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Purification and Characterization of Manganese-Dependent Alkaline Serine Protease from *Bacillus pumilus* TMS55

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The purification and characterization of a Mn^{2+}-dependent alkaline serine protease produced by *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°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 (H_2O_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.

Keywords: *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 *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

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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 stabile 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 *Bergey'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_2HPO_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 1.2 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 unabsorbed 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.

Effects of Metal Ions and Inhibitors on Protease Activity

The effects of metal ions on the purified enzyme were determined by treating the purified enzyme with various metal ions, like Ca^{2+} (calcium chloride), Mg^{2+} (magnesium sulfate), Mn^{2+} (manganese sulfate), Cu^{2+} (copper sulfate), Zn^{2+} (zinc sulfate), Co^{2+} (cobalt chloride), Ba^{2+} (barium chloride), Fe^{2+} (ferric chloride), and Hg^{2+} (mercuric chloride), at concentrations of 1 mM and 5 mM for 30 min at room temperature. The enzyme assay was subsequently performed as described earlier. The percentage of relative enzyme activity was calculated with reference to the activity of the enzyme without these supplements. The effects of inhibitors provide the most reliable information as to the catalytic type of a peptidase. Thus, to investigate the catalytic type of the purified protease, it was pre-incubated with different protease-specific inhibitors from Sigma, namely, AEBSF (serine protease-specific inhibitor), EDTA (metalloprotease-specific inhibitor), E-64 (cysteine protease-specific inhibitor), pepstatin (acid protease-specific inhibitor), and PMSF (serine protease-specific inhibitor), at a concentration of 1 mM in various tubes for 30 min at room temperature. After the pre-incubation, a protease assay was performed. The purified protease without protease specific inhibitors was used as the control and its activity considered as 100%.

Effects of Detergents, and Oxidizing and Reducing Agents on Enzyme Activity

To study the effects of detergents on the enzyme activity, the purified protease was pre-incubated with the detergents Tween 20, Tween 80, Triton X-100, and SDS at 1% and 2% concentrations for 30 min at room temperature in various tubes. After the pre-incubation, a protease assay was performed at 60°C for 15 min at pH 9.5. The detergent-free pre-incubated purified protease was taken as the control and its activity considered as 100%. To investigate the effects of oxidizing and reducing agents on the enzyme activity, β-mercaptoethanol and hydrogen peroxide were added at 1% and 2% (v/v) in various tubes. The enzyme and additive mixture were pre-incubated at room temperature for 30 min and then assayed for protease activity. The activity of the purified protease enzyme pre-incubated under similar conditions without any additives was taken as 100%.

Effects of Organic Solvents on Stability of Purified Enzyme

To determine the effects of solvents on the protease stability, 3.0 ml of the enzyme solution in a 50 mM phosphate buffer (pH 9.5) was mixed with 1 ml of different organic solvents, such as ethanol, acetone, pyridine, *n*-butanol, benzene, chloroform, toluene, xylene, and hexane. The mixtures were then incubated at room temperature for 30 min with constant shaking. The residual activity was measured using the assay method described earlier. All the experiments were conducted three times and the standard errors are reported. The activity of the purified protease enzyme pre-incubated under similar conditions without solvents was taken as 100%.

Evaluation of Purified Protease for Dehairing

A fresh piece of goat skin (about 3 cm×4 cm) was treated with 2 U/g of skin in the presence or absence of 5 mM PMSF and incubated at 37°C for a certain time. The dehairing efficacy of the protease from TMS55 was then compared with that of commercially available dehairing proteases; namely, Biodart from Southern Petrochemical Industries Corporation (SPIC) Limited, and Ezyme-A from Bioscience Ltd. All the proteases were adjusted to an azocasein unit of 2 U/g of skin. At the end of the process, the skin pieces were manually scraped to remove the loose hairs. This procedure was necessary, as rubbing on a laboratory scale would not have been as vigorous as that in industrial drums. The quality of the dehaired skin was estimated according to the appearance observed by the naked eye and under a microscope [6].

