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RESEARCH ARTICLE

A COMPARATIVE STUDY OF PROTEASE PRESENT IN THE LEAF AND LATEX OF *HOLOSTEMMA ADA-KODIEN* SCHULT.

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Abstract

Proteases, an important group of hydrolytic enzyme break the peptide bonds of proteins. *Holostemma ada-kodien* Schult. (Asclepiadaceae) is a medicinally important plant with therapeutic and pharmacological actions. The crude protease extracted from the leaves had an activity of 51.38 U/ml while that from the latex was with an activity of 290U/ml. Native PAGE and Zymogram analysis showed the presence of five different proteases in the leaf and two major proteases in the latex. Both the crude enzymes were subjected to purification by ammonium sulphate $[(NH_4)_2 SO_4]$ fractional precipitation followed by CM-Cellulose and DEAE-Cellulose column chromatography. The biochemical characterisation of major protease of the latex showed optimum temperature as 50^{o}C while optimum pH was 7.0. The enzyme exhibited good thermal and pH stabilities. Effect of inhibitors, metal ions on activity, hydrolysis of protein substrates and effect of substrate concentration on reaction velocity were evaluated. This is the first report on the protease from *H. ada-kodien*.

Introduction:-

Proteases are a group of hydrolytic enzymes that cleave the peptide bonds of proteins. In all organisms growth and development occur as a result of balance between protein synthesis and proteolysis. Proteases are found in all forms of life: plants, animals and microorganism including viruses. In plants, five classes of endoproteases have been described: Serine, Cysteine, Aspartic, Metallo and Threonine (Rawlings *et al*., 2010).

The majority of commercial enzymes have been obtained mainly from microbial sources. However, the latex and leaves of plants are also a rich source of several hydrolytic enzymes in which protease are the key enzyme. Plant protease has recently received a special attention in medicine and food industries because of their broad substrate specificity as well as activity in wide range of pH and temperature. Plant proteases are finding application in various processes such as brewing, meat softening, milk-clotting, cancer treatment, digestion and viral disorder, as well as a food complement (Kleef *et al*., 1996; Priolo *et al*., 2000; Losada, 1999). They are also useful in leather industry for dehairing and bating of hides to substitute toxic chemicals (Foroughi *et al*., 2006).

Proteases have been identified and studied from several plant families such as Asteraceae, Caricaceae, Moraceae, Asclepiadaceae, Apocynaceae and Euphorbiaceae. Latex from the members of Asclepiadaceae has been reported to be a mixture of several hydrolytic enzymes and secondary metabolites (Santos and Van Ree, 2011). Of these hydrolytic enzymes, proteases are known to be the key enzymes for many of the observed pharmacological actions. *H. ada-kodien* is one of the medicinally important plant of the family Asclepiadaceae. The protease activity in this plant has so far been not reported. In this scenario, the present study was focused on the evaluation of proteases from the latex and leaves of this plant.

Materials and methods:-

Pant Material:-

H. ada-kodien is a laticiferous, twining shrub with large conspicuous flowers. This plant is with high medicinal properties and extracts of plants with high content of proteolytic enzymes have been used in traditional medicine since long time. The latex of *Holostemma* has been employed for the treatment of wounds, blisters and the plant is also reported to have anticancer and antitumour properties. Its medicinal properties are attributed to the amino sugars present in the roots. It also contains six amino acids like alanine, aspartic acid, valine, glycine, serine and threonine.

Preparation of Crude Enzyme from the Latex:-

The latex was collected by superficial incision of petioles into 0.1 M citrate phosphate buffer (pH 6.5) containing 0.05 mM ethylene diamine tetra acetic acid (EDTA) and kept overnight in the refrigerator (Liggieri *et al*., 2009). After repeated thawing and freezing, the latex was centrifuged at 12000 rpm for 20 minutes at 4^0C and was used as the crude protease enzyme.

Preparation of Crude Enzyme from the Leaf:-

The crude leaf extract was prepared using 0.1 M phosphate buffer (pH 7.0). 10 gm of leaves were homogenized using motor and pestle in 30 ml of 0.1 M PO_4 buffer and was centrifuged at 10000 rpm for 20 minutes at 4^0C. The supernatant was used as the crude enzyme.

Protease Enzyme Assay:-

The protease activity was estimated using modified method of Tsuchida *et al* (1986). The assay of proteolytic activity was performed using casein as substrate. Enzyme solution and casein were both prepared in 0. 1 M phosphate buffer of pH 7.0. Enzyme action was stopped by adding 1 ml of 10% w/v trichloroacetic acid. Folin's reagent is used to estimate the reaction products at 660 nm. One unit of activity is defined as the microgram of tyrosine released per ml per minute of the reaction. Specific activity was expressed as the number of units of activity per milligram of protein.

