Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; SP1: 2608-2611

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Partial purification and characterization of hide dehairing protease

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Abstract
Partial purification of crude enzyme was carried out by ammonium sulphate precipitation, dialysis and ion exchange chromatography. Alkaline protease was purified upto 2.19 fold as compared with crude enzyme. The apparent molecular weight of partially purified enzyme was determined as 48 kDa by SDS-PAGE and the same was confirmed from zymography. The maximum alkaline protease production was achieved with 7% inoculums size at pH 10, 36 ºC incubation temperature, with impellor tip velocity of 4714 cm/min, and 48hrs of incubation period. Alkaline protease activity was completely inhibited by PMSF; while EDTA showed no inhibition on protease activity that is suggested that the enzyme is a serine protease. The Km and Vmax for the partially purified alkaline protease were 4.343mg and $1mg/min^{-1}ml^{-1}$ respectively. Alkaline protease was used for hide deharing of goat and found that complete deharing was achieved with protease unit 200 U/ml and in 6 hrs having Mn^{2+} concentration of 10 mM with 50 ^{0}C temperature and 10pH.

Keywords: Dehairing, Protease, Goat hide, Purified, Inhibition and Enzyme

Introduction
Bacterial alkaline protease has varied biochemical diversity and thermo-stability; they are active at the broad range of temperature 35 ºC – 80 ºC (Kamran *et al.*, 2015). Bacterial culture submerged fermentation was preferred due to their consistent enzyme production with defined medium composition, better process conditions and improved downstream processing (Prakasham *et al.*, 2006; Dabananda and Kshetri, 2010) [12, 4]. Partial purification of crude enzyme was carried out by ammonium sulphate precipitation, dialysis and ion exchange chromatography (Olutiola and Akintunde, 1979) [11]. The apparent molecular weight of purified enzyme was determined by the SDS-PAGE method. Alkaline protease used in the tannery and detergent industry having optimum pH of 8-12 and temperature range are 45 °C - 70°C (Haddar *et al.*, 2009; Joo *et al.*, 2003) [6, 7]. Bacterial alkaline protease was used as a dehairing agent because they have the ability to remove whole hair including an epidermal layer (Deng *et al.*, 2010) [5]. Conventionally dehairing and depletion of hide in the leather industry was done by lime and sodium sulphide method, which is creating serious environmental pollution, it is important to explore an environmentally friendly enzymatic method to provide an alternative to the chemical method (Awad *et al.*, 2013) [2].

Material and Methods
Bacillus licheniformis SK7 (N43) was previously isolated by the authors. All the chemicals used were analytical grade.

Medium and fermentation conditions
The fermentation medium consisted of skimmed milk 11.6 gm/L, glycerol 1.16 gm/L, fish meal 7.6 gm/L and ferric chloride 0.1 gm/L. The fermentation of *Bacillus licheniformis*SK7 (N43) was carried out in the above said medium at pH at 10.0, 220 rpm 48 hrs of incubation at 37 °C.

Partial purification of protease enzyme
Extracellular protease enzyme was extracted by centrifugation of cell suspension grown in fermentation medium at 10,000 rpm after 48 hrs of incubation. Cell free supernatant was saturated with ammonium sulphate (80%) at 4 ºC and precipitate was collected after centrifugation (10,000 rpm, 15 min, 4 ºC), precipitate dissolved in 10 mMTrisHCl buffer (pH-8.0) and dialyzed overnight at 4 °C against the same buffer. Dialyzedprotease dissolved in 10 mMTris-HCl buffer and loaded onto an ion exchange chromatography column equilibrated

with Tris–HCl buffer 10 mM (pH 8.0) at 4 ºC. After washing with the same buffer, adsorbed proteins were eluted with a linear gradient of NaCl0.1-1.0M in the same buffer at a flow rate of 1ml/min. Protein determination and enzyme activity was performed in collected fraction. Its purity verified using SDS-PAGE and zymography (Laemmli, 1970; Schmidt *et al.*, 1988) [9, 13].

Determination of molecular weight by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and Zymography

Partially purified protease was separated on the basis of molecular weight (MW) using polyacrylamide gels containing SDS as denaturing agent (SDS-PAGE) (Laemmli, 1970).The protease activity was visualized by zymogram, using 0.1% gelatine as copolymerized substratein sodium dodecyl sulfatepolyacrylamide gel (Schmidt *et al.*, 1988).

Determination of optimum pH and temperature

The optimal pH of partially purified protease was determined at different pH ranging from 4 to 12 and optimal temperature was investigated by exposure of enzyme to temperatures in the range of 30-80 °C.

Effect of metal ions and inhibitors

The effect of metal ions was determined in the presence of different metals such as Mn^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Na^{+}, and Mg^{2+}at a concentration range 10 mM - 20 mM. The effect of inhibitors was studied using EDTA, PMSF and 1, 10-Phenanthrolin (10 mM-20 mM) on the protease activity.

Enzyme kinetics

Kinetic constant of purified protease enzyme such as V_{max} and K_m were determined under steady state conditions using various concentration of casein as substrate (1-35 mg/ml) (Schmidt *et al.*, 1988) [13].

