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Full Length Research Paper

Purification and partial characterization of a thermostable alkaline protease from *Bacillus licheniformis* LHSB-05 isolated from hot spring

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An alkaline protease produced from *Bacillus licheniformis* LHSB-05 isolated from Nigerian hot spring was purified in a 2-step procedure by ammonium sulphate precipitation and Sephacryl S-300 gel filtration chromatography. The enzyme was purified 13.4-fold with a yield of 6.6%. The molecular weight of the protease was 16 kDa on SDS-PAGE. The purified protease had optimum temperature of 50°C. The enzyme retained 90% of its maximum activity at 60°C and had 45% relative activity at 70°C. This enzyme exhibited high thermostability with 100% stability at 50°C and almost 80% stability at 60°C after 60 min of incubation. The protease had optimum pH of 9.0 and showed relative activity between 81 and 71% in the pH range of 10.0 to 12.0. The enzyme was stable over a broad pH range (6.0 to 12.0). Ca^{2+} and Mg^{2+} increased protease activity with 27 and 9%, whereas Hg^{2+} strongly inhibited its activity by 83%. The enzyme was relatively stable in the presence of Ca^{2+}, Mg^{2+}, Al^{3+}, Zn^{2+} and ethylene diamine tetra acetic acid (EDTA). These characteristics suggest the suitability of protease from *B. licheniformis* LHSB-05 for use in detergent industries.

Key words: Alkaline protease, *Bacillus licheniformis* LHSB-05, characterization, purification, thermostability.

INTRODUCTION

Proteases (serine protease (EC. 3.4.21), cysteine (thiol) protease (EC.3.4.22), aspartic proteases (EC. 3.4.23) and metallo-protease (EC. 3.4.24) constitute one of the most important groups of industrial enzymes accounting for about 60% of the total worldwide enzyme sales (Nascimento and Martins, 2004; Beg and Gupta, 2003). Proteases have wide applications in pharmaceutical (Vellard, 2003), detergent (Gupta et al., 2002), leather (Huang et al., 2003), food (Pastor et al., 2001) and waste processing industries (Ward, 1985). Alkaline proteases are employed primarily as cleansing additives in detergent industries and ideally, proteases used in detergent formulation should have a high level of activity over a broad range of pH and temperature (Gupta et al., 2002).

Among the various proteases, bacterial proteases are the most significant, compared with plants, animal and fungal proteases because they are mostly extracellular and easily produced in large amount, but their usefulness is limited by various factors, such as enzyme instability at high temperatures, extreme pH, the presence of organic solvents and the need for co-factors (Breithaupt, 2001). Thermostable proteases are advantageous in many applications because higher processing temperatures can be employed, resulting in faster reaction rates, increase in solubility of nongaseous reactants and products and reduced incidence of microbial contamination by mesophilic organisms (Rao et al., 1998).

Alkaline proteases from high yielding strains of *Bacillus* species have been studied extensively and reported, but most of the proteases are thermolabile (Studdert et al., 2001; Tsuchida et al., 1986). Obtaining thermostable alkaline proteases has continued to be a challenging problem. Hence, it is important to search for new proteases with novel characteristics for industrial applications from diverse bacteria isolates. Moreover, geothermal soils are a rich potential source of novel bacteria capable of producing thermostable and thermotolerant enzymes and relatively simple cultivation techniques are practical for

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isolating bacteria from these habitats (Olajuyigbe and Ajele, 2005; Stott et al., 2008)

We report here the purification and partial characterization of a thermostable protease from *Bacillus licheniformis* LHSB-05 isolated from Nigerian hot spring.

MATERIALS AND METHODS

Chemicals

Sephacryl S-300 was purchased from Amersham Biosciences. EDTA (Ethylene Diamine Tetra Acetic acid), acryl amide, TEMED (N,N,N',N' tetra methyl ethylenediamine), APS (ammonium persulfate) and casein were products of Sigma-Aldrich, St Louis, MO. All other chemicals used were of analytical grade and obtained from leading manufacturers including BDH and Sigma-Aldrich.

