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One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp.

Anshu Gupta [a], Ipsita Roy [a], R.K. Patel [b], S.P. Singh [b], S.K. Khare [a,*], M.N. Gupta [a]

[a] *Chemistry Department, Indian Institute of Technology, Delhi, Hauz Khas, New Delhi 110016, India*
[b] *Department of Biosciences, Saurashtra University, Rajkot 360005, India*

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Abstract

An alkaline protease producer haloalkaliphilic bacteria (isolate Vel) was isolated from west coast of India. It was related to *Bacillus pseudofirmus* on the basis of 16S r RNA gene sequencing, lipid profile and other biochemical properties. The protease secreted by this bacteria was purified 10-fold with 82% yield by a single step method on Phenyl Sepharose 6 Fast Flow column. The apparent molecular mass based on the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was estimated to be 29 000 Da. The K_m and V_{max} towards caseinolytic activity were found to be 2 mg ml^{-1} and 289.8 μg min^{-1}, respectively. The enzyme was active over the pH range of 8.5–12.0, the optimum being 10–11.0. The purified enzyme when kept at 45 °C and 50 °C for 40 min retained 92% and 85% protease activity, respectively. Effect of NaCl concentration on protease activity showed that the enzyme was slightly inhibited with high concentration of salt. The proteolytic activity was inhibited by PMSF, suggesting that the enzyme may belong to serine type protease. Interestingly, the activity was slightly enhanced with SDS (0.1%) and Triton X-100 (0.1%) but remained unaffected by Tween 80 (0.1%). The activity was affected by metal ions to varying extent. While Mn^{2+}, Zn^{2+} and Mg^{2+} had no significant effect on protease activity, the enzyme was activated with Ca^{2+} (1 mM) and Cu^{2+} (5 mM). The stability of the enzyme in the presence of detergent components and surfactants is particularly attractive for its application in detergent industries.

Keywords: Alkaline protease; *Bacillus* sp.; Haloalkaliphilic; Hydrophobic interaction chromatography; Protein purification; Surfactant stability

1. Introduction

Proteases are one of the largest selling enzymes accounting about 60% of the total enzyme market worldwide [1]. They find diverse industrial applications in peptide synthesis, protein processing, leather, dairy and detergent industries [2–4]. Their use in detergent formulation makes up a high percentage (89%) of their total sales [5]. Typically, a detergent protease needs to be active and stable in alkaline environment encountered under harsh washing conditions (pH 9–11, temperature 30–60 °C and presence of high salt and surfactant concentration). Proteases in general are not stable under these conditions. Hence there have been extensive studies on screening [6] and protein engineering [7] to obtain proteases suitable for detergent applications.

Although some alkaline proteases have been reported in the literature, most of such preparations are patented [8]. Enzymes derived from halophiles are potential biocatalysts in this regard. Halophilic bacteria are extremophilic microorganisms that can grow optimally in media containing 0.5–4.5 M NaCl. These have received considerable attention because of their potential use in biotechnology [9]. There is an increasing interest in enzymes produced by these halophiles, which are expected to show optimal activities in presence of salts and surfactants. Halophilic alkaline proteases are active at a salt concentration in the range of 0.2–5.2 M (mesophilic enzymes under such conditions show low activity and even

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

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get denatured) [10]. These have been reported to be better catalysts for peptide synthesis, enhanced oil recovery [10] and hypersaline waste treatment [11]. However, their purification has been a major challenge [10]. Hypersaline requirement of some halophilic proteins makes the purification and handling of these enzymes difficult. Therefore, purification procedures that work in the presence of salt need to be developed.

We have isolated a halophilic *Bacillus* sp. strain from coastal Gujarat region of India [12]. This was found to secrete a novel protease active at alkaline pH and high salt concentrations. In the present work we describe a one-step protocol based on hydrophobic interaction chromatography for efficient purification of protease from this halophile. Some limited characterization of this purified protease is also reported.

