Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1069 (2005) 155–161
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Purification and characterization of a solvent stable protease from *Pseudomonas aeruginosa* PseA

Anshu Gupta, Ipsita Roy, S.K. Khare*, M.N. Gupta

Chemistry Department, Indian Institute of Technology, Delhi, Hauz Khas, New Delhi 110016, India

Received 3 November 2004; received in revised form 14 January 2005; accepted 28 January 2005

Abstract

A solvent tolerant *Pseudomonas aeruginosa* PseA strain was isolated from soil. It secreted a novel alkaline protease, which was stable and active in the presence of range of organic solvents, thus potentially useful for catalysis in non-aqueous media. The protease was purified 11.6-fold with 60% recovery by combination of ion exchange and hydrophobic interaction chromatography using Q-Sepharose and Phenyl Sepharose 6 Fast Flow matrix, respectively. The apparent molecular mass based on the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was estimated to be 35,000 Da. The enzyme was stable in the pH range of 6.0–9.0, the optimum being 8.0. The K_m and V_{max} towards caseinolytic activity were found to be 2.7 mg/ml and 3 μmol/min, respectively. The protease was most active at 60 °C and characterized as a metalloprotease because of its sensitivity to EDTA and 1,10-phenanthroline. It was tested positive for elastase activity towards elastin–orcein, thus appears to be an elastase, which is known as pseudolysin in other strains of *P. aeruginosa*. The protease withstands range of detergents, surfactants and solvents. It is stable and active in all the solvents having log *P* above 3.2, at least up to 72 h. These two properties make it an ideal choice for applications in detergent formulations and enzymatic peptide synthesis.

Keywords: *Pseudomonas aeruginosa*; Alkaline protease; Solvent tolerance; Elastase; Pseudolysin; Hydrophobic interaction chromatography

1. Introduction

Proteases constitute one of the most important groups of industrial enzymes [1]. They find diverse industrial applications in peptide synthesis, protein processing, leather, food, pharmaceutical, dairy and detergent industries [2–4] as they hydrolyze peptide bonds in aqueous environments and synthesize them in non-aqueous environments. Use of proteases in peptide synthesis has numerous advantages over chemical synthesis [5]. This approach requires protease preparations stable in the presence of organic solvents. Proteases in general are inactivated or give very low rate of reactions in non-aqueous media [6,7]. Search for solvent stable proteases, therefore, has been an extensive area of research [8]. Several physical and chemical methods viz. chemical modification [9], immobilization [10], protein engineering [11] and directed evolution [12] have been employed for the stabilization of enzymes in the presence of organic solvents [13,14]. However, if enzymes are naturally stable and exhibit high activities in the presence of organic solvents, such stabilization of enzymes is not necessary.

Though solvents are highly toxic to microorganisms, in recent years some organic solvent tolerant microbes have been reported. It is found that enzymes from these microbes are attuned to work under solvent rich environment, thus they produce solvent tolerant enzymes. Solvent stable proteases have been reported from solvent tolerant *Pseudomonas* sp. [8,15] and *Bacillus* sp. [16].

In a similar effort, we have isolated a solvent tolerant *Pseudomonas aeruginosa* PseA strain from the soil by cyclohexane enrichment (unpublished results). As compared to other solvent tolerant *Pseudomonas* sp. [17] this isolate secretes a novel solvent stable protease with higher specific activity. The present paper describes efficient purification of this protease by ion exchange and hydrophobic interaction chromatog-

* Corresponding author. Tel.: +91 11 2659 6533; fax: +91 11 2658 1073.
E-mail address: khare_sk@hotmail.com (S.K. Khare).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.01.080

raphy. The purified protease is characterized and compared with proteases isolated from other bacterial sources.

2. Experimental

2.1. Materials

Phenyl Sepharose 6 Fast Flow, α-chymotrypsin, *Aspergillus oryzae* protease were purchased from Sigma (St. Louis, MO, USA). Q-Sepharose was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Casein was a product of SISCO Research Laboratories (Mumbai, India). Media components were purchased from Hi Media Laboratories (Mumbai, India). All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Isolation of bacterial strain

Soil samples were collected from the proximity of a solvent extraction unit in New Delhi, India. Small amount of soil was suspended in sterilized distilled water and 100 μl of resulting suspension was spread on modified King's B medium agar [18] plate, which contained (g/l): peptone, 20; glycerol, 8; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 4 and agar, 20. pH of the medium was adjusted to 7.0 with 1 M NaOH. The medium plates were overlaid with about 7 ml of cyclohexane and incubated at 30 °C for 24 h. Microorganisms which formed colonies on the surface of plates covered with organic solvent were selected. Growing colonies were further purified by repeated streaking. The protease producers among purified colonies were detected by plating on skim-milk agar [16]. The microbes showing clear zones were identified as protease producers. The strain showing maximum zone diameter was selected as potent producer of the proteolytic enzyme and was maintained on nutrient agar slants at 4 °C for further studies. It was designated as strain PseA, and identified to be *Pseudomonas* sp. based on morphological and biochemical tests by the Microbial Type Culture Collection Facility, Institute of Microbial Technology (IMTECH), Chandigarh, India and *P. aeruginosa* based on further FAME analysis by the Microcheck Microbial Analysis Laboratory, Vermont, USA.