Isolation of microorganisms, screening and identification of proteolytic bacteria

Soil samples were collected from 0 to 5 cm layers at the point of outflow of Lamurde hot spring in North Eastern part of Nigeria and were diluted in sterile saline solution. The temperature of the hot spring at the time of collection of soil sample was 50°C. The diluted samples were plated onto skim milk agar plates containing peptone (0.1% w/v), NaCl (0.5% w/v), agar (2% w/v) and skim milk (10% v/v). Plates were incubated at 37°C. After 24 h, individual colonies were picked and purified by streaking three times onto skim milk agar plates. The isolated bacteria were identified based on cell morphology, growth conditions, gram stain, motility and biochemical tests. The data obtained were compared with standard description provided in Bergey's manual of determinative bacteriology (Bergey and Holt, 1994). Protease production was demonstrated by clear zone of skim milk hydrolysis in the area surrounding isolated colonies growing on the surface. Depending upon the zone of clearance and properties of crude protease expressed by identified *Bacillus* species, *B. licheniformis* LHSB-05 was selected for further experimental studies.

Production of protease

Production of protease from *B. licheniformis* LHSB-05 was carried out in a medium containing 1.5% peptone (w/v), 0.5% glucose (w/v) and 0.5% (w/v) NaCl with pH adjusted to 8.0. The fermentation medium was maintained at 40°C for 72 h at 200 rpm in a shaking incubator (Stuart, UK). At the end of the fermentation period, the broth was centrifuged at 10,000 rpm for 15 min at 4°C. The clear supernatant was recovered as crude enzyme preparation and subjected to purification for further studies

Enzyme purification

Ammonium sulphate precipitation

The clear supernatant (crude protease) was fractionated by precipitation with ammonium sulphate of 80% saturation. The precipitated protein was dissolved in 50 mM Tris-HCl buffer, pH 8.0 and dialyzed against same buffer.

Sephacryl S-300 gel filtration chromatography

The protein pellet obtained after saturation with 80% ammonium sulphate and dissolved in 50 mM Tris-HCl buffer was loaded on to a Sephacryl S-300 column (2.5 $\times$ 60 cm) which had been previously washed and equilibrated with 50 mM Tris-HCl buffer, pH 8.0. The column was eluted at a flow rate of 0.5 ml/min using same buffer. From the elution profile, it was observed that the protease was eluted as a single peak. Fractions (19-31) with high protease activity were pooled and used for further studies. The concentration of protein during purification studies was calculated from the absorbance at 280 nm.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The purity of the protease after Sephacryl S-300 column chromatography was confirmed by subjecting the pooled fractions (19-31) to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed according to Laemmli (1971) using 12% acrylamide. Protein bands were visualized using Coomassie blue staining procedure. The molecular weight of the protease was determined by comparison with the migration distances of standard marker proteins consisting of phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa).

Assay of protease activity and protein quantification

Protease activity was determined by a modified procedure based on Fujiwara et al. (1993) using 1.0% casein in 50 mM Tris-HCl buffer (pH 8.0) as substrate. The assay mixture consisted of 2.0 ml of substrate and 0.5 ml of enzyme solution in 50 mM Tris-HCl buffer, pH 8.0. The reaction mixture was incubated at 40°C for 30 min and the reaction was terminated by the addition of 2.5 ml of 10% trichloroacetic acid (w/v). The mixture was allowed to stand for 15 min and then centrifuged at 10,000 rpm for 10 min at 4°C to remove the resulting precipitate. Protease activity was determined by estimating the amount of tyrosine in the supernatant which was done by measuring the absorbance at 280 nm. One unit of protease activity was defined as the amount of enzyme required to release 1 μg of tyrosine per ml minute per minute under the mentioned assay conditions. Protein was measured by the method of Bradford (1976) with bovine serum albumin (BSA) as standard.

Partial characterization of purified protease

Effect of pH on protease activity and stability

The effect of pH on activity of the purified protease was determined by assaying for enzyme activity at different pH values ranging from 4.0 to 12.0. The pH was adjusted using 50 mM of the following buffer solutions: sodium acetate (pH 4.0 to 5.0), sodium citrate (6.0), Tris-HCl (pH 7.0 to 8.0) and glycine-NaOH (pH 9.0 to 12.0). Reaction mixtures were incubated at 40°C for 30 min and the activity of the protease was measured.