Protein Determination:-

The content of protein was determined following the method of Lowry *et al* (1951) using Bovine serum albumin (BSA) as standard at 640 nm.

Fractional Precipitation by Ammonium Sulphate:-

The 40-70% $(NH_4)_2$ SO_4 fraction of latex and 30-70% fraction of leaves were collected in two different lots. The precipitates were centrifuged at 10,000 rpm for 20min. The precipitates of these two fractions were separately dissolved in 0.1M phosphate buffer (pH 7.0) and dialyzed against the same buffer. The dialysate from the latex was used as the enzyme source for further analysis and electrophoretic studies.

CM Cellulose Column Chromatography:-

The dialysed enzyme concentrate was loaded on to CM Cellulose column pre-equilibrated with 0.05 M phosphate buffer (pH 7). Adsorbed proteins were eluted using gradient elution system and the gradation ranged from 0.0 to 0.5 M NaCl. Each fraction was monitored for protease activity. The optical density of the samples at 280 nm in comparison with protein standard gives the protein concentration. The molarities of the NaCl in the fractions were determined by the ORION Ion Analyser. Fractions showing protease activity were pooled, dialysed using 0.05 M phosphate buffer (pH 7), lyophilized and subjected to further purification.

DEAE Cellulose Column Chromatography:-

CM Cellulose fractions were pooled and subjected to DEAE Cellulose column which was initially equilibrated with five times the volume of column using 0.05 M phosphate buffer (pH 7). Adsorbed proteins were eluted using a gradient elution protocol with a gradation ranging from 0.0 to 0.5 M NaCl. The molarity of NaCl in each fraction was determined by using ORION Ion Analyser. Active fractions were pooled, concentrated by lyophilisation and used for electrophoretic studies.

Native PAGE and Zymogram Analysis:-

In order to study the presence of protease activity bands in the crude and dialyzed fractions obtained following ammonium sulphate fractional precipitation, Polyacrylamide Gel electrophoresis was performed (Laemmli, 1970).

Zymogram analysis of the different electrophoresed samples was performed by gelatin hydrolysis method. Separating gel was mixed with 1.0% gelatin and the samples were loaded. After electrophoresis, the gels were incubated in the buffer at 40^0C for half an hour and stained in Coomassie brilliant blue. Gelatin incorporated gel was used for finding the number of the proteases present in the dialysed samples.

Electrophoresis under Denaturing Conditions:-
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using 12% gels having a thickness of 1.5 mm. The samples were dissolved in sample buffer containing, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% of bromophenol blue, 25% glycerol, 60 mM Tris–HCl (pH 6.8), and 0.9 ml of water. Coomassie brilliant blue was used to stain the protein bands. The samples were run along with the protein markers in order to detect the molecular mass of the proteins. The protein markers used ranged from 14 kDa to 220 kDa.

For zymogram analysis following SDS PAGE, the gel was washed in Triton X 100 followed by buffer wash before staining,

Biochemical Characterisation:-
To study the effect of pH on enzyme activity, partially purified enzymes solution was incubated with casein of various pH values (6.0 to 11.0) at 30 °C for 10 minutes and activities were measured. The pH stability was illustrated by the residual activity of the protease after two hours incubation in respective pH values. Similarly temperature optimum (40-50^o C) and thermal stability was monitored by measuring the residual activity after 2 hrs of incubation at a temperature of 45^0C for a period of two hrs.

Effects of Metal Ions :-
The influence of various metal ions such as Hg, Ca^{2+}, Cu^{2+},Fe^{2+}, Mg^{2+}, $Mn^{2+}$and Ba^{2+} on the proteolytic activity were evaluated by pre incubating the enzyme with metal salts for 30 minutes and then estimating the residual activity.

Effect of Substrate Specificity:-
The protease activity with various protein substrates including casein, gelatine and egg albumin (2mg/ml) was assayed. The reaction mixture contained 0.5 ml protein substrate, 0.2 ml enzyme and 0.3 ml assay buffer and the activity of the enzyme was measured.

Effect of Inhibitors:-
The enzyme solution was pre incubated with inhibitors like EDTA and Phenyl Methyl Sulfonyl Fluoride (PMSF) for 30 minutes at 45^0 C (final concentration 0.01M) and assayed for the protease activity.

Results and discussion:-

Partial Purification of Latex Protease:-
Even though protease is of widespread occurrence in many members of the family Asclepiadaceae (Liggieri 2009; Dubey 2003), they have not been identified from the medicinally important plant *H. ada-kodien*. The protease activity was nearly six times higher in the latex in comparison to leaf. Since the highest activity was shown by the latex, further studies were mainly focused on the latex protease. More over the leaf showed the presence of five proteases as revealed by zymogram analysis (Fig.1). The leaf protease partial purification has been attained using CM Cellulose cation exchange chromatography (Fig.2).

