

5638 *Geotrichum candidum* NRRL Y-553 Lipase: Purification, Characterization and Fatty Acid Specificity

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Lipases from *Geotrichum candidum* NRRL Y-553 are of interest because of their unique specificity for *cis*-9-unsaturated fatty acids relative to both stearic and palmitic acids. The lipases were partially purified by chromatography on Octyl Sepharose, AG MP-1 macroporous anion exchanger, and chromatofocusing resin. The preparation was found to contain multiple, glycosylated lipases varying slightly in pI (pI 4.88, 4.78, 4.65, 4.57 and 4.52) as judged by both activity and silver staining. The molecular mass determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis was 64 kilodaltons for the main species, with minor species of 60 and 57 kilodaltons present as well. The specificity of the crude lipases for hydrolysis of 4-methylumbelliferyl esters of oleic *vs.* palmitic acid was 20-to-1. The specificity of the purified, partially separated lipases was similar to that of the crude preparation. Thus the lipases could be used even in crude form for the hydrolysis and restructuring of triacylglycerols on a large scale. *Lipids* 26, 831-836 (1991).

Lipases (EC 3.1.1.3) from *Geotrichum candidum* are known to show preference for unsaturated fatty acids with a *cis*-double bond at the 9-position (oleic, linoleic, linolenic, palmitoleic acids) (1). The extent of specificity for *cis*-9-unsaturated fatty acids in comparison to saturated fatty acids varies between strains. Among the saturated fatty acids, palmitic acid (16:0) is hydrolyzed more readily by some strains than others. Since palmitic acid and stearic acid are among the most plentiful saturated fatty acids of common fats and oils, a lipase which would preferentially react with unsaturated fatty acids in the presence of these saturated fatty acids could be of commercial use for the splitting and restructuring of natural triacylglycerols. The utility of such a process would ultimately depend on the selectivity of the enzyme employed.

We have previously reported the relative specificity constants for the competitive hydrolysis of oleic and palmitic methyl or butyl esters for several strains of *G. candidum* (2). The relative specificity constants ranged from 1.0 (no specificity shown) to 50 (highly specific). Two preparations showed high specificity: strain NRRL Y-553 and lipase 26557 RP, a proprietary enzyme preparation from Rhône-Poulenc. The original strain of *G. candidum* (3), characterized by Jensen and co-workers (4), but no longer available, released 23 ± 14 times as much oleic acid (18:1) as palmitic acid (average of four triacylglycerols contain-

ing only these two fatty acids). Of the strains available (5-8), *G. candidum* NRRL Y-553 produces one of the lipases that is most specific for oleic acid relative to palmitic acid.

Continuing our efforts to obtain *G. candidum* lipases that are highly selective against both stearic and palmitic acids, we report here the purification and further characterization of *G. candidum* NRRL Y-553 lipases. We have modified our previous purification scheme for separating *G. candidum* lipases (9) to accomplish the separation of a relatively low concentration of lipases from a complex medium.

MATERIALS AND METHODS

Lipase production. *G. candidum* NRRL Y-553 was obtained from the Agricultural Research Service Culture Collection (Peoria, IL). The culture was stored on silica gel dextrose agar (Difco Laboratories, Detroit, MI) as needed. Lipase was produced under the conditions previously optimized (2). A liquid medium was used consisting of 5% peptone (Difco), 0.1% NaNO₃, and 0.1% MgSO₄·7H₂O, adjusted to pH 7.0 before sterilization. Sterile soybean oil (0.75% v/v) was added as a carbon source. A single colony was transferred to 65 mL of medium in a 250-mL Erlenmeyer flask and incubated about 24 hr at 30°C (300 rpm). An aliquot (0.5-1.0 mL) was used to inoculate similar flasks for lipase production. Lipase activity peaked at about 40 hr after inoculation under these conditions.

The crude lipase broth was tested for protease activity using an agar diffusion method with casein as substrate (kit from Bio-Rad Laboratories, Richmond, CA); no protease activity was found even after extended incubation times.

