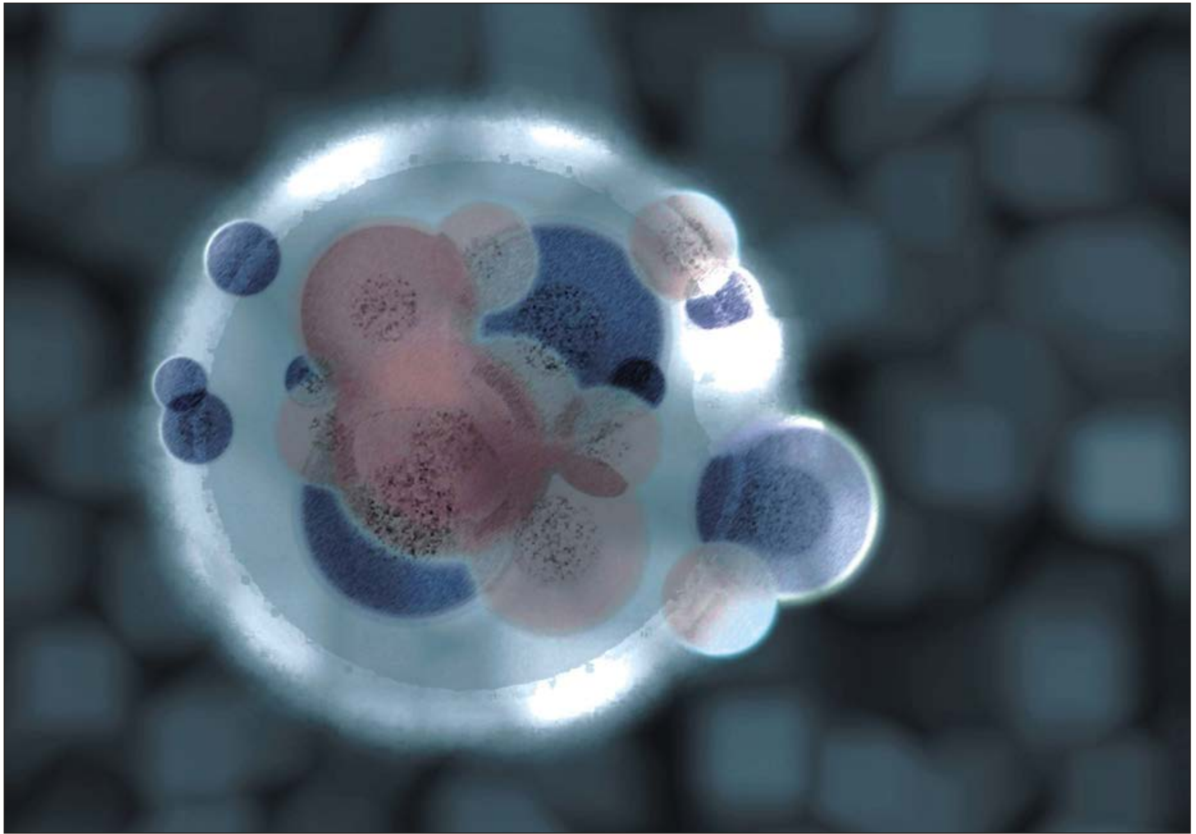

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Purification and Characterization of an Extracellular Protease from *Pseudomonas aeruginosa* Isolated from East Calcutta Wetland

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Abstract: In an earlier study, we had isolated and identified a protease producing bacterium as *Pseudomonas aeruginosa* from the Charakdanga Bheri waters of East Calcutta Wetland. The enzyme was concentrated by lyophilization and finally purified by hydrophobic interaction chromatography using Phenyl Sepharose CL-4B column resulting in 1.2 fold increase in specific activity and 28% recovery. The crude extracellular protein was run on a 12% SDS-PAGE; two bands were found, one between 43 and 66 kDa and another between 29 and 43 kDa. The molecular weight of the purified protein was 36.18 kDa as determined from ESI-Mass Spectroscopy; corresponding to the lower band of the crude sample. The zymogram showed a sharp zone of clearance corresponding to the lower band confirming protease activity. The optimum temperature for the protease activity was 40°C. The protease activity showed a wide range of pH tolerance with almost similar activity ranging from pH 5 to 8. The activity of the enzyme was totally lost in the presence of chelating agent EGTA (10 μM), Phosphoramidone (500 μg mL^{-1}) and 1,10 Phenanthroline (2 μM), suggesting the purified enzyme to be a metalloprotease, while other protease inhibitors had no effect on the enzyme activity. Moreover, since phosphoramidone is a metallo-endoprotease inhibitor, this finding was further confirmed. The endoprotease nature was confirmed through BSA digested banding pattern generation. The kinetics of protease activity revealed that BSA was completely digested within 8 to 9 min and a minimum of 5 μL (6 mg mL^{-1}) of crude protease soup was required for digestion of 50 μg BSA solution.