The crude latex protease contained 27.6 mg/ml of total protein with total activity of 6283.8 U/ml enzyme units with specific activity 232.73 unit/mg protein. The protease activity measured in ammonium sulphate precipitated latex was found to be 4125.6 U/ml and the protein content was 4.8 mg/ml, nearly 34.85% of the activity could be retained at 40-70% level. The precipitate following dialysis and lyophilization was loaded into CM Cellulose column. The elution profile showed protein peaks (Fig. 3) of which two revealed proteolytic activity. The first one was the major protease that eluted in fraction 2-4. The second protease was a minor one that eluted between the fractions 6-8. The fraction I with relatively high proteolytic activity were separately pooled and loaded to DEAE Cellulose column. The fractions showing activity were eluted between 6-8 fractions. They were pooled together, dialyzed and

subjected to lyophilization before doing electrophoresis. The result of the purification step of *H. ada-kodien* latex protease is summarised in the Table.1

Native PAGE and Zymogram Analysis:-

The protease activity bands were detected by zymogram analysis. Gelatin is widely used as the substrate for analyzing protease activity. Gelatin is readily hydrolyzed by different classes of plant protease. The zymogram profile of the leaf showed the presence of five different proteases as revealed by clear zone in the gelatin gel.
The latex showed the presence of two different proteases as revealed by zymogram analysis and native PAGE. In CM Cellulose two activity bands could be observed in the zymogram which was purified into a single activity band in DEAE Cellulose (Fig4).

SDS PAGE:-

The SDS PAGE analysis of the latex protease showed the CM Cellulose fraction I to have two proteases as evidenced by zymogram analysis. The major latex protease could be purified to near homogeneity by DEAE Cellulose chromatography. This protease was estimated to have a molecular weight of 24kDa. The major leaf protease on the other hand possessed a slightly lower molecular weight of 23kDa (Fig.4). Similar to the present observation, papain-like protease isolated from the latex of *Asclepias curassavica* L. is reported to have molecular weight of 23kDa as revealed by SDS PAGE and MALDI/ MS TOF (Liggieri *et al*., 2009).

Characterization of protease:-

The characterization of latex protease has been carried out with respect to their optimum temperature, optimum pH as well as thermal and pH stabilities. The optimum temperature for protease activity of latex was assayed at different temperature from 30 to 70^0C using casein as the substrate at pH 7.0. The protease activity was optimum at the temperature of 50^0C, activity gradually declined at higher and lower temperature (Fig.5). Temperature is an important factor that determines the enzyme activity of purified protein and influences the three dimensional structure of proteins, it may be disrupted at extreme temperatures. The result indicated that the enzyme was completely stable at 50^0C for 120 minutes (Fig.6).

From the latex of *Ervatamia coronaria*, a new well characterized low molecular weight (27.60kDa) cysteine protease i.e. ervatamin A had optimum activity at 50^0C temperature (Nallamsetty *et al*., 2003). A new plant protease, named Asclepian C I of *Asclepias curassavica* latex had been characterized, optimum activity is achieved at 50^0C. (Liggieri, 2004). Relatively similar result of protease having optimum temperature at 52^0C was isolated from the green fruit of *Phytolacca americana* (Uchikoba *et al.*, 1998). The protease from the latex of *Synadenium granti* exhibited the optimum temperature at 60^0C. *Plumeria rubra* showed an optimum temperature at 55^0C with casein as the substrate (Indranil *et al.*, 2011).

Optimum pH and Stability of Latex Protease:-

The optimum pH for protease activity of partially purified latex protease was at pH 7.0 (Fig.7). In order to understand the pH stability of protease was studied for two hours of 40^0C at pH 7 and pH 10. The enzyme was stable at pH 7.0 (Fig.8). There are many reports on protease with activity in the neutral range. A proteolytic enzyme was extracted from the latex of *Ficus hispida* was having optimum pH 7.0 (Dutta *et al*., 1999).

A latex serine protease of *Euphorbia pulcherrima* had molecular mass about 74 kDa, and optimum pH 7.0 (Lynn *et al.*, 1984). A neutral, high molecular weight protease (64 kDa), named raphanin isolated in a homogeneous state from leaves of *Raphanus sativus* (Radish) showed optimum activity at pH 7.0 (Sayed, 2001). Protease isolated from the flesh of *Lycopersicon esculentum*, showed optimum activity at pH 7.0 (Karim *et al*., 1999). A low molecular weight (25 kDa) alkaline serine protease of *Holarrhena antidysentrica* seeds had optimum activity at pH 7.5 (Khan, 2008). The isolated protease from the latex of *Calotropis procera* was found to have optimum at pH 8.0 (Sarot *et al*., 2010).

