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ISOLATION AND CHARACTERIZATION OF AN EXTRACELLULAR PROTEINASE FROM *PSEUDOMONAS TOLAASII*

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Key Word Index—*Pseudomonas tolaasii*; bacterium; proteinase; isolation; characterization.

Abstract—The plant pathogen, *Pseudomonas tolaasii*, produced an extracellular proteinase in 10% (w/v) reconstituted skim milk, which was isolated to homogeneity by ion-exchange chromatography on DEAE-52 cellulose and gel filtration on Sephadex G-150. Ion-exchange chromatography achieved a 65-fold purification and gel filtration increased specific activity a further 2.7-fold. The enzyme was optimally active at pH 7 and 40°; activity decreased rapidly at higher temperatures. The proteinase was almost totally inactivated on heating at 50–55° for 1.5 min but *ca* 50 and 25% residual activity remained after heating for 1.5 min at 90–120° or 140°, respectively. The Z-value for inactivation of the proteinase in the temperature range 80–140° was *ca* 34° and the corresponding activation energy (E_a) was 82 kJ mol^{-1}. Activity was strongly inhibited by EDTA and *o*-phenanthroline and partially restored by Zn^{2+}, Co^{2+}, or Mn^{2+}. The M_r was *ca* 50 000 (by SDS–PAGE) or *ca* 45 000 by gel filtration (TSK G3000 SW).

INTRODUCTION

Pseudomonas tolaasii causes brown blotch disease of the cultivated mushroom [1, 2]. The symptoms are dark brown, often wet and sunken lesions on the caps and stalks which render the crop unsaleable. This bacterium is a member of Pseudomonadaceae, being Gram-negative, oxidase positive, rod shaped and aerobic. It was considered to be a biotype II *Ps. fluorescens*, which means that this biotype may deserve eventual recognition as a separate species [3]. In *Bergey's Manual Of Systematic Bacteriology* [4] it was assigned to the family Pseudomonadaceae under the miscellaneous strains which includes a number of species of *Pseudomonas*, the natural relationships of which with well characterized species of the genus are largely unknown.

Many species of *Pseudomonas* produce very active extracellular enzymes, especially proteinases and lipases which cause spoilage in protein-rich and lipid-rich foods and consequently these enzymes have been well studied and characterized [5–14]. However, it appears that no work has been published so far on the extracellur enzymes produced by *Ps. tolaasii* although a toxin (tolaasin) produced by this organism during the infection of cultivated mushrooms has been studied extensively [15–18]. Since this bacterium is indigenous to soil, like other *Pseudomonas* spp, there is a possibility that it will contaminate milk, eggs, fish and meat products. Hence, it is important to study the extracellular enzymes of this organism. We believe this communication reports the first isolation, purification and characterization of the proteinase of *Ps. tolaasii*.

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RESULTS AND DISCUSSION

Members of the genus *Pseudomonas* are strongly proteolytic and most species produce only one proteinase [19, 20]. The extracellular proteinase of *Ps. tolaasii* is very similar in most respects to those secreted by other *Pseudomonas* species [14, 21]. Proteinase was produced by *Ps. tolaasii* mainly during the exponential phase of growth (Fig. 1), and increased slightly during the stationary phase.

Dialysed cell-free-supernatant (CFS) (30 ml, *ca*, 1 g protein) were applied on a column (80 × 2 cm) of DEAE-52 cellulose in 0.02 M Tris–HCl buffer, pH 8.5, which was eluted by a linear NaCl gradient (0–0.5 M) in the same buffer. A single sharp proteinase peak was eluted at 0.08 M NaCl (Fig. 2). A 65-fold purification was achieved by this procedure, but recovery was *ca* 7%. The CFS contained a lipase which adsorbed on the DEAE-cellulose column under the experimental conditions; it was eluted at *ca* 0.18 M NaCl and was well separated from the proteinase. The proteinase-rich fractions from DEAE-cellulose were pooled, dialysed against distilled water (4°, 24 hr) and freeze-dried. The freeze-dried fraction was dissolved (5 mg ml^{-1}) in 0.02 M Tris–HCl buffer, pH 8.5, and applied to a column (80 × 2 cm) of Sephadex G-150. Gel filtration indicated the presence of three protein peaks, the smallest of which contained proteinase (Fig. 3). The proteinase-rich fractions were pooled, freeze-dried and stored at room temperature. Gel-filtration

