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Purification and characterization of an alkaline protease from *Pseudomonas aeruginosa* MN1

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An alkaline protease produced by *Pseudomonas aeruginosa* MN1, isolated from an alkaline tannery waste water, was purified and characterized. The enzyme was purified 25-fold by gel filtration and ion exchange chromatography to a specific activity of 82 350 U mg^{-1}. The molecular weight of the enzyme was estimated to be 32 000 daltons. The optimum pH and temperature for the proteolytic activity were pH 8.00 and 60°C, respectively. Enzyme activity was inhibited by EDTA suggesting that the preparation contains a metalloprotease. Enzyme activity was strongly inhibited by Zn^{2+}, Cu^{2+} and Hg^{2+} (5 mM), while Ca^{2+} and Mn^{2+} resulted in partial inhibition. The enzyme is different from other *Pseudomonas aeruginosa* alkaline proteases in its stability at high temperature; it retained more than 90% and 66% of the initial activity after 15 and 120 min incubation at 60°C. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 291–295.

Keywords: protease; alkaline protease; *Pseudomonas aeruginosa*

Introduction

Proteases are the most important kind of enzymes from an industrial point of view. Alkaline proteases have applications in leather processing, laundry detergents, production of protein hydrolysates, and food processing [4,7,13]. Several alkaline proteases from alkaliphilic bacteria have been purified and characterized [3,6,8,11]. However, few reports exist on proteolytic enzymes from *Pseudomonas aeruginosa*. Jang *et al* [6] reported alkaline serine protease from an alkaline-resistant *Pseudomonas* sp which has a molecular weight of 29 000 daltons. Protease-producing *P. aeruginosa* K-187 was used for deproteonization of shrimp and shell wastes [12].

Isolation and screening of microorganisms from naturally occurring alkaline habitats or from alkaline waste water was expected to provide new alkaliphilic bacteria producing enzymes active and stable in highly alkaline conditions. This article deals with the purification and characterization of an alkaline protease produced by an alkaliphilic bacterial strain isolated from an alkaline waste water.

Materials and methods

Bacterial strain

The microorganism used was an alkaliphilic bacterium which was isolated from tannery waste water. It was identified as *Pseudomonas aeruginosa* MN1. The classification was based on gram strain (–), catalase (+) and oxidase (+) reactions, and the use of API 20E (Api System SA, Montalieu-Vercieu, France).

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Screening of protease-producing strains

Bacteria producing alkaline protease were obtained as follows: samples of repeated batch cultures were plated on skim milk agar (pH 9.0–11.0). After 24–48 h at 37°C, clones which exhibited the largest cleared zones around their colonies were purified and assayed for protease activity.

Cultivation and media

The medium used for isolation of alkaliphilic bacteria was composed of (g L^{-1}): peptone, 5; yeast extract, 3; skimmed milk, 250 ml and agar, 12. The medium for protease production was composed of (g L^{-1}): peptone 10; $(NH_4)_2SO_4$, 2; K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.4; $MnSO_4 \cdot H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01 and yeast extract, 1. The pH of the culture was 8.0.

The preculture was cultivated in L-Broth (peptone 10 g L^{-1}; yeast extract 5 g L^{-1} and NaCl 5 g L^{-1}) [10].

Cultivations were conducted in 50 ml of medium in 500-ml conical flasks maintained at 30°C. The growth rate of the microorganisms was determined by measuring absorbance at 600 nm.

Protease purification

The bacterium was grown for 24 h at 30°C. The culture was then centrifuged and the cell-free supernatant was precipitated with ammonium sulfate. Ammonium sulfate was added to the solution to 70% saturation which was then kept at 4°C overnight. The precipitate was collected by centrifugation and dissolved in a small volume (1/50) of 20 mM Tris-HCl buffer (pH 8.0), and dialyzed against the same buffer.

The dialyzed enzyme preparation was applied on a Sephadex G-100 column (2 × 85 cm) pre-equilibrated with 20 mM Tris-HCl (pH 8.0). Fractions (3 ml) were collected and those showing protease activity were pooled.