Effect of Metal ions:-

The influence of different metal ions on the proteolytic activity of the enzyme was analysed. It showed that $MnCl_2$ and Hg had the maximum inhibition towards enzyme activity and $MgSO_4$ and Ca showed the least inhibition (Fig.9). Pena *et al.*, (2006) demonstrated the influence of different metal ions like Pb, Al, Ni, Zn, Cu, Co, Cr, Hg and Cd on the protease activity of sunflower cotyledons, of which Pb, Al, and Ni treatments showed decrease in the enzyme activity. But Cd and Hg increased the enzyme activity. Pb, Al and Ni toxicity may be due to the inhibition

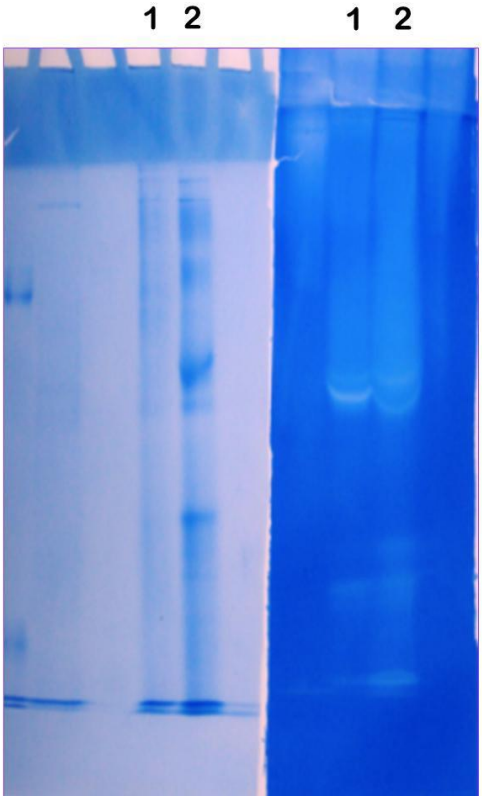
of protease activity, which might be causing the carbonylation of proteins. On the other hand Cd and Hg induce oxidative stress and enhance proteolytic activity.

Effect of Inhibitors on Enzyme Activity:-

The enzyme activity was measured after pre-incubating the enzymes with 10 mM of EDTA and PMSF. The results showed that nearly 70% reduction in protease activity could be observed in EDTA, while PMSF failed to inhibit enzyme activity (Table 2). The reduced enzymatic activity in the presence of EDTA is suggestive that *H. ada-kodien* could be a metallo protease. The effect of various inhibitors like PMSF, iodoacetamide, and EDTA on the protease activity of the seeds of *Holarrhena antidysentrica* was determined by Subhan *et al*., 2008. It showed that 90% of the protease activity was inhibited by 2 mM PMSF suggesting the protease is of serine type.

Substrate Specificity:-

The specificity of the enzyme for different substrates such as casein egg albumin and gelatin were evaluated. The enzyme showed much activity when casein was used as the substrate (Fig.10). Negligible activity was shown when egg albumin was used as the substrate. According to Fahmy *et al*., 2004 a cysteine protease isolated from wheat extract (*Triticum aestivum*) exhibit highest activity towards azocasein than other proteinaceous substrates. Similarly a low molecular weight (25KDa) alkaline serine protease of *Holarrhena antidysentrica* seeds showed optimum activity when 1% casein is used as the substrate (Khan *et al*., 2008). The maximum activity was shown by the isolated amino peptidases of Oat leaves, when the substrates were either azocasien or rubisco (Casano *et al*., 1989). This shows that different plant proteases shows great variation in the preferences of substrates



Fig, 1 Native PAGE and Zymogram analysis of leaf proease.
1. Crude enzyme 2. Ammonium sulphate fraction

