erucic Acid from Rapeseed Oil

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The fatty acid selectivity of several commercial lipases was evaluated in the hydrolysis of high-erucic acid rapeseed oil (HEARO). The lipase of *Pseudomonas cepacia* catalyzed virtually complete hydrolysis of the oil (94–97%), while that of *Geotrichum candidum* discriminated strongly against erucic acid, especially in esterification. A two-step process is suggested for obtaining a highly enriched erucic acid in which the *G. candidum* lipase is employed to selectively esterify the fatty acid residues of unsaturated C-18, and shorter chain acids, from a mixture of HEARO fatty acids obtained from total hydrolysis of the oil with *P. cepacia* lipase.

KEY WORDS: Erucic acid, fatty acid selectivity, lipases.

Considerable effort is currently being expended to study the chemistry of lipases (triacylglycerol hydrolase, EC 3.1.13) for catalyzing transformations involving fats, oils, partial glycerides and fatty acids and their derivatives. More specifically, there has been a recent surge of interest in fatty acid-selective reactions. Haraldsson has provided a timely review that emphasizes reactions involving the "n-3" fatty acids from marine oils (1), and Sonntag has offered information concerning the basis of the industrial interest in particular fatty acids (2). Additionally, useful procedures for measuring lipase fatty acid selectivity have been described (3,4). In some instances it is possible to couple fatty acid selectivity to positional selectivity as, for example, when a natural triglyceride has a high proportion of a particular fatty acid in the primary positions. Thus, *Rhizopus delemar* lipase has been employed in conjunction with a biomembrane reactor to remove erucic acid (*Z*-13-docosenoic acid) from the primary positions of crambe oil (5). Similarly, lesquerolic acid (14-hydroxy-*Z*-11-eicosenoic acid) has been removed from lesquerella oil by means of the positionally selective lipase of *Rhizomucor arrhizus* (6). Contrastingly, a lipase of *Candida rugosa* was reportedly sufficiently fatty acid selective that 1,3-dierucin could be prepared from rapeseed oil, *i.e.*, the lipase is positionally nonselective, but favors hydrolysis of acid residues shorter than that of erucic acid, most of which occur at the 2-position (7). The authors then synthesized trierucin by esterifying their diglyceride product with erucic acid. In related work, rape lipase was shown to discriminate strongly against γ-linolenic acid (*Z,Z,Z*-6,9, 12-octadecatrienoic acid, GLA) (8), *C. rugosa* lipase selected against both erucic acid and GLA (9), and *Geotrichum candidum* lipase also discriminated against erucic acid (10). Here we report the evaluation of fatty acid selectivities of a number of commercial lipase preparations based on the partial hydrolysis of high-erucic acid rapeseed oil (HEARO) as well as a two-step sequence with two different lipases that allows the preparation of a highly enriched erucic acid fraction from that oil.

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MATERIALS AND METHODS

Fatty acid methyl esters, FAMES, were analyzed by gas-liquid chromatography (GLC) on a Hewlett-Packard (Avondale, PA) Model 5895 chromatograph equipped with a split capillary injector, a flame-ionization detector and a Hewlett-Packard Model 3396 integrator. Separations were obtained on an SP-2340 column (0.25 mm i.d. × 30 m) from Supelco, Inc. (Bellefonte, PA). The carrier gas was He with a linear flow velocity of 22.9 cm s^{-1} at an 80:1 split ratio. Elution of FAMES was carried out with temperature programming from 140 to 155°C at 0.5°C min^{-1}; then from 155 to 200°C at 2°C min^{-1}. FAMES were identified by comparison with the standard mix M-100, obtained from Nu-Chek-Prep (Elysian, MN). Florisil was obtained from Fisher Scientific (King of Prussia, PA), and thin-layer chromatography was done on 0.25-mm silica gel 60 plates purchased from Merck (Montreal, Canada). Titrations were conducted with a Radiometer AGU 80 Autoburette (Copenhagen, Denmark). All solvents were high-performance liquid chromatography (HPLC) grade, and all reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Butyl oleate and erucate were prepared by standard procedures, as was a mixture of butyl esters from HEARO, to allow GLC identifications. Enzymes employed were (supplier, trade name if any, identity, specific activity on olive oil in μmol free fatty acids, min^{-1}, mg^{-1}): Enzeco (New York, NY), Lipase R, *C. rugosa*, 9.45; Amano Co. (Troy, VA), GC-20, *G. candidum*,, 6.2; Sigma Chemical Co. (St. Louis, MO), Type II, porcine pancreatic, 2.0; Tanabe (Tokyo, Japan), Food Grade, *R. delemar*, 3.9; Amano Co., Lipase FAP, *Rhizopus oryzae*, 150 (labeled activity); Amano Co., Lipase AP-12, *Aspergillus niger*, 136 (labeled activity); Amano Co., LPL, lipoprotein lipase, 2300; Novo Co., Bagsvaerd, Denmark, Lipozyme, *Rhizomucor miehei*, 1.5; and Amano Co., Lipase P-30, *Pseudomonas cepacia*, 1.55.