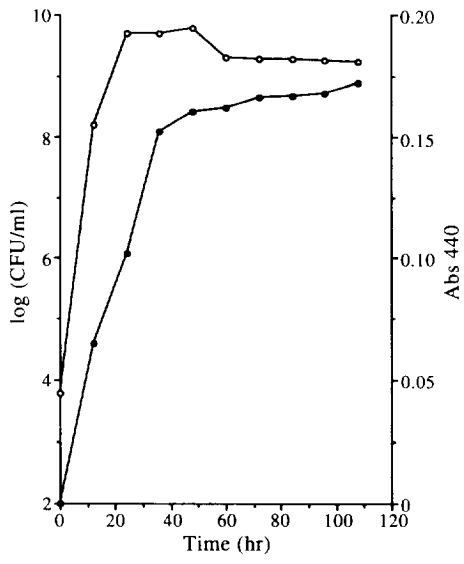

Fig. 1. Growth (○) and proteinase production (●) by *Ps. tolaasii* in reconstituted skim-milk (10%, w/v) at 30°.

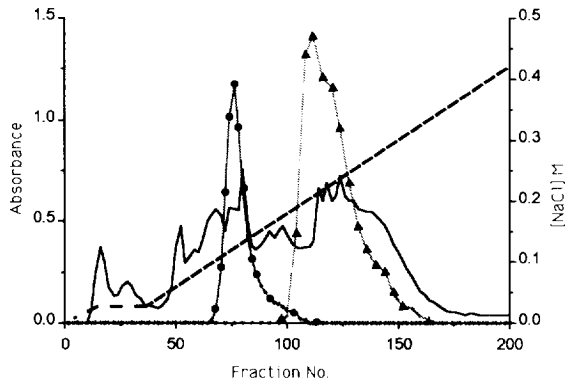

Fig. 2. Purification of extracellular proteinase from *Ps. tolaasii* on DEAE-cellulose using 0.02 M Tris–HCl buffer, pH 8.5, and a linear NaCl gradient 0–0.5 M at 4°. Protein absorbance at 280 nm (—), proteinase absorbance at 440 nm (●), lipase *A* at 540 nm (▲) and NaCl concentration (– –).

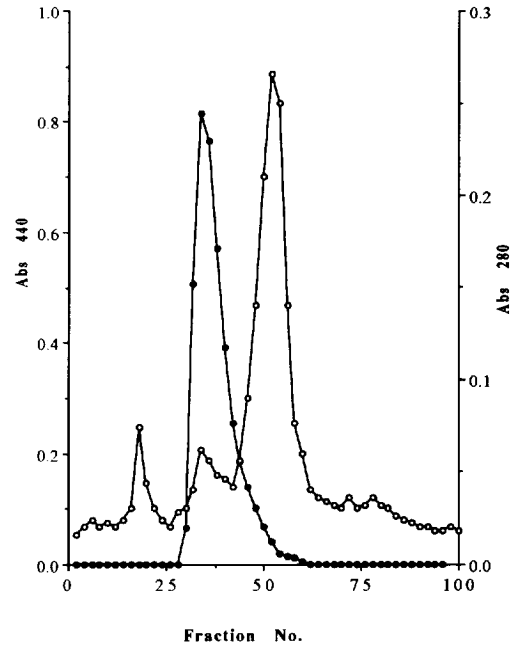

Fig. 3. Chromatogram of the proteinase-rich fractions from DEAE-cellulose on Sephadex G-150 using 0.02 M Tris–HCl buffer, pH 8.5, at 4°. Protein *A* at 280 nm (○) and proteinase *A* at 440 nm (●).

increased the specific activity a further 2.7-fold but only *ca* 2% of the original activity was recovered. The degree of purification and the recovery are summarized in Table 1.