Key words: Bheri, metalloprotease, endoprotease, hydrophobic interaction chromatography, aprA gene

INTRODUCTION

Proteases represent an important class of enzymes which constitute more than 50% of the total enzyme market (Rao *et al*., 1998). Proteases execute a large variety of functions and have important biotechnological applications; such as laundry detergents, leather preparation etc. (Cowan, 1996). The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases (Rao *et al*., 1998). Moreover, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that in turn is desirable for their various applications (Najafi *et al*., 2005). Recently, the application for proteases to the production of certain oligopeptides has received great attention as a viable alternative to the chemical approach (Kunugi and Yoshida, 1996; Murakami *et al*., 1996; Rival *et al*., 1999; Jellouli *et al*., 2008). *Pseudomonas aeruginosa* is a Gram negative bacterium that has already been reported to produce various proteolytic enzymes (Morihara *et al*., 1965; Ogino *et al*., 1999; Shastry and Prasad, 2002; Gupta *et al*., 2005). Among the numerous extracellular products of *P. aeruginosa*, elastase and alkaline protease are two well characterized metalloproteases (Hase and Finkelstein, 1993; Jellouli *et al*., 2008). Metalloproteases are the most diverse of the catalytic types of proteases. They are characterized by the requirement for a divalent metal ion for their activity. About 30 families of metalloproteases have been recognized, of which 17 contain only endopeptidases, 12 contain only exopeptidases and 1 contain both endo and exo peptidase (Rao *et al*., 1998). Among the different extracellular protease produced by *Pseudomonas aeruginosa* elastase (las B) and alkaline protease (apr A) are two important and well characterized enzymes (Kim *et al*., 2006). Both of these proteases are metalloprotease in nature (Marquart *et al*., 2005). These

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two protease are reported to be produced more in iron deficient conditions and are able to destroy transferrin, a major iron-withholding protein in human body fluids (Kim *et al*., 2006). Elastase is a protease responsible for degradation of elastin and collagen (Bever and Iiglewski, 1988). Alkaline protease produced by *Pseudomonas aeruginosa* is reported to be related with corneal infection (Guzzo *et al*., 1990) and can cleave transferrins with the production of siderophores and thus helps itself to overcome iron deficiency during human infection (Kim *et al*., 2006).

This study deals with the characterization and purification of an extracellular protease from *Pseudomonas aeruginosa* strain SRC-002 isolated from East Calcutta Wetland. As evident from the literature cited, there are various reports of protease purification from *Pseudomonas aeruginosa*, but the reason for undertaking this study was the ability of this particular enzyme for dehairing of goat hide at neutral pH (Unpublished data). The strain was found to produce extracellular metalloprotease having size of 36.18 kDa.

MATERIALS AND METHODS

The consumables were funded by Department of Biotechnology as project student fund (2008) and Department of Atomic Energy under the BRNS scheme (2006-2010). This work was conducted during Jan 2008 till April 2009. The fellowship was from IUC-DAE as well as DBT, India.

Isolation of strain and its complete characterization: Water samples were collected from Charakdanga Bheri at East Calcutta Wetland (RayChaudhuri and Thakur, 2006; RayChaudhuri *et al*., 2007, 2008) and spread on milk medium plate containing 10% double toned milk, 0.3% Yeast Extract (Himedia), 1.5% Agar (Himedia) to screen for protease secreting bacteria. Further cultivation and maintenance of pure isolate was done in Luria Bertani (LB) broth containing 1% tryptone (Himedia), 0.5% Yeast Extract (Himedia), 0.5% Sodium Chloride (Himedia) and 1.5% Agar (Himedia). Cultures were grown overnight in an incubator shaker at 37°C with shaking at 150 rpm. Strain was stored at -80°C as glycerol stock and streaked weekly on L.B. agar plates for routine use. Complete characterization of the isolate, namely morphological; biochemical (enzymatic assays chiefly protease, lipase, DNase, catalase and oxidase); physiological (optimum pH and temperature, growth profile, antibiotic sensitivity) and molecular analysis (partial 16S rDNA sequencing followed by phylogenetic analysis using neighbour joining method) was done according to the protocol of Nandy *et al*. (2007) and reported elsewhere (Chowdhury *et al*., 2008).

