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Isolation and characterization of an extracellular lipase from *Pseudomonas tolaasii*

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Pseudomonas tolaasii, a plant pathogen, produced an extracellular, heat-stable lipase in reconstituted skim milk (10%, w/v) which was purified by ion-exchange chromatography on DEAE-52 cellulose and gel filtration on Sephadex G-150. The purified enzyme showed one major protein peak on a TSK G3000 SW column, from which a native molecular weight of ~670 kDa was estimated. Maximum enzyme activity was at pH 7.0 and 35°C when assayed on β-naphthyl caprylate. The enzyme lost no activity at pH 7.0 for 48 h at 21°C and was more stable over the pH range 5–6.5 than in the range 8–11. The lipase was quite heat-stable (D-values ranged from 1171 s at 100°C to 79 s at 140°C; the Z-value in the temperature range 100–140°C was ~31°C and the corresponding activation energy (E_a) for inactivation was 94 kJ mol^{-1}. The enzyme was inhibited more strongly by EDTA than by *o*-phenanthroline but not by phenylmethylsulphonylfluoride (PMSF) and was strongly reactivated by Ca^{2+}. The lipase hydrolysed β-naphthyl esters in the order $C_8 > C_6 > C_4 > C_2 > C_{10}$. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Extracellular proteinases and lipases from psychrotrophic pseudomonads are recognised as major spoilage enzymes. They are quite heat-stable and are generally good indices of the keeping quality of protein- and lipid-rich foods. Extracellular heat-stable proteinases and lipases produced by psychrotrophs have been studied extensively (see Stead, 1986; Fairbairn & Law, 1986; Fox *et al.*, 1989; Sørhaug & Stepaniak, 1991). Most of the studies on extracellular proteinases and lipases from *Pseudomonas* spp, have been on enzymes from *Ps. fluorescens*.

Ps. tolaasii is a plant pathogen which causes brown blotch disease in the cultivated mushroom (Tolaas, 1915; Paine, 1919). It was considered to be a biotype II *Ps. fluorescens* (Doudoroff & Palleroni, 1974) but in Bergey's Manual of Systematic Bacteriology (Palleroni, 1984), it was assigned to the family Pseudomonadaceae under miscellaneous strains. Since it is a soil organism, like other *Pseudomonas* spp, it can contaminate milk, meat, fish and eggs. Thus, it is important to characterize the enzymes produced by this bacterium. Although *Ps. tolaasii* has not been recognised as a spoilage organism, several unclassified *Pseudomonas* spp have been isolated from foods, perhaps one of them is *Ps. tolaasii*. The first isolation and characterization of the extracellular proteinase of *Ps. tolaasii* was reported by Baral *et al.* (1995) and there are no published reports on its lipolytic activity. This paper deals with the isolation and characterization of the extracellular lipase of *Ps. tolaasii*.

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MATERIALS AND METHODS

Culture

Ps. tolaasii (obtained from the Culture Collection of the Laboratorium voor Microbiologie, Rijksuniversiteit Gent, Belgium) was grown in sterilized reconstituted skim-milk (10%, w/v) at 30°C on a shaker for 36 h. The resulting culture was sub-cultured (2%, v/v) into sterilized skim-milk and incubated at 30°C, with shaking, for 72 h.

Proteinase activity

Proteinase activity was assayed on 1% (w/v) sulphanilamide azo-casein at pH 7.0 in universal buffer (6.01 g citric acid, 3.89 g KH_2PO_4, 1.77 g H_3BO_3, 5.27 g diethylbarbituric acid per litre; pH adjusted with 0.2 M NaOH). After incubation at 40°C for 1 h, the reaction was stopped by adding TCA to 2% (w/v). The precipitate was removed by filtration and the absorbance of the filtrate measured at 440 nm.