The purified proteinase from *Ps. tolaasii* appeared as a single band on SDS–PAGE, suggesting that the protein was homogeneous; the M_r of the proteinase was estimated to be *ca* 50 k (Fig. 4). From a TSK G3000 SW column, the elution volume of the proteinase corresponded to a M_r of *ca* 45 000, suggesting that the enzyme occurs as a monomer. The reported M_r of *Pseudomonas* proteinases are within the range 35 000–50 000 [14, 21] with the exception of two proteinases [22, 23].

The optimum pH of the *Ps. tolaasii* proteinase was found to be pH 7 (may be classified as a neutral proteinase). Activity decreased above pH 7, *ca* 10% of maximum activity was detected at pH 10. The proteinase did not lose activity on holding at pH 6.5–7 for 48 hr at 21°; stability decreased at pH values above 7, but the loss of activity was greater in the pH range 3–6 than in the range 7.5–11.

The optimum temperature of the proteinase was 40°. At temperatures greater than 40°, activity decreased rapidly and the proteinase was inactive at 55°. The proteinase was almost completely inactivated by heating at 50–55° for 1.5 min but it was relatively stable at higher temperatures; ~ 50 and ~ 25% of the original activity remained after heating at 90–120° or at 140° for 1.5 min, respectively (Fig. 5). Perhaps the most interesting feature of the extracellular *Pseudomonas* proteinases is their remarkable heat stability. The best studied enzymes are those secreted by strains of *Ps. fluorescens* [24–28]. The heat stability characteristics of this proteinase were typical of those of *Ps. fluorescens* proteinases: it was inactivated at relatively low temperatures (e.g. 55°), consistent with the results reported for proteinases secreted by strains of *Ps. fluorescens* [19, 26–31]. Low temperature (55°) inactivation appears to be due to autolysis [25, 27–29, 31]. On heating to 55°, the enzyme molecules unfold sufficiently to become susceptible to hydrolysis while still retaining sufficient structure to be proteolytically active. On heating to higher temperatures, unfolding continues but the enzyme has now lost its proteolytic (and autolytic) activity and is relatively stable, although inactive [27, 28]. In the present study, chromatography of the heated (55° for 1.5 min) enzyme on a TSK G3000 SW column showed that the enzyme peak disappeared and

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

Fraction	Vol (ml)	Total protein (Mg)	Total activity (U)	Specific activity ($U\ mg^{-1}$)	Yield (%)	(fold)
CFS	1800	54 000	15 000	0.3	100	1
Ultrafiltered, Dialysed CFS	585	15 300	10 100	0.7	65	2.3
DEAE-52	135	20	1 040	45	7	150
Sephadex G-150	45	2.5	308	120	2	400