Characterization of crude protease: The preliminary qualitative detection of the enzyme was by the appearance of clear zone on the milk medium plates due to the degradation of milk protein casein as a result of the action of extracellular enzyme. The protein amount was estimated by standard Lowry method (Lowry *et al*., 1951) and the quantitative estimation of enzyme produced was done by azocasein assay as reported by Malathu *et al*. (2008). One unit of enzyme activity was defined as the amount which yielded an increase in A_{440} of 0.1 in 20 min at 60°C.

The supernatant containing extracellular protease was obtained by centrifugation of overnight grown culture at 17,000 g for 10 min. It was adjusted to different pH (1-12) using HCl or NaOH, incubated for 12 h at 37°C and activity was measured by azocasein assay. Similarly, the effect of temperature on protease activity was studied by overnight incubation of the extracellular supernatant at different temperatures ranging from 4 to 80°C. To understand the nature of protease, the extracellular supernatant was incubated with various inhibitors like Antipain (20 μM), Bestatin (50 μg mL^{-1}), Chymostatin (20 μM), E-64 (2 μM), 1,10 Phenanthroline (2 μM), Pepstatin (1 mM), Phosphoramidone (500 μg mL^{-1}), EGTA (10 μM) and Ebelacetone-B (1 μM) at 37°C for 30 min followed by measurement of protease activity by azocasein assay. This inhibitor study was also repeated with the purified enzyme. In order to determine the proteolytic activity of the enzyme (endopeptidase or exopeptidase), 25 μL of Bovine Serum Albumin (stock concentration 5 mg mL^{-1}) was incubated with 50 μL of crude protease at 60°C for 30 min and run on a 12% SDS-PAGE with BSA as a control. For determining the kinetics of enzyme action, 50 μL of crude enzyme was incubated with the substrate (25 μL of BSA, stock concentration 5 mg mL^{-1}) for 1, 2, 3, 5, 7, 8, 9 min; enzyme action was terminated by heating at 100°C for 5 min followed by 12% SDS-PAGE to analyze the banding pattern. The minimum amount of protease required to degrade BSA was also checked by incubating 5, 10, 15, 20, 25, 30, 35 and 40 μL of crude supernatant with 50 μg of substrate for 15 min and the degradation profile was checked by SDS-PAGE. Twenty five micrograms of BSA was loaded in each lane.

Determination of molecular nature of protease: Genomic DNA was isolated from the strain by modified alkali lysis method (Adarsh *et al*., 2007). PCR amplification of the aprA gene fragment was done using the aprA-specific

primer set (5'-TACTCGCTGGGCAAGTTCAGCG-3' and 5'-GTAGCTCATCACCGAATAGGCG-3') under the following condition: hot start at 94°C for 5 min; 35 amplification cycles (denaturation at 94°C for 30 sec; annealing at 59°C for 30 sec; extension at 72°C for 30 sec); and a final extension at 72°C for 5 min (Kim *et al*., 2006). After PCR the amplicon was run in a 2% agarose gel with a 100 bP DNA ladder (Cat. No. SM0623, Fermentas) and then stained with ethidium bromide (0.01 μg mL^{-1}) before visualization over a transilluminator.

Purification of the enzyme by hydrophobic interaction chromatography: The supernatant (after centrifugation at 17000 g for 10 min at 4°C) was filtered through 0.45 μm pore size Whattman filter to remove any traces of cell. The extracellular supernatant containing protease was lyophilized for 1 h (Maxi drier lyophilizer, heto vacuum centrifuge at 2000 rpm); then dissolved in binding buffer [20 mM phosphate buffer (pH-7) + 50%ammnium sulfate] and loaded on previously pre-equilibrated [20 mM phosphate buffer (pH-7.0)] Phenyl Sepharose CL-4B column (12.21 cm^3 bed volume). The column was washed with 20 mM phosphate buffer and proteins were eluted by decreasing the concentration of salting out ions, i.e., of ammonium sulfate from 50 to 0%, while maintaining a flow rate of 1.5 mL min^{-1}. Each fraction was collected and the protein containing fraction was determined by measuring absorbance at 280 nm; the amount of protein was determined by Lowry *et al*. (1951), while the enzyme activity was measured by azocasein assay.

