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Evaluation of Cysteine Protease from Niger Seeds *(Guizotiaabyssinica)*

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ABSTRACT

Proteolytic enzymes play central role in the biochemical mechanism of germination and intricately involved in many aspects of plant physiology and development. To study the mechanism of protein mobilization, undertaken the task of purifying and characterizing proteases, which occur transiently in seeds of Niger. Cysteine protease (CPRNS) was purified to homogeneity with 118 fold by four step procedure comprising Crude extract, $(NH_4)_2SO_4$ fractionation, DEAE-Cellulose and CM-sephacel chromatography from the 2 day germinating cotyledons of Niger *(Guizotiaabyssinica)* CPRNS is a monomer with molecular mass of 30 k Da, was determined by SDSPAGE and gel filtration. The purified enzyme on IEF showed two isoforms having pI values of 5.85 and 6.1. CPRNS composed of high content of aspartic acid, glutamic acid and serine. The enzyme activity was completely inhibited by pCMB, iodoacetate and DEPC indicating cysteine and histidine residues at the active site. However, on addition of sulfhydryl reagents (cysteine, dithiothreitol, glutathione and β-ME) reverse the strong inhibition by pCMB. The enzyme is fairly stable toward pH and temperature. Immunoblot analysis shows that the enzyme synthesized as zymogen (pre-pro-enzyme with 81 kDa) and processed to a 40 kDa pro-enzyme which was further degraded to give 30 kDa. It appears that the newly synthesized protease is inactive, and activation takes place during germination. CPRNS has a broad substrate specificity and stability in pH, temperature, etc. therefore, this protease may turn out to be an efficient choice for the pharmaceutical, medicinal, food, and biotechnology industry.
Keywords: cysteine protease, Niger, Seeds

Abbreviations used
CPRNS: Cysteine Protease of Niger Seeds; DEAE: Diethyl aminoethylcellulose; CM: Carboxymethyl; pCMB: p-chloromercuribenzoate; NEM: N-ethylmaleimide; PMSF: phenyl methylsulfonylfluoride; DIFP: diisopropylflourophosphate; DEPC: diethyl pyrocarbonate; CBZ: carboxybenzyl; NBRF: National Biomedical Research Foundation; EP: Endopeptidase; EDTA: Ethylene di-amine tetra acetic acid; DTT: Dithiothreitol; STI: Soyabean trypsin inhibitor.

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INTRODUCTION

Proteolytic enzymes are multifunctional enzymes thathave 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. Commercially they are extremely important asmore than 60% of the total enzyme market is made up of proteases; they are isolated from plants, animals, bacteria and fungi. Proteolytic enzymes from the plant sources have received special attention because of their broad substrate specificity as well active in wide range of pH, temperature, and in presence of organic compounds as well as other additives [1,2]. Search for valuable proteases with distinct specificity is always a continuous challenge for varied industrial applications. The mobilization of seed storage proteins represents one of the most important post-germination events in the growth and development of seedling. Proteolytic enzymes play central role in the biochemical mechanism of germination and intricately involved in many aspects of plant physiology and development [2,3]. Numerous reports including our previous data supported the proteases are responsible for protein degradation. 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 vicillin and leguminfamily these are degraded by endoproteases particularly cysteine proteinase. In horse gram, during germination we observed the disappearance of high molecular weight (HMW) polypeptides of globulins and appearance of a new 25 kDa polypeptide [4-9]. 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 [2,10,11]. With renewed interest, there has been proliferation of reports in the last decade concerning purification and characterization of these proteases from germinating leguminous and non-leguminous seeds [12-16]. 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. Horse gram (*Guizotiaabyssinica)* is one of the lesser known, unexploited legume of the tropics and subtropics grown under dry-land agriculture. In our previous studies the pattern of mobilization of seed storage proteins and activation of proteolytic enzymes (endoprotease, carboxypeptidase and leucineaminopeptidase) were investigated in germinating horse gram seeds [7,17]. In the present investigation we report the purification and biochemical characteristics of an endoprotease from the germinating seedlings of horse gram. It is shown that this protease is a cysteine protease (CPRNS) hitherto not known in the genus*Guizotia*with novel cleavage specificities.