To determine the effect of pH on stability of protease, the purified protease was incubated in relevant buffers of varying pH (4.0 to 12.0) without substrate for 30 and 60 min at 40°C. The residual protease activity was determined as described earlier.

Effect of temperature on protease activity and stability

The activity of purified protease at different temperatures was determined by incubating the reaction mixture at temperatures ranging from 30 to 80°C for 30 min before determining protease activity.

To determine the enzyme stability at different temperatures, the purified protease was incubated at temperatures ranging from 30 to 80℃ for 30 and 60 min, respectively and the residual protease activity was determined according to the standard assay procedure.

Effect of metal ions and EDTA on protease activity and stability

Metallic chlorides ($MgCl_2$, $CaCl_2$, $FeCl_3$, $HgCl_2$, $ZnCl_2$ and AlCl3) and ethylene diamine tetra acetic acid (EDTA) were prepared to a final concentration of 1 mM and was added to the reaction mixture of purified protease before determining the enzyme activity. Protease activity was determined using 1% casein in 50 mM Tris-HCl buffer (pH 8) as substrate at 40℃.

The effect of metal ions (Mg^{2+}, Ca^{2+}, Fe^{3+}, Hg^{2+}, Zn^{2+} and Al^{3+}) and EDTA (1 mM) on protease stability was determined by pre-incubating the purified protease with each of the metallic chlorides and EDTA without substrate for 30 min at 40℃, thereafter, the residual protease activity was determined.

RESULTS AND DISCUSSION

Identification of protease producing bacterial isolates

Four soil samples were collected and 27 microbial colonies secreting proteases were isolated. The isolates were purified through repeated streaking onto skim milk agar plates as earlier described under methods. During screening of bacterial isolates for proteolytic activity, isolate LHSB-05 showed highest proteolytic activity on skim milk agar with an average clear zone diameter of 42 mm and was selected for further studies. The culture was maintained on agar slants and stored at 4℃.

Microscopic observation of isolate LHSB-05 showed spore forming, motile, rod shaped bacterium; the bacterium grew aerobically and formed creamish white colonies which were mucoid and undulating. The morphological and biochemical characteristics are presented in Table 1. The phenotypic characteristics based on Bergey's manual of determinative bacteriology (Bergey and Holt, 1994) suggest that the isolate belonged to the genus *Bacillus* and was identified as *B. licheniformis* and designated as *B. licheniformis* LHSB-05.

Purification of extracellular protease from *B. licheniformis* LHSB-05

The protein pellet obtained after 80% saturation with ammonium sulphate was dissolved in 50 mM Tris-HCl buffer, pH 8.0 and loaded onto a column of Sephacryl S-300 (2.5 × 60 cm) equilibrated with same buffer. The elution profile of gel filtration chromatography is shown in Figure 1. The elution profile showed that the protease was eluted as a single peak. Fractions (19-31) with protease activities were pooled and concentrated for further studies. The purification procedure is summarized in Table 2, showing that the enzyme was purified 13.4-fold with a specific activity of 185.6 U/mg protein after Sephacryl S-300 gel filtration chromatography. The yield of the enzyme after purification was 6.6%.

SDS-PAGE of protease from *B. licheniformis* LHSB-05

The enzyme purity was confirmed by the single band obtained on SDS-PAGE with the purified protease from Sephacryl S-300 gel filtration chromatography (Figure 2), which indicated a homogeneous preparation. The molecular weight of purified protease determined by interpolation from a linear semilogarithmic plot of relative molecular mass of standard marker proteins versus the R_f (relative mobility) was calculated to be 16 kDa. This is similar to the reported molecular weights of alkaline proteases in the range of 15 to 30 kDa (Adinarayana et al., 2003; Bhaskah et al., 2007).

Characteristics of purified protease from *B. licheniformis* LHSB-05

pH optimum and *pH stability*

The optimum pH of the purified protease was found to be 9.0 which was the pH at which the protease exhibited 100% activity. The protease retained 81, 78 and 71% of the maximum activity at pH 10.0, 11.0 and 12.0, respectively (Figure 3). Similar results were obtained for some alkaline proteases from *Bacillus* species showing pH optima of 9.0-10.0 (Johnvesly and Naik, 2001; Yu et al., 2006).