Partial hydrolysis of HEARO with various lipases. The equivalent weight (saponification number) of the oil was determined by total hydrolysis with methanolic KOH, isolation of the acids, and titration with 0.100 N NaOH in aqueous ethanol to pH 10.6; then corrected by a blank. The average of two determinations was 316.4; this formed the basis for the determination of percent hydrolysis or esterification in enzyme-catalyzed reactions. For all of the enzyme-catalyzed hydrolyses, excepting that of *G. candidum*, 2 g of HEARO (oil) and 2 mL of 0.05 M phosphate buffer at pH 7.00, containing from 1–20 mg of enzyme according to how reactive or pure the preparation was, were shaken in tubes at 30°C for differing lengths of time. For the *G. candidum* lipase, the buffer was 0.1 M "tris" at pH 8.0 and 10 mM calcium chloride. The tube contents were acidified with 2N HCl, and the mixtures were then extracted with hexane. The hexane extracts were washed with water, dried ($MgSO_4$) and subsequently pumped free of solvent to constant weight. Aliquots of 0.3–0.4 kg were weighed (±0.05 mg) and titrated to determine the free fatty acid (FFA) content, *i.e.*, the degree of hydrolysis. A second aliquot of 0.3 g was treated with methanolic NaOMe to convert the residual glycerides

TABLE 1

Lipase-Catalyed Partial Hydrolysis of HEARO: Fatty Acid Composition of Unhydrolyzed Glycerides Relative to That of Original Oil[a]

Lipase	C[b]	16:0	18:0	18:1[c]	18:2[d]	18:3[e]	20:1	22:1[f]
Aspergillus niger	73.8	0.69	0.98	0.97	1.13	1.18	0.86	0.97
Candida rugosa	69.0	0.69	1.19	0.62	0.25	0.08	1.48	1.57
Geotrichum candidum	70.0	0.39	0.85	0.05	0.04	0.04	2.00	2.10
Pseudomonas cepacia	65.2	0.90	0.96	1.26	1.08	0.74	0.88	0.83
Rhizomucor miehei	72.0	0.79	1.00	1.02	1.07	1.03	0.91	0.95
Rhizopus delemar	59.8	0.82	0.85	1.30	1.06	0.68	0.90	0.86
Rhizopus oryzae	60.2	0.81	0.92	1.24	1.21	0.93	0.80	0.90
Lipoprotein	67.1	0.72	0.94	1.21	0.82	0.79	0.91	1.01
Porcine pancreatic	62.6	0.75	0.94	1.19	1.21	0.91	0.82	0.86

[a]The wt% composition of HEARO (±0.5%) was as follows: wt% (component); 3.1 (16:0); 1.0 (18:0); 2.4 (18:1Δ^7); 23.4 (18:1Δ^9); 14.8 (18:2$\Delta^{9,12}$); 9.6 (18:3$\Delta^{9,12,15}$); 0.8 (20:0); 4.5 (20:1); 0.5 (22:0); 37.5 (22:1Δ^{13}); 0.5 (22:2); 0.7 (24:0); 1.2 (unidentified).
[b]Degree of hydrolysis expressed as percentage.
[c]Δ^9.
[d]$\Delta^{9,12}$.
[e]$\Delta^{9,12,15}$.
[f]Δ^{13}.

to FAMES in the usual fashion (NaOMe, MeOH, 1 h, room temperature), and these were then separated from sodium salts (11). FAMES were analyzed by GLC as described above. A minimum of four determinations at different degrees of reaction were made; the data for 60–75% conversion have been selected for display in Table 1.