Determination of molecular weight and zymography: A 12% Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) was run to determine the protein profile post purification, by comparing the relative mobility of the protein with that of a standard molecular weight marker by Bangalore Genei, India, (PMWss) consisting of phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), bovine carbonic anhydrase (29 kDa), lactoglobulin (18.4 kDa) and bovine α-lactalbumin (14.3 kDa). The details of SDS-PAGE composition, the run conditions were same as reported earlier (Chowdhury *et al*., 2008). For the detection of protease enzyme among the total protein fraction, the crude enzyme as well as the partially purified one, was subjected to an activity gel (zymogram), the detailed procedure was as followed by Chowdhury *et al*. (2008).

Enzyme kinetics study of purified protease: To determine the kinetics of the enzyme, protease assay was done using hide powder azure as substrate. Hide powder azure is a blue fibrous substrate where hide powder is coupled with a dye remazol brilliant blue. Protease degrades the hide powder azure and releases dye-bound peptides into solution. Protease activity is measured by reading the absorbance of the supernatant liquid at 595 nm. The 0.5 mL (1.25 Units) of purified enzyme was added to different substrate concentration (5-100 mg) in 1.5 mL of assay buffer [50 mM Tris-HCl (pH-8), 1 mM $CaCl_2$]. Then they were incubated at 28°C for 1 h. The supernatant was collected by centrifugation at 10000 g for 10 min and absorbance was measured at 595 nm.

RESULTS AND DISCUSSION

Characterization of crude protease: The pH profile of the crude enzyme showed almost similar activity along the entire range of 5-8 with an optimum at 7 (Fig. 1). The enzyme assay at different temperature also demonstrates activity over the entire range of 4 to 50°C with maximum activity at 40°C (Fig. 2).

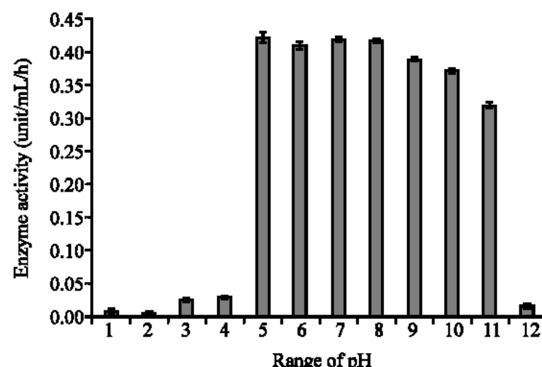

Fig. 1: Graph depicting the variation in protease activity over a wide range of pH (1 to 12) as measured by azocasein assay; after 12 h of incubation of the extracellular supernatant adjusted to the different pH at 37°C

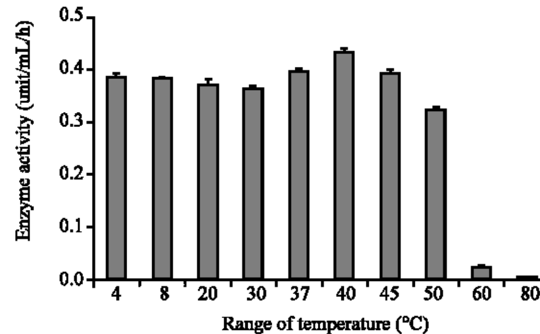

Fig. 2: Graph representing the thermostability of the enzyme; as measured by azocasein assay post overnight incubation of the extracellular supernatant at temperatures ranging from 4 to 80°C

