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Isolation and characterization of Cysteine protease from leguminous cotyledons

Ranajit Kumar Shaha*[1], Nurul Azurin Binti Badruzaman[1], Asrul Afandi[1] and Shyam Sundar Shaha[2]

[1]*Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi, Bangladesh.*
[2]*Bangladesh Institute of Health Science Hospital, Department of Immunology, 125/1 Darussalam Mirpur, Dhaka-1216, Bangladesh.*
[*]*Department of Bio-industrial Technology, Faculty of Agro Based Industry, University Malaysia Kelantan, Jeli Campus, 17600 Jeli, Kelantan, Kelantan, Malaysia.*

Abstract: Proteolytic enzymes play central role in the biochemical mechanism of germination and intricately involved in many aspects of plant physiology and development. The present study was conducted on the compares studies the Cysteine proteases from four varieties of 62 hours germinated leguminous seeds: lentil, green gram, black gram and pea bean. We elaborated the easy procedure for isolation of protease from leguminous germinated seeds by using $(NH_4)_2SO_4$ precipitation followed by Gel-filtration and DEAE-cellulose chromatography from the 72h germinated cotyledons of lentil (*Lens esculenta*), green gram (*Vigna radiata*), black gram (*Vigna mungo*) and pea bean (*Phaseolus vulgaris*). This study revealed that the water-soluble protein concentration of crude extract ranged between 2.03 to 2.36mg/ml in which green gram was highest protein concentration (2.36mg/ml) and lentil accounted was the lowest concentration (2.03mg/ml). Cysteine proteases from all different leguminous seeds show very close monomer with a molecular mass of 29.5–30kDa were determined by SDS-PAGE. The enzyme activities were completely inhibited by pCMB, iodoacetate and DEPC indicating Cysteine and histidine residues at the active site. The enzyme is fairly stable towards pH and temperature. Cysteine protease has broad substrate specificity and stability in pH, temperature, therefore, this protease may turn out an efficient choice for the food, pharmaceutical, medicinal, and biotechnology industries.

Keywords: Protease activity, Characterization, Industrial purpose, Leguminous seeds.

1. Introduction

Proteolytic enzymes are multifunctional enzymes that have many physiological functions in plants and animals including germination, senescence, apoptosis, complement activation, inflammation process etc. and also having commercial importance in food, leather and textile industry. Proteases may be classified into two major groups; exopeptidase and endopeptidase based on their ability to degrade N- or C- terminal peptide bond. Endopeptidase, which have more potent industrial applications than exopeptidase, can be divided into four types (aspartic, cysteine, metallo and serine protease) on the basis of their active site and sensitivity to various inhibitors (Al-shehri and Mostafa, 2004; Sandhya *et al*., 2004)[1,2]. Proteases have been used in the processing of various foods such as calf rennet or chymosin in cheese making (Smith *et al*., 2005)[3]; preparation of soy sauce and other hydrolysates (Wang & Wang, 2004)[4] and used of papain for meat tenderization (Soper, 1998)[5]. In leather processing steps such as soaking, dehairing and baiting, the use of proteases as alternatives to chemical detergent powder has proved successful modification to remove protein and bloodstain from the skin and improving environmental pollution (Najafi and Deobagkar, 2005)[6]. Proteases are mainly obtained from microbial sources for industrial purpose. Proteolytic enzymes from the plant sources have received special attention because of their broad substrate specificity as well active in a wide range of pH, temperature, and in

*Corresponding author:
E-mail: ranajit57@yahoo.com; ranajit@umk.edu.my.

presence of organic compounds as well as other additives (Uhlig, H. 1998; Schaller, A. 2004)[7,8]. The mobilization of seed storage proteins represents one of the most important post-germination events in the growth and development of seedlings. Proteolytic enzymes play central role in the biochemical mechanism of germination and intricately involved in many aspects of plant physiology and development (Schaller, A. 2004; Muntz, K. *et al*., 1997)[8,9]. The legume seeds contain albumin and globulin storage proteins; act as amino acid reserves, which are mobilized to nourish the seedling. Globulins belong to the vicilin and legumin family these are degraded by endoproteases particularly cysteine proteinase. In horse gram, during germination it was observed the disappearance of high molecular weight (HMW) polypeptides of globulins and appearance of a new 25kDa polypeptide (Rotari, V. *et al*., 1997; Tiedemann, I. *et al*., 2001; He, F. *et al*., 2007; Jinka, R. *et al*., 2002; Ramakrishna, V. *et al*., 2006; Zakharov, A. *et al*., 2004)[10-15]. To study the mechanism of protein mobilization process, many have undertaken the task of purifying and characterizing a variety of proteases, some of which occur only transiently in germinating seeds (Schaller, A. 2004; Shutov, A.D. *et al*., 1987; Jones, B.L. 2005)[8,16,17]. Exploration for existence of valuable proteases as well as understanding the appropriate physiological role of such proteases in plants is still an open area of investigation. Studies on the pattern of mobilization of seed storage proteins and activation of proteolytic enzymes (endoprotease, carboxypeptidase and leucine aminopeptidase) were investigated in germinating horse gram seeds (Jinka, R. and Ramakrishna Rao, P. 2002; Rotari, V. 1997)[13,18]. The understanding of such facts influenced us to conduct this study. In the present investigation, we report the purification and biochemical characteristics of an endoprotease from the germinating seedlings of the lintel, green gram, black gram and pea bean. It is shown that this protease is a Cysteine protease (CP).

