Bowman−Birk Type Proteinase Inhibitor Profiles of Horse Gram (*Dolichos biflorus*) during Germination and Seed Development

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Germination induces changes in the Bowman−Birk type proteinase inhibitors (BBIs) of horse gram (*Dolichos biflorus*) at both qualitative and quantitative levels. The original isoinhibitors present in the dry seed almost disappear by the fifth day of germination with the concomitant appearance of two new inhibitor species. The isoinhibitors present in the dry seed are electrophoretically distinct from the isoinhibitors that appear during the early stages of seed development. The two inhibitor species occurring in the early stages of seed development (up to 10 days after flowering) are also present in the flower, husk, and leaf. The two new inhibitor species that appear during the process of germination appear to be electrophoretically similar to the inhibitor species found in the flower, leaf, and early stages of seed development.

Keywords: *Dolichos biflorus; leguminoseae; horse gram; germination; seed development; Bowman−Birk inhibitor; proteinase inhibitor*

INTRODUCTION

Legume seeds contain a number of proteinase inhibitors that suppress the activities of serine proteinases (Laskowski and Kato, 1980; Liener, 1982). Extensive investigations have been carried out on two classes of these inhibitors, the Kunitz and Bowman−Birk (BBI) families of inhibitors (Ikenaka and Norioka, 1986). Information related to the physiological role of plant BBIs is lacking, although they are one of the most well studied classes of proteins with respect to structure and mechanism of action in vitro. Some of the suggested functions of the proteinase inhibitors include their role as storage proteins, their regulation of endogeneous proteinases, or their acting as protective agents against insect or microbial predators (Ryan and Green, 1974). Recently it has been demonstrated that the proteinase inhibitors of pigeon pea have neither a storage role nor a role in controlling the endogeneous proteinase activity (Godbole et al., 1994). Several studies on the appearance or disappearance of electrophoretically distinct proteinase inhibitors during germination have appeared (Pusztai, 1972; Freed and Ryan, 1978; Wilson, 1988). However, information pertaining to the spatial and temporal synthesis of these inhibitors is limited (Ambekar et al., 1996; Harsulkar et al., 1997). To understand the physiological functions of these inhibitors, studies related to their spatial and temporal synthesis are very important since insect attacks can devastate developing and mature seeds. Biosynthesis and degradation of proteins in general, and proteinase inhibitors in particular, play an important role in the regulation of nitrogen sink/source relationships, which are controlled by developmental and environmental factors (Muntz, 1994).

Four BBI type isoinhibitors specific for trypsin and chymotrypsin from horse gram (*Dolichos biflorus*) have been purified and characterized (Sreerama et al., 1997). The complete primary structure of HGI−III, the major isoinhibitor, has been determined (Prakash et al., 1996). In addition, the two independent reactive sites for trypsin and chymotrypsin and the antigenic determinants have been mapped (Sreerama and Gowda, 1997). In this paper, we report the changes that take place in the isoinhibitors of horse gram during germination and the isoinhibitor profile during seed development. During germination there is a rapid appearance of two new active species of the inhibitor while the inhibitor species present in the ungerminated seed rapidly disappears. We further demonstrate that the two new inhibitor forms which appear upon germination are electrophoretically similar to the two inhibitor species present in the flower and early stages of seed development.

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MATERIALS AND METHODS

Plant Material and Reagents. Horse gram (*D. biflorus*) seeds were procured from the local market. Bovine pancreatic trypsin (2× crystallized, type III), bovine pancreatic α-chymotrypsin (3× crystallized, type II), *N*-α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA), *N*-benzoyltyrosine ethyl ester (BTEE), acetyl-DL-phenylalanine β-naphthyl ester (APNE), and tetrazotized *o*-dianisidine were obtained from Sigma Chemical Co. (St. Louis, MO).

Germination of Seeds and Collection of Plant Tissues. Seeds were imbibed overnight in 50 g batches in distilled water, and germination was continued on moist filter paper in the dark. After the desired period of germination, the seeds were rinsed with distilled water and the cotyledons dissected free of the seed coats and axes. Cotyledons were frozen at −20 °C until used. Germination time was recorded relative to the beginning of imbibition.