2.2.2. Culture conditions for protease production

Mother culture was prepared by inoculating a loopful of stock culture of *P. aeruginosa* in the medium (pH 7.0) containing (g/l): peptone, 5.0; yeast extract, 5.0; NaCl, 0.5, followed by incubation at 30 °C and 140 rpm. This overnight grown culture (0.5 ml) was used to inoculate 50 ml of protease production medium in 250 ml Erlenmeyer flask. The protease production media consisted of (g/l): peptone, 5.0; yeast extract, 5.0; NaCl, 0.5; gelatin, 10.0, adjusted to pH 7.0 with 1 M NaOH. The incubation was done at 30 °C in an orbital shaker at 140 rpm. After 48 h of growth, the cells were harvested by centrifugation at 11,000 × *g* and 4 °C for 10 min, and the supernatant thus obtained was used as crude enzyme preparation.

2.2.3. Protease assay

Protease activity was determined as described by Shimogaki et al. [3], using casein as the substrate. Enzyme solution (0.5 ml) was added to 3.0 ml of substrate solution (0.6% casein in 0.1 M Tris–HCl buffer, pH 8.0) and the mixture was incubated at 60 °C for 10 min. The reaction was stopped by addition of 3.2 ml of TCA mixture (containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid) and kept at room temperature for 30 min followed by filtration through Whatman filter paper no. 1. The absorbance of the filtrate was measured at 280 nm. One unit of protease activity is defined as the amount of enzyme required to produce 1 μg of tyrosine per minute under the conditions described above.

2.2.4. Protein estimation

Protein was estimated by dye-binding method [19], using bovine serum albumin as standard protein.

2.2.5. Purification of protease

2.2.5.1. Q-Sepharose anion exchange chromatography. Anion exchange chromatography was performed in batch mode on Q-Sepharose matrix (2.0 ml), which had been equilibrated with 50 ml of 0.02 M sodium phosphate buffer, pH 7.7. The crude protease preparation (2 ml, pH adjusted to 7.7) was added to the matrix and shaken in an orbital shaker (100 rpm) at 25 °C. The shaking was stopped after 1 h, followed by filtration under vacuum. The matrix and supernatant were thus separated. The separated matrix was repeatedly washed with the equilibration buffer (2.0 ml, each time) till no enzyme activity could be detected in the washings. It was observed that the protease did not bind to matrix and most of the activity was recovered in supernatant and washings. The proteolytic active fractions were pooled and subjected to hydrophobic interaction chromatography on Phenyl Sepharose 6 Fast Flow matrix.

2.2.5.2. Phenyl Sepharose hydrophobic interaction chromatography. NaCl was added into active pooled fractions (total volume, 12 ml) from previous Q-Sepharose anion exchange chromatography step described above. The final NaCl concentration in this pooled fraction was adjusted to 2.5 M and pH to 7.7. This was then loaded on a Phenyl Sepharose 6 Fast Flow column (1 cm × 5.5 cm), which had been equilibrated with 0.02 M sodium phosphate buffer, pH 7.7 containing 2.5 M NaCl. The column was washed with equilibration buffer till no enzyme activity could be detected in the washings. The bound protease was eluted with 50% ethylene glycol in 20 mM sodium phosphate buffer, pH 7.7 at 4 °C at a flow rate of 4.8 ml/h using a peristaltic pump (Gilson Inc., Middleton, USA). Fractions (1.2 ml each) were collected by BIO-RAD fraction collector (BIO-RAD, California, USA) and analyzed for protease activity. Active fractions were pooled

and used for further characterization. Removal of ethylene glycol was not found to be necessary since the additive does not affect the measurement of protease activity.

2.2.6. Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [20] using a 12% crosslinked polyacrylamide gel on a Genie gel electrophoresis unit (Bangalore Genie, Bangalore, India). Silver staining was performed to visualize protein bands on the gel.

