

Rapid Release of Protease Inhibitors from Soybeans

IMMUNOCHEMICAL QUANTITATION AND PARALLELS WITH LECTINS

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ABSTRACT

Specific antisera were prepared against the Bowman-Birk trypsin inhibitor and four other trypsin inhibitors of low molecular weight isolated from soybeans (*Glycine max* L. cv. Tracy). These antisera were used to detect the presence and amount of the inhibitors in: (a) seeds and protein extracts of soybean meal; (b) seedlings; and (c) the water surrounding the seeds and roots of seedlings. Lectin activities in seeds, seedlings, and water were also determined at the same time as the protease inhibitor activities. By competitive inhibition of immunoprecipitation, the combined five low molecular weight protease inhibitors were found to constitute the following percentages of proteins (w/w): 6.3% in defatted soybean meal; 8.1% of the protein extracted from the meal by a buffer of pH 8.6; 8.3, 14.7, 15.2, 16.1, 17.2, and 18.9% of the protein in a lyophilisate of water in which seeds were incubated for 4, 8, 12, 16, 20, and 24 hours, respectively; 8.2% in a lyophilisate of water in which roots of seedlings grew for 20 days; 1.5% in cotyledons; and less than 0.1% in epicotyls, hypocotyls, and roots of 12-day-old seedlings. Hemagglutination activities, expressed as the lowest amount of protein required to give a positive agglutination of 0.2 ml of 2% rabbit red blood cells, were as follows: purified soybean lectin, 0.08 μ g; lyophilisate of water in which seeds were incubated for 4, 8, 12, 16, 20, and 24 hours, 10, 2.5, 5, 5, and 2.5 μ g, respectively; lyophilisate of water in which roots grew for 20 days, 5 μ g; 12-day-old cotyledons, roots, epicotyls, and hypocotyls, 12.5, 100, > 1,000, and >500 μ g, respectively. The results indicate that a large amount of protease inhibitors as well as lectins are released from seeds during the first 8 hours of imbibition. Neither lima bean trypsin inhibitor (mol wt, 10,000) nor Kunitz soybean trypsin inhibitor (mol wt, 21,500) showed competitive inhibition in tests with antisera against low molecular weight soybean protease inhibitors.

rhizosphere. In 1973, Ryan (24) summarized the postulated roles of plant proteinase inhibitors: they may act as storage proteins, as regulators of endogenous proteinases, or as protective agents against insect and/or microbial proteinases. Knowledge of their influence on the rhizosphere is scanty or lacking. More recent summaries of the possible roles of lectins may be found in the review by Liener (16) and the paper by Clarke *et al.* (8). Based upon the fact that lectins make up from 2 to 10% of the total protein in most leguminous seeds, Liener suggested that they must play some important physiological role in the plant (16). Several recent studies (8, 9, 16) have contributed data pertinent to the influence of lectins in the rhizosphere. The present paper is an extension of the recent finding in soybean (14, 15) of four new protease inhibitors in the mol wt range of the Bowman-Birk inhibitor (10, 18). The low mol wt protease inhibitors are proteins with very unusual amino acid compositions. They contain about 20% of S-containing amino acids, especially methionine, the most limited essential amino acid in soybean seeds. It is important to find out the distributions and mode of actions of these protease inhibitors in the life cycle of soybeans in order to elucidate the physiological roles of these proteins in the legume seeds. In this report, we describe the quantitative estimation of the low mol wt inhibitors by means of competitive inhibition of immunoprecipitation in the following protein sources: (a) SBM⁴; (b) proteins extractable from SBM by buffer (76 mM tris-HCl [pH 8] containing 5 mM 2-mercaptoethanol); (c) proteins released from seeds during the first 24 hr of imbibition; (d) proteins released from roots of seedlings into the surrounding water over a 20-day period; and (e) various plant parts, *i.e.* epicotyls, hypocotyls, cotyledons, and roots of 12-day-old seedlings. We also extend the recent study of the release of lectins from imbibing seeds (9) to include growing plants by determining hemagglutinating activity not only during imbibition by seeds but also during certain stages of seedling growth.

