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Two-step purification of a highly thermostable alkaline protease from salt-tolerant alkaliphilic *Streptomyces clavuligerus* strain Mit-1

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Abstract

An alkaline protease from a salt-tolerant alkaliphilic *Streptomyces clavuligerus* was purified to homogeneity by 141-fold with a yield of 12% using two-step method of salt precipitation and ion exchange chromatography on DEAE cellulose. The apparent molecular mass was 49 ± 2 kDa and the enzyme appeared as monomer based on SDS and Native-PAGE. The temperature optimum was 70 °C with significant stability at 60–80 °C for more than 60 min. The enzyme was active over the pH range of 8.5–11, with an optimum at 10–11. The serine nature of the protease was confirmed by PMSF inhibition. The enzyme was highly resistant against chemical denaturation and displayed varied effects towards metal ions. The results are significant as extremozymes are difficult to purify and therefore, a two-step purification of alkaline protease from relatively less explored group of actinomycetes is quite appealing.
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Keywords: Two-step purification of alkaline protease; Salt-tolerant alkaliphilic actinomycete; *Streptomyces clavuligerus*; Thermostable alkaline protease

1. Introduction

Driven by increasing industrial demands for biocatalysts able to cope with industrial process conditions, continous efforts are being focused on the search for such enzymes. Despite the fact that large number of different enzymes have been identified and many are being used in various biotechnological applications, the available enzymatic array is still not sufficient to meet the ever increasing demand [1,2]. In addition to their novel applications, the enzymes from extremophiles may also provide unique models for protein stability under extreme conditions.

Purification of extremozymes assumes significance in understanding cellular metabolism and regulatory pathways. It is also significant for commercial production of several industrially and pharmaceutically important enzymes. Since each enzyme requires specific strategy for purification, it is necessary to develop strategies for the purification of individual extremozymes.

Proteases being one of the largest commercial enzymes account for more than 60% of the total enzyme market worldwide [3–7]. Among them, alkaline proteases constitute a very large and complex group of enzymes. While number of alkaline proteases have been characterized from alkaliphilic bacteria, the similar explorations and studies from halophilic and haloalkaliphilic bacteria [8–12] appears to be quite limited. Representations from haloalkaliphilic actinomycetes for such studies are further restricted [13–15].

In view of the above realization, the present study aimed at the purification of alkaline protease secreted by salt-tolerant, alkaliphilic (extremophilic) actinomycete, Mit-1, isolated from west coast of India.

2. Experimental

2.1. Materials

DEAE Cellulose, reagents for SDS-PAGE and media components were purchased from Hi media Laboratories (Mumbai, India). Protein molecular weight markers for SDS-PAGE were obtained from Banglore Genei (Banglore, India). Casein was a product of Sisco Research Laboratories (Mumbai, India). All other chemicals used were of analytical grade.

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2.2. Methods

2.2.1. The organism

A halo-tolerant and alkaliphilic actinomycete, Mit-1, was isolated from saline soil collected from coastal region of Mithapur, Gujarat, India. The saline soil (10 g) was incubated at 45 °C with $CaCl_2$ (1 g) for 1 week. The treated soil was enriched in Actinomyces broth (Hi Media Ltd.) with NaCl (5%, w/v). The pH of the medium was adjusted to 9 by adding separately sterilized Na_2CO_3 (20%, w/v). The enriched culture was streaked on the Actinomyces agar (5% w/v NaCl, pH 9). After the incubation of 6 days at 30 °C, a typical chalky white colony was picked up and re-streaked to ensure the purity of the colony. The culture was maintained at 4 °C on Actinomyces agar slants (5% w/v NaCl and pH 9).

2.2.2. Protease production in liquid culture

The inoculum was prepared by transferring a loop full of culture from the slant into 25 ml sterile gelatin broth (GB) containing, g/l: Gelatin, 10; Peptone, 5; Yeast extract, 5; NaCl, 50; pH 9, followed by incubation at 30 °C on a rotary shaker (100 rpm) for 48 h. Ten percent of this activated culture was inoculated into 100 ml of gelatin broth (5% w/v NaCl, pH 9) and incubated at 30 °C under shaking conditions as described. After 110 h of growth, the cells were harvested by filtering the broth with cellulose filter; the filtrate was used as the crude enzyme.