Dehairing of hides by alkaline protease enzyme

The two approaches of dehairing, dip method and past method were used. The goat hide was cut in pieces and weighing approximately 50 gm. Before enzymatic treatment washed hides were soaked at room temperature for 18 hrs in 0.001 % non-ionic detergent solution. Soaking hides were washed with fresh water three times for removal of non-ionic detergent. Two sets of experiments were carried out at room temperature and 50 °C. For dip method soaked hide were immersed in enzyme solution (50 - 600 U/ml, 10 Mm Mn^{2+} and pH 10). Paste method enzyme solution was mixed with 0.5% xanthum gum and applied on flesh side of hide.

Result and Discussion

Partial purification of protease from *Bacillus licheniformis* SK7 (N43)

Protease enzyme obtained from *Bacillus licheniformis*SK7 (N43) was purified 2.19 fold through ammonium sulphate precipitation (80%), dialysis and ion exchange chromatography. The purification result issummarized in table 1.

Molecular weight determination by SDS-PAGE and Zymography

The molecular mass of the partially purified protease was estimated to be approximately 48 kDa through SDS-PAGE (Figure 1).The digested band (hydrolysis zone) were obtained in zymography was the same range of 48 kDa as the results obtained in SDS-PAGE (Figure 2).In the former studies alkaline protease were obtained by various researchers; Akel*et al.*, 2009[1]reported 49 kDa thermostable protease from Bacillus strain HUTBS71, Yilmaz *et al.*, 2016 [14]. reported 44.55kDathermotolerant alkaline protease from *Bacillus licheniformis* A10.

Effect of pH on protease activity

The maximum activity of partially purified protease was found at pH 10.0, before and after pH 10 the activity of enzyme decreases (Figure 3).

Effect of temperature on protease activity

The maximum activity of partially purified protease was foundat 60 °C (Figure 4). The activity of protease does not decrease significantly at 50 °C.

Effect of metal ions on protease activity

The maximum protease activity was obtained with10 mM concentration of Mn^{2+} ion, enzyme activity enhances nearly double then original enzyme (Figure 5).

Effect of inhibitors on protease activity

Protease activity was completely inhibited by PMSF, but no effect was observed in case of EDTA and 1, 10-phenanthroline. From the above stated observations it was concluded that the enzyme extracted by the fermentation of *Bacillus licheniformis* SK7 (N43) was alkaline serine protease as previously reported by various workers (Yilmaz *et al.* 2016, Mothe and Sultanpuram 2016 [10]. and Cui *et al.*, 2015) [3].

Enzyme kinetics

The Km and Vmax for the partially purified alkaline protease were 4.343mg and 1mg $min^{-1}ml^{-1}$respectively, using casein as substrate (Figure 6).

Dehairing of hides by alkaline protease

The enzymatically treated hide pieces were gently scraped with fingers to remove loose hairs, in case of dip method 100% dehairing of was achieved in 6 hrs with 200 U/ml of working float at 50 ^{0}C temperature, 10 pH and 10 mMof Mn^{2+} concentration (Figure 7). In past method, 100% dehairing was achieved with 400 U/gm of enzyme paste at 50 °C in 12 hrs (Figure 7), while at room temperature complete dehairing was achieved with 500U/gm of enzyme past. The dip method enzymeaticde haired hide was of good quality showing white colour, clean hair pore and clear grain structure, whereas past method treated hide became faded colour, thin and spotted as compare to dip method treated hide.

Table 1: Summary of purification of protease from *Bacillus licheniformis* SK7 (N43)

Preparation	Volume in ml	Total protein in mg	Total protease enzyme units	Specific activity (Unit/mg)	Yield (%)	Fold Purification
Crude Extract	500	2265	331000	146.14	100	1
Ammonium sulphate precipitation (80%)	15	1535	281350	183.29	85	1.25
Concentrate after dialysis	5	906	258180	284.97	78	1.95
Ion exchange chromatography	1	630	202305	321.19	61	2.19

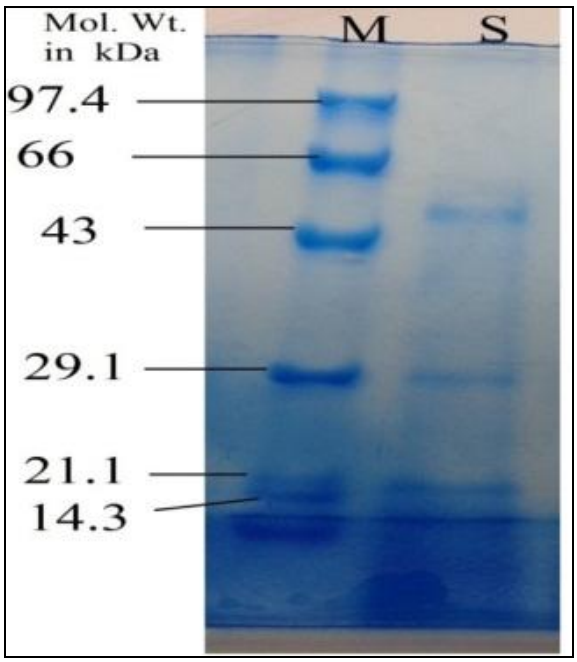

Fig 1: Molecular weight determination by SDS-PAGE,Lane M-low molecular weight marker and Lane S-protease