MATERIALS AND METHODS

Four- to 5-month-old male New Zealand rabbits were purchased from The Jackson Laboratory, Bar Harbor, Maine; goat anti-rabbit γ -globulin was obtained from Cappel Laboratories, Downington, Pa.; lima bean trypsin inhibitor and Kunitz soybean trypsin inhibitor came from Worthington Biochemical Corporation, Freehold, N.J.; and [¹²⁵I]sodium iodide was the product of Amersham/Searle Corp., Arlington Heights, Ill. Purified soybean protease inhibitors of low mol wt (PI I-V), [¹²⁵I]-labeled antigens, and antisera were obtained as described previ-

Although trypsin inhibitors and lectins have been intensively studied in plants, particularly in seeds, relatively little is known of their role in the plant and their influence on the plant's

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⁴ Abbreviations: SBM: defatted soybean meal; PBS: phosphate-buffered saline; PI: protease inhibitors.

ously (15). SBM was prepared from soybeans (*Glycine max* L. cv. Tracy) by grinding the seeds and sieving the meal through a 40-mesh screen. The meal was defatted by hexane in a Soxhlet apparatus overnight and then air-dried. The protein extract consisted of those proteins extracted from SBM by 76 mM tris-HCl buffer (pH 8.6) containing 5 mM 2-mercaptoethanol.

The lyophilisates of (a) the water in which seeds were soaked and (b) the water in which roots grew were prepared as follows. For a test at a given time, 100 g of soybean seeds were surface-washed with 70% ethanol for 10 sec and rinsed immediately with a large excess of sterile distilled H₂O. The sterilized seeds were placed in sterile distilled H₂O (about 2 ml/g of seeds) in sterile Petri dishes and put in a growth chamber at 16.7 to 18.3 C. Samples were taken at 4, 8, 12, 16, 20, and 24 hr. Each seed was then washed three times with 1 ml of distilled H₂O. The water in which the seeds had been soaked and the water in which they were washed were combined and lyophilized. From the above preparations, only those soybean seeds that had been incubated for 24 hr were placed on sterile metal screens (16 × 22 cm, with 0.5 × 0.5-cm holes) which were set on the top of glass dishes (30 × 18 × 4.5 cm) filled with sterile distilled H₂O. These seeds were allowed to germinate and grow in a closed glass chamber at room temperature for 20 days, with the seeds above the water and only the roots in the water contained in the dishes. The water in the dishes was combined and lyophilized.

Nonsterilized soybean seeds were also germinated on rolls of germination paper in a growth chamber for 12 days. The plants were cut into four parts: (a) cotyledons; (b) epicotyls excised at the cotyledonary node; (c) hypocotyls excised at the cotyledonary node and at the most proximal lateral root; and (d) roots excised at the most proximal lateral root and including all of the main and branch root system. Individual pieces of each structure were pooled (cotyledons together, roots together, etc.), homogenized with cold distilled H₂O in a blender, and then lyophilized.

Lyophilisates of the water around the seeds, the water around the growing roots, and the lyophilized plant parts were each extracted with 100 volumes (w/v) of 76 mM tris-HCl buffer (pH 8.6) containing 5 mM mercaptoethanol at room temperature for 1.5 hr. After centrifugation at 16,000g for 30 min, the supernatant was filtered through glass wool and rinsed with distilled H₂O. Each filtrate was dialyzed against running tap water overnight at 4 C and then lyophilized. The protein content of the lyophilized buffer extracts was determined by the method of Bramhall *et al.* (7). The protein content of the purified protease inhibitors was measured by the biuret reaction.