RESULTS

Purification of protease

The cotyledons of day 2 germinating horse gram seeds were used for isolation of endoprotease, since the activity was maximal at this point during the four-day period of germination [7]. Maximum yield of protease activity observed with 0.05 M Tris-HCl buffer pH 7.2, containing 2 mM of β-ME among other buffers (acetate, borate, phosphatebuffers) used. Change in pH, alterations in molarity or omission of β-ME in the extraction buffer substantially lowered the yield of the activity (data not shown). The protease activity was

measured by using the chromogenic substrate, azocasein. The results of the four step purification of CPRNS were summarized in Table 1. The CPRNSwas initially precipitated with (30% - 60%) ammonium sulphate and dialyzed and subjected to DEAE cellulose column. The bound enzyme was eluted with a linear gradient of KCl (0 - 0.5 M). The fractions [14-20] contained 40% of enzyme activity with specific activity of 4.80 were pooled and concentrated by ammonium sulphate (Fig. 1). The final efficient step of purification is the fractionation on CM-Sephacel. Under provided conditions, 12% of the loaded enzyme was not adsorbed to the column and eluted, that was found to be homogeneous on 10% SDSPAGE (Fig. 2).

Table 1: Summary of the purification of CPRNS from germinating Niger seeds

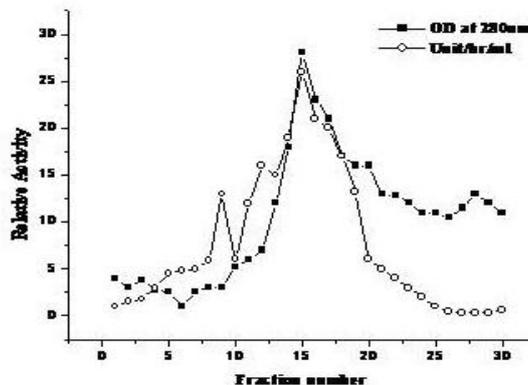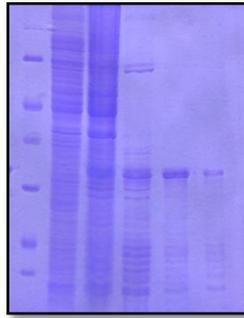| Purification step	Total Protein (mg)	Total activity (Units/hr)	Specific activity (Units/mg protein)	Yield (%)	Enzyme activity Purification fold
Crude extract	9405.00	1578.00	0.168	100.00	1.00
Ammonium sulfate precipitation (30 - 60%)	2713.00	1187.00	0.437	75.22	2.44
DEAE-cellulose	130.80	628.00	4.801	40.00	28.27
CM-Sephacel	3.65	72.58	19.88	4.59	118.36

Figure 1of enzyme on DEAE-Cellulose column

Fractionation of enzyme on DEAE-Cellulose column:

The enzyme fraction obtained from ammonium sulfate precipitation (30-60%) was loaded on to DEAE-Cellulose column equilibrated with equilibrating buffer (0.01 M Tris- HCl buffer, pH 7.0, containing 2 mM β-ME) at a flow rate of 40 ml/hr and 5 ml fractions were collected and assayed for enzyme activity as described in materials and methods. The indicated fractions (14-20) were pooled for further processing.

Figure 2

SDS-PAGE analysis of the fractions from each step of endoprotease (EP-NS) purification from the cotyledons of germinating Niger Seeds and seedlings;

Conc. of protein loaded on each well in gel is 100 ug. Lane 1.Crude extract, Lane 2. Protein from 30-60% saturatedammonium sulfate fraction. Lane 3. Pooled fractions (14-20)after DEAE-Cellulose chromatography. Lane 4. Unboundfraction from CM Sephacel column. Lane 5. Molecular weightmarkers consisting of Phosphorylase b (94 k Da), BSA (67 kDa), ovalbumin (43 k Da), carbonic anhydrase (30 k Da), soybeantrypsin inhibitor (20.1 k Da) and α-lactalbumin (14.4 kDa).

Homogeneity and size of the purified enzyme

The purified CPRNS was found to be homogeneous by the detection of a single polypeptide by SDS-PAGE which is further supported by single precipitin band on immune diffusion of the crude extract (result not shown). Immunoblotanalysis of the purified CPRNS also conform the monoreactivity of the antiserum (Fig 3 lane 5). The size of the purified CPRNS was estimated to be 30.2 K Da on Sephadex G-100 column and the same was also supported by SDS-PAGE (Fig. 2). However, on isoelectrofocussing(IEF) two isoforms were observed with different pI values of 5.85 and 6.1 (Fig 4).