Lipase activity

Lipase activity was routinely assayed on β-naphthyl caprylate (McKellar, 1986). The reaction mixture [1.8 ml buffer (Tris-HCl, 50 mM, pH 7, or universal buffer, pH 7), 0.02 ml β-naphthyl caprylate (100 mM in dimethylsulphoxide: DMSO), 0.1 ml sodium taurocholate (120 mM) and enzyme solution (0.1 ml)] was incubated at 35°C for 30 min; 0.02 ml of freshly prepared fast blue BB salt (100 mM in DMSO) were then added to the mixture and incubated for a further 5 min. TCA (0.2 ml, 0.72 N) was added to stop the reaction. Then, 5 ml ethyl acetate were added and the coloured complex extracted by vigorous mixing. The mixture was centrifuged at 3000 g for 10 min and the absorbance of the top layer read at 540 nm using a Varian spectrophotometer. Lipase activity was expressed as units, one unit being the amount of enzyme that caused an increase in absorbance at 540 of 0.05 per h at pH 7 and 35°C.

Protein concentration

Protein concentration was determined by measuring absorbance at 280 nm, assuming that $A_{280}^{1\%}$ was 10.

Purification of lipase

A 72 h culture was centrifuged at 12000 g for 1 h at 4°C. The cell-free supernatant was diafiltered using polysulfone membranes (Millipore Corp., Milford, MA; nominal molecular weight cut-off, 10 kDa). The retentate was dialyzed against H_2O at 4°C for 48 h and a sample (30 ml, ~1 g protein) applied on a column (80×2 cm) of DEAE-cellulose (DE-52; Whatman Laboratory Division, Maidstone ME14 2LE, UK) previously equilibrated with 0.02 M Tris–HCl buffer, pH 8.5, at 4°C and eluted with a linear NaCl gradient (0–0.5 M) in the same buffer at 60 ml/h. Fractions (10 ml) were collected and assayed for proteolytic and lipolytic activity. The lipase-rich fractions from DEAE-cellulose were pooled, dialyzed against distilled water (4°C, 24 h) and freeze-dried. The freeze-dried preparation (20 mg) was dissolved in 2.5 ml 0.02 M Tris–HCl buffer, pH 8.5, and applied to a column (80×2 cm) of Sephadex G-150 (Pharmacia LKB Biotechnology, Uppsala, Sweden) using the same buffer at room temperature. Fractions (5 ml) were collected and assayed for enzyme activity. Fractions with high activity were pooled and freeze-dried and used to characterize the enzyme.

Molecular weight

The molecular weight of the purified lipase was estimated by chromatography on a TSK G3000 SW column calibrated with α-lactalbumin (MW = 14 kDa), β-lactoglobulin (MW = 18 kDa), ovalbumin (MW = 44 kDa), bovine serum albumin (MW = 66 kDa), immunoglobulin-G (MW = 158 kDa) and thyroglobulin (MW = 669 kDa) using 0.1 M Na–phosphate buffer, pH 7, containing 0.1 mol/l Na_2SO_4. Eluate was monitored at 280 nm using an LC 75 spectrophotometric detector (Perkin–Elmer) interfaced with a Shimadzu C-R4AX chromatopack integrator. To establish if the enzyme was a monomer, it was (i) treated with 10 mM 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) or 6 M guanidine–HCl and chromatographed on the above column, and (ii) analyzed by SDS-PAGE (Laemmli, 1970) using a 13.9% separation and 5% stacking gel in a protean IIxi, vertical slab cell (Bio–Rad Laboratories Ltd, Watford) using standard proteins (Sigma).

Effect of pH

The effect of pH on lipolytic activity was determined at various pH values in universal buffer (pH 4.5–11) using β-naphthyl caprylate as substrate. pH stability was determined by incubating solutions of enzyme in the above buffer (in the pH range 5–11) at 21°C for 48 h and assaying for residual activity.

Effect of temperature

The effect of temperature on purified lipase was determined in 0.05 M Tris–HCl buffer at pH 7 using β-naphthyl caprylate in the temperature range 20–75°C for 30 min. The lipolytic activity at each temperature was determined and the results expressed as percent of maximum activity.

