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Purification and Characterization of Extracellular Lipases from *Ophiostoma piliferum*

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Abstract—Interest in lipases from microorganisms, animals, and plants has greatly increased in the past decade due to their applications in biotransformations and organic syntheses. We are reporting the purification and characterization of two lipases from the fungus, *Ophiostoma piliferum*, a saprophytic organism commonly found on wood. A major and a minor lipase have been co-purified by hydrophobic interaction chromatography on octyl sepharose FF, followed by ion exchange chromatography on Q sepharose FF. The lipases bound very tightly to octyl sepharose resulting in greater than 100-fold purification in this one step. The major lipase has a molecular weight of approximately 60 kDa, a pI of 3.79, and is glycosylated as determined by PAS staining. The minor lipase, which composes 10% of the total protein, has a pI of 3.6, and molecular weight of approximately 52 kDa and did not stain with the PAS reagent. Deglycosylation of the major lipase produced two proteins of lower molecular weight, a 55 kDa protein and a 52 kDa protein. The deglycosylated protein at 52 kDa co-migrates with the minor lipase on SDS-PAGE gels. N-terminal amino acid sequencing of the major and minor lipases indicated both lipases have the same N-termini and MALDI-TOF mass spectral analysis showed similar peptide patterns. Available data indicate that the lipases are derived from the same protein and appear to differ in their post-translational modification as evidenced by their pIs and molecular weight difference. The pH rate profile and thermal stability were determined for the purified *O. piliferum* lipase and were consistent with a mesophilic lipase. In aqueous solution, the lipases exhibited a higher rate of hydrolysis for *p*-nitrophenylbutyrate (C4) than for *p*-nitrophenylstearate (C18), which is an unexpected result.

Introduction

Lipases (triacylglycerol acylhydrolase; EC 3.1.1.3) are ubiquitous in nature, catalyzing the hydrolysis of water-insoluble triacylglycerides to di- and mono-acylglycerides, free fatty acids, and glycerol. Lipases have been utilized for chiral resolutions, transesterification reactions, synthesis of structured triglycerides for nutrition, and ester synthesis.[1–7] Due to the utilitarian value of the reactions catalyzed, organic chemists have created a toolbox of lipases with different properties. The various properties include thermotolerance, reactivity and stability in organic solvents, regiospecificity, and enantioselectivity. To expand the repertoire of catalytic possibilities in their toolbox, chemists continue to search for new lipases from nature or to engineer the desired properties into available enzymes.[8–11]

In previous work, we reported that *Ophiostoma piliferum*, a saprophytic Ascomycete, is among the early colonizing wood fungi which assimilate the readily available sugars, starches, and lipids located in ray parenchyma cells and resin canals of softwoods.[12] We demonstrated that in solid wood substrate treated with *O. piliferum* the concentration of wood triglycerides decreased with a concomitant increase in free fatty acids.[13] Lipase activity was subsequently identified in liquid cultures of *O. piliferum*. Extracellular lipase activity has been reported in the sapwood-staining fungus, *O. piceae*, by Gao.[14] Here, we report the purification of two extracellular lipases isolated from *O. piliferum*. We are investigating the potential of these lipases as additional tools for biocatalysis.

Results

Fungal cultivation

O. piliferum grew as mycelia in shake flask cultures containing olive oil as the carbon source, and lipase activity was detected within 20 h in the culture medium.

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At 60 h incubation, the activity appeared to peak, and the lipases were separated from the fungal cells by centrifugation.

Purification

Lipases have been purified to homogeneity from mammals, bacteria, fungi, and plants by a variety of purification methods.[15,16] A purification from culture supernatants of *O. piliferum* is summarized in Table 1. Clarification of the supernatant was achieved by filtration through a series of membranes of decreasing pore size. Once the residual oil was removed, the crude enzyme solution was concentrated by ultrafiltration. We found that the crude supernatant, immediately after centrifugation, could be frozen and later thawed without loss of activity. In addition, freezing coalesced the oil, thereby making its removal much easier. The level of lipase produced was sufficiently low that recovery by traditional precipitation methods would have given significantly lower yields.

Hydrophobic interaction chromatography (HIC) of the crude enzyme allowed separation of the lipases from the colored components and other extracellular proteins in the spent culture broth. The lipases bound very tightly to the octyl sepharose and could not be eluted with salt-free buffers alone. Lipase elution was achieved by the addition of 0.2% Triton X-100 in piperazine buffer, and the activity was found to co-elute with the leading edge of the detergent. The octyl sepharose gave both a high recovery and greater than 100-fold purification of the lipase from the crude sample (Table 1). Chromatography on butyl sepharose gave a similar profile as the octyl sepharose with the exception that the activity appeared to slowly elute from the column with the salt-free buffer, and again detergent was required to elute the main peak of activity. Often, pH has been found to play an important role in HIC separation of proteins, and that increasing the pH weakens hydrophobic interactions.[17,18] This premise was tested by increasing the pH of the elution buffer from 5 to 7, but this change had no effect on the enzyme elution profile from either butyl or octyl sepharose. Chromatography of the lipase on the less hydrophobic phenyl sepharose, resulted in the lipases co-eluting with the colored spent medium components in the salt gradient over a large volume. Complete separation from the spent broth was not achieved with the less hydrophobic resin.