2. Methods and Materials

2.1 Materials

The present study was conducted in the laboratory of the Faculty of Agro Based Industry, University Malaysia Kelantan on four kinds of leguminous seeds collected from the Rajshahi University, Agriculture Centre, Bangladesh. These seeds were lentil (*Lens esculenta*), green gram (*Vigna radiata*), black gram (*Vigna mungo*) and pea bean (*Phaseolus vulgaris*). Sephadex G-100, LMW markers were procured from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine serum albumin (BSA), azocasein, was collected from E. Merck (Germany) and Trichloroacetic acid (TCA) from BDH (England). DEAE-cellulose, p-chloromercuribenzoate (pCMB), CM-sephacel, N-ethylmaleimide (NEM), iodoacetamide, soybean trypsin inhibitor were purchased from Sigma Chemical Company. The other chemicals and reagents used in this experiment were analytical grade and obtained from Faculty of Agro Based Industry, Jeli campus, UMK, without further purification.

2.1.1 Germination conditions

The seeds (lentil, green gram, black gram and pea bean), approximately five hundred grams (500g) in amount were surface sterilized with 0.1% $HgCl_2$ solution for 5 minutes, washed repeatedly with distilled water and soaked in 10 volumes of distilled water for 6 hours. The imbibed seeds were set to germinate at room temperature (28 - 30^0C) at 12 hours dark and 12 hours light cycle for 96 hours in sterile Petri dishes lined with four layers of Whatman-41 filter paper. Sterile conditions were maintained by including 15ppm of streptomycin sulphate in the water, which was used to wet the filter paper every day. The cotyledons harvested for three days (62 hours germination) were used for isolation of Cysteine protease (CP).

2.2 Methods

2.2.1 Isolation and preparation of protease enzyme (crude extract)

Each variety of 62h germinated Cotyledons (100g) were ground thoroughly in a mortar adding four volumes of chilled 50mM Tris-HCl buffer, pH 7.1, containing 2M NaCl for 2 hours. The extract was filtered through the gauge and the filtrates were centrifuged at 10000g for 10 min. at 4°C. The collected supernatant was used for the estimation of extracellular concentration of protein enzyme and further purifications. All separation procedures were carried out at 4°C unless otherwise stated.

2.2.2 Purification of Cysteine protease from germinating cotyledons

1. $(NH_4)_2SO_4$ precipitation

The crude enzyme extracts were prepared as described above from 100g of each different leguminous cotyledons (62h germinated) were subjected to ammonium sulphate precipitation. The collected supernatants were saturated with 65% solid $(NH_4)_2SO_4$ for overnight precipitation in a 4^0C refrigerator. After precipitation, they were centrifuged at 10,000g for 20 min. below 4^0C. The collected precipitated were dissolved in a minimal amount of extraction buffer and extensively dialyzed against 10mM Tris-HCl buffer, pH 7.2 at 4°C (change dialysis water frequently; 4 h change up to 12 h) and finally centrifuged at 5000rpm for 10 minutes and lyophilized for column chromatography. The lyophilized supernatant was used as a crude enzyme for the assay of specific activity of enzyme and characterization.

2. Protein measurement

Protein concentration was determined by the method of Lowry *et al.*, 1951[19] using bovine serum albumin (BSA) as standard protein. The amount of the soluble protein was calculated from the standard curve as mg of protein per ml of the test sample.

3. Assay of protease enzyme: Determination of catalytic activity of protease

Assay of protease activity was measured by using chromomeric substrate, azocasein, following the method described by Sarath *et al.*, (1989)[20] with slight modifications. 0.25ml of 1% azocasein (prepared in 20mM sodium acetate buffer, pH 5.5 containing 2mM β-ME) was mixed with 0.15ml of enzyme extract or 150µg of purified enzyme (1mg/ml) and incubated at 37°C for 90 minutes. The reaction was stopped by the addition of 1.2ml of 10% TCA and mixed thoroughly. For the control, the substrate was precipitated with 1.2ml 10% TCA before adding the enzyme solution and then treated as described above. The contents were allowed to stand for 15 min. and centrifuged for 5 min. at 3000rpm. 1.2ml of the supernatant was transferred to a tube containing 1.4ml of 1M NaOH, mixed and the absorbance was read at 440nm against the reagent blank. One unit of protease activity was defined as the amount of the enzyme required to produce an absorbance change of 1.0 in a 1cm cuvette under the conditions of the assay.

4. Germinating Time course study

5gm germinated cotyledons of lentil, green gram, black gram and pea bean, were collected at 24h, 40h, 50h, 60h, 72h, 96h, and 120h respectively and homogenized with 20ml of 10mM Tris-HCl buffer, pH 7.2. After filtration and centrifugation, the clear supernatant "crude extract" use to estimate protease activity. The crude extracted protease from different cotyledons and azocasein were taken as described earlier and incubated at 37°C for 1.5 hours. The maximum enzyme activity was observed at 60-62h germinated cotyledons (Fig. 1). So for our further research works, we use 62 hours germinated cotyledons only.