Competitive hydrolysis and esterification of oleic and erucic acid residues. Weighed amounts of about 0.3–0.35 g (this did not need to be accurate) of a 1:1 equimolar mixture of butyl oleate and butyl erucate were allowed to react at 30°C in a shaker tube with 2.0 mL of buffer containing the enzyme (2–4 mg) as described above. Samples were worked up at varying times (1–8 h) by extraction as described above. The mixture of acids and esters was separated either by florisil column chromatography or by extraction of the butyl esters from the sodium salts of the acids with ether. The acid fraction was esterified with methanolic BF_3 to be analyzed as methyl esters. The excess of butyl erucate over butyl oleate, and the excess of methyl oleate over methyl erucate were used to calculate the percent hydrolysis and the relative reactivity of the two acyl groups in hydrolysis. The relevant equations are (12):

$$C = \text{excess SM}/(\text{excess SM}) + (\text{excess P}) \text{ and} \quad [1]$$

$$S_R = \ln(1 - C)(1 - \text{excess SM})/\ln(1 - C)(1 + \text{excess SM}) \quad [2]$$

where C is the fraction conversion. Excess SM and P refer to the excess of slower reacting material present in the unreacted starting material expressed as a fraction (SM), and the excess of faster reacting material in the product (P); and S_R is the relative reactivity of the competing acyl groups (Substrate Ratio, by analogy to the established use of the term "Enantiomeric Ratio" in biocatalytic resolutions). For example, if A and B are present in a 75:25 ratio, the excess of A over B is 50% or 0.50 expressed as a fraction.

Esterification was conducted analogously: a 1:1 equimolar mixture of the acids, *ca.* 25–50 mg each, was allowed to react with 0.50 mL of *n*-butanol in 10 mL of hexane saturated with the appropriate buffer and to which the enzyme had been added as a powder (0.25 g) while magnetically stirred at 30°C for 4–24 h. Alternatively, the acids were allowed to react with *n*-butanol, as above, but with 2.0 mL of the appropriate buffer containing 0.1–0.2 g of lipase. These mixtures were shaken at 30°C for 3–24 h and then worked up as described above (filtering the acidified mixture through celite with suction) to determine the relative reactivity of the two acyl groups. The relative reactivities are shown in Table 2.

Evaluation of a two-step procedure for high-erucic acid content FFA. HEARO, 50 g of a second sample higher in erucic acid content, was shaken at 300 rpm for 100 h with 50 mL of 0.05 M phosphate buffer at pH 7.0 containing 1.0 g of lipase from *P. cepacia.* The reaction was worked up by adding 50 mL of 2N HCl and extracting the resultant mixture with 2 × 50 mL hexane. The organic phase was washed with 3 × 50 mL H_2O and stripped of solvent. Titration of an aliquot indicated 97% hydrolysis.

The FFA from the hydrolysis, 2.0 g, was treated directly with 2.0 mL *n*-butanol and 4 mL of 0.1 M Tris buffered at pH 8.0 and containing 10 mM calcium chloride and 2.0 g of lipase from *G. candidum.* The mixture was shaken at 100 rpm and 30°C for 21 h. The mixture was worked up by acidification with 10 mL of 2N HCl and extraction of the organics into hexane as above. Filtration with suction through Celite aided the separation of phases. The crude product was pumped free of residual *n*-butanol (20 min at 60°C, 0.1 Torr), and an aliquot was analyzed as described above to determine the percent esterification (51.9% in this case). The acid and ester fractions were separated on Florisil, and the acid fraction was converted to methyl esters as before. The results are given in Table 3.

RESULTS AND DISCUSSION

Biocatalytic resolutions of various racemic alcohols and acids have been discussed in some detail with an emphasis on the use of triacylglycerol lipases (12), and it has been pointed out that fatty acid selectivity can be re-

TABLE 2

Selectivity of *Candida rugosa* and *Geotrichum candidum* Lipases for Oleic *vs.* Erucic Acid

Lipase	FFA[a]	Ester[b]	C[c]	S_R[d]
Hydrolysis of 1:1 butyl esters				
C. rugosa	0.276	0.172	38.4	2.1
	0.578	0.286	33.1	4.9
G. candidum	0.796	0.252	24.1	11.1
	0.728	0.234	22.0	13.5
Esterification of 1:1 acids				
C. rugosa				
oil-water	0.386	0.340	53.2	2.6
wet hexane	0.150	0.198	43.1	1.7
G. candidum				
oil-water	0.592	0.990[e]	37.4	400
wet hexane[f]	—	—	—	—

[a]Excess of oleic over erucic acid expressed as a fraction (calculated as one does for enantiomeric excess as when resolving racemates by biocatalysis; see Materials and Methods). FFA, free fatty acids.
[b]Excess of butyl erucate over oleate expressed in the product esters as a fraction.
[c]Degree of conversion expressed as a percentage.
[d]S_R = Substrate Ratio, *i.e.*, relative reactivity of the two fatty acyl groups in the reaction in question. Oleic acid reacts faster than erucic.
[e]At 37% conversion the (product) ester contained less than 1% butyl erucate.
[f]Essentially no reaction occurs.

