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Full Length Research Paper

Purification of acidic protease from the cotyledons of germinating Indian bean (*Dolichos lablab* L. var lignosus) seeds

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The positive correlation between the developments of acid, neutral and alkaline proteases (azocaseinolytic) with protein depletion suggest the involvement of these proteases in the degradation of proteins in germinating Indian bean. These proteases increased in the early stages of germination and decreased later. However, the activity of acid proteases was higher throughout the germination period compared with the activities of neutral and alkaline proteases. The acid protease from the cotyledons of 4-day old germinating Indian bean seedlings was purified to 152 folds by a five step procedure comprising - crude extract from cotyledons, $(NH_4)_2SO_4$ fractionation, DEAE-cellulose, CM-cellulose and finally casein-agarose affinity chromatography. The molecular mass of acidic protease is 32 kDa.

Key words: *Dolichos lablab*, proteolytic enzymes, acidic protease, purification, protein mobilization, germination.

INTRODUCTION

The plant seed is not only an organ of propagation and dispersal but also the major plant tissue harvested by humankind. The amount of protein present in seeds varies from ~10% (in cereals) to ~40% (in legumes), forming a major source of dietary protein. The mobilization of seed storage proteins represents one of the most important post-germinative events in the growth and development of seedling. Proteolytic enzymes play central role in the biochemical mechanism of germination (Bewley and Black, 1994; Shewry et al., 1995; Muntz 1996). Numerous reports in which increase in activity of proteases are correlated with the breakdown of storage proteins support that these proteases are responsible for protein degradation (Storey and Beevers, 1977; Nandi et al., 1995; Senyuk et al., 1998; Rajeswari and Ramakrishna Rao, 2002; Ramakrishna and Ramakrishna Rao, 2004). 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 (Ashton, 1976; Davy et al., 1998; Shutov and Vaintraub, 1987). 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. Hence, in the present paper we report the pattern of

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changes in the protease (azocaseinolytic) activity during germination and purification of acidic protease, the major storage protein degrading endoprotease from the germinating Indian bean (*Dolichos lablab* L. var lignosus) seeds.

MATERIALS AND METHODS

Indian bean (*Dolichos lablab* L. var lignosus) seeds were procured from the 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 10 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. Seedlings were withdrawn at designated time intervals and used for analysis. Each experiment was carried out at least five times and each analysis was carried out in duplicate and averaged, unless otherwise stated.

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 containing 2 mM -mercaptoethanol. 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 (1951). The results were expressed as mg/2 cotyledons.

Assay of proteolytic enzymes

Endopeptidase activity was measured by using chromogenic substrate, azocasein, following the method described by Sarath et al. (1989) with slight modifications. 0.25 ml of 1% azocasein (prepared in 0.02 M sodium acetate buffer pH 5.5 containing 2 mM -mercaptoethanol) was mixed with 0.15 ml of enzyme extract. The reaction mixture was incubated at 40°C for 1 h. The reaction was arrested by adding 1.2 ml of 10% TCA and mixed thoroughly. The contents were centrifuged, 1.2 ml of 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 is defined as the amount of the enzyme required to produce an absorbance change of 1.0 in 1 cm cuvette under conditions of the assay.

Purification of an acidic protease

A procedure for the purification of acidic 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 4^{th} day cotyledons of Indian bean was homogenized in 0.2 M tris-Hcl buffer pH 7.2, containing 2 mM -mercaptoethanol. A clear homogenate was obtained after centrifugation at 15000 g for 15 min. (ii) Cotyledonary extract was concentrated by ammonium sulphate fractionation and the 40 – 60% saturated fraction, dissolved in minimum volume of tris buffer and dialyzed extensively with 0.2 M acetate buffer pH 5.5. The dialyzed protein sample subjected to ion-exchange chromatography on DEAE–cellulose column (3 cm X 25 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 – 0.5 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. This purified protein is passed through a CM-cellulose column (3cm X 25 cm) and eluted the protein with equilibrating buffer and KCl gradient. The eluted protein was monitored at 280 nm and colleted fractions containing enzyme activity. (iv) The pooled fractions were again subjected to $(NH_4)_2SO_4$ precipitation and dialysis and pass through an affinity column of casein-agarose. 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.