Thermal stability

Solutions of the purified enzyme (100 μl in 0.05 M Tris–HCl buffer, pH 7) were sealed in melting point capillary tubes and heated for 1.5 min at temperatures in the range 40–140°C in a thermostatically controlled water bath or oil bath. After cooling in ice, residual activity was determined on β-naphthyl caprylate, pH 7, 35°C and expressed as % of activity of an unheated control. Thermal denaturation-time curves for the enzyme were prepared by heating solutions of the enzyme for 1, 2, 5, 10 or 20 min at temperatures in the range 50–140°C, followed by determination of residual activity. A log plot of residual activity yielded a straight line, the reciprocal of the slope of which gave the D-value (time necessary at a particular temperature to cause 90% inactivation) at that temperature. From the D-values at several temperatures, a decimal reduction time (DRT) curve was prepared by plotting log D-values against temperature. The reciprocal of the slope of the curve was the Z-value (change in temperature that causes a 10-fold change in D-value). The activation energy for denaturation was determined from an Arrhenius plot of the D-values.

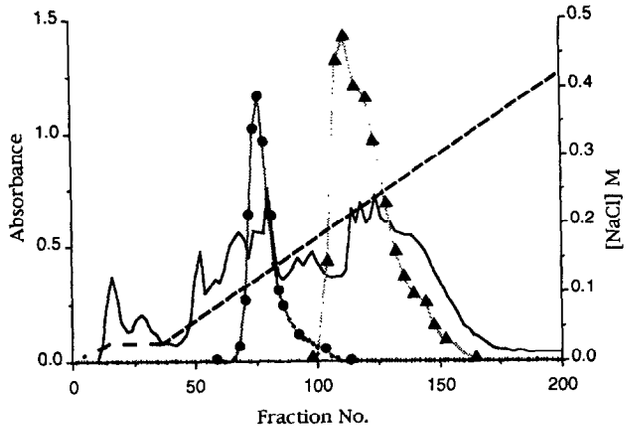

Fig. 1. Chromatogram of the cell free supernatant from *Ps. tolaasii* on DEAE-52 cellulose using 0.02 M Tris–HCl buffer, pH 8.5, and a linear NaCl gradient, 0–0.5 M, at 4°C. Protein, absorbance at 280 nm (–), proteinase activity, absorbance at 440 nm (●), lipase activity, absorbance at 540 nm (▲) and NaCl concentration (---).

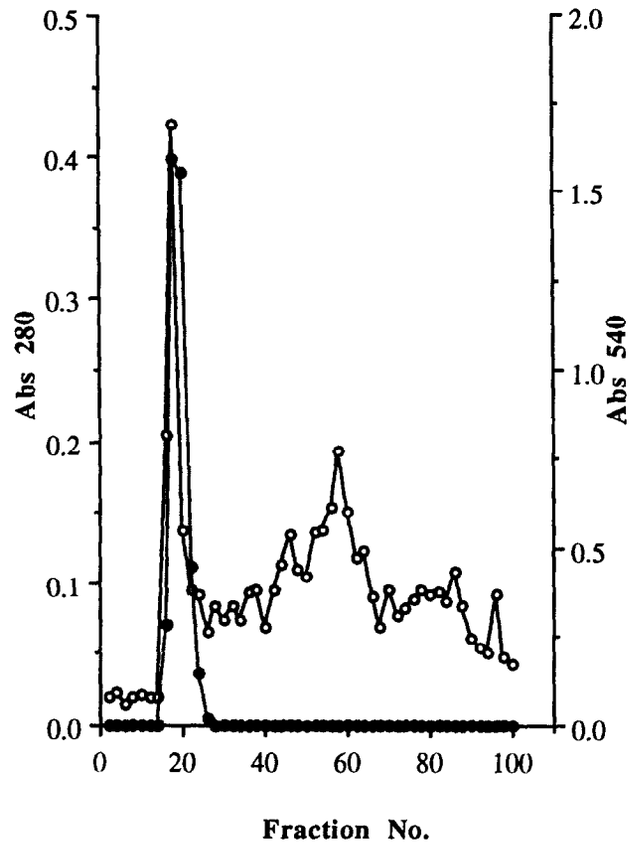

Fig. 2. Chromatogram of the lipase-rich fractions from DEAE-cellulose on Sephadex G-150 using 0.02 M Tris–HCl buffer, pH 8.5, at room temperature. Protein, absorbance at 280 nm (○) and lipase activity, absorbance at 540 nm (●).

Effect of inhibitors

Equal volumes of enzyme solution and PMSF (0.1–1 mM) or EDTA (1–25 mM) or *o*-phenanthroline (0.25–50 mM) were incubated at 20°C for 15 min and then assayed on β-naphthyl caprylate, pH 7, 35°C. The results were expressed as % of the activity of an untreated control.