Materials. Endoglycosidase F/N-glycosidase (Endo F), N-glycosidase F and the glycan detection kit were obtained from Boehringer Mannheim (Indianapolis, IN). Purified olive oil, 4-methylumbelliferyl (4-MUMB) esters and concanavalin A (Con A) labeled with peroxidase were obtained from Sigma Chemical Company (St. Louis, MO). Chromatography media were obtained from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ) or Bio-Rad. All other materials were reagent grade or better.

Assays. Lipase activity was quantitated titrimetrically by an initial rate assay at pH 8.2 using an olive oil-gum arabic emulsion containing 11 mM CaCl₂, as described previously (11). A unit of lipase activity (U) was defined as the release of one micromole of free fatty acid (FFA)/min at room temperature. Lipase activity of column fractions and native electrophoretic gels were qualitatively measured on Rhodamine-olive oil-agar plates (12), as previously described (9).

Protein was assayed by the bicinchoninic acid (BCA) method (13) (Pierce, Rockford, IL) when detergent was present, or by the Bradford method (14) (Bio-Rad), with bovine serum albumin (BSA) (Sigma) as standard.

The specificity of lipase for oleate *vs.* palmitate was

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Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; Con A, concanavalin A; Endo F, endoglycosidase F/N-glycosidase; FFA, free fatty acids; IEF, isoelectric focusing; 4-MUMB, 4-methylumbelliferyl; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; U, unit of lipase activity.

calculated from the initial rate of non-competitive hydrolysis of the respective esters of 4-methylumbelliferone (15), as previously described (9). To avoid loss of enzyme activity, enzyme samples were diluted as needed with cold buffer [0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris), 5 mM CaCl₂, pH 8.0] containing 0.1 mg/mL BSA.

The specificity for one substrate (A) *vs.* another substrate (B) at one minute reaction time was calculated using Eq. [1] (16):

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

where the rate, *V*, was multiplied by the substrate concentration at one min. Low values of specificity (around 1) can be measured much more accurately than large values (around 100). This method produces the same values of specificity 18:1/16:0 as obtained by non-competitive hydrolysis of emulsified methyl esters of oleic and palmitic acids (measured by initial rate titration at 35°C), but is much more rapid and sensitive.

Electrophoresis. Polyacrylamide gel electrophoresis was performed using the PhastSystem (Pharmacia) and the gels were silver stained (17) or activity stained as previously described (9,11). Standards for pI measurements were obtained from Sigma (pI 3.6–6.6) and molecular weight standards were from Diversified Biotech (Newton Centre, MA). Molecular weight measurements have a standard deviation of 2 kilodaltons (kDa).

Carbohydrate analysis. Carbohydrate was removed by treatment of native lipase with Endo F (pH 6.0) (18) or of denatured lipase with N-glycosidase F (pH 7.5) according to the instructions of the manufacturer. Two to three units of glycosidase activity were added to the lipase sample in aliquots over an extended incubation period. After IEF or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), samples were transferred to Immobilon transfer membranes (Millipore Corp., Bedford, MA) by diffusion blotting, and glycosylation was detected with the glycan detection kit according to the manufacturer's instructions, or by peroxidase-labeled Con A (18).

Purification. The procedure previously used for purifying commercial *G. candidum* lipases (9) was modified to accommodate dilute lipases in a broth of high peptone concentration. All steps were performed at room temperature (21–25°C). When lipase activity was maximal (about 14 U/mL), the broth was centrifuged for 200,000 *g* × min, decanted, and filtered under a slight vacuum through Whatman No. 2 filter paper. The supernatant (approximately 1 L) was incubated with Octyl Sepharose in an affinity rather than hydrophobic interaction mode (80 mL preequilibrated in 0.01 M imidazole buffer, pH 6.8) for 30 min with gentle agitation. The resin was then placed in a Buchner funnel with a fritted disc, and washed with the imidazole buffer to remove the colored peptone; negligible lipase activity was washed from the column by this procedure. A limited amount of water (250 mL) was used to wash the resin, which was then placed into a 1.5 × 50 cm column, and briefly packed with water (flow rate of 25 mL/hr). Lipase activity was eluted by a linear gradient of 250 mL each of water and 0.5% polyoxyethylene

10-tridecyl ether (Emulphogene BC-720, Sigma) in water.

