Purification and Characterization of Manganese-Dependent Alkaline Serine Protease from \textit{Bacillus pumilus} TMS55

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The purification and characterization of a Mn$^{2+}$-dependent alkaline serine protease produced by \textit{Bacillus pumilus} TMS55 were investigated. The enzyme was purified in three steps: concentrating the crude enzyme using ammonium sulfate precipitation, followed by gel filtration and cation-exchange chromatography. The purified protease had a molecular mass of approximately 35 kDa, was highly active over a broad pH range of 7.0 to 12.0, and remained stable over a pH range of 7.5 to 11.5. The optimum temperature for the enzyme activity was found to be 60$^\circ$C. PMSF and AEBSF (1 mM) significantly inhibited the protease activity, indicating that the protease is a serine protease. Mn$^{2+}$ ions enhanced the activity and stability of the enzyme. In addition, the purified protease remained stable with oxidants ($\text{H}_2\text{O}_2$, 2%) and organic solvents (25%), such as benzene, hexane, and toluene. Therefore, these characteristics of the protease and its dehairing ability indicate its potential for a wide range of commercial applications.

\textbf{Keywords:} \textit{Bacillus pumilus}, alkaline serine protease, dehairing, manganese dependent, enzyme purification

Microorganisms are an excellent source of many commercial enzymes, as they can be cultured in large quantities within a relatively short time using established fermentation methods, resulting in an abundant, regular supply of the desired product [28]. The majority of microbial enzymes with industrial applications are derived from terrestrial microorganisms, particularly from bacteria and/or fungi [5].

However, the marine environment harbors millions of species of microorganisms that play an active role in the mineralization of complex organic matter; degradation of dead organisms; degradation of pollutants and toxicants; and diverse metabolites. In addition, marine microorganisms have a diverse range of enzymatic activities and are capable of catalyzing various biochemical reactions with novel enzymes. For example, aerobic bacteria are capable of producing enzymes, such as amylases, deoxyribonucleases, lipases, and proteases. The majority of previous reports on these enzymes are with reference to secondary production in the marine environment [31], and there are a few findings that describe their potential industrial applications. Thus, marine bacteria can provide a wealth of new enzymes.

Protease is a hydrolytic enzyme that cleaves peptide bonds to produce small peptides and amino acids. Since proteases are physiologically necessary for all living organisms, they are ubiquitous, being found in a wide diversity of sources, including plants, animals, and microorganisms. Therefore, studying this enzyme is important for physiology, and pathology, as well as commercial applications. Proteases from \textit{Bacillus} species are the major industrial workhorses, and the use of proteases from these sources has increased in various industrial areas, including leather processing, detergents, food, waste treatment, and peptide synthesis, due to their increased production capacities, high catalytic activities, and high degree of substrate specificity [8, 13, 16]. Leather processing involves several steps, such as soaking, dehairing, bating, and tanning, and almost 70% of the related pollution originates from the pre-tanning operations [27]. The conventional method of dehairing consists of creating extremely alkaline conditions using lime, followed by treatment with sulfide to solubilize the proteins in the hair root. The sulfide emissions resulting from the dehairing operations [20] then create problems of pollution and effluent disposal. However, it is now possible to reduce the sulfide at its source using enzyme-assisted processes [2]. Enzymatic dehairing generally uses alkaline proteases along with small amounts of sulfide and lime [9]. In addition, when alkaline enzymes are used in the hide-dehairing process, the dehairing is carried out at pH values of between 8 and 10 [12]. Yet, even though
enzymes can assist in the dehairing process and, to some extent, reduce the pollution load, a technology based on enzymes alone, without the use of sulfide and other chemical inputs, has yet to be explored for commercialization. The success of alkaline proteases in dehairing depends on such properties as having a wide pH activity range; being stable under high alkaline conditions; exhibiting high activity and stability in the presence of surfactants, chelating reagents, and bleaching agents; and having high activity over a wide temperature range and long shelf-life.

For maximum activity, alkaline proteases require a divalent cation, like Ca\(^{2+}\), Mg\(^{2+}\), or Mn\(^{2+}\), or a combination of these cations. It is believed that these cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures [34]. The requirement of these cations varies from organism to organism. Although the majority of reported proteases require either Ca\(^{2+}\) or Mg\(^{2+}\) for activity, a few enzymes have been reported to require Mn\(^{2+}\) [1, 36]. Accordingly, the present paper attempted to purify and characterize a Mn\(^{2+}\)-dependent protease with a dehairing property from *B. pumilus* isolated from marine sediment and compare it with proteases from other bacterial sources.