Reversibility of EDTA inactivation

A sample of enzyme solution was completely inactivated by treatment with 25 m mol/l EDTA for 30 min at 20°C, followed by dialysis against deionized H_2O at 4°C for 24 h. Then, solutions of chlorides of Ca, Mg, Ba, Mn, Cu or Zn (1–50 mM) were added to the apo-enzyme and activity determined on β-naphthyl caprylate (pH 7, 35°C). The extent of reactivation by these metals was expressed as % of the activity of an untreated control.

Substrate specificity

The specificity of the purified lipase was determined on various β-naphthyl esters (from C_2 to C_{10}, 100 mM). Hydrolysis was assayed in Tris–HCl buffer (0.05 M, pH 7) containing 120 m mol/l Na taurocholate at 35°C for 30 min.

RESULTS AND DISCUSSION

Purification of lipase

On ion-exchange chromatography on DEAE-52 cellulose using 0.02 M Tris–HCl buffer, pH 8.5, with a linear NaCl gradient, 0–0.5 M, the lipase was eluted at ~0.2 M NaCl and was well separated from the proteinase which was eluted at 0.12 M NaCl (Fig. 1). On Sephadex G-150, the enzyme eluted close to the void volume as a single, narrow, highly active peak (Fig. 2). Ion-exchange chromatography gave 115-fold purification while gel filtration increased the specific activity a further 8.7-fold with a recovery of 9% of total lipolytic activity. The recovery and degree of purification at each purification step are summarized in Table 1. A high degree of purity was achieved, as indicated by a single protein peak by chromatography on a TSK G-3000 SW column (not shown).

Molecular weight

Lipases from many *Pseudomonas* spp form aggregates of various size (Fox *et al.*, 1989; Stepaniak & Sørhaug, 1989). A lipase from *Ps. fluorescens* had a MW 450 kDa on SDS-PAGE (Sztajer *et al.*, 1991). In this study, the native molecular weight of *Ps. tolaasii* lipase was estimated to be ~670 kDa by chromatography on a TSK G-3000 SW column. Chromatography of the enzyme treated with 6 M guanidine–HCl or 10 mM CHAPS gave the same profile on the TSK G-3000 SW column, suggesting that the lipase preparation was homogeneous and monomeric, although it was inactivated by these

Table 1. Purification and recovery of the extracellular lipase from *Ps. tolaasii*

Fraction	Vol (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
CFS	1800	54000	9000	0.2	100	1
Diafiltered, Dialysed CFS	585	15300	4972	0.3	55	1.5
DEAE-52	100	54	1250	23	14	115
Sephadex G-150	20	4	800	200	9	1000

reagents. No protein band appeared on SDS-PAGE under the experimental conditions (Laemmli, 1970) suggesting that the enzyme did not migrate into the gel because of its high molecular weight.

Effect of pH

The pH optimum of the lipase was ~7, which is within the reported range, 7–9, for lipases from other *Pseudomonas* spp (Stead, 1986; Fox *et al.*, 1989). The enzyme showed >60% of maximum activity over the pH range 6–8, outside of which activity decreased sharply and was almost completely inactive at pH 10.5 (Fig. 3). The enzyme lost no activity at pH 7 on incubation at 21°C for 48 h but it was more unstable in the range 8–11 (~35–2%) than in the pH range 5–6.5 (~50–80%).

Effect of temperature

The lipase was most active on β-naphthyl caprylate in the temperature range 30–40°C, with a slight optimum at 35°C, which is within the range of reported temperature optima (30–50°C) for lipases from other *Pseudomonas* spp (Stead, 1986; Fox *et al.*, 1989). Above 40°C, activity decreased sharply but even at 75°C, ~7% of maximum activity was observed (Fig. 4).

