Purification and characterization of a thermostable neutrophilic metalloprotease from *Pseudomonas* sp. DR89

Ahmad Asoodeh[1,2*], Hossein Mohammadian Musaabadi[1]

[1]Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, P.O. Box 9177948974, Mashhad, I.R. Iran [2]Cellular and Molecular Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, P.O. Box 9177948974, Mashhad, I.R. Iran

Abstract
A novel neutrophilic metalloprotease was isolated from *Pseudomonas* sp. DR89 isolate which was identified in a mineral spring in Iran. The enzyme was purified from the isolate to 21-folds in a three-step procedure involving ammonium sulfate precipitation, Q-Sepharose ionic exchange and Sephadex G-100 gel filtration chromatography. Resuts showed that the enzyme was active at high temperatures and in a wide-range pH of 5-11 with the optimum of 8.0. The zymogram and sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis revealed the presence of one protease with a molecular weight of 74 kDa. The enzyme activity was decreased by Zn^{2+}, Mn^{2+}, H_2O_2 and cetyl trimethylammonium bromide (CTAB), whereas its activity was increased by Ca^{2+}, Mg^{2+}, Cu^{2+} and dimethyl sulfoxide (DMSO). Na^+, phenylmethyl sulfonylfluoride (PMSF), β-mercaptoethanol, sodium dodecyl sulfate (SDS), and Triton X-100 did not show a considerable effect on its activity. Casein was a better substrate than bovin serum albumin (BSA) and gelatin for this enzyme. The kinetic parameters (K_m and V_{max}) of the purified protease towards caseinolytic activity were also determined. These properties of the enzyme make it suitable for use in food industries.
Keywords**:** Neutrophilic protease; phylogenetic tree; thermostability; purification; characterization

INTRODUCTION

Proteases are one of the most important classes of hydrolytic enzymes which are classified into serine-, cysteine-, aspartic-, and metalloproteases based on their catalytic activity. They are further categorized depending on their optimal pH of activity such as acidic, neutral and alkaline proteases. Bacterial proteases constitute one of the largest classes of industrial enzymes and accounting for 40% of the total worldwide enzyme sales (Rao *et al*., 1998).

These enzymes have a large variety of applications in industrial processes, such as detergent, food, paper, leather and pharmaceutical industries. Besides, they are used in waste treatment, peptide synthesis, diagnostic reagents and silver recovery from x-ray/photographic films (Patil and Chaudhari, 2009; Haki and Rakshit 2003; Rao *et al*., 1998).

Metalloproteases are the most diverse kind of catalytic proteases. The neutral metalloproteases show specificity for hydrophobic amino acids generating less bitterness in hydrolyzed food proteins and hence are valuable in food industries, the low thermo-stability being an advantage for controlling their activity (Rao *et al*., 1998; Barrett, 1995).

Many of the thermo-stable proteases, namely, thermolysin, caldolysin and alkaline proteases from *Streptomyces* need calcium for their stability (Cowan and Daniel 1982; Zamost *et al*. 1990). Proteases are usually inactive or show a low activity in non-aqueous media (Gupta 1992; Vulfson *et al*., 2001). Therefore, finding solvent stable proteases has made an extensive area of research (Geok *et al*., 2003). Several low-water stable proteases have been identified in *Pseudomonas* sp (Geok *et al*., 2003; Ogino *et al*., 1995; Aono *et al*., 1992; Cruden *et al*., 1992; Nakajima, 1992). In this

Correspondence to: **Ahmad Asoodeh, Ph.D.**
Tel/Fax: +98 511 8795457
E-mail: Asoodeh@um.ac.ir

study, *Pseudomonas* sp. DR89 was isolated from a mineral water spring. We purified and characterized a metalloprotease with a broad range of pH activity being more active at neutral pH. This enzyme is stable at the high temperatures as well as the presence of several surfactants.

Materials and methods: Nutrient broth medium, nutrient agar medium, ammonium sulfate, Tris, citrate and other buffering salts were purchased from Merck KGaA Co. (Darmstadt, Hesse, Germany). Q-sepharose and Sephadex G-100 were purchased from Pharmacia (GE Healthcare Life Sciences, Uppsala, Sweden). Amicon ultrafiltration membrane system was purchased from Millipore (Bedford, MA, USA). All other chemicals used were of analytical grade.

