

Purification and Specificity of Lipases from *Geotrichum candidum*¹

Mary Welch Ballargeon

U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, Pennsylvania 19118

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A crude, commercial *Geotrichum candidum* lipase (EC 3.1.1.3) preparation (Amano GC-20) was purified by hydrophobic interaction chromatography on Octyl Sepharose. The purified enzyme is a microheterogeneous glycoprotein containing isozymes varying in molecular weight, pI and specificity. It consists of 64, 62 and 59 kDa species as determined by denaturing polyacrylamide gel electrophoresis. Five isozymes (pI 4.40, 4.47, 4.58, 4.67 and 4.72) are detected by isoelectric focusing using both silver and activity stains. Chromatofocusing was used to separate the isozymes according to pI. Although all the isozymes are specific for oleate *vs* stearate esters, one isozyme (pI 4.72) is also specific for oleate *vs* palmitate. The number of isozymes is reduced to two (pI 4.67 and 4.72) after carbohydrate removal using endoglycosidase F/N-glycosidase. These isozymes may be products of two lipase genes. *Lipids* 25, 841-848 (1990).

Lipases (EC 3.1.1.3) catalyze the hydrolysis of ester bonds at a lipid-water interface. Lipases may exhibit specificity for the position or type of fatty acid in a triglyceride, their natural substrate (1). Lipases also exhibit stereochemical specificity when reacting with a wide variety of substrates in organic solvents (2).

Lipases from *Geotrichum candidum* are known to show specificity for the hydrolysis of unsaturated fatty acids with a *cis*-double bond at the 9-position (oleic, linoleic, linolenic acids) *vs* the corresponding saturated fatty acid (stearic) (3). Some differences in specificity among different strains have been reported (3-8). The molecular bases for this specificity are unknown, although partial purifications of various lipases from *G. candidum* (9-12) have been reported. A low resolution X-ray structure (13) and a DNA sequence (14) have been reported for strain ATCC 34614 (9). The existence of two genes for *G. candidum* lipase from this strain has been reported very recently (15). A second lipase was found upon further purification of a preparation (14) previously believed to be homogeneous. The two lipases had the same substrate specificities, and similar amino acid compositions, but showed slight differences in molecular mass (15).

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Correspondence should be addressed at U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Philadelphia, PA 19118.

Abbreviations: BCA, bicinchoninic acid; con A, concanavalin A; endo F, endoglycosidase F/N-glycosidase; FFA, free fatty acids; IEF, isoelectric focusing; kDa, kilodalton; 4-MUMB, 4-methylumbelliferyl; O, free or esterified oleic acid; P, free or esterified palmitic acid; PAS, periodic acid-Schiff reagent; S, free or esterified stearic acid; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFMS, trifluoromethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane hydrochloride.

Unfortunately, the strain of *G. candidum* originally characterized (3) is no longer available (7). We have previously studied crude lipase from several strains of *G. candidum* (7) and found that while all the lipases were specific for the hydrolysis of oleate (O) *vs* stearate (S) esters, the fatty acid specificity for oleate *vs* palmitate (P) varied from 1.0 to 50 (that is, from no selectivity to high selectivity). In order to investigate the bases for these differences, we chose to study a commercially available strain, GC-20 (Amano), in detail. The purification, characterization and specificity of isozymes of GC-20 lipase are reported here.

MATERIALS AND METHODS

Materials. *G. candidum* lipase GC-20 lot 80106TS20, was a generous gift from Amano International Enzyme (Troy, VI). *G. candidum* lipase was also obtained from Germe (Marseille, France). Endoglycosidase F/N-glycosidase (endo F) was obtained from Boehringer Mannheim (Indianapolis, IN). Commercial olive oil was purified according to the procedure of Linfield *et al.* (16), or purified olive oil was obtained from Sigma (St. Louis, MO). Chromatography media were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ) or Bio-Rad Laboratories (Richmond, CA). 4-Methylumbelliferyl (4-MUMB) esters were obtained from Sigma. All other materials were reagent grade or better.

Assays. Lipase activity was quantitated titrimetrically by an initial rate assay using an olive oil-gum arabic emulsion containing 11 mM CaCl₂ at pH 8.2, as described previously (17). A unit of lipase activity is defined as the release of one micromole of free fatty acid (FFA)/min at room temperature.