RESULTS AND DISCUSSION

Isolation of Alkaline Protease-Producing Strain TMS55

The marine sediment samples were collected from the east coastal region of Thondi, Palk Bay, India. Among the isolates screened for alkaline protease activity, strain TMS55 was selected for further study because of its potential dehairing efficacy.

Isolate TMS55 was characterized as *B. pumilus* based on its physiological and biochemical characteristics and a 16S rRNA sequence analysis. A similarity search using BLAST at the NCBI revealed a high similarity with *B. pumilus* strains. The 16S rRNA gene sequence of strain TMS55 has been deposited in the GenBank database under Accession No. DQ988522. Moreover, strain *B. pumilus* TMS55 has been deposited in the Microbial Type Culture Collection and Gene Bank (MTCC), India under Accession No. MTCC 5357.

Purification of Protease

The protease produced by *B. pumilus* TMS55 was concentrated by ammonium sulfate (50%) precipitation and purified consecutively with gel filtration and ion-exchange

Table 1. Summary of the purification procedures for alkaline serine protease from *B. pumilus* TMS55.

Fraction	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Purification factor[a]	Overall yield[b] (%)
Crude	250	3.2	802.5	5.8	1,460.0	1.8	1.0	100.0
Ammonium sulfate	12	22.4	268.2	40.9	491.0	1.8	1.1	33.6
Gel filtration	2	7.9	15.8	71.6	143.1	9.1	4.9	9.8
CM-Sepharose	1	0.4	0.4	100.8	100.8	288.1	31.7	6.9

[a]Defined as purification factor=(specific activity of fraction/specific activity of crude).
[b]Defined as overall yield=(total activity of fraction/total activity of crude).

chromatography. The results of the purification procedure are summarized in Table 1. After the final purification step, the enzyme was purified 31.72-fold with a recovery of 6.9% and specific activity of 288.11 U/mg of protein. The protein purification yield and specific activity were comparable with other previously purified alkaline serine proteases [8, 16].

When comparing the relative mobility of the marker proteins, the molecular mass of the purified protease was estimated to be approximately 35 kDa (Fig. 1). The enzyme purification homogeneity was confirmed by an SDS–PAGE analysis as a single band, and the molecular mass of the purified protease was found to be approximately 35 kDa. A 34 kDa serine protease was previously reported from *B. pumilus* CBS [14]. In addition, a 38 kDa organic solvent- and detergent-stable protease was also reported from *Bacillus* sp. RKY3 [29].

Effect of pH on Activity of Enzyme

The enzyme produced by *B. pumilus* TMS55 exhibited its optimum activity at pH 9.5, indicating that this enzyme belongs to the alkaline protease group. When varying the pH of the reaction mixture, this caused a reduction of the catalytic activity (Fig. 2). When increasing the alkalinity of the reaction mixture, the relative activities of the purified enzyme were 78% and 73.15% at pH 11.5 and 12.0, respectively. However, a progressive reduction was observed when decreasing the alkalinity of the reaction mixture towards neutral, indicating a robust nature within a pH range of 7–12.0. The pH stability is very important, and probably the most important factor, for the industrial application of protease. In the case of the protease from *Bacillus* sp. RKY3, its optimum activity is at pH 7.0 and it only exhibits 48% relative enzyme activity at pH 9.0 [29]. In general, bacteria belonging to *Bacillus* species are known to secrete two types of extracellular protease; a neutral or metalloprotease with a pH optimum at 7.0, or an alkaline protease with a pH optimum between 9.0 and 11.0 [8].

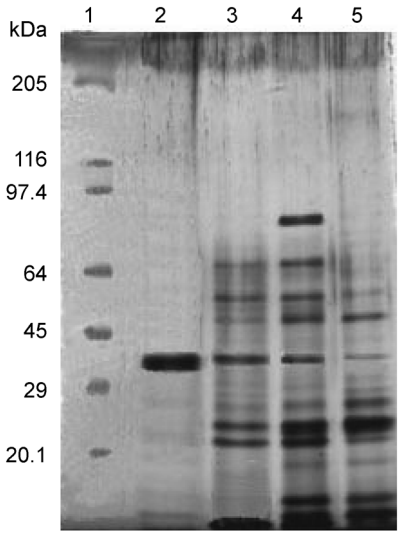

Fig. 1. SDS–PAGE showing purity at different stages of purification.
Lane 1: Marker; Lane 2: Purified protein from ion-exchange column; Lane 3: Protein from Sephadex G-75 column; Lane 4: Ammonium sulfate precipitation; and Lane 5: Cell-free culture supernatant.