Development of Seeds and Collection of Plant Tissues. The flowers were tagged on the day of opening. The pods were harvested 3, 6, 10, 15, 20, 30, and 40 days after flowering (DAF). The vegetative pods along with the seeds were used up to 10 DAF. Seeds were separated from pods of 15, 20, 30, and 40 DAF stages. Leaves were removed from the mature

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plant. The seeds, husks, and other plant materials were frozen at −20 °C until used.

Preparation of Plant Extracts. Crude inhibitor fraction was prepared from the seeds, cotyledons, flowers, husk, and leaves. The cotyledons and other plant tissues were homogenized with 5 volumes (w/v) of 0.1 M glycine−HCl buffer, pH 2.5, in a Waring blender. The meal was then extracted by stirring overnight at 4 °C. The extract was filtered through several layers of cheesecloth and then centrifuged at 10000*g* at 4 °C for 30 min. The pH of the supernatant was brought to 7.5 with ammonia. Extracts of unimbibed seed were prepared in a similar manner, except that the whole seeds were ground to a fine meal and then extracted with 250 mL of buffer/50 g of seeds.

Protein and Proteinase Inhibitor Assay. Protein concentrations were measured according to the method of Bradford (1976). The amidolytic activity of trypsin and its inhibition was assayed using BAPNA as the substrate (Kakade et al., 1969). One trypsin unit is defined as an increase in absorbance of 0.01 at 405 nm under the conditions of assay. The inhibitory unit is defined as the number of trypsin units inhibited under the assay conditions. Esterolytic activity of chymotrypsin and its inhibition were determined using BTEE as the substrate (Humel, 1959). One unit of chymotrypsin activity is defined as the activity resulting in the hydrolysis of 1 μmol of BTEE/min under the conditions of assay. One inhibitory unit is defined as 1 unit of enzyme inhibited.

Electrophoretic Analysis. Extracts of developing and germinating horse gram seeds and extracts of leaves, flowers, and husk were analyzed by native PAGE (10% T) using a Davis buffer system (Davis, 1964). Staining of inhibitory activity was performed following the procedure of Uriel and Berges (1968) with some modifications. After electrophoresis, the gel was immersed in trypsin or chymotrypsin (5 mg/mL in 0.1 M sodium phosphate buffer, pH 7.4), for 20 min at 37 °C. The gel was then rinsed with water and stained in a solution containing 0.7 mM acetyl-DL-phenylalanine β-naphthyl ester (APNE) and tetrazotized *o*-dianisidine (0.5 mg/mL) in 0.1 M sodium phosphate buffer, pH 7.4, and allowed to develop.

RESULTS

Appearance of New Bowman−Birk Proteinase Inhibitors during Germination. The inhibitory activity retained after 24 h of germination (≈80% for trypsin and chymotrypsin) has already begun to decrease, as compared to that of the imbibed seeds (Figure 1) (overnight imbibed seeds have been considered as 0 h of germination). However, the inhibitory activity fell dramatically to ≈60% after 48 h of germination. Thereafter, there was a steady decline, with ≈5% of activity retained in the 5-day-old seedlings. In contrast, the decline in total protein content was gradual over the entire period of germination.

Discontinuous PAGE of the crude extracts shows that a number of changes occurred in the inhibitor proteins. The gels stained for trypsin and chymotrypsin inhibitory (Figure 2) activity indicate that the imbibed seed (0 h of germination), like the dry seed, contains four inhibitor bands (R_f 0.41, 0.49, 0.55, and 0.74) (Figure 2A). Of these, the inhibitor having an R_f of 0.55 is the major isoinhibitor, HGI−III, the primary structure of which has been determined (Prakash et al., 1996). After 48 h of germination, there is a decrease in the intensity of the major isoinhibitor (R_f 0.55) and a nearly complete disappearance after 120 h. The isoinhibitor band with the highest R_f, 0.74, declines to a very low level after 120 h of germination. The appearance of a new inhibitor species (R_f 0.33) occurs at 24 h, and thereafter there is an increase in the staining intensity between 96 and 120 h. The second new inhibitor, with R_f 0.20, is observed at 48 h and thereafter. The two slow-moving inhibitor species (R_f 0.33 and 0.20) are the major forms in the 5-day cotyledons.

