

## CHARACTERIZATION OF AN INTRACELLULAR PROTEASE FROM PSEUDOMONAS AERUGINOSA

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### ABSTRACT

An intracellular protease was extracted and purified from *Pseudomonas aeruginosa* by ion-exchange chromatography on DEAE-cellulose followed by CM-cellulose and rechromatography on DEAE-cellulose. The purified protease was found to be homogeneous as judged by polyacrylamide disc gel electrophoresis (PAGE). The molecular mass of the protease as determined by gel filtration on G-150 was about 48,000 and about 49,000 on SDS-PAGE. The enzyme is monomeric in nature. The purified protease is a glycoprotein with neutral sugar content of 0.6%. The Km value of the protease was found to be 0.48% against casein as substrate. The enzyme is stable up to 60°C and showed maximum activity around 50°C. The enzyme activity was affected with the changes of pH and the maximum proteolytic activity was observed at pH 8.0. The protease activity was inhibited in the presence of EDTA, Cu<sup>2+</sup>, Mn<sup>2+</sup> and Hg<sup>2+</sup> whereas the presence of Ca<sup>2+</sup>, K<sup>+</sup>, Na<sup>+</sup> and ascorbic acid enhanced the activity.

**KEY WORDS:** Pseudomonas aeruginosa, Intracellular protease, Purification and Characterization.

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### INTRODUCTION

*Pseudomonas aeruginosa* possesses a variety of exoproducts (enzymes, toxins) which are responsible for direct tissue destruction in lung and may be important for bacterial dissemination.<sup>1</sup> Proteases play a crucial role in

numerous pathologic processes. Arthritis, tumor invasion and metastasis, infection and a number of degenerative diseases have been linked with the involvement of one or more proteolytic enzymes.<sup>2</sup> Microbial proteases have been proposed as virulence factors in a variety of diseases caused by microorganisms. The virulence of *Pseudomonas aeruginosa* is multifactorial, but it is partly determined by exoproducts such as alkaline protease and elastase that are responsible for the damage of tissues by degrading elastin collagen and proteoglycans. These enzymes have been also shown to degrade proteins that function in host defense *in vivo*.<sup>3</sup>

Identification and characterization of microbial proteases are prerequisites for understanding their role in the pathogenesis of infection diseases as well as to improve their application in biotechnology. For this purpose, rapid and sensitive techniques for the detection and characterization of microbial proteases are highly desirable.<sup>4</sup> The present study has been undertaken to isolate and characterize protease from *Pseudomonas aeruginosa* from clinical cases.

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## MATERIALS AND METHODS

Clinical isolates (600) were collected with aseptic precaution from suspected cases of different types of infections from both hospitalized and non hospitalized patients during the period of July 1999 to September 2003. Isolation of *Pseudomonas* has been done by culture in nutrient agar by aerobic incubation at 37° C for overnight and identification has been done by production of pyocyanin, fluorescein, arginine dihydrolase test, gelatin liquifaction test and carbohydrate utilization tests.

Organisms grown in appropriate media for 18 hours were preserved in nutrient agar slant at 2-8 °C in a refrigerator and this culture was used within two weeks for routine laboratory works. For long term preservation, selected and identified bacterial strains were stored in Brain-heart infusion broth with 16% glycerol and stored frozen without significant loss of viability at -20° C until further study.

*Pseudomonas aeruginosa* isolate (strain no.14) used in the present study was a most virulent and multidrug resistant bacterium isolated, characterized and identified from clinical cases in a previous study.<sup>5</sup>

**Preparation of crude enzyme extract:** Bacterial cells (10g approximately) were lysed by ultrasonication with cold 0.1 M sodium phosphate buffer, pH 7.0. The temperature was maintained at 4°C by keeping the sample container in plastic bucket containing ice. The suspension was then centrifuged at 10,000 g for 8 min. at 4°C. The clear supernatant was collected after dialysis against 10 mM Tris-HCl buffer, pH 7.4 for 24 hours at 4°C and was used as crude enzyme extract.

### **Purification of protein:**

**DEAE-cellulose(Diethyl amino ethyl) Chromatography:** The crude enzyme extract was dialyzed against distilled water for 12 h and against 10 mM Tris-HCl buffer, pH 8.4 for 18 hours at 4°C and then loaded onto the DEAE-cellulose column previously equilibrated with the same buffer. The protein was eluted from the column with the same buffer containing different concentrations of NaCl. The fractions were collected on an automatic fraction collector and monitored for protein at 280 nm.