Isolation of bacterial strain: Water sample from mineral spring of "Dig Rostam" on the Tabbas-Kerman Road, Lut desert, Iran was collected. The sample was then cultivated on nutrient agar plates and incubated at 37 °C for 24 h to identify possible existing microorganisms. Protease activity was observed in one of the three types of colonies grown in nutrient broth containing 10% gelatin. Bacterial isolate, producing the extracellular protease, was grown in a medium constituting of nutrient broth (0.8% w/v), sucrose (1% w/v), yeast extract (0.5% w/v), NaCl (0.5% w/v), and incubated at 37°C in a shaker at 120 rpm for 24 h.

Biochemical tests: The isolate was investigated for morphological, physiological and biochemical characteristics according to the methods described in "Bergey's Manual of Determinative Bacteriology" (Bergey *et al*., 1994). Analyses such as: Gram staining, sensitivity to KOH, O_2 requirement, glucose fermentation, catalase test, gelatinase test, IMViC (Indole, Methyl red, Voges-Proskauer, Citrate, H2S, motility) tests, were performed using the existing protocols (Reynolds, 2011).

PCR amplification and 16S rDNA sequencing: Genomic DNA was extracted according to Sambrook *et al*. (Sambrook and Russell 2001). The 16S rDNA gene of the isolate was amplified using theuniversal 16S rDNA PCR forward primer (5'-AGTTTGATCCTGGCTCAG-3') and reverse primer (5'-GGCTTACCTTGTTACGACTT-3'). Amplification of DNA was performed by means of the Techne thermocycler and programmed as follows: denaturation at 93°C for 5 min followed by 35 cycles at 93°C for 45 s, 50°C for 45 s, 72°C for 90 s and final extension at 72°C for 5 min to allow any incomplete products to be extended (Asoodeh *et al*., 2010). The DNA nucleotide sequencing was performed using Amersham auto sequence. Sequence similarity searches were carried out with the BLAST program and analyzed via RDPII using SEQUENCE MATCH (Version 2.7) to identify the most closely related database sequences. Multiple alignments of nucleotides were performed with ClustalX. The phylogenetic tree was constructed using the neighbor-joining method. The 16S rDNA sequence obtained from DR89 has been deposited in GenBank under accession no. HQ871688.

Culture conditions for enzyme production: To optimize enzyme production, a specific medium containing fructose (1% w/v), yeast extract (1%), peptone (0.5%), K_2HPO_4 (0.1%), $MgSO_4.7H_2O$ (0.01%) and $CaCl_2.2H_2O$ (0.01%) was used. The pH of the medium was adjusted to 7.0 by 10% (w/v) Na_2CO_3 solution. The medium was mixed with 5% of the basic medium described above and the obtained mixture was incubated at 37°C with agitation rate of 120 rpm for 72 h.

Purification of the protease: The specific culture medium (500 ml) was precipitated using ammonium sulfate saturation of 85% by slow continuous stirring at 4°C (cold room). The obtained solution was centrifuged, and the sediment was dissolved in a minimum amount of 20 mM Tris-HCl buffer, pH 8.5 containing 2 mM $CaCl_2$, and dialyzed against the same buffer for 18 hours with three times of buffer exchange. The dialyzed sample was poured onto Q-sepharose column (1.6×10 cm) which had previously been reached to equilibrium with 20 mM Tris-HCl, pH 8.5. The bonded protein was eluted using a continuous NaCl gradient (0-0.5 M). The unbound fractions having enzyme activity were loaded onto a Sephadex G-100 column (1×100 cm). The elution was achieved by a flow rate of 0.4 ml/min using a solution of 20 mM Tris-HCl, 0.1 M NaCl, 2 mM $CaCl_2$, pH 8.5. The most active were pooled and concentrated by ultrafiltration with the use of a 10,000-Da membrane cut-off (Amicon, Bevery, MA, USA) and were lyophilized for further characterization of the enzyme.