2.2.7. Activity staining of purified protease

Casein or gelatin zymography was conducted following the procedures of Heussen and Dowdle [21] and Caballero et al. [22]. Briefly, SDS-PAGE of the enzyme was carried out as described by Laemmli [20] using a 10% polyacrylamide gel at 4 °C. A suspension of soluble casein or gelatin (0.1%) was incorporated into the separating gel before polymerization. The electrophoresed gels were soaked twice for 30 min in 2.5% Triton X-100 (to remove SDS) and incubated for 30 h at 37 °C in either casein gel substrate buffer (0.05 M Tris, pH 7.6, 0.1 M NaCl) or gelatin gel substrate buffer (0.05 M Tris, pH 8.0, 0.15 M NaCl, 0.01 M $CaCl_2$). The gels were then stained with 0.1% Coomassie blue R-250 in methanol–acetic acid–water (40:10:50) followed by destaining with methanol–acetic acid–water (5:10:85).

2.2.8. Determination of pH optimum and pH stability

Effect of pH on pure enzyme was studied by assaying the enzyme at different pH values.

pH stability was studied by preincubating the enzyme in buffers of different pH values (pH 5–11), at 25 °C for 1 h. The remaining activities were determined under standard assay conditions.

2.2.9. Determination of temperature optimum and thermal stability

To determine the optimum temperature for purified protease, the activity values of protease were measured at various temperatures using casein as the substrate. The thermal stability was studied by incubating the enzyme at 60, 65 and 70 °C. Appropriate aliquots were withdrawn at different time intervals and the residual activities determined at assay temperature.

2.2.10. Determination of K_m and V_{max}

K_m and V_{max} values of the pure enzyme were determined by measurement of enzyme activity with various concentrations of casein substrate. Kinetic constants were calculated using the Leonora software program [23].

2.2.11. Effect of metal ions, surfactants and protease inhibitors

The enzyme was incubated with different compounds at varying concentrations at 25 °C for 1 h in case of metal ions and 10 min in case of surfactants and inhibitors. The residual activities were measured following the assay procedure.

2.2.12. Substrate specificity

The substrate specificity of *Pseudomonas* protease towards different native and synthetic substrates was also examined.

2.2.13. Organic solvent-stability of enzyme

Three milliliters of various protease solutions (758 U/ml) namely purified protease preparation from *P. aeruginosa* PseA, α-chymotrypsin (EC 3.4.21.1, Type II, Sigma Chemical Co.), *A. oryzae* protease (Type XXIII, Sigma Chemical Co.) were incubated in the absence or presence of 1.0 ml of organic solvent, at 30 °C with constant shaking at 160 rpm. The residual activities were estimated by the assay procedure described above.

3. Results and discussion

In the present work, soil samples from the sites near to the solvent extraction unit were screened for solvent tolerant microbes. Cyclohexane was enriched in the medium right at the beginning of soil inoculation so that only solvent tolerant microbes were able to grow. Protease producers among these were selected on the basis of zone formation on skim-milk agar plate. The isolate showing maximum zone diameter was selected for further studies. Based on biochemical tests by the Microbial Type Culture Collection Facility, Institute of Microbial Technology (IMTECH), Chandigarh, India and FAME analysis by the Microcheck Microbial Analysis Laboratory, Vermont, USA, it was identified as *P. aeruginosa* and designated as PseA.

This strain secreted considerable amount of extracellular solvent stable protease with higher specific activity as compared to other solvent tolerant *Pseudomonas* proteases [17].

The protease was purified in two steps by anion exchange chromatography on Q-Sepharose followed by hydrophobic interaction chromatography on Phenyl Sepharose 6 Fast Flow matrix. The supernatant of 48 h grown culture was used as crude source of enzyme and firstly applied to the Q-Sepharose matrix. Protease activity did not bind to matrix and recovered in flow-through and washings, while most of other contaminating proteins were bound to the matrix. Similar kind of behavior is also reported in case of *Bacillus* sp. protease, which did not bind to DEAE cellulose [3]. The crude extract was found to contain a lipase activity in addition to the protease activity characterized in the present work. This step, while did not result in any purification (and reduced the enzyme yield), removed this lipase activity (A. Gupta, I. Roy, S.K. Khare, M.N. Gupta, unpublished results).

The active fractions (flow-through fraction and washings) recovered from Q-Sepharose step were pooled together and loaded on Phenyl Sepharose 6 Fast Flow matrix. The enzyme with the proteolytic activity was completely adsorbed on the

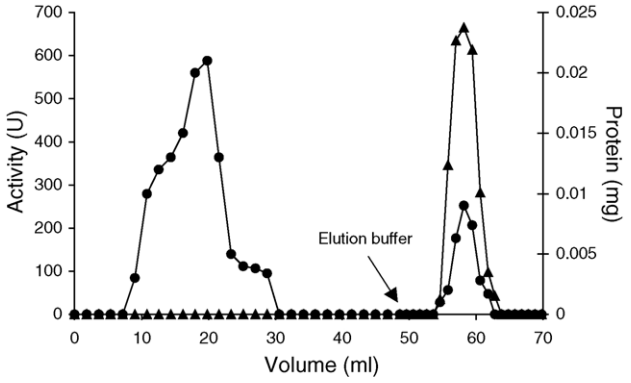

Fig. 1. Hydrophobic interaction chromatography of *P. aeruginosa* protease on Phenyl Sepharose 6 Fast Flow. The details of the experiment are described in the text. Elution of the bound protease was carried out with 20 mM phosphate buffer, pH 7.7 containing 50% (v/v) ethylene glycol (elution buffer). Symbols (▲) and (●) represent the protease activity and protein, respectively.