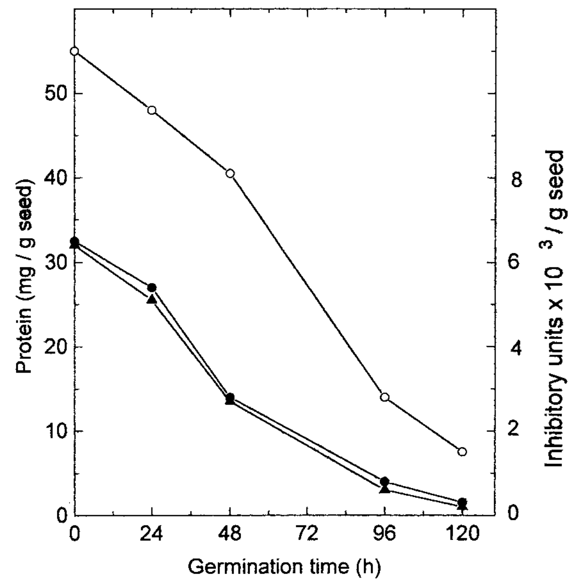

Figure 1. Effect of germination on the trypsin and chymotrypsin inhibitory activities of horse gram inhibitors: (○) protein as milligrams per gram of seed; (●) trypsin inhibitory activity; (▲) chymotrypsin inhibitory activity.

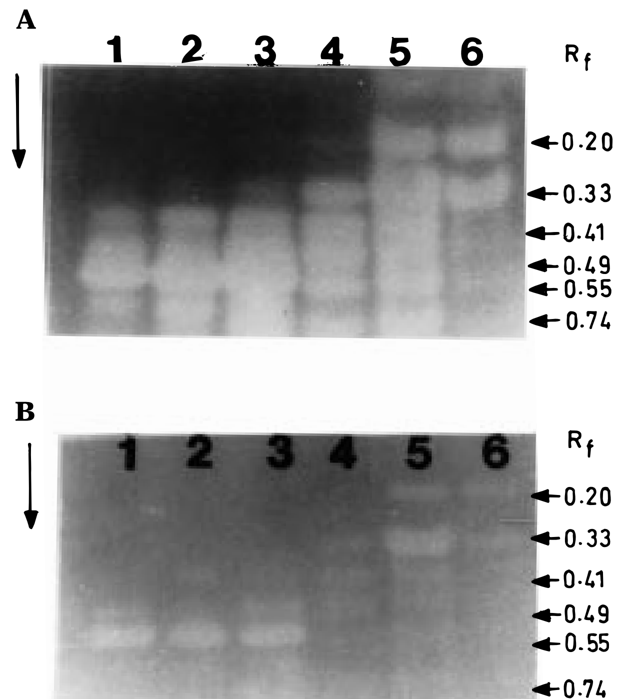

Figure 2. Native PAGE of the crude extracts from germinating horse gram seed stained for inhibitory activity: (A) trypsin inhibitory activity; (B) chymotrypsin inhibitory activity. Lanes 1−6 are extracts from unimbibed seeds and from seeds after 0, 24, 48, 96, and 120 h of germination, respectively (∼5 μg of protein was loaded in each lane). The inhibitory activity staining shows clear bands against a dark pink background.

Analysis of the Inhibitor during Seed Development. A steep increase in the inhibitory activity with a concomitant increase in the protein content is observed from 10 DAF (Figure 3). In contrast, there is no significant change in the inhibitory activity and a marginal increase in the protein content during the intial stages of development (0−10 days, with flower

