ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF ALKALINE SERINE PROTEASE FROM SEEDS OF CUCUMIS MELO VAR AGRESTIS

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Abstract

The present study shows the isolation, partial purification and characterization of alkaline protease from the seeds of Cucumis melo var agrestis, by a four step purification process. Its molecular weight was estimated to be 54KDa. Enzyme showed maximum activity at p^H 9.0 and optimum temperature at40^{o}Cwith casein as substrate. The enzyme exhibited homogeneity as attested by a single protein band on both native PAGE and SDS PAGE. It is a monomeric enzyme and nonglycoprotein in nature. The k_m value of enzyme for casein as determined by double reciprocal plot was 2.5 mg/ml. It was strongly inhibited by PMSF but not by EDTA. The results indicate that the alkaline protease is a serine protease, similar to cucumisin from sarcocarp of melon fruit.

Keywords: *Cucumis melo var agrestis, purification, characterization, monomeric, homogeneity, serine protease, cucumisin.*

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1. INTRODUCTION

Proteases are group of enzymes which catalyse the cleavage of peptide bonds. They are also generally referred to proteolytic enzymes or proteinases. They are widely distributed in plants animals and microorganisms. Many proteases have been isolated from latex , fruits and seeds (Boller,1986). They have importance in both commercial and physiological fields. Nearly half of commercially available proteases are frequently used in food processing, tenderization of meat, brewing, cheese elaboration, bread manufacturing, leather and textile industry (Kaneda *et. al.;* 1997).

Serine proteinases are class of proteases which come under endopeptidases, which have been purified from a number of plants in the past 30years (Antao & Malcata 2005). In general these enzymes are active at 40ºC and above, however, optimum pH vary greatly, from acidic to neutral and alkaline environments and show broad substrate specificity for proteins. Most serine proteases are inhibited by a class of specific inhibitors, such as DFP (diisopropyl-fluorophosphate) and PMSF (phenylmethylsulfonylfluoride). Alkaline proteases are ubiquitous in nature, found in animals, plants and microorganisms. Proteases which are active above p^H 7 are called as Alkaline proteases. Alkaline proteases are involved in the mobilization of seed storage proteins in *Dolichous lab lab* during germination (Padmakar *et al.*, 2005).Proteases are involved in the processing of pre proteins and maintenance of intracellular protease levels in seeds of horse gram (Jinka *et al.,* 2009). In barley, hordolisin , a subtilisn like serine protease is not involved in degradation of hordein storage protein of barley (Nina Trep *et al.,* 2000). Another protease SEP-1 a subtilisin like serine endopeitidase was isolated from germinated seeds of *Hordeum vulgare* L. cv. Morex. It was reported to be not involved in storage degradation (Debora Fontanini and Berne L. Jones, 2002).The proteases isolated from germinating finger millet were stable at pH 5 (Vidyavathi *et al.,* 1983).

The most popular plant protease Cucumisins are a family of subtilisin like endopeptidases found in Cucurbitaceae (Kaneda and Tominaga, 1975; Arima *et al* ., 2000a; Uchikoba *et al.,* 1995). Kaneda and Tominaga (1975) purified cucumisin for the first time from the sarcocarp of melons (*Cucumis melo*). Several cucumisin-like enzymes have also been isolated from other members of Cucurbitaceae family and from rice, maize, wheat and barley (Antao and Malcata 2005). Most of these enzymes show molecular mass in the range 50-80 kDa and exhibit broad specificity, preferring hydrophobic amino acid residues in the PI position (Arima et al., 2000b). Mature cucumisin (54 kDa) shows optimum pH in the range of 8-10 and is stable at 60ºC. Cucumisin is not affected by trypsin inhibitor from soybean (SBTI), Ovomucoid, cysteine proteinase inhibitors or EDTA, but is strongly inhibited by chloromethyl ketone derivatives of peptide substrates, PMSF and DFP (Kaneda and

Tominaga 1975; Uchikoba *et al.,* 1995) indicating association with serine protease family.

2. MATERIALS AND METHODS

The plant species of the present study were collected from Visakhapatnam district. The fruit wild melon *Cucumis melo* var *agrestis* was collected from local farms of Visakhapatnam. The fruit can be even obtained from farm or local market .The seeds were removed washed, cleaned and shade dried.

2.1 Preparation of Seed Extract

One gram of seeds were weighed and ground in a chilled mortar and pestle with 10ml buffer solution, Tris-HCl (0.1M) pH7.2. The extract was centrifuged at 4^0C for 20 minutes at 10,000rpm and the supernatant obtained was used as the enzyme source to assay the activities of proteases.