Assay for Competitive Inhibition of Immunoprecipitation. The amounts of the low mol wt protease inhibitors present in each of the lyophilized buffer extracts were determined by competitive inhibition of immunoprecipitation against ¹²⁵I-labeled PI IV or PI V with rabbit anti-PI IV or -PI V serum, respectively. The protein concentration of ¹²⁵I-labeled antigen was determined by competition assays with the corresponding nonlabeled antigen. The assay was done as follows: 10 μl of ¹²⁵I-labeled antigen (diluted 1:40 in 0.5 mg of BSA/ml of PBS) was added to each siliconized glass tube (10 × 75 mm) containing 10 μl of the nonlabeled antigen (at appropriate dilution) or buffer-extracted proteins. This was mixed with a Vortex mixer and 100 μl of 1:100 dilution (in PBS) of normal rabbit serum was added to each tube and mixed again. Then 100 μl of the corresponding antiserum (diluted 1:100 in PBS) was added. The mixture was incubated at 37 C for 30 min; then 100 μl of goat anti-rabbit γ-globulin (diluted 1:4 in PBS) was added to each tube. The mixture was incubated at 37 C for an additional 30 min, then overnight at 4 C. To bring the total volume to 1 ml, 0.68 ml of PBS was added to each tube. The mixture was centrifuged at 16,000g for 10 min. Half of the supernatant (0.5 ml) was taken from each tube and transferred to the same size of siliconized tube, and the precipitate was left with the remain-

ing (bottom) half of the reaction mixture. The assay was run twice and the results were averaged.

Both the top and the bottom halves of the reaction mixture were assayed for radioactivity by a Tracerlab Gamma/Guard 150 gamma counter. The difference between the bottom and top was calculated in cpm and plotted *versus* the various amounts of protein contained in the reaction mixture. At the point where radioactivity is 50%, that of control (no nonlabeled antigen added), the amount of nonlabeled antigen involved in competitive inhibition is the same as ¹²⁵I-labeled antigen.

Assay for Hemagglutination Activity. Hemagglutination activity was determined by a standard visual assay (9). Serial dilutions of protein in PBS were made in a total volume of 0.2 ml in siliconized glass tubes, and 0.2 ml of a 2% PBS washed, rabbit red blood cell suspension was added to each tube. The tubes were incubated at 37 C for 30 min. After incubation overnight at 4 C, the agglutination pattern was observed. Hemagglutination activity is expressed as the lowest amount of protein in μg required for a positive value of agglutination. At least two determinations were made for each hemagglutination assay. The value which could be reproduced was taken for the calculation of the hemagglutination activity.

RESULTS

Immunochemical Properties of Soybean Low Mol Wt Protease Inhibitors. In a previous study, five low mol wt protease inhibitors (PI I-V) of Tracy soybean seeds were isolated and purified and rabbit antisera were obtained against them (15). PI I to IV showed complete immunological cross-reactivity among themselves. Immunological studies also showed that PI I to IV reacted poorly with antiserum against PI V, and PI V likewise reacted poorly with antiserum against PI I to IV. The immunoprecipitation of ¹²⁵I-labeled PI IV or PI V by the specific antiserum was inhibited competitively by nonlabeled protein of the same inhibitor, but not by the nonlabeled protein of the other (Fig. 1). In this study the highest titer anti-PI IV serum and PI IV antigen were used as representative of PI I through PI IV.

Figure 1 shows that neither lima bean trypsin inhibitor nor Kunitz soybean trypsin inhibitor competes with either PI IV or PI V antigen in the binding reaction to their corresponding antibodies.

By the method of competitive inhibition of immunoprecipitation, 10 μl of ¹²⁵I-labeled antigen PI IV used in this study was equal to 0.049 μg of nonlabeled antigen PI IV, while 10 μl of ¹²⁵I-labeled antigen PI V was equal to 0.050 μg of nonlabeled antigen PI V (Fig. 1).

Protease Inhibitors in Soybean Meal and Extracted Protein. The protein content of the SBM was determined by the Kjeldahl method (N × 6.25) to be 41%, and that of the tris-HCl buffer extract of SBM was determined by the biuret reaction to be

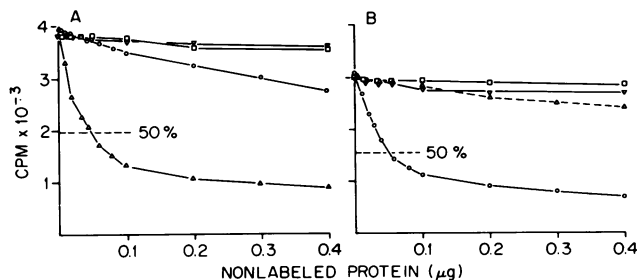


Fig. 1. Competitive inhibition of radioimmunoprecipitation in the reaction of (A) ¹²⁵I-labeled antigen PI IV with rabbit anti-PI IV serum; and of (B) ¹²⁵I-labeled PI V with rabbit anti-PI V serum, by nonlabeled antigen PI IV (Δ), PI V (○), lima bean trypsin inhibitor (▽), and Kunitz soybean trypsin inhibitor (□).

