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Purification and Characterization of Serine Protease from Seeds of *Holarrhena antidysenterica*

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Abstract: Low molecular weight serine protease has been purified from the seeds of *Holarrhena antidysenterica* to electrophoresis homogeneity by the combination of size exclusion and ion exchange chromatography. The molecular mass was estimated by SDS-PAGE to be about 25 kDa. The enzyme showed optimum activity at pH 7.5 and exhibited its highest activity at 35°C using 1% casein as a substrate. The enzyme was strongly inhibited by 2 mM PMSF but not by EDTA and cysteine protease inhibitors, suggesting the presence of serine residues at the active site. The enzyme had a K_m of 1.1 mg mL^{-1} and V_{max} of 389.71 units min^{-1} mg^{-1} of protein.

Key words: Apocyanaceae, *Holarrhena antidysenterica*, seeds, serine protease, characterization, purification

INTRODUCTION

Proteases are enzymes which potentially hydrolyze anything containing a peptide bond, from a dipeptide up to a large protein containing thousands of amino acids. Many proteases show specificity towards protein substrates, but some are more specific towards short peptides or even just dipeptides (Smith *et al*., 1997). Proteolytic enzymes play significant roles in numerous cellular and extra cellular processes, just like regulating the cell cycle, cell growth, antigen processing and angiogenesis (Antao and Maclcata, 2005). In addition it is becoming apparent that the aberrant functioning of certain proteases may be involved in several disease states, including Alzheimer's disease, in cancer metastasis and in inflammation. An understanding of the role played by proteases in these processes may provide the opportunity for therapeutic intervention and inhibitors of certain proteases have already proved to be effective therapeutic agents in hypertension, heart failure and some forms of cancer (Hooper, 2002).

Proteolytic enzymes that depend on serine residue for their catalytic activity are commonly spread in nature. Serine proteases include a large class of enzymes and have been grouped into six clans, on the basis of their three dimensional structures (Rawlings and Barret, 1994). They provide much information on enzyme catalysis (Hedstrom, 2002). Catalytic triad and oxyanion hole are important for enzyme activity of this category (Blow, 1997; Henderson, 1970). In addition to the biological role played by digestive enzymes such as trypsin, serine proteases also function broadly as regulators through the proteolytic activation of precursor proteins (Neurath, 1989; van de Ven *et al*., 1993). Examples of this regulation include the processing of trypsinogen by enteropeptidase to produce active trypsin (Huber and Bode, 1978) and the cascades of prothrombin activation to thrombin that control blood clotting (Davie *et al*., 1991). Several serine proteases have been isolated from distinct parts of plants ranging from seeds to latex and fruits. Plant serine proteases are involved in many physiological processes such as microsporogensis, protein degradation, signal transduction and differentiation and in hypersensitive response (Antao and Maclcata, 2005). In the present study we have purified and characterized serine protease from seeds of *Holarrhena antidysenterica* for the first time.

MATERIALS AND METHODS

The harvested *Holarrhena antidysenterica* seeds were obtained from local market. Sephadex G-75, G-25 and DEAE-Sepharose Cl- 6B were purchased from Pharmacia. Dithioerythrytol (DTE), Dithiothrytol (DTT), Urea and Bovine Serum Albumin (BSA) were purchased from Fluka. Casein, β-lactoglobuline, lysozyme and Phenylmethylesulphonyl Fluoride (PMSF) were purchased from Sigma.

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Preparation of crude extract: The grinded seeds were defatted by homogenizing in 4 volumes of cold acetone (-20°C) for 15 min and kept in open air for vaporization of acetone over night.

Ten gram dry defatted seeds powders of Holarrhena antidysenterica were suspended in 100 mL of 100 mM Tris-HCl buffer pH 8.5 containing 0.01% sodium azide, gently stirred for about 24 h at 4°C. The crude extract, obtained after filtration and centrifugation at 10,000 x g for 20 min, w as stored at 4°C. Proteins were precipitated from crude extract using 30 and 70% ammonium sulfate saturation. 30% precipitates were centrifuged at 10,000 x g for 20 min, while the 70% precipitates were centrifuged at 12,000 x g for 20 min. Both the pellets were re-suspended in 50 mM Tris-HCl buffer pH 8.5.