2.2 Assay of Alkaline Protease (EC 3.4.21.25):

Protease activity was assayed by the procedure of Kakade *et al.,* (1969) using casein as substrate. The reaction mixture contained 2ml of casein in (0.1M) phosphate buffer, pH 7.6 and 0.5ml of enzyme extract. After 20 minutes of incubation, the reaction was arrested by adding 5ml of 5% TCA (w/v). The mixture was mixed well and allowed to stand for some time and the mixture was filtered through Whattman filter paper 1.

2.3 Optimum pH

The activity of alkaline protease was determined by using different buffers in pH range of 3-12 by the method described earlier. Buffers used were sodium acetate, 0.1M pH (3-4), sodium citrate, 0.1M pH (5-6), phosphate buffer, 0.1M pH (7-8) and Glycine-NaOH, 0.1MpH (9-12).

2.4 Optimum Temperature

Optimum temperature was determined for alkaline protease by measuring its activiy in temperature range 20º-70ºC using the activity assay procedure.

2.5 Stability of Alkaline Protease

2.5. pH

In order to determine the pH stability of alkaline protease, the enzyme (2ml in 0.1M buffer) was kept at 5ºC for 12h, aliquots were then taken and assayed for enzyme activity. Buffers used were sodium acetate, 0.1M pH (3-4), sodium citrate, 0.1M pH (5-6), phosphate buffer, 0.1M pH (7-8) and Glycine-NaOH, 0.1M pH (9-12).

2.5.2 Temperature

In order to determine the temperature stability of alkaline protease, the enzyme (2ml in 0.1M buffer) was incubated for 30 min at different temperatures (20-100ºC). After cooling for 2h, enzyme activities were determined from the aliquots taken by the method described earlier.

2.6 Effect of Substrate on Alkaline Protease Activity

Alkaline protease activity was determined at different concentrations of casein. Km value was calculated using Lineweaver-Burkplot.

2.7 Determination of Proteins

Protein content of the enzyme extract was determined by the method of Lowry *et al.,* (1951) using crystalline BSA as standard.

3. PURIFICATION OF PROTEASE FROM SEEDS OF CUCUMIS MELO VAR.AGRESTIS.

3.1Partial Purification with Acetone

The seeds of *Cucumis melo* var.*agrestis* were collected and cleaned and air dried. The seeds were homogenized in cold with extraction buffer (4ºC) and centrifuged at 10000 rpm and the supernatant was taken .To this mixture two volumes of prechilled acetone was added and the entire mixture was well stirred and kept aside for 30 minutes and then centrifuged for 10 to 15 minutes at 5000 rpm. The pellet is resuspended in the extraction buffer.

3.2 Ammonium Sulfate Fractionation:

To the fraction from acetone purification, solid ammonium sulfate was added slowly to 60% saturation. It was stirred for some time and allowed to stand for overnight. The precipitated protein was separated by centrifugation at 5000 rpm for 20 to 30min. The pellet was suspended in 10 ml extraction buffer and was dialysed against the phosphate buffer for nearly 8 to 10 hours.

3.3 DEAE-Cellulose Ion - Exchange Chromatography:

The dialyzed protein sample was subjected to Ion-exchange chromatography on DEAE-Cellulose column (20×1.5cm). The column was washed with 100 ml of the initial buffer, 0.1M Tris - HCl buffer pH 7.2. There after gradient elution was carried out using 90 ml of 0.1 M Sodium chloride in the same buffer in the reservoir. Similarly 0.2, 0.3, 0.4 and 0.5 M Sodium chloride in the same buffer, gradient elution was carried out. Absorbance of the fractions at 280 nm was measured in spectrophotometer. Fractions containing enzyme activity were pooled.

3.4 Gel Chromatography on Sephadex G-100:

The pooled samples were placed in a dialysis sac and concentrated in sucrose crystals and the further subjected to dialysis. Then the sample was placed on the top of SephadexG-100 column then was eluted with 0.1M Tris-HCl buffer, which was monitored through spectrophotometrically at 280 nm.

3.5 Molecular Weight Determination by Gel Filtration on Sephadex G-200

Gel filtration of the alkaline protease on sephadex G-200 was carried out as described earlier . Alkaline protease was eluted out as a single protein with a corresponding activity. The enzyme obtained was pure and homogeneous. Calibrating proteins were also used for determining the molecular weight of the enzyme. The plot of Ve/Vo versus log molecular weight for these proteins was drawn and the molecular weight of the enzyme was calculated.