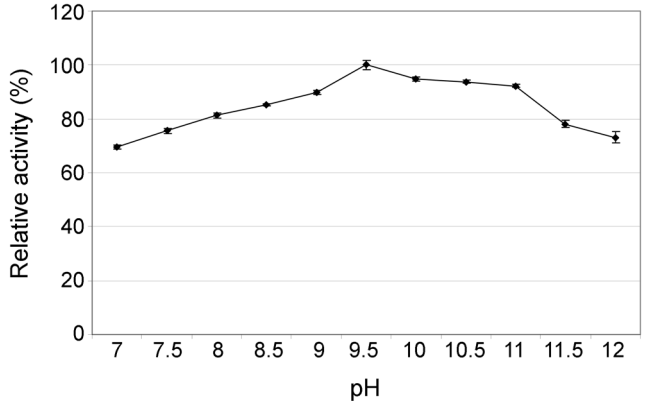

Fig. 2. Effect of pH on protease activity.
The optimum pH of the enzyme was found to be 9.5.

Effects of Metal Ions on Protease Activity

Metal ions help in maintaining the active conformation of an enzyme at high temperatures and thus protect the enzyme against thermal denaturation. The majority of alkaline serine proteases require Ca^{2+} or Mg^{2+}. In this study, the addition of Mn^{2+} increased the protease activity by 125.85% and 136.23% at 1 mM and 5 mM concentrations, respectively. The dehairing proteases previously reported by Huang *et al*. [13] and Nilegaonkar *et al*. [21] required Ca^{2+}, but not Mn^{2+}. Moreover, in the current study, Cu^{2+} and Hg^{2+} inhibited the enzyme activity by 70% and 90%, respectively, at a 5 mM concentration (Fig. 3). In terms of the inhibition of protease activity by heavy metals, certain bacterial serine proteases are repressed by metal ions like Zn^{2+}, Hg^{2+}, and Cu^{2+}, whereas others are not [21].

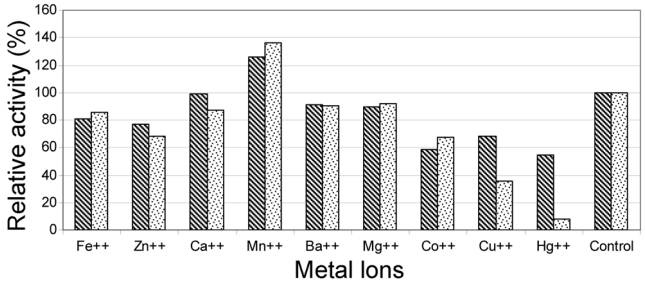

Fig. 3. Effects of metal ions at concentrations of 1 mM (▨) and 5 mM (▩) on protease activity.