Protease assay and determination of protein concentration: Protease activity was determined by the method of Moradian *et al*., (2009). The reaction mixture consisting of 0.5 ml casein 0.5% (w/v) was dissolved in 20 mM, Tris buffer, pH 8.0. The reaction

started once the 0.25 ml enzyme solution was added. The reaction mixture was then incubated at 37°C for 30 min and the reaction was stopped by adding 0.75 ml trichloroacetic acid (10%, w/v). This mixture was further incubated at 4°C for 1 h. After incubation, the mixture was centrifuged at 12,000 rpm for 10 min and the absorbance of the supernatant was measured at 280 nm. The same reaction mixture free of enzyme was used as a blank. One unit of protease activity was defined as the amount of enzyme releasing soluble fragments equivalent to 0.001 absorbance of 280 nm per min under the standard conditions described above. Protein concentration was determined by Bradford's method (Bradford, 1976) using bovine serum albumin (BSA) as standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli's method (Laemmli, 1970) to determine the purity and molecular weight of the protease. Separating gel (10%) and stacking gel (5%) were used in this work. The molecular weight was estimated using a standard protein marker.

Zymogram analysis: Zymogram was performed adding a suspension of soluble casein (0.1%) into the separating gel before polymerization. Samples were mixed with sample buffers empty of β-mercaptoethanol and were loaded into the gel without heating. The electrophoresis was run at 4°C and the gel was incubated in renaturing buffer (2.5% v/v Triton X-100) at room temperature with a gentle agitation for 30 min. The developing buffer (Tris 20 mM, NaCl 0.2 mM, $CaCl_2$ 5 mM and Brij 0.02%) was then replaced with the renaturing buffer. The gel was equilibrated at room temperature with gentle agitation for 30 min. The old buffer was replaced with the fresh developing buffer and was incubated at least at 37°C for 4 hours. The gel was stained with coomasie brilliant blue R-250 (0.5%, w/v) for 30 minutes and then destained with a destaining solution containing 50% methanol, 40% water and 10% acetic acid (Biró *et al*., 2003).

Characterization of purified enzyme

Effect of pH on activity and stability of protease: Activity of the purified protease was measured based on standard conditions at various pH values using a mixed buffer containing 50 mM concentration of acetic acid-sodium acetate, Tris and Glycin-NaOH. The pH stability of the protease was also examined incubating the enzyme in the pH range of 4.0-12.0 at room temperature for 48 h. Then the remaining enzyme activity was measured under standard assay conditions.

Effect of temperature on activity and stability of protease: To determine on optimum temperature of enzyme activity, the reaction mixture containing casein (0.5%), dissolved in buffer Tris-HCl 20 mM pH 8.0 was tested at different temperatures ranging from 20 to 80°C for 30 minutes. Then reaction mixture was incubated according to standard assay conditions for monitoring its activity levels. Enzyme temperature stability was determined incubating the purified enzyme in buffer Tris-HCl (20 mM, pH 8.0) at three temperatures of 50, 60 and 70°C. Enzyme remaining activity was determined by harvesting some of the enzyme solution and the control sample (no enzyme) at each temperature in regular intervals.

Substrate specificity: Substrate specificity of the purified protease was analyzed for casein, bovine serum albumin and gelatin. A 0.5 ml fraction of each substrate (0.5% w/v), prepared in 20 mM Tris-HCl, pH 8.0, was incubated at 62°C for 30 min. The enzyme activity was examined under the standard assay conditions.

Determination of kinetic features of the enzyme: The reaction rate of the purified protease was determined at various concentrations of casein (0.05 to 0.5 % w/v), prepared in 20 mM, Tris-HCl buffer, (pH 8.0), at 62°C. The kinetic parameters (K_m and V_{max}) of the purified protease for casein were determined subsequently (Bisswanger, 2008).

Effect of metal ions on enzyme activity: The effect of various metal ions, namely Mg^{2+}, Na^{+},Ca^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+}, on protease activity was examined. For this purpose, metal ions with 5 mM of final concentration were added to 0.5 ml of casein (0.5%) dissolved in 20 mM Tris-HCl buffer, pH 8.0, and the purified protease was pre-incubated with the mentioned chloride salts at 62°C for 1 h. Finally, the results were compared with a control (without metal ion).

Effect of inhibitors: Inhibitors including, phenylmethylsulphonyl fluoride (PMSF), a classical serine protease inhibitor; 2-mercaptoethanol, a cystein protease inhibitor; and ethylenediamine tetra acetic acid (EDTA), a chelator of divalent cations (metalloprotease inhibitor) were pre-incubated with the enzyme

for 1 h at room temperature, and the residual activity-was measured under standard assay conditions.