Purification of enzyme: Ammonium sulphate precipitates (70%) were loaded to Sephadex G-75 gel filtration column (2.5 × 70 cm) equilibrated and eluted with 50 mM Tris-HCl buffer pH 8.5. The flow rate was maintained at 5 mL. 15 min^{-1}, proteins content were monitored at 280 nm and fractions of 5 mL were collected. The activity of each collected fraction was measured as described later in enzyme assay. The proteolytically active peak-II of Sephadex G-75 showing protease activity was subjected to an anion exchange DEAE Sepharose Cl-6B (2.5 × 24 cm) equilibrated with 50 mM Tris-HCl buffer pH 8.5. The bound proteins were eluted with 500 mL of a salt gradient of 0-0.5M NaCl in the same buffer at a flow rate of 5 mL 15 min^{-1}. The proteins content were monitored at 280 nm in each fraction and fractions of 5 mL were collected. The fractions having protease activity were pooled together.

Electrophoresis: Polyacrylamide gel electrophoresis (PAGE) of the enzymes was performed by the method of Lammli (1970) with 12 and 10% polyacrylamide gel in the presence of Sodium Dodecyl Sulfate (SDS) and 2-mercaptoethanol (SDS-PAGE), with 8% polyacrylamide gel in the absence of SDS (native-PAGE). The protein in the gels were stained with Coomassie Brilliant blue R-250 and silver staining method (Helmut and Beier, 1987). Protein contents were estimated, according to the method of Bradford (1976), using Bovine Serum Albumin (BSA) as the standard protein.

Protease activity detection by zymogram: Gelatin zymography was performed in polyacrylamide slab gel containing SDS and gelatin (1%) as co-polymerized substrate, as described by Anson (1938). After electrophoresis the gel was washed in Triton X-100 (2.5%) three times with 10 times volume of the gel to remove the SDS for 20 min, incubated in 50 mM Tris-HCl buffer pH 8.0 containing 0.1 M NaCl and 1 mM $CaCl_2$ for 5 h at 37°C and then stained with Coomassie Brilliant blue R-250. The activity band was observed as a clear colorless area depleted of gelatin in the gel against the blue back-ground.

Enzyme assay: In each purification step, the enzyme was assayed by (Heussen and Dowdle, 1980) with slight modification. The reaction mixture consisting of 100 μL of enzyme solution, 100 μL of 1% casein (w/v) in 50 mM Tris-HCl buffer pH 8.0 and 1 mL of 50 mM Tris-HCl buffer pH 8.0 was incubated at 37°C for 30 min. The reaction was stopped by the addition of 200 μL 40% TCA, incubated on ice for 40 min and then centrifuged at 14000 x g for 10 min. The control was prepared in the same quantity but the enzyme was added to the mixture of substrate and TCA. The TCA soluble fractions and control were measured at 280 nm. The protease activity was expressed as the difference of absorbance at 280 nm between the control sample and the test sample. One unit of proteolytic activity was defined as the amount of enzyme necessary to increase absorbance by 0.001 under the conditions previously described.

Protease activity by agar plate assay: The protease activity was determined by using agar plate assay. An agar plate was prepared in 20 mM Tris-HCl buffer pH 7.6 containing 2 mM $CaCl_2$ and 0.5% casein solution. An agar casein medium was used for the protease activity assay. The crude extract, 70% ammonium sulfate precipitates, peaks of Sephadex G-75 and unbound peak of DEAE-sepharose Cl- 6B were applied on casein agar plate. After 20 h incubation, the casein was precipitated by saturated ammonium sulfate solution. The peaks of Sephadex G-75 and unbound DEAE-sepharose Cl- 6B digested the casein in agar medium and showed clear zone.