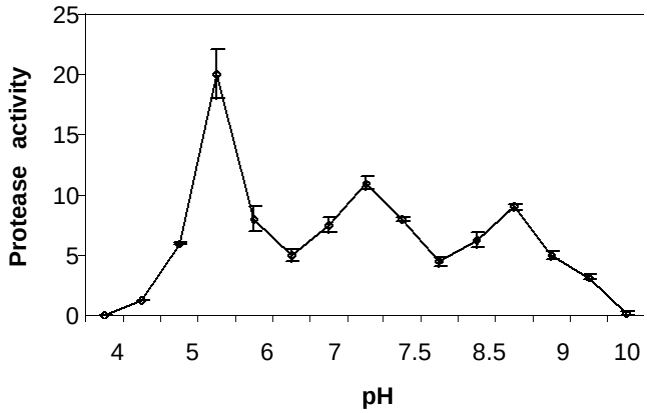

Figure 1. Effect of pH on the protease (azocaseinolytic) activity.

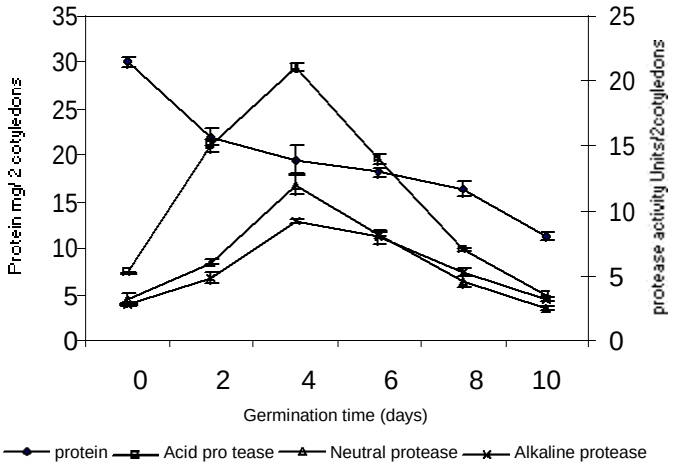

Figure 2. Proteolytic activity and protein profile during germination of Indian bean seeds.

Table 1. Summary of the purification of an acid protease from the cotyledons of germinating Indian bean seeds.

Purification Step	Total protein (Mg)	Total activity (Units)	Specific activity (Units/mg)	Yield (%)	Relative purification
Crude extract	1080	460	0.42	100	1.0
$(NH_4)_2SO_4$ Fractionation	375	404	1.06	89	2.5
DEAE-cellulose	42	154	3.65	34	8.6
CM-cellulose	2.4	89	37.08	20	88
Casein-agarose	0.9	58	64.5	12	152

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.

RESULTS

Identification of major classes of proteases

In order to standardize a suitable assay system for the proteases, preliminary studies were carried out using a 4-day germinated Indian bean cotyledonary extract as source of enzyme. Various substrates, such as casein, azocasein, Bovine serum albumin (BSA), and haemoglobin, were tested as possible proteolytic enzyme substrates. Azocasein was found to be a versatile and sensitive substrate, suitable for routine assays. With this assay system, the pH vs. protease activity relationship was mapped out for the proteases from 4-day germinated Indian bean seeds. The three major classes of proteolytic activity can be distinguished: 1st in acidic, 2nd in neutral and 3rd in alkaline region (Figure 1). DTT and -mercaptoethanol both enhance the acidic protease activity several fold. The activity of acid endopeptidase was not affected by preincubation with Mg^{2+}, Ca^{2+}, and EDTA (data not shown).

Changes in the levels of protease(s) during germination

The developmental profile of three proteases (acid, neutral and alkaline) is depicted in Figure 2. The activities of all the three proteases increased uniformly up to day 4 and then gradually fell. However, the activity of acid proteases was higher throughout the germination period compared with the activities of neutral and alkaline proteases. To investigate any possible relationship between the germination profiles of the acidic proteases in the process of storage protein mobilization, we purified the acid protease of the germinating Indian bean seeds.