81.3%. The amounts of the low mol wt protease inhibitors present in SBM are 2.3% for PI I to PI IV and 4.0% for PI V; those in the buffer extractable proteins are 3.3% for PI I to PI IV and 4.8% for PI V (Fig. 2).

Release of Proteins, Protease Inhibitors, and Lectins during Imbibition and Early Germination Periods. It has been reported (9) that certain proteins can be released from seeds during early phases of imbibition. The mol wt of the released proteins were determined by SDS-polyacrylamide gel electrophoresis, which showed several bands, including those in the mol wt ranges of lectins as well as low mol wt protease inhibitors. Tracy soybeans (harvested in 1976) were used for the protein release studies. The total proteins, total protease inhibitors, and total hemagglutination units (hemagglutination activity $\mu\text{g}^{-1} \times$ total released proteins) released by the seeds into the water during various imbibition periods (*i.e.* 4, 8, 12, 16, 20, and 24 hr) are shown

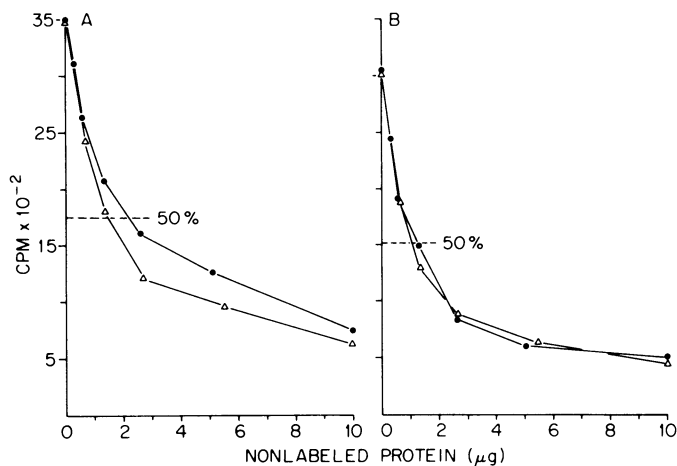


FIG. 2. Competitive inhibition of radioimmunoprecipitation in the reaction of (A) ^{125}I -labeled antigen PI IV with rabbit anti-PI IV serum; and of (B) ^{125}I -labeled antigen PI V with rabbit anti-PI V serum, by proteins in SBM (●) and proteins extracted by buffer from SBM (Δ).

in Figure 3A. There was 0.58 g of protein released from 100 g (fresh wt) of soybean seeds during the 24-hr imbibition (Table I). Although the proteins released at that time constituted less than 2% of the total proteins in the seeds, 18.9% of that 2% consisted of protease inhibitors. The protease inhibitors released at 24-hr imbibition accounted for 5.67% of the total protease inhibitors in the seeds. The release of proteins and protease inhibitors showed a linear increase with time over a 24-hr period. The release of lectins reached a peak at 8 hr after soaking, decreased at 12 hr, and then rose again after 20 hr.

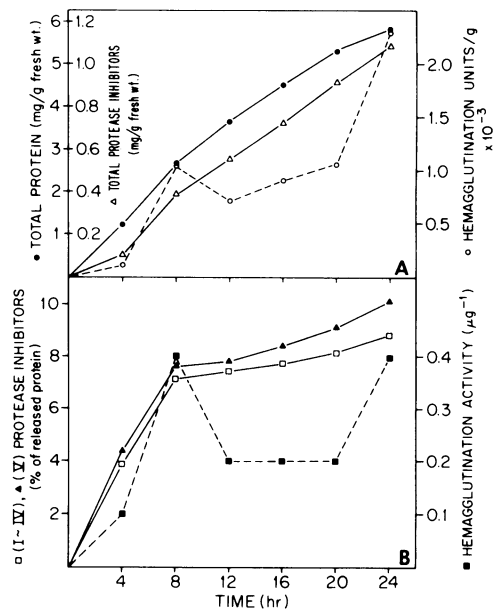


FIG. 3. Time course of (A) total protein release (●), total protease inhibitors release (Δ), and total hemagglutination units released (○) from fresh soybean seeds; and (B) percentage of protein inhibitors PI I to IV (□), PI V (▲), and hemagglutination activity (■) present in released proteins.