Effect of temperature and pH on enzyme activity: The optimum temperature of purified protease was determined by conducting the assay at different temperatures in 50 mM Tris-HCl pH 8.0. The reaction mixture containing enzyme was heated for 30 min in a water bath at various temperatures (20-90°C). The pH stability of the protease was measured after preincubation of the protease in the activity buffer for 30 min at different temperatures (20-90°C) and proceeded for the enzyme activity, as mentioned earlier.

The optimum pH of the purified protease was determined by conducting the assay at various pH. The reaction mixture-containing enzyme was incubated for 30 min at 37°C in a water bath at various pH: 50 mM

sodium acetate (pH 4.5-5.5), 50 mM sodium phosphate (pH 6-7) and 50 mM Tris-HCl (pH 7.5-9). The pH stability of the protease was measured after preincubation of the protease in the different buffers 50 mM sodium acetate (pH 4.5-5.5), 50 mM sodium phosphate (pH 6-7) and 50 mM Tris-HCl (pH 7.5-9), after incubation in each buffer for 30 min at 37°C, the reaction was proceeded for the enzyme activity, as mentioned above in enzyme assay.

Effect of protease inhibitors, compounds and metal ions on enzyme activity: The effect of various protease inhibitors as phenylmethylsulfonyl fluoride (PMSF), iodoacetamide and ethylendiaminetetracetic acid (EDTA) and compounds as urea, 1, 4-dithio-L-threitol (DDT), 2-mercaptoethanol and sodium dodecyl sulfate (SDS), on the peptide hydrolyzing activity of purified protease were accessed.

The purified enzyme (5 μg) was preincubated at 37°C for 30 min with inhibitors and compounds at a given concentration (Table 1). This was followed by addition of 1% casein and 50 mM Tris-HCl buffer of pH 8.0. The residual protease activity was then measured.

For determining the influence of metal ions, the following salts were used: $ZnCl_2$, $MgCl_2$, KCl, $CuSO_2$, LiCl, NaCl, $CoCl_2$ and $CaCl_2$, The residual protease activity was then measured as described above. The activity without inhibitors or metal ions was considered to be 100%.

K_m determination: The K_m value of the enzyme were determined by using increasing substrate concentration and plotted $1/V_o$ against 1/[S] the so called Linewaever-Burk or double reciprocal plot with error bars of $\pm 0.05\ V_{max}$.

Table 1: Effect of metals, detergent and inhibitors on protease activity

Inhibitors	Concentration (mM)	Residual activity (%)
None	---	100
PMSF	0.5	70
	1	40
	2	20
	5	5
Iodoacetamide	0.5	90
	1	76
	2	71
EDTA	1	85
	2	88
	5	71
DTT	1	73
2-Mercaptoethanol	1	71
Urea	5	50
SDS	5	43
Metals		
$ZnCl_2$	2	60
$MgCl_2$	2	121
KCl	2	102
$CuSO_2$	2	112
LiCl	2	109
NaCl	2	108
$CaCl_2$	2	131
$CoCl_2$	2	105

RESULTS AND DISCUSSION

Purification of serine protease: In the present study, we have purified and characterized alkaline serine protease from seeds of *Holarrhena antidysenterica* by ammonium sulfate precipitation, gel filtration and ion exchange chromatography.

The crude extract was fractionated by solid ammonium sulfate; step wisely, first, 30% ammonium sulfate precipitates contained high molecular mass proteins and 70% ammonium sulfate precipitates contained low molecular masses proteins. The precipitated proteins were redissolved in 50 mM Tris-HCl buffer pH 8.5. Protease activity was not seen in crude extract and 70% ammonium sulfate precipitates (Asif *et al*., 2006).