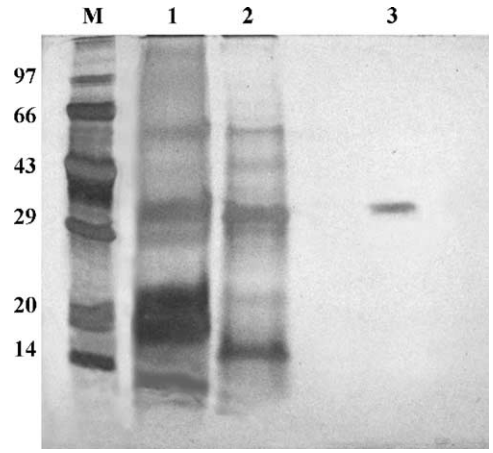

Fig. 2. SDS-PAGE pattern of purified protease. Electrophoresis was carried out using a 12% crosslinked polyacrylamide gel. Lane 1: crude extract of *P. aeruginosa* (7 μg protein); lane 2: pooled protease fractions from anion exchange chromatography (7 μg protein); lane 3: purified protease (7 μg protein); lane M: molecular mass marker proteins. Values: $M_r \times 10^{-3}$.

matrix and no activity was detected in washings (Fig. 1). The bound enzyme was eluted with 50% ethylene glycol (in 20 mM phosphate buffer, pH 7.7) by isocratic mode. The results of the protease purification are summarized in Table 1. Total 60% of the activity units could be recovered and approximately 11.6-fold purification of the protease was achieved. The specific activity of finally purified enzyme was 99,613 U mg^{-1}. Proteases from other strains of *Pseudomonas* sp. have been purified by various combinations of chromatographic procedures. Ogino et al. [17] obtained 25.6% yield and 102-fold purification of *P. aeruginosa* PST-01 protease by ammonium sulphate fractionation and hydrophobic interaction chromatography. By employing ammonium sulphate precipitation, gel filtration and ion exchange chromatography, 37.7% yield and 25-fold purification was reported in case of *P. aeruginosa* MN1 by Bayoudh et al. [24].

SDS-PAGE of the crude and purified enzyme preparations is shown in Fig. 2. In silver-stained gel the purified preparation showed single band corresponding to molecular mass of 35,000 Da, which is in good agreement with other *Pseudomonas* sp. proteases [17,25,26]. Zymogram activity staining also revealed one clear zone of proteolytic activity against the blue background for purified sample. The pure protease (elastase) degraded gelatin (Fig. 3A) and casein (Fig. 3B), used as the substrates in zymography and aggregated into a complex of high molecular mass (approximately 160,000 Da). Caballero et al. [22] have also reported the same high molecular-mass complexes for *P. aeruginosa* elastase B on gelatin and casein zymograms.

Although *P. aeruginosa* is reported to produce several proteolytic enzymes, the predominant proteases secreted by this bacterium are alkaline protease and elastase. Both are metalloproteases in nature [25]. Elastase from *P. aeruginosa* was first time isolated and characterized by Morihara [27] and the name elastase relates to its ability to degrade elastin [28]. It is also called as pseudolysin.

The purified preparation was also found to be a metalloprotein. The effects of divalent cations on protease activity were tested at a 5 mM concentration. Protease activity was markedly reduced by Ni^{2+} and Cu^{2+} (to 11.9 and 21.2%, respectively) and moderately by Zn^{2+}, Mn^{2+}, Mg^{2+} and Ca^{2+} (Table 2). No precipitation was observed in any of the samples.

Metal-complexing agents such as EDTA and 1,10-phenanthroline at 5 mM concentration inactivated the proteolytic activity to a great extent (75 and 100% inhibition, respectively) confirming it to be a metalloprotease [17,29].

Inhibitors of serine protease (phenylmethanesulfonyl fluoride) and thiol protease (iodoacetic acid and *p*-chloromercuribenzoate) had no effect on enzyme activity (Table 3). Proteolytic activity was not affected by some surfactants like Triton X-100, Tween 80 (at 0.1 and 0.5% concentration) but there was 47 and 39% reduction in protease activity in the presence of 0.1% cetyltrimethylammonium bromide (CTAB) and 0.1% SDS, respectively.