Figure 3

Western blot analysis of crude extracts and purified CPRNS against anti- CPRNS polyclonal antibodies

8% SDS-PAGE, 100 μg loaded on the each lane of the gel.Lanes 0-4: Cotyledon extracts at days 0,1,2,3 and 4, respectively,from germinating horse gram seedlings. Lane 5: PurifiedCPRNS.

Figure 4

Two dimensional (2D) gel electrophoresis of CPRNS

The first dimension isoelectric focusing was performed in a tube gel using wide range of ampholytes (pH 3- 10). Second dimension was on 10% SDS-PAGE and was stained with coomassie brilliant blue.

Amino acid composition

The amino acid analysis of CPRNS was shown relatively fewer numbers of Histidine, sulfur containing amino acids (Cys and Met) and higher number of aspartic acid (Asp), glutamic acid (Glu) and serine (Table 2).
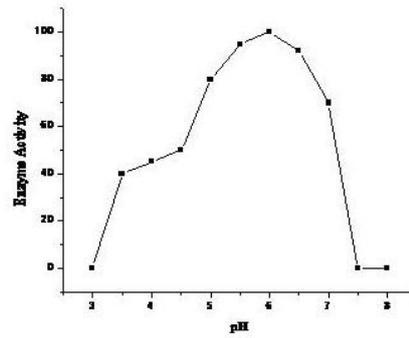
Table 2: Amino acid composition of purified CPRNS

Amino acid	No. of residues
Asx	33
Thr	20
Ser	30
Glx	25
Pro	16
Gly	19
Ala	24
Cys	2
Val	19

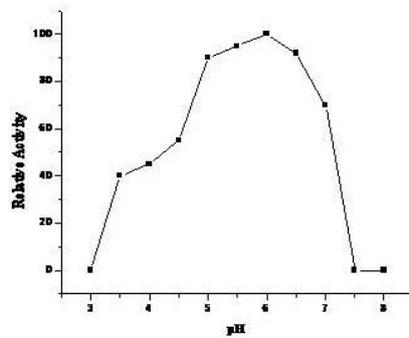## Effect of pH, temperature, time and storage stability

Effect of pH on CPRNS was carried out using azocasein as substrate and found the enzyme was active at acidic pH optimum, 5.5 with azocasein (Fig 5), 4.0 with haemoglobin and 5.2 with gelatin and BSA (data not shown). The activity was stable in the pH range 5.0-6.0 (Fig 6) andalso observed gradual loss of activity outside this pHrange. However, the temperature vs activity profile showed an increased activity with temperature and exhibited maximal activity at 40°C (Fig 7). The enzyme was fairly stable at 40°C (Fig 8) and also exhibits linear proteolyticactivity up to 4 h at this temperature (Fig 9).
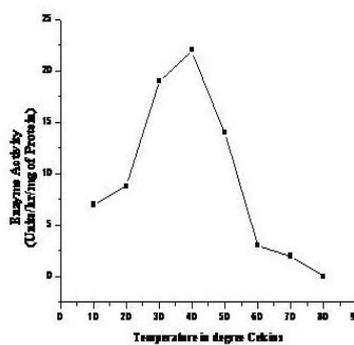
Figure 5

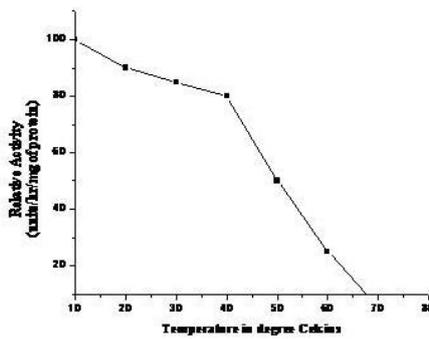**Effect of pH on the activity of CPRNS**.Azocasein is the substrate for enzyme assay.

Figure 6

PH stability of CPRNS. Azocasein is the substrate for enzyme assay.

Figure 7

Temperature- activity profile of CPRNS. Azocasein is the substrate for enzyme assay.

Figure 8

Thermal stability of CPRHG. Azocasein is the substrate for enzyme assay. The activity was expressed as per cent of activity at 10°C.