2. Experimental

2.1. Materials

Phenyl Sepharose 6 Fast Flow was purchased from Sigma (St. Louis, MO, USA). 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. Bacterial strain

A haloalkaliphilic bacteria (isolate Vel) used in this study was isolated from saline soil located in south coastal Gujarat region, India. 1.0 g of soil was suspended in sterilized distilled water and 2 ml of the resulting suspension was transferred to 100 ml of the screening medium which contained ($g\,L^{-1}$): glucose, 10; peptone, 5; yeast extract, 5; KH_2PO_4, 10 and NaCl, 200. pH of the medium was adjusted to 10.0 with 20% (w/v) Na_2CO_3. The culture was incubated at 30 °C with constant shaking at 100 rpm for 72 h. The culture fluid was spread on screening medium agar plate and the isolates were purified by repeated streaking. The protease producers among purified colonies were detected by plating on gelatin agar (pH 9.0), which contained ($g\,L^{-1}$): gelatin, 30; peptone, 10 and NaCl, 100. 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 slants at 4 °C for further studies. This strain was characterized on the basis of 16S r RNA gene sequencing, lipid profile and other biochemical properties [12].

2.2.2. Culture conditions for protease production

For protease production, the bacteria was grown at 37 °C in a medium (pH 8.0) consisting of ($g\,L^{-1}$): glucose, 10.0; KH_2PO_4, 10.0; yeast extract, 5.0; peptone, 5.0; casein acid hydrolysate, 5.0 and NaCl, 100.0. This 24 h grown mother culture (10 ml) was used to inoculate 100 ml of production medium containing ($g\,L^{-1}$): gelatin, 10.0; casein acid hydrolysate, 10.0 and NaCl, 100. The pH of the medium was adjusted to 8.0 with 20% Na_2CO_3. The sample was incubated at 37 °C in an orbital shaker at 100 rpm. After 66 h of growth, the cells were harvested by centrifugation at $5500 \times g$ and 4 °C for 10 min, and the supernatant thus obtained was used as crude enzyme preparation.

2.2.3. Enzyme purification by hydrophobic interaction chromatography

Hydrophobic interaction chromatography was performed on a Phenyl Sepharose 6 Fast Flow column (1 cm × 6.5 cm), which had been equilibrated with 0.1 M sodium phosphate buffer, pH 7.5 containing 1 M ammonium sulfate. The crude protease preparation (26.0 ml, containing 1 M ammonium sulfate, pH adjusted to 7.5) was loaded onto this column at a flow rate of $0.7\,ml\,min^{-1}$ using a peristaltic pump (Gilson Inc., Middleton, USA). The column was washed with equilibration buffer until the absorbance of the effluent at 280 nm reached base line. The bound enzyme was then eluted from the column (4 × 3.5 ml) using 0.1 M sodium phosphate buffer, pH 7.5 containing a decreasing step gradient of ammonium sulfate (0.5 M, 0.2 M, 0.1 M). The final fraction was eluted with 0.1 M sodium phosphate buffer, pH 7.5 containing no ammonium sulphate. Fractions at a flow rate of $0.7\,ml\,min^{-1}$ 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.

2.2.4. Measurement of enzyme activity

Protease activity was determined as described by Shimogaki et al. [4], using casein as the substrate. Enzyme solution (0.5 ml) was added to 3.0 ml of substrate solution (0.6% casein in 20 mM borax–NaOH buffer, pH 10.0) and the mixture was incubated at 37 °C for 20 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.

2.2.5. Estimation of protein

Protein was estimated by dye-binding method [13], using bovine serum albumin as standard protein.

2.2.6. Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [14] using 12% crosslinked polyacrylamide gel on a Genie gel electrophoresis unit (Bangalore Genie, Bangalore, India). Silver staining was used to visual-

ize protein bands on the gel [15]. Since sensitivity of "silver staining" is much higher than "coomassie staining", the purified preparation was checked by it to ensure homogeneity and absence of even minor contaminant bands.