The active fractions pooled from Sephadex G-100 were resolved on a DEAE-cellulose column (2 × 9.5 cm) equilib-

rated with 20 mM Tris HCl (pH 8.0). The unadsorbed protein fraction was eluted with the same buffer (150 ml). The enzyme was eluted with a linear gradient of 0–0.75 M NaCl in the same buffer at a flow rate of 0.66 ml min^{-1}.

Fractions (2 ml) were collected. Active fractions that contained (80%) of the enzyme activity were pooled, concentrated by lyophilisation and subsequently used for characterization. All steps were conducted at 4°C.

Polyacrylamide gel electrophoresis

SDS-PAGE was carried out for determination of purity and molecular weight of the enzyme.

pH and temperature optimum and thermal stability

The optimum pH was determined over a range of pH 5.5–12 with casein as substrate. The effect of temperature on the reaction was examined at various temperatures for 15 min at pH 8.

Thermal stability of *P. aeruginosa* protease was determined by incubating the enzyme at 60°C in the absence and presence of various polyols. Aliquots were withdrawn at desired time intervals to test the remaining activity at standard conditions.

Effect of inhibitors on protease activity

EDTA, PMSF, iodoacetate and different metal ions were tested for ability to inhibit protease activity. Enzyme was preincubated with each inhibitor for 15 min at 20°C, then the residual activity was tested using casein as substrate.

Assay of proteolytic activity

Protease activity was measured by the method of Asha *et al* [1] using casein as substrate. Enzyme solution (0.5 ml) suitably diluted was mixed with 100 mM Tris-HCl (pH 8.0) containing 1% casein, and incubated for 15 min at 60°C. The reaction was stopped by addition of 0.5 ml TCA (20%). The mixture was allowed to stand at room temperature for 30 min and then centrifuged at 10 000 × *g* for 10 min to remove the precipitate. The absorbance was measured at 280 nm. A standard curve was generated using solutions of 0–50 mg L^{-1} tyrosine. One unit of protease activity was defined as the amount of enzyme which liberated 1 μg tyrosine in 1 min at 37°C.

Protein concentration

Protein concentration was determined by the method of Lowry *et al* [9] with bovine serum albumin as a standard.

Results and discussion

Isolation of alkaline protease-producing strains

Screening of alkaliphilic microorganisms that produced alkaline protease was done on cultures acclimated by repeated transfer on undiluted and non inoculated tannery alkaline waste water, since organic pollution is essentially composed of proteins. Protease production strains were selected by growth on skim milk agar, as described in Methods.

Bacteria showing maximum zones of clearance around their colonies were selected (>20). The purity of the isolated bacteria was ascertained through repeated streaking. Cells of each isolate were inoculated in conical flasks containing 20 ml of production medium and incubated at 37°C for 24 h. The cultures were then centrifuged and the supernatants were assayed for protease activity. Strain MN1 was selected as the most potent producer of protease and was identified as *P. aeruginosa*.

Protease purification

The initial step of purification was the concentration of proteins by precipitation using ammonium sulfate and organic solvents. Precipitation by 70% ammonium sulfate yielded a recovery of more than 82%. This treatment did not result in an increase in the specific activity (Table 1). However, treatment of the protease preparation with acetone or ethanol (60% v/v) resulted, respectively, in 5.5- and 5-fold purification, with only 66% recovery. On the basis of these results, precipitation was carried out using 70% ammonium sulfate.

After centrifugation, the precipitate was dissolved in 20 mM Tris-HCl pH 8.0 and then purified by gel permeation on a column (2 cm × 85 cm) of Sephadex G-100. The elution profile is shown in Figure 1. This step resulted in 7.6-fold purification of the enzyme with 55.7% recovery.

The third and final step was purification by ion exchange chromatography on DEAE-cellulose using a linear sodium gradient. This procedure yielded a single peak of protease activity. The chromatogram obtained is shown in Figure 2. The protease activity was eluted from the column with 0.16 M NaCl. The active fraction eluted was essayed for activity and analyzed on a 15% SDS-polyacrylamide gel. The MW was estimated to be 32 000 by SDS-PAGE (data not shown) and by gel filtration.