In vitro digestion of endogenous and exogenous proteins

Table 3 showed the comparative hydrolytic activity of the purified CPRNS with endogenous (seed proteins) and exogenous proteins (BSA, casein, gelatin and hemoglobin).Seed protein degradation was remarkably higher than the gelatin, casein, BSA and hemoglobin. The enzyme was unable to cleave Leu-p-nitroanilide and CBZL-phenylalanine. Digestion of endogenous proteins by CPR HG on time course study through SDS-PAGE shows no appreciable degradation within first 90 min. However, the disappearance of polypeptides of 96, 81, 66, 60, 58 and 21 k Da and appearance of new polypeptides of 73 and 59 k Da and intensification of 56 and 29 kDa bands were observed on SDS-PAGE with increasing digestion time (Fig 10).

Effect of metal ions, selective inhibitors and sulfhydryl reagents on CPRNS

The protease activity was clearly inhibited at lower concentrations of Zn^{2+}, Hg^{2+} and Cu^{2+}(0.5 mM). However, metal ions like Ni^{2+}, $Co^{2+}$and Pb^{2+}were shown an inhibition on above 1 mM. Mn^{2+}at higher concentrations (above 1 mM) stimulated the activity. Metal chelating agents such as 1, 10phenanthroline and EDTA had no effect (Table 4 &5). The CPRNS activity was neither inhibited by aspartyl modifying reagent (pepstatin) nor by serine modifying reagents (DIPF, soybean trypsin inhibitor, serine, 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 CPRNS is a cysteine protease, with cysteine and histidine residues at the active site (Table 5). Addition of sulfhydryl reagents (cysteine, dithiothreitol, glutathione and β-ME) reverses the strong inhibition by pCMB (Table 6 &7).

Table 4: Effect of various metal ions on purified CPRNS

Reagent	Relative activity (%)				
	Conc. of reagents (mM)				
	0.5	1.0	2.0	5.0	10
Control	100	100	100	100	100
$NiCl_2$	81	86	68	63	57
$CoCl_2$	73	76	63	57	56
Cupric acetate	19	13	11	7	4
Manganese chloride	101	101	111	136	158
Lead acetate	89	90	89	64	34
Zinc acetate	58	35	20	8	2
Magnesium Chloride	116	102	102	102	95
Mercuric Chloride	20	15	7	3	0

Table 5: Effect of various inhibitors on CPRNS activity

Inhibitor	Conc	Relative activity (%)
pCMB	10 mM	0
NEM	100 mM	61
Iodoacetamide	100 mM	8
DTNB	0.2%	40
DIPF	10 mM	93
STI	0.05%	99
Eserine	0.01%	96
pMSF	100 mM	81
Bestatin	0.1 mg/ml	92
Aprotinin	0.001%	96
Pepstatin	0.01%	88
EDTA	10 mM	95
1, 10-Phenanthroline	10 mM	99
DEPC	10 mM	0
Control -		100

Table 6: Effect of various Sulfhydryl reagents on CPRNS

Conc. of reagents (mM)	Relative activity (%)			
	Glutathione	Cysteine	β-ME	Dithiothretol
0.5	100	106	107	112
1.0	121	114	120	116
2.0	115	110	115	131
5.0	119	105	98	127
10.0	172	103	92	106
Control	100	100	100	100

Table 7: Reactivation of pCMB treated CPRNS by sulfhydryl reagents

Substance added	Concentration (mM)	Relative activity (%)
Control	-	100
pCMB	10	0
pCMB + β-ME	5	53
	10	77
pCMB + DTT	5	73
	10	75
pCMB +Glutathione	5	85
	10	104
pCMB + Cysteine	5	71
	10	83

The hydrolyzing activity is expressed as per cent of control (19.73units/h/mg enzyme)

mmunoblotting of CPRNS development

The polyclonal anti-CPRNS could recognize 81, 41, 30 and 27 k Da polypeptides from crude extract of germinating cotyledons. Presence of HMW 81 k Da protein in the first 2 days of germination and disappeared by day 3. The 41 k Da polypeptide was noticed in the extracts from days 0-3. The intensity of 30 k Da endoprotease (CPRNS) in the cotyledons of germinating seeds increased up to day 2 and declined later. Similar profile was also noticed for 27 k Da polypeptides (Fig 3). These results revealed that the enzyme CPRNS synthesized as zymogen (pre-pro-enzyme with 81 K Da) and processed to a 40 K Da proenzyme and then to the 30 K Da active enzyme which is furtherdegraded to a 27 K Da product.