**CM-cellulose (carboxy methyl) Chromatography:** The active fraction of enzyme obtained after DEAE-cellulose chromatography was dialyzed 12 h against distilled water and then overnight against 5 mM sodium phosphate buffer, pH 6.5 at 4°C. The dialyzed sample was loaded onto the CM-cellulose column at 4°C. The proteins were eluted from the column with the same buffer as well as with the buffer containing NaCl.

**Polyacrylamide disc gel electrophoresis:** The homogeneity of the protein was judged by polyacrylamide disc gel electrophoresis conducted at room temperature, pH 8.5 on 7.5% gel as described by Ornstein.<sup>6</sup> The gel was stained with 1% Amido black 10B in 7.5% acetic acid for an hour at room temperature and destaining was performed by washing the gel in 7% acetic acid (v/v) solution.

**Assay of protease activity:** Protease activity was measured following the method of Kunitz<sup>7</sup> using casein as substrate. The activity was determined by detecting the release of amino acids (tyrosine) and the amount of tyrosine released was calculated from the standard curve constructed with tyrosine. One unit of protease activity was defined as the amount required for liberating 1 mg of tyrosine per minute at 45°C.

### **Characterization of protein**

**Molecular mass determination by gel filtration:** The molecular mass of the protein was determined by gel filtration on Sephadex G-150 (0.75 × 100 cm) according to the method of Andrews<sup>8</sup> using b-galactosidase from *E. coli*, bovine serum albumin, a-amylase from *Bacillus subtilis*, ovalbumin, trypsin inhibitor from corn kernels and lysozyme as reference proteins.

**Molecular mass determination by SDS-PAGE:** SDS-PAGE was conducted on 10% polyacrylamide gel according to Weber and Osborn<sup>9</sup> and the marker proteins used were b-galactosidase from *E. coli*, Bovine serum albumin, a-amylase from *Bacillus subtilis*, trypsin inhibitor from corn kernels and lysozyme. Dissociation and reduction of proteins were performed by heating for 5 min at 100 °C in 0.1% SDS with 0.1% 2-mercaptoethanol and the proteins were stained with Coomassie Brilliant Blue R-250.

*Protein content and estimation of sugar:* The concentration of protein was measured by the method of Lowry<sup>10</sup> using BSA as the standard and the protein in column eluate fractions was also monitored spectrophotometrically at 280 nm. The total neutral carbohydrate content of the purified proteins was determined by the phenol-sulfuric acid method of Dubois<sup>11</sup> with D-glucose as the standard.

*Determination of  $K_m$  value of the protease:* The initial velocity is equal to the amount of product formed per unit time. The initial velocity ( $V_0$ ) was determined by measuring quantitatively the amount of one of the products at various time intervals by the method of Robyt and White.<sup>12</sup> The supernatant were collected separately and their absorbances were taken at 275 nm against the reagent blank.

*Determination of optimum pH of the protease:* For the determination of the optimum pH, the activity of protease was measured at different pH values (4 -10) at 45°C following the procedure as described above. The buffer used was of 0.1M concentration.

*Determination of effect of temperature on protease activity:* The activity of the protease was measured at different temperatures using 10mM Tris-HCl buffer, pH 8.0, following the procedure as described earlier. The reaction mixtures were incubated at 20°C, 30°C, 40°C, 50°C, 60°C, 70 °C, 80 °C, 90 °C, and 100°C for 30 mins and after cooling at room temperature, the activity of protease was measured at 45°C as described in materials and methods.

*Effect of metallic salts and denaturants on activities of protease:* Metallic salts and denaturants of different concentrations were added to the enzyme solutions (0.5%) in 10 mM Tris-HCl buffer, pH 8.0 and after one hour incubation at 50°C. The residual protease activities were measured as described earlier.