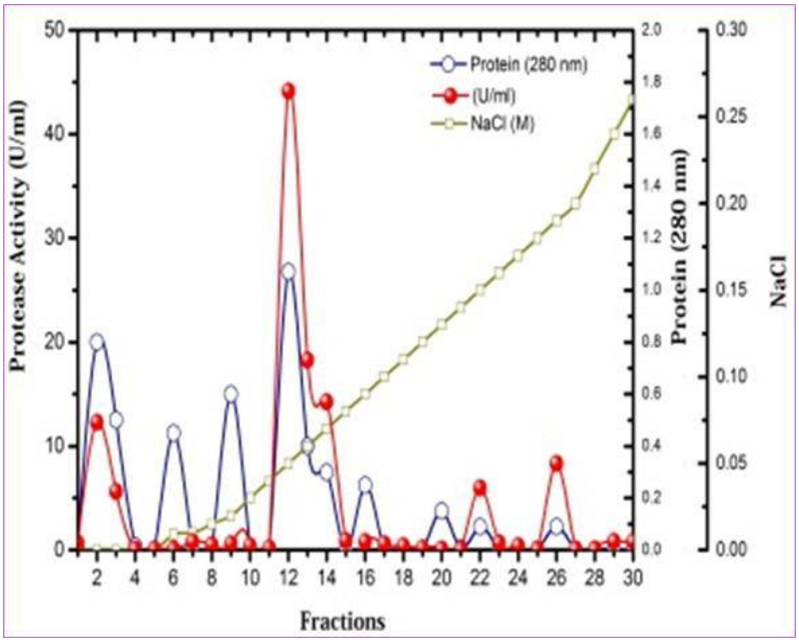


Fig.2 Cation exchange chromatography using CM cellulose of the leaf extract.

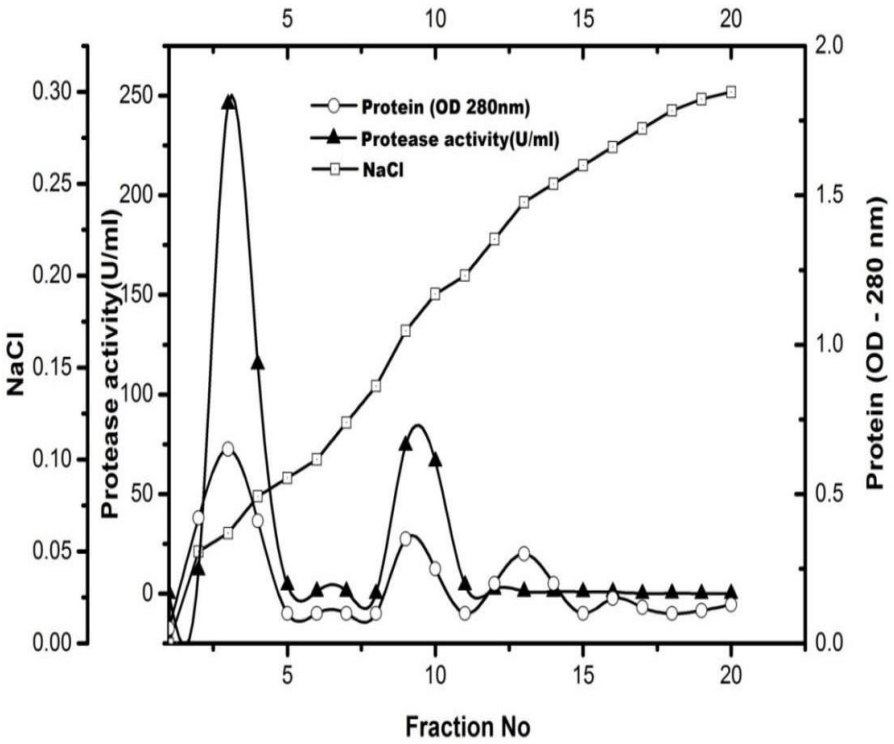


Fig .3 Cation exchange column chromatography of latex protease using CM Cellulose.

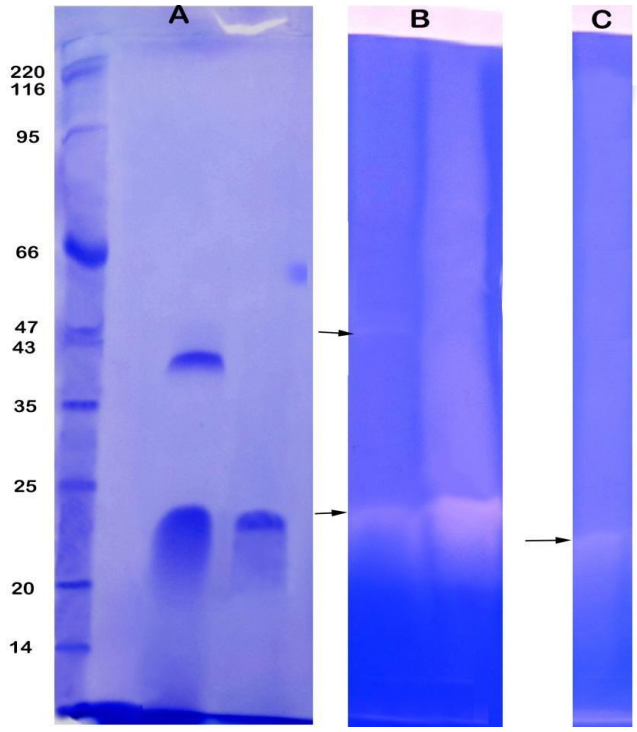


Fig. 4

A. SDS PAGE of latex protease showing CM cellulose Fraction I and DEAE Fraction I
B. Zymogram of the latex protease lane 1 CM cellulose fraction Lane 2 DEAE fraction I
C. Zymogram analysis of leaf protease fraction II