Lipase activity was qualitatively measured by detection of fluorescence on a Rhodamine-olive oil-agar plate (18). This method is more sensitive than the titrimetric method, and was used to monitor column fractions and native electrophoretic gels. An unstained native gel was laid on the surface of a Rhodamine-olive oil plate. Bands due to active lipase were visible under fluorescent light after about 30 min.

The method of protein assay was chosen to avoid interfering substances in the sample buffer. The bicinchoninic acid (BCA) method (19), available as a kit from Pierce (Rockford, IL), was used when detergent was present, and the Bradford method (20) (kit from Bio-Rad) was used when chromatofocusing buffer was present. Bovine serum albumin (Sigma) was used as standard.

The specificity of lipase for oleate *vs* stearate or palmitate was calculated from the initial rate of hydrolysis of the respective esters of 4-methylumbelliferone. Slight modifications were made to the procedure of Dooijewaard-Kloosterziel and Wouters (21). A stock solution of 1 mM substrate in *N,N*-dimethylacetamide (Alfa, Danvers, MA) was diluted to a final concentration of 50 μ M in a buffer (pH 8.0) of 0.1 M

tris(hydroxymethyl)aminomethane hydrochloride (Tris), 5 mM CaCl₂. Fluorescence of the product was measured on a Sequoia-Turner Model 450 Fluorometer (360 nm excitation filter, 415 nm emission filter), standardized with 4-methylumbelliferone. The specificity for one substrate (A) *vs* another substrate (B) at one min reaction time was calculated using the Eq. [1] (22):

$$\text{Specificity}_{A/B} = \frac{V_A [B]}{V_B [A]} \quad [1]$$

where the rate, *V*, was multiplied by the substrate concentrations at one minute. Note that low values of specificity (around 1) can be measured much more accurately than large values (around 100).

Electrophoresis. Polyacrylamide gel electrophoresis was performed using the pHast system (Pharmacia) with 7.5% or 12.5% acrylamide gels or isoelectric focusing (IEF) gels (pH 4–6.5) according to instructions provided by the manufacturer. Two-dimensional electrophoresis was run on the pHast system using native IEF gels (pH 4–6.5) and 12.5 acrylamide denaturing gel. Mini-slab (8 × 10 cm) vertical electrophoresis was also performed. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was run according to Laemmli (23). Molecular weight standards were obtained from Diversified Biotech (Newton Centre, MA). pI Standards were obtained from Sigma (pH 3.6–6.6). Molecular weight measurements are averages of three to six denaturing gels of both 7.5 and 12.5% acrylamide; all measurements had a standard deviation of 1 kDa. Proteins were silver stained according to the method of Morrissey (24). Lipase activity was detected in native gels by an esterase stain (17) which was more sensitive than silver stain. It was confirmed that the esterase stain identifies lipase activity by comparing the position of esterase-positive bands with the position of active, fluorescent lipase bands from a duplicate gel placed on a Rhodamine-olive oil-agar plate. Periodic acid-Schiff reagent (PAS) (25) was used for staining carbohydrate.

Carbohydrate was also detected by concanavalin A (con A) labeled with peroxidase (Sigma). After SDS PAGE, samples were transferred to nitrocellulose by diffusion blotting. The nitrocellulose was blocked with deglycosylated bovine serum albumin (26), incubated with con A-peroxidase, and washed. Peroxidase activity was detected with 4-chloro-1-naphthol/H₂O₂.

Carbohydrate analysis. The amount of sugar in the purified lipase was measured by the phenol-sulfuric acid procedure (27), using mannose as the standard. Carbohydrate was removed by hydrolysis with trifluoromethanesulfonic acid (TFMS) for 2 hr (26) or by treatment with endo F (26) for 24 hr.

Purification. All procedures were performed at room temperature (21–25°C). GC-20 (3.6 g) was dissolved in a solution (pH 6.8) of 1 M ammonium sulfate, 10 mM imidazole-Cl, and 0.1 mM ethylenediaminetetraacetic acid, to a final concentration of 50 mg/mL. The sample was applied to an Octyl Sepharose column (1.5 × 48 cm, flow rate of 25 mL/hr) previously equilibrated in the same buffer, and the unbound proteins were washed off. When the absorbance at 280 nm returned to baseline,

the buffer was switched to 10 mM imidazole-Cl (pH 6.8), and additional contaminants were eluted. Lipase was eluted by a linear gradient of 365 mL each of 10 mM imidazole and 10 mM imidazole with 0.5% polyoxyethylene 10-tridecyl ether (Emulphogene BC-720, Sigma). This is a non-ionic detergent very similar to Triton X-100, but non-absorbing at 280 nm (28).