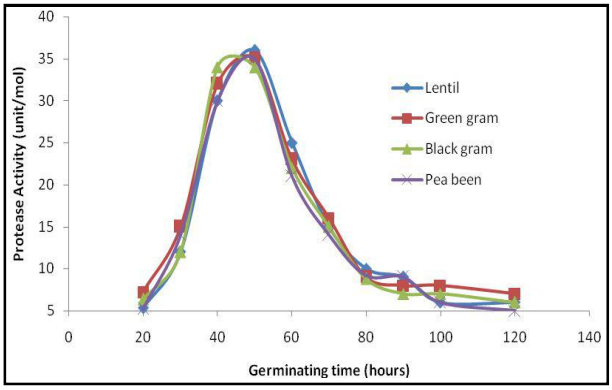

Fig. 1. Germinating time course studied of four different leguminous seeds at 25^{0}C.

2.2.2 Purification by Gel and Ion Exchange Chromatography

Among four varieties of leguminous seeds *viz* lentil, green gram, black gram and pea bean, only germinated lentil cotyledons enzyme purification steps by Gel and DEAE-cellulose are described and shown here (Fig. 2 and 3).

1. Gel filtration: [Lentil]

The lyophilized material was dissolved in 10ml of 50mM phosphate buffer, pH 7.2 and loaded into a Sephadex G-100 column (2.5 x 120cm) pre-equilibrated with 50mM phosphate buffer, pH 7.2, for 3 column volumes. The column was eluted with the same buffer at a flow rate of 1ml min^{-1}. The enzymatically active fractions (Fig. 2, fractions no. 13-20) were collected together, dialysis and lyophilized for further Ion exchange chromatography.

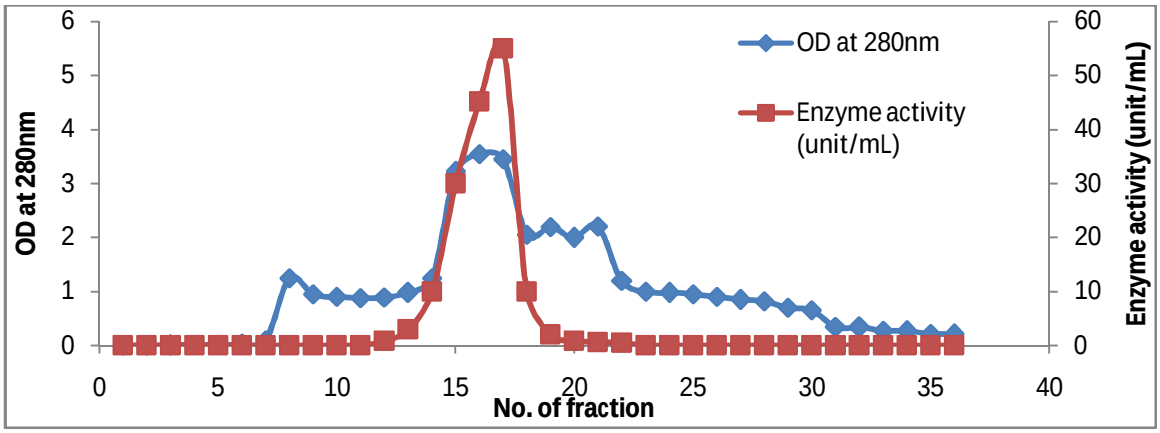

Fig. 2. Chromatography of the Lentil Cysteine protease from lentil cotyledons on Sephadex G-75 Column (2.0 x 20cm). The column was eluted with 50mM sodium acetate buffer (pH 5.0) at a flow rate 15mL/h. Fraction of 3mL was collected.

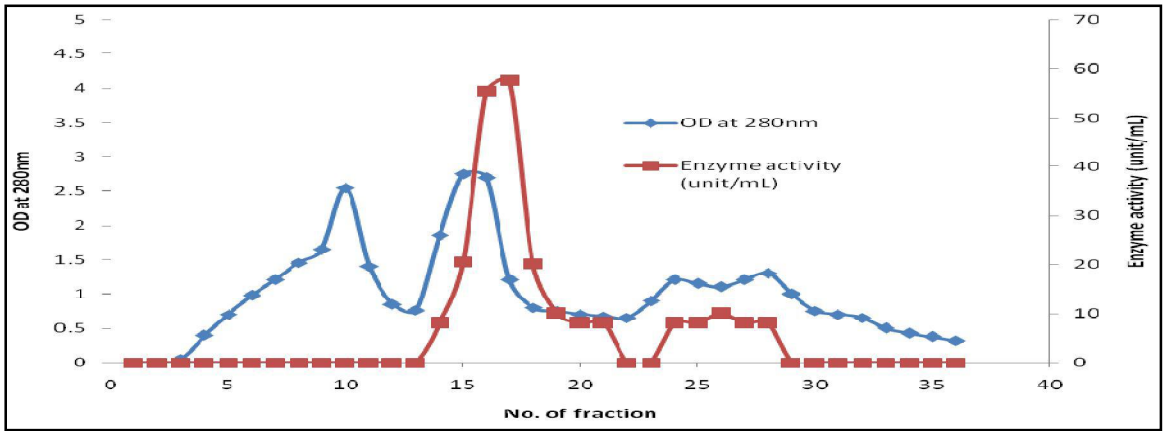

Fig. 3. Chromatography of Lentil protease after Gel filtration (combined fractions no. 13-20) on DEAE-cellulose column (1.8 x 40cm). The column was eluted with 10mM Tris-HCl buffer, pH 7.3 containing 2mM β-ME with sodium chloride (0.1-1.0M) gradient at a flow rate of 24mL/ h. Fraction of 3mL was collected.