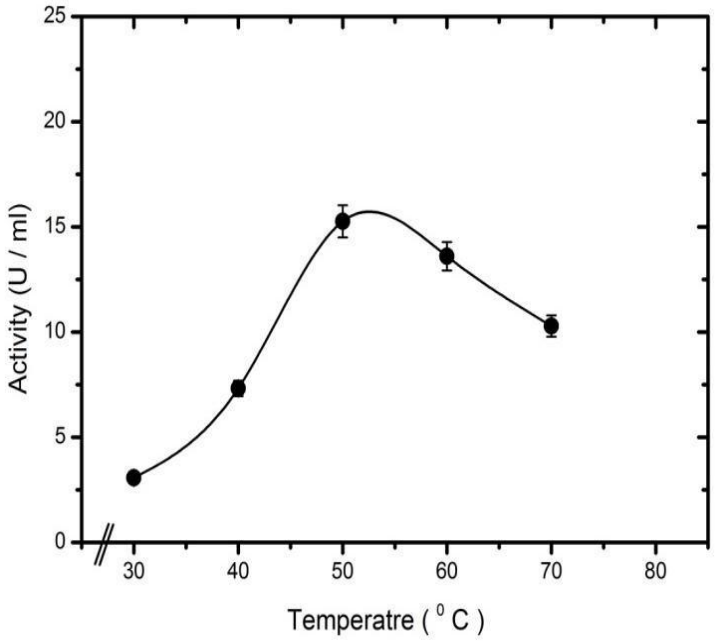


Fig .5 Optimum temperature of latex protease.

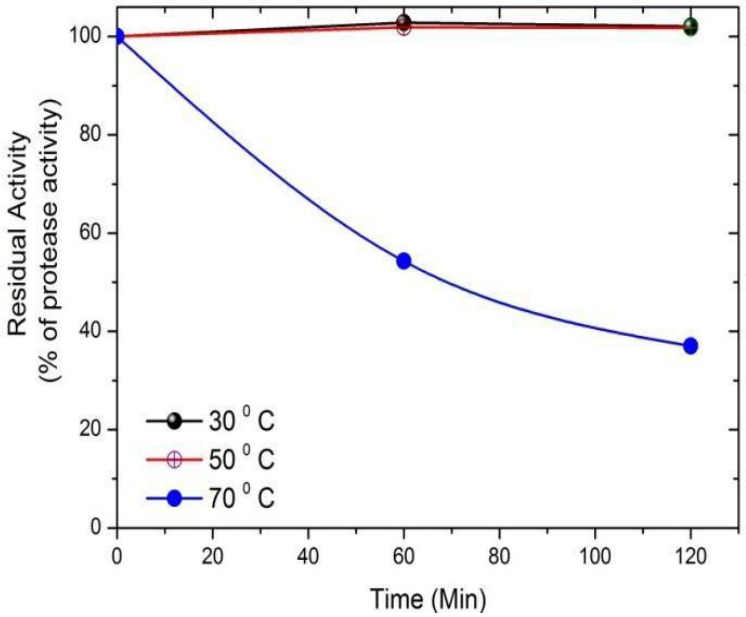


Fig.6 Thermal stability of latex protease.