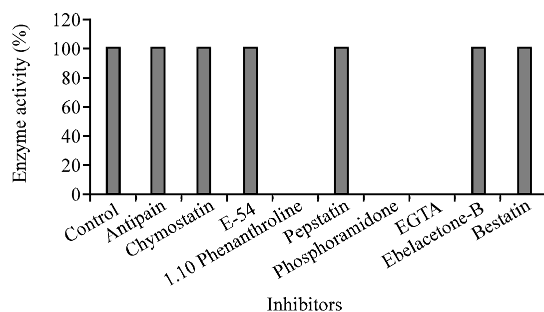

Fig. 3: The influence of various inhibitors on the activity of the enzyme. The extracellular supernatant was incubated with different concentrations of inhibitors at 37°C for 30 min followed by measurement of protease activity by azocasein assay. The enzyme activity was completely lost in presence of metalloprotease inhibitors

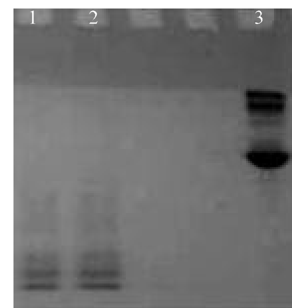

Fig. 4: Distinct banding pattern as evident from protease digested BSA samples in lane 1 and 2 suggests that the enzyme is an endopeptidase. Lane 3 contains only BSA. 25 μL (5 mg mL^{-1}) of BSA was incubated with 50 μL crude protease at 60°C for 30 min and run on a SDS-PAGE using BSA as a control

In case of inhibitor study, EGTA (10 mM); 1,10 Phenanthrolin (2 mM) and Phosphoramidone (500 μg mL^{-1}) completely inhibited protease activity (Fig. 3) while Antipain (20 μM); Bestatin (50 μg mL^{-1}); Chymostatin (20 μM); E-64 (2 μM); Pepstatin (1 μM) and Ebelacetone-B (1 μM) had almost no effect on the activity of protease. As all three inhibitors which inhibit protease activity are metalloprotease inhibitors, thus the protease was considered to be a metalloprotease. This experiment was also repeated using purified enzyme, which yielded exactly similar results. This is in confirmation with the earlier report indicating the production of metalloprotease by *Pseudomonas aeruginosa* (Jellouli *et al*., 2008). The protease digested BSA banding pattern reveals it to be an endopeptidase and from the kinetics study it is clear that BSA was completely digested within 8-9 mins and a minimum of 5 μL (6 mg mL^{-1}) of crude supernatant was required for digestion of 50 μg of BSA solution (Fig. 4).

Determination of molecular nature of protease: The aprA gene amplification was evident for the isolate. The size of the amplicon was around 400 bp as compared to 100 bp DNA ladder (Fig. 5a). The gene was sequenced and found to be novel and was submitted to Genbank under the accession number GQ202011 (Fig. 5b). From phylogenetic analysis it was observed that the strain was closely related to *Pseudomonas aeruginosa*.

Purification of the enzyme by hydrophobic interaction chromatography: The *P. aeruginosa* protease was purified by hydrophobic interaction chromatography; the graph (Fig. 6) of absorbance at 280 nm indicates that the wash fraction as well as fraction numbers 11 to 14 show

Table 1: Summary of the purification of *P. aeruginosa* protease

Methods	Total enzyme unit (U)	Total protein (mg)	Activity yield (%)	Specific activity (U mg^{-1})
Crude	1850	300	100	6.16
Lyophilization	755	294	40	2.56
Phenyl sepharose column	370	50	28	7.40

an increase in absorbance, i.e., these fractions contain protein. The azocasein activity showed that the wash fraction has no activity but fraction 11 and 12 have distinct azocasein activity, i.e., presence of protease (Fig. 7). The results of the purification process were summarized in Table 1. The enzyme was purified 1.2 fold with a recovery of 28% and a specific activity of 7.4. The gel showed two distinct bands in case of crude sample, one was between 66 and 43 kD and second was between 43 and 29 kD. However the purified protein obtained after hydrophobic interaction chromatography was homogeneous on SDS-PAGE and its molecular weight was estimated to be around 36 kDa (corresponding to the lower band of crude sample) (Fig. 8) and also showed a clearing zone (Fig. 9) in the activity gel containing gelatin as a substrate; while the upper band which was present in the crude extracellular supernatant did not show any clearing zone in the activity gel. The molecular weight of the purified protein was further confirmed to be 36.18 kDa by ESI Mass Spectroscopy.