TABLE 3

Esterification of HEARO Acids with *n*-Butanol Catalyzed by *Geotrichum candidum* Lipase

		C = 45.9[b]		C = 51.9[b]	
Acid	HEARO[a]	Esters[c]	Acids[d]	Esters[c]	Acids[d]
14:0	<0.1	<0.1	<0.2	<0.1	<0.1
16:0	2.7	6.4	1.3	7.4	0.2
16:1	0.2	0.3	<0.1	0.5	<0.1
18:0	1.0	1.6	0.5	0.2	0.2
18:1[e]	12.8	31.1	1.5	32.3	1.7
18:1[f]	1.0	2.0	<0.1	2.3	0.2
18:2	12.3	30.1	0.7	28.2	1.0
18:3	7.4	18.0	0.6	16.2	0.3
20:0	0.7	0.2	0.6	0.2	0.7
20:1	9.6	2.9	5.6	5.1	6.5
22:0	0.6	0.1	0.8	0.1	0.8
22:1	47.5	6.8	83.5	7.5	85.4
22:2	0.7	0.2	2.2	<0.1	1.0
24:0	0.9	0.2	2.3	<0.1	1.9

[a]Composition of this particular sample of higher grade high-erucic acid rapeseed oil (HEARO) fatty acids (see Materials and Methods) normalized for unidentified fatty acids. Values constitute wt% and are reliable to ±0.2.
[b]C = % converted to esters.
[c]Determined as the product butyl esters from gas-liquid chromatography (GLC) areas.
[d]Determined after transesterification to methyl esters from GLC areas.
[e]Δ^9.
[f]Δ^7.

garded as a biocatalytic resolution as well (3). Accordingly, these processes are expected to share the same essential features. Developing a product based upon the selective reaction of particular fatty acids with a lipase, whether that process is hydrolysis, esterification or any other acyl transfer reaction, places a great emphasis on relative reactivity (12). As the faster-reacting acid residues are consumed, the enzyme is exposed to an increasing amount of the slower reacting fatty acids. Consequently, as the reaction progresses, the product mixture increases in the content of the unwanted, slower-reacting acid residues. Accordingly, discrimination "against" desired acid residues seems the better strategy for resolution. That is to say, one should gear a projected enzyme-catalyzed reaction to remove a less desirable faster-reacting material. Secondly, one may expect that the selectivity of an acyl transfer reaction would be greater in esterification and transesterification than in hydrolysis. One possible reason for this may be that the intermediate acylated enzymes when reacting with alcohols are exposed to nucleophiles larger than water, which may offer an added basis for discrimination. Indeed, esterification has been found

superior to hydrolysis in separating unsaturated fatty acids (13,14). Finally, and this pertains uniquely to selectivity for fatty acids, because hydrolyses are reversible, once the equilibrium (total esters:total acids) has been achieved, the ratios of the fatty acids in the acid fraction would change until they reflected the ratios of the acyl groups in the original triglyceride mixture. A reaction may be highly selective, but once equilibrium is achieved the skewed ratios of acid residues in the product will gradually change to become less skewed as one eventually achieves a true thermodynamic equilibrium.

Using this information as background, we elected to screen a number of commercially available lipases for the hydrolysis of HEARO. The composition of the oil was ascertained by GLC of the corresponding methyl esters and is given in a footnote to Table 1. The oil was then methodically exposed to the lipases at pH 7.0 and 30°C in a phosphate buffer. For the lipase of *G. candidum*, however, a tris buffer (pH 8.0) with added calcium ion was used because these are known to be conditions for optimal activity (15). It is also known that these conditions do not appear to significantly alter selectivity. Reactions were interrupted at varying degrees of hydrolysis, and the fatty acid composition of the unreacted glycerides was determined. Analyses were consistent for each lipase with degrees of hydrolysis varying from 20–80%. For comparison, Table 1 presents the fatty acid compositions of unreacted glycerides obtained at 60–74% conversions relative to that of the original oil. Values >1.0, for example, indicate slower hydrolysis for that particular fatty acid, while values less than 1.0 indicate faster hydrolysis. Both *G. Candidum* and *C. rugosa* lipases seem to hydrolyze fatty acids of chain length >C-18 quite slowly. The *G. candidum* lipase was particularly intriguing: at 70% conversion the content of each of the unsaturated C-18 fatty acids had declined to 5% or less. Initially, fatty acids >C-18 in chain length comprised 44.5% of the HEARO; after 70% hydrolysis the remaining glycerides contained 92.1% of these acids.