Further purification of the HIC eluate was achieved by anion exchange chromatography using Q sepharose. The pooled, concentrated lipase activity from the octyl sepharose column bound to the Q sepharose resin. Approximately 75% of the activity eluted at 0.3 M NaCl, while the remaining 25% of the activity remained tightly bound to the column (Fig. 1). The addition of 1 M NaCl failed to elute this activity, as did addition of the detergent *n*-octyl-β-D-glycopyranoside at 0.5% concentration. The residual activity could be eluted from the resin with 0.1% Triton X-100. Analysis of the two activity peaks by SDS-PAGE showed that protein of the same molecular weight eluted in both peaks.

Physical characterization

SDS-PAGE analysis of an octyl sepharose purified sample showed a major protein band at approximately 60 kDa and four minor proteins with approximate molecular weights of 54, 52, 45 and 41 kDa (Fig. 2, lane B). However, isoelectrofocusing (IEF) of the same sample showed only 3 proteins with pIs 3.88, 3.75 and 3.60 (Fig. 3, lane C). All three IEF bands were demonstrated to have hydrolyase activity upon incubation of the IEF gel in a buffered solution of α-naphthyl acetate and staining with pararosaniline diazonium salt (Fig. 3, lane A).

SDS-PAGE analysis of the Q sepharose purified lipase showed a major protein band at approximately 60 kDa and a minor band at approximately 52 kDa (Fig. 2, lane C). An IEF gel of the Q sepharose purified enzymes showed two protein bands: a major band at a pI 3.79 and a minor band at a pI 3.60 (Fig. 3, lane D). Densitometric scanning of the IEF gel showed that the major

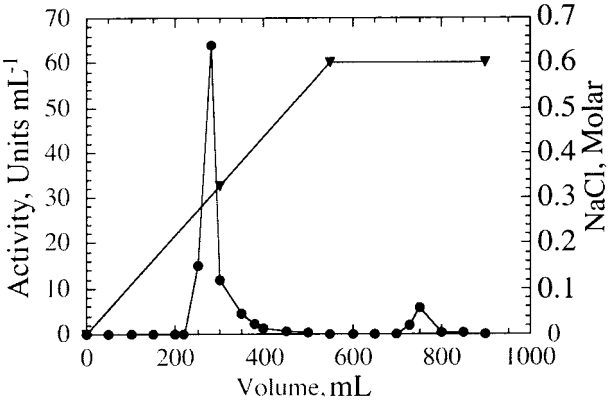

Figure 1. The extracellular lipases from *O. piliferum* were chromatographed on Q sepharose FF. Activity was measured with the *p*-nitrophenylcaproate assay, and the major peak of activity (●) eluted with 0–0.6 M NaCl (▼) gradient. A smaller peak of activity eluted when the column was washed with piperazine buffer containing 0.1% Triton X-100.

Table 1. Purification of lipase from culture supernatant of *O. piliferum*

Purification step	Total activity (units)[a]	Total protein (mg)	Specific activity (units/mg)	Cumulative yield (%)	Cumulative purification factor
Crude supernatant	6836	3400[b]	2.0	100	
Concentrated crude supernatant	4005	1400[b]	2.9	59	1.45
Octyl sepharose	3609	11.0	330	53	165
Q sepharose	2270	4.4	520	33	260

[a] Lipase activity was determined with *p*-nitrophenylcaproate.
[b] Medium components interfered with accurate protein determination resulting in an artificially high number.

band represented 90% of the total protein load and the minor band was 10% of the total detectable protein. An α-naphthyl acetate activity-stained IEF gel of the Q sepharose sample showed both bands to have lipase activity (Fig. 3, lane B). The third lipase active band at pI 3.88 was removed by Q sepharose chromatography.

The N-termini of the major and minor lipases were determined by Edman degradation and were shown to be identical (Table 2). A BLAST search indicated that the sequence did not show significant homology with any other proteins in the database.[19] The major and minor lipases, electroblotted from an IEF gel onto Immobilon-P membrane, were digested in situ with trypsin and the digestion mixture analyzed directly by MALDI-TOF mass spectrometry. Mass maps for the two proteins were nearly identical (Fig. 4).

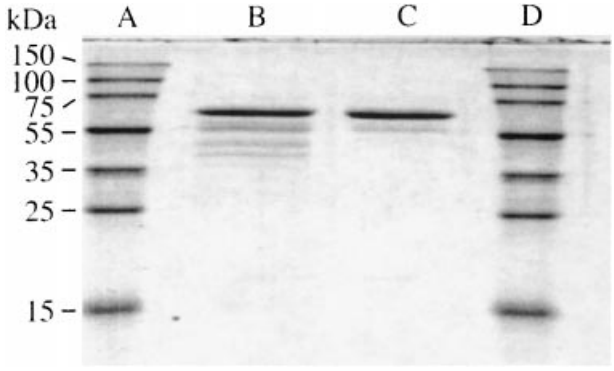

Figure 2. SDS-PAGE analysis of the column purified *O. piliferum* lipase on a Coomassie stained 12% acrylamide gel. Lanes A and D are Novagen markers; lane B is pooled lipase from the octyl sepharose; lane C is pooled lipase from the Q sepharose.