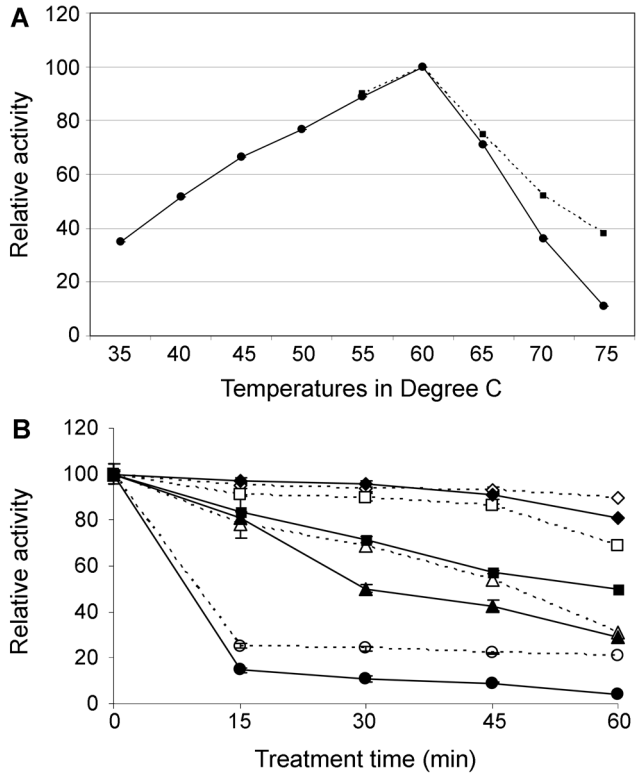

Fig. 4. Effect of temperature on the activity (**A**) and stability (**B**) of purified protease in the presence of Mn^{2+}.

A. The temperature profile was determined by assaying the enzyme activity in the absence (●) and presence (■) of 5 mM Mn^{2+} at various temperature values (35–75°C) using a 50 mM phosphate buffer, pH 9.5. The maximum activity obtained at 60°C was considered as 100% activity. **B**. The enzyme was pre-incubated in the absence or presence of Mn^{2+} at various temperatures; 50°C with 5 mM Mn^{2+} (◇), 50°C without Mn^{2+} (◆), 55°C with 5 mM Mn^{2+} (□), 55°C without Mn^{2+} (■), 60°C with 5 mM Mn^{2+} (△), 60°C without Mn^{2+} (▲), 65°C with 5 mM Mn^{2+} (○), and 65°C without Mn^{2+} (●). The activity of the non-heated enzyme in the presence of 5 mM Mn^{2+} was considered as 100%. Each point represents the mean of three independent experiments. The vertical bars indicate the standard error of the mean.

Effect of Temperature on Protease Activity

When investigating the effect of different temperatures between 35 and 75°C at pH 9.5, the optimum temperature was found to be 60°C (Fig. 4A), and the activity decreased rapidly above 65°C. Alkaline serine proteases have been found to be active in different temperature ranges. Annapurna *et al*. [3] reported two groups of alkaline serine proteases, one active within a temperature range of 28–90°C, and the other active within a temperature range of 20–50°C with an optimum activity at 37°C. The alkaline serine protease previously reported by Huang *et al*. [13] exhibited an optimum temperature at 55°C and was completely inactivated at 70°C. Thus, the protease produced by *B. pumilus* TMS55 would seem to be more thermotolerant than previously reported proteases.

Table 2. Effects of oxidizing agents, reducing agents, detergents, and inhibitors on the activity of the purified enzyme.

Components	Concentration	Residual Activity (%)
Detergents		
Triton X-100	1%	79.4 (±0.8)
Tween 20	1%	87.5 (±1.5)
Tween 80	1%	85.3 (±0.6)
SDS	1%	35.8 (±2.0)
Oxidizing agent		
H_2O_2	1%	127.4 (±2.4)
Reducing agent		
β-Mercaptoethanol	1%	40.4 (±2.4)
Inhibitors		
E-64	1 mM	82.9 (±2.3)
Pepstatin A	1 mM	74.8 (±1.3)
EDTA	1 mM	69.4 (±1.2)
AEBSF	1 mM	16.6 (±2.4)
PMSF	1 mM	7.2 (±0.7)

Effect of Metallic Ion on Thermal Stability

The activity of the enzyme treated with 5 mM Mn^{2+} was unaffected by temperatures up to 60°C, indicating that the activity of the protease was independent of this metallic ion. At higher temperatures of 65°C, 70°C, and 75°C, the enzyme treated with 5 mM Mn^{2+} retained 75%, 52%, and 38% of its activity, whereas the untreated enzyme retained only 71%, 36%, and 11%, respectively (Fig. 4A), providing a strong indication that the metallic ion stabilizes the enzyme at temperatures above 60°C. Furthermore, in the absence of Mn^{2+}, the purified enzyme only retained 81% and 50% of its activity for 60 min at 50°C and 55°C, respectively, whereas the enzyme treated with Mn^{2+} retained almost 90% and 70% of its activity for 60 min at 50°C and 55°C, respectively (Fig. 4B).