3.6 Polyacrylamide Gel Electrophoresis:

Polyacrylamide gel electrophoresis (PAGE) of the enzyme was performed by the method of Lammli (1970) with 10% polyacrylamide gel in the presence of Sodium Dodecyl Sulfate (SDS) and 2- mercaptoethanol (SDS-PAGE), with 12% polyacrylamide gels in the absence of SDS (native-PAGE). The protein in gels were stained with Coomassie Brilliant blue R -250 and silver staining method (Blum *et. al.;*1987) and specific staining is also done(Heussen andDowdle,1980).

3.7 Effect of Denaturants on Alkaline Protease Activity:

In order to determine the effect of denaturants on alkaline protease activity, the enzyme solution was preincubated for 6h with the denaturants (urea, guanidine hydrochloride and SDS, each 0.1% at 5^0C and then assayed for enzyme activity by the methods described earlier.

3.8 Effect of Metals on Alkaline Protease Activity:

In order to determine the effect of metal ions on alkaline protease activity, the enzyme solution was pre incubated for 30 min with metal chlorides (final concentration 0.01M) at 5^0C and assayed for the protease activity.

3.9 Effect of Inhibitors on Alkaline Protease Activity:

In order to determine the effect inhibitors on alkaline protease activity, the enzyme solution was pre incubated for 30 min with inhibitors (final concentration 0.01M) at 5^0C and assayed for the protease activity.

3.10 Substrate Specificity:

Substrate specificity of protease was determined by incubating enzyme with various substrates at 37^0C in 0.1M Tris –HCl buffer, pH 7.2 for 30 min. After incubation, TCA (10%) was added and protease in the filtrate was estimated quickly and at low temperatures by the method described earlier . Each substrate had its own blank sample consisting of buffer solution instead of enzyme.

4. RESULTS

4.1 Purification of Alkaline Protease

Preliminary studies revealed that the alkaline protease from melon seeds was stable at pH 7-10 and at temperature 40^0C. All the purification steps were carried out under controlled conditions i.e. pH 9 and temperature 4^0C.

Step 1: Extraction
Crude enzyme extract was prepared from 20g of melon seeds by grinding the seeds with acid washed sand in a mortar and pestle with ice cold 200ml of 0.1M Tris-HCl buffer, pH7.2 at 4^0 C using seed buffer ratio 1:10. The extract was centrifuged at 10,000 rpm for 20 min at 4^0 C and the supernatant was collected and then subjected to ammonium sulphate fractionation.

Step 2: Ammonuim sulphate fractionation
To the enzyme solution (200ml) from step 1, solid ammonium sulphate was added gradually with constant stirring at 4^0 C to obtain 60% saturation. The mixture was allowed to stand overnight at 5^0 C. The precipitate was collected by centrifugation at 8,000 rpm for 20min at 4^0 C, dissolved in 50ml of 0.1M Tris-HCl buffer pH 7.2 and dialyzed against the same buffer. The enzyme solution was concentrated and subjected to chromatography on DEAE – cellulose.

Step 3: DEAE-cellulose Ion exchange Chromatography
The dialyzed and concentrated ammonium sulphate fraction (480mg) was loaded on DEAE-cellulose (20×1.5) previously equilibrated with 0.1M Tris-HCl buffer, pH7.2. The unbound proteins were eluted with 100ml of the equilibrating buffer and the bound material was then eluted with 90ml of the same buffer containing 0.1M NaCl and 65ml of the same buffer containing 0.2M NaCl and 75ml of same buffer containing 0.3ml NaCl. Fractions of 5ml were collected at a flow rate of 30ml/h. The fractions were assayed for protein by measuring their absorbance at 280nm as well as for the enzyme activity. The active fractions were pooled, dialyzed at 4^0 C and lyophilized. The elution profile of DEAE-cellulose chromatography for alkaline protease is shown in figure1.

The enzyme activity was, however, associated with a protein eluted from the column with buffer containing 0.2M NaCl.

Eluents from 0.4M and 0.5M NaCl did not show any protease activity (results not shown). About 70% of the protein present in ammonium sulphate fraction was unbound to DEAE-cellulose and appeared in the fractions 5-22.

Step 4: Gel filtration on Sephadex G-100

The sample from step 3 (145mg) was dissolved in 0.1M Tris-HCl buffer, pH 7.2 and was loaded on Sephdex G-100 column (20×1.5cm). The column was previously equilibrated with 0.1M Tris-HCl buffer, pH 7.2 and eluted with the same buffer. Fractions of 2ml were collected at a flow rate of 12ml/h and the protein was monitored by measuring the absorbance at 280nm. The enzyme activities of the fractions were assayed using casein as the substrate. The elution profile of gel permeation chromatography is shown in figure 2. The fractions (31-43) containing the enzyme activities were pooled, dialyzed against distilled water at 4^0 C and then lyophilized.