Enzyme kinetics study of purified protease: Enzyme kinetics study revealed the presence of either two isozyme of the protease or two different protease enzymes. One of the enzyme acts at low substrate concentration while the other was found to be active at higher substrate

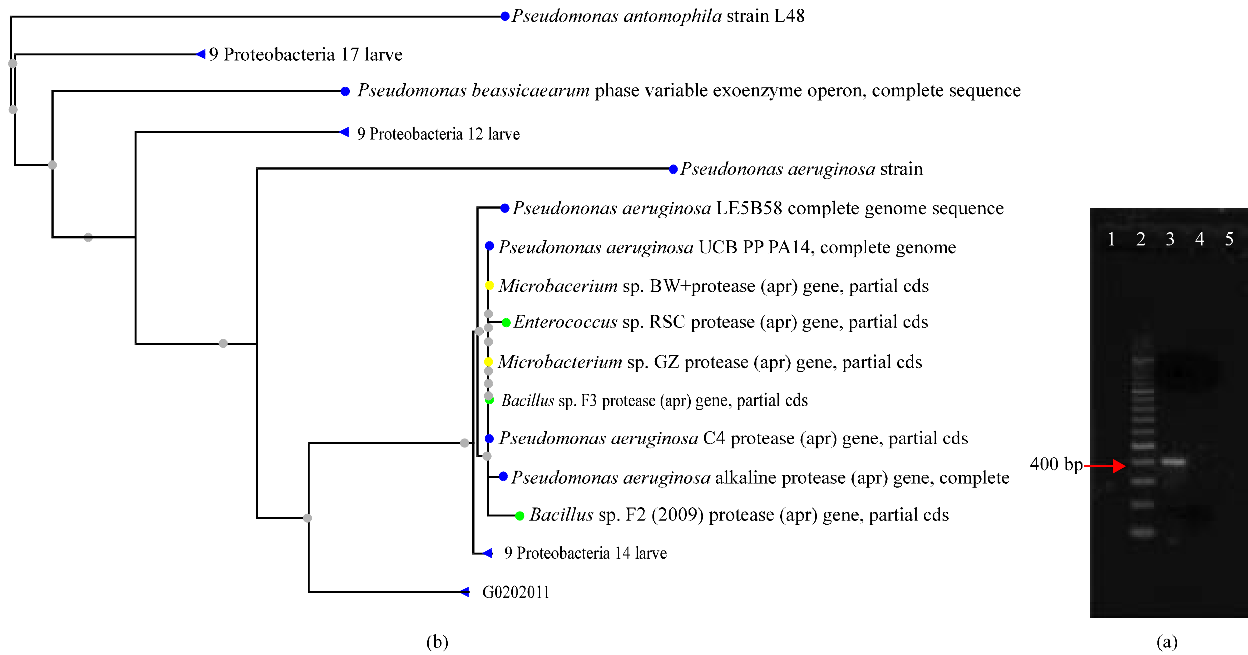

Fig. 5: (a) Photograph of ethidium bromide stained 2% agarose gel run at 100 V cm^{-2} for 1.5 h showing the amplification product of aprA gene generated using aprA specific primer sets. The primer specifications and the amplification conditions were as reported by Kim *et al*. (2006). The samples were loaded as follows: Lane 2-100 bp DNA ladder (Cat. No. SM0623, Fermentas), lane 3-SRC-002 amplified product, lane 4-negative control in which the DNA template was absent. The 400 bp product corresponding to apr A gene was amplified in SRC-002 strain. (b) Phylogenetic analysis of isolate based on sequencing of aprA gene. The phylogenetic trees were constructed using neighbour joining method

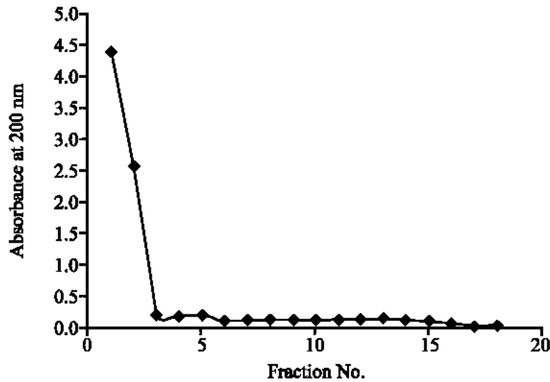

Fig. 6: Protein estimation of each fraction after hydrophobic interaction chromatography (using Phenyl Sepharose CL-4B column) was done by measuring the absorbance at 280 nm. The graph indicates that washout as well as fraction number 11 and 12 show an increase in absorption i.e., these fractions contain protein