Effects of Protease Inhibitors

North [22] has already classified proteases based on their sensitivity to various inhibitors. Thus, in the present study, EDTA (metalloprotease inhibitor), E-64 (cysteine protease inhibitor), and pepstatin (acid protease) had a minimal effect on the protease activity (Table 2). In contrast, AEBSF and PMSF (serine protease inhibitors) significantly inhibited the enzyme activity at a very low concentration, suggesting that the protease produced by *B. pumilus* TMS55 belongs to the serine protease group.

Effects of Oxidizing Agents, Reducing Agents, and Detergents on Activity of Purified Enzyme

The purified enzyme remained stable with most of the tested compounds. In the presence of nonionic detergents, Triton X-100, Tween-80, and Tween-20, around 80% residual

activity was retained with 1% (v/v). In the presence of 1% of the strong anionic surfactant SDS, the enzyme retained 35% of its initial activity. According to an earlier study, proteases belonging to the *Bacillus* species are unstable in the presence of oxidants and bleaching agents [4]. However, in the current study, the enzyme showed a slightly enhanced activity in the presence of 1% hydrogen peroxide (Table 2). However, the reducing agent β-mercaptoethanol had a significant inhibitory effect. In contrast, the protease produced by *Bacillus* sp. RKY3 showed a high stability and compatibility with strong anionic surfactants (SDS), oxidizing agents (H_2O_2), and reducing agents (β-mercaptoethanol). Moreover, Kobayashi *et al*. [17] reported that the alkaline protease produced by *Bacillus* sp. KSM-K16 retained approximately 75% of its original activity after treatment with 5% (w/v) SDS for 4 h. Earlier reports on the stability of alkaline proteases with oxidants indicated that the alkaline protease from *Bacillus* sp. RGR-14 exhibited a 40% loss of enzyme activity with 1% (v/v) H_2O_2, whereas the subtilisin-like protease from *Bacillus* sp. KSMKP43 exhibited little or no enzyme activity after treatment with 10% H_2O_2 for 30 min [30, 32]. However, there are relatively for reports on the stability of proteases with SDS and H_2O_2. Gupta *et al*. [10] reported that the protease produced by *Bacillus* sp. SB5 retained about 60% and 95% of its activity after treatment for 1 h with 1% (w/v) SDS and 5% (v/v) H_2O_2, respectively, whereas the protease from *Bacillus* sp. JB-99 retained 75% and less than 95% of its activity after treatment for 1 h with 0.5% (w/v) SDS and 5% (v/v) H_2O_2, respectively [15]. Similar results were obtained for the alkaline protease from an alkali-tolerant *B. patagoniensis* [25].

Effects of Organic Solvents on Stability of Purified Enzyme

The effects of organic solvents on protease activity differ among proteases. Organic solvents with a different log P between −0.235 and 3.5 were used in this study. The solvent parameter, log P, which is an index of biological toxicity, is used to measure the solvent tolerance of organisms. Log P, the logarithm of the partition coefficient of a solvent in a defined octanol–water mixture, is commonly used as a measure of the lipophilicity of a solvent. In the present study, the purified protease remained stable after 30 min of pre-incubation with most of the tested organic solvents, except for pyridine, acetone, ethanol, and butanol. In particular, as shown in Fig. 5, the purified protease remained stable in the presence of organic solvents with log P values almost equal to or less than 4.0 (benzene 2.0, toluene 2.5, and hexane 3.5). Hydrophobic solvents are usually superior to hydrophilic solvents, as the latter have a greater tendency to strip tightly bound water, which is essential for catalytic activity. Similarly, the half-life of the organic solvent-stable PST-01 protease in the presence of water-insoluble organic solvents, such as toluene, benzene, *n*-heptane, *p*-xylene, *n*-hexane, *n*-decane, and cyclohexane, was found to be shorter than that in the absence of an organic solvent [23, 24]. Thus, the main disadvantage of employing organic solvents as the media for enzymatic reactions is that the enzymes are easily inactivated or denatured. Therefore, proteases that are naturally stable in the presence of organic solvents are very useful for synthetic reactions. A few reports on the purification and characterization of organic solvent-tolerant proteases have been published [7, 26]. One important reason for diminished enzymatic activities in organic solvents stems from the reduced structural flexibility of enzymes. In aqueous environments, enzymes possess the conformational mobility or flexibility necessary for optimal catalysis. In contrast, organic solvents lack the ability of water to engage in multiple hydrogen bonds, and also have lower dielectric constants, which lead to stronger intraprotein electrostatic interactions. Consequently, enzyme molecules become more rigid [26].