The protease was stable over a broad pH range of 6.0 to 12.0 after 1 h incubation of the enzyme in buffers of different pH. The protease had 100% residual activity at pH 9.0. Surprisingly, the enzyme had 98% relative residual activity at pH 10.0 and 97% at pH 11.0. The protease retained 90% of its relative activity at pH 12.0 (Figure 3). These results indicate that the protease produced from *B. licheniformis* LHSB-05 is a highly stable alkaline protease. However, alkaline protease from some *Bacillus* species have been reported to exhibit sharp decline in stability at pH 11.0 and 12.0, respectively (Banerjee et al., 1999; Patel et al., 2006),

Optimum temperature and thermostability

The activity of the purified protease from *B. licheniformis* LHSB-05 was determined at different temperatures ranging from 30 to 80℃. The purified protease was most active at 50℃ which was recorded as 100% activity. The enzyme had 90% relative activity at 60℃ (Figure 4). Interestingly, the protease was still active at 70℃ having a relative activity of 45%, but drastically lost about 80% of its activity at 80℃. The loss of enzyme activity between

Table 1. Characteristics of *B. licheniformis* LHSB-05.

Morphological and biochemical test	Result
Colony morphology	Creamish white, mucoid and undulating
Cell morphology	Rods
Motility test	Positive
Spores	Ellipsoidal
Aerobic growth	Positive
Temperature (30 - 90℃)	Positive
Growth on MacConkey	Positive
Nutrient agar + 5% NaCl	Positive
Nutrient agar + 10% NaCl	Positive
Nutrient agar buffered at pH 8-12	Positive
Anaerobiosis	Positive
Biochemical tests	
Gram reaction	Positive
Catalase test	Positive
Oxidase test	Negative
Indole production	Negative
Methyl red test	Negative
Voge's proskaver test	Positive
Citrate utilization	Positive
Urease activity	Negative
Starch hydrolysis	Positive
Gelatin hydrolysis	Positive
NO^{-3} reduction	Positive
Glucose	Positive
Arabinose	Positive
Xylose	Negative
lactose	Negative
Sucrose	Positive
Raffinose	Negative
Galactose	Positive
Salicin	Negative
Maltose	Positive
Mannitol	Positive
Identification of organism	***Bacillus licheniformis***

70 and 80℃ could be due to denaturation of enzyme structure at such temperatures. Proteases from *Bacillus subtilis* and *Bacillus pumilus* have also demonstrated optimum activity at 50℃ (Yu et al., 2006; Aoyama et al., 2000; Yang et al., 2000). Lower optimum temperature has been reported for proteases from some *Bacillus* species (Feng et al., 2001) and protease isolated from an haloalkaliphilic *Bacterium* sp had optimum activity at 37℃ (Dodia et al., 2008).

The protease exhibited 100% stability up to 50℃ and retained 78% stability at 60℃ after 60 min of incubation (Figure 4). These contradict earlier reports of alkaline proteases from some bacteria strains which were completely denatured within 5 min of incubation at 60℃ (Dodia et al., 2008; Gessesse et al., 2003) and protease from *B. pumilus* which had relative activity of 47% at 50℃ after 30 min of incubation (Feng et al., 2001). Comparing these results, the alkaline protease from *B. licheniformis* LHSB-05 is relatively more stable at high temperatures. Currently, used detergent enzymes are alkaline and thermostable in nature. The most commercially available subtilisin-type protease is also active at temperature ranging from 50 to 60℃, respectively (Ghorbel et al., 2003; Saeki et al., 2007).