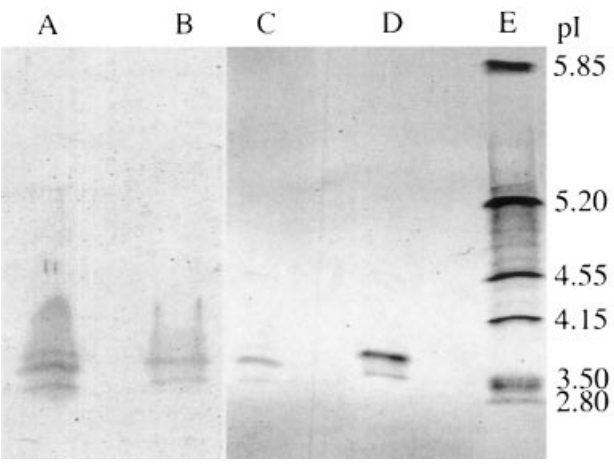

Figure 3. IEF gel analysis of the purified lipases on an ampholine PAG gel, pH gradient 3.5–9.5, electrophoresed at 5°C. The activity-stained gel, shown by lanes A and B, was incubated with α-naphthyl acetate in MES buffer, pH 6, and stained with pararosaniline diazonium salt; lane A is the octyl sepharose purified sample; lane B is the Q sepharose purified sample. The Coomassie stained gel is shown by lanes C to D: lane C is the octyl sepharose purified lipase; lane D is the Q sepharose purified lipase; lane E is Pharmacia low pI markers.

Table 2. N-terminal amino acid sequence of *O. piliferum* lipases

Mol. wt.(kDa)	N-terminal sequence found
60	TTVDVDYPEGKVTGLSALGI
52	TTVDVDYPEG

The major lipase stained positive for carbohydrate with Periodic Acid-Schiff (PAS) reagent. The minor lipase was not observed to be stained by the PAS reagent. To determine if the 60 kDa and 52 kDa proteins on the SDS-PAGE gel of the Q sepharose purified sample had different levels of glycosylation, we attempted to collapse the bands onto each other by deglycosylating them. Q sepharose purified peak fractions were used in the deglycosylation experiment (Fig. 5, lane C). After treatment with *N*-glycosidase F, two bands were observed at 55 and 52 kDa (Fig. 5, lane B). The *N*-glycosidase F deglycosylation activity was confirmed by incubation with bovine α_1-acid glycoprotein resulting in a molecular weight shift of the α_1-acid glycoprotein from 39 kDa (Fig. 5, lane G) to several bands below 35 kDa (Fig. 5, lane F).

The 55 and 52 kDa proteins from the deglycosylated major lipase were characterized by "Mass Mapping" analysis. The mass maps of the two proteins were nearly indistinguishable (Fig. 6). To determine if residual carbohydrate remained on the lipase, the deglycosylated sample was analyzed using Oxford GlycoSystem's GlycoTrack-Carbohydrate Detection kit. Results indicated that the 52 kDa protein did not contain carbohydrate, and were inconclusive on the 55 kDa protein.

Fatty acid selectivity

Selectivity for long or short chain fatty acid substrates was examined with the Q sepharose purified enzyme using *p*-nitrophenyl esters with fatty acid chain lengths from C4 to C18. Under the experimental conditions tested, the lipases hydrolyzed the C4 ester approximately 18×faster than the C16 ester (Fig. 7).

Effect of temperature and pH on lipase activity

Temperature and pH stability were measured by incubating the lipase in 100 mM buffer at designated temperatures and pHs and measuring the residual activity over 2 h with *p*-nitrophenylcaproate at pH 6.9 and 35°C. The lipase demonstrated no loss of activity over 2 h upon incubation from pH 4 to 8. An increasing loss of activity was observed as the temperature increased to 55°C (Table 3). After 30 min at 55°C, no activity was detected. Incubations of the lipase for 1 h at increasing temperatures showed the activity was stable up to 25°C, at 35°C the activity began to decline and at 55°C no activity was detectable.

Using olive oil as the substrate, lipase activity was accessed at various temperatures and pHs. The release of fatty acids from the olive oil was measured by titrimetry. Olive oil is composed of mixed triglycerides with oleic acid constituting 83.5% of the fatty acid. Maximal activity for the lipase was observed at 45°C (Fig. 8). The lipase activity dropped off rapidly above 45°C with only 27% of the activity remaining at 60°C. Lipase activity