Chromatofocusing was used for isozyme isolation. Active fractions were dialyzed against 0.025 M piperazine-Cl (pH 5.4), and applied to a column (0.7 × 50 cm, flow rate of 13 mL/hr) of Polybuffer exchanger 94 equilibrated in the same buffer. The amount of protein applied to the column was kept minimal in order to maintain high resolution. The column was later scaled-up (1 × 111 cm, flow rate of 28 mL/hr) to accommodate larger samples. Lipase was eluted with Polybuffer 74, pH 3.5 (1 column volume Polybuffer 74 diluted with 12 column volumes of water). Polybuffer was removed by desalting on Bio-Gel P-30 in water or 10 mM ammonium bicarbonate. The active fractions were lyophilized.

In a separate procedure, Con A-Sepharose was also used to partially purify the crude lipase. A Con A-Sepharose column was equilibrated in a buffer (pH 6.0) of 0.1 M sodium acetate, 0.2M NaCl, 0.01% thimersol and 1 mM each of MnSO₄, MgCl₂, and CaCl₂. Lipase was dissolved in the equilibrating buffer, applied, and subsequently eluted with 0.2 M α -methyl D-mannoside. However, due to tailing and poor recovery of activity, this method of purification was not pursued. Lipase that had been partially purified on the Con A-Sepharose column ("con A lipase") was used as a standard marking the position of the lipase isozymes in isoelectric focusing; it was very similar to the material referred to as lipase A below.

Initial attempts to purify the lipase by gel filtration on Sephacryl S-200 or Bio-Sil TSK 250 were abandoned because of a tendency of the lipase to be retained and to elute in broad bands on both columns.

Amino acid analysis. Lipase was hydrolyzed in 6 N HCl at 110°C for 24, 48 and 72 hr. The hydrolyzed amino acids were analyzed on a Beckman 119Cl amino acid analyzer (Beckman, Fullerton, CA). Serine and threonine values were determined by extrapolation to 0 hr. Cysteine and cystine were determined as cysteine acid, and methionine as methionine sulfone, after performic acid oxidation. Tryptophan was determined by pyrolysis gas chromatography (29).

pH Stability. Purified lipase was dissolved (0.5 mg/mL) in deionized water or 0.2 M buffer containing 5 mM CaCl₂. Citrate-phosphate buffer was used at pH 4, 5, 6 and 7, and Tris buffer at pH 7, 8 and 9. The samples were incubated at 30°C, and aliquots were assayed for activity titrimetrically at pH 8.2 as a function of time. The control was incubated in water at 4°C.

RESULTS

Purification. Conventional methods of enzyme purification showed the lipase separation to be rather complex. We have developed a rapid and relatively simple two-step procedure for purification of lipases involving Octyl Sepharose and chromatofocusing.