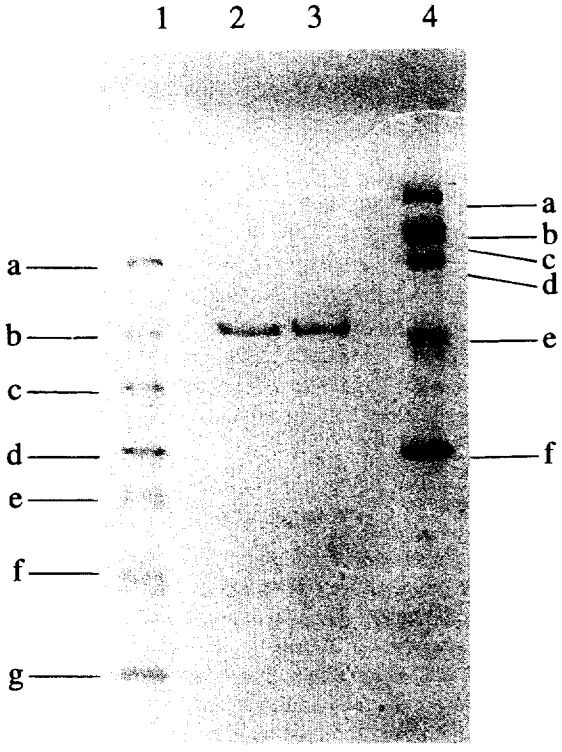

Fig. 4. M_r determination for purified proteinase of *Ps. tolaasii* by SDS–PAGE. Slot 1: M_r ($\times 10^3$) standard mixture (Sigma, SDS-7): (a) 66, (b) 45, (c) 36, (d) 29, (e) 24, (f)20.1, (g) 14.2, slots 2 and 3: purified proteinase and slot 4: high M_r ($\times 10^3$) standard mixture (Sigma, SDS-6H): (a) 205, (b) 116, (c) 97.4, (d) 66, (e) 45, (f) 29.

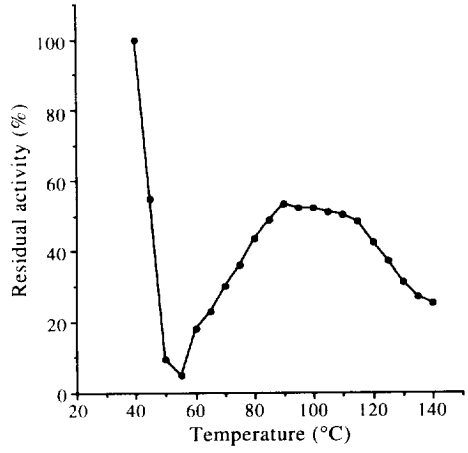

Fig. 5. Thermal inactivation of *Ps. tolaasii* proteinase on heating for 1.5 min in 0.02 M Tris–HCl buffer, pH 8.5.

was replaced by two peaks with elution times corresponding to M_rs of 8000–12 000. Thermal denaturation curves for *Ps. tolaasii* proteinase were prepared by determining the residual activity on azo-casein (1% w/v, pH 7, 40°) after heating a solution of the proteinase (100 µl in melting point capillary tubes) at temperatures in the range 40–140° for 1, 2, 5, 10 and 20 min. The result showed that the enzyme lost activity very rapidly at 50–60°. The D-values (time necessary at a given temperature to reduce enzyme activity to 10% of its original value) for inactivation of the proteinase were determined at various temperatures; the D-value was very low (4–2 min) in the temperature range 50–60° (data not shown). From the plot of D-values against temperature, the Z-values (the change in temperature which causes a 10-fold change in D-value) were calculated. The thermal denaturation curves for *Ps. tolaasii* proteinase were strongly biphasic at lower temperatures, which made it impossible to determine a single Z-value throughout the temperature range 40–140°; Z-values for the temperature range 80–140° and 40–50° were calculated to be *ca* 34° and *ca* 6°, respectively (Fig. 6). The biphasic shape of the inactivation curves might be due to rapid denaturation as the enzyme passed through temperatures around 50–60° and to slower denaturation at higher temperatures. The E_a for inactivation of the proteinase calculated from a best-fit plot over the temperature range 80–140° was 82 kJ mol^{-1}, which is in good agreement with values of E_a reported for other *Pseudomonas* proteinases [27, 28].

Phenylmethylsulphonylfluoride (PMSF) at 1 mM did not inhibit the proteinase, indicating that it was not a serine proteinase. The enzyme was strongly inhibited by EDTA and *o*-phenanthroline, and thus it can be classified as a metalloproteinase. At 25 mM EDTA, the proteinase was completely inactivated. *o*-Phenanthroline was found to be more inhibitory than EDTA and completely inactivated the enzyme at 5 mM (Fig. 7). *o*-Phenanthroline binds Zn^{2+} strongly but Ca^{2+} weakly [32]. In this study, *o*-phenanthroline was a more effective inhibitor than EDTA, which suggests that the proteinase contains Zn.