DISCUSSION

Maximum endoprotease activity was observed in day 2 cotyledons of Niger seeds during 4 day germination period and also shown pH optima in acidic region suggesting that the enzyme is located in the vacuoles [3,7,10,17,18]. The role(s) of specific proteases in seed protein degradation is unclearly understood, indeed, the overall role has been focus for direct investigation. We made an attempt to purify the endoprotease (CPRNS) from the cotyledons of Niger and studied its properties and physiological role in the degradation of storage proteins. The purification procedure yielded an essentially homogeneous preparation with an overall recovery of 4.59% and 118 fold purification. The final recovery of CPRNS (4.59%) was similar to that of other plant cysteine proteases i.e., vicilinpeptidohydrolase (8.0%)from mung bean seedlings [19] and GA3- induced protease (3.38%) from barley aleurone layers [20], 15% acidic protease from germinating winged bean [12] and 12% from Indian beans [16]. The specific activity of the purified aspartic protease was 0.64 U/mg with a recovery of 20% and existed as a single form from *Ficusracemosa* [15]. CPRNS appeared to be monomeric protein with molecular weight of 30 k Da (Fig 2) similar to other cysteine proteases isolated from germinating *Viciasativa*[5], soyabean [21], barley [22] and wheat [23]. However, the molecular weights of cysteine proteases isolated from *Vignamungo*seeds varied between 20-30 k Da [24] and in barley 30-37 k Da [25,26]. PurifiedCPRNS was found to contain two isoforms with iso-electric points, pI 5.85 and 6.1 (Fig 4) as that of aleurain, isolated from aleurone cells, with two isoforms of pI values 6.0 and 6.1 and purified thiol protease from barley also had multiple forms including EP-A and EP-B [20]. CPRNS exhibits acidic pH optima by showing higher activity at 5.5. Discrepancy in pH optima was noticed for various protein substrates used in the assay, since a given substrate will have numerous ionizable groups with similar pKa values. The pH optimum obtained with a protein substrate reflects more about the pH - mediated susceptibility of the substrate to proteolysis than about the influence of pH on the protease as a catalyst. A similar susceptibility to pH had been described for endo-peptidase from *Phaseolous vulgaris* [27], barley [20] and wheat [28].

Since the purified CPRNS was 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 [6,7,10-13,29]. The enzyme (CPRNS) exhibited surprisingly high temperature optima at 40°C and the catalytic reaction was linear with time for at least 4 h at this temperature, which reflects resistance to autolysis and the results were correlated to the proteases of lentil seeds [30] and barley [20,22].CPRNS showed a high degree of specificity towards the natural substrates i.e., seed proteins from Nigerseeds and had completely an endoproteolytic activity,which suggested an important role in the mobilization of seed proteins during germination. *In vitro* digestion of seed proteins by purified CPRNS led to the gradual disappearance of high molecular weight polypeptides. An aspartic endoprotease from wheat also exhibited the similar endoproteolytic activity with no exopeptidase activity [28]. Studies with *Phaseolous vulgaris* [31], *Phaseolus mungo*[32], mung bean [33], *Glycine max* [13,34], wheat [35] and winged bean [12] also indicated the role of cysteine endoprotease in the mobilization of stored proteins. We have employed various inhibitors specific to each class of proteinases (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 enzyme by the addition of sulfhydryl reagents clearly established the thiol nature of the enzyme. However, the partial inhibition by other sulfhydryl blocking reagents suggests the involvement of other residue(s) also in enzyme activity. The strong inhibition of CPRNS by DEPC suggested the involvement of histidine at the active site along with cysteine as proposed for papain [36]. However, thiol proteases purified from winged bean [12] and *Phaseolous vulgaris*[37] did not belong to the papain family. Generally all thiol proteases have three conserved pairs of cysteine residues in their mature domain. However CPRNS contained only one pair of cysteine residues (Table 2). This data suggested that CPRNS showed homology to other proteases, such as EP-C1 of *Phaseolous vulgaris* [37] and EP-A of barley [20]. Temporal changes in the levels of CPRNS in the cotyledons of germinating Niger seeds were observed by immunoblotting. In addition to 30 k Da protein (CPRNS), the other three polypeptides with 81, 41 and 27 k Da was also cross-reacted with the antiserum. These results are similar to the development of endoproteaseactivity in germinating Niger seeds, where increased development from day 0 to day 2 and decreased further [7]. Information obtained from NBRF protein sequence data bank and translated Gen Bank database indicated that several cysteine proteases are produced as zymogens [26]. The polypeptide (CPRNS) with ≥ 30 k Da in the immunoblot appeared corresponding to the putative enzyme and the 81 and 41 k Da polypeptides to the corresponding prepro- and pro-enzymes. The 27 k Da polypeptide probably originated from the 30 k Da protease. The endoproteses in the seeds of *Vignamungo*[38], castor beans [39] and barley [20,40] were found to be synthesized as proenzymes and processed to the active forms during germination.