2.2.3. Purification of Mit-1 alkaline protease

The partial purification of Mit-1 protease was carried out by precipitation of the crude enzyme with ammonium sulphate between 30 and 60% saturation. The precipitates were collected by centrifugation (10,000 × *g* at 4 °C) and resuspended into a minimum volume of 20mM NaOH–Borax buffer (pH 10). This preparation was used as a partially purified protease.

The partially purified enzyme was desalted by conventional dialysis against 20 mM NaOH–Borax buffer (pH 10) at 4 °C for over night. The enzyme was then loaded onto DEAE cellulose column (10 cm × 2.5 cm) pre-equilibrated with 20 mM NaOH–Borax buffer (pH 10). The column was washed with the same buffer to remove unbound proteins. The bound protease was eluted by applying a gradient of increasing concentrations of KCl (0–0.5 M). The fractions were analyzed for the protease activity and protein content was measured by Lowry's method [16] using bovine serum albumin (BSA) (20–200 μg) as standard.

2.2.4. Enzyme assay for protease

Anson–Hagihara's method [17] was used to measure the alkaline protease activity using casein as substrate. The enzyme (0.5 ml) was added to 3.0 ml casein solution (0.6%, w/v casein solution prepared in 20 mM Borax–NaOH buffer, pH 10). The reaction was incubated at 70 °C for 10 min and then the enzyme reaction was terminated by the addition of 3.2 ml of TCA mixture (containing 0.11 M trichloro acetic acid, 0.22 M sodium acetate and 0.33 M acetic acid) and incubated at 37 °C for 30 min. The precipitates were removed by filtration through Whatman No.1 filter paper. The absorbance of the filtrate was measured at 280 nm. One unit of alkaline protease was defined as the amount of enzyme liberating 1 μg of tyrosine per min under assay condition. Enzyme units were calculated using tyrosine (0–100 μg) as standard.

2.2.5. Gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [18] using 12% polyacrylamide gel. Protein bands were visualized by coomassie brilliant blue R-250 staining. Native gel electrophoresis was also performed to determine the molecular weight of the protein.

2.2.6. Temperature profile and thermal stability

The optimum temperature for protease activity was determined by incubating the reaction mixture at different temperatures in the range of 37–90 °C. The thermal stability of the purified Mit-1 protease was investigated by incubating it at different temperatures (50–90 °C). The aliquots were withdrawn at 20 min intervals followed by the measurement of enzyme activity at 70 °C.

2.2.7. Determination of pH optimum and pH stability

The effect of pH on protease activity was determined at 70 °C by using substrate prepared in various buffers (20 mM) of different pH (citrate phosphate, pH 5–7; Tris–HCl, pH 8–9; Glycine–NaOH, pH 9–10; NaOH–Borax, pH 10–11 and KCl–NaOH, pH 12–13). Similarly, the enzyme stability at different pH was determined by incubating the enzyme in the pH range of 5–13 in respective buffers as listed above. After incubating the enzyme for 30, 60 and 120 min in respective buffers, the residual activities were measured as per assay procedure.

2.2.8. Effect of inhibitors

Protease was incubated for 2 h with various protease inhibitors at 5 and 10 mM concentrations. The inhibitors were: *p*-chloromercuribenzoic (*p*-CMB), dithiothritol (DTT), phenyl methane sulfonyl fluoride (PMSF), thiourea and ethylene diamine tetra acetic acid (EDTA). The percent residual activities were calculated by considering the activity of the untreated enzyme as 100%.

2.2.9. Chemical denaturation by urea

Urea was added in the enzyme solutions at the final concentrations of 8 M. The aliquots were withdrawn at definite time intervals and subjected to the determination of the residual enzyme activities. The residual activities were calculated as mentioned in Section 2.2.8.

2.2.10. Effect of metal ions

The effect of metal ions on enzyme was assessed by carrying out enzyme assay in the presence of 5 mM of $CaCl_2$, $AgCl_3$, $CoCl_2$, $CdCl_2$, $FeCl_3$, NaCl, $HgCl_2$, $SrCl_2$, $NiCl_3$ and $CuSO_4$. The relative activities were estimated with reference to control.

Table 1
Purification of Mit-1 protease with salt precipitation and ion exchange chromatography

Purification steps	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	187787	3912.5	47.99	–	100
60% $(NH_4)_2SO_4$ precipitation	110562	85.5	1293.12	26.94	58
DEAE cellulose (Ion-exchange chromatography)	20400	3.0	6800	141.66	11

Details of the experiment are described in the text.