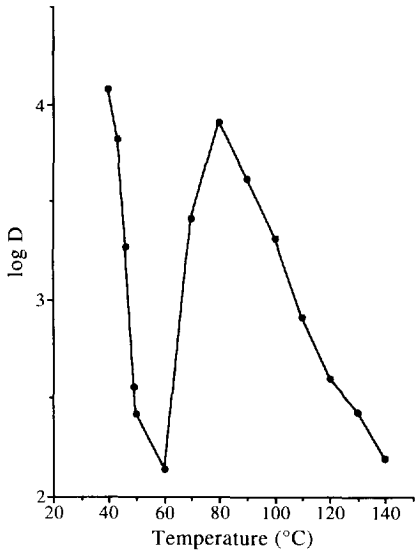

Fig. 6. Decimal reduction time (Z-value) curves for *Ps. tolaasii* proteinase in 0.02 M Tris–HCl buffer, pH 8.5.

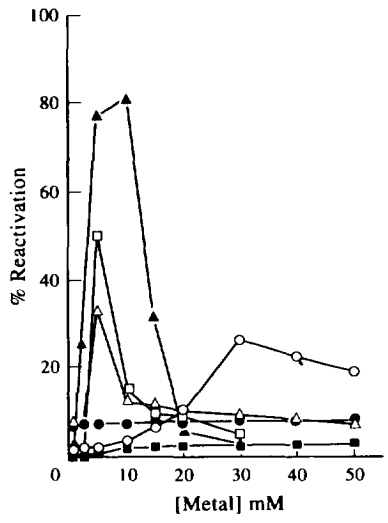

Fig. 8. Reactivation of *Ps. tolaasii* proteinase by Ca^{2+} (○), Mg^{2+} (●), Ba^{2+} (■), Mn^{2+} (△), Co^{2+} (□) or Zn^{2+} (▲).

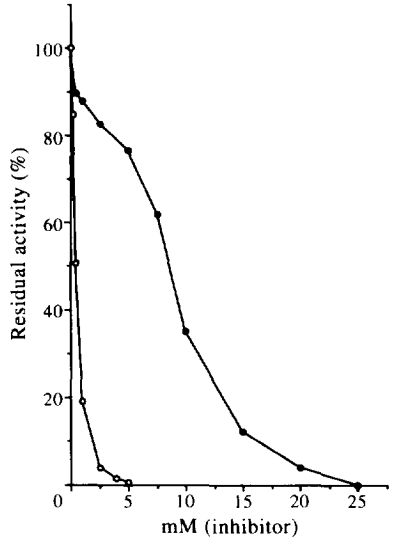

Fig. 7. Effect of EDTA (●) and *o*-phenanthroline (○) on the activity of *Ps. tolaasii* proteinase.

Zn has been shown to be an integral part of many *Pseudomonas* proteinases, occurring at one atom/molecule and often together with Ca, which is involved in maintaining the integrity of the active site [33]. The greater inhibitory effect of *o*-phenanthroline than EDTA was also reported by Porzio and Pearson [34], Juan and Cazzulo [35], Azcona *et al.* [36], Mitchell *et al.* [12] and Richardson [25].

Reactivation of the apoenzyme by various metal ions (after treatment with 25 mM EDTA) was examined (Fig. 8). Zn^{2+}, Co^{2+} and Mn^{2+} were more effective than Ca^{2+}, Mg^{2+} or Ba^{2+}; at 10 mM, Zn^{2+} reactivated the apo-enzyme to 80% of its original activity. Azcona *et al.* [36] reported that 90% reactivation of the apoenzyme of *Ps. fluorescens* AH-70 proteinase was achieved by Zn^{2+}. Mg^{2+} and Ba^{2+} gave very little reactivation even at 50 mM, while Ca^{2+} gave low reactivation up to 15 mM but at 30 mM, gave 25% reactivation.

EXPERIMENTAL

Culture. Pseudomonas tolaasii, strain LMG 2342T (obtained from the Culture Collection of the Laboratorium voor Microbiologie, Rijksuniversiteit, Gent, Belgium) was grown in sterilized (110° × 10 min), reconstituted skim-milk (10%, w/v) at 30° under shaken conditions for 36 hr. The resulting culture was inoculated at 2% (v/v) into sterilized skim-milk and incubated at 30° on a shaker for 72 hr. Periodically during the growth period, the cell population was determined by plate count on skim-milk agar (incubated at 30° for 48 hr) and proteinase activity by assay on azo-casein.

