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Characterization of the Proteinase that Initiates the Degradation of the Trypsin Inhibitor in Germinating Mung Beans (*Vigna radiata*)[1]

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ABSTRACT

The proteinase (proteinase F) responsible for the initial proteolysis of the mung bean (*Vigna radiata*) trypsin inhibitor (MBTI) during germination has been purified 1400-fold from dry beans. The enzyme acts as an endopeptidase, cleaving the native inhibitor, MBTI-F, to produce the first modified inhibitor form, MBTI-E. The cleavage of the Asp76-Lys77 peptide bond of MBTI-F occurs at a pH optimum of 4.5, with the tetrapeptide Lys-Asp-Asp-Asp being released. Proteinase F exhibited no activity against the modified inhibitor forms MBTI-E and MBTI-C. Vicilin, the major storage protein of the mung bean, does not serve as a substrate for proteinase F between pH 4 and 7. Proteinase F is inhibited by phenylmethylsulfonyl fluoride, chymostatin, *p*-hydroxymercuribenzoate, and *p*-chlorophenylsulfonate, but not by iodoacetate and $CuCl_2$. It is not activated by dithiothreitol, and is stable for extended periods of time (10 months, 4°C, pH 4.0) in the absence of reducing agents. An apparent molecular weight of 65,000 was found for proteinase F by gel filtration. Subcellular fractionation in glycerol suggests that greater than 85% of the proteinase F activity is found in the protein bodies of the ungerminated mung bean. The same studies indicate that at least 56% of the MBTI of the seed is also localized in the protein bodies.

During the development of the dicot seed on the mother plant, reserves in the form of storage molecules such as proteins, lipids, and polysaccharides are accumulated. Upon germination of the seed and early growth of the seedling these reserves are mobilized to supply energy and biosynthetic intermediates to the growing plant. The seeds of the Leguminosae are well known for their high levels of protein proteinase inhibitors, particularly those belonging to the Bowman-Birk family of inhibitors (24, 27). It has been suggested that the Bowman-Birk type proteinase inhibitors, because of their large content of half-cystine (approximately 20 residue percent), may serve as a major sulfur depot in the seed (22). We have demonstrated that the Bowman-Birk type trypsin inhibitor of the mung bean (*Vigna radiata* [L.] Wilczek) is indeed degraded during germination and seedling growth. This proteolysis, at least initially, proceeds along a discrete pathway of limited specific proteolytic events (16, 28, 30). The carboxypeptidase which appears during germination of the mung bean (carboxypeptidase II) has been demonstrated to be involved in the later stages of this degradation (29). This enzyme is, however, inactive in the degradation of the native MBTI,[2] MBTI-F. In this paper we describe the partial purification and characterization of the proteinase present in dry mung bean seeds which initiates the degradation of MBTI.

MATERIALS AND METHODS

Plant Materials and Reagents. Mung bean seeds cv Jumbo (*Vigna radiata* [L.]Wilczek) were purchased from Johnny's Selected Seeds, Albion, ME. DEAE- and CM-Trisacryl M were obtained from LKB Produkter AB (Bromma, Sweden), while Sephacryl S-200 was from Pharmacia Fine Chemicals. Leupeptin, pepstatin A, antipain, chymostatin, azocoll, Z-phenylalanyl-alanine, Z-alanyl-phenylalanine, BAPA, tBOC-L-asparagine and tBOC-L-glutamine *p*-nitrophenyl esters, Na trinitrobenzenesulfonate, and oxidized chain A of bovine insulin were from Sigma Chemical Co. PMSF and Z-aminoacyl *p*-nitrophenyl esters were from Research Organics (Cleveland, OH), while E-64 (N-[N-(L-3-transcarboxy-oxiran)-2-carbonyl)-L-leucyl]-agmatine) was from Boehringer Mannheim (Indianapolis, IN). MBTI-F, -E, -E′, and -C were prepared as previously described (28). The tryptic peptide T-IIIA, representing residues 61-80 of MBTI-F, was prepared from reduced carboxymethylated MBTI-F by the method of Wilson and Chen (28). Mung bean vicilin was prepared from ungerminated seeds (9). All other chemicals were reagent grade or better. All pH adjustments were performed at room temperature (21 ± 1°C), and twice distilled H_2O was used throughout.