2.2.7. Determination of pH optimum

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

2.2.8. 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 different temperatures and activities were determined after cooling the mixture to the assay temperature.

2.2.9. 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 [16].

2.2.10. Effect of inhibitors and surfactants

The effect of inhibitors and surfactants on enzyme activity was carried out under standard assay conditions where the assay cocktail was supplemented with PMSF (5 mM), EDTA (1 mM), cysteine (1 mM), SDS (0.1%), Tween 80 (0.1%) and Trition X 100 (0.1%). The effect was assessed by comparing the control having no effectors.

2.2.11. Effect of NaCl on enzyme activity

The enzyme was preincubated with various concentrations of NaCl (0–0.17 M) for 30 min and the enzyme assay was carried out under standard assay conditions.

2.2.12. Effect of metal ions

To study the effect of metal ions on the purified enzyme, the assay was carried out where the assay mixture was supplemented with 1 mM and 5 mM of $CaCl_2$, $MgCl_2$, $CuSO_4$, $ZnCl_2$, $MnCl_2$ and $Ni(NO_3)_2$.

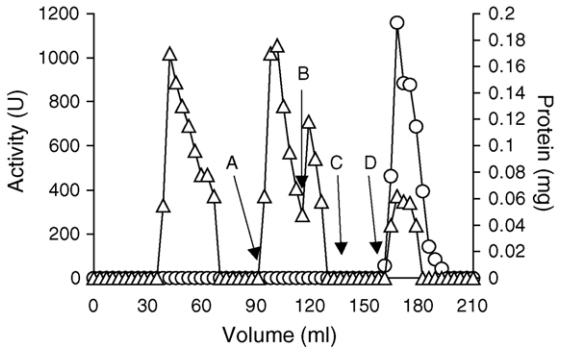

Fig. 1. Purification of *Bacillus* sp. protease on Phenyl Sepharose 6 Fast Flow. The details of the experiment are described in the text. Elution of the bound enzyme was carried out in a stepwise manner as follows: 0.1 M sodium phosphate buffer, pH 7.5 containing 0.5 M ammonium sulfate (A), 0.1 M sodium phosphate buffer containing 0.2 M ammonium sulfate (B), 0.1 M sodium phosphate buffer, pH 7.5 containing 0.1 M ammonium sulfate (C), 0.1 M sodium phosphate buffer, pH 7.5 (D). Symbols (◯) and (△) represent the protease activity and protein, respectively.

3. Results and discussion

One of the haloalkaliphlic isolates Vel, from south coastal Gujarat state of India was used for alkaline protease production, purification and further characterization. The organism based on the 16S r RNA gene homology was related to a recently described, *Bacillus pseudofirmus* FTU [12].

Alkaline protease was purified by hydrophobic interaction chromatography on a Phenyl Sepharose 6 Fast Flow column. Fig. 1 illustrates the purification profile and steps involved in the elution of the desired protein. The crude broth (containing 1 M ammonium sulfate, pH adjusted to 7.5) was loaded on the column. The elution, after washing the column with the equilibration buffer consisted of four stages where a decreasing stepwise gradient of ammonium sulfate was used. The eluting steps (A–C) with buffer containing 0.5–0.1 M ammonium sulfate did not elute the desired protein (Table 1). However, during these steps, significant amount of contaminating proteins were removed (Table 1 and Fig. 1). In the last step (D), the bound protein was eluted with just buffer without any ammonium sulfate and the fractions contained the protease activity with a symmetrical peak corresponding to the active protein. As can be seen from Table 1, a small amount

Table 1
Purification of *Bacillus* sp. protease on Phenyl Sepharose 6 Fast Flow column

Steps	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery[a] (%)	Fold purification
Crude	5806	3.20	1814	–	–
Wash	0	0.90	–	0	–
Step elution					
(A)	0	0.54	–	0	–
(B)	0	0.47	–	0	–
(C)	0	0.00	–	0	–
(D)	4788	0.26	18415	82	10

Details of the experiment are described in the text. The symbols used for step gradient are described in legend to Fig. 1.
[a] All recovery values are expressed in terms of activity units in the crude taken as 100%.

