Isolation, Purification, and Characterization of a Cold-Active Lipase from *Aspergillus nidulans*

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Aspergillus nidulans WG312 strain secreted lipase activity when cultured in liquid media with olive oil as carbon source. Highest lipase productivity was found when the mycelium was grown at 30 °C in a rich medium. The new enzyme was purified to homogeneity from the extracellular culture of *A. nidulans* by phenyl-Sepharose chromatography and affinity binding on linolenic acid−agarose. The lipase was monomeric with an apparent M_r of 29 kDa and a p*I* of 4.85 and showed no glycosylation. Kinetic of enzyme activity versus substrate concentration showed a typical lipase behavior, with K_M and K_{cat} values of 0.28 mM and 494 s^{-1} and 0.30 mM and 320 s^{-1} for the isotropic solution and for the turbid emulsion, respectively. All glycerides assayed were hydrolyzed efficiently by the enzyme, but this showed preference toward esters of short- and middle-chain fatty acids. The optimum temperature and pH for the lipolytic activity were 40 °C and 6.5, with high activity in the range 0−20 °C and reduced thermal stability.

Keywords: *Lipase; Aspergillus nidulans; triacylglycerol hydrolases; enzyme purification; cold-active lipase; cold-adapted enzymes*

INTRODUCTION

Lipases (triacylglycerol hydrolases EC 3.1.1.3) form a large group of enzymes that catalyze the hydrolysis of a wide range of carboxyl esters. From a kinetic point of view, lipases are characterized by the interfacial activation phenomenon, which differentiates them from esterase or cutinase (Ferrato et al., 1997; Miroslaw and Schrag, 1997).

Although lipases have so far found many industrial uses (detergents, oils and fats, dairy, pharmaceutical, and textile industries) and can be produced on a large scale by growth of different microorganisms (Björkling et al., 1991; Godtfredsen, 1993; McKay, 1993; Birschbach, 1994), there is still a great interest in new enzymes with commercially useful properties. Lipases with high activity at low temperature are particularly attractive for some industrial applications, such as in detergent formulations, fine chemistry catalysis, or food processing, for which substrate or product stability and energy savings are critical (Gerday et al., 1997; Marshall, 1997).

Most microbial lipases exhibit high activity between 30 and 50 °C, with a maximum around 45 °C (Birschbach, 1994). At lower temperatures, their catalytic efficiency is severely reduced and the reaction rate falls to zero at ∼0 °C. Cold-adapted enzymes, produced by psychrophilic organisms, show, however, high specific activity in the range 0−20 °C, with optimal activity at 30−40 °C, and reduced thermal stability compared with their temperate counterparts (Feller et al., 1994; Marshall, 1997; Gerday et al., 1997; Feller and Gerday, 1997). These enzymes are therefore obvious candidates for low-temperature processes. Nevertheless, the lack of industrial knowledge about psychrophilic organisms sometimes makes the biotechnological application of cold-adapted enzymes difficult (Marshall, 1997).

Fungi are an important source of enzymes for the industry. Among them, members of the genus *Aspergillus* have been widely used for biotechnological processes. In this work we present the isolation and characterization of a novel lipase from the fungus *A. nidulans*. This enzyme acts preferentially over micelles and short fatty acid chains and presents high catalytic activity over a temperature range of roughly 0−40 °C and a marked heat lability. These enzymatic properties, similar to those displayed by cold-adapted enzymes, make this enzyme of great biotechnological potential. The influence of nutritional factors and culture conditions on the enzyme production has been also studied.

MATERIALS AND METHODS

Microorganism and Culture Media. *A. nidulans* WG312 strain (*pyrG89, pabaA1*) was maintained on complete agar medium slants (Pontecorvo et al., 1953). Spores were inoculated (10^5 spores/mL) from a conidial suspension in Tween 80 (1:10000). Growth was performed on *Aspergillus* minimal medium (Pontecorvo et al., 1953) or rich medium (1% peptone as nitrogen source). Olive oil or glucose was used as carbon source at the indicated concentrations. In some experiments, yeast extract, gum arabic, $CaCl_2$, KH_2PO_4, starch, Tween 20, Nonidet P-40, sodium deoxycholate, or Triton X-100 (0.1% in all cases) was added to the minimal medium. Growth experiments were carried out in 500 mL Erlenmeyer flasks with 100 mL of medium at 30 or 37 °C and 200 rpm.