Effect of detergents and other chemical agents on protease activity: The effect of (a) chemical surfactants [(sodium dodecyl sulphate (SDS), Triton X-100 and Cetyltrimethylammonium bromide (CTAB)], (b) oxidizing agent (H_2O_2) and (c) organic solvent dimethyl sulfoxide (DMSO) on enzyme activity were determined with incubation of different concentrations of these agents at room temperature for 1 h, and then the residual activity was measured.

RESULTS

Identification of microorganism: Our results showed that the isolated bacterial strain was a non-spore forming, Gram-negative and rod-shape bacterium. Furthermore, the strain was gelatinase, oxidase and catalase positive and capable of using citrate as the carbon source, as well as negative for glucose fermentation. Other properties of the strain were observed as its motility, indole negative, no sulfur reduction, MR negative and VP positive. Therefore, according to these morphological, physiological, and biochemical tests, the isolate was identified as a *Pseudomonas* species. To confirm its identity, PCR amplification and 16S rDNA sequence analysis was done. The 16S rDNA sequence was multiple sequence alignment with fourteen 16S rDNAs from *Pseudomonas* species obtained from National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). The homology was searched with other *Pseudomonas* species; the construction of the phylogenetic tree was performed using the neighbor-joining method (Fig. 1). Our data revealed that the isolated bacterium is a strain belongs to *Pseudomonas* species.

Isolation and purification of the protease: The

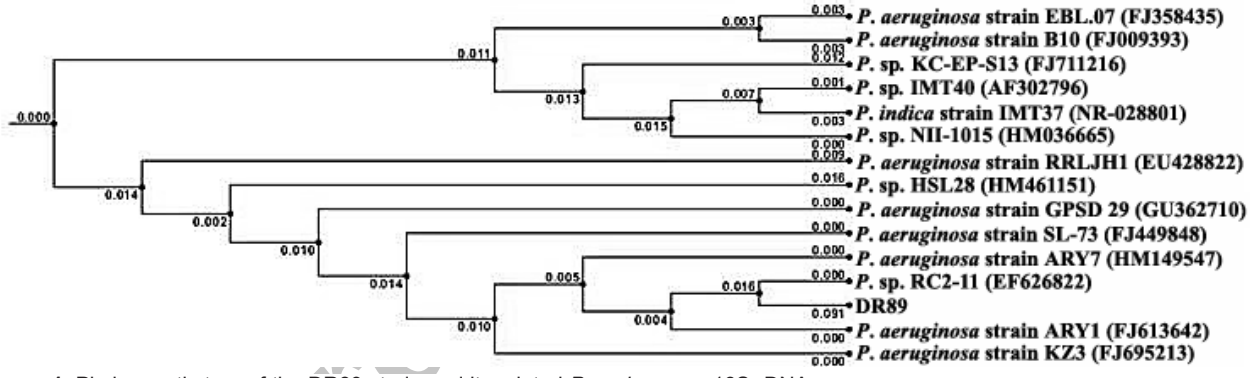

Figure 1. Phylogenetic tree of the DR89 strain and its related *Pseudomonas* 16S rDNA gene sequences.

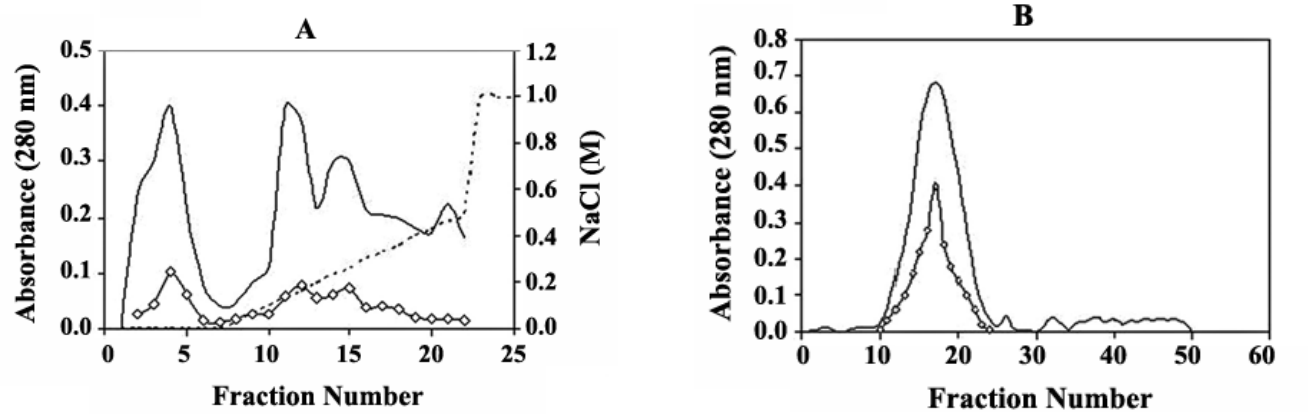

Figure 2. A: Purification profile of protease from Pseudomonas sp.DR89 on Q-Sepharose and B: gel filtration on Sephadex G-100 columns. Protein content and protease activity (◊) in fractions were determined by absorbance of 280 nm; (- - -) NaCl gradient.