Preparation of Crude Proteinase. Mung beans were ground to a fine meal using repeated short pulses in a blender. The resulting meal, 25 g, was extracted with 125 ml 50 mM Na formate (pH 4.0) for 1.5 h with stirring. This and all subsequent operations were carried out at 0 to 4°C. The resulting brie was filtered through four layers of buffer-washed cheesecloth, and clarified by centrifugation at 39,000*g* for 30 min. After holding overnight, the extract was adjusted to pH 8.0 with 1 N NaOH. The resulting light precipitate was removed by centrifugation as above to yield the crude proteinase preparation.

Assay of Inhibitor Proteolysis. The assay of the proteolysis of MBTI-F to MBTI-E (proteinase F activity) at pH 4.0 was carried out as previously described (29, 30). In assays of column fractions activities were expressed on a scale of 0 to 1.0, indicating the fraction of MBTI-F converted to MBTI-E. For the more careful quantitation of activity at the various stages of purification, one

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[2] Abbreviations: MBTI, mung bean trypsin inhibitor; BAPA, α-*N*-benzoyl-L-aginine *p*-nitroanilide; pCMPS, *p*-chloromecuriphenylsulfonate; pHMB, *p*-hydroxymercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; tBOC-, *tert*-butyloxycarbonyl-; Z-, benzyloxycarbonyl-.

unit of enzyme was defined as converting 1 mg of MBTI-F to MBTI-E in 24 h under the conditions of the assay. The assay was performed with varying amounts of the sample of interest to ascertain that a linear relationship was achieved between the amount of MBTI-F converted to MBTI-E and the amount of sample in the assay. Assays of activity with MBTI-E, -E′, and -C as substrates were carried out in a similar manner.

Assays of Other Proteolytic Activities. Assays for carboxypeptidase activity were carried out as previously described (30) using a modification of the method of Mikola and Kolehmainen (21). Carboxypeptidase I and II were assayed using Z-alanyl-phenylalanine and Z-phenylalanyl-alanine, respectively. One unit of activity was defined as hydrolyzing 1 μmol of substrate per min in the described system. Activity against Z- or tBOC-L-aminoacyl *p*-nitrophenyl esters was carried out as previously described by Wilson and Tan-Wilson (29).

Hydrolytic activity towards BAPA was assayed by a modification of the method of Erlanger *et al.* (10). The substrate solution consisted of 1 mM substrate in 50 mM Na phosphate (pH 7.3). Sample, 25 μl, was mixed with 125 μl of substrate solution in the well of a plastic microtiter plate (Corning). After 1 h at 37°C, the reaction was stopped by the addition of 50 μl of 30% (v/v) acetic acid. The *A* at 405 nm was determined using a Bio-Tek Instruments model EL307 microplate reader. One unit of activity was defined as producing a change in absorbance of 1.0 in this assay system.

General proteolytic activity was assayed using 5 mg/ml azocoll (6) in citrate/phosphate buffer (18), pH 4.0 to 7.0, as substrate. Mung bean vicilin was also tested as a possible substrate for proteinase F. Vicilin, 48 μg in 5 μl 50 mM Tris-Cl + 0.1 M NaCl (pH 8.0), was mixed with 20 μl of enzyme (0.050 units) and 35 μl citrate/phosphate buffer at pH 3, 4, 5, 6, or 7. After incubation at 24 h at 37°C, the reactions were terminated by the addition of 60 μl of treatment buffer and SDS-PAGE carried out by the method of Laemmli (15) in 12.5% (w/v) gels. Controls where 20 μl of water was substituted for the enzyme were performed at the same time.