Enzyme Purification. *A. nidulans* was cultivated in rich medium with 2% olive oil as carbon source. After 48 h of growth, the culture broth was filtered through nylon gauze to remove mycelia. Solid ammonium sulfate was added to the filtrate (700 mL) to reach a final concentration of 1 M, and the pH was adjusted to 7. The solution was applied onto a 70 mL bed volume column of phenyl-Sepharose (Pharmacia

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Biotech) equilibrated with 50 mM phosphate buffer (pH 7). The column was washed with 70 mL of 1 M ammonium sulfate in the equilibration buffer and then with 50 mM Tris-HCl buffer (pH 7.5) until the absorbance of the effluent at 280 nm was zero. Finally, lipase was eluted with 5 mM Tris-HCl buffer (pH 7.5) containing 20% 2-propanol. The active fractions were pooled, lyophilized, and dissolved in 17.5 mL of water. The concentrated lipase solution was put onto a 2 mL bed volume column of agarose−linolenic acid (Sigma Chemical Co.) previously equilibrated at 4 °C with 25 mM Tris-HCl buffer (pH 8). The matrix was washed with 3 bed volumes of the equilibration buffer, and lipase was eluted with the same buffer containing 6 M urea. The active fractions were pooled, dialyzed against distilled water, freeze-dried, and resuspended in water. A final volume of 11.9 mL of purified lipase solution was obtained and used for later studies.

Lipase Activity Assays. Regular lipase activity assay was performed using *p*-nitrophenyl palmitate (pNPP) as described by Winkler and Stuckmann (1979), with some modifications. In brief, the reaction mixture consisted of 405 μL of buffer (200 mg of Triton X-100, 50 mg of gum arabic in 50 mL of 50 mM phosphate buffer, pH 6.5) and 45 μL of substrate (15 mg of pNPP in 10 mL of 2-propanol), emulsified for 5 min in an ultrasonic bath. The mixture was prewarmed at 40 °C, and then 50 μL of enzyme solution was added. After 5 min of incubation, the reaction was stopped by the addition of 0.5 mL of 2% Trizma base. The solution was centrifuged at 14000 rpm for 3 min, and the optical density of the clear supernatant was measured at 410 nm. One unit (U) of lipase activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol/min under the above conditions. The activity of lipase against other pNP derivatives, pNP-valerate, pNP-caprylate, pNP-laurate, and pNP-stearate, was determined according to the standard procedure described above.

Lipase activity was also determined by measuring the fatty acids released by the hydrolysis of mono-, di-, or tripalmitoylglycerol (40 mM in Cl_3CH/CH_3OH; 2:1, v/v) in a biphase system composed of 250 μL of substrate and 250 μL of enzyme solution (0.9 mU/μL, determined with pNPP) in 50 mM phosphate buffer (pH 6.5). After 2 h of reaction at 40 °C, 200 μL of the organic phase was withdrawn and evaporated, and the residue was resuspended in 200 μL of a solution containing 6% ethanol and 6% Triton X-100. After centrifugation, the free fatty acids were determined in the supernatant as described by Shimizu et al. (1979).

Kinetic Determinations. Kinetic constants of *A. nidulans* lipase were determined by direct regression of the Michaelis−Menten hyperbola obtained experimentally. The assays were carried out according to the standard procedure described above using a pure lipase sample (0.25 μg of protein/mL) and pNPP in a range of concentration from 0.02 to 0.71 mM. Under these reaction conditions the enzyme activity was linear within 13 min.

For the inhibition studies, enzyme−inhibitor mixtures consisting of 40 μL of 50 mM phosphate buffer (pH 6.5), 5 μL of inhibitor, and 5 μL of pure lipase (2.5 mg/mL) were preincubated at 30 °C for 15 min. Then 450 μL of a prewarmed (40 °C) reaction mixture prepared with 45 μL of pNPP solution, 45 μL of inhibitor, and 360 μL of reaction buffer was added to each tube, and the reaction was performed at 40 °C for 5 min.