Recoveries and relative purification at each step for a typical purification from 20g of melon seeds is shown in table - 1. By this procedure, about 120 mg of the enzyme was obtained and the final yield of enzyme was about 29.8%.

Table 1: Summary of purification of alkaline protease from 20g of melon seeds

Preparation	Volume ml	Total protein mg	Total activity units *	Specific Activity units/mg protein	Yield %	Fold purification
Crude extract	200	1220	2,72,200	223.11	100	1
60 % Ammonium sulphate	30	480	1,20,424	250.80	44.2	1.12
DEAE-Cellulose	20	145	85,420	589.27	31.38	2.63
Sephadex G-100	15	120	81,215	676.8	29.8	3.03

Yield and fold purification were calculated on the basis of total activity units and specific activity units respectively.

* µmoles of tyrosine liberated under experimental conditions

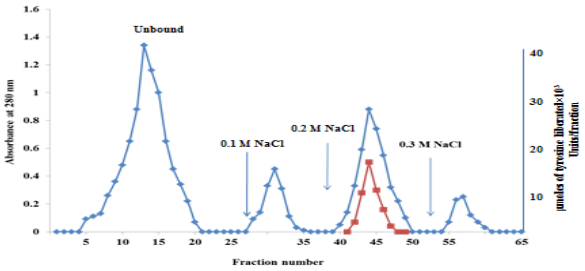


Fig 1: DEAE-cellulose Ion exchange chromatography of alkaline protease from melon seeds

480 mg of protein from Ammonium sulfate fraction (0-60%) was loaded on to the column (20×1.5 cm) in 0.1M Tris-HCl buffer, pH 7.2 and the adsorbed proteins were eluted with 0.1M, 0.2M and 0.3M NaCl in the buffer. Fractions were collected at a flow rate of 30ml/h.

Protein was monitored through absorbance at 280nm (♦----------♦)
Protease activity (■----------■)

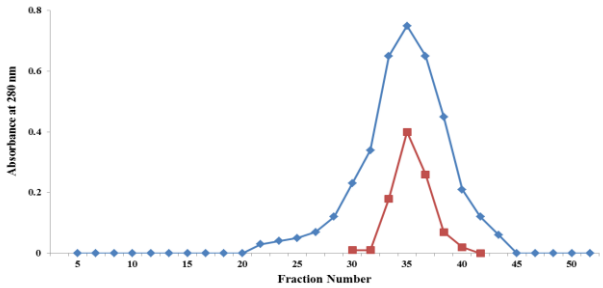


Fig 2: Gel filtration on Sephadex G-100 of alkaline protease from melon seeds

145mg of the lyophilized preparation was applied to the column (20×1.5 cm) in 0.1M Tris-HCl buffer, pH7.2 and eluted with the same buffer. Fractions, each 2ml, were collected at a flow rate of 12m/h.

Protein was monitored through absorbance at 280nm (♦----------♦) Protease activity (■----------■

4.2 Homogeneity and Physico–Chemical Properties of Serine Protease

4.2.1 Polyacrylamide Gel Electrophoresis

PAGE was carried out under non-denaturing conditions using slab gels. A single protein band was obtained in 12% slab gel. Specific stain of protease also showed a single band corresponding to the coomassie blue and silver stainable bands (figure 3). The enzyme was not positive towards PAS stain suggesting the absence of carbohydrate moieties in the

protein. A single protein band obtained with protease suggesting that the preparation was pure (results not shown).

4.2.2 Gel Filtration on Sephadex G-200

Gel filtration of the alkaline protease on Sephadex G-200 was carried out as described under methods. Alkaline protease was eluted out as a single protein with corresponding activity peak. The results obtained clearly indicate that the enzyme preparation was pure and homogenous (figure 4). Calibrating proteins were also used for determining the molecular weight of the enzyme. The plot Ve/Vo versus log molecular weight for these proteins was shown. The molecular weight of the enzyme, as calculated from the plot was 54 kDa (figure 5).

4.2.3 SDS-PAGE

When subjected to SDS-PAGE, alkaline protease showed single band on 12% slab gels indicating the monomeric nature of the enzyme s(figure 6). From the plot of distance migrated in cm versus log molecular weight for standard proteins. The molecular weight of the alkaline protease was determined to be 54.5 kDa (figure 7).