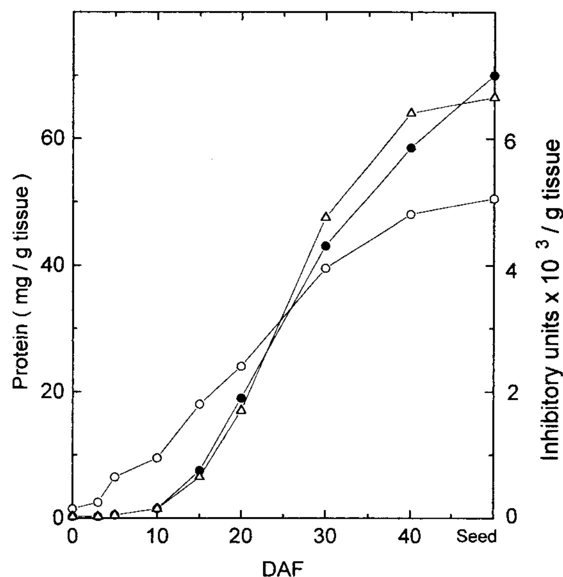

Figure 3. Trypsin and chymotrypsin inhibitory activities during the course of seed development of horse gram: (○) protein as milligrams per gram of initial dry weight; (△) trypsin inhibitory activity; (●) chymotrypsin inhibitory activity.

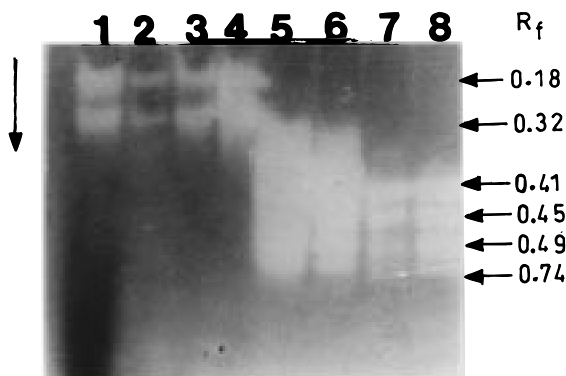

Figure 4. Development of the isoinhibitors in horse gram, as studied by native PAGE, trypsin inhibitory activity. Lanes 1−8 are extracts from the flower and 3, 6, 10, 15, 20, 30, and 40 DAF (the whole vegetative pod was used for 3, 6, and 10 DAF and only seeds thereafter), respectively (~5 μg of protein was loaded in each lane). The inhibitory activity staining shows clear bands against a dark pink background.

being considered 0 day). Using specific substrates, the inhibitory activity of trypsin was measured in the presence of excess chymotrypsin and vice versa (Figures 1 and 3). The inhibitors from the flowering stage inhibit both trypsin and chymotrypsin simultaneously and independently.

The extracts of the flower exhibit two inhibitors (R_f 0.18 and 0.32) as revealed by the inhibitory activity staining technique (Figure 4). The electrophoretic mobilities of these two species appear to be similar to those of the only two inhibitor species present after 5 days of germination (Figure 2). During the initial stages of seed development (up to 10 days) only these two forms of the inhibitor are visible (Figure 4; R_f 0.18 and 0.32). The extracts at these stages were made from vegetative pods that contained the immature seeds. The mature isoinhibitor forms (as seen in the dry seed) appear only after 15 DAF, with the intensity of inhibitor stain increasing as the seed matured, concomitant with the disappearance of the inhibitor forms initially present in the vegetative pods. It is also observed that among

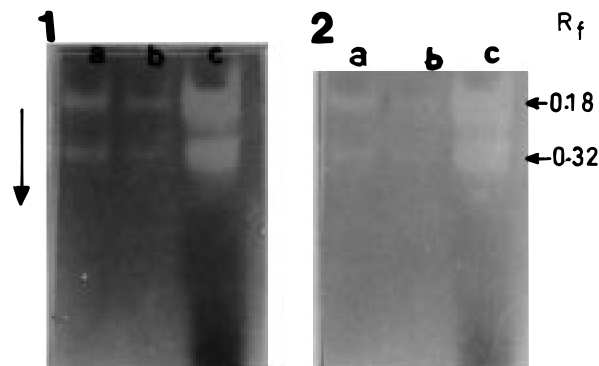

Figure 5. Native PAGE of the crude extracts of leaf, husk, and flower stained for inhibitory activity: (1) trypsin inhibitory activity; (2) chymotrypsin inhibitory activity. Lane a, leaf; lane b, husk; lane c, flower. The inhibitory activities were detected as clear bands on a dark pink background (~5 μg of protein was loaded in each lane).

the mature inhibitor forms (30 and 40 DAF) the major isoinhibitor is that with an R_f 0.49, which is HGI−III (Figure 4).

