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The Appearance of New Active Forms of Trypsin Inhibitor in Germinating Mung Bean (*Vigna radiata*) Seeds[1]

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ABSTRACT

Ungerminated seeds of mung bean contain a single major species (F) of trypsin inhibitor with five minor species (A-E) separable on diethylaminoethyl-cellulose. During germination the level of trypsin inhibitory activity decreases from 1.8 units/grams dry weight in ungerminated cotyledons to 1.2 units/grams in cotyledons from seeds germinated 5 days. This decrease is accompanied by major changes in the distribution of inhibitory activity among the inhibitor species. By 48 hours of germination, inhibitor F has largely disappeared with an accompanying rapid increase in inhibitor C. Similarly, though less rapidly, inhibitor E decreases while inhibitor A increases. A similar sequence of changes is found *in vitro* when purified inhibitor F is incubated with extracts from seeds germinated 96 hours. The combined *in vivo* and *in vitro* data suggest a conversion sequence of: F → E → C → A. The *in vitro* conversion is inhibited by phenylmethyl sulfonyl fluoride but not by iodoacetamide, indicating that at least the initial phases of inhibitor conversion are not catalyzed by the mung bean vicilin peptidohydrolase.

The seeds of the Leguminosae generally contain large quantities of proteinase inhibitors active against mammalian serine proteinases such as trypsin, chymotrypsin, and elastase (9, 13). The molecular structure of these inhibitors and the mechanism of their *in vitro* reaction with proteinases have been well studied (10). Their *in vivo* role remains poorly understood with suggested functions including acting as storage proteins, regulation of endogenous proteinases, or acting as protective agents against insects or microbial predators (14). A decline in seed inhibitor during germination is commonly observed (1, 15). In part, this decrease may be explained by the physical loss of active inhibitor from the seed into the medium (16). It is generally assumed that the bulk of this decrease is due to proteolysis of the inhibitor by seed proteinases. Definitive experimental evidence supporting this is lacking with the possible exception of the germinating adzuki bean (*Phaseolus angularis*) (21). We show here that during germination there is a rapid appearance of two new active species of trypsin inhibitor, while the two major species present in the ungerminated seed rapidly disappear. We further demonstrate that this change is due to the posttranslational modification by limited proteolysis of the inhibitor species initially present.

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MATERIALS AND METHODS

Plant Material and Reagents. Mung bean seeds (*Vigna radiata* (L.) Wilczek) were obtained from Johnny's Selected Seeds, Albion, Maine, and had a germination rate of 95%. Bovine trypsin (EC 3.4.21.4, thrice crystalized), and α-*N*-benzyol-L-arginine ethyl ester were obtained from Sigma. Sephadex G-75 and DEAE-Sephadex A-25 were from Pharmacia Fine Chemicals. All other chemicals were reagent grade or better. All pH adjustments were made at room temperature, and twice distilled H_2O was used throughout.

Trypsin Inhibitor Assays. Trypsin inhibitor was determined by the method of Kassell *et al.* (8) using bovine trypsin. The trypsin used was approximately 56% active by active site titration (3). All values of trypsin inhibitor have been corrected for the inactive trypsin in the assay.

Germination of Mung Bean Seeds. Seeds, in 100-g aliquots, were imbibed for 24 h at room temperature (20 C) in distilled H_2O. The seeds were then drained, rinsed twice with distilled H_2O, and germination continued on moist filter paper in the dark. After the desired period of germination the seeds were rinsed with distilled H_2O, and the cotyledons dissected free of the seed coats and axes. Cotyledons were frozen at −20 C until needed. Germination time was reckoned from the beginning of inhibition.