Table 1. Summary of the purification procedures of the protease from Pseudomonas sp. DR89.

Step	Total activity (unit)	Total protein (mg)	Specific activity (u/mg protein)	Purification fold	Yield (%)
Crude extraction	77090	850	90.7	1.0	100
$(NH_4)_2SO_4$ precipitation	18800	138	136.2	1.5	24.4
Q-sepharose	7020	4.8	1451.2	16	9.1
Sephadex G-100	3000	1.6	1904.7	21	3.9

results obtained from ion-exchange chromatography of Q-Sepharose and gel filtration on Sephadex G-100 are presented in Figure 2. Summary of the purification steps are given in Table 1. The purified enzyme exhibited a 21-fold purification and 3.9% recovery of the enzyme. The zymogram analysis showed the presence of one protease with higher molecular weight than that of BSA (66.7 kDa) as a control (Fig. 3A). The exact molecular weight of the protease was then estimated to be 74 kDa using SDS-PAGE (Fig. 3B).

pH activity and pH stability profiles: The protease was active in a broad range of pH from 5 to 11 with an optimum pH of 8.0. The enzyme also showed 85% activity at pH 10.0 (Fig. 4A). The stability of protease was determined by pre-incubation of the enzyme at various buffers in the pH range of 4.0 to 12.0 at 37°C for 48 h. Our findings showed that the most pH stability of the enzyme belongs to pH 8.0 (Fig. 4B).

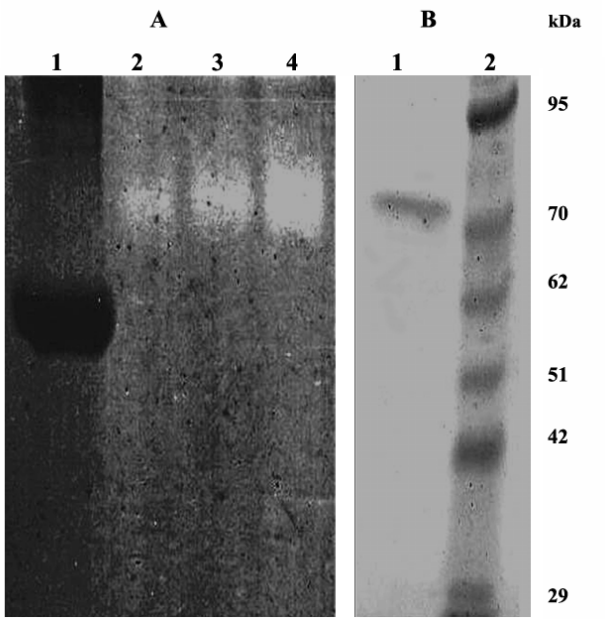

Figure 3. Polyacrylamide gel electrophoresis of the purified protease from *Pseudomonas* sp.DR89. A: Zymogram analysis. Lane 1: BSA; Lane 2: crude extract; Lane 3: active fraction eluted from Q-Sepharose; Lane 4: active fraction eluted from Sephadex G-100. B: SDS-PAGE. Lane 1: purified enzyme Lane 2: molecular weight standard proteins.

Effect of temperature on enzyme activity and stability: The activity of the purified enzyme was tested at various temperatures. The protease showed more than 70 percent of its activity at temperatures from 30 to 70°C, with an optimum temperature around 60°C (Fig. 4C). The thermal stability of the protease was tested at temperatures of 50, 60, and 70°C for different incubation times (30 to 90 min) (Fig. 4D). The enzyme had a half-life of 70 min at 50°C, less than 60 min at 60°C and 45 min at 70°C.