Effects of Temperature and pH on Enzyme Activity and Stability. The relative activity of *A. nidulans* lipase at several temperatures (0−80 °C) was determined by using the standard procedure described above. Thermal stability experiments were conducted from 20 to 70 °C. The enzyme samples were incubated in 50 mM phosphate buffer (pH 6.5) at the chosen temperature for 30 min and cooled on ice, and the residual activity was measured as before.

To determine the optimum pH, 0.05 M solutions consisting of phthalate (pH 3−6), phosphate (pH 6.5−7.5), and Tris-HCl (pH 8) buffers were used in the standard assay. To test pH stability, 5 μL of the enzyme was incubated at 30 °C for 1 h with 45 μL of the following buffers: phthalate (pH 2−6), phosphate (pH 6−7), and Tris-HCl (pH 7−9). Samples were then diluted (final dilution = 1/10) in 50 mM phosphate buffer (pH 6.5), and the remaining activity was assayed as before.

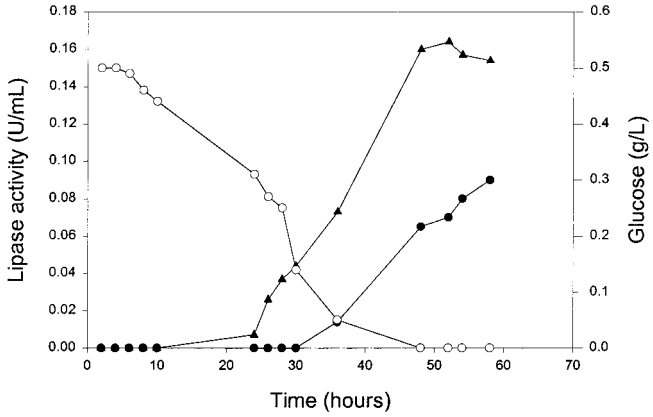

Figure 1. Lipase biosynthesis by *A. nidulans* in minimal media containing 2% olive oil (▲) or 2% olive oil plus 0.5% glucose (●). Residual glucose (○) and lipase activity were determined as described under Materials and Methods.

Biochemical Assays. Residual glucose in the culture medium was assayed as described by Entian and Fröhlich (1984).

Total protein was determined according to the method of Lowry et al. (1951), using rabbit IgG as standard.

Electrophoretic Analyses. Native PAGE and SDS−PAGE were performed on 6 and 13% polyacrylamide gels, using the buffer systems described by Davis (1964) and Laemmli (1970), respectively. Zymogram analysis was done by running the protein samples on a PAGE gel containing 1% olive oil (Yourno and Mastropaolo, 1981). The gel was then incubated overnight at 37 °C in 50 mM phosphate buffer (pH 6.5) and treated with an aqueous solution of 1% Rhodamine B to improve the visualization of the bands.

Isoelectric focusing was performed with a Phast-System unit (Pharmacia). Gels with a pH range from 3.0 to 9.0 were used.

Glycoprotein Staining. Proteins were run on SDS−PAGE, electroblotted to nitrocellulose paper, and stained by using the concanavalin A−peroxidase method (Hawkes, 1982; Millette and Scott, 1984). Glycoprotein analysis was also carried out by dot blot assay using digoxigenin-labeled lectins (PNA, DSA, SNA, GNA, and MAA) according to the Boerhinger GmbH (Mannheim, Germany) digoxigenin glycan differentiation kit instructions (catalog no. 1210 238).

RESULTS AND DISCUSSION

Lipase Biosynthesis by *A. nidulans*. Influence of the Medium Composition and Culture Conditions. *A. nidulans* WG312 strain secreted lipase activity when cultured in liquid media with olive oil as carbon source. Enzyme activity was detected throughout the micellar growth, with maximal values around 48 h of culture (Figure 1). Lipase activity was not detected in media with glucose as sole carbon source, whereas in a medium containing both glucose (0.5%) and olive oil (2%), the enzyme activity started to be detected later (Figure 1). Therefore, lipase biosynthesis by *A. nidulans* appears to be repressed by glucose, and it requires oil as inducer. Similar results has been previously reported for lipase of different origin (Baillargeon et al., 1989; Del Río et al., 1990; Ohnishi et al., 1994a; Pokorny et al., 1994).