Purification of an acidic protease

The 4- day cotyledons of germinating Indian bean used to isolate acidic protease since the activity levels were high at this point. The activity measured by using the chromogenic substrate, azocasein, absolute and specific activities calculated at each stage of purification. The results of the purification summarized in Table 1. The crude extract contains about 460 units with a specific activity of 0.42 units/mg. The precipitate obtained between 40 – 60% saturation recovered nearly 89% of the enzyme activity. In the next step, the recovered enzyme binds to DEAE-cellulose and eluted by the KCl gradient. The purified enzyme was about nine fold by this step. The unbound protein fractions of CM-cellulose showed the maximal protease activity than bound protein, which eluted through KCl linear gradient. The CM-cellulose bound protein fraction was not used in further investigation as it was contaminated with other proteins. The final efficient step in the purification procedure is the fractionation on casein-agarose affinity column, the specific activity of the enzyme is very high (64.5 units/mg) and recovered 12% 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 (results not shown). The molecular weight of the purified enzyme was estimated to be 32 kDa.

DISCUSSION

The positive correlation between the developments of acid, neutral and alkaline proteases (azocaseinolytic) with protein depletion suggest the involvement of these proteases in the degradation of proteins in germinating Indian bean. The mobilization of storage proteins in germinating Indian bean seeds, 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 amino acids by exopeptidases (Callis, 1995; Mikola, 1983; Shutov and Vaintraub, 1987). 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 (Ryan and Walker-Simmons, 1981; Rajeswari,

1997; Usha and Singh, 1996; Schlereth et al., 2001). Most of the cysteine proteases have acidic pH optima *in vitro* suggesting that they are localized in the vacuole *in vivo* (Muntz et al., 1985; Wilson, 1986; Shutov and Vaintraub, 1987).

In the present study, the most widely used ammonium sulphate fractionation was carried out directly with the crude extract. Considerable amount of enzyme was recovered in the precipitate obtained by fractionating with $(NH_4)_2SO_4$ between 40 - 60% saturation. DEAE-cellulose and CM-cellulose and casein-agarose columns purifications resulted in a higher activity recovery with lesser contamination of other proteins. The enzyme fraction from each step of purification was concentrated by $(NH_4)_2SO_4$ and followed by dialysis. Poulle and Jones (1988) used fractionation on CM-cellulose effectively for purification of endoprotease from germinating barley and could recover more than 40% of the activity which was not adsorbed in the column effluent. The enzyme preparation recovered in each step was judged by PAGE and SDS- PAGE, in which the enzyme gave a single band (not shown). The present purification procedure yields overall recovery of 12% acid protease. Similar or even lower yields are common for acidic proteases from other germinating seeds, such as vicillin peptidohydrolase (8.0%) from mung bean seedlings (Baumgartner and Chrispeels, 1977), EP-HG (4.5%) from horse gram seedlings (Rajeswari, 1997), acidic protease (15%) from germinating winged- bean seeds (Usha and Singh, 1996) and EP-1 (1.6%) from barley seedlings and GA_3-induced cysteine protease (3.38%) from barley aleurone layers (Koehlar and Ho, 1988).

The molecular weight of the purified enzyme from Indian bean germinating seeds (32 kDa) is in close agreement with that of the cysteine proteinases isolated from germinating barley (Poulle and Jones, 1988; Philips and Wallace, 1989), wheat (Bottari et al., 1996) and from some other leguminous seeds (Rajeswari, 1997; Usha and Singh, 1996). However, the molecular weight of the cysteine endoproteases isolated from *Vigna mungo* varied between 20-30 kDa (Mitsuhashi et al., 1986), and is 34 kDa from buck wheat seeds (Belozersky et al., 1990). In barley, proteases of 30, 31, 32, and 37 kDa have been obtained (Wrobel and Jones, 1992). Further work is under progress in the characterization of the acidic protease and identification of natural substrates with in seeds and their utilization during germination of Indian bean.

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