The 70% ammonium sulfate precipitates were fractionated into five peaks by Sephadex G-75, the protease activity was traced spectrophotometrically in Sephadex G-75 fractions using 1% casein as substrate. Enzyme activity profile showed, Protease was concentrated to Fractions of Peak-2 of Sephadex G-75 (Fig. 1A). Fractions of Sephadex G-75 were electrophoresed on SDS-PAGE showing group separation. Zymography (activity gel electrophoresis) of different fractions of peak-1 and peak-2 from Sephadex G-75 showed more than one proteases of different molecular mass in the seeds of *Holarrhena antidysenterica*. Peak-2 of Sephadex G-75, exhibited protease activity was further fractionated by DEAE-Sepharose CL- 6B (Fig. 1B). The fractions eluted after initial concentration of gradient contained a protease when analyzed by activity gel electrophoresis (Fig. 2B). The bounded fractions showed the partially purified protease migrated as a single band in both reducing and non reducing condition. The molecular mass of the partially purified protease was estimated to be about 25 kDa by SDS-PAGE (Fig. 2A).

The molecular weight of serine protease from *Holarrhena antidysenterica*, is similar to that of the serine protease hevain 1 (80 kDa), isolated from latex of Hevea brasiliensis (Suarez *et al*., 2004; Lynn and Clevette Radford, 1986). The molecular weight of the other latex proteases from the Euphorbia species ranges from 25 to 80 kDa (Patel *et al*., 2007; Lynn and Clevette Radford, 1988), molecular weight of well-known plant serine proteases of the cucumisin family range from 30 to 70 kDa (Uchikoba *et al*., 1995; Tornero *et al*., 1996). The plant serine proteases known at present vary from 19 to 110 kDa (Antao and Maclcata, 2005).