Polyacrylamide gel electrophoresis of the 15 DAF husk extracts revealed the presence of only two inhibitors (R_f 0.18 and 0.32), which also occur in the flower and leaf (Figure 5). However, these inhibitors disappear completely from the husk between 20 and 30 DAF (results not shown).

DISCUSSION

Germination has been suggested as an inexpensive and effective technology for improving the quality of legumes, by enhancing their digestibility (Reddy et al., 1989), increasing the level of amino acids (Chang and Harrold, 1988), and reducing the contents of antinutritional factors [Ghorpade and Kadam, 1989; Vidal-Valverde and Frias, 1992; reviewed by Liener (1994)]. Studies of legume proteinase inhibitor metabolism during seed germination and seed maturation are hindered by the multiplicity of homologous inhibitors (Haynes and Feeney, 1967; Wilson and Laskowaski, 1973; Odani and Ikenaka, 1977; Domoney et al., 1995). Four isoinhibitor species in ungerminated horse gram seed have been purified and characterized (Sreerama et al., 1997). On germination, these four inhibitors disappear with the appearance of new inhibitor species with reduced mobility (Figure 2). The retarded mobility of these inhibitors reflects the decrease in the net negative charge of the polypeptides. Comparison of the determined sequences of HGI−III and other BBIs (Prakash et al., 1996) shows that the amino- and carboxy-terminal ends are "ragged", which reflects proteolytic processing at both ends. The amino-terminal sequences of horse gram inhibitors typically contain large quantities of aspartyl and glutamyl residues (Sreerama et al., 1997), the loss of which would decrease the net negative charge. Sequence determination of the electrophoretically distinct inhibitors (MBI-E and MBI-F), which appear upon mung bean germination, are reported to be products of limited carboxy-terminal proteolysis of the major inhibitor MBI-F present in the dormant seed (Wilson and Chen, 1983). Similar results have been reported for soybean BBI (Tan-Wilson et al., 1982). It seems possible that both amino-terminal processing and carboxy-terminal processing of HGIs occur during germination by the action of nonspecific

endoproteinases. Recently, an endoproteinase from germinating horse gram has also been reported (Rajeswari et al., 1996).

Domoney et al. (1995) reported the presence of a relatively stable trypsin inhibitor mRNA in the dry pea seeds. However,whether such stored mRNA can be utilized and translated into the new inhibitor forms on germination as observed in horse gram (Figure 2) is not clear. The two inhibitor species detected in the flower and pod (up to 10 DAF) could be newly synthesized proteins, expressed by the genes other than those that code for the inhibitor species found in dry seed or are products of the same gene, from which the inhibitors of the mature seed are derived by post-translational modifications. The inhibitor formation during seed maturation in horse gram, similar to that in kidney bean (Pusztai, 1972) mung bean (Lorensen et al., 1981), and adzuki bean (Yoshikawa et al., 1979), appears to follow the latter event. A steep increase in trypsin inhibitor activity during the very late stages of horse gram embryogenesis (Figure 3) is associated with the appearance of four isoforms (Figure 4).

The proteinase inhibitors in pigeon pea (Ambekar et al., 1996) and chickpea (Harsulkar et al., 1997) are late-synthesizing proteins, appearing only after 11 and 20 DAF, respectively. In contrast, two distinct proteinase inhibitor species are present as early as the flower itself in horse gram (Figure 5). Most insects attack pods at an early stage when they contain developing seeds. The presence of these distinct inhibitors at the very early stages of seed maturation and vegetative parts of the *D. biflorus* plant (Figure 5) may be vital to the natural plant defense and for their potential usefulness in protecting the developing seeds against herbivores.

The two new inhibitor forms that appear after germination (Figure 2; R_f 0.20 and 0.33) and the two forms present in the leaf, flower, husk, and early stages of seed development (R_f 0.18 and 0.32) have apparently similar electrophoretic mobilities and inhibit both trypsin and chymotrypsin (Figure 5). It is not clear whether these forms are products of the same gene or not. This paradox can be resolved only by purification and structural characterization of these new inhibitor forms, which are in progress.

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