Preparation of the Crude Inhibitor Fractions. Frozen cotyledons were partially thawed in the refigerator overnight before extractions. All subsequent manipulations were carried out at 0 to 4 C with the exception of column chromatography which was run at room temperature (approximately 20 C). The cotyledons derived from 100 g of seeds (unimbibed weight) were homogenized with 150 ml of 50 mM Tris-Cl (pH 8.0), in a Waring Blendor. The homogenate was filtered through several layers of cheesecloth washed in distilled H_2O, followed by centrifugation at 23,400*g* for 30 min. The appropriate quantity of formic acid (1 M) was added to the clarified extract to give a final concentration of 50 mM, and the pH was adjusted to 3.75 with 6 N HCl, with rapid stirring. After standing overnight at 4 C, the precipitate was removed by centrifugation. Solid ammonium sulfate was then added to a final concentration of 85% saturation at 0 C. After 18 h at 0 C the precipitate was recovered by centrifugation and redissolved in a minimal volume of 50 mM Tris-Cl (pH 8.0). Extracts of unimbibed seed (0 time) were prepared in a similar manner, except that the whole seeds were ground to a fine meal, and then extracted with 250 ml buffer/100 g seeds. Overall recovery of trypsin inhibitory activity in the final crude inhibitor preparation averaged 85% (range 81–92%) of that in the first Tris extract.

Estimation of the Percentage of Each Inhibitor Species in Total Inhibitor. The elution pattern from DEAE-Sephadex ion exchange chromatography for each germination time was traced on graph paper and each peak cut out and weighed. The percentage of each inhibitor species present was then calculated from its individual weight and the total weight of all inhibitor peaks combined. It should be noted that this method may tend to overestimate small

peaks occurring as shoulders on larger peaks, and underestimate such larger peaks.

Polyacrylamide Disc Gel Electrophoresis. Polyacrylamide disc gel electrophoresis at basic pH was performed by the method of Davis (6) using 10% acrylamide gels run at 5 mamp/gel. Gels were stained for protein with Amido Schwartz, and destained with 7.5% acetic acid. To locate trypsin inhibitors, unstained gels were cut into 1.5 mm slices using a Yeda (Rehovot, Israel) gel slicer. The slices were individually eluted overnight with 150 μl of 50 mM Tris-Cl (pH 8.0), and the extracts assayed for inhibitory activity in the normal manner. Migration rates, R_F, are given relative to the tracking dye bromphenol blue.

Purification of Inhibitor Species E and F. Mung bean seeds, previously soaked overnight in distilled H_2O, were homogenized with 25 mM Na-citrate (pH 5.4) (500 ml/100 g dry seeds). After centrifugation at 8,000*g* for 1 h, the clarified extract was adjusted to 75% saturation with ammonium sulfate by addition of the solid salt at 0 C. The precipitate was recovered by centrifugation and redissolved in a minimal volume of buffer. The resulting solution was heated in 1-liter batches in a boiling water bath to a temperature of 70 C (approximately 8 min). The resulting precipitate was removed by centrifugation and the supernatant lyophilized after exhaustive dialysis against distilled H_2O. Overall recovery of inhibitory activity was 70%, with a 5-fold purification based on protein determination by *A* at 280 nm. The crude inhibitor preparation was next subjected to ion exchange chromatography on a 2.5 × 50 cm column of DEAE-Sephadex A-25 equilibrated to 50 mM Tris-Cl (pH 8.0). The column was eluted at 100 ml/h with a linear gradient or NaCl, 0–0.5 M in 50 mM Tris-Cl (pH 8.0), 1500 ml/bottle. The inhibitor peaks corresponding to species E and F were pooled separately, dialyzed against distilled H_2O, and lyophilized.

Chromatography of each inhibitor on Sephadex G-75 (5 × 90 cm) in 50 mM Tris-Cl (pH 8.0), resulted in single symmetrical peaks of inhibitor activity which were pooled. However, the preparation of F was contaminated with E, and *vice versa*, as judged by disc gel electrophoresis. For further purification each peak was chromatographed on SP-Sephadex C-25 (2.5 × 83 cm). Isocratic elution was with 50 mM Na formate + 0.33 M NaCl (pH 3.75) at 60 ml/h. Under these conditions, species F and E elute as symmetrical peaks at 2.3 and 3.0 column volumes of eluent, respectively. These preparations, after dialysis and lyophilization, were used in the studies below. Both were judged to be more than approximately 95% pure by disc gel electrophoresis.