3. Results and discussion

The organism Mit-1, isolated from coastal region of Gujarat (India), was gram-positive having filamentous structure. It was identified as *Streptomyces clavuligerus* based on the morphological, physiological and biochemical characteristics. The culture was assigned MTCC 7037 as accession/strain number by Microbial Type Culture Collection and *Gene Bank*, IMTECH, Chandigarh (India).

Mit-1 alkaline protease was purified by the combination of ammonium sulphate fractionation and ion exchange chromatography. Table 1 illustrates the steps involved in the purification process. The ammonium sulphate precipitation at 60% saturation yielded 27-fold purification enhancing the specific activity to 1293 U/mg protein with a yield of 58%. After dialysis, the 60% ammonium sulphate fraction was subjected to ion exchange chromatography on DEAE-Cellulose leading to 141-fold purification with the specific activity of 6800 U/mg of protein. The results are quite encouraging in comparison to an alkaline protease from Thermoactinomycetes in which only limited (20-fold) purification was achieved by DEAE-SepharoseCL-6B and Toyopearl 650 column chromatography [19].

The enzyme was successfully purified to the homogeneity as evident by a single band on SDS-PAGE. The apparent molecular mass of purified protease was estimated as 49–50 kDa by using Rf values of the reference proteins (Fig. 1). A single band on the Native-PAGE indicated that Mit-1 protease would be a monomeric protein (Fig. 2). The molecular mass of alkaline proteases from alkaliphilic *Thermoactinomyces* sp. HS682 and E79 was quite comparable with our values [20,21]. It also corresponded to some halophilic and alkaline proteases from haloalkaliphilic bacteria, where the molecular weight ranged from 40 to 130 kDa [22–24].

The temperature optima of purified Mit-1 protease was 70 °C (Fig. 3) which was comparable to alkaline proteases from *Bacillus subtilis* NCIM 2713 [5] and *Streptomyces albidoflavus* [25]. Interestingly, the optimum temperature for the Mit-1 protease was nearly 2-fold higher than those reported from haloalkaliphilic bacteria recently from our laboratory [26,27]. Besides high temperature requirement for the activity, Mit-1 protease also displayed greater stability at higher temperatures, a feature quite desirable for biotechnological applications. The enzyme was quite stable at 50 °C for 60 min, but lost 50% of the original activity during the same time of incubation at 60 °C. At 70 °C, however, the enzyme retained 80% activity after 20 min

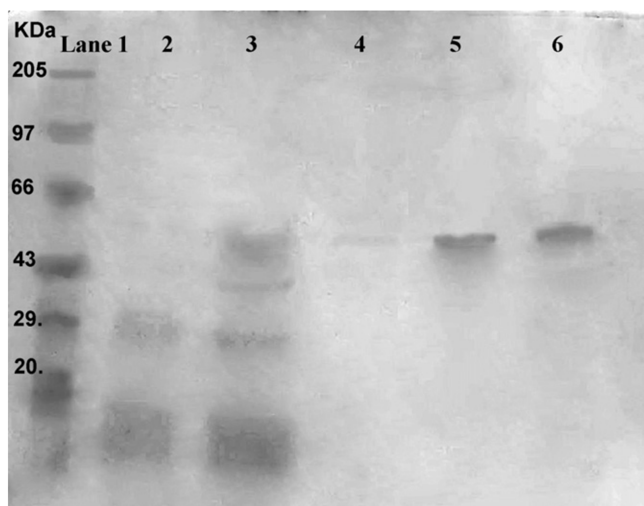

Fig. 1. SDS-PAGE pattern of purified protease. Electrophoresis was carried out using a 12% cross linked polyacrylamide gel. Lane 1: protein marker, lane 2, 3: electrophoretic separation of partially purified Mit-1 protease, lane 4, 5, 6: purified protease on 12% polyacrylamide gel stained with coomassie brilliant blue.