The protease was further characterized for its K_m and V_{max} towards casein as a substrate. It showed K_m of 2.69 mg/ml

Table 1
Purification of *P. aeruginosa* protease

Steps	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery[a] (%)	Fold purification
Crude	5147	0.600	8578	100	1
Q-Sepharose chromatography	3088	0.409	7550	60	0.9
Phenyl Sepharose chromatography	3088	0.031	99,613	60	11.6

[a] All recovery values are expressed in terms of activity units in the crude taken as 100%. The last step had 100% recovery.

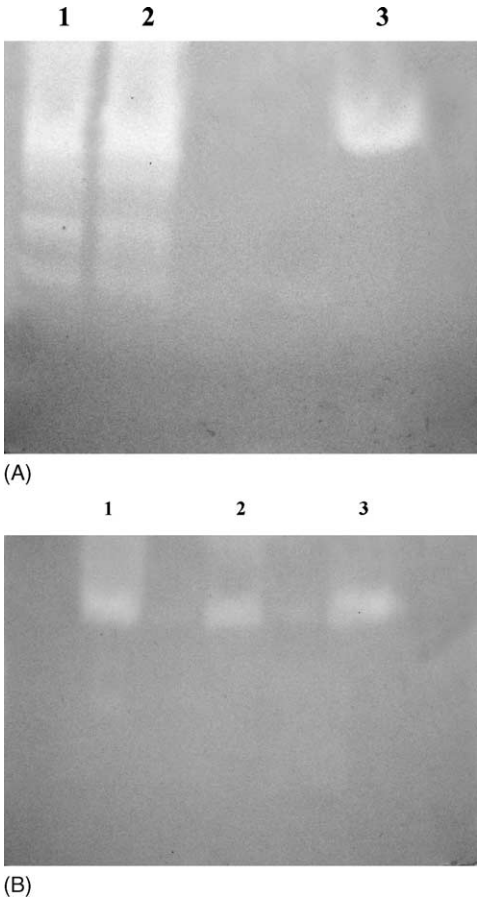

Fig. 3. Zymography of *P. aeruginosa* protease. Samples were electrophoresed under non-denaturing conditions in a 10% gel containing 0.1% gelatin (A) or 0.1% casein (B). The gels were washed in 2.5% Triton X-100, incubated for 30 h at 37 °C in substrate buffer, and processed for visualization as described under Sections 2.2.7. Lane 1: crude extract of *P. aeruginosa* (7 μg protein); lane 2: pooled protease fractions from anion exchange chromatography (7 μg protein); lane 3: purified protease (7 μg protein).

and V_{max} of 3.03 μmol/min. The enzyme was active in the pH range of 7–9 and showed optimum pH of 8.0 (Fig. 4). *Pseudomonas* proteases are reported to have pH optima 8.0 [27,30]. As regards to stability of enzyme at different pH shown it was found to be stable in the pH range 6–9 (Fig. 5).

Table 2
Effect of metal ions on protease activity

Sample	Protease activity (%)
Control	100
Ca^{2+}	53.9
Ni^{2+}	11.9
Mg^{2+}	56.0
Zn^{2+}	63.2
Mn^{2+}	40.4
Cu^{2+}	21.2

Purified protease preparation was incubated with salts of different metal ions (5 mM) at 25 °C for 1 h and remaining enzymatic activity was determined under normal assay conditions. The protease activity of the sample without any metal ion (control) has been taken as 100%.

Table 3
Effect of inhibitors and surfactants on protease activity

Reagent	Concentration	Activity (%)
None		100
EDTA	5 mM	25
1,10-Phenanthroline	1 mM	92
	5 mM	0
Phenylmethanesulfonyl fluoride (PMSF)	1 mM	100
	5 mM	94
p-Aminobenzamidine	1 mM	88
	5 mM	88
p-Chloromercuribenzoic acid	1 mM	99
	5 mM	95
Iodoacetic acid	1 mM	93
	5 mM	93
1,4-Dithio-DL-threitol (DTT)	1 mM	32
	5 mM	0
Glutathione	1 mM	90
	5 mM	90
β-Mercaptoethanol	1 mM	98
	5 mM	80
Urea	1 mM	110
	5 mM	100
Cetyltrimethylammonium bromide (CTAB)	0.1%	53
	0.5%	51
SDS	0.1%	61
Triton X-100	0.1%	110
	0.5%	106
Tween 80	0.1%	106
	0.5%	99

Purified enzyme was preincubated with the various reagents at 25 °C for 10 min and residual protease activity was determined as described in the text. One hundred percent was assigned to the activity in absence of reagent. The experiment was carried out in duplicate and the difference between individual set of readings was less than 3%.

Protease was most active at 60 °C, though it showed considerable activity over the range of 40–65 °C (Fig. 6). Similar temperature optimum (60 °C) for other *Pseudomonas* proteases has been observed by many researchers [29,31].