Studies with Synthetic Proteinase Inhibitors. Crude extract from beans germinated for 96 h (50 μl) was incubated with (a) 10 μl of 50 mM Tris-Cl (pH 8.0); (b) 10 μl of 50 mM iodoacetamide in 50 mM Tris-Cl (pH 8.0); (c) 10 μl of 5% (v/v) dioxane-50 mM Tris-Cl (pH 8.0); or (d) 10 μl of 12.5 mM phenylmethyl sulfonyl fluoride in 5% (v/v) dioxane-50 mM Tris-Cl (pH 8.0). After incubation at 25 C for 1 h, 40 μl (approximately 40 μg) of inhibitor species F in 1 mM HCl was added and incubation continued for an additional hour. Controls containing inhibitor F or crude extract along with either iodoacetamide or dioxane and phenylmethyl sulfonyl fluoride were also run. At the end of the incubation the reactions were immediately subjected to disc gel electrophoresis.

RESULTS

During the first 5 days of germination under the conditions used here there is a gradual decrease in extractable trypsin inhibitor in the mung bean seed (Table I). Examination of the crude inhibitor fraction from ungerminated seeds by gel filtration (Fig. 1) revealed a single inhibitor peak. The crude inhibitor fraction from seeds germinated for varying lengths of time gave a similar single peak eluting at approximately the same position. There was a slight decrease in the apparent molecular weight of the inhibitor between 0 and 120 h of germination. In addition, a number of the other UV absorbing peaks do show significant changes in size with time (Fig. 1). Notably, peaks 1, 2, 5, and 7 decrease during germination, while peak 4 increases in size.

Table I. *Variation in Trypsin Inhibitor Content During Mung Bean Seed Germination*

Germination Time	Seeds	Specific Activity
h	*U/g*	*U/A*$_{280}$ *unit*[b]
0[a]	1.83	0.036
24	1.49	0.036
48	1.58	0.030
96	1.48	0.021
120	1.22	0.018

[a] Unimbibed seeds.

[b] One A_{280} unit of protein, when dissolved in 1 ml of solution, gives an *A* at 280 nm of 1.0 with a 1 cm light path.

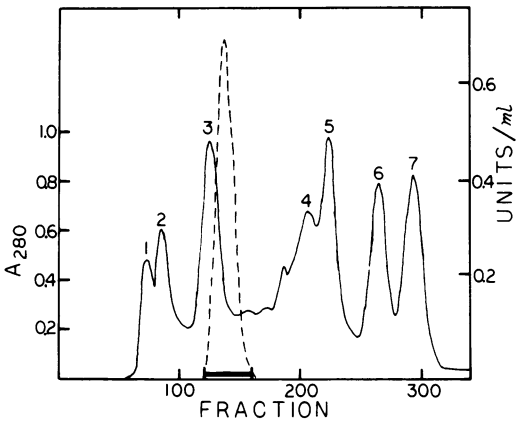

FIG. 1. Gel filtration of the crude inhibitor preparation from 100 g (dry weight) of ungerminated mung bean seeds. The inhibitor was applied to a 5 × 95 cm column of Sephadex G-75 equilibrated to 50 mM Tris-Cl (pH 8.0). Elution was at 100 ml/h with 10 ml fractions collected. (——), A_{280}; (- - -), trypsin inhibitor, U/ml. Fractions containing inhibitor were pooled as indicated. Arabic numerals indicate the protein peaks referred to in the text.