One of the most important properties of *Pseudomonas* proteinases and lipases is their remarkably high heat stability. Heat-resistant proteinases from various *Pseudomonas* spp have been studied more extensively (Kroll, 1989) than heat-stable lipases. Most studies (see Fox *et al.*, 1989) on the properties of psychrotroph lipases have been made on culture supernatants, which introduces the possibility of interaction between proteinases and lipases during characterization. In this study, the purified enzyme was found to be very heat-stable; ~6% of the original activity remained after heating at 140°C for 1.5 min (Fig. 5). However, it was very unstable in the temperature range 70–90°C, where >95% of the initial activity was lost in 1.5 min. This phenomenon, which is termed low temperature inactivation (LTI), is typical of most heat-stable *Pseudomonas* lipases, although reports on the temperature of minimum stability are not consistent (Dring & Fox, 1983; Fox & Stepaniak, 1983;

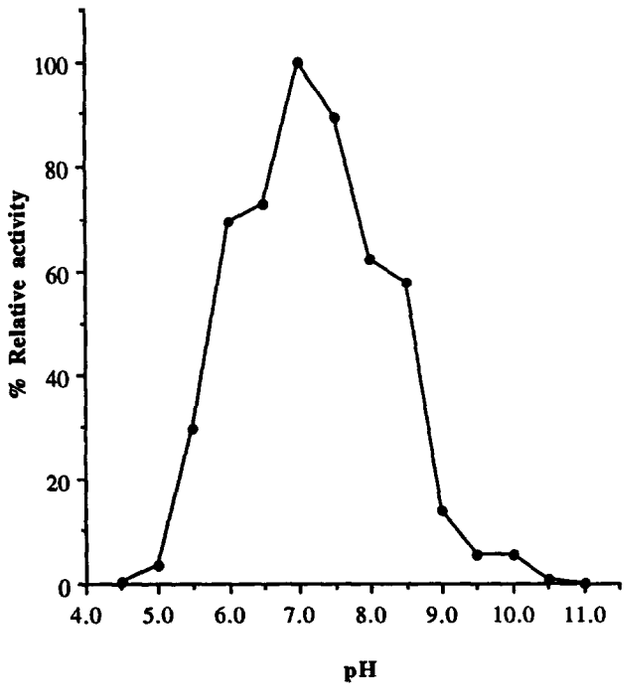

Fig. 3. Effect of pH on the activity of purified lipase from *Ps. tolaasii* in the pH range 4.5–11 in universal buffer, assayed on β-naphthyl caprylate at 35°C for 30 min.

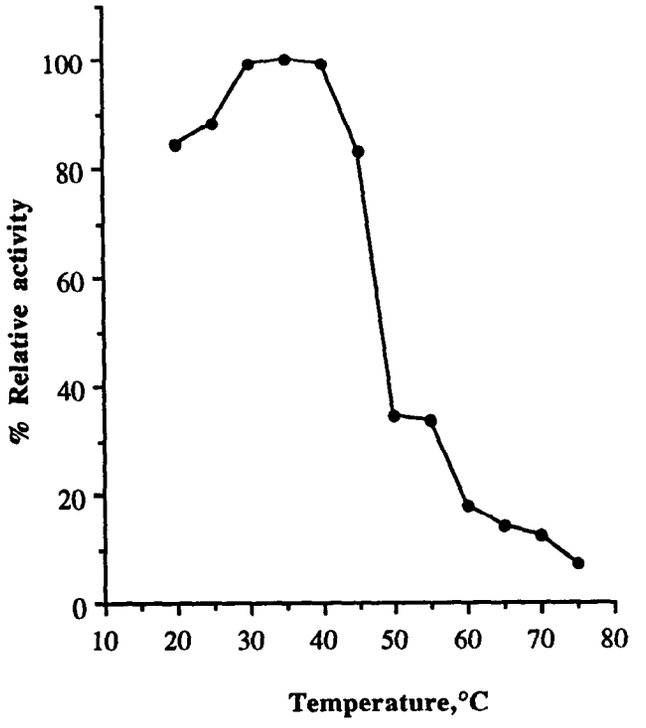

Fig. 4. Effect of temperature on the activity of purified lipase from *Ps. tolaasii* assayed on β-naphthyl caprylate at pH 7 for 30 min.