Reaction of Proteinase F with Peptide Substrates. Two peptides of known sequence were examined as possible substrates for proteinase F. The S-oxidized A chain of bovine insulin, 400 μg, was reacted with 0.50 units of proteinase F in 300 μl of 33 mM NH_4 formate (pH 4.5), for 22 h at 37°C. The tryptic peptide T-IIIA of reduced, S-carboxymethylated MBTI-F was also used as a substrate. Peptide T-IIIA, 35 nmol and 0.041 units of proteinase F were incubated for 22 h at 37°C in 0.1 ml of 50 mM Na formate (pH 4.0). In both cases reactions containing only the peptide, or only the enzyme, were run at the same time. After incubation the reaction mixtures were examined by reversed phase HPLC as described below.

Characterization of the Product Formed by the Action of Proteinase F on MBTI-F. To confirm that the product of the action of the enzyme on MBTI-F was indeed MBTI-E the following experiment was carried out. MBTI-F, 450 μg in 700 μl of 50 mM NH_4 formate (pH 4.5), was incubated with 250 μl of proteinase F (0.63 units) which had been dialyzed against 5 mM ammonium formate (pH 4.0). After 24 h at 37°C, a sample equivalent to 5.2 nmol of MBTI-F was removed, lyophilized, and subjected to amino-terminal analysis by dansylation (11). The remaining material was examined by ion exchange HPLC.

Electrophoresis. PAGE of the purified proteinase preparation was performed in 10% (w/v) gels by the method of Davis (8), and stained using the Gelcode color silver stain system (Pierce Chemical Comp., Rockville, IL).

High Performance Liquid Chromatography. A single pump gradient HPLC system consisting of a model 2150 pump, a model 2152 LC gradient controller and proportioning valve, and a model 2151 variable wavelength UV-visible detector from LKB was used throughout. All solvents and samples were filtered (0.45 μm) before use. Solvents were degassed by sparging with helium. For the examination of mixtures from the reactions of proteinase F with MBTI-F ion exchange HPLC was performed. A 7.5 × 75 mm TSK DEAE-5PW column (Toyo Soda Co.) with a 6 × 10 mm guard column of the same resin was used. Buffer A was 20 mM Tris acetate (pH 8.0), while buffer B was 20 mM Tris acetate + 0.5 M Na acetate (pH 8.0). Elution was at 0.5 ml/min using a linear gradient from 0 to 100% buffer B in 15 min. The eluting MBTI peaks were detected by absorption at 260 nm. Pure MBTI-F, -E, -C, and -E′ were used to calibrate the system.

Reversed phase (C18) HPLC was used to examine the reactions between proteinase F and the A chain of insulin and tryptic peptide T-IIIA from MBTI-F. A TSK ODS-120T column (4.6 × 250 mm) and a Lichrosorb RP18 precolumn (4 × 30 mm), both obtained from LKB, were used. Solvent A was 0.1% (v/v) TFA in water. Solvent B was 0.1% (v/v) TFA in acetonitrile. The column was eluted at 1 ml/min with a linear gradient of 10 to 40% buffer B in 30 min followed by one of 40 to 80% buffer B in 2 min. Detection was by absorption at 230 nm.

Mol Wt Determination. Gel filtration was used to estimate the mol wt of proteinase F. An aliquot of the final preparation, 2 ml, was applied to a 1.5 × 105 cm column of Sephacryl S-200 equilibrated with 50 mM Tris-Cl (pH 8.0). The column was eluted with the same buffer at 20 ml/h, with 1.35 ml fractions. The fractions were assayed for proteinase F activity as described above. The column was calibrated with apoferritin, sweet potato β-amylase, yeast alcohol dehydrogenase, BSA, chicken ovalbumin, carbonic anhydrase, and Kunitz soybean trypsin inhibitor. Mol wt of 443,000, 200,000, 150,000, 66,200, 45,000, 29,000, and 21,500 respectively were assumed for these standard proteins.