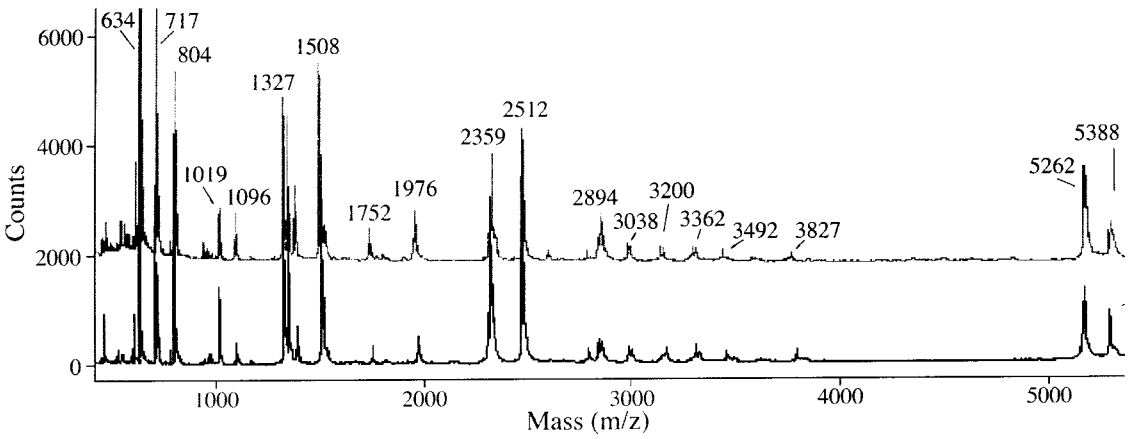

Figure 4. MALDI-TOF peptide mass fingerprinting maps of the major (in black) and minor (in light gray) *O. piliferum* lipases. The proteins, blotted onto Immobilon-P from an IEF gel, were digested in situ with trypsin and the digestion mixture analyzed directly by MALDI-TOF mass spectrometry. Mass maps for both proteins were nearly identical.

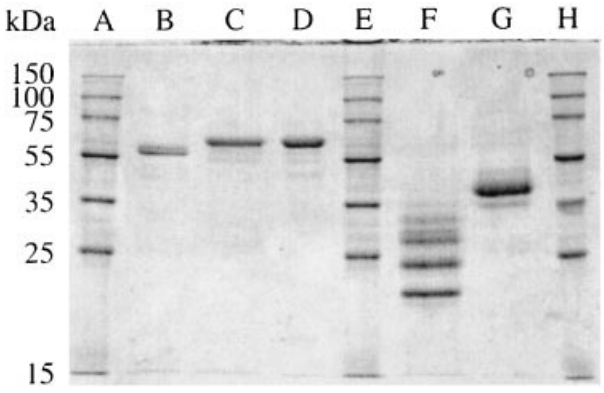

Figure 5. A 12% SDS-PAGE gel of the deglycosylated *O. piliferum* lipase shows the major lipase at 60 kDa and the two resulting deglycosylated proteins at 52 and 55 kDa: lanes A, E, and H are Novagen molecular weight markers; lane B is the deglycosylated lipase sample; lanes C and D are Q sepharose purified lipase sample. The deglycosylation activity of *N*-glycosidase F was confirmed using bovine α_1-acid glycoprotein as a positive control: lane F is the deglycosylated bovine α_1-acid glycoprotein; lane G is the untreated glycoprotein control.

was determined from a pH range of 3–8, the maximal rate of hydrolysis was observed at a pH of 6.9 (Fig. 9), activity dropped sharply at pHs above 7.

Discussion

Purification schemes from the literature utilize ion exchange, gel filtration and affinity chromatography as the more frequently used chromatographic methods.[20] Cell-free solutions of crude lipase extracts are typically concentrated by ammonium sulfate, extraction with organic solvents or ultrafiltration as a first isolation step.[15] The low concentration of lipase secreted by *O. piliferum* dictated that we employ ultrafiltration as the concentration step.

Detergent was required to elute the tightly bound *O. piliferum* lipases from octyl sepharose. Similarly, complete elution of the major and minor lipases from the anion ion exchange resin also required detergent. Triton X-100 was necessary in the elution buffer to displace the remaining 25% of the activity that could not be eluted with 1 M NaCl. The application of detergents, such as CHAPS, to break up high molecular weight lipase aggregates has been previously reported for purification of enzymes from *Pseudomonas aeruginosa*.[16] Treatment of the enzyme from *P. aeruginosa* with the zwitterionic detergent CHAPS solubilized lipopolysaccharides and separated the aggregates. After this treatment, the lipase monomer molecular weight was determined to be 29 kDa. In our experiments, the aberrant behavior of the *O. piliferum* lipase on both the anion exchange column and hydrophobic column could be explained by aggregation phenomena. These data indicate that the *O. piliferum* lipase has hydrophobic areas on the surface of the protein that associate strongly with the octyl ligands in HIC medium and the linker sequences in the anion exchange resin. Triton X-100 most likely disrupts the hydrophobic interactions that bind the protein to the column. We would therefore propose that the DEAE and Q sepharose resins gave similar chromatographic results because both resins contain hydrophobic linker domains with which the protein interacts and the binding interactions were predominately hydrophobic rather than ionic.