2. DEAE-cellulose column: [Lentil]

The enzymatically active protein fractions after gel filtration ware collected then dialysed and concentrated to its 1/4th volume (on polyethylene glycol, MW 50,000) at 4^{0}C in a refrigerator. The concentrated dialysate was loaded on DEAE-cellulose column (40 x 2.5cm) equilibrated with 10mM Tris-HCl buffer, pH 7.3 containing 2mM β-ME. The bound proteins were eluted with a linear gradient of increasing ionic strength of sodium chloride (0.5M) in equilibration buffer and 5ml fractions were collected. In Fig. 3 the fractions (16-19) with higher enzymatic activity were pooled and brought to 70% saturation with pulverized ammonium sulphate at 4°C and centrifuged. The pellet was dissolved in a minimal volume of 20mM sodium acetate buffer, pH 5.6 containing 2mM β-ME and dialyzed against the same buffer at 4°C for 12 hr. Then concentrated by lyophilization (freeze dryer, Japan) and stored at -20°C. A criterion of purity at each stage was checked by 10% SDS-PAGE.

2.2.4 Effect of pH and temperature on enzyme

The effect of pH on enzyme activity was determined by carrying experiment at different pH using sodium acetate buffer (pH 3.5 - 5.5), sodium phosphate buffer (pH 5.6 - 7.0) and Tris-HCl buffer (pH 7.2 - 9.2). The enzyme activity was assayed as described[20] (Fig. 5). The effect of temperature on the enzyme activity was also determined at different temperatures ranging from 10 - 80°C for 1 hr in 0.02M sodium acetate buffer pH 5.5, containing 2mM β-ME uses the same assay as described above[20] (Fig. 4).

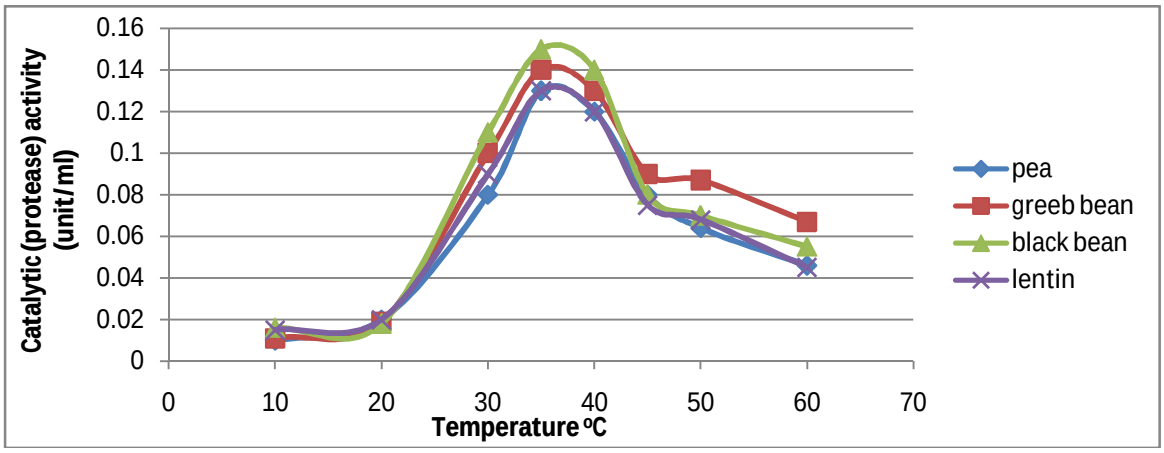

Fig. 4. Temperature-activity profile of four different leguminous enzymes at pH 6.8 [Azocasein is the substrate for enzyme].

Table 1. Concentration of protease (crude extract) from leguminous seeds.

Common name	Botanical name	Volume of ammonium salt precipitation (ml)	Protein concentration (mg/ml)
Lentil	*Lens esculenta*	97.0	2.02
Green gram	*Vigna radiata*	96.5	2.34
Black gram	*Vigna mungo*	97.0	2.29
Pea bean	*Phaseolus vulgaris*	97.0	2.38

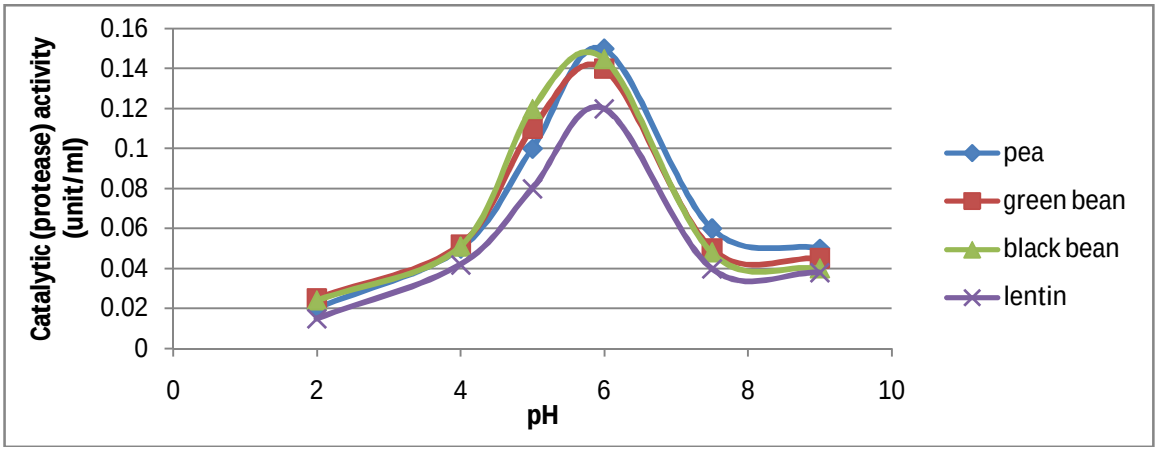

Fig. 5. pH stability of four different leguminous seeds enzymes at 25^{0}C [Azocasein is the substrate for enzyme].