The fractions containing the main lipase activity were pooled and applied directly to an AG MP-1 column (1.5 × 12 cm) equilibrated in 0.05 M histidine buffer (pH 6.0). After washing with the histidine buffer (flow rate of 50 mL/hr), the lipase activity was eluted by a linear gradient of 50 mL each of histidine buffer and histidine buffer with 0.4 or 0.5 M NaCl.

Chromatofocusing was used for separation of the molecular forms of lipases with varying charge as previously described (9), with a pH gradient from 5.4 to 3.8. The pH gradient was later narrowed (pH 5.2 to pH 4.1), resulting in increased resolution of the multiple lipase species.

Attempts to desalt the chromatofocusing buffer from column fractions by gel filtration on Bio-Gel P-30 were abandoned because lipase activity was retained on the column and trailed severely. Activity was also lost during dialysis; this was attributed to binding to the dialysis membrane.

RESULTS

Isoelectric focusing. Analytical IEF gels of *G. candidum* NRRL Y-553 lipases are shown in Figure 1. The lipases consisted of three major forms of pI 4.78, 4.65 and 4.57, named b, c, d; and of two minor forms of pI 4.88 and 4.52, named a and e, respectively. Additional minor species are also identifiable in more concentrated samples. All forms were identified as lipases by lipase activity stain, and were also stained with silver stain and esterase stain. Analysis by two-dimensional electrophoresis (not shown) showed charge heterogeneity among lipases of the same or very similar molecular mass. Glycosylation of the lipases was confirmed with the glycan detection kit and Con A-peroxidase.

Purification. The high concentration of peptone in the growth medium interfered with the purification procedure used previously (9). The peptone bound to the Octyl Sepharose at salt levels that promoted hydrophobic interactions (1 M ammonium sulfate), reducing the capacity of the column. The peptone also bound to the AG MP-1 anion exchanger. We therefore developed a rapid and relatively simple procedure for purification of lipases in peptone broth using Octyl Sepharose, AG MP-1, and chromatofocusing. Affinity chromatography on Octyl Sepharose using low ionic strength buffer allowed lipase to bind while the peptone was washed off. This was followed by anion exchange on AG MP-1, accomplishing a rapid concentration and at least partial removal of the detergent. Chromatofocusing was then used to separate the multiple lipase forms.

Elution of lipases from Octyl Sepharose by a detergent gradient is shown in Figure 2. A contaminant and a small amount of lipase activity are eluted at low detergent concentrations. The main lipase activity is eluted in a broad peak from about 0.25 to 0.50% detergent. Fractions 63 to 121 were pooled and applied directly to the AG MP-1 anion exchanger.

Chromatography on AG MP-1 is shown in Figure 3. The lipase activity eluted as a sharp peak (fractions 40–48), with some trailing which included contaminating proteins. Fractions 40 to 48 were pooled, dialyzed against 0.025 M piperazine (pH 5.4), applied to the chromatofocusing