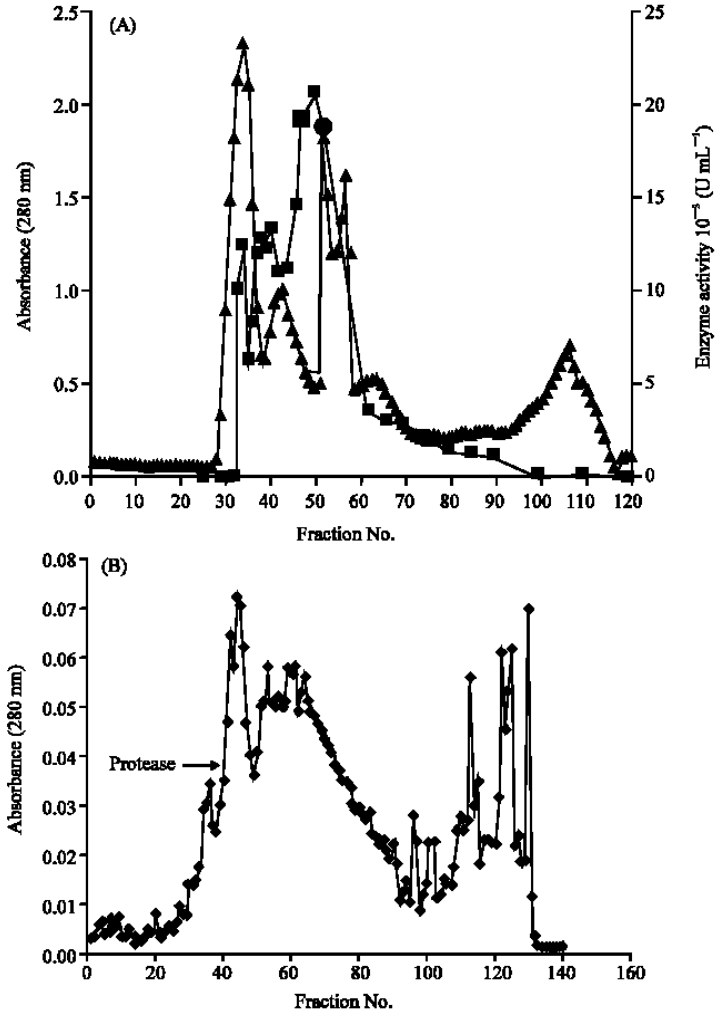

Fig. 1: Purification of serine protease (A) 70% ammonium sulfate precipitates loaded on Sephadex G-75 column (2.5 × 115 cm), equilibrated with 50 mM Tris-HCl buffer (pH 8.5). The fraction having casein digesting activity were put on (A) and (B) DEAE-Sepharose Cl-6B column (2.5×24 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.5) and the bound proteins were eluted with linear gradient of 0-0.5 M NaCl in the same buffer. Protein was monitored at 280 nm (■) and the protease activity was assayed by Anson method's at 280 nm (●) using 1% casein as a substrate

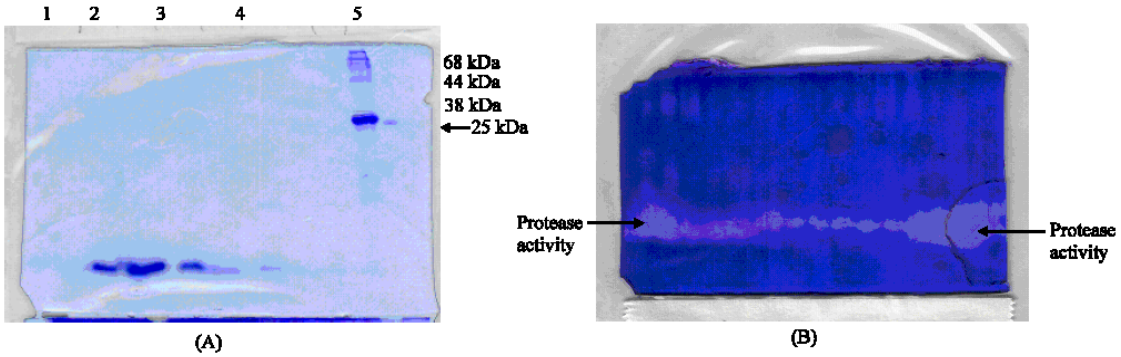

Fig. 2: Purified serine protease of *Holarrhena antidysenterica*, (A) Lane 1-2: purified protease non-reducing form, Lane 3-4: purified protease reducing form Lane Standard proteins BSA (67 kDa) and (B) Protease activity on zymogram

Effect of pH and temperature on enzyme activity and stability: The alkaline serine protease of *Holarrhena antidysenterica* was characterized by using 1% casein as substrate at pH 8.0. Figure 3A depicted the alkaline Serine protease optimum pH and pH stability, respectively. The enzyme showed maximal activity at pH 7.5 and was found to be relatively stable in the pH range 7.0-8.0 relatively similar to already reported the latex proteases from Euphorbia species, which showed optimal activity between pH 6 and 8 (Heussen and Dowdle, 1980). In contrast, Cucumisin-like proteases possess an optimum pH in the range 8-10 (Patel *et al*., 2007; Lynn and Radford, 1988; Yonezawa *et al*., 1997). Most of the plant serine proteases showed optimum pH ranges from 7-11 (Antao and Maclcata, 2005) The optimum temperature of *Holarrhena antidysenterica* protease was found to be 35°C. *Holarrhena antidysenterica* protease was stable up to 30°C (Fig. 3B) relatively similar to protease having optimum temperature 45°C, isolated from *Neurospora crassa* (Lindberg, 1981). and to protease isolated from digestive tract of sea cucumber having optimum temperature 37°C, while alkaline protease isolated from *Alcaligenes faecalis*, exhibited its highest activity at 55°C. The optimum temperature for plant serine proteases are variable, from 30°C up to 80°C, but most plant serine proteases usually act best in the range 20 to 50°C (Antao and Maclcata, 2005).