Table 2a. Purification of leguminous protease by the method[20] [52h germinated 5g cotyledons].

Purification steps	Total protein (mg)	Total activity (Units)	Specific activity (units mg^{-1})	Yield (%)	Purification (fold)
Crude extract					
Lentil	1234.8	2189	1.77	100	1
Green gram	1545.5	2245	1.45	100	1
Black gram	1545.9	2325	1.50	100	1
Pea bean	1456.8	2125	1.46	100	1
Salting out and dialysis					
Lentil	560.0	1789	3.20	31.3	1.8
Green gram	455.0	1456	3.2	31.25	2.2
Black gram	435.2	1432	3.29	30.39	2.2
Pea bean	451.8	1567	3.47	28.83	2.38
Sephadex G-100 gel					
Lentil	120.7	1255.8	10.40	9.61	5.9
Green gram	165.3	1344.0	8.13	12.29	5.6
Black gram	178.9	1198.9	6.7	14.9	4.5
Pea bean	189.7	1278.9	6.74	14.83	4.6
DEAE-cellulose					
Lentil	16.3	1109.8	68.08	1.47	38.46
Green gram	20.6	1089.9	52.9	1.9	36.48
Black gram	22.5	989.8	43.9	2.27	29.27
Pea bean	18.6	1145.5	61.92	1.62	42.41

2.2.5 Time course study

The purified extracted protease from different cotyledons and azocasein were taken as described earlier and incubated at 37°C for 4 hr. Aliquots were withdrawn from the incubation mixture at different intervals of time from 0 - 4 hrs and the azo compound liberated was measured as described[20]. Maximum enzyme activity was found at 1.5 to 2 hours incubation time (Data & Fig not shown).

2.2.6 Effect of metal ions, selected inhibitors and sulfhydryl reagents

150µg of extracted protease (lentil, green bean, black bean & pea) in 0.02M sodium acetate buffer pH 5.5 was pre-incubated with 10µl of different metal ions, selected inhibitors and sulfhydryl reagents at varied concentrations for 1 hr at room temperature and the assay was initiated by the addition of azocasein and the activity assayed as described[20].

2.2.7 Effect of -SH reagents on pCMB treated EP-HG

pCMB (10mM) treated protease was re-incubated prior to the assay with sulfhydryl reagent (5mM and 10mM) for 1 hr at room temperature and the activity was assayed as described. The proteolytic activity was compared with the control experiment containing all the components except the SH-reagent.

2.2.8 Determination of MW of extracted protease

The molecular weight of the purified protease was determined by SDS-PAGE (Sambrook, J. *et al.*, 1989)[21].

Table 2b. Purification steps of leguminous protease [52h germinated 5g cotyledons].

Purification Steps	Total Protein (mg)	Total activity (Units)	Specific activity (unit mg-1)	Yield (%)	Purification (folds)
Lentil					
Crude extract	1234.8	2189.0	1.70	100	1
Salting out and dialysis	560.00	1789.0	3.20	31.3	1.8
Sephadex G-100	120.70	1255.8	10.40	9.61	5.90
DEAE-cellulose	16.30	1109.8	68.08	1.47	38.46
Green gram					
Crude extract	1545.5	2245.0	1.7	100	1
Salting out and dialysis	455.00	1456.0	3.2	31.25	2.2
Sephadex G-100	165.30	1344.0	10.40	12.29	5.60
DEAE -cellulose	20.60	1089.9	68.08	1.90	36.48
Black gram					
Crude extract	1545.9	2325.0	1.7	100	1
Salting out and dialysis	435.20	1432.0	3.2	30.39	2.2
Sephadex G-100	178.90	1198.9	10.40	14.9	4.50
DEAE-cellulose	22.50	989.8	68.08	2.27	29.27
Pea bean					
Crude extract	1456.8	2125.0	1.7	100	1
Salting out and dialysis	451.8	1567.0	3.2	28.83	2.38
Sephadex G-100	189.7	1278.9	10.40	14.83	4.60
DEAE-cellulose	18.6	1145.5	68.08	1.62	42.41

2.2.9 Hydrolysis of endogenous and exogenous substrates

The relative digestibility of endogenous and exogenous proteins by the purified extracted protease was measured by incubating 1ml of 1% protein (dry seed protein, BSA, casein, hemoglobin, gelatin) prepared in an appropriate buffer mixed with 0.15ml of the purified protease (1mg/ml) and incubated at 37°C for 1 hour and the amino acids released were estimated by the ninhydrin method (Rosen, R. 1957)[22].

2.2.10 Effect of metal ions, selective inhibitors and sulfhydryl reagents on extracted protease

The protease activity was clearly inhibited at lower concentrations of Zn^{2+}, Hg^{2+} and Cu^{2+} (0.5mM). However, metal ions like Ni^{2+}, Co^{2+} and Pb^{2+} were shown an inhibition on above 1mM. At higher concentrations (above 1mM) of Mn^{2+} stimulated the activity. Metal chelating agents such as EDTA had no effect (Table 3).

