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Purification of Alkaline Protease from the Cotyledons of Germinating Indian Bean (*Dolichos lablab* L. var *lignosus*) Seeds

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Abstract: The present study reported the purification, characterization of alkaline protease and its possible involvement in moblization of storage proteins. The mobilization of seed storage proteins represents one of the most important post-germinative events in the growth and development of seedling. The proteolytic enzymes play a central role in the biochemical mechanism of germination. The alkaline protease from the cotyledons of 4-day old germinating Indian bean seedlings was purified to 198 folds by a four step procedure comprising-crude extract, $(NH_4)_2SO_4$ fractionation, DEAE-cellulose and finally casein-alginate affinity chromatography. The alkaline protease was shown to be homogeneity, as attested by a single protein band on both native PAGE and SDS-PAGE. It is a monomeric enzyme with molecular mass of 40 kDa and exhibited sharp pH optima at 8.8 with casein. It has been characterized by seeing the effect of various inhibitors and metal cofactors on the alkaline protease activity. The enzyme activity was markedly increased with Zn and significantly inhibited by the metal chelating agents-EDTA and 1, 10-phenanthroline. These results suggest that the alkaline protease is a metalloenzyme.

Key words: *Dolichos lablab,* proteolytic enzymes, alkaline protease, purification, characterization, protein mobilization, germination

INTRODUCTION

Seeds are endowed with food reserves required for providing nutrients for seedling growth and are mobilized during germination and post-germinative growth. In legumes 80% of the seed proteins may be of storage proteins. The mobilization of seed storage proteins represents one of the most important post-germinative events in the growth and development of seedling. The proteolytic enzymes play a central role in the biochemical mechanism of germination. Solvation of insoluble proteins, activation of pre-existing enzymes and/or *de novo* synthesis of enzymes, and degranulation of storage proteins are apparently a chain of events occurring mainly in the cotyledons of germinating seeds, leading to the transport of the products to the growing axis for the synthesis of new proteins and other nitrogenous compounds[1-4]. Numerous reports in which increase in activity of proteases are correlated with the breakdown of storage proteins support that these proteases are responsible for storage protein degradation[5-9]. Recent studies have also revealed the existence of more than one system of proteases in the same seed for the storage protein degradation, as there is a multiplicity of storage proteins in many seeds[10-12]. To study the mechanism of protein mobilization process, many have undertaken the task of purifying and characterizing a variety of proteases and peptidases, some of which occur only transiently in germinating seeds[4,13]. 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. For each enzyme, it is important to establish its role in protein degradation and the natural substrates need to be study *in vitro*. The realization of such approaches obviously requires purification of seed proteases, or at least their separation from each other.

The Indian bean (*Dolichos lablab* L. var *lignosus*) is a lesser known legume which has not received due attention by biochemists and nutritionists. We have previously presented the storage protein degradation with developmental profile of three (acidic, neutral and alkaline) types of proteolytic enzymes with different pH optima during 10 day germination period and also the purification of acidic thiol protease from the germinating Indian bean

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seeds[7,14,15]. In the present study we report the purification and characterization of alkaline protease and look at its possible involvement in the mobilization of storage proteins.

MATERIALS AND METHODS

Indian bean (*Dolichos lablab* L. var *lignosus*) seeds were obtained from the Agricultural form of Andhra Pradesh Agricultural University, Rekulakunta, Anantapur, Andhra Pradesh. Healthy seeds were sorted and surface sterilized with 0.1% $HgCl_2$ and rinsed thoroughly with sterile distilled water. The water imbibed seeds (12 h) were germinated in dark and light cycle at room temperature for 4 days in sterile petri dishes lined with moist filter paper. Sterile conditions were maintained by including 20 ppm of streptomycin sulphate in the incubation medium (sterile distilled water). Seedlings were withdrawn at the end of 4th day and used for further analysis.

Preparation of cotyledonary extract: The cotyledons were ground thoroughly in a pre-chilled mortar with chilled 0.05 M tris-HCl buffer, pH 7.2. The extract was filtered and centrifuged at 10000 rpm for 15 min. The supernatant was used for the estimation of proteins and assay of proteolytic enzymes.

Estimation of proteins: Protein content in the cotyledonary extract was estimated by the method of Lowry *et al.*[16]. The results were expressed as mg mL^{-1}.