While the inhibitors isolated from seeds germinated for differing lengths of time appear similar by gel filtration, they are distinctly different when examined by polyacrylamide disc gel electrophoresis. The extracts of ungerminated seeds exhibit three inhibitor bands (R_F 0.36, 0.43, and 0.50) as revealed by the slicing technique described under "Materials and Methods" and the inhibitor staining technique of Uriel and Berges (22). These correspond to protein bands staining with Amido Schwartz (Fig. 2). The major inhibitor band at R_F 0.50 decreases throughout germination. After 48 h, the only inhibitor species present migrate at R_F 0.36 and 0.43, with the species at 0.50 absent. An increase in the staining intensity of the R_F 0.36 band is also noted between 0 and 48 h.

This radical change in the inhibitor complement of the germinating seed is confirmed by ion exchange chromatography on DEAE-Sephadex of the pools from gel filtration. At all time points the majority of the protein (60% or greater) emerged unretarded from the column. This material was found to correspond to the major protein in the pools (equivalent to peak 3 of Fig. 1), and migrates in disc gel electrophoresis at R_F 0.15. Elution with a linear NaCl gradient reveals six inhibitor species in ungerminated seeds (Fig. 3), emerging at 0.12, 0.14, 0.15, 0.17, 0.18, and 0.21 M NaCl (species A through F, respectively). Species E and F together constitute approximately 65% of the total trypsin inhibitory activity of the ungerminated seed (Fig. 4). After 48 h of germination, species F has largely disappeared, with species E showing a smaller decrease. In the same time period species C greatly increases, with

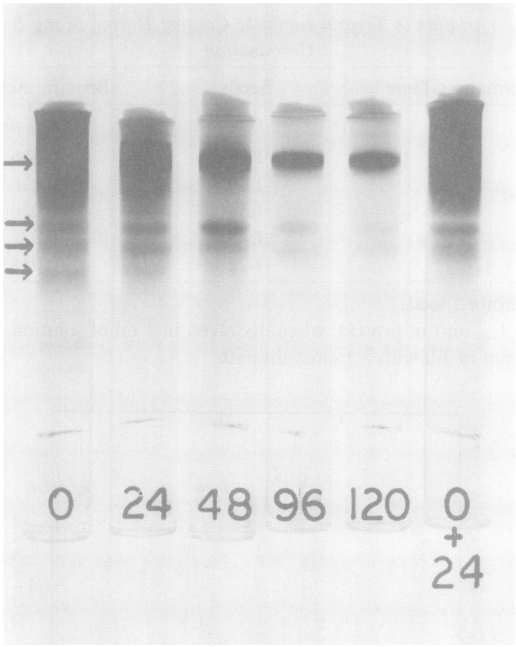

FIG. 2. Polyacrylamide disc gel electrophoresis of the crude extracts from germinating mung bean seeds. The first five gels (left to right) are extracts from unimbibed seeds, and seeds germinated 24, 48, 96, and 120 h, respectively. Each gel represents the extract derived from approximately 40 mg of seeds. The location of the Bromphenol Blue tracking dye was marked prior to staining with an India ink coated needle. The first and last gels contain the same amount of crude extract from unimbibed seeds. For the last gel, the extract of unimbibed seeds was incubated with an equal volume of 50 mM Tris-Cl (pH 8.0), at 25 C for 24 h prior to electrophoresis. The arrows indicate the position of bands with R_F values of 0.15, 0.36, 0.43, and 0.50, respectively, from top to bottom.

a smaller increase in species A (Figs. 3 and 4). By 120 h species E and F have entirely disappeared, with A and C constituting approximately 70% of the total inhibitor present.

Purified inhibitors F and E were found to correspond to the inhibitor bands at R_F 0.50 and 0.43, respectively, on disc gel electrophoresis, while a partially purified preparation of inhibitor C (from DEAE-Sephadex) revealed a single major band at R_F 0.36. We have been unable to unambiguously assign the R_F value of species A as we find two bands at R_F 0.36 and 0.43 in our partially purified preparations of this inhibitor.