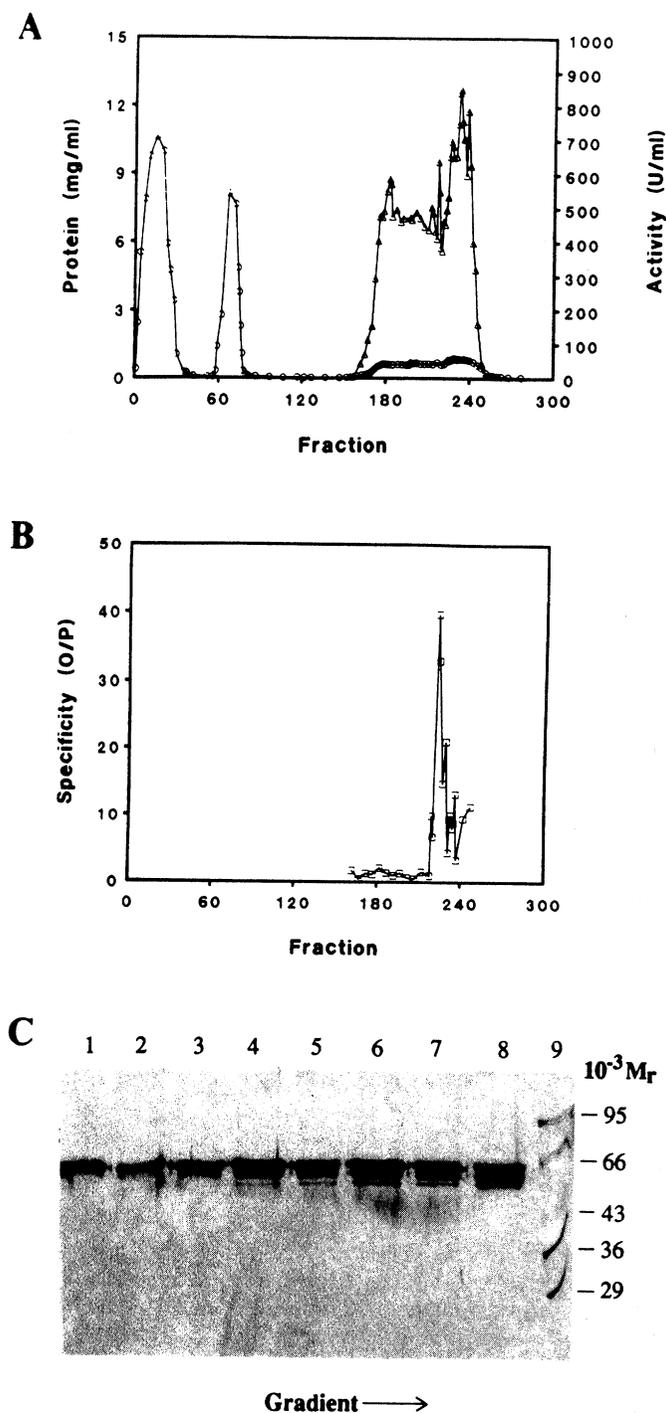


FIG. 1. Chromatography on Octyl Sepharose. Crude lipase was applied as described in Materials and Methods. Low ionic strength buffer was started at fraction 50, and the detergent gradient was started at fraction 120. Lipase B started eluting at fraction 211. Shown are (A), lipase activity (Δ), protein (O); and (B), specificity O/P (\square). (C) SDS PAGE of detergent gradient fractions showing the transition from lipase A (lanes 1-3) to lipase B (lanes 4-8). Lane 9, molecular weight standards.

Chromatography of lipase on Octyl Sepharose is shown in Figure 1. Colored, nonactive material which reacts strongly in the BCA protein assay is not bound

to Octyl Sepharose. Additional colored material is eluted with the low ionic strength buffer. Total lipase activity is eluted by the detergent gradient as two relatively broad peaks (Fig. 1A). However, lipase specific for oleate *vs* palmitate is found primarily in a narrow region of the second peak (Fig. 1B). All the fractions contain a number of components on SDS PAGE. The first peak (fractions 165 to 211) contains one main species of 64 kDa and a minor one of 62 kDa—it is referred to as lipase A. The second peak (fractions 212 to 246) contains the components in lipase A plus an additional species at 59 kDa—it is referred to as lipase B. SDS PAGE of fractions from a representative Octyl Sepharose column at the transition between lipase A (lanes 1-3) and lipase B (lanes 4-8) is shown in Figure 1C. The lipases contain no other contaminating bands by SDS electrophoresis at loadings of 0.5 to 3 μ g, with silver staining. The purification achieved is shown in Table 1.

In order to investigate the identity of the multiple molecular weight species in lipases A and B, the samples were studied by IEF. Lipase A contains four main components by both silver stain and activity stains. Lipase B contains the same four components plus an additional component at more basic pI.

Chromatofocusing was then used to separate the components of lipase A and lipase B. In theory, this method separates proteins according to pI; however, proteins do not necessarily elute at their pI. The separation of representative aliquots of lipases A and B is shown in Figures 2 and 3, respectively. Lipase A contains 5 isozymes eluting at pH 4.25, 4.11, 3.99, 3.93 and 3.83 (minor). The aliquot of lipase B shown in Figure 3 contains 4 isozymes eluting at pH 4.24 (shoulder), 4.20, 4.09 and 3.93 (minor). The reproducibility of peak position *vs* pH among different chromatofocusing runs is good.