**Materials and Methods**

**Bacterial Strain**

The marine sediment samples were collected using a core sampler at four different depths (1.5 m, 2.4 m, 3 m, and 4.5 m) in the east coastal region of Thondi, Palk Bay, India (latitude 9°45’ N and longitude 79°13’ E). The bacterial isolates were screened on skim milk agar plates incubated at 37°C for 24 h and, depending on the zone of clearance, the isolates showing the maximum activity on the plate and liquid cultures were selected for further dehairing studies.

**Identification and Taxonomical Studies**

The isolate TMS55 was biochemically characterized according to the method described in *Berger’s Manual of Determinative Bacteriology* [11] and identified based on its 16S rRNA gene sequence. The 16S rRNA gene amplification, cloning, and sequencing of the 16S rRNA gene were conducted as described by Syed Ibrahim *et al.* [35]. The sequence data were analyzed based on comparison with 16S rRNA genes in the GenBank database.

**Enzyme Production**

The culture medium (pH 7.5) used for protease production in this study contained MgSO\(_4\) (0.2%), K\(_2\)HPO\(_4\) (0.5%), maltose (0.5%), NaCl (0.5%), beef extract (0.5%), and soya bean meal (1%). The culture medium (50 ml in 250-ml Erlenmeyer flasks) was inoculated at 1% with 24-h seed culture and incubated at 28°C for 48 h. The cells were then separated by centrifugation and the cell-free supernatant was used as the crude enzyme preparation.

**Protease Assay and Total Protein Estimation**

The enzyme activity was assayed using azocasein as the substrate, according to the method of Sarath *et al.* [33]. The reaction mixture consisted of 0.25 ml of a 50 mM sodium phosphate buffer (pH 7.8), containing 2.0% (w/v) azocasein and 0.15 ml of the enzyme solution. After incubating at 37°C for 15 min, the reaction was stopped by the addition of 1.2 ml of 10.0% (w/v) TCA and incubated at room temperature for an additional 15 min. The precipitate was then removed by centrifugation at 8,000 × *g* for 5 min. Next, 1.4 ml of 1.0 M NaOH was added to 12.0 ml of the supernatant and the absorbance measured at 440 nm. One unit of enzyme activity was expressed as giving an absorbance of 1.0 under the above conditions. The protein content was estimated using the method of Lowry *et al.* [19] with bovine serum albumin as the standard. During the chromatographic purification steps, the protein concentration was estimated as a function of its absorbance at 280 nm.

**Protein Purification and SDS–PAGE**

The cell-free culture supernatant (250 ml) was precipitated by ammonium sulfate saturation (up to 50%). The saturated solution was then centrifuged at 13,000 × *g* for 20 min at 4°C and the pellet suspended in a minimum amount of a 50 mM phosphate buffer (pH 7.8). The pellets and supernatants were checked for protease activity using the azocasein assay. Any insoluble material present after the suspension was removed by centrifugation at 13,000 × *g* for 20 min at 4°C and the supernatant then collected. The concentrated protease after desalting was applied to a Sephadex G-75 column (1 cm × 50 cm) equilibrated with a 20 mM phosphate buffer (pH 7.8). The flow rate was 0.8 ml/min and 1 ml fractions were collected. The active fractions were pooled and subjected to ion-exchange chromatography using the AKTApurifier FPLC (GE Healthcare, Sweden). The sample from the gel filtration was then loaded at a flow rate of 0.5 ml/min onto a CM-Sepharose Fast flow column (1 cm × 30 cm) that had been equilibrated with a 20 mM phosphate buffer (pH 7.8). The unasorbed materials were washed from the column using the same buffer. The protease was eluted using a 20 mM phosphate buffer (pH 7.8) containing 0.1 M NaCl at a flow rate of 0.8 ml/min. The active fractions were pooled and concentrated by ultrafiltration with the use of 10-kDa cut-off membrane (Amicon, Beverly, MA, USA) and used for further characterization of the enzyme. The homogeneity and molecular weight of the purified protease was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to the method of Laemmli [18]. The protein bands were visualized by silver staining. The molecular weight of the purified protease was determined by SDS–PAGE using a commercial protein marker from Sigma-Aldrich (Cat. No. S8445).

**Effects of pH and Temperature on Activity**

The activity of the purified protease was measured at different pH values (7 to 12) using azocasein (2% (w/v)) as the substrate. The pH of the reaction mixture was adjusted to the desired value using the following 50 mM buffers; potassium phosphate (pH 7.0–9.5) and glycine-sodium hydroxide (pH 10.0–12.0). The relative activities were quantified under standard assay conditions. The effect of temperature on the enzyme activity was examined at various temperatures at pH 9.5. The thermal stability was determined by incubating the purified enzyme at various temperatures, in the absence or presence of 5 mM Mn\(^{2+}\). Aliquots were withdrawn at certain time intervals to test the remaining activity under the optimum conditions. The non-heated enzyme was considered as the control and assumed to have 100% activity.