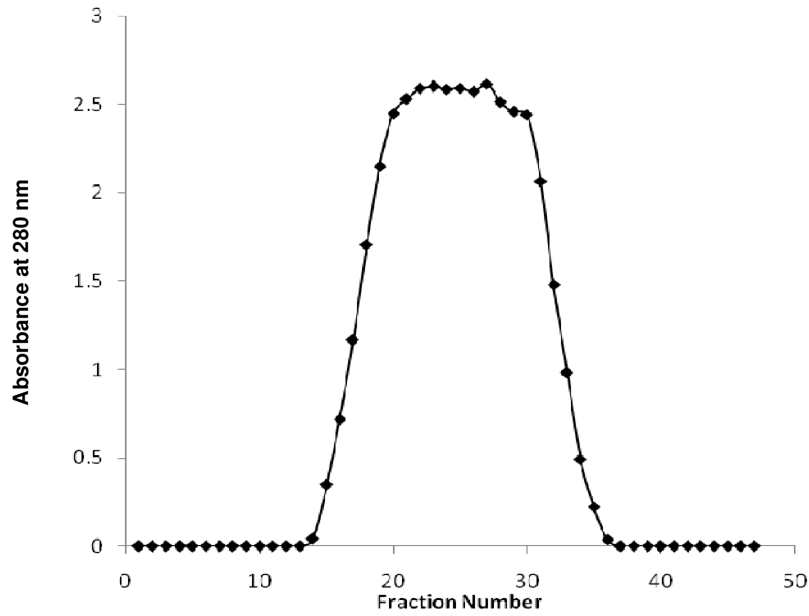

Figure 1. Elution profile of protease from *B. licheniformis* LHSB-05 on Sephacryl S-300 column.

Table 2. Summary of purification steps of alkaline protease from *B. licheniformis* LHSB-05.

Purification step	Total enzyme activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	35000	2523	13.8	1	100
$(NH_4)_2SO_4$ precipitation, dialyzed	20960	585.3	16.8	1.2	60
Sephacryl S-300	2320	12.5	185.6	13.4	6.6

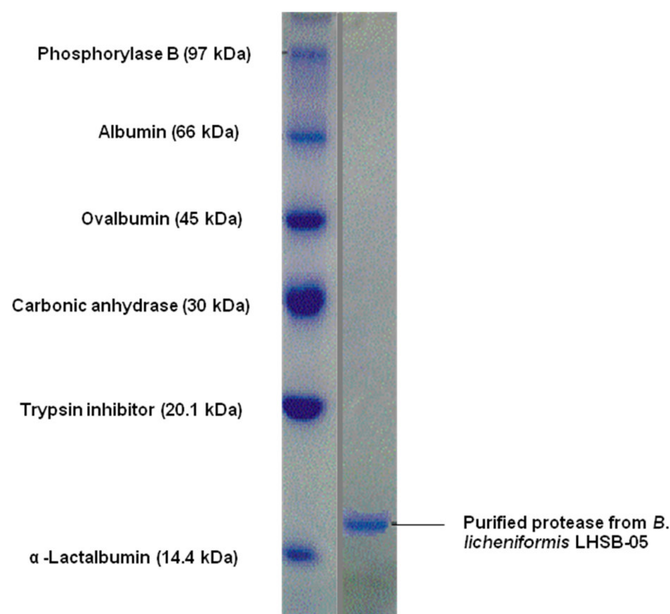

Figure 2. SDS-PAGE of the purified protease from *B.licheniformis* LHSB-05.