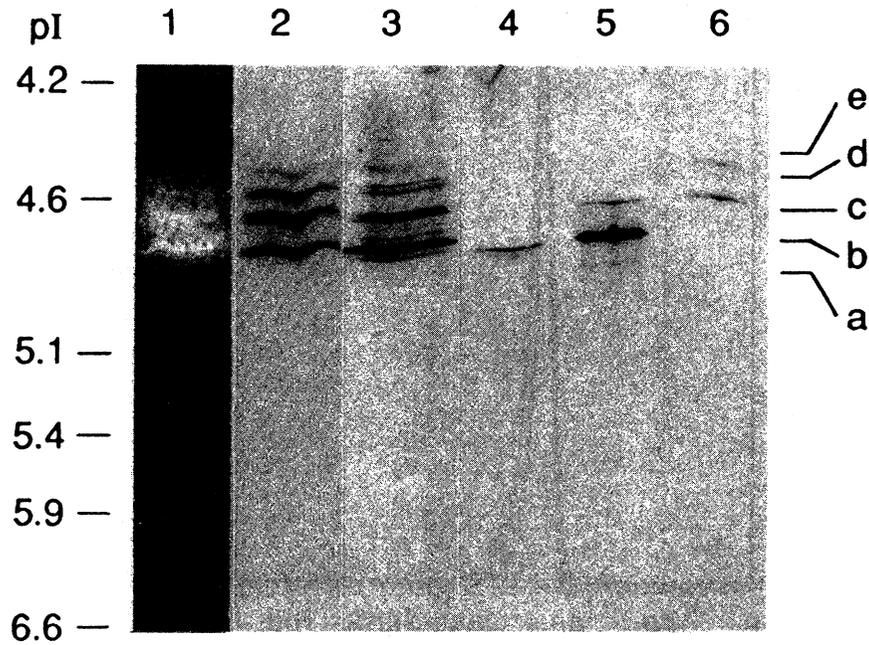


FIG. 1. Isoelectric focusing of *G. candidum* NRRL Y553 lipases at 15°C. Lipases a through e are identified. Lane 1, AG MP1 fraction 49 with lipase activity stain photographed under ultraviolet light; the scale is slightly reduced compared to lanes 2-6. Lipases b and c are of much stronger intensity. Lane 2, same sample with esterase stain; lane 3, same sample with silver stain; lane 4, chromatofocusing, fraction 44; lane 5, chromatofocusing, fraction 48; lane 6, chromatofocusing, fraction 57. Lanes 4 through 6 are silver stained.

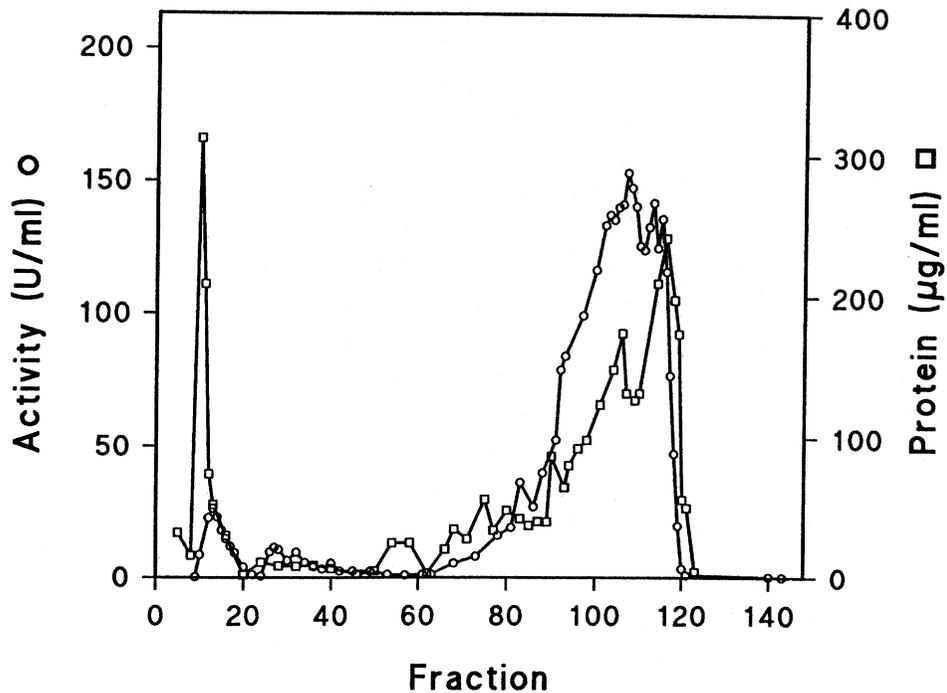


FIG. 2. Chromatography of *G. candidum* NRRL Y553 lipases on Octyl Sepharose. Lipases were eluted with a detergent gradient from 0 to 0.5% which was started at fraction 1. See text for details.