The overall purification scheme showing the specific activity, the recovery and the purification effected at each purification step is summarized in Table 1. After the final purification step the enzyme was purified 25-fold with a specific activity of 82350 U mg^{-1} and a recovery of 37.7% from the spent medium.

Effect of inhibitors on protease activity

The effect of chelating agent and group-specific reagents (PMSF, iodoacetate) on protease activity is summarized in Table 2. The enzyme was resistant to inhibition by PMSF and iodoacetate; however, EDTA affected the activity considerably. These data indicate that the enzyme is a metalloprotease.

Effect of pH and temperature on protease activity

The optimum pH for protease activity determined at 60°C, was 8.0. Activity declined rapidly above pH 8.0 and was 70% and 45% of the maximal activity at pH 9.0 and 10.0, respectively. The enzyme was active even at pH 12.0 (Figure 3).

The optimum temperature for protease activity was determined by varying the reaction temperature at pH 8.0. Enzyme activity was detected between 30 and 80°C. The temperature optimum of the proteolytic activity was 60°C with a sharp decrease in activity above 65°C. The enzyme had 60% and 15% of the maximum activity at 70 and 80°C, respectively (Figure 4).

Table 1 A summary of the purification of alkaline protease from *Pseudomonas aeruginosa* MN1

Purification steps	Total activity ($U \times 10^3$)	Total protein (mg)	Specific activity $\times 10^3$ ($U\ mg^{-1}$)	Recovery (%)	Purification (fold)
Cell-free culture supernatant	877.3	264	3.32	100	1
Ammonium sulfate fraction (70%)	725.4	209	3.46	82.7	1.04
Sephadex G-100	488.8	19.37	25.24	55.7	7.6
DEAE-cellulose chromatography	330.8	4.02	82.35	37.7	24.8

All operations were carried out at 4°C.

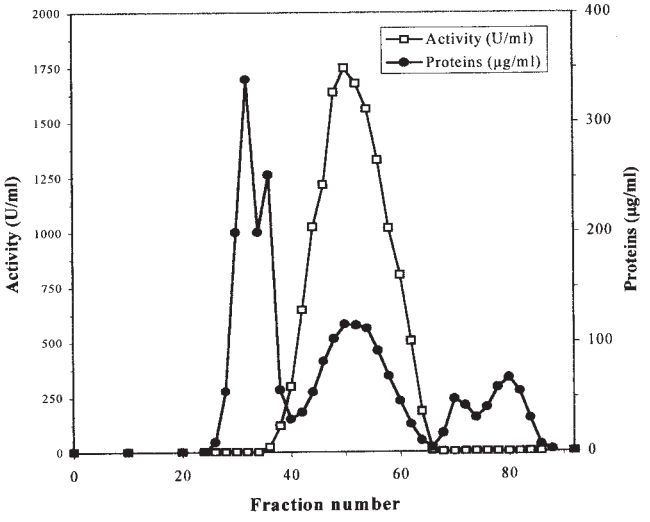

Figure 1 Purification of *P. aeruginosa* protease by gel filtration on Sephadex G-100. The enzyme preparation was applied to a 2 × 85-cm column, equilibrated and eluted with 20 mM Tris-HCl. Fractions (3 ml) collected from the column were assayed for protease activity. Flow rate 0.23 ml min^{-1}.