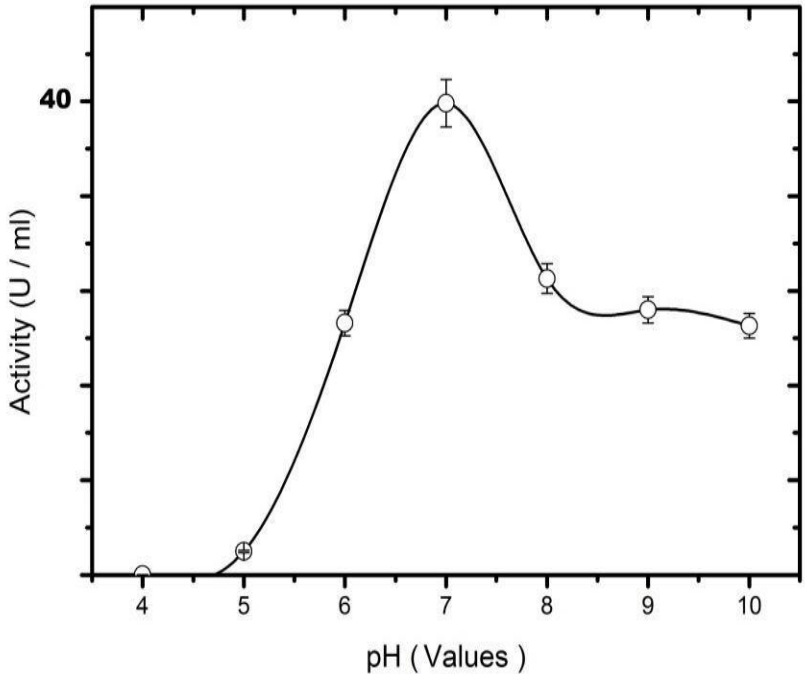


Fig.7 Optimum pH of major latex protease.

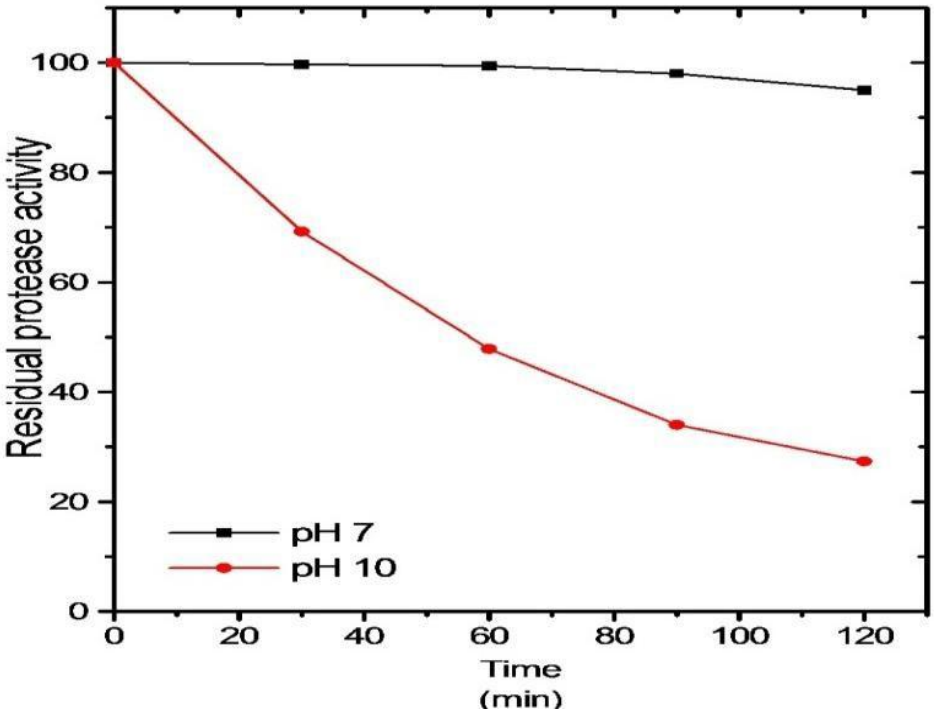


Fig. 8 pH stability of latex protease.