The purification factors for the chromatofocusing columns are shown in Table 1. The recovery is based on the sum of all peaks. A small increase in the specific activity was achieved. In order to obtain pure isozyme, the fractions were pooled according to isozyme content. Yield was sacrificed for purity.

Analytical IEF of the five isozymes of *G. candidum* lipase is shown in Figure 4. The isozymes have pIs of 4.72, 4.67, 4.58, 4.47 and 4.40, and are identified by the numbers 0, 1, 2, 3 and 4, respectively. Lipase A contains isozymes 1 to 4. Lipase B contains isozyme 0 in addition to isozymes 1 to 4. Con A lipase contains mainly isozymes 1-4; isozyme 0 is also found after extended incubation or with higher concentration samples.

The isozymes elute from the chromatofocusing column approximately, but not exactly, in order of decreasing pI; although band 0 is the most basic isozyme (pI 4.72), it elutes at pH 4.20, after isozyme 1 (pI 4.67, pH 4.25). This suggests that isozyme 0 differs in surface charge distribution and/or solubility as compared to the other isozymes.

Specificity. The specificity of GC-20 lipase at various stages of purification is summarized in Table 2. *G. candidum* lipase favors reaction with oleate over stearate at all stages; for the crude lipase, the specificity for 4-MUMB esters (specificity O/S) is 17. For lipase A the

tris(hydroxymethyl)aminomethane hydrochloride (Tris), 5 mM CaCl₂. Fluorescence of the product was measured on a Sequoia-Turner Model 450 Fluorometer (360 nm excitation filter, 415 nm emission filter), standardized with 4-methylumbelliferone. The specificity for one substrate (A) vs another substrate (B) at one min reaction time was calculated using the Eq. [1] (22):

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RESULTS

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TABLE 1

Purification of *G. candidum* Lipase

Step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Recovered activity (%)	Purification factor
Octyl Sepharose					
Applied	1,030	81,828	79	100	1
Recovered	127	91,584	721	112	9
Chromatofocusing					
Lipase A					
Applied	11.1	8,748	790	10.78 ^a	10
Recovered	7.7	7,869	1,023	9.6	13
Lipase B					
Applied	10.9	8,335	763	10.2 ^a	10
Recovered	2.5	3,602	1,464	4.4	18

^aChromatofocusing was run on aliquots to avoid overloading the column.

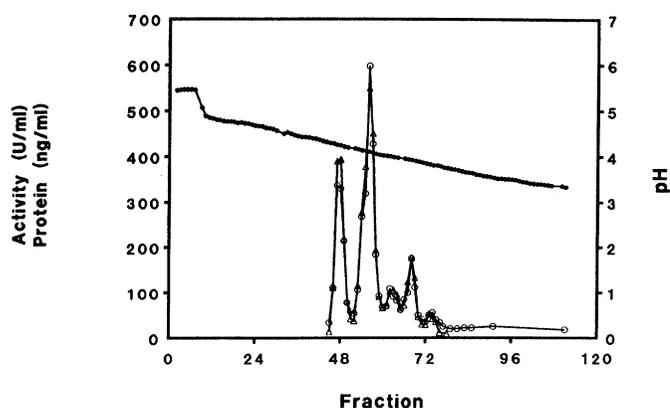


FIG. 2. Chromatofocusing of lipase A. Lipase A from the Octyl Sepharose column (21 mL of pooled fractions 165 to 197) was applied to a 0.7×50 cm polybuffer exchanger 94 column. Elution conditions are given in Materials and Methods. Shown are pH, (●); lipase activity (Δ); and protein, (○). The specificity O/P is 1 for all peaks.

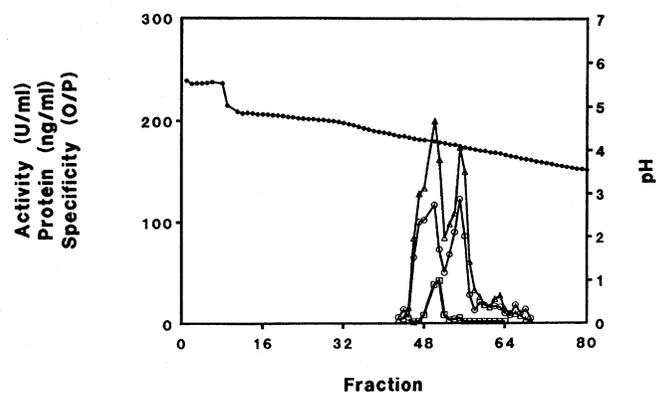


FIG. 3. Chromatofocusing of lipase B. Fractions 220 to 229 from the Octyl Sepharose column were applied to the column. Conditions as in Figure 2. Also shown is specificity O/P, (□).

specificity O/S is 15 ± 11 [averaged for 12 pure or mixed samples of isozyme(s) 1 to 4 after chromatofocusing]; for lipase B, it is 128 ± 105 (11 samples).