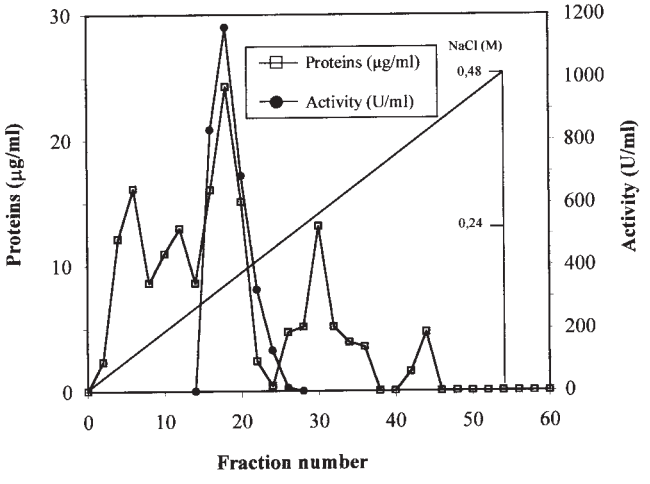

Figure 2 Chromatography of *P. aeruginosa* protease, previously purified by gel filtration, on DEAE-cellulose. The column (2 × 9.5 cm) was equilibrated with 20 mM Tris-HCl (pH 8.0), loaded with enzyme preparation and eluted with a linear gradient (0–0.75 M NaCl) at a flow rate of 0.66 ml min^{-1}. No proteins were eluted after 0.48 M NaCl.

Table 2 Effect of inhibitors on protease activity

Inhibitors	Concentration (mM)	Residual activity (%)
None		100
PMSF	2	100
	5	100
Iodoacetate	2	100
	5	100
EDTA	2	50
	5	0

EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.
Enzyme activity measured in the absence of any inhibitor was taken as 100%. The remaining protease activity was measured after preincubation of enzyme with each inhibitor at 20°C for 15 min.

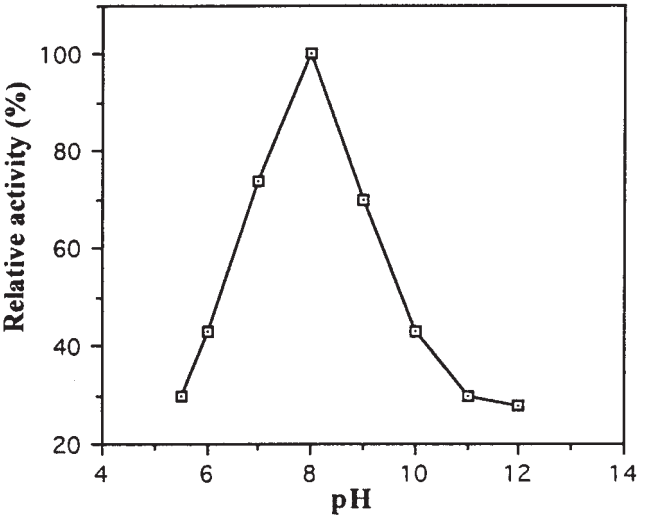

Figure 3 Effect of pH on proteolytic activity. The pH profile was determined in different buffers by varying pH values at 60°C.

Thermal stability

In the first experiment, thermal stability of the protease was determined by heating the purified enzyme for 15 min at different temperatures in 100 mM Tris-HCl (pH 8.0). The data presented in Figure 5a show that up to 50°C the enzyme was very stable followed by a rapid loss of activity above 70°C. The enzyme retained more than 90% and 15% of its activity at 60 and 70°C, respectively. However, the enzyme was completely inactivated at 80°C.

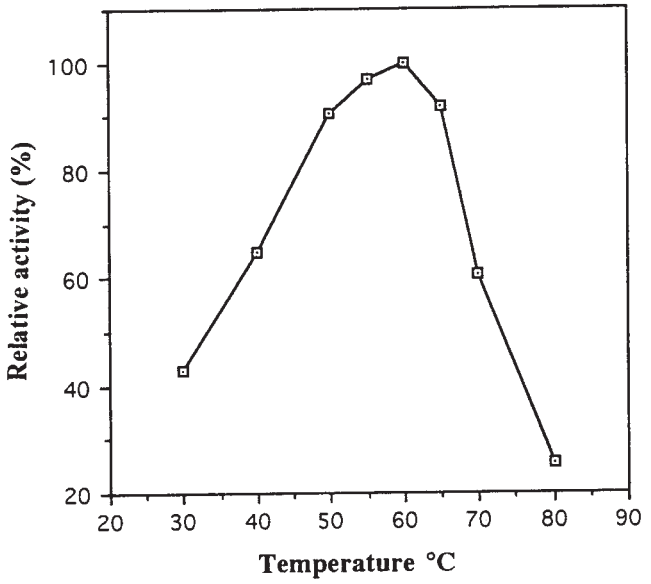

Figure 4 Effect of temperature on proteolytic activity. The temperature profile was determined by assaying protease activity at different temperature values (30–80°C).