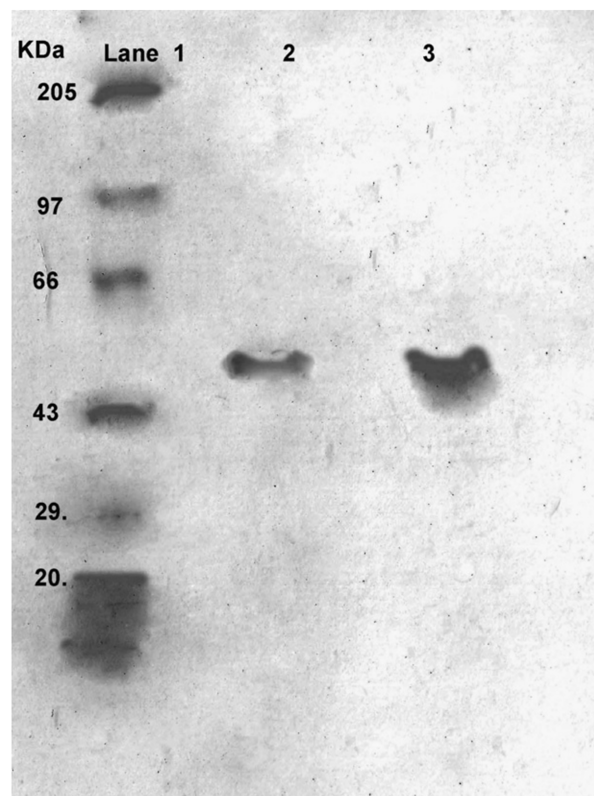

Fig. 2. Native-PAGE analysis of purified Mit-1 protease. Lane 1: protein marker, lane 2, 3: electrophoretic separation of purified protease on 12% polyacrylamide gel stained with coomassie brilliant blue.

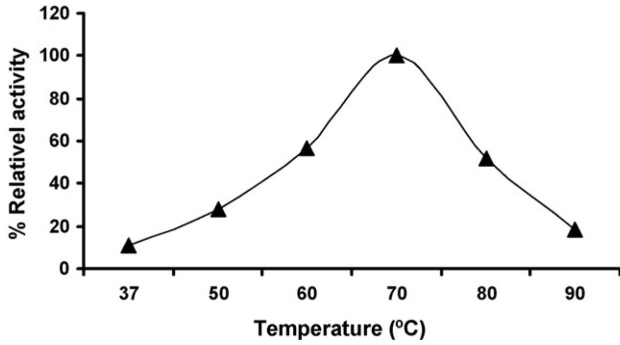

Fig. 3. Temperature optimum of purified Mit-1 protease. The purified enzyme was incubated with the substrate at different temperatures. The activity at 70 °C has been taken as 100%.

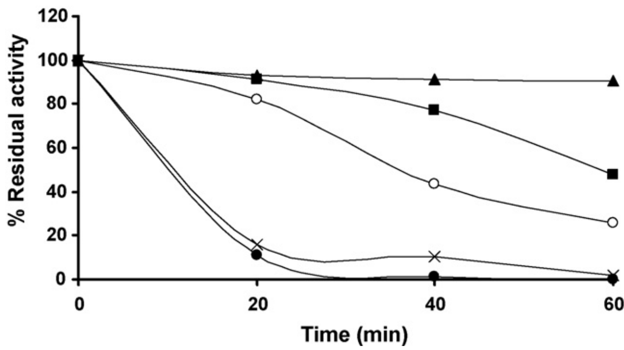

Fig. 4. Thermal stability of purified Mit-1 protease. The pure enzyme was incubated at temperature 50 °C (▲); 60 °C (■); 70 °C (◯); 80 °C (×) and 90 °C (●) for 20, 40 and 60 min. The remaining protease activity was determined as described in Section 2.2. The activity at 0 min is taken as 100%.

which declined further to 35 and 5% after 40 and 60 min incubation, respectively. The enzyme was completely denatured within 20–40 min at 80–90 °C (Fig. 4). Han and Damodaran [1] reported an alkaline protease from alkaliphilic *Bacillus pumilus* that was stable only for 20 min at 55 °C.

The purified alkaline protease was active in the pH range of 9–11 with optimum at 10–11 (Fig. 5). The findings are in accordance with several recent reports showing pH optima of 10–11 [6–8,28]. While a serine protease from the keratin degrading *Streptomyces pactum* DSM 40530 was optimally active in a pH range of 7–10 [29], a thermostable alkaline protease from *Thermoactinomycetes* sp HS682 was active at pH 11 [20]. The Mit-1 enzyme was stable over a relatively narrower pH range 8–10 and displayed lower stability at acidic pH; for instance, the enzyme lost all its activity at pH 6 within 6 h of incubation. On the other hand, it was highly stable at alkaline pH and retained nearly 90–100% of the activity at pH 8–11 even after prolong incubation (Fig. 6). In order to judge the role of certain stabilizing proteins or factors on enzyme stability, the above studies were also performed with crude preparations. The results indicated that the enzyme stability at higher pH was marginally better in crude preparation as compared to purified enzyme (data not shown). Nevertheless, the greater stability in the alkaline range reflected the alkaliphilic nature of the enzyme.