TABLE I. The percentage of low molecular weight protease inhibitors and hemagglutination activities in various protein sources (based on 100 g fresh weight of soybean seeds)

Protein source	Dry weight of protein source (g)	Dry weight of buffer extract (g)	Percent protein (w/w)	Weight of protein (g)	Percent PI I-PI IV (w/w)	Percent PI V (w/w)	Total percent PI (I-V) (w/w)	Total PI in protein (g)	Percent total PI in 100 g of seeds	Hemagglutination activity* (μg)	Total hemagglutination units† × 10 ⁻³
Defatted soybean meal (SBM)	74.80	—	41.0	30.67	2.3	4.0	6.3	1.932	100.00	—	—
Protein extracted from SBM	—	26.40	81.3	21.46	3.3	4.8	8.1	1.738	89.96	—	—
Water surrounding:											
4-hr	1.16	0.31	38.7	0.12	3.9	4.4	8.3	0.010	—	10.00	12
8-hr	2.02	0.57	46.0	0.26	7.1	7.6	14.7	0.038	—	2.50	104
12-hr	2.92	0.77	46.4	0.36	7.4	7.8	15.2	0.055	—	5.00	72
16-hr	3.35	0.95	47.4	0.45	7.7	8.4	16.1	0.073	—	5.00	90
20-hr	3.75	1.05	50.0	0.53	8.1	9.1	17.2	0.091	—	5.00	106
24-hr	4.01	1.13	51.3	0.58	8.8	10.1	18.9	0.110	5.67	2.50	232
20-day growing roots	1.97	0.71	21.4	0.15	4.2	4.0	8.2	0.012	0.64	5.00	30
12-day cotyledons	28.70	5.55	49.7	2.76	1.1	1.4	2.5	0.069	3.57	12.50	221
12-day epicotyls	12.61	2.44	39.3	0.96	< 0.1	< 0.1	< 0.1	—	—	> 1000.00	—
12-day hypocotyls	15.33	1.15	29.3	0.34	< 0.1	< 0.1	< 0.1	—	—	> 500.00	—
12-day roots	12.42	2.04	32.1	0.66	< 0.1	< 0.1	< 0.1	—	—	100.00	6.6
Purified soybean lectins	—	—	—	—	—	—	—	—	—	0.08	—

* Expressed as the lowest amount of protein required to give a positive value of agglutination in the experiment described.

† Hemagglutination activity (μg^{-1}) × total protein (μg).

The percentages of protease inhibitors of the PI I to PI IV type and of PI V in the released proteins in each 4-hr incubation period are shown in Figure 3B. Both of these types of protease inhibitors were released very rapidly during the first 8 hr of incubation (7.1 and 7.6%, respectively, of total protein). The rate of release declined thereafter, with final values of 8.8% for PI I to PI IV and 10.1% for PI V at the end of the 24-hr incubation period.

The hemagglutination activity released into the water reached a peak at 8 hr after soaking ($0.4 \mu\text{g}^{-1}$), followed by a decrease in the activity up to the 20th hr of incubation ($0.2 \mu\text{g}^{-1}$), and then increased thereafter ($0.4 \mu\text{g}^{-1}$) (Fig. 3B). Figure 4 shows the competitive inhibition of radioimmunoprecipitation of the proteins released at 24-hr incubation against antigens PI IV and PI V with corresponding antiserum. The percentages of released proteins, protease inhibitors of soybean seeds, and the hemagglutination activity of the released proteins at every 4-hr interval over the first 24-hr period are summarized in Table I.