A number of microorganisms are known to produce lipases with differences in post-translational modifications. *Aspergillus niger, Penicillium cyclopium*, and *Candida rugosa* are a few of the organisms that produce multiple lipase forms,[15,16,21] so it was not unexpected to detect more than one form of the lipase expressed by *O. piliferum*. In addition, *Rhizomucor miehei* was reported to produce two lipases that differed in the degree of glycosylation and pI values, although the lipases were found to have the same primary structure as deduced from their DNA sequences.[22,23] For our *O. piliferum* lipases, N-terminal sequencing of the major and minor lipase showed the N-termini to be identical, and removal of the bound carbohydrate by *N*-glycosidase F collapsed the major lipase protein into two proteins of lower molecular weights at 55 and 52 kDa. The deglycosylated protein at 52 kDa appeared to co-migrate with the minor band at 52 kDa in the sample not treated with *N*-glycosidase F. Increasing the incubation time of the major lipase with additional *N*-glycosidase F to 48

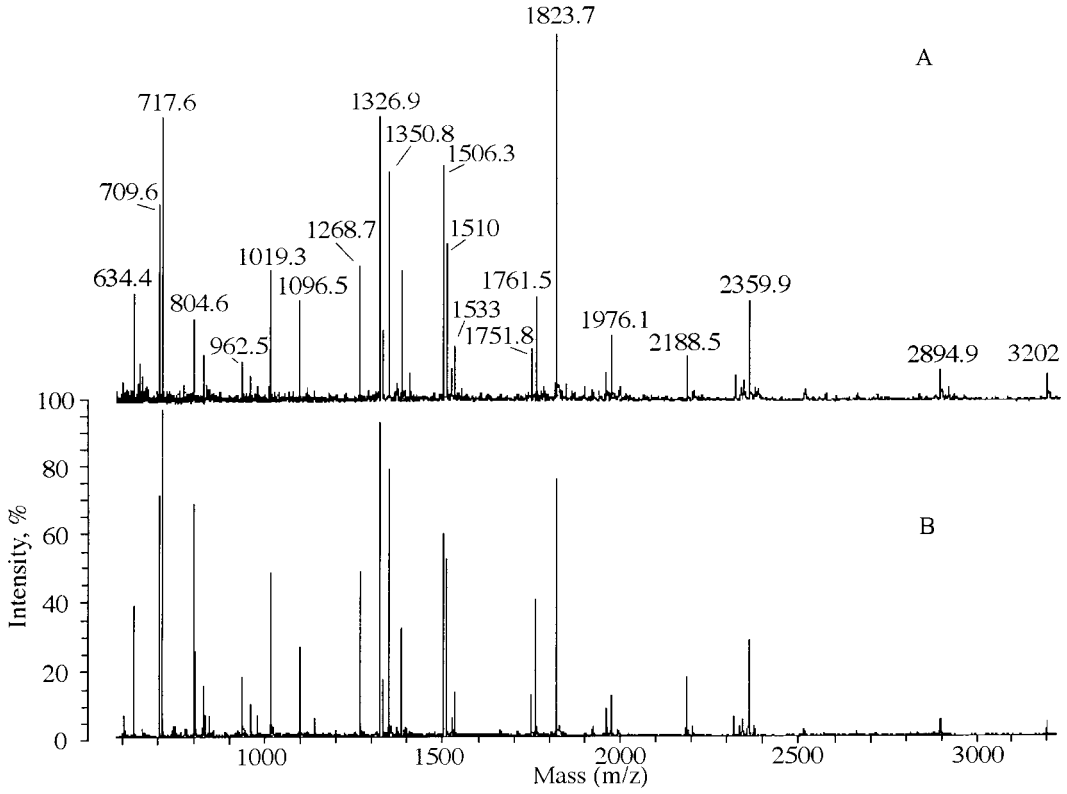

Figure 6. MALDI-TOF peptide mass fingerprinting maps of the two proteins at 52 kDa (A) and at 55 kDa (B) which arose from deglycosylation of the major lipase. The deglycosylated sample was electrophoresed on a 10% SDS-PAGE gel, electroblotted onto immobilon-P, digested in situ with trypsin and the digestion mixture analyzed by MALDI-TOF mass spectrometry. The mass maps are nearly indistinguishable for both proteins.

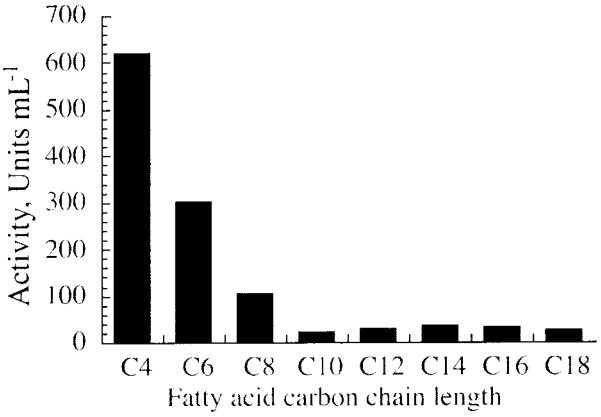

Figure 7. Fatty acid specificity profile of the *O. piliferum* lipase determined with *p*-nitrophenyl esters in aqueous solution. The lipase was incubated with 2.5 mM *p*-nitrophenyl ester, 2% Triton X-100, 50 mM buffer, pH 6.9 at 24°C, and the rate of release of *p*-nitrophenol was measured. The rate of hydrolysis of the C4 ester was 18× higher than the hydrolysis rate of the C16 ester.

hours gave the identical result suggesting that no additional carbohydrate could be removed from the protein. The major lipase was not checked for *O*-glycosylation. It is possible that the deglycosylated lipase at 55 kDa still contains some carbohydrate that was inaccessible to *N*-glycosidase F.