It seems likely that the disappearance of inhibitor species E and F and the appearance of species A and C is due to an enzymic process, presumably proteolytic. Incubation of crude extract from ungerminated seeds at 25 C at pH 8.0 results in the rapid disappearance of inhibitor F (Fig. 2). Similar results are obtained if an aliquot of ungerminated crude extract is incubated with extracts from 48 and 96 h germinated seeds. Purified inhibitor F under similar conditions is stable in the absence of any added crude extract. Incubation of inhibitor F with the crude extract from seeds germinated 96 h leads to the rapid appearance of an electrophoretic species with an R_F value identical to E, with the subsequent appearance of one species identical to C as well as a lesser species not noted before with R_F 0.45 (Fig. 5). Similarly, incubation of purified inhibitor E with the same extract leads to the appearance of a species equivalent to inhibitor C, with no production of inhibitor F (not shown). The presumed enzyme(s) involved in these conversions is insensitive to iodoacetamide, but is somewhat inhibited by phenylmethyl sulfonyl fluoride (Fig. 6). Significantly, when inhibitor F is incubated with 96 h extract reacted with iodoacetamide a new electrophoretic species (R_F 0.29) is observed. With longer incubation (48 h) this new species

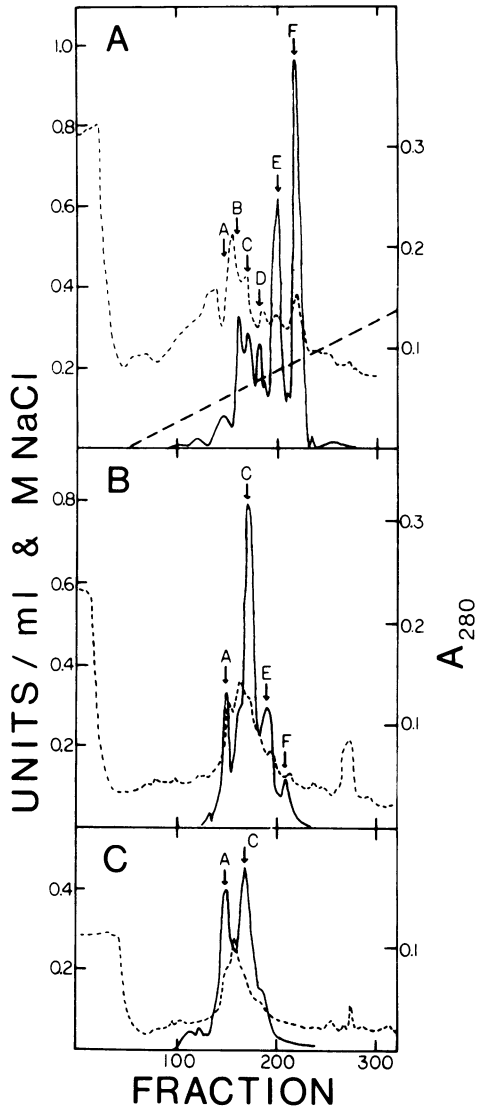

FIG. 3. Ion exchange chromatography of inhibitor pools from gel filtration. The material from the indicated germination times was applied to a 2.5 × 48 cm column of DEAE-Sephadex A-25 equilibrated to 50 mM Tris-Cl (pH 8.0). The column was then eluted with 100 ml of the same buffer, followed by a linear gradient of 0 to 0.4 M NaCl in 50 mM Tris-Cl (pH 8.0), 750 ml/bottle. Elution was at 50 ml/h, with 5 ml fractions collected. Frames A, B, and C: gel filtration pools from 0, 48, and 120 h germinated beans, respectively. (——), trypsin inhibitor, U/ml; (·····), A_{280}; (-----), NaCl, M (same in frames A, B, and C) determined by conductivity. The elution positions of inhibitor species A through F are indicated by lettered arrows.

increases in amount, while species E decreases and F disappears. This species is not evident after incubation with either untreated extract or phenylmethyl sulfonyl fluoride-treated extract, or when either inhibitor F or 96 h extract are treated separately with iodoacetamide and incubated at 25 C.