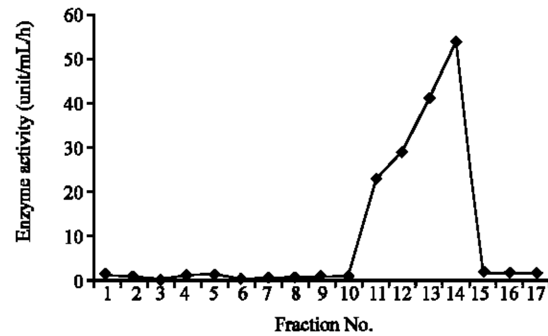

Fig. 7: The enzyme activity of the fractions of Phenyl Sepharose CL-4B determined by azocasein assay. The graph of azocasein assay clearly shows that the wash fraction has protein but no enzyme activity while fraction number 11 and 14 have definite protease activity

concentration. The K_m value of these two protease determined from Lineweaver-Burk plot were 18.18 and 46.5 mg mL^{-1} (Fig. 10).

The study reports the purification and characterization of an extracellular protease from an isolate of *Pseudomonas aeruginosa* SRC-002 from Charakdanga Bheri (waste water fed fish pond) at East Calcutta Wetland in India. Its pH tolerance is within pH 5 to 8 with

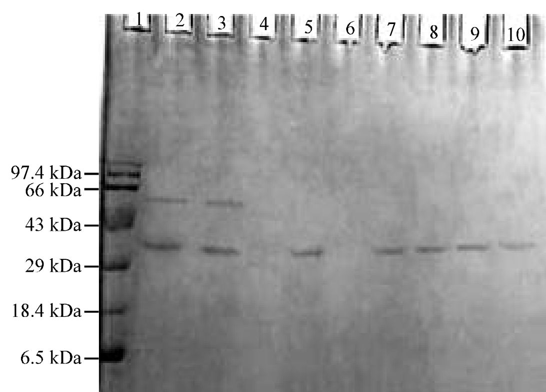

Fig. 8: Twelve percent SDS-PAGE of crude supernatant (lane 2 and 3); and purified sample (lane 4, 7, 8, 9, 10) after hydrophobic interaction chromatography. The gel image clearly depicts that the sample before purification has 2 bands [one between 66 to 43 kDa (upper band) and another between 43 to 29 kDa (lower band)], while after chromatography it shows a single band corresponding to the lower band of the crude supernatant, i.e., lying between 43 and 29 kDa (equal to 36.18 kDa approximately) as compared to the ladder in lane 1 from Bangalore Genei (PMWss)

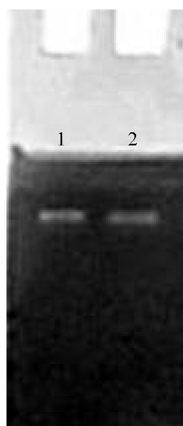

Fig. 9: Activity gel of purified sample (lane 1 and 2) using gelatin showing clearing zone corresponding to the lower band of the crude sample (approximately 35 kDa, as determined by SDS-PAGE analysis of the purified sample). The activity gel confirms the fact that the purified protein has protease activity

optimum at pH 7. Most proteases from *P. aeruginosa* exhibit optimum pH within the range of 7 to 9 (Ogino *et al*., 1999; Gupta *et al*., 2005). *Pseudomonas aeruginosa* PST-01 was reported to have optimum pH 8.5 (Ogino *et al*., 1999) while strain PseA was found to be