Lipase production by *A. nidulans* was influenced by nutritional factors and growth conditions. As can be expected, the level of extracellular lipase activity was larger in rich than in minimal media (Table 1), whereas the oil concentration (1−3%) hardly varied the lipase productivity (data not shown). In agreement with previ-

Table 1. Influence of the Nitrogen Source and Growth Temperature on the Level of Lipase Activity during the Culture of *A. nidulans* on Olive Oil

	lipase activity[a] (mU/mL)			
	minimal medium		rich medium	
time (h)	30 °C	37 °C	30 °C	37 °C
23	nd	36	38	245
44	34	146	1011	283
50	29	112	1077	199
62	16	97	1121	79

[a] Enzyme activity at the indicated times was determined as described under Materials and Methods.

Table 2. Effect of the Addition of Different Compounds in the Culture Media on the Production of Lipase by *A. nidulans*

	lipase activity[b] (mU/mL)		
culture media[a]	28 h	40 h	48 h
MM−control	33	102	99
MM−yeast extract	147	337	154
MM−arabic gum	49	87	96
MM−starch	95	35	30
MM−$CaCl_2$	3	21	6
MM−KH_2PO_4	82	24	19
MM−Nonidet P-40	57	93	115
MM−Triton X-100	30	91	117
MM−Tween 20	100	157	192
MM−sodium deoxycholate	5	5	33

[a] Micelia were grown in minimal media supplemented with 2% olive oil as carbon source and 0.1% of the indicated compound. [b] Lipase activity was determined as described under Materials and Methods.

ous reports for the lipase production by *A. oryzae* (Ohnishi et al., 1994a), a higher lipase productivity was obtained when the culture temperature in rich medium was lower than the optimum for growth (Table 1). Thermal lability of the exoenzyme and/or alterations in the secretory pathway could account for this effect as it has been described for enzymes of different organisms (Feller et al., 1994).

The effect of the addition of different compounds in the culture media on the production of lipase by *A. nidulans* was also tested (Table 2). Only the presence of yeast extract or Tween 20 increased the level of activity observed, whereas the addition of other compounds had a detrimental effect (sodium deoxycholate or Ca^{2+} ions) or no effect (Table 2). In this respect, it should be pointed out that a functional role of the Ca^{2+} ions as activators or inhibitors of lipase activity has been reported previously (Ohnishi et al., 1994b; Paquot et al., 1996).

Isolation and Purification of Lipase from *A. nidulans*. Lipase activity from 0.7 L of *A. nidulans* WG312 culture filtrate was bound to phenyl-Sepharose and eluted with 20% 2-propanol. Approximately 36% of the initial lipase activity was recovered, giving a purification factor of 170-fold (Table 3). Binding of hydrophobic interaction lipase activity on agarose−linolenic acid and later elution with 6 M urea in 25 mM Tris-HCl buffer (pH 8) resulted in a pure fraction of enzyme

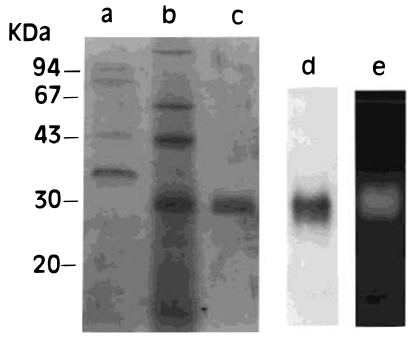

Figure 2. Electrophoretic analysis of protein samples at different steps of purification of *A. nidulans* lipase. Culture filtrate (lane a), hydrophobic interaction chromatography (lane b) and affinity chromatography (lane c) protein samples were run on SDS−PAGE and stained with Coomassie blue. PAGE (d) and Zymogram analyses on olive oil (e) of affinity chromatography protein samples (0.46 μg) were carried out as described under Materials and Methods.

