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RESEARCH ARTICLE

Isolation, purification and characterization of serine protease from latex of *Euphorbia prunifolia* Jacq.

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Abstract

A new serine protease named "prunifoline" was purified from the latex of *Euphorbia prunifolia*, a medicinally important indigenous plant of Euphorbiacae family. "Prunifoline" was isolated using acetone precipitation method and purified by using DEAE cellulose column chromatography. The molecular weight of the purified protease was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). "Prunifoline" a single protein band detected on SDS-PAGE and calculated molecular weight was approximately 57.44 kDa. It remained active over a broad range of temperature but had optimum activity at 55^0C and pH 7.0, when casein was used as a substrate. Thus enzyme "prunifoline" may be considered as a strong candidate for various applications in the food and biotechnological industries.

INTRODUCTION

Proteolytic enzymes or proteases are a class of proteins ubiquitously found in all organisms from microbes to higher organisms, and it acts as catalysts to participate in diverse vital function [1]. In plants, proteases are participated in all aspects of the life cycle ranging from the mobilization of storage proteins during seed germination to the initiation of cell death [2]. Generally, proteases are produced as inactive proenzymes to prevent protein degradation at a wrong site of time. Such peptide segments that keep the enzyme inactive are always found at N-terminal ends of the precursor molecule. These properties also function as chaperones in the proper folding and compartmentalization of the enzymes. Most of the reported plants proteases are cysteine endopeptidase, where as serine or other endopeptidases are highly rare [3].

Proteases are one of the most important enzymes used in industries and hold about 60% of the total worldwide enzyme market demand [4]. Proteases are also habitually used in food processing, brewing, cheese preparation [5], tenderization of meat, bread manufacturing, leather and textile industries. Proteolytic enzymes from plant sources have also received greater attention in the pharmaceutical and biotechnology industry due to their property of being active in a wide range of temperature and pH.

Thus the arduous search for new potential plant proteases still continues in order to make them industrially applicable and cost effective [6, 7]. The plant derived serine proteases are stable and more active under the unpalatable conditions are become more useful and economical for industrial application and forced the inviting search of new plant serine protease [8]. Therefore, an attempt has been made to purify novel thermostable serine protease from the latex of medicinally important plant *Euphorbia prunifolia* belongs to the family of Euphorbiacae and is commonly distributed throughout India and other parts of the world [9]. *Euphorbia prunifolia* is a medicinally important flowering plant, and its latex is used as toothaches, nervine diseases, dropsy, palsy, deafness to kill the maggot in wounds. We purified protease from the latex of *Euphorbia prunifolia* which was named as "Prunifoline" according to the nomenclature of proteases [10].

Materials and Methods

Chemicals

All chemicals were of the highest purity, analytical HPLC grade purchased from Sigma Chemicals, USA; Himedia Laboratories, Mumbai; SRL Chemicals, Bangalore, Qualigen Fine Chemicals, Mumbai Merck Chemicals, India Bangalore Genie, India. Skimmed milk powder was thankfully received from Central Dairy, Aarey Milk Colony, Mumbai, India. Sequencing grade Hybond-PVDF membrane (Amershyam) was gifted by Department of Biotechnology, TMC.

Plant material and collection of latex

The plant latex sample of *Euphorbia prunifolia* was collected early in the morning by superficial incision of stem or leaves of healthy plants and allowing the milky latex to drain in clean glass vials separately, brought to the laboratory and kept in refrigerator till the experiment started [11].

Preparation of crude enzyme

All operations were carried out at 0.5^0C. Latex was homogenized in a homogenizer under chilled condition and filtered through four folds of muslin cloth. Filtrate latex sample was centrifuged at 15,000 rpm for 45 minutes at 4^0C. The resulting supernatant of latex enzyme called "Crude enzyme" or "Centrifugal fraction" which was used for further investigation of protease enzyme assay [12].

Protease assay

Proteolytic activity was determined by the colorimetric assay using 1% casein as a substrate as described by Khan et al., [10]. The protease activity was expressed as amount of enzyme required to produce peptide equivalent to μg of tyrosine/min/mg protein at 37^0C and protein content was determined according to Lowry's method [13] using Bovine serum albumin as the standard protein.