Contrastingly, the lipase of *P. cepacia*, which is a positionally random lipase, was also indiscriminate with respect to fatty acid structure and was therefore deemed a candidate for catalyzing complete hydrolysis of this oil. The positionally selective lipases, namely those of the fungal genera *Rhizopus* and *Rhizomucor*, unfortunately showed no selectivity for or against the longer chains. This could have proven useful because the erucic acid residues of this oil reportedly reside exclusively at the primary positions of the glycerol (16).

On the basis of the data of Table 1, the lipases of *C. rugosa* and *G. candidum* were selected for further evaluation as catalysts for discriminating oleic from erucic acids. Competitive hydrolysis of 1:1 mixtures of the butyl esters of oleic and erucic acids was conducted. The degree of hydrolysis was determined from the relative amounts of the unreacted butyl esters and the relative amounts of the produced FFA. A calculation of the relative reactivity of the two fatty acids can then be made with the equations developed by Sih and Wu (12) for biocatalytic kinetic resolutions of racemates (see Materials and Methods) (Table 2). The lipase of *C. rugosa* reacted about 2–5 times faster with butyl oleate than with butyl erucate, while the selectivity of the *G. candidum* lipase was 11–14 (oleate > erucate). Esterifications of 1-butanol were conducted with 1:1 mixtures of the acids either in wet hexane or as oil-water mixtures without any organic solvents. It has been reported several times that lyophilized *G. candidum* lipase is relatively unreactive, but not denatured, in hydrocarbon solvents; and that lipases in aqueous solution may be used to esterify hydrophobic alcohols (17). The selectivity of the *G. candidum* lipase was greater for esterification (>100:1 in favor of oleic over erucic acid) than for hydrolysis (11:–14:1). The selectivity of the *C. rugosa* lipase, on the other hand, remained the same as in hydrolysis. Attempted transesterifications with butyl esters and 1-propanol conducted in wet hexane or as oil-water mixtures were too slow to be useful.

A two-step sequence was envisioned for obtaining a mixture of esters enriched in erucic acid from the HEARO in which the lipase from *P. cepacia* would be used to produce essentially complete hydrolysis as the initial step. The crude fatty acids would then be exposed to *G. candidum* lipase in the presence of 1-butanol under appropriate conditions to selectively esterify the acids. Through trial and error with a grade of HEARO containing several percent more erucic acid (see Materials and Methods), it was determined that 20 mg of *P. cepacia* powder/gram of HEARO produced 94–97% hydrolysis at pH 7.0 (phosphate buffer) and 30°C in 96 h. This was accomplished without added surfactants, thereby facilitating separation of the oil and water phases. These are not regarded as optimized conditions, but are intended to indicate the utility of the procedure. The esterification was monitored so that it could be interrupted when the unreacted acid fraction contained the optimum proportion of erucic acid. Trial esterifications of the mixture of acids (97% acid) from the HEARO with *n*-butanol and the aqueous buffer described above were conducted. The reactions were allowed to proceed for a fixed length of time with differing quantities of Amano's lipase "GC-20", which is obtained from *G. candidum*. Results are shown in Table 3 for reactions conducted to 45.9% and 51.9% conversion. Again, the conditions have not been optimized, but indications are that the procedure is highly selective for retaining erucic acid in the FFA fraction. The data indicate, as one expects, that the higher the degree of conversion, the more erucic acid will have been esterified. At 51.9% conversion to butyl esters, the product esters contain 7.5% butyl erucate, and the residual acids contain 85.4% erucic acid. It is also clear that the enzyme generally discriminates against acids of chain length > 18. At 45.9% conversion the esters contain 10.4% of C >18, and the content of this acid blend in the recovered acid fraction is 95.4%. For the higher degree of conversion, namely 51.9%, the content in the ester product has increased to 13.0%, and that of the acid has increased to 96.3% (they both increase because there is net conversion of acid to ester from an acid mixture already high in the slower reacting acids).

In summary, *P. cepacia* lipase is a suitable catalyst for total hydrolysis of HEARO. The enzyme exhibits neither positional, nor fatty acid selectivity. By contrast, lipase from *G. candidum* discriminates strongly against certain fatty acids in esterification reactions and can be used to provide a relatively high-grade erucic acid that contains less than 4% oleic, linoleic and linolenic acids. To address the issue of synthetic utility, it would be necessary to consider immobilization schemes and various optimizations of reaction parameters. It may, in fact, be possible to

obtain a more selective lipase catalyst, or devise a reaction strategy for which the lipase would be yet more selective. These ideas are currently under investigation in our laboratories and are complementary to the current research intended to enhance the erucic acid content of rapeseed oil itself.

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