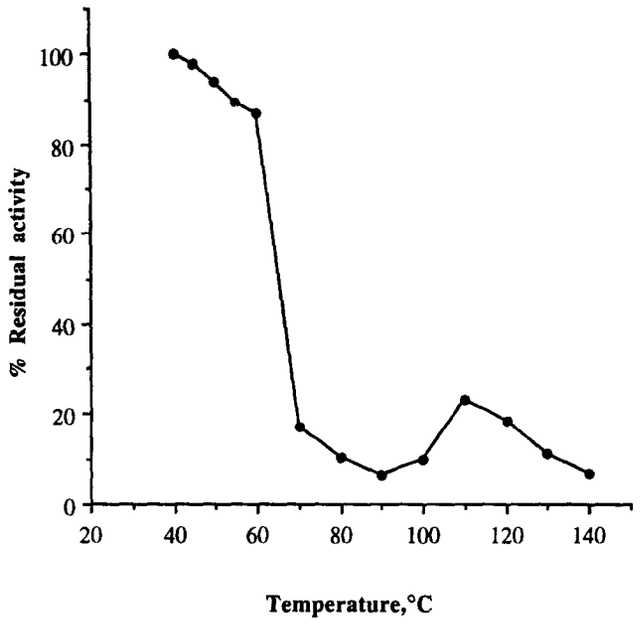

Fig. 5. Thermal inactivation of *Ps. tolaasii* lipase; aliquots of enzyme solution in 0.05 M Tris–HCl buffer, pH 7 were incubated for 1.5 min at temperatures in the range 40–140°C; residual activity was then determined on β-naphthyl caprylate at pH 7, 35°C for 30 min.

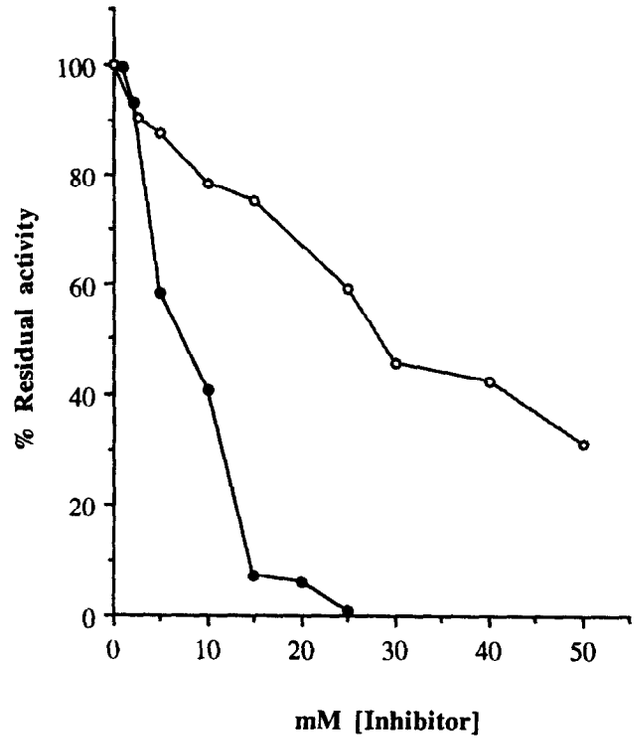

Fig. 6. Effect of EDTA (●) or *o*-phenanthroline (○) on the activity of *Ps. tolaasii* lipase; equal volumes of EDTA/*o*-phenanthroline and lipase solutions were incubated at 20°C for 15 min and then assayed on β-naphthyl caprylate at pH 7, 35°C for 30 min.

Table 2. D-values for *Ps. tolaasii* lipase in 0.05 M Tris–HCl buffer, pH 7.0

Temperature, °C	D-value, min
50	146
60	139
70	16
75	15
80	14
90	12
100	19
110	21
120	8.3
130	2.9
140	1.3

Swaisgood & Bozoglu, 1984; Christen & Marshall, 1985; Bucky *et al.*, 1987; Fox *et al.*, 1989; Kroll, 1989). The thermal denaturation curves of *Ps. tolaasii* lipase were strongly biphasic in the temperature range 70–130°C (results not shown). The biphasic inactivation curves can be explained by instability as the enzyme passed through the temperature zone 70–90°C (Fox & Stepaniak, 1983). As the temperature was increased, the enzyme molecules may have undergone an irreversible conformational change in the temperature range 70–90°C, leading to unfolding, aggregation and loss of activity. At temperatures >90°C, inactivation may have been due to a simple chemical reaction for which an enthalpy of ~60 kJ mol^{-1} is common. The D-values ranged from 1171 s at 100°C to 79 s at 140°C (Table 2), which differed slightly from the reported D-values for some other *Pseudomonas* lipases (Dring & Fox, 1983; Fox & Stepaniak, 1983; Fox *et al.*, 1989), possibly due to different experimental conditions such as a different assay procedure, composition of the heating medium, buffer and pH. The Z-value in the temperature range 100–140°C was calculated to be ~31°C, which is in good agreement with the reported Z-value for other *Pseudomonas* lipases (Fox *et al.*, 1989).