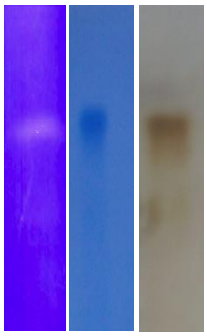


Fig 3: Polyacrylamide gel electrophoretic pattern at pH 8.3 in 12% slab gel

1. Coomassie blue staining
2.Silver staining
3. Specific staining

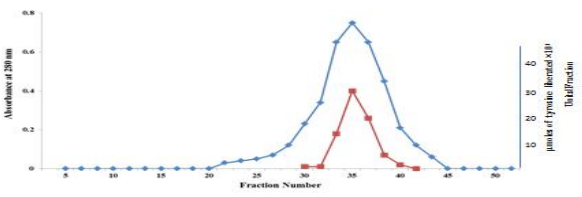


Fig 4: Gel filtration of alkaline protease on Sephadex G-200.

25mg of purified protease in 0.1M Tris-HCl buffer, pH 7.2 was loaded on Sephadex G-200 column (20×1.5 cm). Elution was done with the same buffer and fractions of 2ml were collected at a flow rate of 12ml/h.

Protein was monitored through absorbance at 280nm (♦----------♦)

Protease activity (■----------■)

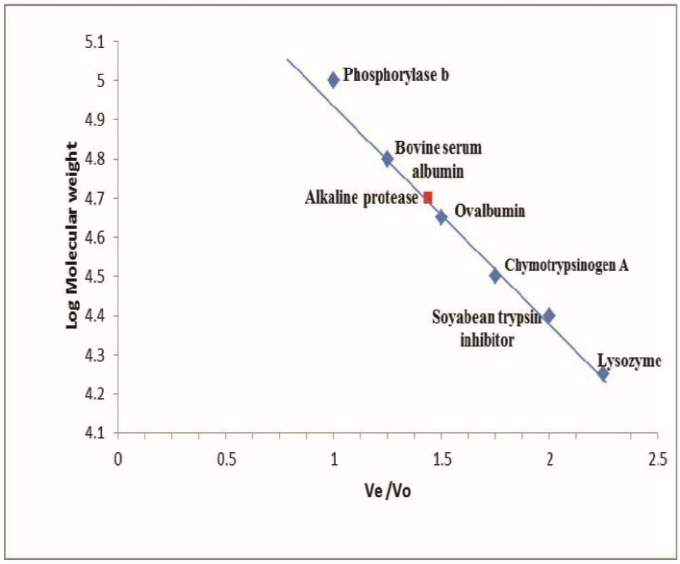


Fig 5: Molecular weight determination of protease by gel filtration on Sephadex G-200.

Plot of elution volume against log molecular weight of standard proteins(■) and Alkaline protease(■)

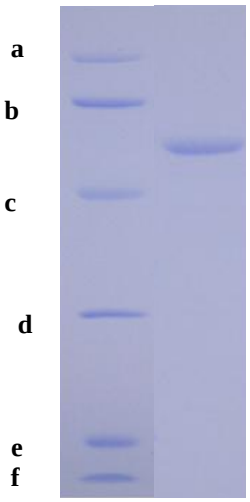


Fig 6: Molecular weight determination of Alkaline protease by SDS-PAGE

1. Standard protein markers
a) Phosphorylase b, 97 kDa
b) Bovine serum albumin, 67 kDa
c) Ovalbumin, 44 kDa
d) Chymotrypsinogen A, 25 kDa
e) Soybean trypsin inhibitor, 20.1 kDa
f) Lysozyme, 14 kDa
2. Purified alkaline protease

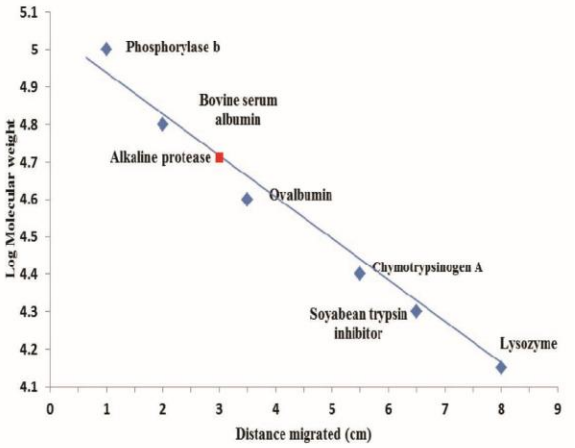


Fig 7: Molecular weight determination of alkaline protease by SDS-PAGE

Plot of distance migrated against log molecular weight of standard protein (■) and Alkaline protease(■).