The effect of temperature on enzyme stability was also determined by preincubating at 60°C and the activity remaining was then measured as a function of reaction time as described in Materials and methods (Figure 5b). The non-heated enzyme was considered as control (100%). The data presented in Figure 5b show that protease activity decreased with heating time and the enzyme retained 66% activity after 2 h incubation. When incubated 2 h at 50°C, the enzyme retained 100% of its initial activity (data not shown).

The activity and stability of our enzyme were higher than those reported for alkaline protease from *Pseudomonas* sp [6]. Jang *et al* [6] reported total inactivation of alkaline protease activity during the 15-min incubation at 70°C.

Effect of polyols on protease thermostability

Several reports showed that the thermostabilization of enzymes could be enhanced by modification of the environment, such as addition of various additives (polyols, PEG) [2,5]. These protective effects were explained by the strengthening of hydrophobic interactions inside protein molecules and by indirect action of polyols on water structure.

In a first experiment polyols such as glycerol, mannitol, sorbitol and xylitol, were added to the reaction medium (final concentration 5% w/v). Most polyols used improved protease activity (Figure 6). The best results were obtained with glycerol.

In a second experiment the influence of polyols on thermostability of the protease was studied. Enzyme was first incubated at 60°C in 50 mM Tris-HCl buffer pH 8.0 in the absence or the presence of polyols (10% w/v). After 150 min incubation, the remaining activity was measured. As shown in Figure 7, thermostabilization was enhanced with polyols. Thermostabilization was more effective with glycerol. After 150 min incubation at 60°C, in the presence of glycerol or in the absence of any additive, 84.5% and

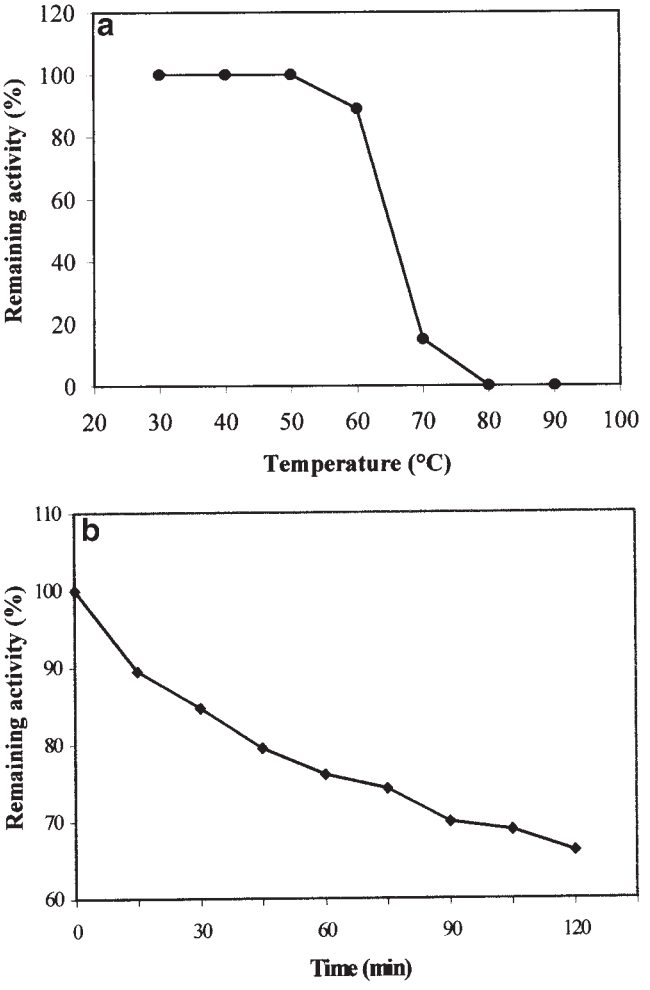

Figure 5 Effect of temperature on thermal stability of the protease. (a) The enzyme was incubated 15 min in 100 mM Tris-HCl pH 8.0 at different temperatures, and the residual activity was determined as described in Materials and methods. (b) The enzyme was incubated at 60°C and residual enzyme activity was determined from 0 to 120 min at 15-min intervals.