From the Arrhenius plot, the E_a for inactivation of the enzyme in the temperature range 100–140°C was calculated to be 94 kJ mol^{-1}.

Effect of inhibitors

The enzyme was not inhibited by PMSF but was strongly inhibited by EDTA and less effectively by *o*-phenanthroline, suggesting that the lipase is a metalloenzyme. Activity was completely inhibited by 25 mM EDTA while *o*-phenanthroline at 50 mM caused ~70% inactivation (Fig. 6). The inhibitory effect of EDTA on *Pseudomonas* lipases is well documented (Dring & Fox, 1983; Bozoglu *et al.*, 1984; Roussis *et al.*, 1988).

Reactivation by metals

The apo-enzyme (dialysed enzyme after treatment with 25 mM EDTA) was strongly reactivated by Ca^{2+} and

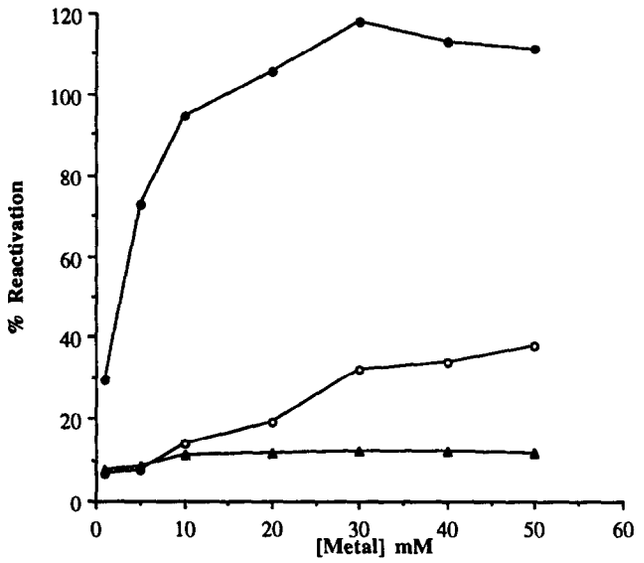

Fig. 7. Reactivation of *Ps. tolaasii* lipase by Ca^{2+} (●), Mg^{2+} (○) or Ba^{2+} (▲) following treatment with 25 mM EDTA at 20°C for 30 min and dialysis against deionized water at 4°C for 24 h. Reactivated enzyme was assayed on β-naphthyl caprylate at pH 7, 35°C for 30 min.

less effectively by Mg^{2+} or Ba^{2+} (Fig. 7) but not by Mn^{2+}, Cu^{2+} or Zn^{2+}. At 10 mM, Ca^{2+} gave 100% reactivation, Mg^{2+} gave ~40% reactivation at 50 mM but Ba^{2+} gave little reactivation (< 10%) either at low or high concentration. Most *Pseudomonas* lipases are inhibited by heavy metal ions, e.g., Zn^{2+}, Hg^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, and activated by Ca^{2+} or Mg^{2+} (Stead, 1986; Fox *et al.*, 1989).

Effect of substrate

A comparison of the hydrolysis of several β-naphthyl esters (C_2–C_{10}) showed that β-naphthyl caprylate was the best substrate for *Ps. tolaasii* lipase; taking activity on β-naphthyl caprylate as 100, the relative activity on β-naphthyl caproate, β-naphthyl butyrate, β-naphthyl acetate and β-naphthyl caprate was 80, 65, 35 and 33%, respectively. Bozoglu *et al.* (1984) reported that the lipase from *Ps. fluorescens* MC50 was most active on eight-carbon esters and McKellar (1986) reported that β-naphthyl caprylate was the best substrate among β-naphthyl esters for *Ps. fluorescens* B52 lipase.

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