Effect of various metal ions and inhibitors: The effect of different metal ions on the purified enzyme was examined by pre-incubating at 62°C for 1 h. A slightly enhanced activity was observed in the presence of $CaCl_2$, $MgCl_2$ and $CuCl_2$ compared to the original activity, while $FeCl_2$, $MnCl_2$ and $ZnCl_2$ could inhibit the activity about 10%, 20% and 50%, respectively (Fig. 5). Among the inhibitors tested, EDTA was able to inhibit the protease almost completely and its inhibitory effect somewhat declined at the presence of 10 mM $CaCl_2$ (Table 2). The other inhibitors namely PMSF and β-mercaptoethanol had no considerable effects on protease activity. Therefore, the DR89 protease is suggested to be a metalloprotease.

Effect of detergents, and other chemical agents on protease activity: Two concentrations of CTAB (cetyltrimethylammonium bromide), SDS (sodium dodecyl sulphate), Triton X-100, and H_2O_2 were incubated with DR89 protease and the residual activity was determined after 1 h at room temperature (Table 3). Our results showed that the effect of anionic and natural surfactants (SDS and Triton X-100) on protease activity was less than that of cationic surfactant (CTAB). Furthermore, the high concentrations of H_2O_2

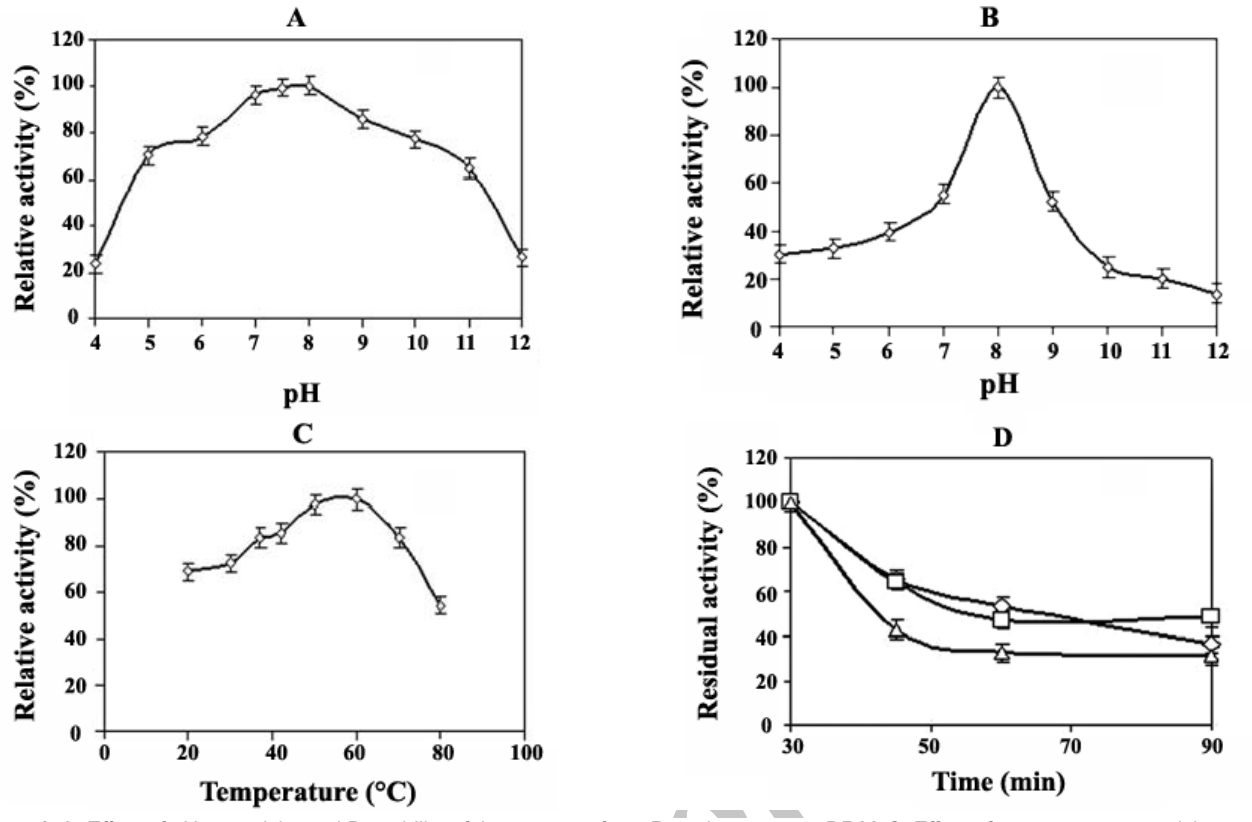

Figure 4. A: Effect of pH on activity and B: stability of the protease from *Pseudomonas* sp. DR89 C: Effect of temperature on activity and D: stability of the DR89 protease at 50 (◊), 60 (□), and 70°C (Δ). For thermo-stability assay at different times, the original activity at 30 min after the beginning of reaction (according to standard assay conditions) was taken as 100% for each temperature (the standard errors were less than 5% of the means).