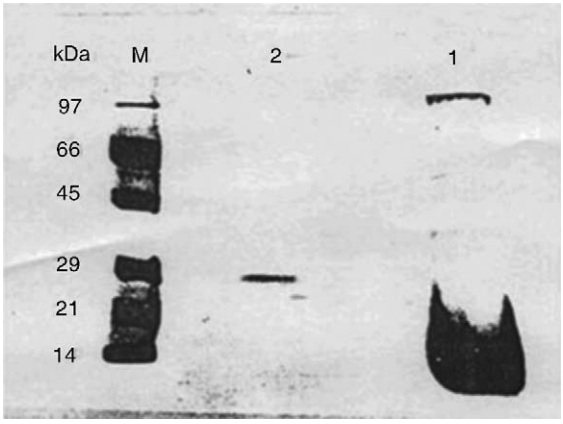

Fig. 2. SDS-PAGE pattern of purified protease. Electrophoresis was carried out using a 12% crosslinked polyacrylamide gel. Lane 1: Crude extract of *Bacillus* sp. (10 μg protein); lane 2: purified protease (pooled fractions from elution step D, 7 μg protein); lane M: molecular mass marker proteins.

of contaminating proteins were retained on the column after the elution of the target protein.

Table 1 shows that 82% of the total activity units could be recovered with 10-fold purification. SDS-PAGE of the crude enzyme and purified enzyme preparations is shown in Fig. 2. The purified preparation showed single band with molecular mass of 29 000 Da. In an earlier instance, molecular mass of a protease from alkalophilic *Bacillus* sp. has been reported to be 30 000 Da [17]. The purified preparation was characterized for its K_m and V_{max} towards casein as a substrate. It showed K_m of 2 mg/ml and V_{max} 289.8 μg/min. K_m value of 2 mg/ml is quite less in comparison to 7.4 mg/ml (towards casein) observed in the case of alkaline protease from the moderate halophile *Halomonas* sp. ES-10 [18].

The enzyme was active in the pH range of 10–12 with broad optimum pH range 10–11 (Fig. 3). This pH range of optimum catalysis, however, is narrower as compared to many recent reports for alkaline proteases active in much broader alkaline range [19–21]. Protease was most active at 37 °C, though it showed considerable activity over the range

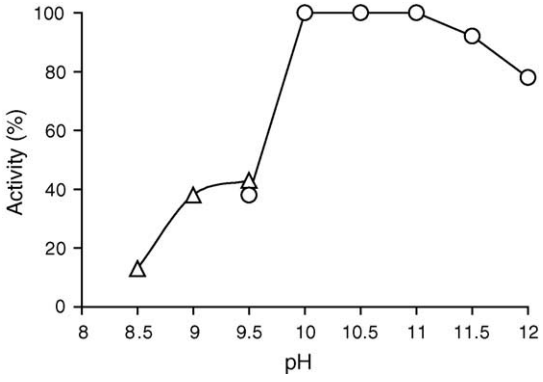

Fig. 3. pH optimum of purified protease. pH optima was measured by incubating the enzyme with the substrate at different pH values. The buffers used were 0.05 M glycine–NaOH (△) (pH 8.5–9.5) and 0.02 M borax (◯) (pH 9.5–12). The activity at pH 10.0 is taken as 100%.