The specificity for 4-MUMB oleate *vs* 4-MUMB palmitate (specificity O/P) of the crude lipase (1.5 ± 0.4) is the same as that of isozymes 1 to 4 (1.4 ± 0.4); there is very little selectivity between oleate and palmitate. However, in the lipase B samples, the specificity O/P increases as the isozyme O content increases. The specificity O/P is 29 ± 8 in samples of isozyme 0 pure or mixed with a small amount of isozyme 1 (5 samples). Thus, the specificity for oleate *vs* palmitate characteristic of lipase B appears to be found only in isozyme 0.

Comparison of lipase A and lipase B. Lipases A and B were initially separated because of the presence of an additional, lower molecular weight component in SDS PAGE of lipase B (Fig. 1C). Lipase B also shows an additional, higher molecular weight component compared to lipase A on native PAGE with silver or activity stains (not shown). Lipase B has an additional, basic isozyme in IEF, and has higher specificity for

4-MUMB oleate compared to lipase A. However, amino acid analysis (Table 3) shows no significant difference between lipases A and B. Originally, it appeared that Con A-Sepharose separated lipases A and B, but small amounts of isozyme 0 (characteristic of lipase B) are indeed found in con A lipase.

The contribution of carbohydrate side chains to lipases A and B was investigated. Carbohydrate was removed from lipases A and B enzymatically by treatment with endo F. The endo F-treated lipases no longer stain with con A-peroxidase, indicating successful removal of the carbohydrate sites responsible for con A binding. Figure 5 shows native and endo F-treated lipases A and B after native IEF and incubation on a Rhodamine-olive oil plate. Activity is maintained after endo F treatment. Isozymes 2 to 4 have collapsed into isozyme 1 in both lipase A and B. Thus isozymes 2 to 4 appear to differ from isozyme 1 only by extent of glycosylation. Isozyme 0 appears to be unaffected by endo F according to IEF.

These conclusions were extended by two-dimen-

GEOTRICHUM CANDIDUM LIPASES

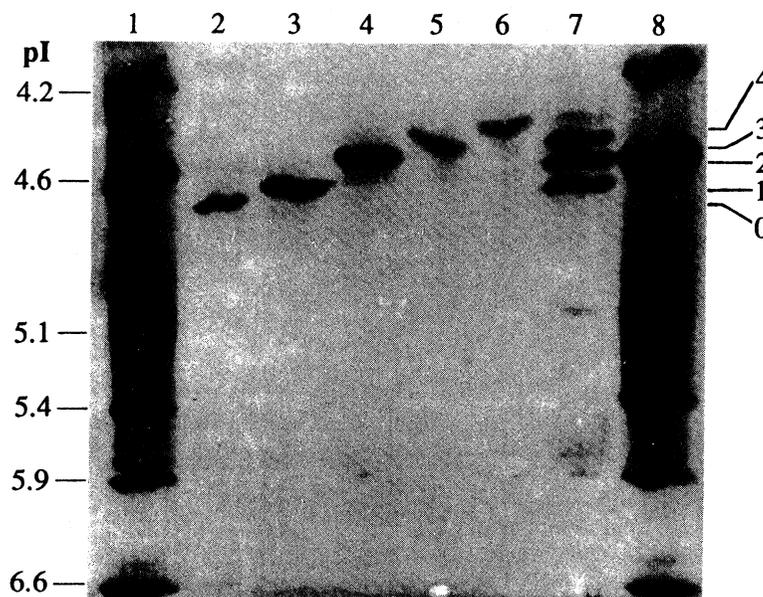


FIG. 4. IEF of lipase isozymes. The gel was run at 15°C and silver stained. Lanes 1 and 8, pI standards; lanes 2 to 6, isozymes 0 to 4, respectively; lane 7, con A lipase.