(Figure 2). The procedure gave a final purification of 1186-fold, with a recovery of ~10% of the initial activity (Table 3).

Biochemical Characterization. Figure 2 shows the SDS−PAGE profile at different stages (lanes a−c) of purification of *A. nidulans* lipase. The purified lipase fraction was homogeneous and contained a single Coomassie-stained band (lane c) with an apparent molecular size of 29 kDa. This molecular mass was confirmed by gel filtration (Sephadex G-100), indicating that the enzyme is a monomer (data not shown). A single band was also detected by PAGE (lane d) and by Zymogram analysis on olive oil (lane e).

Staining of nitrocellulose blots containing purified lipase with concanavalin A revealed that the enzyme did not present noticeable glycosylation (data not shown). Similar results were obtained using a set of different lectins (SNA, MAA, PNA, GNA, and DSA; see Materials and Methods).

Isoelectric focusing analysis of the purified lipase revealed one band that moved to the cathode with apparent isoelectric point of 4.85 (data not shown).

Similar biochemical characteristics have been previously reported for lipases from other filamentous fungi (Sztajer et al., 1992; Hoshino et al., 1992; Destain et al., 1997; Toida et al., 1998; Ibrik et al., 1998).

Enzymatic Characterization. Lipases show a different kinetic behaviors depending on the substrate concentration, which determines their physical−chemical state (Ferrato et al., 1997; Miroslaw and Schrag, 1997). Below the critical micellar concentration (cmc) the substrate forms an isotropic solution, whereas above the cmc exists as a turbid emulsion. Therefore, we assayed the kinetics of the *A. nidulans* lipase using pNPP as substrate in a wide range of concentrations. As seen in Figure 3, the enzyme showed a typical lipase behavior, with two Michaelis−Menten hyperbola. The K_M and K_{cat} values at 40 °C and pH 6.5 were 0.28 mM and 494 s^{-1} and 0.30 mM and 320 s^{-1} for the isotropic

Table 3. Purification of the Lipase from *A. nidulans* WG312

step	vol (mL)	total protein (mg)	total lipase (U)	specific activity (U/mg)	yield (%)	purification factor (fold)
culture filtrate	700	2765	952	0.3		
phenyl-Sepharose	17.5	6.7	342.1	50.9	36	170
linolenic acid−agarose	11.9	0.3	97.5	355.7	10	1186

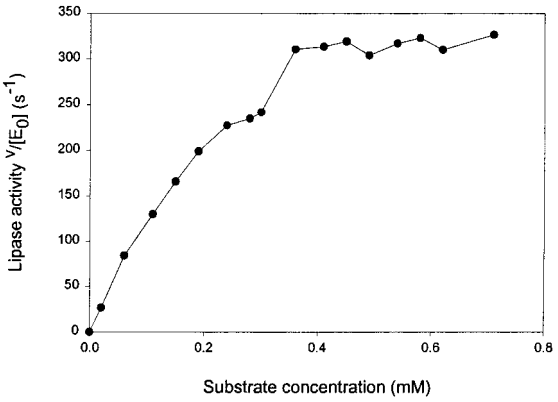

Figure 3. Kinetics of *A. nidulans* lipase activity versus *p*-nitrophenylpalmitate concentration.

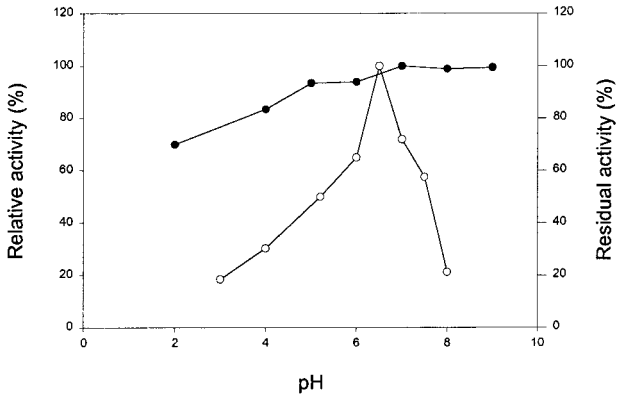

Figure 4. Optimum pH and stability curves of lipase. For each pH, activity was assayed at 40 °C as described under Materials and Methods and expressed as relative activity (○). The pH stability curve (●) represents the residual activity after a preincubation period of 30 min at the indicated pH.

solution and for the turbid emulsion, respectively. Hence, the enzyme produced by *A. nidulans* is a true lipase.