2.2.11 Effect of inhibitors and sulfhydryl reagents on partial purified Cysteine protease

The Cysteine protease (CP) activity was inhibited by serine modifying reagents (DIPF, soybean trypsin inhibitor, eserine, PMSF and aprotinin). However, the activity was completely inhibited by sulfhydryl modifying reagents (pCMB and iodoacetamide). We also observed the complete inhibition by histidyl modifying reagent (DEPC). These results suggested that the CP is a cysteine protease, with cysteine and histidine residues at the active site (Table 4). The addition of sulfhydryl reagents (cysteine, dithiothreitol, glutathione and β-ME) reverses the strong inhibition by pCMB (Table 5).

3. Results

3.1 Purification of protease

The cotyledons of 62h germinating seeds (lentil, green gram, black gram and pea bean) were used for isolation of endoprotease, since the activity was maximal at 62h and then declined rapidly (Fig. 1) during the four-day period of germination. This finding is in good agreement with previous studies by (Jinka, R., *et al*., 2002)[13]. The protein concentrations of extracellular proteases isolated from four leguminous seeds were shown in Table 1. The concentration of water-soluble protein ranged between 2.02 to 2.38mg/ml in which pea bean showed highest protein concentration (2.38mg/ml) and lentil accounted lowest concentration (2.02mg/ml). Dahot, M.U. (1992)[23] investigated on proteases present in some plant seeds and found the ranged of protein concentration between 1.10 to 2.76mg/ml with highest value in soybean seed (*Glycine max*, 2.67mg/ml), which is in a good agreement with our value reported. Maximum yield of protease activity observed with 50mM Tris-HCl buffer pH 7.2, containing 2mM of β-ME among other buffers (acetate, borate, and phosphate buffers) used. The protease activity was measured by using the chromogenic substrate, azocasein. The results of the four step purification of CP from four varieties of leguminous seeds: lentil, green gram, black gram and pea bean were summarized in Table 2. The CP was initially precipitated with (60%) ammonium sulphate and dialyzed and subjected to Gel and DEAE cellulose column. The enzyme from gel filtration was eluted with the same buffer 10mM Tris-HCl, pH 7.2. The fractions (Beilinson *et al*., 2002; Devaraj, K.B. *et al*., 2008; Ramakrishna *et al*., 2005; Karunagaran *et al*., 1990;

Jiang *et al*., 1998; Baumgartner *et al*., 1977; Koehler *et al*., 1990)[24-30] contained 35% of enzyme activity with specific activity of 3.80 were pooled and concentrated by freeze dryer/polyethylene glycol (MW 50,000). The final efficient step of purification is the fractionation on DEAE-cellulose column. The bounded protein band eluted with a linear gradient of NaCl / KCl (0 - 0.5M). All enzymatically active fractions are collected together, dialysis, concentrated and stored at -80^{0}C for further works.

Table 3. Effect of metal ions on partial purified Cysteine proteases from leguminous seeds.

Metal Ion	Lentil Remaining Activity (%)	Green gram, Remaining Activity (%)	Black gram, Remaining Activity (%)	Pea bean, Remaining Activity (%)
None	100	100	100	100
Hg^{2+} (0.5mM)	54	57	51	52
Hg^{2+} (1.0mM)	96	92	93	91
Hg^{2+} (1.5mM)	89	88	86	89
Cu^{2+} (0.5mM)	61	63	62	63
Cu^{2+} (1.0mM)	88	87	89	90
Cu^{2+} (1.5mM)	86	88	89	85
Pb^{2+} (0.5mM)	89	90	92	91
Pb^{2+} (1.0mM)	69	68	61	64
Pb^{2+} (1.5mM)	67	69	65	67
Co^{2+} (0.5mM)	91	94	93	90
Co^{2+} (1.0mM)	66	69	64	62
Co^{2+} (1.5mM)	59	62	60	59
Ni^{2+} (0.5mM)	88	89	92	94
Ni^{2+} (1.0mM)	66	61	65	62
Ni^{2+} (1.5mM)	67	61	64	62
Mn^{2+} (0.5mM)	93	90	92	90
Mn^{2+} (1.0mM)	99	105	109	99
Mn^{2+} (1.5mM)	120	125	135	124
EDTA (0.5mM)	95	93	92	95
EDTA (1.0mM)	92	91	90	93
EDTA (1.5mM)	95	95	92	91
Zn^{2+} (0.5mM)	59	54	52	50
Zn^{2+} (1.0mM)	87	82	86	81
Zn^{2+} (1.5mM)	88	86	89	90

Table 4. Effect of various inhibitors on Cysteine protease activity.

Inhibitor	Conc.	Relative activity			
		(%)[1]	(%)[2]	(%)[3]	(%)[4]
pCMB (p-chloromercuribenzoate)	10mM	0	2	0	3
NEM (N-ethylmaleimide)	100mM	61	64	52	49
Iodoacetamide	100mM	8	9	10	9
DTNB	0.2%	40	34	39	42
STI (Soybean trypsin inhibitor)	0.05%	99	93	98	95
pMSF (phenylmethylsulfonyl fluoride)	100mM	81	85	81	82
DEPC	10mM	0	4	5	0
EDTA (Ethylene diamine tetra acetic acid)	10mM	95	92	95	93
Control	-	100	100	100	100

1= Lentil; 2= Green gram; 3= Black gram & 4= Pea Been.

Table 5. Activation of pCMB treated Cysteine protease by sulfhydryl reagents.