Effect of protease inhibitors, compounds and metal ions on enzyme activity: Table 1 shows the effect of various protease inhibitors and metal ions on the activity of *Holarrhena antidysenterica* protease. The protease inhibition profile showed that the enzyme activity was 90% inhibited by 2 mM PMSF similar to serine protease isolated from *Alcaligenes faecalis* (Thangam and Rajkumar, 2002). while metalloprotease inhibitor such as EDTA and cysteine protease inhibitors iodoacetamide has no significant effect on enzyme activity. The results clearly indicate that the enzyme is serine protease having serine residue in its active site.

The enzyme was further characterized by treating with different metal ions in 50 mM Tris-HCl pH 8.0 using 1% casein as a substrate (Table 1). These results indicated that the enzyme activity was enhanced in the presence of most divalent metals ions such as Ca^{++} and Mg^{++}, whereas the activity of protease was decreased in the presence of Zn^{++}.

Km and substrate specificity: Line weaver Burk analysis showed that *Holarrhena antidysenterica* protease has a K_m of 1.1 mg mL^{-1} using casein as the substrate and V_{max} of 389.71 units min^{-1} mg^{-1} of protein.

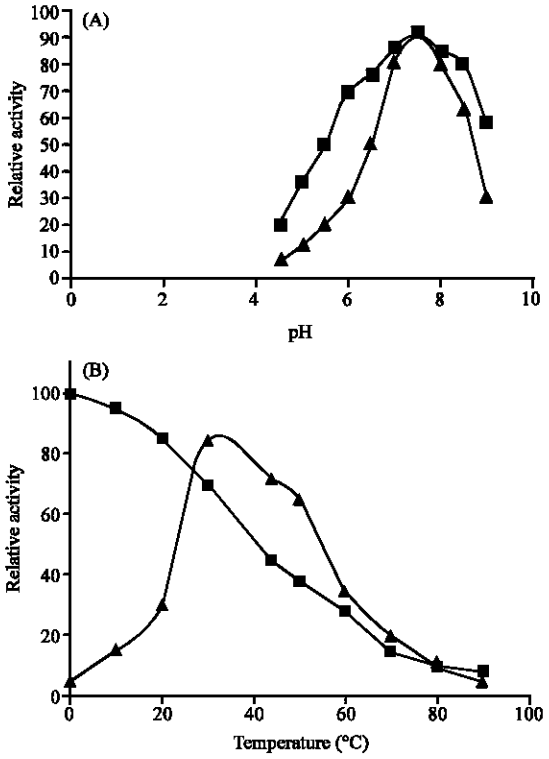

Fig. 3: (A) Effect of pH and (B) temperature on activity (-▲-) and stability (-■-) of alkaline serine protease from *Holarrhena antidysenterica*

Table 2: Substrate specificity of the alkaline serine proteases from *H. antidysenterica*

Substrate	Concentration (%)	Monitoring wavelength	Relative
Casein	1	280	100
Hemoglobin	1	280	90
BSA	1	280	70
Gelatin	1	280	60
Azocasein	1	440	114
Azoalbumin	1	440	107

The substrate specificity of *Holarrhena antidysenterica* protease showed that it was active on a variety of modified substrate (azoalbumin and azocasein) and natural proteins (BSA, casein and gelatin) as shown in (Table 2). *Holarrhena antidysenterica* protease exhibited the highest activity towards azoalbumin. The enzyme also hydrolyzes fibrous proteins like gelatin.

CONCLUSION

Plant serine proteases are widespread among taxonomic groups, from trees and crops to legumes and herbs. They are also present in almost all plant parts, but seem to be more abundant in fruits. In present research work we have studied about serine protease. The results

indicate that serine protease is highly sensitive to towards temperature. The temperature stability varies from low to high temperature, but in case of serine protease from seeds of *Holarrhena antidysenterica* the enzyme is mostly active near at 40°C. The pH stability of serine protease varies from acidic to neutral to basic, but we have seen that enzyme was stable and reactive in alkaline pH, isolated from the seeds of *Holarrhena antidysenterica* In general, they show broad substrate specificity for proteins and casein in particular and for oligopeptides, although many studies have been conducted using only synthetic substrates.

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