CONCLUSION

The temporal variations in CPRNS activity, its acidic pH optimum and its ability to degrade native seed storageproteins preferentially fulfilled the criteria laid down for aprotease to be involved in seed storage protein degradation during germination. It appears that the newly synthesized protease is inactive, and activation takes place during germination. The reported enzyme has broad substrate specificity and stability in pH, temperature, etc., therefore, this protease may turn out to be an efficient choice in pharmaceutical, medicinal, food, and biotechnology industry.

METHODS

Chemicals

Sephadex G-100, CM-Sephacel, LMW markers were procured from Pharmacia Fine Chemicals, Uppsala, Sweden and DEAE-cellulose, aprotinin, p-chloromercuribenzoate(PCMB), N-ethylmaleimide (NEM), phenylmethylsulfonylfluoride(PMSF), iodoacetamide, Soybean trypsin inhibitor, eserine, diisopropylflourophosphate (DIFP), bestatin and 1,10- phenanthroline, diethylpyrocarbonate(DEPC), CBZ-L-phenyl alanine and L-Leu-p-nitroanilidewere purchased from Sigma Chemical company, USA. All other chemicals, unless otherwise specified, were of analytical grade and purchased from Specrochem (India) and Qualigens (India).

Plant Material and Germination conditions

Niger (*Guizotiaabyssinica)* seeds were procured from National Seed Corporation University of Agricultural Sciences Bangalore, India.Niger seeds were surface sterilized with 0.1% $HgCl_2$solution for 5 min, washed repeatedly with sterile water and soaked in 10 volumes of water for four hours. The imbibed seeds were set to germinate at room temperature (30 ± 2°C) at 12 h dark and 12 h light cycle for four days in sterile petri dishes lined with four layers of filter paper. Sterile conditions were maintained by including 20 ppm of streptomycin sulphate in the incubation medium (water). The cotyledons harvested for two days were used for the isolation of CPR HG.

Preparation of the enzyme extract

Cotyledons were ground thoroughly in a mortar adding four volumes of chilled 0.05 M Tris-HCl buffer, pH 7.2,containing 2 mM β-ME. The extract was filtered through four layers of cheesecloth and the filtrate was centrifuged at 10000 g for 15 min at 4°C. The supernatant was used for isolation of the enzyme. All separation procedures were carried out at 4°C, unless otherwise stated.

Purification of CPRNS from germinating Niger cotyledons

The crude enzyme (endo-proteinase) extract was preparedas described above from 100 g of cotyledons (day 2) was subjected to ammonium sulfate precipitation. The precipitate obtained between 30% - 60% ammonium sulfate saturation was collected and dissolved in minimal amount of extraction buffer and extensively dialyzed against 0.01 M Tris- HCl buffer, pH 7.2 containing 2 mM β-ME at 4°C (1:200 volume ratio with 2 h change upto 12 h). The dialysate was loaded on DEAE-cellulose column equilibrated with 0.01 M Tris- HCl buffer, pH 7.0 containing 2 mM β- ME. The bound proteins were eluted with a linear gradient of increasing ionic strength of potassium chloride (0.5 M) in equilibration buffer and 5 ml fractions were collected. The fractions (14-20) with higher enzymatic activity were pooled and brought to 60% saturation with pulverized ammonium sulfate at 4°C and centrifuged. The pellet was dissolved in minimal volume of 0.02 M sodium acetate buffer, pH 5.5 containing 2 mM β-ME and dialyzed against the same buffer at 4 °C for 12 h. The dialysate was loaded on CM-Sephacelcolumn equilibrated with 0.02 M sodium acetate buffer, pH 5.5 containing 2 mM β-ME. The unbound fractions were collected and concentrated by lyophilization and stored at -20°C. Criteria of purity at each stage were checked by 10% SDS-PAGE.