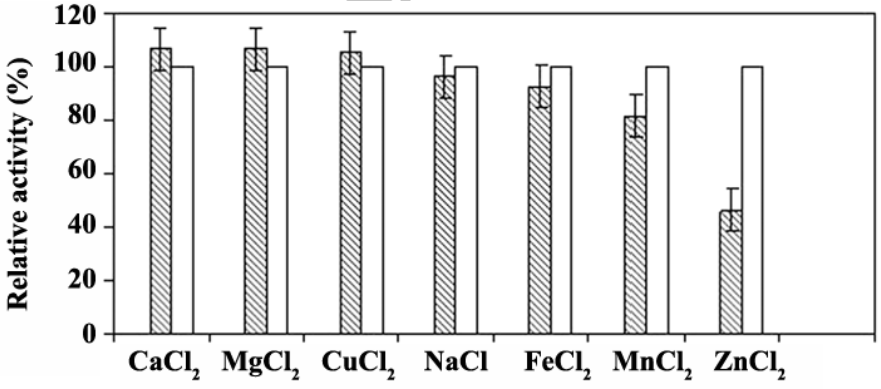

Figure 5. Effect of various metal ions on enzyme activity; the enzyme was incubated in the presence of various metal ions and then the residual activity was determined in standard assay conditions. The unhatched columns: control (without metal ions) and the hatched ones represent the treated samples (The standard errors were less than 5% of the means).

(2.5%) led the DR89 protease to be 65% inactive. The protease was exposed to three different concentrations of DMSO. The protease DR89 was activated by increasing concentration of DMSO (Table 3).

Substrate specificity: Among the tested substrates, the purified protease showed a significant activity toward casein (100%). The activity toward BSA and gelatin was 71% and 53%, respectively. Therefore, the protease isolated from *Pseudomonas* sp. DR89 has a higher affinity for hydrolysis of casein than other proein substrates.

Determination of kinetic parameters: The kinetic parameters including, K_m and V_{max} values towards

Table 2. Effect of some inhibitors on protease activity.

Inhibitors	Concentration (mM)	Residual activity (%)
PMSF	5 mM	95%
PMSF	10 mM	92%
EDTA	2.5 mM	6.7%
EDTA+$CaCl_2$*	2.5 mM	7.3%
EDTA	5 mM	6.4%
EDTA+$CaCl_2$*	5 mM	6.7%
β-Mercaptoethanol	5 mM	77%
β-Mercaptoethanol	10 mM	73%

Table 3. Table 3. Effect of laboratory surfactants, oxidizing agent and organic solvent on protease activity.

Detergents/oxidizing agent/organic solvent	Concentration (%)	Residual activity (%)
CTAB	0.05	69%
	0.25	54%
SDS	0.05	80%
	0.25	70%
Triton X-100	0.5	82%
	2.5	73%
H_2O_2	0.5	81%
	2.5	35%
DMSO	10	73%
	20	86%
	30	90%

casein as a substrate was determined. The K_m and V_{max} values for the DR89 protease were to be 0.79 mg/ml and 4.5 mU/min, respectively. The Lineweaver-Burk curve of enzyme activity is represented in Figure 6.

DISCUSSION

In this study, we investigated the purification and characterization of a novel thermostable neutraphilic metalloprotease from a *Pseudomonas* strain, named *Pseudomonas* sp. DR89. The protease was purified in three steps included: ammonium sulfate precipitation, ion-exchange chromatography and gel filtration. Finally, the purified protease recovered 21-fold with 3.9% yield. The remarkable reduction in the yield of purification could be mainly due to the decrease in the number of total protease and inactivation owning to the auto digestion. The molecular weight of the protease was estimated to be 74 kDa, based on the zymogram analysis and SDS-PAGE.