## RESULTS

### *Purification of intracellular protease*

#### *Ion-exchange chromatography on DEAE-cellulose*

The crude enzyme extract after dialysis against 10 mM Tris-HCl buffer, pH 8.4 at 4°C for 24 h. was applied to a DEAE-cellulose column at 4°C, previously equilibrated with the

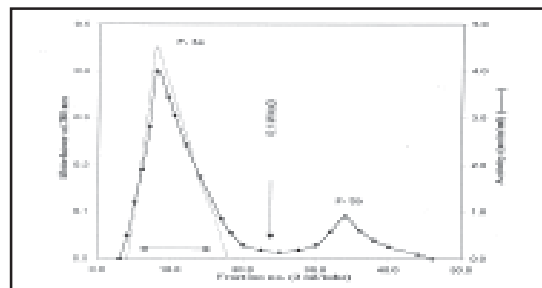


Fig-1: Ion-exchange chromatography of crude cell extract on DEAE-cellulose. The crude extract solution (40 mg) was applied to the column (2.1 × 20 cm) pre-equilibrated with 10 mM Tris-HCl buffer, pH 8.4 at 4°C and eluted by stepwise increase of NaCl concentrations in the same buffer. Flow rate: 45ml/hour

same buffer. The column bound proteins were eluted stepwisely with different concentrations of NaCl in the same buffer. As shown in Fig-1, the components of crude cell extract were separated into five major peaks; F-1, F-2, F-3, F-4 and F-5. Of these fractions, F-1 was eluted by the buffer only while F-2, F-3, F-4 and F-5, were eluted by the buffer containing 0.05 M, 0.11M, 0.22M, and 0.4 M NaCl respectively. Only F-3 fraction as indicated by solid bar possessed protease activity were pooled and used for further purification process. The remaining fractions were not used for experimental purposes as they contained no such property.

*CM-cellulose chromatography of F-3 fraction:* Pooled fractions of F-3 were dialyzed against distilled water and the eluting buffer (5 mM sodium phosphate buffer, pH 6.5) for 12 hr at 4°C. After centrifugation, the clear supernatant was applied to CM-cellulose column, previously equilibrated with the same buffer at 4°C and eluted with the same buffer also. As shown in the Fig-2, the fraction F-3 was separated mainly into one major fraction, F-3a which was eluted by the buffer only, while another minor fraction, F-3b was eluted from the column by the same buffer containing 0.1M NaCl. Significantly the fraction F-3a contained only protease activity and the area as indicated by solid bar was pooled separately and its purity was checked by polyacrylamide disc gel electrophoresis. This fraction was impure as it produced more than one bands on the gel and therefore was subjected to rechromatography on DEAE-cellulose.

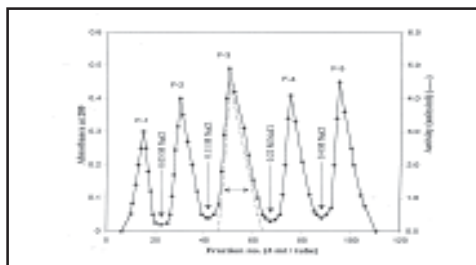


Fig-2: CM-cellulose chromatography of F-3 fraction obtained after ion-exchange chromatography on DEAE cellulose. Fraction F-3(8mg) was applied to the column (1.5×10cm) pre-washed with 5m M sodium phosphate buffer, pH 6.5 at 4°C & eluted by the buffer as well as with the buffer containing 0.1M NaCl. Flow rate: 45ml/hour

**Rechromatography on DEAE- cellulose:** The F-3a fraction was dialyzed first against distilled water for 12 h and then 10 mM Tris-HCl buffer, pH8.4 for 12h and was then rechromatographed on DEAE-cellulose under identical condition as described previously. The protein of F-3a fraction was found tightly bound to the column and eluted as three sharp peaks with different concentrations of NaCl (Fig-3 a). Only the fraction F-3a<sub>ccc</sub> contained protease activity, which was eluted from the column by the buffer containing 0.11M NaCl. This fraction contained pure protein as it produced a sharp single band on 7.5% polyacrylamide gel (Fig-3 b) The data pertaining to the purification of protease from *Pseudomonas aeruginosa* has been summarized in (Table-I). Although the activity as well as yield of the protein was decreased at each subsequent purification steps but their purification fold were increased and finally the purification of protease activity was increased to 9.23 fold.

**Characterization of Protease**

**Determination of molecular weight by gel filtration:** The molecular weight of the protease purified from *Pseudomonas aeruginosa*, as determined by gel filtration on Sephadex G-150, was estimated to be about 48,000.

**Molecular weight and subunit structure of the protease:** The molecular weight of the protease and its subunit were also determined by SDS-PAGE at pH 7.2 on 10% gel and was estimated to be about 49,000. Photographic representation of the electrophoretic patterns of the marker proteins and the purified protein have presented in Fig-4.