Assay of *endoprotease*

Endoprotease (EP-NS) activity was measured by using chromogenic substrate, azocasein, following the method described by Sarath et al [41] with slight modifications. 0.25 ml of 1% azocasein (prepared in 0.02 M sodium acetate buffer, pH 5.5 containing 2 mM β-ME) was mixed with 0.15 ml of enzyme extract or 150 µg of purified enzyme (1 mg/ml) and incubated at 40°C for 1 hr. The reaction was arrested by adding 1.2 ml of 10% TCA and mixed thoroughly. The contents were allowed to stand for 15 min and centrifuged for 5 min at 3000 rpm. 1.2 ml of the supernatant was transferred to a tube containing 1.4 ml of 1 M

NaOH, mixed and the absorbance was read at 440 nm 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 1 cm cuvette under the conditions of the assay.

Determination of amino acid composition

The protein was hydrolyzed with 6N HCl for 24 h at 110°C in an evacuated sealed tube. The hydrolysate was filtered through Whatman No.1 filter paper and the filtrate was evaporated to dryness in a flash evaporator and the amino acid composition was analysed on model 119 CL Beckman, amino acid analyser. Cysteine residues were measured by performic acid method [42].

Iso-electric focusing and MW of CPRNS

IEF and 2D electrophoresis of purified enzyme was carriedout according to the method of O' Farrel [43].The molecular weight of the purified protease was determined by Sephadex G-100 gel filtration (2.4 × 132 cm column, flow rate 2 ml/min) and also through SDS-PAGE [44].

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. The effect of temperature on the enzyme activity was also determined at different temperatures ranging from 10-80°C for 1 h in 0.02 M sodium acetate buffer pH 5.5, containing 2 mM β-ME using the same assay as described.

Storage stability of enzyme

The storage stability of the of the purified enzyme (CPRNS) was investigated by storing the enzyme at different temperatures, -10°C, 4°C and 30°C and its stability for a week was tested by withdrawing an aliquots of the enzyme at different intervals of time and was assayed for the enzyme activity.

Time course study

The purified CPRNS and azo-casein were taken as described earlier and incubated at 40°C for 4 hr. Aliquots were withdrawn from incubation mixture at different intervals of time from 0 - 4 hrs and the azo compound liberated was measured as described.

Antisera preparation and Western blot analysis for developmental pattern of CPRNS in the cotyledons of germinating Niger seedlings

Polyclonal antibodies against the CPRNS were raised in rabbits by injecting 100 μg of the purified protein. Antibodies were used for immune inhibition, immune diffusion and

immunoblot to check their specificity (44).Extracts of the cotyledons from germinating seeds harvested at daily intervals for 4 days were subjected to SDSPAGE and the proteins were transferred on to a nitrocellulose paper. The proteins on nitrocellulose paper were treated with primary antibody (anti-CPRNS) and successively with secondary antibody (Ig-alkaline phosphotase). The bands were visualized by using NBT and BCIP in Tris HCl buffer, pH 9.0, containing 10 mM $MgCl_2$and 100 mM NaCl [44].

Effect of metal ions, selected inhibitors and sulfhydryl reagents

150 µg of CPRNS in 0.02 M sodium acetate buffer pH 5.5 was pre-incubated with 10 µl of different metal ions, selected inhibitors and sulfhydryl reagents at varied concentrations for 1 h at room temperature and the assay was initiated by the addition of azocasein and the activity assayed as described.

Effect of -SH reagents on pCMB treated EP- HG

pCMB (10 mM) treated protease was re-incubated prior to the assay with sulfhydryl reagent (5 mM and 10 mM) for 1 h 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.

Hydrolysis of endogenous and exogenous substrates

The relative digestibility of endogenous and exogenous proteins by the purified CPRNS was measured by incubating 1 ml of 1% protein (dry seed protein, BSA, casein, hemoglobin, gelatin) prepared in an appropriate buffer mixed with 0.15 ml of the purified CPRNS (1 mg/ml) and incubated at 40°C for 1 h and the amino acids released were estimated by ninhydrin method [45]. Presence of exopeptidase activity was checked by using L-Leu-pnitroanilideand CBZ-L-phenylalanine as described by Chrispeels and Boulter [46] and Sarath et al. [41], respectively.

In vitro digestion of seed proteins (SDS-PAGE analysis)

Suitable volumes of dry seed protein extract (1%) and CPRNS (1 mg/ml) were incubated as described, at various intervals aliquots were withdrawn from the digestion mixture, mixed with 2× sample buffer, boiled for 3 min and subjected to SDS-PAGE.

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