Substance added	Concentration (mM)	Relative activity (%) Lentil	Relative activity (%) Green gram	Relative activity (%) Black gram	Relative activity (%) Pea Been
Control	-	100	100	100	100
pCMB	10	0	2	0	2
	5	52	61	51	53
pCMB + β-ME	10	74	67	49	55
	5	71	76	72	74
pCMB + DDT	10	75	72	71	71
	5	87	78	78	77
pCMB + Cysteine	10	84	81	77	81

4. Discussion

Maximum endoprotease activity was observed in 62h cotyledons of leguminous seeds during 120h germination period (Fig. 1) and also shown pH optima in the acidic region suggesting that the enzyme is located in the vacuoles (Jinka *et al*., 2002; Shutov *et al*., 1987; Muntz *et al*., 2001)[13,16,31]. We made an attempt to purify the endoprotease (CP) from the cotyledons (62h) of peas, green gram, black gram, lentil germinated seeds, study its properties and physiological role in the degradation of storage proteins. The purification steps yielded a homogeneous preparation with a recovery of about 3% and 29–43 fold purification. The final recovery of CP from leguminous pulses less than studied by 10% from Indian beans (Ramakrishna *et al*., 2005)[26] and purified aspartic protease from *Ficus racemosa* (Devaraj *et al*., 2008)[25]. This loss may be due to poor cooling system during handling at the main three extracted procedures time and properties of enzyme studies.

CP from leguminous pulses exhibits acidic pH optima by showing higher activity at pH 5.6 (Fig. 4). Since the purified CP were found to be stable in mildly acidic pH range 5.0 – 6.0, the enzyme may be localized *in vivo* in protein bodies like in other legume seeds (He *et al*., 2007; Jinka, *et al*., 2002; Jones, B.L. 2005; Liu *et al*., 2001)[12,13,17,32]. Table 6 showed the comparative hydrolytic activity of purified CP from four leguminous cotyledons with endogenous (seed proteins) and exogenous proteins (BSA, casein, gelatine, and haemoglobin). Seed protein degradation was remarkably higher than the gelatine, casein, BSA and haemoglobin.

We have employed various inhibitors specific to each class of proteases (metallo-, asp-, serine- and cys-) in order to investigate the amino acid residue(s) contributing to the active site of the enzyme. Inhibition of the enzyme activity by heavy metal ions and inhibitors, sulfhydryl blocking reagents, and the reactivation of pCMB- treated by the addition of sulfhydryl reagents clearly established the thiol nature of the enzyme. However, the partial inhibition by the sulfhydryl blocking reagents suggests the involvement of other residue(s) also in enzyme activity. The strong inhibition of CP by DEPC suggested the involvement of histidine at the active site along with Cysteine as proposed for papain (Dunn, M.J., 1989)[33].

The purified CP from four leguminous pulses was found to be homogeneous by the detection of a single polypeptide by 10% SDS-PAGE [gel filtration (Fig. 6a,b) & DEAE-cellulose chromatography (Fig. 7)]. The sizes of the purified CP's were estimated to be 29.5 kDa on Sephadex G-100 column (Data & Fig. not shown) and nearly the same was also supported by SDS-PAGE (Fig. 7). Cysteine protease purified by DEAE-cellulose from four leguminous pulses appeared to be monomeric protein with molecular weight of 29.5 kDa (Fig. 7) similar to other Cysteine proteases isolated from germinating *Vicia sativa* (Tiedemann *et al*., 2001)[11], soybean (Seo *et al*., 2001)[34].

5. Conclusion

Partially purified Cysteine protease from lentil, green gram, black gram and peas show nearly similar optimum pH, optimum temperature and its ability to degrade native seed storage proteins preferentially fulfilled the criteria laid down for a protease to be involved in seed storage protein degradation during germination. It appears that enzyme activation takes place during germination. The reported enzyme may turn out to be an efficient choice in pharmaceutical, medicinal, food and biotechnology industry.

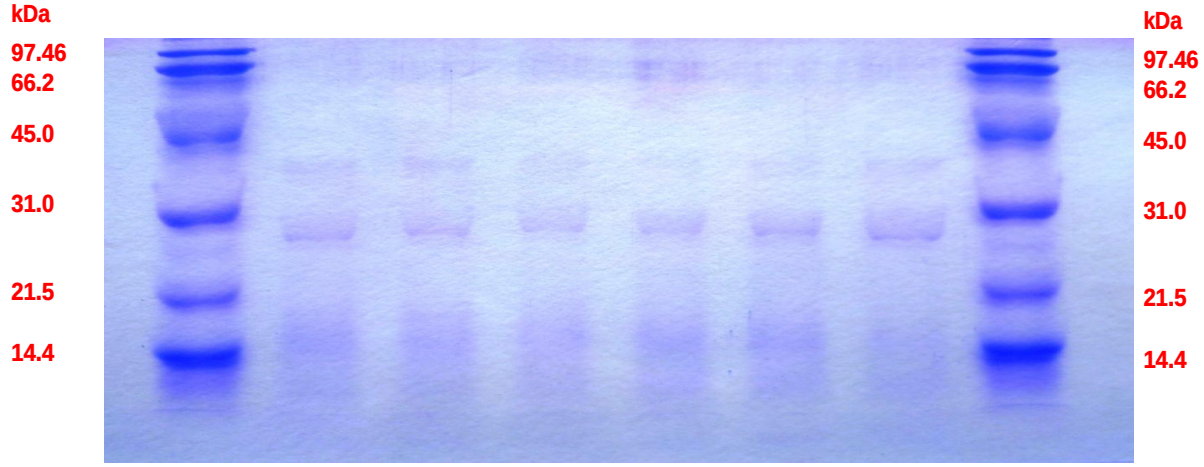

Fig. 6a. SDS-PAGE Electrophoresis of four different leguminous enzymes (After gel filtration).