Based on the nearly identical spectra from the MALDI-TOF mass analysis of the major and minor *O. piliferum* lipases and the spectra of the deglycosylated proteins, the data indicate that the major and the minor lipases

Table 3. Temperature stability of the *O. piliferum* lipase: residual activity at 1 and 2 h

Temperature (°C)	Relative activity at 1 h (%)	Relative activity at 2 h (%)
5	100	100
25	100	80
35	89	74
45	61	47
55	0	0

are the same protein which differ only in their post-translational modification patterns. The mass maps of the two proteins at 55 and 52 kDa arising from deglycosylation of the major lipase suggest that the primary sequence of the proteins is the same. We would propose that the differences in molecular weights and pIs of the major and minor lipases could result from heterogeneity in the glycosylation patterns during post-translational modification of the lipase.

Under the experimental conditions tested, the lipases preferentially hydrolyzed the C4 and C6 *p*-nitrophenyl esters over the C10–C18 esters, which was a surprising result. In previous experiments with solid wood substrates, such as Southern Yellow pine, treatment of the wood with *O. piliferum* resulted in almost complete hydrolysis of the wood's triglycerides.[13] These triglycerides are composed of predominantly C16 and C18 fatty acids. Based on these results we would have expected the *O. piliferum* lipases to preferentially

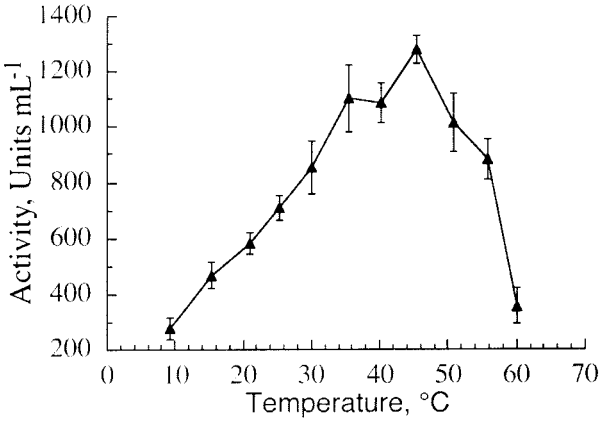

Figure 8. The effect of temperature on the reaction rate of the purified lipase was determined by incubating the enzyme at various temperatures with 20% olive oil emulsion in 50 mM acetate, pH 5.7 and measuring the concentration of fatty acid liberated in 10 min.

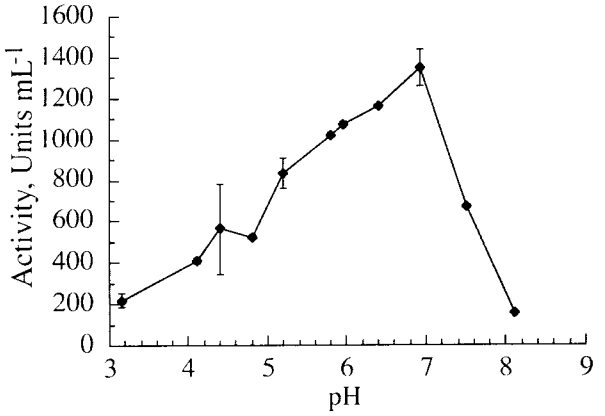

Figure 9. The effect of pH on the reaction rate of the purified lipase was determined by incubating the enzyme at various pHs with 20% olive oil emulsion at 35°C and measuring the amount of fatty acid released in 10 min.

hydrolyze the longer chain fatty acid esters over the short-chained fatty acid esters. The necessary presence of Triton X-100 in the assay may have effected the hydrolysis of the *p*-nitrophenyl esters. Triton X-100, present at 2% concentration in the reaction, was needed to solubilize the long chain fatty acid esters. Helistö et al. reported that Triton X-100 at a concentration of 0.01% in *p*-nitrophenylbutyrate assays inhibited lipases from *Candida cylindracea, Penicillium* sp. by 60–80%, and from *Aspergillus carneus* by 40%.[24] However, they found *Chromobacterium viscosum, Pseudomonas fluorescens*, and *Bacillus* sp. were activated by Triton X-100 at concentrations of 0.01–0.0125%. They concluded that the interaction between lipase, substrate, and detergent appears to be complex, inhibiting some enzymes and activating others. It is possible that Triton X-100 inhibited the *O. piliferum* lipase reaction with the longer chained fatty acid substrates or interacted with the long chained fatty acid esters and prevented their accessibility to the lipase. Furthermore, the *p*-nitrophenyl ester assay may not be a reliable method to analyze lipase substrate specificity, since activity with an ester substrate does not predict lipolytic activity with a triacylglyceride.[25] However, the use of *p*-nitrophenyl esters does provide a quick method for locating enzyme activity during purification and is more sensitive then titrimetry.