4.3 Effect of pH and Temperature on Purified Alkaline Protease

The purified enzyme showed maximum activity at pH 9 (figure 8) and at temperature 40^0C (figure 9). The results are in agreement with those obtained with the crude preparation. The stability of the purified alkaline protease was tested at different pH and temperatures and the results obtained are shown in figures 10 & 11. The enzyme was found to be more stable around pH 8-10.

The alkaline protease was stable at 40^0C and at 60^0C there was a loss of about 33%of its activity .The enzyme was completely inactive when exposed to 90^0C for 30 min (result not shown).

4.4 Effect of Substrate Concentration on Alkaline Protease Activity

Alkaline protease activity was determined varying its substrate concentration from 0.25-1.5%. The results obtained are presented in the form of Line weaver-Burk plot. The Km value for alkaline protease was determined to be 2.5mg/ml from the reciprocal plot (figure 12).

4.5 Effect of Different Substrates on Alkaline Protease Activity

A number of compounds were tested as substrates for alkaline proteases. Casein, Ovalbumin, Gelatin, BSA were used as substrates in 1% concentration. Casein showed 100% relative activity, BSA showed 51%, Gelatin exhibited 33.5% and Ovalbumin showed 24.7% of relative activity (Table 2).
Stability of alkaline protease towards denaturants and inhibitors

Alkaline protease activity was decreased by 50% when it was exposed to 5M Urea and 5M SDS seperately. With 2mM β-Mercaptoethanol it showed 30% inhibition and 5mM EDTA exhibited 20% inhibition. Alkaline protease activity was inhibited by 95% by treating with 5mM PMSF.

By the above result it is concluded that the alkaline protease is a serine protease as it is completely inhibited by PMSF (Table 3).

4.6 Effect of Metals on Alkaline Protease Activity

The effect of various metal ions on alkaline protease activity at pH 9 is shown in the Table 4. None of the metals were effective either in activating or inhibiting the enzyme.

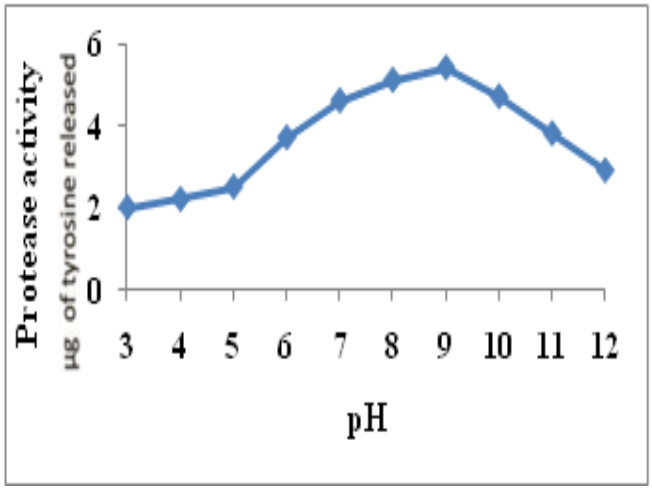


Fig 8: Optimum pH of purified alkaline protease

Buffers used were sodium acetate, 0.1M pH (3-4), sodium citrate, 0.1M pH (5-6), Tris-HCl, 0.1M pH (7-8), Glycine-NaOH, 0.1MpH (9-12)