Fig 2: Zymograph of protease for molecular weight determination

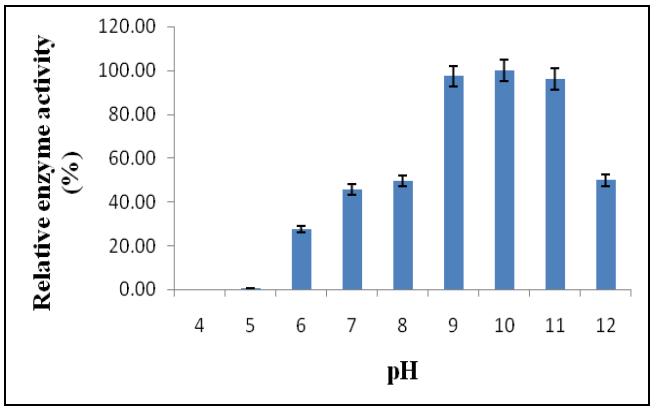

Fig 3: Effect of pH on protease activity

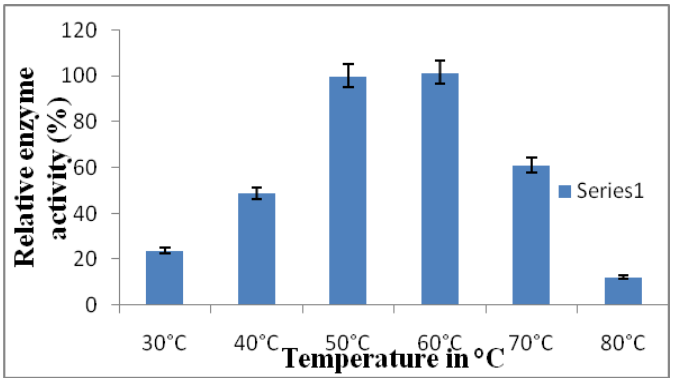

Fig 4: Effect of temperature on protease activity

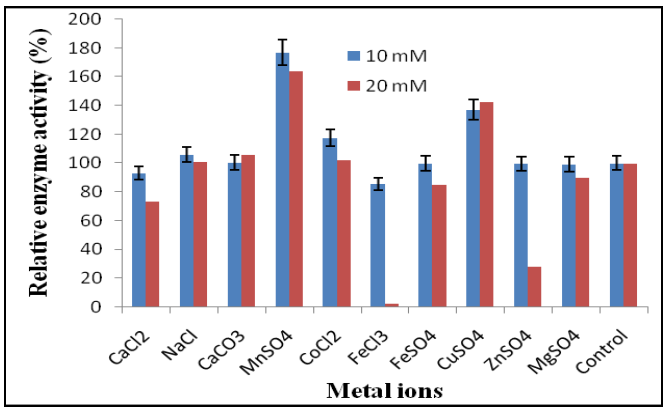

Fig 5: Effect of metal ions on protease activity

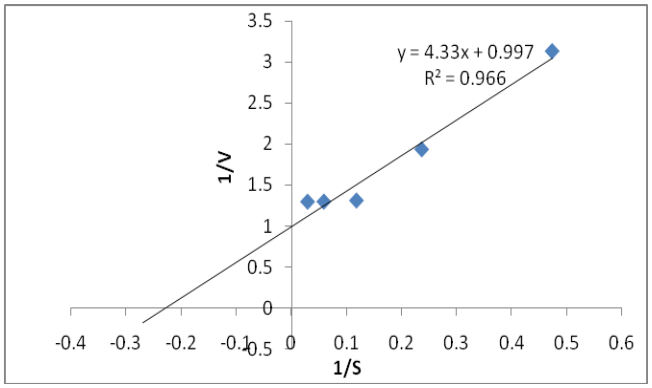

Fig 6: Determination of *Vmax* &*Km* of partially purified protease.

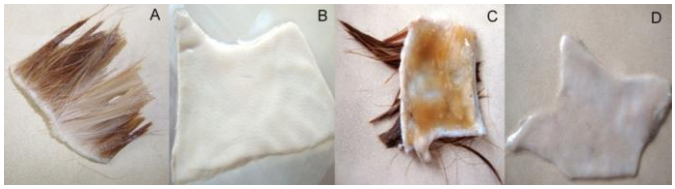

Fig 7: Dehairing of goat hide by dip method and paste method (A: Control for dip method, B: Dehaired goat hide by dip method, C: Goat hide pasted with enzyme paste on flesh side, D: Dehaired goat hide by paste method.

Conclusion

In this study the alkaline protease produced by *Bacillus licheniformis* SK7 (N43) was partially purifiedand characterized. The alkaline protease activity inhibited by PMSF and exhibiting high activity andstability at high temperatures and pH, will likely to have a potential of use in leather industry.

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