Fig. 7 shows thermal stability of enzyme at 60, 65 and 70 °C. The purified enzyme when kept at 60 °C for 30 min retained 100% protease activity. At 65 and 70 °C, however, about 33 and 80% activity was lost in 10 min of incubation. Almost similar observations have been reported by Morihara [27] for protease from *P. aeruginosa*.

The substrate specificity of *Pseudomonas* protease indicated that it was active on a variety of natural (BSA, casein, elastin–orcein and haemoglobin) proteins. The protease exhibited the highest activity towards casein. Various synthetic substrates such as benzoyl arginine *p*-nitroanilide (BAPNA), benzoyl arginine ethyl ester (BAEE), *p*-toluenesulfonyl-L-arginine methyl ester, hippuryl-L-arginine, acetyl-L-tyrosine

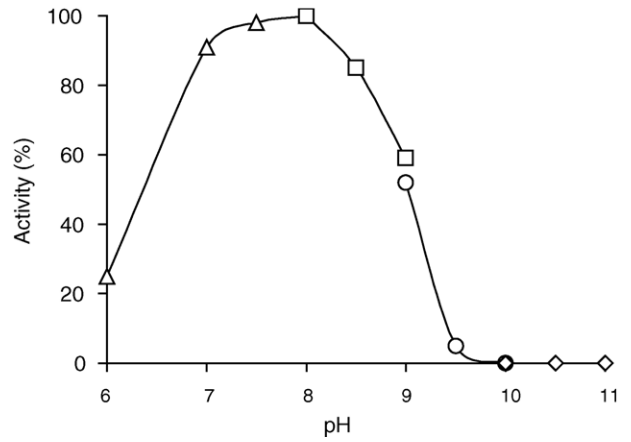

Fig. 4. pH optimum of purified protease. pH optima was measured by incubating the enzyme with the substrate at different pH values. The buffers used were 0.03 M phosphate (△) (pH 6.0–8.0); 0.1 M Tris–HCl (□) (pH 8.0–9.0); 0.1 M glycine–NaOH (◯) (pH 9.0–10.0) and 0.03 M borax–NaOH (◊) (pH 9.5–11). The activity at pH 8.0 is taken as 100%.

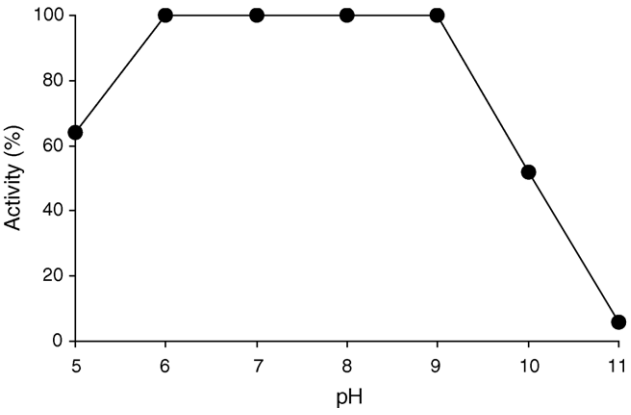

Fig. 5. pH stability of protease. Purified enzyme was incubated in buffers of different pH values (pH 5–11) at 25 °C for 1 h and remaining activity was determined at pH 8.0 under standard assay conditions. The maximum activity of the enzyme has been taken as 100%.

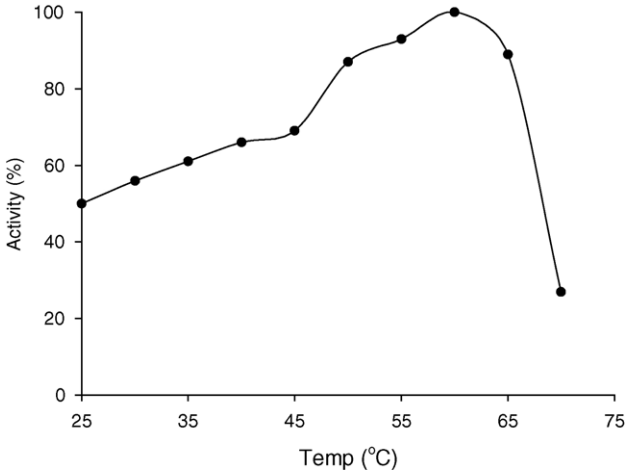

Fig. 6. Temperature optimum of protease. The purified enzyme was incubated with the substrate at different temperatures. The activity at 60 °C has been taken as 100%.