The activity of the *A. nidulans* lipase showed a high dependence with the pH (Figure 4). Optimum pH was found to be 6.5; at pH 6 or 7 the activity decreased ~30−35%. The pH stability profile revealed that the activity was especially conserved at alkaline pH values over a 1-h period.

The apparent optimal activity temperature of the enzyme was observed at 40 °C, when assayed at pH 6.5, although the enzyme showed the ability to keep high reactivity at lower temperatures (Figure 5). Even at 0 °C, the *A. nidulans* lipase conserved almost 30% of relative activity. Furthermore, the lipase from *A. nidulans* showed also a pronounced heat lability. Thus, the enzyme lost activity in an exponential fashion when the temperature exceeded 40 °C (Figure 5). These thermal properties are similar to those displayed by cold-adapted enzymes (Marshall, 1997; Gerday et al., 1997; Feller and Gerday, 1997). Although reduced thermal stability appears to be a common property among lipase isolated from many fungal species (Hoshino et al., 1992; Toida et al., 1995, 1998; Ibrik et al., 1998), no previous reports have been found about their catalytic activity at lower-than-usual temperatures.

The enzyme activity was hardly affected by the presence of 1 mM (Cu^{2+} or Ni^{2+}) or 10 mM (Ca^{2+} or Mg^{2+}) levels of divalent cations. Nevertheless, monovalent cations had different effects. Thus, 1 mM Ag^{+} or Na^{+} inhibited the activity (41 and 86% of residual

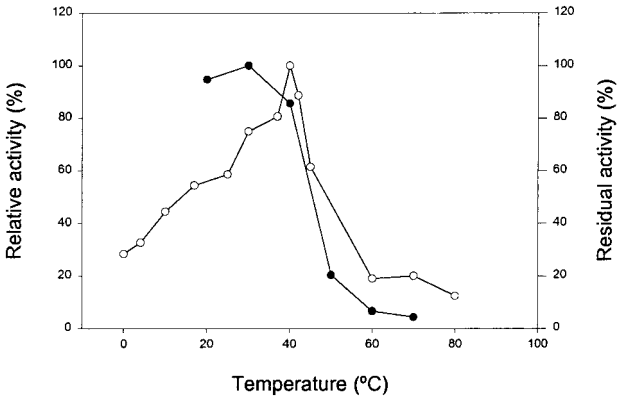

Figure 5. Effect of temperature on the activity and stability of *A. nidulans* lipase. Enzyme activity was assayed for each temperature after an incubation period of 5 min (○). For thermal stability experiments (●), the enzyme extract was prewarmed at the indicated temperature for a 1 h period, and then the remaining activity was determined.

activity, respectively), but no effect was found when 1 mM K^{+} was tested. The influence of different detergents, Tween 20 or SDS, on the lipase activity was almost insignificant, and only the presence of 0.01% Nonidet P-40 or sodium deoxycholate resulted in a slight depletion of activity (84 and 90% of residual activity, respectively).

Substrate specificity was examined using several *p*-nitrophenyl derivatives of fatty acids varying in chain length. Lipase from *A. nidulans* catalyzed more efficiently the hydrolysis of esters of short- and middle-chain fatty acids, with relative hydrolysis rates of 100, 76, 29, 11, and 3% for the C5:0, C8:0, C12:0, C16:0, and C18:0 derivatives, respectively. Hydrolysis by *A. nidulans* lipase was also determined on mono-, di-, and tripalmitoylglycerol. Although the enzyme showed some preference for monoacylglycerols, all glycerides assayed were hydrolyzed efficiently with relative activities of 100, 81, and 70%, respectively.

All of these properties make this enzyme very interesting for a number of biotechnological applications in which the use of cold-active lipase is required (Feller et al., 1996; Marshall, 1997).

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