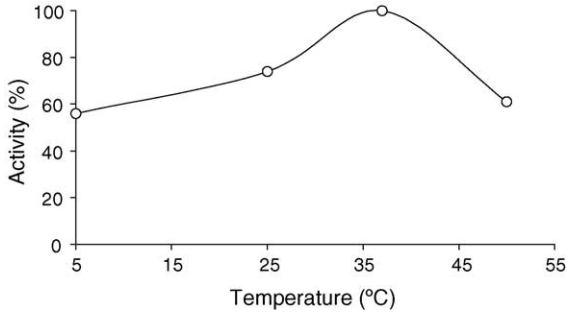

Fig. 4. Temperature optimum of protease. The purified enzyme was incubated with the substrate at different temperatures. The activity at 37 °C has been taken as 100%.

of 5–50 °C (Fig. 4). The purified enzyme when kept at 45 °C and 50 °C for 40 min retained 92% and 85% protease activity, respectively. The protease activity decreased to 18% after incubating the enzyme for 40 min at 55 °C, 60 °C and 70 °C while there was complete loss in activity at 80 °C and 90 °C after 40 min incubation (Fig. 5).

The purified enzyme was not inhibited at all by cysteine (1 mM) but was slightly inhibited (10%) by EDTA (1 mM) and strongly (94%) by phenylmethanesulfonyl fluoride (5 mM) indicating that this protease may belong to serine type protease (Fig. 6). Different surfactants did not inhibit enzyme activity; instead a marginal enhancement was evident with SDS (0.1%) and Triton X-100 (0.1%). Tween 80 (0.1%) did not affect the activity at all (Fig. 7). This behavior in the presence of these surfactants agrees with other reports concerning halophilic protease [18,22].

To study the relationship of NaCl with enzyme activity and the role of contaminating proteins present (in the crude) on the stability in the presence of salt, the enzyme activity of both crude and purified enzyme preparations was monitored in the presence of 0–0.17 M salt concentration. The enzyme activity was slightly increased at 0.03 M NaCl. However, on increasing the salt concentration some reduction was observed (Fig. 7). The salt requirement of this protease is relatively less in comparison with other halophilic proteases [19,23,24].

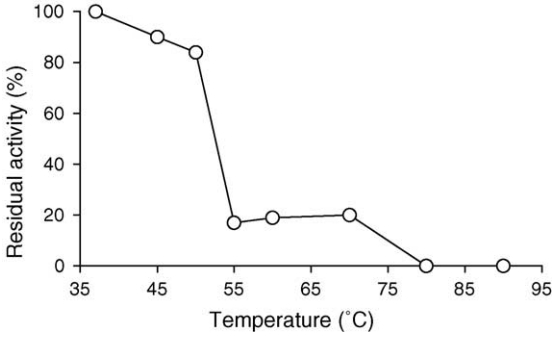

Fig. 5. Thermal stability of purified protease. The purified protease was incubated at different temperatures for 40 min. The remaining protease activity was determined as described in Section 2.2. The activity at 37 °C (with prior incubation of the enzyme at 37 °C for 40 min) is taken as 100%.

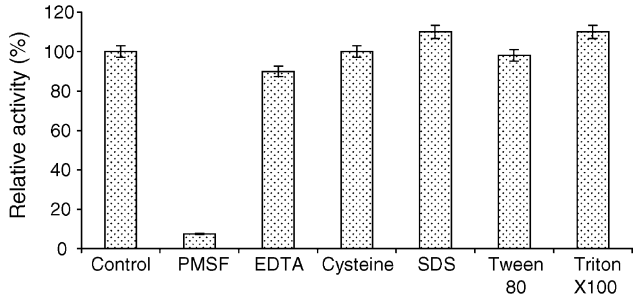

Fig. 6. Effect of inhibitors and surfactants on the activity of the purified protease. The activity of the protease was measured in the presence of phenylmethanesulfonyl fluoride (PMSF, 5 mM), EDTA (1 mM), cysetine (1 mM), SDS (0.1%), Tween 80 (0.1%) and Triton X-100 (0.1%). The activity of protease, without any effectors, is taken as 100%. The experiment was carried out in duplicates and the difference in the individual results was less than 3%. Bars correspond to percentage variation.