Assay of proteolytic enzymes: Endopeptidase (alkaline) enzyme activity was measured by the modified method of Beevers[17] using casein as substrate. The reaction mixture containing 1 mL of diluted enzyme extract, 1 mL of 1% casein (prepared in 0.1 N NaOH, pH adjusted to 7.0 with 0.1 N HCl) and 1 mL of 0.025 M borate buffer pH 8.8. Incubation was carried out for 1 h at 40°C. The reaction was arrested by the adding 1 mL of 20% TCA. The contents of the tube were kept at 4°C for 15 min and centrifuged at 3000 rpm for 15 min and an aliquot of the supernatant was used for the determination of amino acids by ninhydrin method[18]. The enzyme units were expressed as μmoles of amino acids released per mL under experimental conditions.

Purification of an alkaline protease: A procedure for the purification of alkaline protease from Indian bean seeds has been standardized. Starting with seeds that had been germinated for 4 days and the following steps were carried out at 4°C. (i) 5 g of 4th day cotyledons of Indian bean was homogenized in 0.2 M tris-HCl buffer pH 7.2. A clear homogenate was obtained after centrifugation at 10000 rpm for 15 min. (ii) Cotyledonary extract was concentrated by Ammonium sulphate fractionation and the 20-40% saturated fraction, dissolved in minimum volume of tris buffer and dialyzed extensively with 0.025 M borate buffer pH 8.8. The dialyzed protein sample subjected to Ion-exchange chromatography on DEAE-Cellulose column (3X25 cm). The equilibrating buffer (tris buffer) was used for preliminary washing of unbound proteins. The protein elution pattern with linear gradient of increasing ionic strength of KCl (0-1 M) in equilibrating buffer was monitored spectrophotometrically at 280 nm. Fractions containing enzyme activity were pooled. (iii) The pooled fractions of DEAE-cellulose were collected and concentrated by $(NH_4)_2SO_4$ precipitation and dialysed. (iv) The pooled fractions were again subjected to pass through an affinity column of casein immobilized sodium alginate beads. The protein elution pattern KCl linear gradient was monitored through spectrophotometrically at 280 nm and fractions containing enzyme with high specific activity were pooled. All the enzyme activity recovered in the fractions were concentrated by lyophilization and stored.

Statistical analysis: Each value presented in results represents the arithmetic mean of five independent trials unless otherwise stated. The levels of significance were calculated by t-test[19].

RESULTS

It is previously reported that the extracts of 4th day germinating seedlings of Indian bean showed the three major classes of proteolytic activity with casein as substrate with pH optima 5.5, 7.3 and 8.8. The developmental profile of three proteases increased uniformly up to day 4 and then gradually fell with lesser rate of fall in alkaline protease and suggests that the alkaline protease involved in later stages of 10 day germination period in the storage protein mobilization. To investigate any possible relationship between the germination profiles of alkaline proteases in the process of storage protein mobilization, we purified the alkaline protease of the germinating Indian bean seeds.

Purification of an alkaline protease from germinating Indian bean seed: The cotyledons of 4 day germinating Indian bean used to isolate alkaline protease since the activity levels were high at this point. The activity measured by using the casein, absolute and specific activities calculated at each stage of purification.

Table 1: Purification of an alkaline protease from the cotyledons of Indian bean seeds

Purification step	Total protein	Total activity	Specific activity	Yield (%)	Purification fold
Crude extract	980.0	3120	3.18	100	1.0
$(NH_4)_2SO_4$ fractn	237.0	2548	10.75	82	3.4
DEAE-cellulose	28.0	1254	44.78	40	14.2
Casein-alginate	1.8	385	198.60	13	62.2

Cotyledons (5 g) obtained from 4th day germinating Indian bean seeds free from seed coats and embryonic axis, were used. Each value is the average of three purification steps

Table 2: Effect of various inhibitors, thiols and metal ions on alkaline protease

Agents	Concentration (mM)	Relative activity (%)
Control	--	100
DTNB	5.0	95
$HgCl_2$	5.0	92
EDTA	5.0	38
DEPC	1.0	89
DIPF	0.1	86
1, 10-phenanthroline	10.0	42
B-Mercaptoethanol	2.0	89
DTT	1.0	92
Zinc acetate	5.0	156
$MnCl_2$	5.0	115
$MgCl_2$	5.0	126
$CaCl_2$	5.0	118

Enzyme assays were carried out at 40°C for 1 h in a reaction mixture containing the indicated concentrations various inhibitors, thiol agents and metal ions in 0.025 mM borate buffer pH 8.8 and casein as a substrate. The results were expressed as percent inhibition or activation to control. Each value is the average of five independent values

Extraction of the enzyme from the cotyledons was carried out using acetate, borate, phosphate and tris buffer and the yield of the enzyme in terms of the absolute activity and specific activity maximum with 0.05 M tris-HCl buffer pH 7.2. The crude extract contains about 3120 enzyme units with a specific activity 3.18 enzyme units/mg. The precipitate obtained between 20-40% saturation recovered nearly 82% of the enzyme activity (Table 1). In the next step, the recovered enzyme binds to DEAE-cellulose and eluted by the KCl gradient. The purified enzyme was about 14 fold by this step. The final efficient step in the purification procedure is the fractionation on casein-alginate affinity column, the specific activity of the enzyme is very high (198 enzyme units/mg) and recovered 13% of enzyme. PAGE and SDS-PAGE analysis of the proteins at each step of purification shows that substantial qualitative difference between the proteins profiles of crude extract (data not shown). The homogeneity of the alkaline protease was demonstrated by non-denaturing and denaturing PAGE with single band.