TABLE 2

Specificity of GC-20 Lipase at Various Stages of Purification

Sample	Specificity O/P	Specificity O/S
Crude lipase	1.50	17.0
Con A lipase	1.33	15.8
Lipase A	2.04	47.0
Lipase B	14.5	302
	8.68	68.8
Isozyme 0	36.7	246
Isozymes 0, 1	31.6	124
	24.7	51.6
	32.9	303
	17.4	95.8
Isozymes 0, 1, (2) ^a	14.9	47.1
Isozymes 0, 1, 2	20.2	92.6
Isozymes (0), 1	2.07	34.6
Isozymes (0), 1, 2	1.82	36.8
	3.28	64.4
	4.26	154
Isozymes (0), 1, 2, 3	3.27	25.0
Isozymes 1	1.00	7.4
	2.15	6.8
Isozymes 1, 2	1.68	12.3
Isozymes 1, 2, 3	1.04	9.0
Isozymes 2, 3	0.96	11.1
	1.55	19.2
Isozyme 3	1.41	7.6
	1.28	19.0
Isozymes 3, 4	0.82	9.1
	1.46	10.4

^aIsozymes in parentheses are minor components. Multiple entries refer to different preparations, which may differ slightly in the ratio of isozymes present.

sional electrophoresis (Fig. 6). After endo F treatment, the four chain spots of lipase A are reduced to one. The five chain spots of lipase B are reduced to two, one equivalent to lipase A and an additional, more basic

component. Endo F-treated lipase A has a main component of 60 kDa, while endo F-treated lipase B has species of 60 and 58 kDa (Fig. 7).

Characterization of lipase A. Lipase A was further studied because it appears to be a single protein varying only in degree of glycosylation, and is readily available. Lipase A contains 11.4% sugar by the phenol-sulfuric acid method. The carbohydrate was also hydrolyzed with TFMS; after this treatment, lipase A no longer stained with PAS stain or con A-peroxidase, indicating successful removal of the carbohydrate side chains. The hydrolysis results in a decrease in size to 60 kDa, similar to endo F treatment. Lipase A is compared to TFMS- and endo F-treated A in Figure 7.

Lipase A was found to be N-terminal-blocked when sequencing was attempted. Shimada *et al.* (14) also reported N-terminal blocking, and determined the N-terminal amino acid to be pyroglutamic acid. Lipase A and *G. candidum* lipase from Germe are very similar in molecular weight. The Germe lipase contains 2 isozymes apparently corresponding to isozymes 2 and 3 in lipase A (not shown).

Lipase A proved to be quite stable. After 24 hr at 30°C, 99 ± 4% of the initial activity remains in the control and in samples incubated in buffers at pH 5, 6, 7, 8 and in H₂O. After 90 hr, 86 ± 5% activity remains. At pH 4 and 9, activity is 90 ± 2% of initial at 24 hr, and after 90 hr, 58% at pH 4 and 73% at pH 9.

DISCUSSION

We have previously reported (7) significant variations among different strains of *G. candidum* lipase in the specificity for oleic *vs* palmitic acid esters of methanol or butanol. We can only speculate on the molecular bases for these differences and on their utility for the organism. At present, the mechanism of lipase catalysis and substrate binding is unknown. Information on the

TABLE 3

Amino Acid Composition (mole %) of <i>G. candidum</i> Lipase						
Amino acid	Lipase A	Lipase B	Tsuji-saka <i>et al.</i> (9)	Shimada ^a <i>et al.</i> (14)	Germe ^b	Vandamme <i>et al.</i> (11)
Lys	4.0	4.3	3.8	4.0	4.0	6.6
His	2.5	2.2	2.4	2.0	2.6	1.9
Arg	3.3	3.0	3.7	3.7	3.9	3.8
Asx	12.8	12.3	13.1	12.1	14.8	12.3
Thr	5.1	6.0	5.2	4.8	5.5	6.1
Ser	8.1	8.8	6.8	8.1	7.7	7.1
Glx	7.7	7.9	7.4	7.0	7.7	4.7
Pro	6.4	6.1	6.5	6.1	3.0	3.3
Gly	9.1	9.2	9.8	10.1	9.5	11.3
Ala	7.8	9.0	8.2	8.1	8.2	7.5
Cys	0.8 ^c	0.9 ^c	0.0	0.9	0.0	0.0
Val	5.4	5.7	6.0	5.7	5.2	9.4
Met	2.4 ^c	2.2 ^c	0.0	2.2	2.1	2.8
Ile	4.4	4.7	4.3	4.6	3.9	5.7
Leu	9.1	8.7	9.9	9.0	9.6	9.4
Tyr	4.8	3.9	4.4	4.8	4.8	4.2
Phe	5.4	4.9	6.6	5.5	5.7	2.8
Trp	0.8	ND ^d	1.8	1.3	ND	0.9