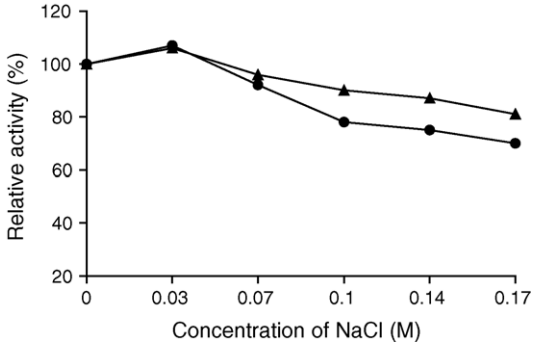

Fig. 7. Effect of NaCl on protease activity. The crude (▲) and purified (●) enzymes were incubated with different concentrations of salt (0–0.17 M) and enzyme assay was carried out as described in Section 2.2.

Some cations are known to enhance the enzyme activities. The effects of metal ions on purified protease activity were tested at 1 mM and 5 mM concentration of metal ions in 20 mM borax–NaOH buffer (pH 10.0). The protease activity was reduced by 1 mM and 5 mM Ni^{2+} (to 75% and 50%, respectively), whereas Mn^{2+}, Zn^{2+} and Mg^{2+} had no significant effect. Addition of Ca^{2+} to the assay medium at the level of 1 mM enhanced the protease activity to 140% while higher concentration (5 mM) reduced the enzyme activity (to 71%). In case of Cu^{2+}, the protease activity decreased to 78% with 1 mM Cu^{2+} but was slightly enhanced (121%) with 5 mM Cu^{2+} (Fig. 8). Enhancement of activity in presence of Ca^{2+} and inhibitory effect of Cu^{2+} in present case is in agreement with protease from moderately halophilic marine *Pseudomonas* sp. that is reported to be activated by Ca^{2+} and Mg^{2+} but strongly inhibited by Fe^{2+}, Cu^{2+} and Mn^{2+} [25].

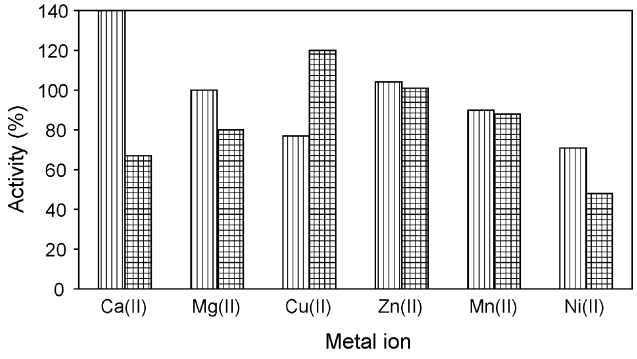

Fig. 8. Effect of metal ions on the activity of the purified protease. The activity of the protease was estimated in the presence of 1 mM (vertically hatched) and 5 mM (crosshatched) metal ions. The activity of the protease, without any metal ions, is taken as 100%.

4. Conclusion

The possibility of using enzymes from haloalkaliphilic bacteria in industrial process has the advantage of optimal activities at alkaline and high salt concentrations. A novel protease from moderately halophilic *Bacillus* sp. is reported in present work. The purification of enzymes from these sources is considered to be tedious. In this case, it is achieved to homogeneity in one-step hydrophobic interaction chromatography. The stability of protein at alkaline pH and in the presence of salts and surfactants is amply demonstrated.

To sum up, a facile one step purification of a *Bacillus* sp. protease fairly stable in a range of salts, detergents and at higher temperatures and being optimally active at pH 10–11 is described. Thus, this protease seems to be potentially useful for biotechnological applications.

The one step purification procedure developed in present study may be useful for purification of proteases from halophilic microbial sources.

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