Malcata et al. reported that most microbial lipases exhibit maximum activity in the pH range from 5.6–8.5 and temperature range of 30–40°C.[26] The optimum activity at 45°C and pH 6.9 observed for the *O. piliferum* lipase is in agreement with activity profiles of other lipases isolated from mesophilic organisms. The *O. piliferum* lipase was observed to be stable from pH 4 to 8 when held at 5°C in the absence of substrate. Based on this result, the decrease in activity observed when assayed at pH 8 does not appear to be from irreversible unfolding of the enzyme structure, but from changes in K_m and V_{max} for particular substrates. These changes are due to factors such as ionization of residues in the binding or catalytic sites or substrate ionization. The lipase does exhibit inactivation in the absence of substrate as the temperature is increased from 5 to 55°C.

The lipase is currently under investigation as to its utility in biocatalysis for chiral resolution. Preliminary data suggest that the enzyme may be useful for lactone hydrolysis. Further analysis of the minor lipase will be conducted pending the results of the investigation for chiral resolution.

Experimental

Materials

Olive oil emulsion (cat. # 800-1), highly purified olive oil, bovine α_1-acid glycoprotein, and Sigma Diagnostics Kit 180 were purchased from Sigma. *N*-Glycosidase F was obtained from Boehringer Mannheim. Octyl sepharose 4 fast flow, butyl sepharose 4 fast flow, phenyl sepharose (low substitution), Q sepharose fast flow, DEAE sepharose, ampholine PAGplates (pH 3.5–9.5), and isoelectric focusing markers were purchased from Amersham Pharmacia Biotech. ProtoGel acrylamide solution, ProtoGel buffer and ProtoGel stacking buffer were from National Diagnostics. Novagen supplied molecular weight markers. Oxford GlycoSystems supplied the carbohydrate detection kit, GlycoTrak. All other chemicals were of reagent grade quality.

Cultivation of *O. piliferum*

The isolate of *O. piliferum* used in this work can be obtained from NRRL #18917. A seed culture of *O. piliferum* was grown in 25 mL of basal medium containing 2% glucose for 30 h at 25°C.[27] A 2% seed inoculum was used to inoculate 500 mL of basal medium containing 1% olive oil in 2 L flasks and cultivated at 25°C with shaking at 150 rpm for 60 h.

Enzyme purification

The culture broth was centrifuged at 7880×*g* (5°C) for 20 min to pellet the fungal cells. The centrifuge bottles

were carefully removed from the centrifuge to prevent re-mixing of the residual olive oil, and the supernatant was recovered from beneath the oily layer by siphoning with a peristaltic pump leaving behind the oil and fungal cells. The supernatant was filtered through Whatman microfiber glass GF/D (2.5 μm) filters and then through GF/F (0.7 μm) filters. Final clarification of the supernatant was achieved by filtration through Filtron's tangential flow minisette unit with an Omega open channel membrane (0.3 μm). The clarified enzyme solution was concentrated with a YM-10 membrane in an Amicon stirred cell at 5°C.

Concentrated ammonium sulfate and piperazine buffer were added to the crude enzyme solution to a final concentration of 0.7 M and 20 mM, respectively, and the pH adjusted to 5. The solution was centrifuged to remove any precipitate, and loaded onto an octyl sepharose 4 FF column (4.5×12 cm) which was pre-equilibrated with 1 M ammonium sulfate in 20 mM piperazine–HCl, pH 5 at room temperature. The column was washed with the equilibration buffer until an OD_{280} of <0.1 was achieved, and then a reverse gradient of 1–0 M ammonium sulfate was applied; column flow rate was 4 mL min^{-1}. The lipases were eluted with 0.2% Triton X-100 in 20 mM piperazine–HCl, pH 5. Active fractions were pooled, concentrated by ultrafiltration with a YM 10 Amicon membrane and the detergent removed by repeated dilution with piperazine buffer and reconcentration. The protein solution was loaded onto a pre-equilibrated Q sepharose FF column (4.5×12 cm), washed with 20 mM piperazine–HCl, pH 5, and the lipases eluted with a linear gradient of 0–0.6 M NaCl in 20 mM piperazine–HCl, pH 5 at flow rate of 4 mL min^{-1}. To elute the remaining lipase activity the column was washed with 20 mM piperazine–HCl, pH 5 containing 0.1% Triton X-100 and 0.6 M NaCl. The active fractions were pooled, concentrated, and buffer exchanged to remove the NaCl. The concentrated enzyme was frozen at −20°C. The lipases were chromatographed at room temperature, but were otherwise maintained at 5°C, to prevent potential proteolysis.

Protein assay

Protein concentration was determined with bicinchoninic acid (BCA) protein assay reagent kit (Pierce) with bovine serum albumin as the standard.

Enzyme assays

One Unit of activity is defined as that amount of enzyme that can release 1 μmol of product per minute.

Spectrophotometric assay. A modification of the spectrophotometric assay according to Chang et al. was followed during the purification steps.[21] The enzyme was incubated at 24 ± 1°C for 10 min in an assay mixture containing 50 mM PIPES, pH 6.9, 2% Triton X-100, and 2.5 mM *p*-nitrophenylcaproate in final volume of 1 mL. To stop the reaction 250 μL of 0.1 M sodium carbonate with 5% Triton X-100 was added, and the ΔOD_{410} was read immediately against a blank reaction without enzyme.