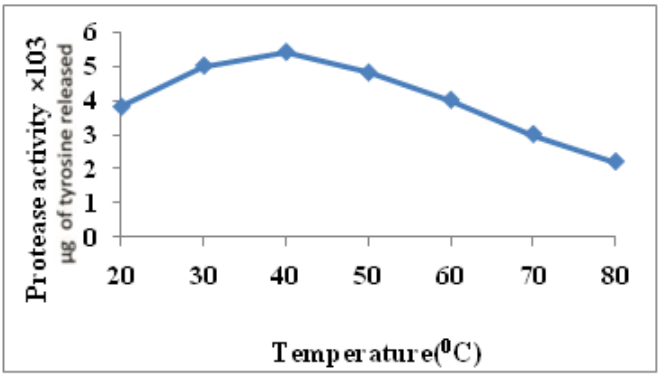


Fig 9: Optimum temperature of purified alkaline protease

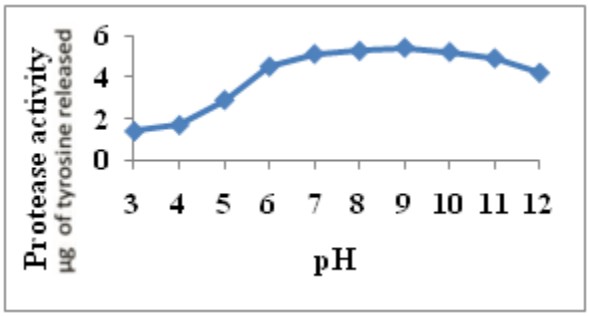


Fig 10: Stability of the purified Alkaline protease at different pH

Buffers used were sodium acetate, 0.1M pH (3-4), sodium citrate, 0.1M pH (5-6), phosphate buffer, 0.1M pH (7-8), Tris-HCl, 0.1MpH (9-12).

Figure 11: Stability of the purified alkaline protease at different temperatures

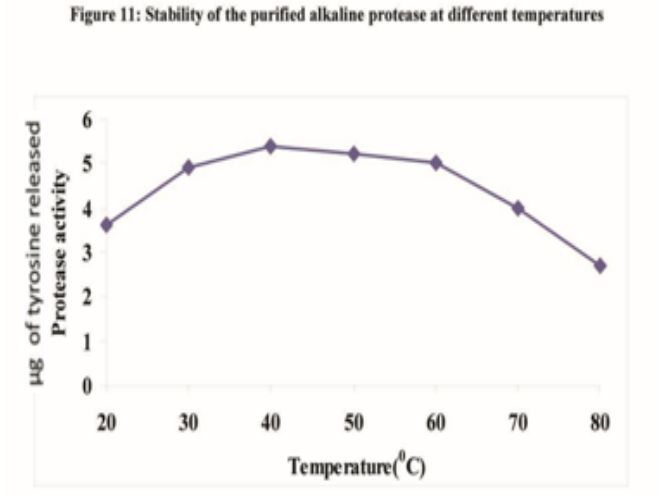
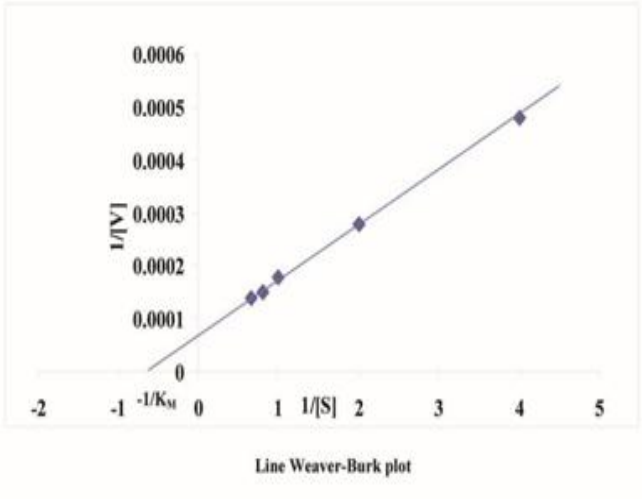


Figure 12: Effect of substrate concentration on alkaline protease activity



Line Weaver-Burk plot

Table-2: Effect of different substrates on alkaline protease activity

Substrate	Relative activity (%)
Casein	100
BSA	51
Gelatin	33.5
Ovalbumin	24.7

Table-3: Stability of alkaline protease towards denaturants and inhibitors

Denaturants and Inhibitors	Relative activity (%)
Control	100
PMSF(5mM)	5
Urea(5mM)	50
SDS(5mM)	47
β-Mercaptoethanol(2mM)	91
EDTA(5mM)	93

Table 4: Effect of metals on alkaline protease activity

Metals (5mM	Relative activity (%)
Control	100
$ZnCl_2$	97
$MgCl_2$	93
$CaCl_2$	98
$LiCl_2$	94
$CoCl_2$	96
KCl	93
NaCl	91

5. DISCUSSION

Alkaline protease has been obtained from the seeds of *Cucumis melo* var *agrestis* in an apparently homogeneous form. The enzyme has been isolated and purified, following ammonium sulfate (60%) fractionation, DEAE- Cellulose ion exchange chromatography and Sephadex G-100 gel filtrati on. Ammonium sulfate (60%) saturation removed 61% of unwanted proteins and gave a yield of 44% of the enzyme. The enzyme in this fraction was adsorbed on to DEAE cellulose column and was eluted at 0.2M NaCl concentration where 31% yield was achieved. The final step in the purification on sephadex G-100 provided a yield of 29% with 3.03 fold purification of the enzyme. The steps involved for the purification of alkaline protease from seeds of wild melon are similar to those used for purification of the serine protease from seeds of *Holarrhena antidysentrica* (Hidayatullah khan *et al.,* 2008).