^aDeduced from the nucleotide sequence.

^bAs reported in Vandamme *et al.* (11).

^cDetermined after performic acid oxidation.

^dNot determined.

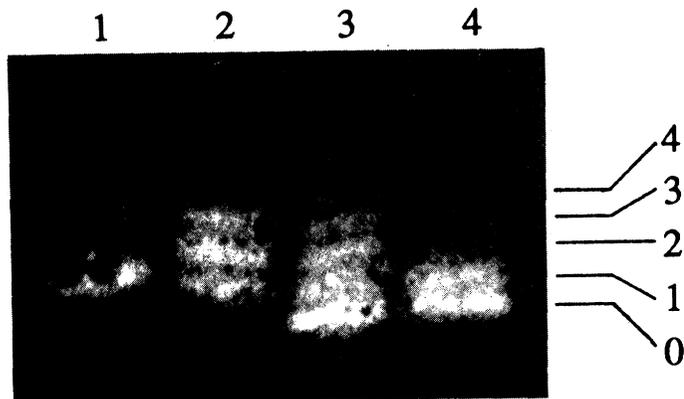


FIG. 5. IEF of native and endo F-treated lipases A and B with lipase activity stain. Lane 1, endo F-treated lipase A; lane 2, lipase A; lane 3, lipase B; and lane 4, endo F-treated lipase B.

actual differences among these strains and the isozymes present would contribute to the understanding of lipase and enzyme structure/function in general. As a means to investigate the biochemical basis for specificity differences, we undertook the purification of Amano GC-20 lipase.

Upon discovering the presence of isozymes in the crude lipase, we looked for, and found, specificity differences among the isozymes. Although the crude Amano *G. candidum* lipase does not select for oleate over palmitate, isozyme 0 (pI 4.72) does, while also retaining specificity against stearates. We will continue to study the basis for this specificity in this, and other, strains and with additional substrates.

The amino acid compositions of lipases A and B from Amano and those previously reported (9,11,14) are quite similar. The composition of the patented li-

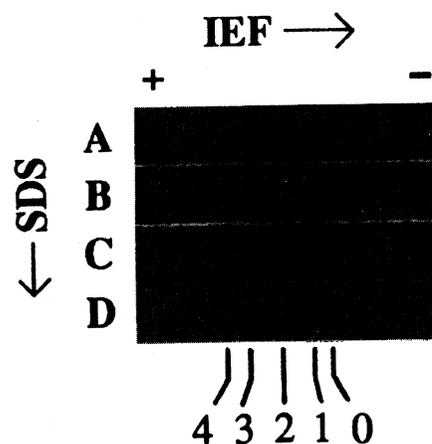


FIG. 6. Two-dimensional PAGE of (A) lipase A; (B) endo F-treated lipase A; (C) lipase B; (D) endo F-treated lipase B. The position of isozymes 0 to 4 is indicated.

pase (11) varies in Lys, Glx, Gly, Val and Phe content. Cysteic acid was found in lipases A and B after performic acid oxidation; no cysteines had been reported previously (9,11). The presence of cysteine is confirmed by the DNA sequence (14). Tsujisaka *et al.* (9) found no methionine, while greater than 2 mole percent is reported for each of the other lipases.

The lipase isozymes isolated here and the lipase from Germe are quite similar in molecular weight to those recently reported (12,14,15). Tsujisaka *et al.* (9) and Kroll *et al.* (10) report lipases of significantly lower molecular weights, as determined by gel filtration. A tendency for the lipase to be retained by gel filtration was observed; this would result in lower molecular weight determinations by this method. The molecular weight reported for the patented *G. candidum* lipase (11) is significantly lower, and suggests that either an

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