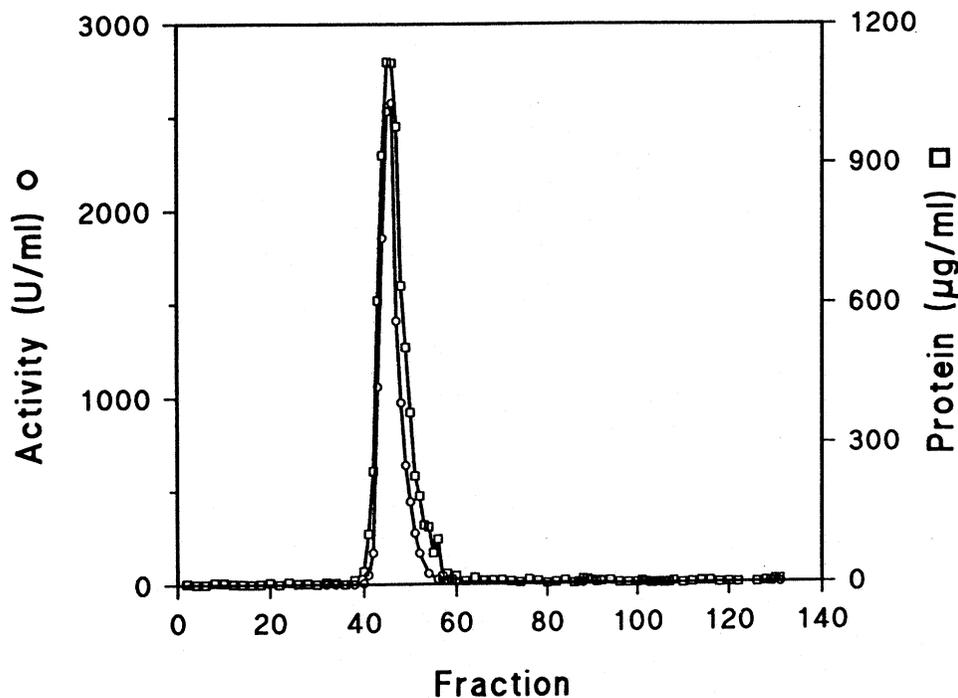


FIG. 3. Anion exchange chromatography of *G. candidum* NRRL Y-553 lipases on AG MP1. Lipases were eluted with a salt gradient from 0 to 0.5 M NaCl starting at fraction 1.

column, and eluted with Polybuffer (pH 3.8) (Fig. 4). Lipases a and b eluted first (fractions 41–45), followed by lipases b and c at the main peak (fractions 46–52). The small second peak (fractions 53–62) contained lipases c, d, e, and a minor amount of lipase b. The lipases trail together such that no single form is completely isolated from others close in pI. Elution using a narrower pH gradient increases the resolution; however, the lipases are so similar that it is not possible to isolate each one.

The purification achieved is shown in Table 1. A 14-fold purification with 96% recovery of activity was obtained with the Octyl Sepharose column. The anion exchange and chromatofocusing steps resulted in a 23-fold purification with a 46% total recovery of activity.

Figure 5 shows SDS PAGE of the lipases at each purification step. The purified lipases contain a main component of 64 kDa, and minor components of 60 and 57 kDa; glycosylation of all three components was confirmed using both the glycan detection kit and Con A-peroxidase.

Carbohydrate analysis. The various species of *G. candidum* NRRL Y-553 lipase separated by either SDS PAGE or IEF were all found to be glycosylated. In order to study the contribution of glycosylation to lipase heterogeneity, lipases purified by Octyl Sepharose and anion exchange was subjected to enzymatic deglycosylation (data not shown). Endo F was used to deglycosylate the lipases under native conditions. The susceptibility of proteins to deglycosylation by endo F varies; indeed, even after extended incubation, only partial deglycosylation of NRRL Y-553 lipases could be achieved, as measured with Con A-peroxidase detection. The partially deglycosylated lipases

contained lipases b and c, with a small amount of lipase d, as detected by IEF gels using lipase and esterase activity stain, and silver stain; however, only lipases b and c were detected with Con A-peroxidase. Two-dimensional electrophoresis of the partially deglycosylated native lipases confirmed the presence of lipases b and c, respectively, at two slightly different molecular masses at constant pI, indicating partial removal of uncharged carbohydrate. The concentration of lipase d was too low to be detected on the 2D gel.

N-Glycosidase was used to deglycosylate denatured *G. candidum* NRRL Y-553 lipases; more complete deglycosylation would be expected under these conditions. Approximately 90% of the lipases were converted to species of lower molecular mass which were no longer detected by Con A-peroxidase or the glycan detection kit, indicating removal of the carbohydrate. A decrease of molecular mass of 2.5 kDa, representing 4% by weight of the main lipase component on SDS PAGE, was obtained. It appeared that the reaction was incomplete, with about 10% of the lipase remaining with unchanged molecular mass and carbohydrate intact.