Substrate preference assay

The substrate preference was determined as proteolytic activity of crude enzyme according to method described by Badgujar 2010 using casein, bovine serum albumin, keratin, gelatin, haemoglobin and egg albumin. Assay was made at 37^0C in 0.01 M phosphate buffer (pH 7.0) [14]. A 2.0 ml aliquot of each substrate (2% was prepared in 0.01 M phosphate buffer (pH 7.0). Each substrate individually was treated with crude enzyme. The resulting reaction mixture was incubated at 37^0C for 1 hour. The reaction was terminated by the addition of 3 ml of ice chilled 5% trichloroacetic acid and maintained for 30 minutes at room temperature followed by filtration through Whatman filter paper number 01. Then the residual protease enzyme activity was calculated as per Khan et al., method [9].

Milk clotting activity

The enzyme was assayed as described by Greenberg method [14] with some modification. The enzyme source (0.2 ml crude enzyme) was added to 2 ml of substrate solution (12% skim milk powder in 0.01 M $CaCl_2$). The time necessary for the formation of milk clot was measured and its validity was confirmed by using pointed curve needle. Milk clotting activity is expressed in terms of soxhlet unit.

Effect of pH on enzyme activity

The effect of pH on proteolytic activity of crude enzyme was measured with casein as substrate (pH 4.5 - 9.5) using 0.01 mM sodium salts of buffer. The buffers used were acetate buffer (pH 4.5 - 5.5) phosphate buffer (pH 5.5 - 7.5) and carbonate buffer (pH 7.5 - 9.5). Due to precipitation of casein at pH 4.5, haemoglobin was used as substrate. Also effect of pH on milk clotting activity was studied by the method similar to that of followed by Greenberg with some modification [15]. Twelve per cent solution of skimmed milk powder i.e. substrate was adjusted to various pH with the help of appropriate buffer (pH between 3 and 8). Milk clotting activity of crude enzyme with casein having different pH values was determined. At each pH a control assay was run without enzyme and treated as blank [16].

Effect of temperature on enzyme activity

The effect of temperature on enzyme activity was studied by using casein. The crude enzyme was incubated at the desired temperature, in the range of 10-80 ^{0}C, for 15 minutes in sodium phosphate buffer (pH 7) and an aliquot was used for the activity measurement at the respective temperature [17].

Additionally, the milk clotting activity of crude enzyme was determined within the temperature range between 20 ^{0}C to 80 ^{0}C and 0.2 ml aliquot of enzyme fraction was treated with the pre incubated skimmed milk substrate at various temperatures for milk clotting enzyme assay. The time required for clotting of milk was noted and

residual milk clotting activity was calculated according standard assay procedure as mentioned above. A suitable control without the addition of enzyme was run simultaneously [18].

Effect of inhibitors on proteolytic activity

Inhibition of the hydrolysis of casein by crude enzyme was investigated using 5 mM PMSF (Phenyl methane sulfonyl fluoride) 0.1 mM pepstatin-A, 1 mM Phenathroline, 5 mM EDTA (Ethylene diammine tetra acetic acid), 50μM IAA (Iodoacetic acid) and 40μM $HgCl_2$. The enzyme preparation was incubated with inhibitors individually at room temperature for 60 minutes [18]. The residual proteolytic activity against casein was determined by the standard assay procedure. Controls were prepared by pre incubating the enzyme fraction with the appropriate solvent used to dissolve the inhibitors. A control assay of the enzyme activity was done without inhibitor and the resulting activity was taken as 100%.

Purification of serine protease from *Euphorbia prunifolia*

Solvent precipitation

For the initial purification step, the crude enzyme was precipitated with ice chilled acetone (20 – 40%) at 4 ^{0}C. The resulting precipitate was separated by centrifugation, washed with acetone and dissolved in 0.01 M ice chilled phosphate buffer (pH 6.0). This preparation was named "partially purified preparation" of enzyme [19].

Chromatographic procedures

The partially purified preparation was applied (3 ml) on to a column (1.8x30 cm) of DEAE. Cellulose pre-equilibrated with 0.01 M phosphate buffer at pH 6.0. Elution of protein was carried out by batch wise addition of 40 ml portions of increasing molarities (0.0-0.5 M) of NaCl in 0.01 M phosphate buffer (pH 6.0) Effluents were collected in 5.0 ml fractions at a flow rate of 20 ml/h. The absorbance at 280 nm as well as the proteolytic activity in all fractions was tested [20]. The fractions showing a peak activity were pooled and dialyzed using dialysis tubing with a cut off of (12000-14000 Da) against three 2 L changes of 0.01M phosphate buffer (pH 6.0) over 16 hours. The dialysate was centrifuged at 10,000 rpm for 20 minutes at 4 ^{0}C to remove any insoluble material and was immediately subjected to re-chromatography on DEAE cellulose column of same dimension and finally eluted with a 0.2 - 0.3 M NaCl linear gradient in the same buffer [21].