The isolated enzyme was active in a wide range of temperature activity (30-70°C). Having a high molecular weight is one of the most prominent features of the DR89 protease in comparison with other investigated *Pseudomonas* metalloproteases. Gupta *et al*. (2005) and Patil and Chaudhari (2009) reported a 35 kDa protease from *P. aeruginosa* PseA and *P. aeruginosa* MTCC 7926, respectively. Ogino *et al*. (1999) isolated a 38 kDa protease from *P. aeruginosa* PST-01. A 18 kDa enzyme was purified from *P. aeruginosa* san-ai strain by Karadzic *et al*. (2004). The half-life of our enzyme was more than metalloproteases of different *P. aeruginosa* sp. (Patil *et al*., 2009 and Gupta *et al*., 2005). The enzyme exhibited a high activity in a wide range of pH (i.e., from 5 to 11, particularly at pH 8.0). The optimum temperature for enzyme activity was 60 °C which indicated that this temperature is the optimum temperature for metalloproteases isolated from different *Pseudomonas* sp. bacteria (Patil and Chaudhari, 2009; Gupta *et al*., 2005; Karadzic *et al*., 2004; Geok *et al*., 2003; Ogino *et al*., 1999; Morihara 1963). *Pseudomonas* metalloproteases were previously reported to have a pH optimum of 8.0 for *Pseudomonas aeruginosa* strains (Gupta *et al*., 2005; Ogino *et al*., 1999) which accommodate our results.

We observed that Ca^{2+} affected on the enhancement of the thermal stability, and the enzyme activity which these findings are approved by other reports (Ghorbel *et al*., 2003; Johnvesly and Naik, 2001). Some alkaline proteases have been reported to be significantly stabilized by Ca^{2+} (Keay *et al*., 1970; Horikoshi and Akiba 1982) Two extracellular enzymes produced by thermophilic microorganisms required to Ca^{2+} for their stability (Arulmani *et al*., 2007; Peek *et al*., 1992). The effect of Ca^{2+} on the thermal stability of the enzyme can be explained through strengthening

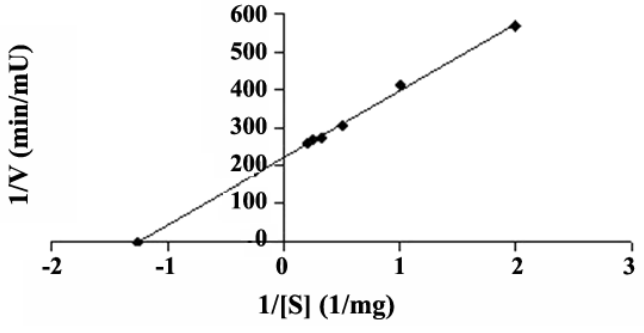

Figure 6. Lineweaver-Burk curve of the enzyme activity.

of ionic-interactions, and the binding of Ca^{2+} to the autolysis site of the enzyme (Johnvesly and Naik, 2001). Besides, decreasing effects of Mn and Zn ions on other *Pseudomonas* strains have been proved (Patil and Chaudhari 2009; Gupta *et al*., 2005). Among the few inhibitors tested on the proteases from *Pseudomonas* sp. DR89, EDTA was the most potent, while the enzyme retained approximately 90% and 70% of its activity in the presence of PMSF and 2-mercaptoethanol, respectively. Our results indicated that this protease belongs to a metalloprotease family (Patil and Chaudhari, 2009; Gupta *et al*., 2005; Karadzic *et al*., 2004). DR.89 protease activity influenced by SDS slightly than CTAB at the same concentrations, and Triton X-100 also meagerly influenced protease activity, indicating that this protease could not be a lipoprotein (Patil and Chaudhari, 2009). Investigations for finding proteases, naturally stable in the presence of organic solvents, have become a new area in enzymology (Gupta *et al*., 2005; Rahman *et al*., 2005; Geok *et al*., 2003). Furthermore, several existing reports have shown that peptide synthesis could be enhanced under low-aqueous conditions because of reduction by-product reactions (Ogino and Ishikawa, 2001; Klibanov, 1997; Bell *et al*., 1995).

In conclusion, the DR.89 protease may be a good choice for application in food industries and peptide synthesis. Future studies should be towards the molecular genetic analysis of this protease in order to understand the mechanism of action, and to improve the stability of the enzyme through molecular genetic analysis.

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