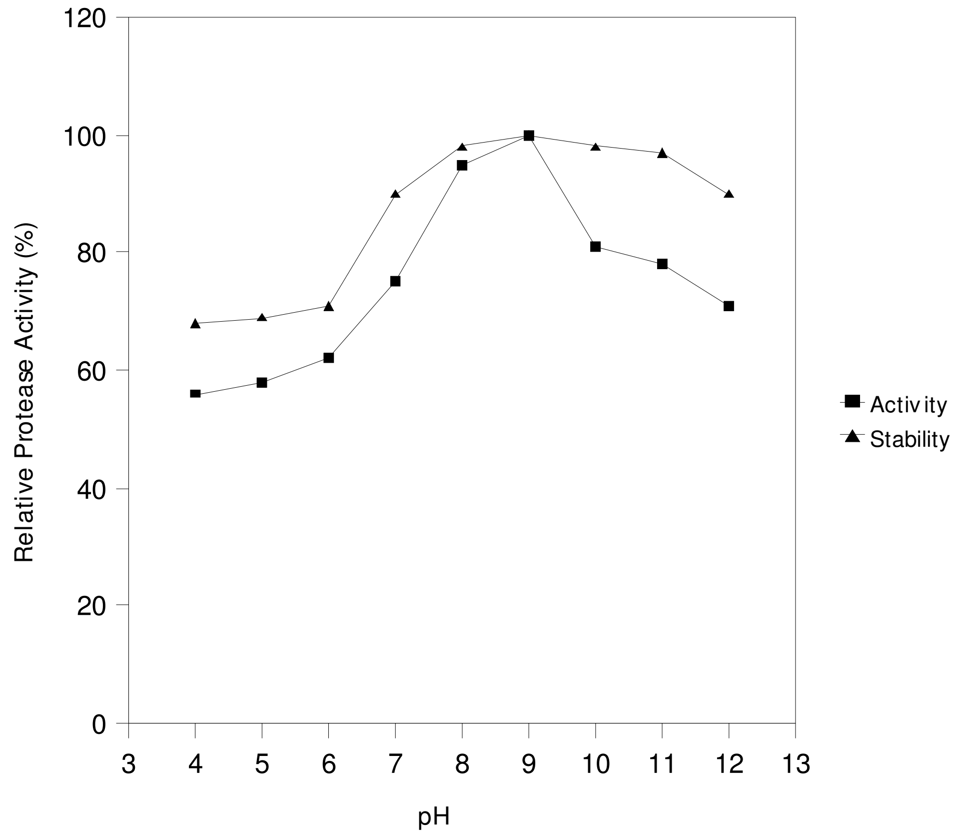

Figure 3. Effect of pH on activity and stability of protease from *B. licheniformis* LHSB-05.

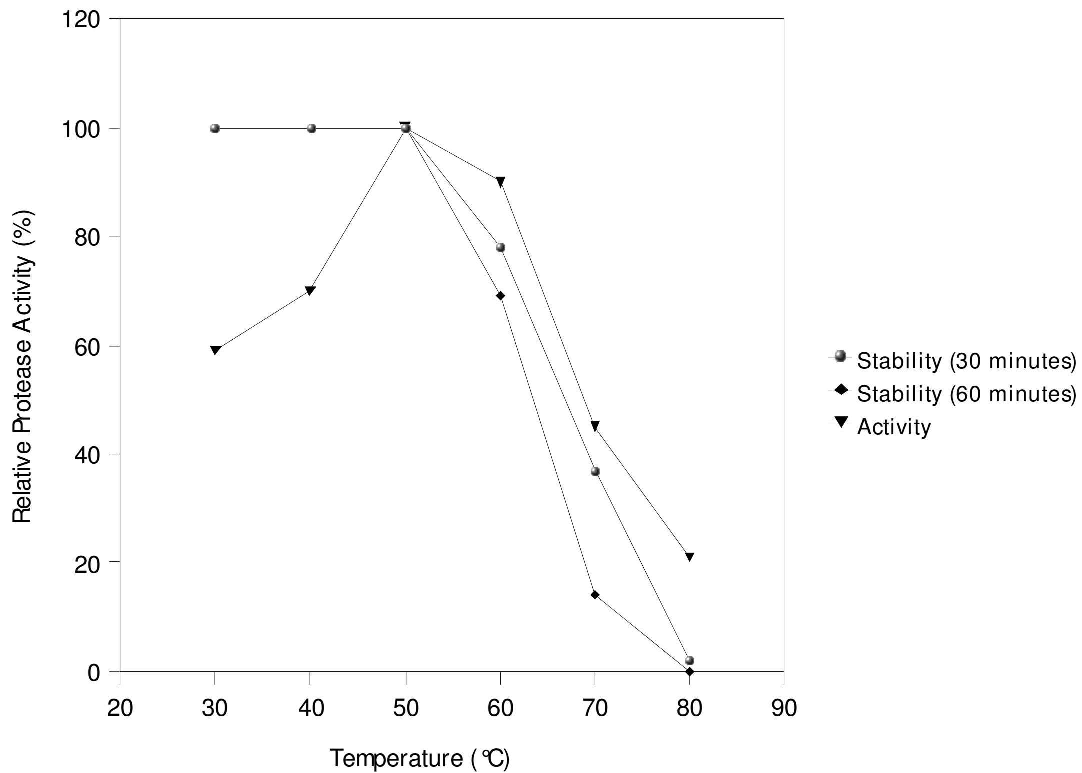

Figure 4. Effect of temperature on activity and stability of protease from *B. licheniformis* LHSB-05.