Subcellular Fractionation Studies. The subcellular fractionation of ungerminated mung bean cotyledon cells was carried out at pH 5.0 in glycerol by the method of Harris and Chrispeels (13). The final centrifugation yielded three fractions, the 80% glycerol layer (fraction I, nominally representing the cytosol), the 90% glycerol layer (fraction II), and the 'protein body' pellet (fraction III). The latter was resuspended in 50 mM Tris-Cl (pH 8.0) for further assay. These fractions were assayed for inhibitor proteolysis as above, α-mannosidase (26), dipeptidase (alanylglycine) (21), and NADH-Cyt *c* reductase (3). Protein was determined by the dye-binding assay of Bradford (4) with BSA as standard. Leucine aminopeptidase was determined using 2 mM leucine *p*-nitroanilide in 10 mM HCl as substrate. Sample, 0.1 ml, 1.6 ml 50 mM Na phosphate (pH 7.3), and 0.2 ml substrate were mixed and incubated for 30 min at 30°C. The reaction was terminated with 1 ml 1 M acetic acid, and A at 410 nm determined. One unit of activity was defined as producing an increase in absorbance of 1.0 in this assay.

MBTI was determined immunochemically using a specific antiserum and radial immunodiffusion as previously described (25). MBTI-F was used as a standard. The vicilin content of subcellular fractions was determined by SDS-PAGE (15). Varying amounts of each fraction were electrophoresed in 1.5 mm thick 12.5% (w/v) gels. After staining with Coomassie blue the amount of vicilin present in each sample was determined by densitometry of the major (approximately 45,000 mol wt) polypeptide band using a Hoefer model GS300 scanning densitometer. Samples of purified vicilin ranging from 1 to 5 μg were included in each gel for calibration of the method.

RESULTS

Purification. The crude proteinase preparation was highly active in converting MBTI-F to MBTI-E. The preparation also contained significant amounts of activity hydrolyzing BAPA and carboxypeptidase I hydrolyzing Z-alanyl-phenylalanine. Gel fil-

tration on Sephacryl S-200 resulted in the removal of a large amount of inert material, as well as a partial resolution of these activities (Fig. 1). The proteinase F activity and the activity hydrolyzing BAPA each eluted as single, coincident peaks of approximately 60,000 mol wt. The carboxypeptidase I activity eluted with a somewhat lower apparent mol wt. The proteinase F pools from gel filtration were further fractionated by ion exchange chromatography on DEAE-Trisacryl M (Fig. 2). Proteinase F eluted as a rather broad peak between 0.03 and 0.10 M NaCl. The BAPA hydrolyzing activity slightly overlapped the trailing edge of the inhibitor degrading activity, peaking at 0.12 M NaCl. Carboxypeptidase I eluted as a third peak at 0.18 M NaCl completely separated from proteinase F and the activity hydrolyzing BAPA.

Rechromatography of proteinase F on CM-Trisacryl M (Fig. 3) separated the proteinase activity from a large portion of inert protein. The majority of activity eluted near the beginning of the salt gradient, corresponding to a very small protein peak eluting at 0.04 M NaCl. Much smaller amounts of activity were also associated with larger protein peaks at 0.06 and 0.09 M NaCl. The specific activities of these last two peaks were much lower than that of the first peak. All further studies described below were carried out on the material pooled from the first peak as indicated in Figure 3. A yield of 34% of the activity in the initial crude proteinase preparation was obtained in the final pool, with an overall purification of greater than 1400-fold (Table I).