Characterization of serine proteases

Thermal stability

The thermal behavior of the partially purified enzyme was evaluated by measuring the residual proteolytic activity at 37 ^{0}C (pH 6.7) for 2 minutes after incubation of samples for 20, 40, 60, 80 and 100 minutes at 40 – 70 ^{0}C[22].

Electrophoresis (SDS-PAGE)

Chromatographically active fraction of protease enzyme was analyzed by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) with Tris - glycine buffer (pH 8.2) in 12% polyacrylamide gel according to Laemmli, 1970 [23]. Detection was done by the Coomassie Brilliant Blue R. 250 staining method and molecular weight was estimated by comparing relative mobility of marker proteins, namely Bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and Lysozyme (14.3 kDa). Substrate polyacrylamide gel electrophoresis was performed to detect the band with protease activity on a 4% stacking gel and 0.2% casein incorporated in to 10% separating gel as per the method described by Heussen and Dowdle [14].

Carbohydrate content

Carbohydrate content of chromatographically active fractions of protease enzyme was determined by phenol Sulphuric acid method using glucose as the standard sugar [24].

Glycoprotein detection

The PAS method of Kapitany and Zebrowski [16] was adopted in order to detect glycoprotein's electro blotted on to a PVDF membrane. After electro transfer the PVDF membrane was washed in 50 ml of 12% TCA for 5 minutes. All the further treatments of the membrane were performed in the dark at 4^{0}C. The membrane was treated by 50 ml of 1% periodic acid for 15 minutes. Three washing with 75 ml of 15% acetic acid were performed for 5 minutes each. A volume of 50 ml of Schiff's reagent was then added for 30 minutes and the membrane was washed 6-8 times with 200 ml of 7.5% acetic acid for more than 60 minutes [25].

Results and Discussion

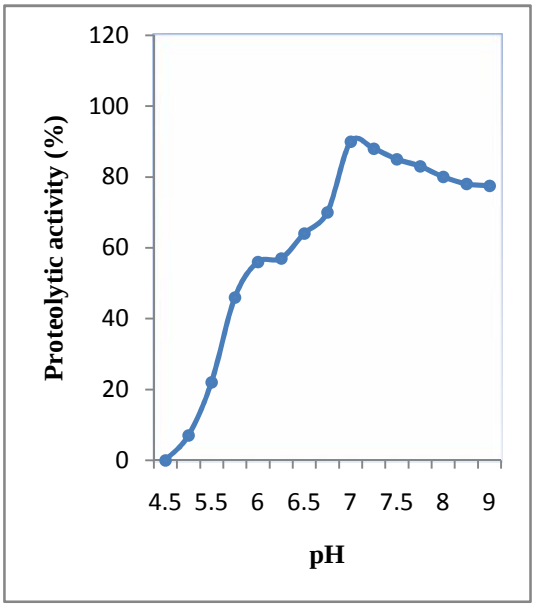


Figure 1 Effect of pH on the proteolytic activity of latex protease of *Euphorbia prunifolia*

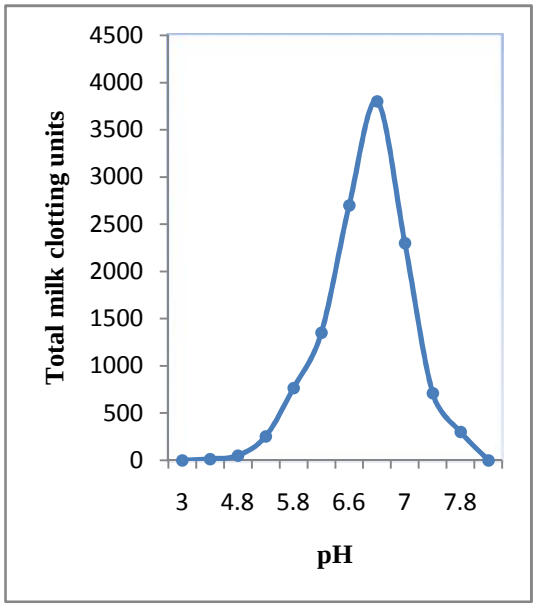


Figure 2 Effect of pH on the milk clotting activity of latex protease of *Euphorbia prunifolia*.