Specificity. The specificity of *G. candidum* NRRL Y-553 lipases at various stages of purification is summarized in Table 2. The specificity of lipases partially separated by chromatofocusing is given and graphed in Figure 4. The crude lipases have a specificity of 20 ± 4 . Fractions eluting from the chromatofocusing column at pH 4.4 (the beginning of lipase elution) and composed of lipases a and b consistently have a slightly higher specificity 18:1/16:0. However, the specificity values are the same within two standard deviations for all stages of purification.

GEOTRICHUM CANDIDUM NRRL Y-553 LIPASE

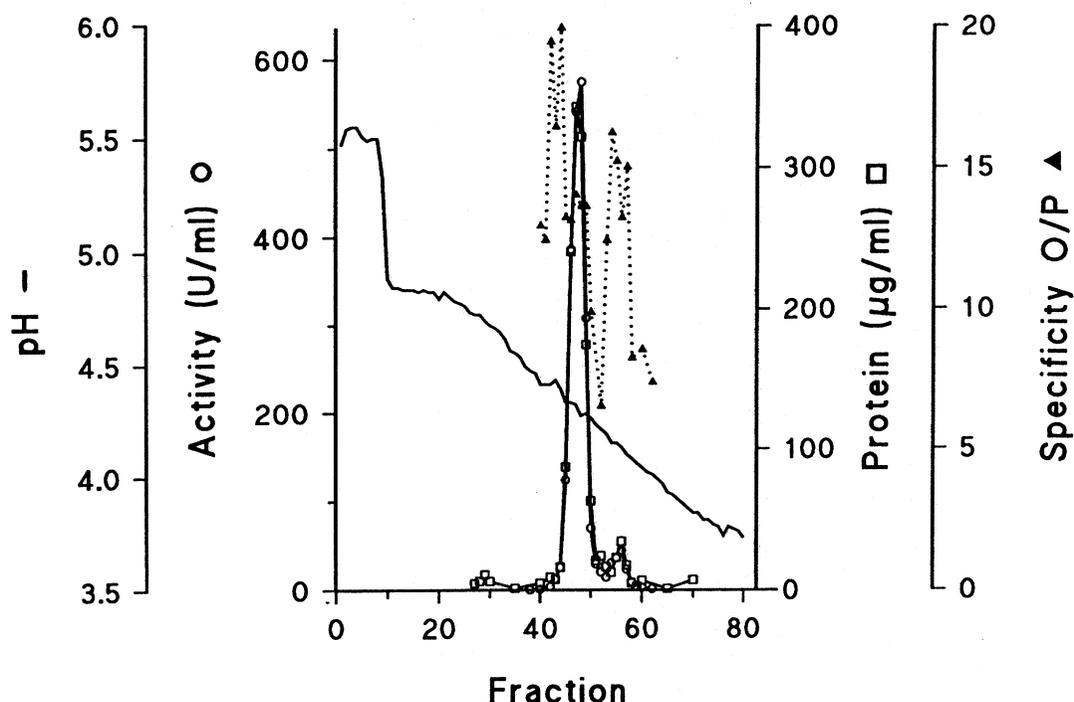


FIG. 4. Chromatofocusing of *G. candidum* NRRL Y-553 lipases. Elution conditions are given in Materials and Methods. The lipase activity closely coincides with the protein concentration.

TABLE 1

Purification of *G. candidum* NRRL Y-553 Lipase

Purification step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Recovered activity (%)	Purification factor ^a
Crude broth	194 ^b	13,400	69	100	1
Octyl Sepharose	13.1 ^c	12,834	980	96	14
AG MP-1	5.2 ^b	6,852	1318	51	19
Chromatofocusing ^d	3.9 ^b	6,133	1572	46	23

^aBased on Bradford assay results (ref. 14).

^bDetermined by Bradford assay (ref. 14).

^cDetermined by BCA assay (ref. 13).

^dSum of all lipase peaks.