Table-I: Protease activity in the course of purification steps from *Pseudomonas aeruginosa*.

Steps of purification	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification fold
Crude cell extract	40	104	2.6	100	1
DEAE-cellulose chromatography	8	75	9.4	72.1	3.62
CM-cellulose chromatography	5	66	13.2	63.5	5
DEAE-cellulose rechromatography	2	48	24	46.2	9.23

Further, the protease gave single band on the SDS gel when the enzyme was treated with or without  $\beta$ -mercaptoethanol and the molecular weight of the protease was found to be identical, i.e., about 49,000, indicating that the protease contained only one subunit.

**Optical density versus concentration relation of the purified protease:** The absorbance of 1.0 at 280nm for protease was found to be equal to 0.575mg of protein, as determined by the Lowry method<sup>8</sup> using BSA as standard. This value was slightly higher when the amount of protein was determined after drying the protein solution by heating under vacuum (0.6mg). **Sugar content of the purified protease:** The purified protease gave slight yellow orange color in the presence of phenol-sulfuric acid<sup>11</sup>, indicat-

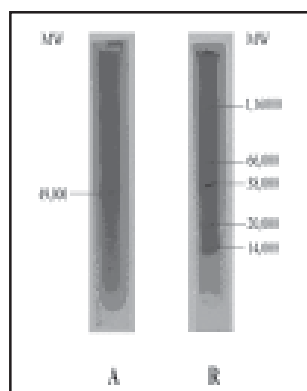


Fig-3(a): Rechromatography of F-3a fraction on DEAE-cellulose obtained after CM-cellulose chromatography. F-3a fraction (5 mg) was applied to the column (1.5 × 10 cm) pre-washed with 10 mM Tris-HCl buffer, pH 8.4 at 4°C and eluted by stepwise increase of NaCl concentrations in the same buffer. Flow rate : 45 ml / hour. 3(b): Polyacrylamide disc gel electrophoretic pattern of purified protease (F-3a<sub>ccc</sub> fraction) on 7.5% gel. The experimental conditions have been described in Materials and Methods.

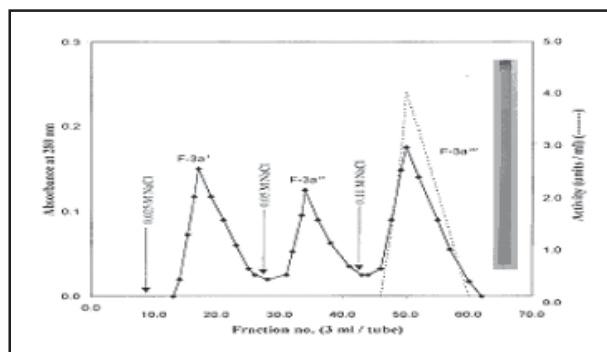


Fig-4: SDS-polyacrylamide disc gel electrophoretic pattern of purified protease and Reference protein on 10% gel. The experimental conditions have been described in Materials and Methods. A=Purified protease, R=Reference proteins.

ing that it contained sugar and the percentage of neutral sugar was calculated about 0.6%.

*Km value of the intracellular protease:* Protease activity was tested using different concentrations of casein solution as substrate and the Km value of the purified enzyme was determined by Lineweaver-Burk double reciprocal plot was calculated to be 0.48%. (Figure not shown)

*Effect of pH:* The activity of intracellular protease from *Pseudomonas aeruginosa*, was determined using various buffers such as sodium acetate (pH 4-5), sodium phosphate (pH 6-7) and Tris-HCl (pH 8-10) of 0.1M concentrations. As shown in Fig.5 (a), the enzyme is more active near neutral to slightly alkaline pH and gave maximum activity around pH 8.0. The activity of the enzyme decreased rapidly at the acidic as well as more alkaline pH values and the enzyme lost its activity about 90% and 64% at pH 4 and 10 respectively.

*Effect of temperature:* As presented in Fig. 5(b), the activity of protease increased rapidly with the rise of temperature and gave optimum activity at 50°C. Further, the enzyme was found to be more than 80% active up to the temperature 60°C. With further rise of temperature the activity was destroyed drastically and the enzyme lost more than 95% of its activity at 90°C.