DISCUSSION

Studies of legume proteinase inhibitor metabolism during seed germination have been hindered by the multiplicity of homologous inhibitors in these plants (7, 11, 18, 20). For this reason we have chosen the mung bean as our experimental system. Previous studies have indicated one, and at the most two, trypsin inhibitors in the ungerminated mung bean (4, 5). In addition, the germination of mung beans has been well studied, especially with regard

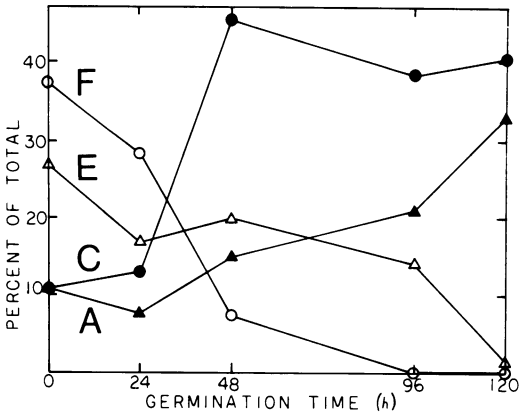

FIG. 4. Contribution of inhibitor species A, C, E, and F to the total inhibitory content during germination. The percent contribution of each was estimated as described in the text. The results shown are of a single experiment, which has been repeated several times with similar values. (▲), species A; (●), C; (△), E; (○) F.

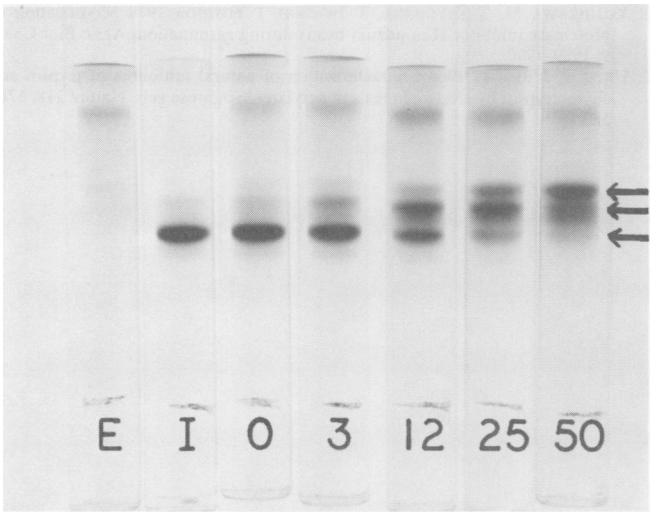

FIG. 5. Conversion of purified inhibitor F to species E and C *in vitro*. Aliquots of 40 μl of F in 1 mM HCl (approximately 40 μg) were incubated with 50 μl of extract (in 50 mM Tris-Cl [pH 8.0]) from beans germinated 96 h. After the desired lengths of time at 25 C the samples were frozen, and later subjected to disc gel electrophoresis as described under "Materials and Methods." Controls containing inhibitor + Tris buffer, or 1 mM HCl + extract, were treated in the same manner. The gels are (from left to right); extract and inhibitor controls at 0 time (E and I, respectively), and inhibitor + extract reactions incubated for 0, 3, 12, 25, and 50 h, respectively. Controls for 25 and 50 h were identical to gels E and I. The arrows indicate the positions of inhibitor species C, E, and F (from top to bottom) migrating at R_F values of 0.36, 0.43, and 0.50, respectively.

to proteolysis (2).