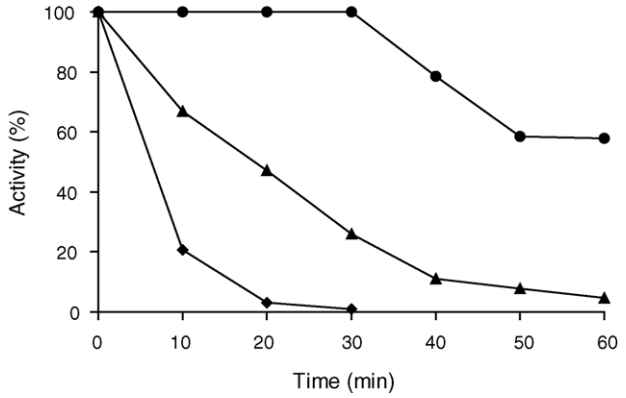

Fig. 7. Thermal stability of protease at 60 (●), 65 (▲) and 70 °C (♦). Aliquots were withdrawn at regular time intervals after incubating the protease at 60, 65 and 70 °C, cooled rapidly to 60 °C and assayed for enzyme activity.

ethyl ester are not hydrolyzed (data not shown). Metalloprotease from *P. aeruginosa* is reported to exhibit similar behavior towards different substrates by Morihara [29]. Considering the elastase activity and other characteristics of the enzyme, this protease appears close to pseudolysin reported from other *P. aeruginosa* strains [17].

Enzymes are usually inactivated by the addition of organic solvents to the reaction solution. The effect of different organic solvents on stability of various proteases was studied. Purified protease preparation from *P. aeruginosa* PseA strain was found to be stable in the presence of range of organic solvents (Fig. 8). However, the relation between the stability against organic solvent and solvent polarity (log *P* value) of the added organic solvent was not found. Similar observations are reported by Ogino et al. [17] in case of purified

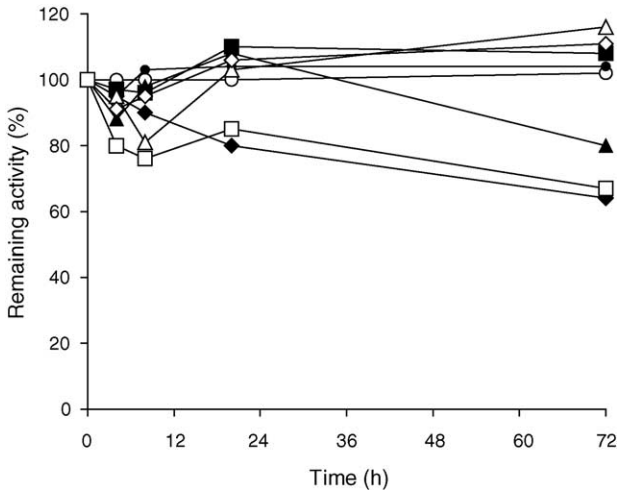

Fig. 8. Effect of organic solvents on stability of purified protease. Purified protease was incubated at 30 °C with constant shaking in the absence (◯) or presence of 25% (v/v) 1-butanol (□); benzene (△); toluene (◊); cyclohexane (●); hexane (■); heptane (▲); isooctane (♦) for 72 h. The protease activity of the non-solvent containing control has been taken as 100%.

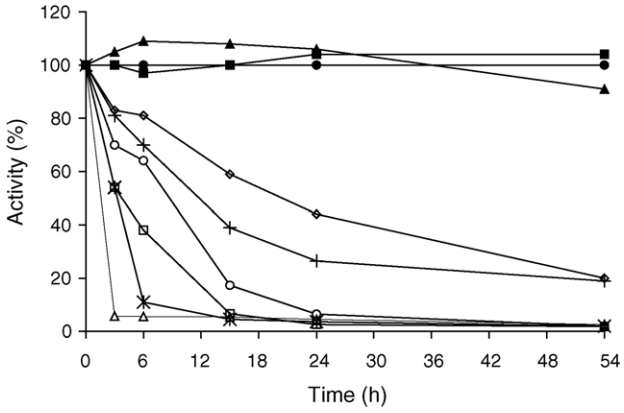

Fig. 9. Effect of organic solvents on stability of various proteases. Various protease solutions were incubated at pH 8.0 and 30 °C with constant shaking in the absence or presence of 25% (v/v) cyclohexane and DMF as described in text. Purified protease from *P. aeruginosa*: without solvent (●), with cyclohexane (■), with DMF (▲); α-chymotrypsin: without solvent (◯), with cyclohexane (□), with DMF (△); *A. oryzae* protease: without solvent (◊), with cyclohexane (+), with DMF (✳). The protease activity of the non-solvent containing control at 0 h has been taken as 100%. Each experiment was carried out three times and the difference in the individual results in each set of experiments was less than 5%.

PST-01 protease. Fig. 9 shows the remaining activity of various proteases in the absence or presence of cyclohexane and DMF. Purified protease was found to be highly resistant to these solvents as compared to α-chymotrypsin and *A. oryzae* protease.