The purified protease was completely inhibited by PMSF (10 mM), a specific inhibitor of serine proteases (Fig. 7). Other specific protease inhibitors such as *p*-chloro mercuri benzoate (*p*-CMB), thiourea and ethylene diamine tetra acetic acid, did not have any significant effect on the enzyme. However, there was marginal increase in the activity by DTT. With reference to serine

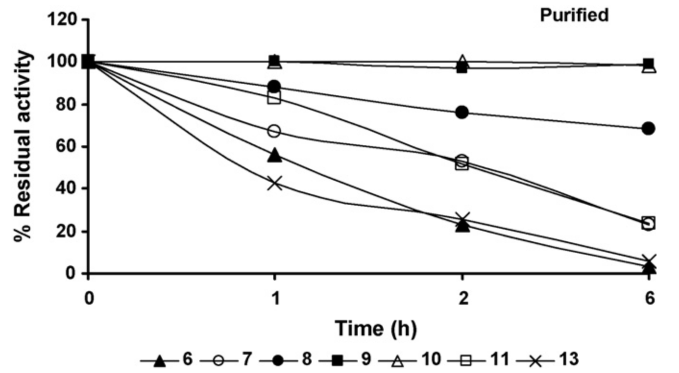

Fig. 6. pH stability of purified protease. The buffers used are described in Section 2.2. The pH range used: pH 6 (▲); pH 7 (◯); pH 8 (●); pH 9 (■); pH 10 (△); pH 11 (□) and pH 13 (×). The activity at time 0 (prior to the incubation of enzyme with buffer) is taken as 100%.

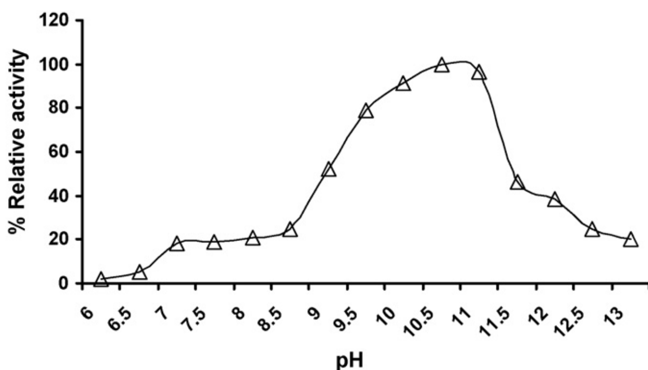

Fig. 5. pH optimum of purified Mit-1 protease. pH optimum was measured by incubating the enzyme with the substrate at different pH values. The buffers used are citrate phosphate (pH 6–7); Tris–HCl (pH 8–9); Glycine–NaOH (pH 9–10); NaOH–Borax (pH 10–11) and KCl–NaOH (pH 12–13). The activity at pH 10.5 is taken as 100%.

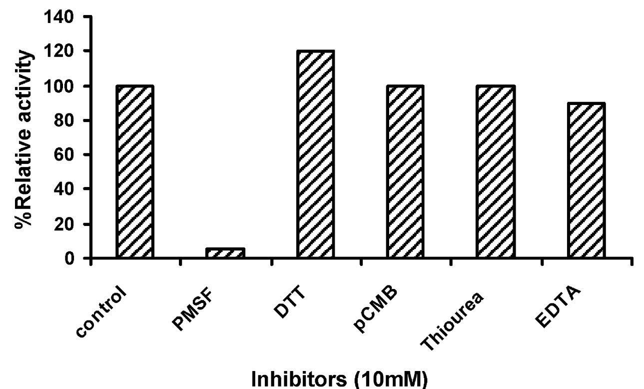

Fig. 7. Effect of inhibitors on the activity of the purified protease. The activity of protease was measured in the presence of phenylmethanesulfonyl fluoride (PMSF), *p*-chloromercuribenzoic (*p*-CMB), dithiothritol (DTT), thiourea and ethylene diamine tetra acetic acid (EDTA) at the concentration of 10 mM. The activity of protease, without any effectors, is taken as 100%.