Protease Inhibitors and Lectin Activities in Germinating Seedlings. It has been reported (9) that the release of lectins is independent of seed viability and insensitive to azide. Germination of this batch of Tracy seeds was 70%. Of seeds soaked for 24 hr, 50% developed into seedlings with roots which grew into the water. The damaged seeds and the roots which did not grow into the water during germinating seedlings were removed. For convenience in comparison, all of the results reported have been converted to 100 g (fresh wt) of soybean seeds as the starting material.

The competitive inhibition of the proteins of various plant parts (12-day-old), and of the proteins in the water in which roots grew for 20 days, in the reactions of anti-PI IV serum against PI IV antigen, and in that of anti-PI V serum against PI V antigen, is shown in Figure 4. Of the proteins in a lyophilisate of the water in which roots grew for 20 days, 8.2% are low molecular wt protease inhibitors (4.2% for PI I-PI IV, and 4.0% for PI V), which represent 0.64% of the total protease inhibitors in the seeds. Of all of the plant parts, only the cotyledons showed a significant amount of protease inhibitors (3.57% of total protease inhibitors in the seeds) and lectins (Table I).

Although specific antisera were used for the quantitation of protease inhibitors, the immunological activities of the protease inhibitors may not be correlated with their biological activities. Thus, the inhibitory activity of each immunochemical active sample was also determined by the biochemical method as described previously (15). The protease inhibitors purified from

soybean seeds were used as standards. The results of biochemical assay are in good agreement with those of the immunochemical assay for those protease inhibitors released from seeds during the first 20 hr of imbibition. The inhibitory activity declined to 85% for the protease inhibitors obtained from the surrounding water of incubated seeds for 24 hr, and declined to 20% for that obtained from the water in which roots grew for 20 days. However, the inhibitory activity we measured may be partly attributed to the Kunitz trypsin inhibitor which is not detected by our immunochemical assay.

DISCUSSION

Of the five low mol wt protease inhibitors purified from Tracy soybeans, four of them (I-IV) are immunologically cross-reactive among themselves (15) but little cross-reaction occurs between PI V and the group of PI I to PI IV, indicating that there are two sorts of these inhibitors present in soybean seeds. One of these is the well known Bowman-Birk inhibitor (PI V); PI II of the other sort (PI I-IV) is similar to the D-II inhibitor described by Odani and Ikenaka (19), as reported previously (15). Cross-reactivity of the inhibitors from Tracy soybeans with either the C-II or D-II inhibitors isolated from the Japanese variety (19) has thus far not been tested. Such an investigation would be of interest, especially for comparison with potato protease inhibitors, which have different physicochemical and inhibitory properties, but are immunochemically similar to one another (25). Birk (5) has reported that rabbit anti-Bowman-Birk inhibitor serum could cross-react with lima bean-type inhibitor antigen. However, none of our rabbit antiserum preparations against the two sorts of soybean protease inhibitors showed cross-reactivity with lima bean trypsin inhibitor or with the Kunitz inhibitor (Fig. 1). The total low mol wt protease inhibitor (PI I-IV) content of Tracy soybean seeds is about 63 mg/g of soybean meal, as detected by our immunochemical assay (Table I). The content of trypsin inhibitors in the soybean seed is actually even higher than this, since the Kunitz inhibitor has not been included in this study.

There was a good correlation between the inhibitory and immunological activities of the protease inhibitors obtained from fresh soybean plants and from surrounding water of incubated seeds up to 20 hr. Thereafter, based on the immunochemical assay, the biological activity declined to 85% for the protease inhibitors released from seeds after 24-hr imbibition and to 20% for that obtained from the water in which roots grew for 20 days. The decline of the biological activity may be due to the natural degradation and denaturation of the protease inhibitor during the prolonged experimental condition. The inhibitory activities of the protease inhibitors may include that from Kunitz trypsin inhibitor. However, due to the lower content and higher mol wt of the Kunitz trypsin inhibitor, the continuation of the inhibitory activity from this inhibitor is estimated to be less than 10% of the total inhibitory activities.