To sum up, a facile purification of a protease fairly stable in a range of organic solvents, surfactants and at higher temperatures is described. The usefulness of this solvent stability has already been discussed in the introduction and elsewhere [32,33]. Considering its surfactant and thermal stability, this *P. aeruginosa* protease may also find potential application in laundry detergents [2].

Acknowledgements

The financial support provided by the Department of Biotechnology, Government of India organization, is gratefully acknowledged. A.G. is grateful to the Council for Scientific and Industrial Research (CSIR) for Senior Research Fellowship grant.

References

[1] A. Anwar, M. Saleemuddin, Bioresour. Technol. 64 (1998) 175.
[2] B.B. Samal, B. Karan, Y. Stabinsky, Biotechnol. Bioeng. 35 (1990) 650.
[3] H. Shimogaki, K. Takeuchi, T. Nishino, M. Ohdera, T. Kudo, K. Ohba, M. Iwama, M. Irie, Agric. Biol. Chem. 55 (1991) 2251.
[4] R. Gupta, Q.K. Beg, P. Lorenz, Appl. Microbiol. Biotechnol. 59 (2002) 15.
[5] M.V. Sergeeva, V.M. Paradkar, J.S. Dordick, Enzyme Microb. Technol. 20 (1997) 623.
[6] M.N. Gupta, Eur. J. Biochem. 203 (1992) 25.
[7] E.N. Vulfson, P.J. Halling, H.L. Holland, Enzymes in Nonaqueous Solvents, Methods and Protocols, Humana Press, Totowa, NJ, 2001.
[8] L.P. Geok, C.N.A. Razak, R.N.Z.A. Rahman, M. Basri, A.B. Salleh, Biochem. Eng. J. 13 (2003) 73.
[9] Y. Jiang, H. Dalton, Biochim. Biophys. Acta 1201 (1994) 76.
[10] M. Persson, E. Whetje, P. Adlercreutz, Chem. Biochem. 3 (2002) 566.
[11] P. Martinez, F.H. Arnold, J. Am. Chem. Soc. 113 (1991) 6336.
[12] F.H. Arnold, J.C. Moore, Adv. Biochem. Eng. Biotechnol. 58 (1997) 1.
[13] J.S. Dordick, Biotechnol. Prog. 8 (1992) 259.
[14] M.N. Gupta, I. Roy, Eur. J. Biochem. 271 (2004) 2575.
[15] H. Ogino, K. Yasui, T. Shiotani, T. Ishihara, H. Ishikawa, Appl. Environ. Microbiol. 61 (1995) 4258.
[16] B. Ghorbel, A.S. Kamoun, M. Nasri, Enzyme Microb. Technol. 32 (2003) 513.
[17] H. Ogino, F. Watanabe, M. Yamada, S. Nakagawa, T. Hirose, A. Noguchi, M. Yasuda, H. Ishikawa, J. Biosci. Bioeng. 87 (1999) 61.
[18] E.O. King, M.K. Ward, D.E. Raney, J. Lab. Clin. Med. 44 (1954) 301.
[19] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[20] U.K. Laemmli, Nature 227 (1970) 680.
[21] C. Heussen, E.B. Dowdle, Anal. Biochem. 102 (1980) 196.
[22] A.R. Caballero, J.M. Moreau, L.S. Engel, M.E. Marquart, J.M. Hill, R.J. O'Callaghan, Anal. Biochem. 290 (2001) 330.
[23] A.C. Bowden, Analysis of Enzyme Kinetic Data, Oxford University Press, Oxford, 1995, p. 27.
[24] A. Bayoudh, N. Gharsallah, M. Chamkha, A. Dhouib, S. Ammar, M. Nasri, J. Ind. Microbiol. Biotechnol. 24 (2000) 291.
[25] M.J. Parmely, in: R.B. Fick (Ed.), *Pseudomonas aeruginosa* the opportunist: pathogenesis and disease, CRC Press, UK, 1993, p. 79.
[26] S. Shastry, M.S. Prasad, Process Biochem. 37 (2002) 611.
[27] K. Morihara, J. Bacteriol. 88 (1964) 745.
[28] N.D. Rawlings, A.J. Barrett, Methods Enzymol. 248 (1995) 183.
[29] K. Morihara, Biochim. Biophys. Acta 73 (1963) 113.
[30] D.V. Qua, U. Simidu, N. Taga, Can. J. Microbiol. 27 (1981) 505.
[31] M.M. Sexton, A.L. Jones, W. Chaowagul, D.E. Woods, Can. J. Microbiol. 40 (1994) 903.
[32] H. Ogino, M. Yamada, F. Watanabe, H. Ichinose, M. Yasuda, H. Ishikawa, J. Biosci. Bioeng. 88 (1999) 513.
[33] S.T. Chen, S.Y. Chen, S.C. Hsiao, K.T. Wang, Biomed. Biochim. Acta 50 (1991) S181.