Determination of substrate specificity. Substrate specificity assays were conducted with *p*-nitrophenyl esters with fatty acid chain lengths from C4 to C18. The substrate solution was prepared by dissolving 2.64 mM *p*-nitrophenyl ester in 190 mL of 52.6 mM PIPES, pH 6.9 containing 2.1% Triton X-100 at 60°C. The reaction was initiated by addition of 50 μL of enzyme into 950 μL of the substrate solution, and carried out as described above. The final concentration of reactants was 2.51 mM *p*-nitrophenyl ester, 50 mM PIPES and 2% Triton X-100.

Effect of temperature and pH on lipase activity. The effect of temperature and pH on the lipase reaction rate was measured by pH-stat assay with a Radiometer TitraLab 11. Purified lipase was added to Sigma olive oil emulsion and buffer, which had been pre-incubated at the designated temperatures, stirred briefly, and incubated for 10 min in a final volume of 5 mL. The reaction was stopped with 3 mL of ethanol and titrated with 0.05 N NaOH volumetric solution (J. T. Baker Cat. # 5664-02). A blank reaction was measured for each set of data points under identical conditions as the enzyme reaction, except the enzyme was added after the stop reagent. The activity was corrected for any background acid, which was present at the start of the reaction. For the pH study, the reaction was incubated for 10 min at 35°C in the presence of 50 mM buffers: acetate for pH 3.5, 4, 4.5, 5, 5.5; MES for pH 5, 6, 6.5; HEPES for pH 7, 7.5, 8; Tris for pH 8. At pH 3, the olive oil emulsion was titrated down to 3 with 0.2 M HCl, and no buffer was used for this pH point. For the temperature study the pH was maintained at 5.7 with 50 mM acetate, and the reaction incubated at the indicated temperatures. All assays were carried out in triplicate for both the spectrophotometric assay and the titrametric assay.

The pH and temperature stability of the purified lipase was measured with the spectrophotometric assay with final concentration of 2.5 mM *p*-nitrophenylcaproate, 50 mM buffer, pH 6.9, 2% Triton X-100 in a 1 mL volume. The reaction was incubated for 10 min at 35°C and stopped as described above with 0.1 M sodium carbonate. For the pH stability the purified lipase was held at pH 4, 5, 6, 7, and 8 in 100 mM of the above buffers for 2 h at 5°C; aliquots were removed and lipase activity was measured. For the temperature stability study, the purified lipase was held at 5, 25, 35, 45, and 55°C for 2 h in 100 mM MES, pH 6 and aliquots were removed and the residual lipase activity measured.

Gel electrophoresis

SDS-PAGE acrylamide gels were prepared according to the protocol from Laemmli.[28] Isoelectric focusing (pH 3.5–9.5) was performed with Pharmacia Ampholine PAGplate at 5°C, according to the manufacturer's recommendations (Instruction sheet 18-1016-67). Gels were stained with PhastGel Blue R (Pharmacia) according to manufacturer's recommendations. Detection of

hydrolytic activity was performed with α-naphthyl acetate using a protocol adapted from the Sigma Diagnostic kit # 180-B. After electrophoresing the protein on an IEF gel, the gel was incubated in 50 mL of 200 mM MES buffer, pH 6 with α-naphthyl acetate and pararosaniline diazonium salt at 37°C. When dark bands appeared, the gel was removed from the reaction solution, soaked in water for 5–10 min to remove excess reagent, then fixed and simultaneously destained to remove background color in 3% acetic acid.

Determination of molecular weight, pI, and N-terminal sequence

The molecular weight of the proteins was determined by densitometry using Molecular Dynamics™ FragmeNT™ Analysis Software, Version 1.1a. To determine the N-terminal sequence the purified lipase was electrophoresed on either a 10% SDS-PAGE gel, or an IEF gel, and electroblotted with a semi-dry electroblotter (integrated separation systems) onto immobilon-P transfer membrane (Millipore). Blotted proteins were sequenced by automated Edman degradation with an ABI Model 492A Procise protein sequencer (Perkin–Elmer) by Dr. John Leszyk at the Worcester Foundation for Biomedical Research, Shrewbury, MA. Peptide mass fingerprinting of the major and minor lipases was performed by matrix-assisted-laser-desorption-ionization time-of-flight mass spectrometry (MALDI-TOF) by Dr. John Leszyk using the Linear Voyager spectrophotometer by Perseptive Biosystems. The proteins, blotted onto immobilon-P, were digested in situ with trypsin and the digestion mixture analyzed directly by MALDI-TOF mass spectrometry.

Detection of glycoproteins and deglycoslyation

The purified lipase was electrophoresed on 12% acrylamide gels and stained for glycoproteins with the Periodic Acid-Schiff stain protocol according to Gerard.[29] For the deglycosylation experiment, the purified lipase was digested with *N*-glycosidase F according to the established methods and electrophoresed on 12% acrylamide gels.[30] Carbohydrate detection was carried out according to the protocol in the GlycoTrack — carbohydrate detection kit by Oxford GlycoSystem.

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