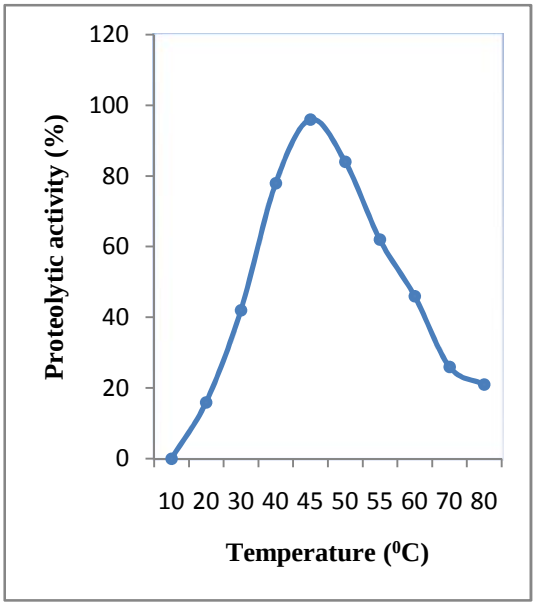
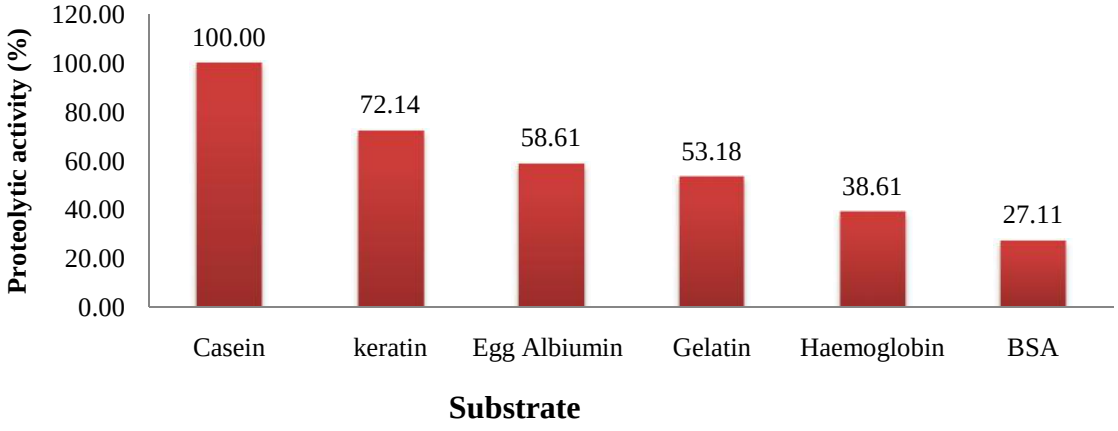


Figure 3 Effect of temperature on the proteolytic activity of latex protease of *Euphorbia prunifolia*.

The total protein concentration of the crude enzymes of milky latex of *Euphorbia prunifolia* was *22.25* mg/g. The ratio of milk clotting to proteolytic activity was recorded as 2:1 for crude enzymes of *Euphorbia prunifolia*. The ratio of milk clotting to proteolytic activities (MCA/PA) of enzyme is an important standard for replacement of calf rennet. Therefore, it is likely to be considered MCA/PA ratio as searching calf rennet replacer. Thus the higher MCA/PA ratio enzyme which has higher milk clotting activity and lower proteolytic activity is suitable for cheese making. Therefore, this ratio is a key index for deciding the suitability or role of protease in traditional cheese production [14]. The proteases of *Euphorbia prunifolia* latex exhibit high proteolytic activity within a pH range from 6.5 to 8.0 and optimum activity was observed within pH 7.0 (Figure 1). The hydrolytic enzymes of *Euphorbia prunifolia* latex exhibits maximum milk clotting activity was observed within a pH range from 6.0 - 6.5 (Figure 2). The optimum milk clotting activity was observed at pH 6.3. The optimum temperature of this enzyme is 55 ^{0}C (Figure 3).

Figure 4 Substrate specificity of the proteolytic activity of crude enzyme of *Euphorbia prunifolia*



Crude enzyme of *Euphorbia prunifolia* latex exhibited the highest substrate specificity with 2% casein at 37 ^{0}C and pH 7.0 compared to 72.14% proteolytic activity with keratin, 58.61% activity with egg albumin, 53.18% activity with gelatin, 38.61% activity with hemoglobin and 27.51% activity with bovine serum albumin (Figure 4). Casein was found to be the most specific substrate where as bovine serum albumin was relatively low substrate for proteolytic activity of protease enzyme of *Euphorbia prunifolia.* Electrophoretic behaviour of purified protease enzyme of *Euphorbia Prunifolia* latex, SDS PAGE and Substrate-PAGE (Zymogram) of purified enzyme fraction and molecular weight marker is illustrated in figure 5 and 6. Isolated serine protease showed positive reaction with PAS. Therefore, evidently it is a glycoproteinin. Highly stable glycosylated serine protease from the medicinal plant *Euphorbia milli* [26].