DISCUSSION

The lipases produced by the fungus *G. candidum* are a complex mixture varying in molecular mass, pI and specificity. The *G. candidum* NRRL Y-553 lipases partially isolated here are quite similar in molecular weight and pI to those recently reported for three other strains—ATCC 34614 (5,19), commercial GC-20 lipases from Amano (9) and an isolate *G. candidum* Link ex Pers. emend. Carmichael from waste water (8,20). However, the specificity varies among the strains—lipases from ATCC 34614 show very little specificity 18:1/16:0 (2,5); while lipase from *G. candidum* Link ex Pers. emend. Carmichael shows intermediate specificity (8). We previously found that although crude GC-20 contains little specificity 18:1/16:0, it contains a low level of an isozyme which is specific (specificity 18:1/16:0 is 29 ± 8) (9). *G. candidum* NRRL

Y-553 produces a group of specific lipases (specificity 18:1/16:0 is 20 ± 4) whose specificity approaches that of the original strain of *G. candidum* (specificity 18:1/16:0 is 23 ± 14) (4).

G. candidum NRRL Y-553 lipases consist of five glycosylated species of molecular mass 64 kDa (major component), and 60 and 57 kDa (minor components). Heterogeneity in size due to non-specific proteolytic degradation seems unlikely, since no proteases were detected in the crude lipase broth. Differential glycosylation contributes to the heterogeneity, since partial deglycosylation reduces the molecular mass of the main lipase component to about that of the smaller species, and reduces the number of species from five to three. Additional heterogeneity could be due to actual differences in amino acid sequence; the presence of two genes coding for two closely-related lipases, each containing two potential

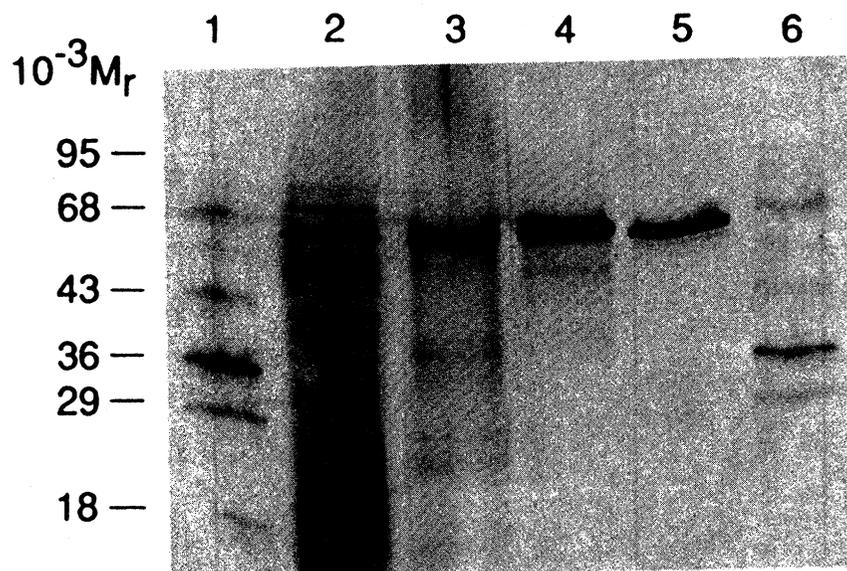


FIG. 5. SDS PAGE of *G. candidum* NRRL Y-553 lipases at various stages of purification with silver staining. Lanes 1 and 6, molecular weight standards; lane 2, crude lipase broth; lane 3, fraction 68 of Octyl Sepharose column (114 ng); lane 4, fraction 46 of AG MP1 column (140 ng); lane 5, fraction 48 of chromatofocusing column (160 ng).

TABLE 2

Specificity of *G. candidum* NRRL Y-553 Lipases at Various Stages of Purification

Sample	Specificity 18:1/16:0
Crude lipase	20.0 ± 4.4
Lipases (a), b ^a	18.6 ± 1.9
Lipases (a), b, c	13.5 ± 0.4
Lipases b, c	8.2 ± 2.4
Lipases (b), c, d	14.4 ± 1.6

^aLipases in parentheses are minor components. Purified lipases are from the chromatofocusing column.

sites for *N*-glycosylation, has recently been reported for *G. candidum* ATTC 34614 (5,21).

G. candidum NRRL Y-553 lipases could be useful for large-scale splitting and restructuring of unsaturated fatty acids from triacylglycerols, and the lipases could be used in crude form without prior purification. We are currently testing the lipases for such practical applications.

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