There is a linear relationship with time of both total protein release and of total protease inhibitor release from imbibing seeds for a 24-hr period (Fig. 3A). The low mol wt protease inhibitors (PI I-V) constitute 18.9% of the proteins which diffused out of the seeds during the first 24 hr of imbibition. Of this 18.9%, 14.7% was released during the first 8 hr of imbibition. Thereafter the rate of release slowed, but continued to rise until the end of the 24-hr imbibition period (Fig. 3B). The hemagglutination activity and the total hemagglutination units detected in the 24-hr period of imbibition showed a peak at 8 hr, followed by a decline with a subsequent rise, which continued to the end of the observation period (Fig. 3). This suggests that there may be two lectins or isolectins or the same lectins released by two different mechanisms during the early phase of germination. These data also confirm the observation of an early peak of lectin release reported by Fountain *et al.* (9)

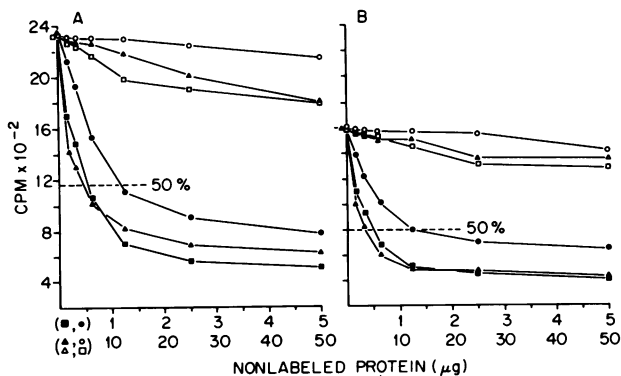


Fig. 4. Competitive inhibition of radioimmunoprecipitation in the reactions of (A) ¹²⁵I-labeled antigen PI IV with rabbit anti-PI IV serum; and of (B) ¹²⁵I-labeled antigen PI V with rabbit anti-PI V serum, by proteins in a lyophilisate of water in which seeds were incubated for 24 hr (■), in a lyophilisate of water in which roots of seedlings grew for 20 days (●), and by proteins of cotyledons (▲), epicotyls, (○), hypocotyls (Δ), and roots (□) of 12-day-old seedlings.

for other cultivars of soybean. The difference in the patterns of released hemagglutination activity of these two reports may be due to differences in varieties, age and viability of seed, or a combination of factors. The evidence that protease inhibitors, as well as lectins, can be detected in the water surrounding soybean seeds indicates that the release of both of these types of proteins may be the result of simple diffusion from a barrier-free pool during early germination of the seed (9). This suggests that these proteins may function as protective agents against insect and/or microbial invasion.

As germination proceeds, there is a concomitant disappearance of both the protease inhibitors and lectins from seedlings, so that in 12-day-old plants only the cotyledons contain an appreciable quantity of both kinds of substances (Table I), although far less than in seeds. Cotyledons of seedlings of this age are beginning to turn from green to yellow, indicating a general deterioration. The occurrence of detectable amounts of protease inhibitors and lectins in the water in which roots grew for 20 days would account for at least some of the loss from seeds and seedlings. The disappearance of protease inhibitors from seeds of legumes and their reappearance in young seeds have been previously reported (2, 3, 6, 12); likewise, the disappearance of lectins from seedlings of legumes and their reappearance in young seeds have also been previously reported (13, 22, 23). These phenomena again suggest that protease inhibitors and lectins may play some important physiological roles during germination of legumes.

There are about equal percentages of PI I to PI IV and PI V present in the water surrounding either imbibing seeds or growing roots, and in cotyledons of 12-day-old soybean plants (Table I). By contrast there is more PI V than PI I to PI IV detectable in soybean meal and in proteins extracted from the meal. This observation is consistent with the findings of Pusztai (20) of independent changes in contents of individual trypsin inhibitors during germination in kidney beans.

The parallels between protease inhibitors and lectins in their distribution in the plant during the life cycle, and in their rapid release into the rhizosphere, may provide evidence for the determination of their physiological roles in the plant (1, 4, 11, 13, 16, 17, 20, 21, 24). It would indeed be interesting if the lectins and protease inhibitors had functions that interact with one another, particularly in the rhizosphere.

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