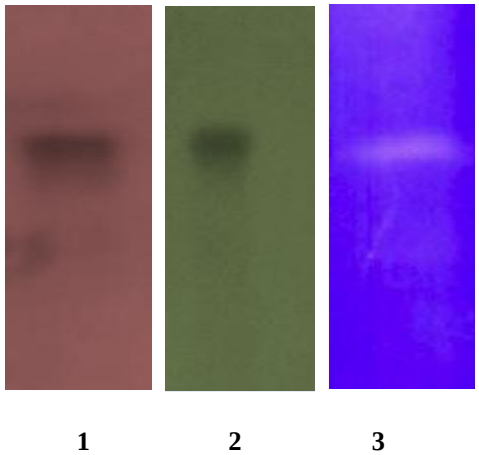


Figure 5 Electrophoretic behaviour of purified protease enzyme of *Euphorbia Prunifolia* latex SDS-PAGE.

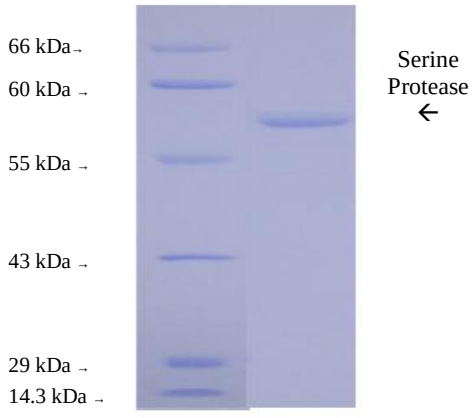


Figure 6 Molecular weight determination of protease by SDS-PAGE

Table 1 Effect of various types of inhibitors on the activity of prunifoline

Type of Inhibitor	Inhibitors	Concentration of inhibitors	Residual activity(%)
	Control (W/O inhibitor)		100%
Serine protease	PMSF	5 mM	10.31
	DFP	5 mM	9.48
Cysteine protease	Iodoacetic acid	50 μM	89.36
	Mercuric Chloride	40 μM	87.93
Metalloprotease	EDTA	5 mM	76.63
	Phenanthroline	1 mM	76.03
Aspartic protease	Pepstatin A	0.1 mM	81.16

In order to identify the classes of protease enzymes of *Euphorbia prunifolia* plant latex, the effect of different proteases inhibitors have been evaluated. Table1 shows the residual activity of the protease enzyme of latex after its inhibitions with the following class specific inhibitors mercuric chloride and iodoacetic acid (inhibitors of cysteine proteases), PMSF and DFP (inhibitors of serine proteases), EDTA and phenanthroline (inhibitors of metalloproteases) and pepstatin A (inhibitors of aspartic proteases) using casein substrate. Maximum inhibition of 90 to 91.1 of proteolytic activity of plant latex containing enzyme occurs in presence of PMSF and DFP. Enzymes did not show remarkable inhibition in the presence of Iodoacetic acid mercuric chloride, EDTA, phenanthroline and pepstatin A on the activity of latex enzymes.

Table 2 Purification scheme of serine protease of *Euphorbia purinifolia* latex.

Sr. No.	Step	Total protein (mg)	Total activity (Units/ min)	Specific activity (Units/ mg)	Yield (%)	Purification fold
1.	Crude enzyme	14.80	14.712	1.19	100	1.00
2.	Acetone precipitation	6.80	8.740	1.54	61.18	1.29
3.	DEAE Cellulose	1.405	3.181	2.28	27.12	2.22
4.	Rechromatographed on DEAE Cellulose	0.58	1.714	3.21	13.64	3.12

Purification profile of purification of prunifoline from latex of *E. prunifolia* is given in table 2. We could achieve 3.12 fold purification using Acetone precipitation and DEAE cellulose chromatography.

Conclusion

The occurrence of proteolytic activity in the latex of the medicinal plant *Euphoriba Prunifolia* is reported for the first time in this investigation. During the period of work, a serine protease was purified from the latex using simple purification procedures. Thermostable serine glycoprotein, "Prunifoline" retains its activity and high stability over a broad range of pH and temperature. Hence this enzyme could be serve as potential for various biotechnological applications such as in the field of food, pharma and textile industries; based on results obtained in our laboratory for degumming of raw silk threads and cheese preparation. Thus, easy availability of the latex and simple purification procedure makes this enzyme an ideal tool to study its application in medicine , agriculture and allied industries.

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