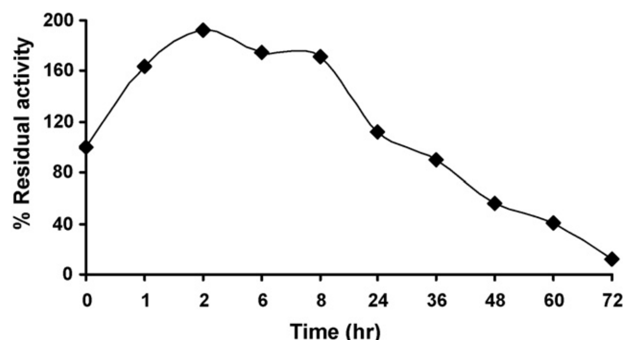

Fig. 8. Denaturation of purified protease by 8 M urea. The activity prior to the addition of urea is taken as 100%.

nature of the protease, it corresponds with most of the alkaline proteases from halophilic and alkaliphilic organisms reported so far [21,23,27,1,30]. Our results are also in agreement with an alkaline protease from alkaliphilic Thermoactinomycetes sp. HS682 in which the enzyme was inhibited by DFP and PMSF, but not by EDTA [20].

To study the effect of chemical denaturant, the enzyme was incubated with 8 M urea and monitored for the residual activity. Surprisingly, instead of getting denatured, the activity of purified protease increased upon incubation with 8 M urea up to 36 h. Within 72 h, however, the enzyme lost 90% of its original activity (Fig. 8). The resistance of Mit-1 protease towards urea denaturation was in contrast with one of the previous studies from our own laboratory, where an alkaline protease from a haloalkaliphilic *Bacillus* sp. was highly sensitive to urea denaturation [27]. However, in comparison to pure enzyme, partially purified preparation of Mit-1 protease displayed greater resistance against denaturation by urea when assessed for the stability on long term basis (data not shown). The phenomenon of extreme resistance against chemical denaturation appears to be quite rare among the alkaline proteases reported from extremophilic actinomycetes.

The metal ions had varied effect on partially purified protease activity. There was significant increase in protease activity with Ca^{2+} and Cd^{2+}. The maximal activity of the enzyme reached to 140% with these metal ions (Fig. 9). While others, such as Fe^{2+}, Cu^{2+}, Sr^{2+}, and Na^{+} did not show any appreciable effect on enzyme activity; marginal decrease was evident with Co^{2+} and NI^{3+}. However, the enzyme lost more than 80% activity in the presence of Hg^{2+} and Al^{3+}**.** The findings gained further ground on the basis of a recent report on two novel halotolerant extracellular proteases from *Bacillus subtilis* strain FP-133 which were activated in the presence of $Ca^{2+}$and inhibited by Hg^{2+} [6].

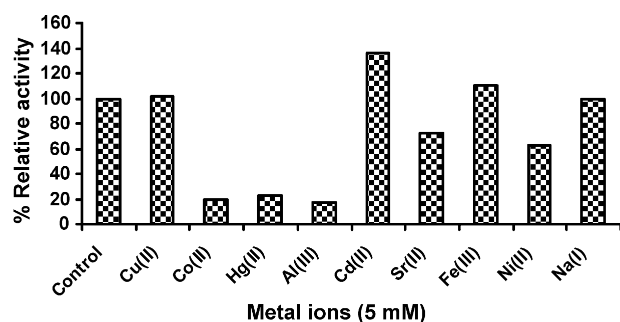

Fig. 9. Effect of metal ions on the activity of purified protease. The activity of the protease was estimated in the presence of 5 mM metal ions. The activity of the protease, without any metal ions, is taken as 100%.

4. Conclusion

Studies on the enzymes adapted to extreme conditions may provide indispensable tool in designing the strategies for their rational improvements. Enzymes are so fine-tuned with nature that they often do not coincide with the desired properties and activities useful for biotechnological applications. Therefore, it was of significant interest to focus on protease purification from rarely explored group of halo-tolerant alkaliphilic actinomycetes.

The present report focused on two-step purification of novel alkaline protease from a salt-tolerant alkaliphilic actinomycete; *Streptomyces clavuligerus* strain Mit-1. Since, the extremozymes are rather difficult to purify, the simple and inexpensive purification procedure developed in the present study would be quite useful for purification of protease from other extremophilic microbial sources. Besides, the enzyme displayed significant stability at higher temperatures, alkaline pH and a range of metal ions. The enzyme also displayed extreme resistance against urea denaturation, a finding which is rather unique and restricted to only few proteins.

In summary, the enzyme stability at two extremities of pH and temperature offers unique avenues for biotechnological applications. The findings are also important in view of the fact that only few enzymes have been purified and biochemically characterized from extremophilic actinomycetes. Therefore, characterization of more enzymes from these groups would help establishing the biochemical and structural basis of the molecular stability under extremities.

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