Table 3. Effect of metal ions on alkaline protease activity.

Metal ions (1 mM)	Relative protease activity (%)
Control	100
Mg^{2+} ($MgCl_2$)	109
Ca^{2+} ($CaCl_2$)	127
Fe^{2+} ($FeCl_3$)	43
Hg^{2+} ($HgCl_2$)	17
Zn^{2+} ($ZnCl_2$)	51
Al^{3+} ($AlCl_3$)	80
EDTA	61

*EDTA refers to ethylene diamine tetra acetic acid. The activity in absence of metal ions refers to 100% relative activity.

Table 4. Effect of metal ions on alkaline protease stability.

Metal ion (1 mM)	Relative residual activity (%)
Control	100
Mg^{2+} ($MgCl_2$)	87
Ca^{2+} ($CaCl_2$)	102
Fe^{2+} ($FeCl_3$)	47
Hg^{2+} ($HgCl_2$)	42
Zn^{2+} ($ZnCl_2$)	62
Al^{3+} ($AlCl_3$)	76
EDTA	60

*EDTA refers to ethylene diamine tetra acetic acid. The activity in absence of metal ions refers to 100% residual relative activity for stability of protease.

Effect of metal ions and EDTA on activity and stability of protease

The protease activity was enhanced with an addition of Mg^{2+} and Ca^{2+} resulting in the relative activity of 109 and 127%, respectively (Table 3). The increase in enzyme activity by 27 and 9% with Ca^{2+} and Mg^{2+} in this study suggests that these cations activated the protease and have the capacity to protect the enzyme from *B. licheniformis* LHSB-05 against denaturation. This result correlates with the findings of Adinarayana et al. (2003) that Mg^{2+}, Ca^{2+} and Mn^{2+} increased the activity of protease from *B. subtilis* PE-11. Nascimento and Martins (2004) also reported that activity of protease from *Bacillus* sp. SMIA-2 was enhanced by Mn^{2+} and Ca^{2+}. Some metal ions have been found to protect the protease against thermal denaturation and maintain active conformation of the enzyme at high temperatures (Donaghy and McKay, 1993). Hg^{2+} had strong inhibitory effect on protease activity with relative activity of 17%, while the enzyme had relative activity of 43 and 51% in the presence of Fe^{3+} and Zn^{2+} (Table 3). A similar type of result was obtained by other investigators, where alkaline protease from *Bacillus brevis* lost its activity completely in the presence of Hg^{2+} (Banerjee et al., 1999).

The purified protease from *B. licheniformis* LHSB-05 was very stable in the presence of Mg^{2+} and Al^{3+} with relative residual activity of 87 and 76%, respectively (Table 4). Ca^{2+} increased the stability of the enzyme by 2%, with the protease having relative residual activity of 102%. This suggests that the protease requires Ca^{2+} for its maximum activity and stability. A universal feature among the subtilisin type proteases is the presence of calcium-binding sites, which largely contributes to their stability against thermal denaturation and autolytic digestion (Smith et al., 1999). The activity of the protease was inhibited by ethylene diamine tetra acetic acid (EDTA) (Table 3) which shows that the enzyme is a metalloprotease (Kuddus and Ramteke, 2008). However, stability studies reveal that the protease was relatively stable in the presence of EDTA (Figure 4).

Conclusion

The protease isolated from *B. licheniformis* LHSB-05

demonstrated unique activity and stability within alkaline pH, extremes of temperature and in the presence of some cations which suggest its suitability for use in detergent industries where alkaline conditions, higher processing temperatures and certain additives containing metal ions are necessary to boost the effectiveness of detergents. These findings provide more insights into the biotechnological potential of microbial flora of hot springs and industrial applications of the enzymes they produce.

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