Our own studies above indicate that there is a single major inhibitor species (F in our nomenclature) in the ungerminated mung bean. There are five other, lesser, inhibitor species also present (A–E). The mung bean is thus similar to other legumes in having multiple isoinhibitor species present in the ungerminated seed (17). In any system containing such isoproteins, the question of the relationship between the multiple forms arises. Such a group of proteins may arise as the gene products of a like number of different genes (or alleles), as the products of a single gene with a number of different posttranslational modifications, or as some combination of the first two possibilities. The experiments described above indicate that in large part the multiplicity of mung bean trypsin inhibitors is due to the second mechanism, though

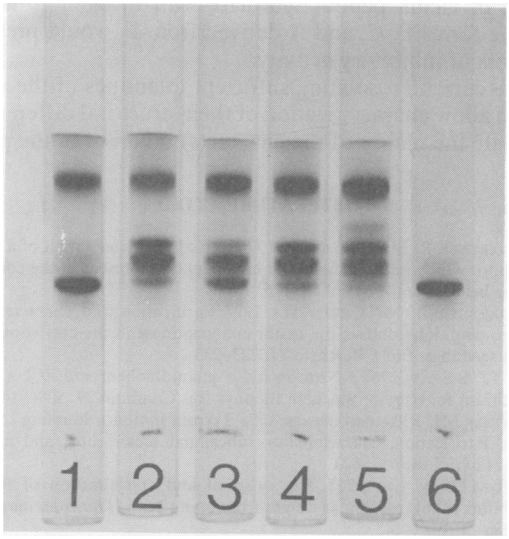

FIG. 6. Effect of synthetic proteinase inhibitors on the conversion of inhibitor into other species *in vitro*. Purified inhibitor F and reacted with crude extract from beans germinated 96 h or crude extract treated with iodoacetamide or phenylmethyl sulfonyl fluoride as described under "Materials and Methods." (1), Inhibitor F + extract, no incubation; (2), F + extract reacted with Tris + 5% dioxane; (3), F + extract reacted with phenylemethyl sulfonyl fluoride in Tris + 5% dioxane; (4), F + extract; (5), F + extract reacted with iodoacetamide; (6), F incubated with Tris buffer. The controls with inhibitor F incubated with iodoacetamide or phenylmethyl sulfonyl fluoride and dioxane were similar to gel 6. All reactions were for 24 h except as noted for gel 1.

the third can not be entirely ruled out. This assessment is based on the sequential changes in these inhibitors during germination, and the evidence that most of the inhibitor species may be produced *in vitro* from the major species F. From these data we deduce a pathway of:

$$F \rightarrow E \rightarrow C \rightarrow A$$

This is a minimal pathway; it does not account for the occurrence or fate of the inhibitor species B and D present in the ungerminated seed.

The conversion of F to these other forms is marked by a sequential decrease in the net negative charge of the molecule and some decrease in apparent molecular weight. It seems likely that some of this change is caused by the cleavage of terminal fragment(s) from the molecule, as has been noted in Bowman-Birk inhibitors from some other legume species (12, 19, 21). Both the amino-terminal and carboxyl-terminal sequences of Bowman-Birk inhibitors typically contain large quantities of aspartyl and glutamyl residues. The loss of such sequences would produce the decrease in charge and size noted.

It seems unlikely that the initial stage of the inhibitor conversion are due to the action of the sulfhydryl-dependent proteinase mung bean vicilin peptidohydrolase (2). This proteinase is absent from ungerminated seeds, and is present at low levels in seeds germinated up to three days (1). However, nearly all of species F has disappeared by this time, with a concomitant increase in species C. The conversion of F to E and C by extracts of beans germinated 96 h is insensitive to inhibition (and indeed seems to be activated) by iodoacetamide, but is at least partially inhibited by phenylmethyl sulfonyl fluoride. Such sensitivity to phenylmethyl sulfonyl fluoride seems to indicate the action of a serine proteinase or possibly a plant carboxypeptidase (13). It is possible that the vicilin peptidohydrolase is involved in the further metabolism of the inhibitor, especially after the third day of germination when trypsin inhibitory activity rapidly declines in the seed (1, 15).

Reaction of vicilin peptidohydrolase with F, or more likely the still active forms E, C, and A derived from F, would presumably lead to loss of inhibitory activity.

We are currently isolating sufficient quantities of the inhibitor species to allow characterization of their structural differences and to elucidate the nature of the enzyme(s) involved in the process.

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