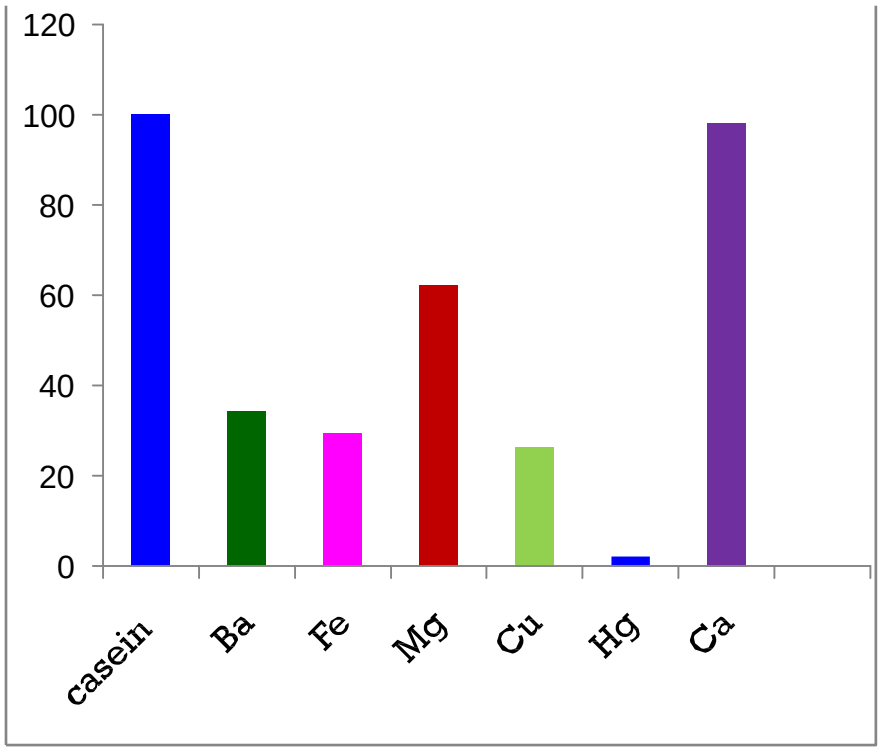


Fig .9 Effect of metal ions on latex protease activity.

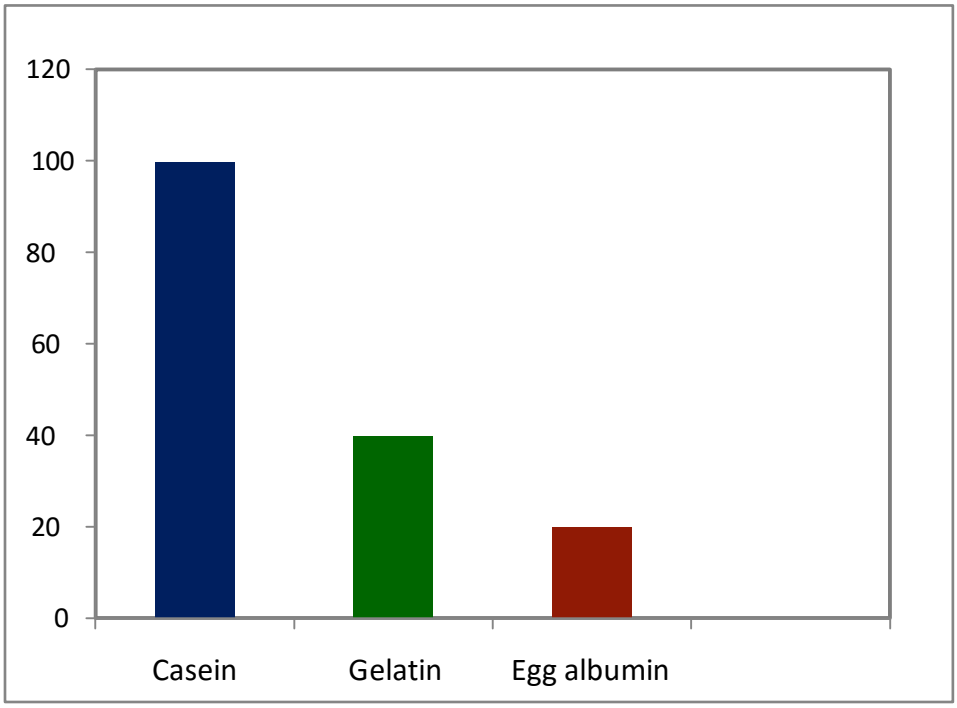


Fig.10 Effect of different substrates on latex protease.

1. Table1: Summary of Partial purification data of latex:-

Sample	Vol (ml)	Total activity	Total protein	Specific activity	Recovery	Purification fold
Crude Enzyme	30	6283.8	27.0	232.73	100%	1
40-70% $(NH_4)_2SO_4$	12	4125.6	4.8	859.5	65.65%	3.69
CM Cellulose	6	2190	2.16	1016.5	34.85%	4.36
DEAE Cellulose	4	1670	1.12	1491.1	26.57%	6.40

1. Table .2 Effect of inhibitors on latex protease:-

INHIBITOR	ACTIVITY(%)
PMSF	99.8
EDTA	31.2
CONTROL	100

Conclusion:-

Proteases constitute the largest and important group of hydrolytic enzymes of plant origin. Latex possesses the highest percentage of several hydrolytic enzymes of which protease are the most important. *H. ada-kodien* has been traditionally used in different ailments. The latex of the plant is particularly used in the treatment of wounds but are not characterised so far with respect to the hydrolytic enzymes present in them. The partial purification of the latex followed by zymogram analysis revealed the latex to possess two different proteases. The major protease purified to near homogeneity had optimum pH at 7.0 and temperature optimum at 50^0C with comparable thermal and pH stability. The molecular weight is 24kDa. The characteristics studied showed that the protease in the latex of this plant has unique features and it possess very high proteolytic activity in comparison to leaf protease. Such proteases are having important applications in healing of wounds, ulcers and blisters. This is the first report on the proteases present in *H. ada-kodien.*

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