Partial characterization of alkaline protease: The purified alkaline protease shows the maximal activity a sharp at pH 8.8. A temperature-activity profile indicated that the activity of the purified alkaline protease increased with temperature and exhibiting maximal activity at 50°C and then further increase in temperature resulted greater loss of activity. The enzyme activity was markedly increase by Zn. The metal chelating agents-EDTA and 1, 10-phenanthroline, showed a significant inhibition in the absolute activity (Table 2). These results suggest that the alkaline protease is a metalloenzyme.

DISCUSSION

The mobilization of seed storage proteins represents one of the most important post-germinative events in the growth and development of seedling and the proteolytic enzymes play a central role in the protein mobilization process. To study the mechanism of this physiologically vital process, many have undertaken the task of purifying and characterizing a variety of proteases and peptidases, some of which occur only transiently in germinating seeds[4,20-22]. We previously reported the positive correlation between the developments of acid, neutral and alkaline proteases with protein depletion suggest the involvement of these proteases in the storage protein degradation during germination of Indian bean seeds[7]. The mobilization of storage proteins in germinating Indian bean, as well in other plant sources, is initiated by endoproteases which convert the water insoluble storage proteins into soluble peptides that can be further hydrolyzed to aminoacids by exopeptidases[2,4,23]. Although all four classes (cys, ser, metallo and aspartic proteases) have been shown to occur in plant seeds, most described to date are cysteine proteases[4,11,15,19,24] and less information is available on the alkaline proteases purification and their role in protein mobilization.

In the present study, the most widely used ammonium sulphate fractionation was carried out directly with the crude extract and considerable amount of enzyme was recovered in the precipitate obtained by fractionation in between 20-40% saturation. This enzyme further on DEAE-cellulose and casein-alginate columns, purification resulted in a higher activity recovery with lesser contamination of other proteins. The enzyme preparation recovered in each step was judged by native PAGE and SDS-PAGE. The present purification procedure yields overall recovery of 13% alkaline protease (Table 1). Similar or even lower yields are common for proteases from other germinating seeds, i.e., vicillin peptidohydrolase (8.0%) from mung bean seedlings[21], EP-HG (4.5%) from horse gram seedlings[19], acidic protease (15%) from germinating winged-bean seeds[11] and 11% recovery of acidic protease from germinating Indian bean[15]. Separation of alkaline protease from the crude extract directly on casein-alginate affinity column by avoiding all purification

steps, increases the maximum recovery (48%) with high specific activity 283 compared to all purification steps.

The molecular weight of the purified alkaline protease from Indian bean germinating seeds was 40 kDa. However, in our previous paper we reported Mr 32 kDa for acidic protease in the same seeds[15]. A group of four proteinases isolated from germinating maize seeds were having Mr range 28-33 kDa[25]. A serine protease with 50-60 kDa and the Mr of the cysteine endoproteases isolated from *Vigna mungo* varied between 20-30 kDa[22], from buck wheat seeds 34 kDa[26]. A temperature-activity profile indicated that the alkaline protease has optimal activity at 50°C. Temperature profiles for the proteases from *P. vulgaris*[27] and barley[28] with temperature optima at 45°C. The loss of activity with increases in temperature at 55°C and above is possibly due to denaturation of both the substrate and the enzyme. Inhibitors specific to each class of proteinases were employed in order to investigate the amino acid residues contributing to the active site of the purified enzyme. The findings (Table 2) that the enzyme was either activated by metal ions like Zn, Mg^{2+} or inactivated by EDTA and 1, 10-phenanthroline led to the conclusion that the enzyme is a metalloenzyme. A few metalloproteases have been identified in plant seeds like buck wheat and pumpkin[26,29]. The Mg^{2+} activated protease was observed in bean[30] and Ca^{2+} activated protease was also observed in winged-bean seeds and root culture of Arabidopsis[11,31]. Further research is under progress in the characterization of the alkaline protease and identification of natural substrates with in seeds and their utilization during germination of Indian bean.

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