*Effect of EDTA and metallic salts:* Some enzymes that require metal ions for activity are inhibited non-covalently by agents capable of binding the essential metal. The present data indicated that the activity of intracellular protease decreased sequentially with the increase in

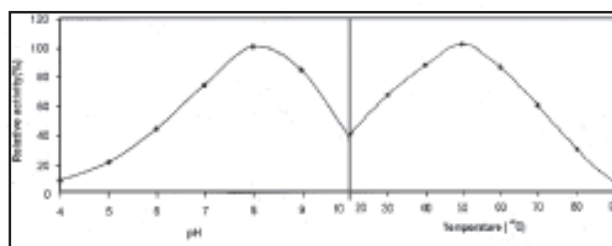


Fig-5(a): Effect of pH on protease activity.

Fig-5 (b): Effect of temperature on protease activity.

concentration of EDTA and the enzyme lost about 92% of its activity at 4 mM EDTA concentration (Table-II).

*Effect of metallic salts on protease activity:* Table-II also represents the effect of various metallic ions on the activity of protease. From data presented in the table, it is evident that the presence of  $Hg^{2+}$  and  $Cu^{2+}$  potentially inhibited proteolytic activities of the protease while activities were inhibited moderately in the presence of  $Mn^{2+}$  salts. The activities of the protease was found to be increased greatly at lower concentration of ascorbic acid but at higher concentration the activity was not increased further while decreased greatly. Proteolytic activity

Table-II: Effect of various metallic salts on protease activity

Salts	Concentration in molar	Relative activity (%)
None	—	100
EDTA	0.001	54.9
	0.002	29.5
	0.004	8.0
$CaCl_2$	0.001	102.2
	0.002	104.4
	0.004	91.2
$CuCl_2$	0.001	38.5
	0.002	33
	0.004	12
$K_2SO_4$	0.001	137.4
	0.002	148.4
	0.004	166
$MnSO_4$	0.001	74.7
	0.002	71.4
	0.004	58
NaCl	0.001	126.4
	0.002	131.8
	0.004	164.8
Ascorbic acid	0.001	208.8
	0.002	181.3
	0.004	137.4
$HgCl_2$	0.001	33
	0.002	1.0
	0.004	0.0

was also found to be increased sequentially with increase in concentration of  $\text{Na}^+$  and  $\text{K}^+$  salts but in the presence of  $\text{Ca}^{2+}$  salt the activity was found to be more or less very similar.

### DISCUSSION

An intracellular protease was isolated and purified to homogeneity from cells of *Pseudomonas aeruginosa* strain no 14 by ion-exchange chromatography using DEAE-cellulose, followed by CM-cellulose and rechromatography on DEAE-cellulose. The purified protease is a monomeric protein as it gave single band under both denaturing and reducing conditions in SDS-PAGE. The molecular weight of purified protease was estimated to be about 49,000 on SDS-PAGE and about 48,000 by gel filtration on Sephadex G-150. The purified protease was glycoprotein in nature as it gave yellow-orange colour in the presence of phenol-sulphuric acid.<sup>11</sup> The optimum pH and temperature for the proteolytic activity were pH 8.0 and 50°C, respectively, but the enzyme activity was significantly destroyed at pH 4.0 and pH 10 as well as above temperature of 90°C, suggesting that the enzyme might be denatured at these conditions or the structure of the enzyme disorganized at these conditions so that the enzyme can not bind with its substrate. The enzyme activity was strongly inhibited by the presence of  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Hg}^{2+}$ . Enzyme activity was also abolished sequentially with increasing concentrations of EDTA, indicating the importance of metallic ions being associated with the activity of the enzyme. This was also confirmed from the findings that the activity of the purified protease was increased in the presence of some metallic salts such as  $\text{K}^+$  and  $\text{Na}^+$ . From the inhibition of protease activity in the presence of  $\text{Hg}^{2+}$  it might be suggested that -SH group containing amino acids are located in or near the active site. Remarkably, we noticed much higher amount of proteolytic activity in the presence of ascorbic acid. An alkaline protease produced by *Pseudomonas aeruginosa* MN1, isolated from an alkaline tannery wastewater, was purified and characterized by Bayoudh.<sup>13</sup> The molecular weight

of the enzyme was estimated to be 32,000. 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 contained a metallo-protease. Enzyme activity was strongly inhibited by  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Hg}^{2+}$  (5 mM), while  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  resulted in partial inhibition. Another extracellular protease from *Pseudomonas aeruginosa* was purified by Elliott and Cohen.<sup>14</sup> The protease was a monomeric polypeptide of MW of about 30,000 and the pH optimum was 8-9.

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