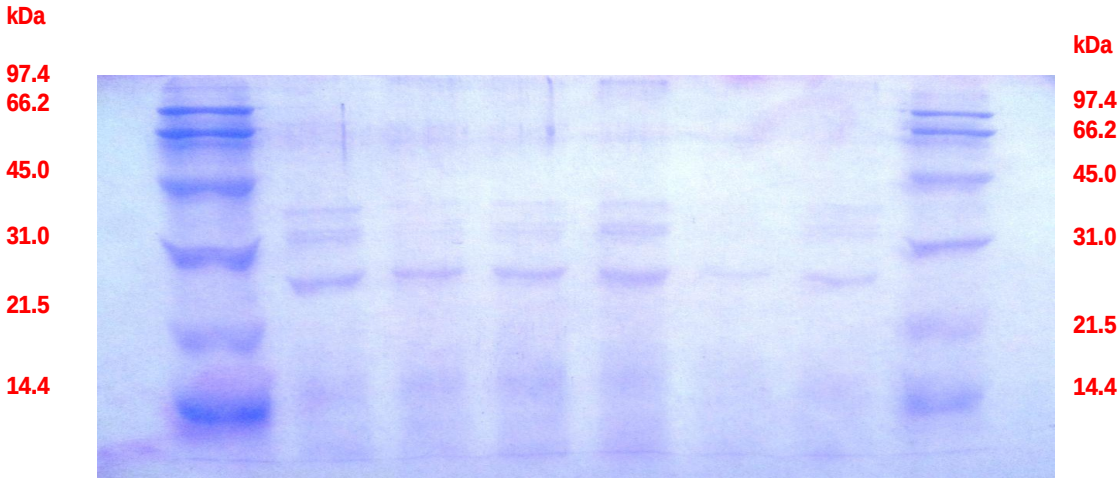

Fig. 6b. SDS-PAGE Electrophoresis of four different leguminous enzymes (Gel filtration).

Table 6. Hydrolytic activity of Cysteine protease with endogenous and exogenous proteins.

Substance added	Concentration (mM)	Relative activity (%) Lentil	Relative activity (%) Green gram	Relative activity (%) Black gram	Relative activity (%) Pea Been
Control	-	100	100	100	100
Exogenous Proteins					
BSA	5	78	87	79	82
	10	72	81	81	83
Casein	5	74	77	69	75
	10	71	76	72	74
Gelatin	5	75	72	71	71
	10	87	78	78	77
Hemoglobin	5	74	81	77	81
	10	79	76	75	77
Endogenous proteins					
(seed proteins)	5	127	111	120	123
	10	124	109	121	120

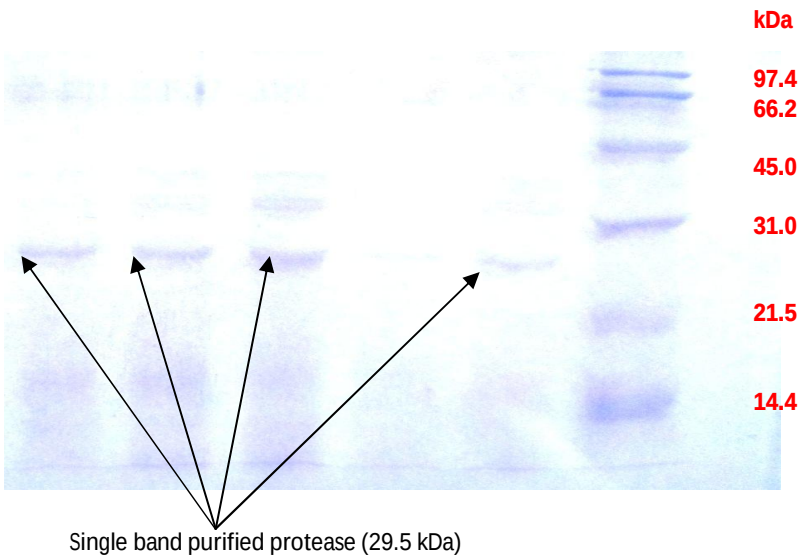

Fig. 7. SDS-PAGE Electrophoresis of four different leguminous enzymes (after DEAE-cellulose purification).

Acknowledgment

Some of the research experiments procedure followed and compared with Jinka *et al., BMC Biochemistry*, 2009. **10**:28 doi:10.1186/1471-2091-10-28.

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Abbreviations used:

- CP: Cysteine Protease;
- DEAE: Diethylaminoethyl cellulose;
- CM: Carboxymethyl;
- pCMB: p-chloromercuribenzoate;
- NEM: N-ethylmaleimide;
- PMSF: phenylmethylsulfonyl fluoride;
- DIFP: diisopropylflourophosphate;
- DEPC: diethyl pyrocarbonate;
- CBZ: carboxybenzyl
- EP: Endopeptidase;
- EDTA: Ethylene diamine tetra acetic acid;
- DTT: Dithiothreitol;
- STI: Soybean trypsin inhibitor.