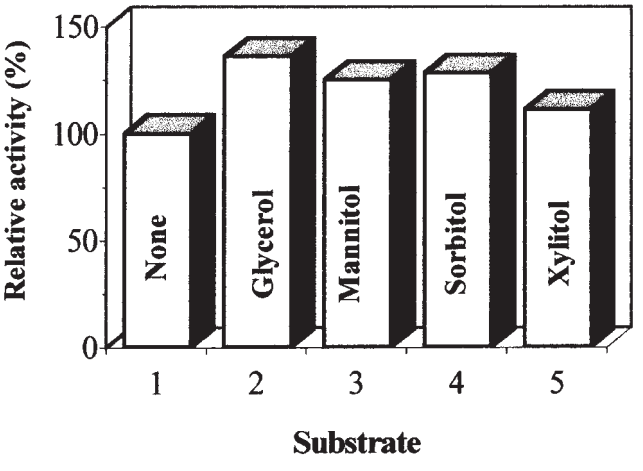

Figure 6 Effect of polyols (5% w/v) on enzyme activity.

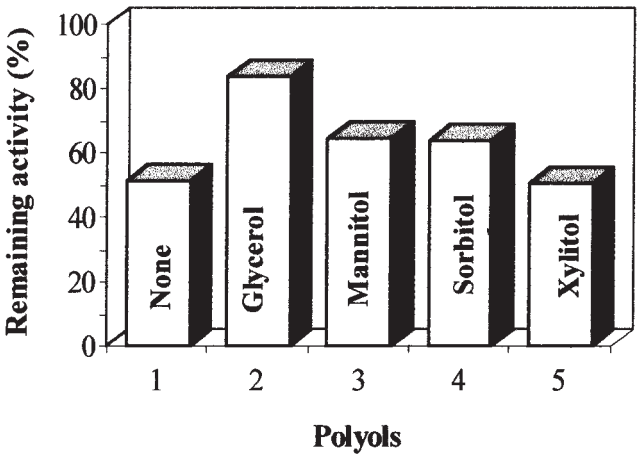

Figure 7 Effect of polyols on protease thermostability. A protease preparation was heated at 60°C in the absence or presence of polyols. After 150 min incubation, the remaining activity was measured.

Table 3 Effect of metal ions on protease activity

Metal ion	Concentration (mM)	Relative activity (%)
None		100
Mg^{2+}	5	100
	10	89
Ca^{2+}	5	87
	10	59
Mn^{2+}	5	41
	10	27
Cu^{2+}, Zn^{2+} or Hg^{2+}	5	0
	10	0

The activity remaining was measured after pre-incubating the enzyme with the metal ion.

51%, respectively, of the initial activity remained. No protective effect was observed with xylitol (Figure 7).

Effect of metal ions on enzyme activity

The effect of different metal ions on enzyme activity was studied. After pre-incubation of enzyme with metal ions at 20°C for 15 min, the remaining protease activity was measured. As shown in Table 3 Mg^{2+} did not affect activity, whereas, Ca^{2+} and Mn^{2+} (5 mM) decreased the activity to 87 and 41% of the control, respectively. The enzyme was completely inhibited by Zn^{2+}, Cu^{2+} and Hg^{2+}.

The *P. aeruginosa* strain isolated in this study produce a thermostable alkaline metalloprotease. The optimum temperature and pH for protease activity were 60°C and pH 8.0. The enzyme retained more than 70% and 45% of the maximal activity at pH 9.0 and 10.0, respectively. The enzyme had a high thermostability with 100% and 66% activity retained at 50 and 60°C, respectively, after 2 h incubation. Considering these properties the *P. aeruginosa* protease may find potential application in the detergent industry.

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