Proteinase activity. Assayed with 1% (w/v) sulphanilamide azo-casein at pH 7 (6.01 g critic acid, 3.89 g KH_2PO_4, 1.77 g H_3BO_3, 5.27 g diethylbarbituric acid per l; pH adjusted with 0.2 M NaOH). Enzyme soln (1 ml) was added to 1 ml of the azo-casein solution and the mixture incubated at 40° for 1 hr. The reaction was stopped by adding 1 ml 6% (w/v) TCA. The precipitated protein was removed on Whatman No. 1 filter paper and the A of the filtrate measured at 440 nm. A blank was made by substituting H_2O for the enzyme soln. Activity was expressed as units, 1 enzyme unit being defined as the amount of enzyme that gave an increase in A_{440} of 1 per hr.

Lipolytic activity. Activity in chromatography fractions was assayed by a colorimetric method using β-naphthyl caprylate as substrate [37].

Protein concentration. This was determined in culture supernatants and chromatographic fractions by A at 280 nm, assuming that the A_{280} of a 1% protein soln was 10.

Purification of proteinase. The 72 hr culture was centrifuged at 12 000 g for 1 hr at 4°. The CFS was ultrafiltered using polysulphone membranes (Millipore Corp., MA, U.S.A.; nominal M_r cut-off, 10 000), followed by diafiltration. The retentate was dialysed against H_2O at 4° for 48 hr. The proteinase was purified by ion-exchange chromatography (DEAE-52 cellulose) and gel-filtration (Sephadex G-150) using 0.02 M Tris–HCl buffer, pH 8.5. Fractions with high activity (gel filtration) were freeze-dried and used to characterize the enzyme.

The M_r of the purified proteinase. Estimated (i) by SDS–polyacrylamide gel electrophoresis (PAGE) using a 13.9% separation and 5% stacking gel in a Protean IIxi, vertical slab cell (Bio-Rad), essentially as described in ref. [38], using standard proteins (Sigma) and (ii) by chromatography on a TSK G3000 SW column calibrated with α-lactalbumin (14 000), β-lactoglobulin (18 000), ovalbumin (44 000), bovine serum albumin (60 000), immunoglobulin-G (158 000) and thyroglobulin (669 000) using 0.1 M Na-Pi buffer, pH 7, containing 0.1 M Na_2SO_4.

Effects of pH. The relative proteinase activity, using 1% (w/v) azo-casein, was determined at various pH values in universal buffer (pH 4.5–10). To assess pH stability, samples of purified enzyme were mixed with the above buffer in the pH range 3–11, incubated at 21° for 48 hr and then assayed for residual activity.

Effect of temperature. Activity was determined with azo-casein (1%, w/v, pH 7) on incubation in the range 10–60°. Aliquots (100 μl) of the purified proteinase in 0.02 M Tris–HCl buffer, pH 8.5, were also sealed in melting point capillary tubes and heated in the range 40–140° for 1.5 min. After cooling in ice, the residual activity was determined on azo-casein (1%, w/v, pH 7, 40°) and expressed as % of activity of an unheated control.

Effect of inhibitors. The effect of PMSF, EDTA and *o*-phenanthroline on the proteinase was determined on azo-casein, after incubating equal vols of solns of PMSF, EDTA, *o*-phenanthroline and enzyme solns at room temp for 15 min at various concns (PMSF: 0.05–1 mM: EDTA: 0.05–25 mM: *o*-phenanthroline: 0.25–5 mM).

To determine the reversibility of the inactivation by EDTA, the proteinase was completely inactivated by treating with EDTA (25 mM). After incubation for 2 hr at room temp., the mixture was dialysed against deionized H_2O at 4° for 24 hr. Then, solns of chlorides of Ca, Mg, Ba or Mn (1 to 50 mM) or Co or Zn (1 mM to 30 mM) were added to the apo-enzyme and assayed for residual activity. The extent of reactivation by the above metals was expressed as a % of the activity of an untreated control.

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