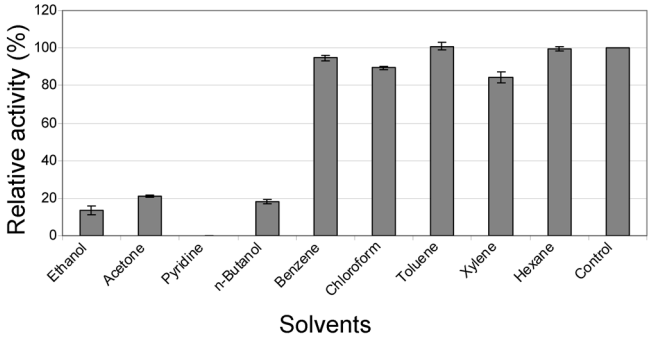

Fig. 5. Effect of organic solvents on protease stability.
The protease showed tolerance to benzene, chloroform, toluene, and xylene.

Evaluation of Purified Protease for Dehairing

Complete dehairing to a clean white pelt was achieved with 2 units of enzyme/g of hide after 9 h (Fig. 6). In the control, no hair loosening was observed even when using the mechanical action of a forceps. Furthermore, when the protease preparation was used along with 5 mM PMSF, no depilation occurred, thereby confirming that the serine protease alone was responsible for the dehairing (Fig. 6B). Therefore, these results indicate that the bacterial depilatory system tested includes the desirable characteristics for the dehairing process. The dehairing was achieved with a low number of units of the enzyme (2 U/g of skin) when compared with the dehairing reported by Huang *et al*. (300 U) [13]. Moreover, when compared with commercial enzymes, the dehairing was comparable to that of Ezyme-A and even better than that of Biodart (Fig. 6E). Thus, the results indicate that the purified enzyme can be a better option for dehairing applications. The use of enzymes as

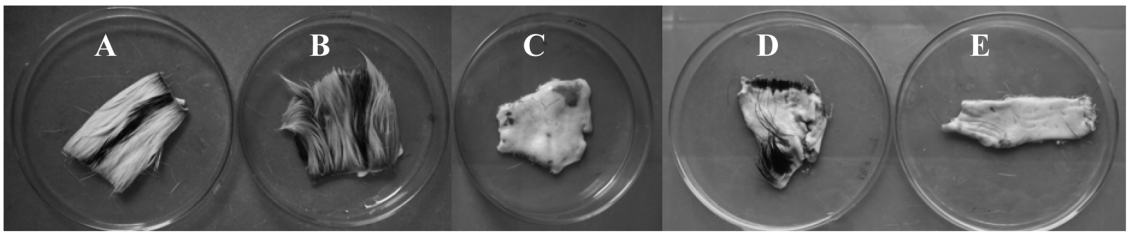

Fig. 6. Evaluation of dehairing with purified and commercial enzymes.
(A) Control (uninoculated medium), (B) purified enzyme (2U) in 50 mM phosphate buffer treated with PMSF (5 mM), and (C) purified enzyme (2 U). (D) Biodart (2U) and (E) Ezyme-A (2U).

an alternative to chemicals not only reduces environmental pollution, but also improves the leather quality.

In conclusion, this study presented an Mn^{2+}-dependent alkaline serine protease from *B. pumilus* TMS55 with dehairing ability. Furthermore, this enzyme shows potential to be investigated for a range of commercial applications.

Acknowledgments

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