Alkaline protease isolated by the purification procedure described appears to be homogenous by PAGE, SDS-PAGE and gel filtration on sephadex G-200. The presence of a single band on the gels also suggested that the preparation was pure and free from any isoenzymic forms. It was reported that serine protease from sarcocarp of snakegourd , sarcocarp of yellow snakegourd and coconut endosperm (Tominaga *et al.*,1986; Uchikoba *et al*.,1990 ;Usha *et al.*,2009) occur in isoenzymic forms. The molecular weight of serine protease was determined to be 54 kDa by SDS-PAGE and on sephadex G-200 gel filtration, the enzyme gave a molecular weight 54.4 kDa and was found to be similar to that of isolated from sarcocarp of *Trichosanthes cucumeroides*, sarcocarp of *Trichosanthes kirlowii*, sarcocarp of honey dew melon, fruit of *Maclura pomifera*, latex of *Cryptolepis buchanani*, latex of *Wrightia tinctoria*, latex of *Euphorbia milii* (Tominaga *et al.*,1986; Uchikoba *et al*.,1990; Kaneda *et al.*,1993; Rudenskaya *et al.*,1995; Pandey *et al.*,2006; Tomar *et al.*,2008; Yadav *et al*.,2008).

Purified alkaline protease was stable in the range of pH 7.0-11.0 with an optimum pH of 9.0 similar to the enzyme isolated from *Cucurbita ficifolia*, sarcocarp of honey dew melon plant, leaves of common beans, latex of *Cryptolepis buchanani*, coconut endosperm and latex of *Euphorbia neriifolia* (Emilia curotto *et al*., 1989; Kaneda *et al*.,1993; Pandey *et al.*,2006; Usha *et al.*,2009; Ravi Prakash *et al.*,2011).

The enzyme was stable in the temperature range of 40-60^{0}C. However it lost its activity completely at 90^{0}C when incubated for a period of 30 min possibly due to thermal denaturation. The optimal temperature, 40^{0}C of the enzyme was similar to the enzymes reported from seeds of soybean, sprouts of *Pleioblast hindsii*, roots of *Ipomea batatus*, seeds of *Nelumbo nucifera* and coconut endosperm (Shimpei *et al.*,1994; Arima *et al.,* 2000c; Tzeng *et al.*,2004; Ahamad *et al.*,2007; Usha *et al*.,2009).

The Km value of serine protease as determined from Lineweaver-Burk plot was found to be 2.5mg/ml using casein as substrate. Many of serine proteases from germinated seeds of barley, seeds of *Nelumbo nucifera* , latex of *Euphorbia neriifolia* , with their Km 2.6×10^2mM, 2.8×10^4mM, 1.39±8mM respectively are deduced using casein as substrate (Debora and Jones ,2002; Ahmad *et al*.,2007; Ravi Prakash *et al.*,2011).

Of the metals tested, no metal was either effective or ineffective in enhancing or decreasing the activity of serine protease. On most of the serine proteases metals are quite ineffective. The enzyme had lost 50% of activity with 5mM Urea and 60% activity with 5mM SDS for 30 min of incubation, while β-mercaptoethanol and EDTA exhibited no effect on the enzyme activity. Complete inhibition was observed when treated with 5mM PMSF. Serine proteases from *Cucurbita ficifolia*, seeds of soybean, germinated seeds of barley, common bean leaves, storage roots of *Ipomea batatus*, latex of *Cryptolepis buchanani*, seeds of *Cucumis trigonus* roxburghi, latex of *Ipomea carnea*, seeds of *Nelumbo nucifera*, seeds of *Solanum dubium* , coconut endosperm, latex of *Ficus benghalensis*, latex of *Ficus religiosa* (Emilia curotto *et al*., 1989; Shimpei *et al.*,1994; Debora Fontanini,2002; Tatjana popovic *et al*.,2002; Tzeng *et al.*,2004; Pandey *et al.*,2006; Mufti-Asifullah *et al.*,2006; Ashok kumar *et al.*,2007; Ahamad *et al.*,2007; Bassem jaoudia *et al.*,2008; Usha *et al.*,2009) also showed inhibition with PMSF.

6. CONCLUSIONS

Serine protease of *Cucumis melo* seeds is a monomeric protein with a molecular weight 54 kDa, active at pH9.0, temperature 40^{0}C preferentially using casein as substrate and significantly inhibited by PMSF.

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