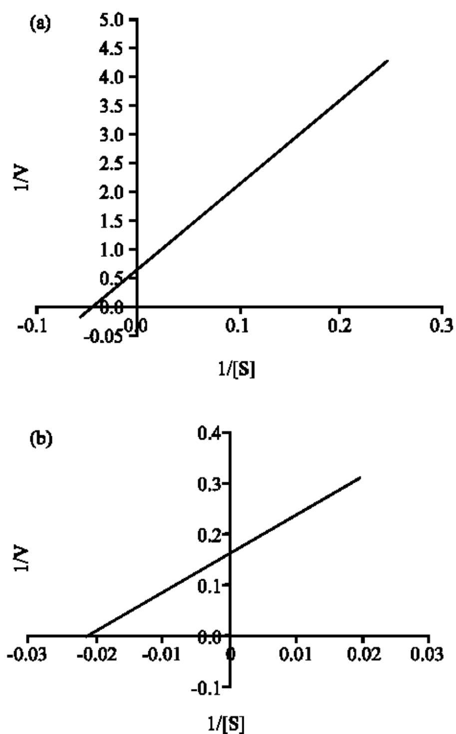

Fig. 10: Graphs representing double reciprocal (Lineweaver-Burk) plot of enzyme kinetics. Inverse of initial rate of reaction (expressed in terms of optical density at 595 nm due to the release of dye remazol brilliant blue as a result of substrate (hide powder azure degradation due to enzyme substrate reaction) was plotted on Y axis against the inverse of initial substrate concentration (mg mL^{-1}) along the X axis. 0.5 mL (1.2 Unit) of partially purified enzyme was added to different substrate concentrations (5-100 mg) in 1.5 mL assay buffer. The reaction mixture was incubated at 28°C for 1 h, supernatant were collected by centrifugation at 10000 g for 10 min and absorbance were measured at 595 nm. A wide range of substrate concentrations (5-100 mg) were selected for the assay. (a) represented the activity of protease at lower substrate concentration while (b) represented the activity of protease at higher substrate concentration. Data obtained from enzyme kinetics study were suggestive of the presence of either two isozymic forms the protease or two different enzyme. One of the enzyme acts at low substrate concentration while the other was found to be active at higher substrate concentration. The K_m value of these two protease determined from Lineweaver-Burk plot were 18.18 and 46.5 mg mL^{-1}, respectively

stable within pH 6.0-9.0 with optimum activity at pH 8.0 (Gupta *et al*., 2005). A wide pH resistance of the protease indicates the absence of ionic interactions in the catalytic site as well as conformational stability of the enzyme. On the other hand, enzyme activity over a wide pH range would be important for commercial application of this enzyme. This optimum temperature (40°C) and range of temperature tolerance (4 to 50°C) is much lower than the values reported earlier for proteases from *P. aeruginosa* strains (Ogino *et al*., 1999; Gupta *et al*., 2005). *Pseudomonas aeruginosa* PST-01 was reported to have optimum temperature of 55°C (Ogino *et al*. 1999) and the optimum temperature for protease activity from strain PseA was 60°C (Gupta *et al*., 2005). *Pseudomonas aeruginosa* strain DN1 was reported to grow at 42°C (Ningthoujam and Shovarani, 2008). The strain was found to have the gene for alkaline proteases. Though the extracellular protease from this strain works in neutral pH range, it contains the gene for alkaline protease.

The protease was purified through hydrophobic interaction chromatography. Tang *et al*. (2010) recently purified an organic solvent tolerant, alkaline metalloprotease from *Pseudomonas aeruginosa* PT121 in a single step by hydrophobic interaction chromatography on a phenyl sepharose matrix. The molecular weight of the purified protein was found to be 36.18 kDa as confirmed by ESI Mass Spectroscopy. Many of the reported protease from *Pseudomonas aeruginosa* was found to have similar size range. Nafaji and his coworker described thermostable, alkaline proteases of size 36 and 38 kDa from *Pseudomonas aeruginosa* strain PD100 (Najafi *et al*., 2005). Ogino *et al* reported metalloprotease having molecular mass of 38 kDa from *Pseudomonas aeruginosa* PST-01 (Ogino *et al*., 1999). An intracellular protease from *Pseudomonas aeruginosa* having size of about 48-49 kDa was purified by Begum and co workers (Begum *et al*., 2007). The protease from *Pseudomonas aeruginosa* PseA strain was reported to have molecular mass of 35 kDa (Gupta *et al*., 2005). Inhibitor study on protease from this strain indicated it to be a metalloprotease. There are several reports available of production of metalloprotease from a number of different *Pseudomonas*. *Pseudomonas aeruginosa* san-ai strain was reported to produce an alkaline metalloprotease having Zn at its active center having size of 18 kDa (Karadzica *et al*., 2004). An alkaline zinc metalloprotease from *Pseudomonas aeruginosa* was reported by Baumann *et al*. (1993).

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