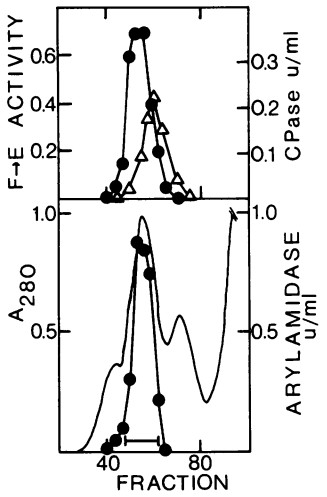

FIG. 1. Gel filtration of the crude proteinase F preparation. The crude proteinase, 40 ml, was applied to a 2.5 × 88 cm column of Sephacryl S-200 equilibrated to 50 mM Tris-Cl (pH 8.0). Elution was at 30 ml/h with the same buffer, with 150 drop fractions (4.75 ml) collected. Top frame: (●), proteinase F (F→E) activity; (△), carboxypeptidase I (unit/ml). Bottom frame: (——), A_{280}; (●), activity hydrolyzing BAPA (unit/ml). The active fractions of proteinase F were pooled as indicated.

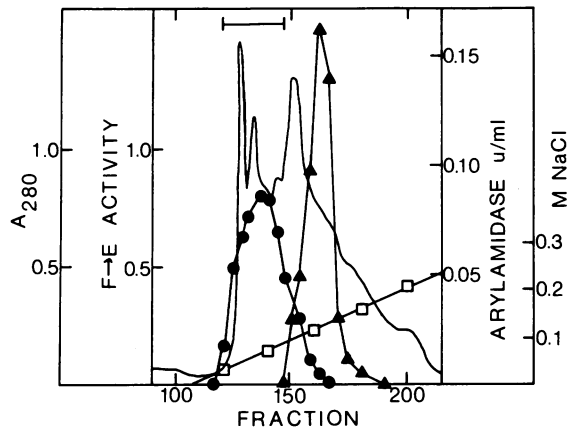

FIG. 2. Ion exchange chromatography of the proteinase F pools from gel filtration. The proteinase F pools from five gel filtration runs as shown in Figure 1 were combined and applied to a 2.5 × 44 cm column of DEAE-Trisacryl M equilibrated to 50 mM Tris-Cl (pH 8.0). The column was eluted with 50 ml of the same buffer, followed by a linear gradient of NaCl, 0 to 0.5 M, in 50 mM Tris-Cl (pH 8.0). Total gradient volume was 1200 ml. Elution was at 52 ml/h, with 160 drop fractions collected. (——), A_{280}; (●), proteinase F; (▲), activity hydrolyzing BAPA; (□), M NaCl. Proteinase F containing fractions were pooled as indicated.

Purity of the Proteinase F Preparation. Electrophoresis in the Davis PAGE system followed by silver staining (not shown) revealed one major protein species, R_F 0.22, and two minor bands of R_F 0.30 and 0.39 in the final proteinase F preparation. The major band constituted approximately 90% of the material based upon staining intensity.

Analytical gel filtration of the final preparation on Sephacryl S-200 at pH 8.0 yielded a single peak with an apparent mol wt of 65,000. The purity of the final preparation from other peptidases and proteinases is also indicated by the lack of activity against the panel of synthetic substrates, mung bean vicilin, and the peptide substrates tested (see below).

Stability of Proteinase F. The purified proteinase was completely inactivated by freezing at pH 4.0 (50 mM Na formate + 40 mM NaCl). It was, however, very stable at 4°C at pH 4.0 (*e.g.* in 50 mM Na formate + 0.04 M NaCl, or 5 mM ammonium formate), with over 80% of the initial activity retained after 10 months. The enzyme was also stable to freezing when dissolved in 50 mM Tris-Cl (pH 8.0).

Activity of Proteinase F toward MBTI Species and Other Proteins. While the crude proteinase preparation exhibited a pH optimum of 4.0 for the conversion of MBTI-F to MBTI-E (data not shown), the purified enzyme displayed a somewhat higher pH optimum between pH 4.5 and 5.0 for the same conversion (Fig. 4). Proteinase F was inactive toward MBTI-E and -C under the same conditions. However, proteinase F did act on MBTI-E′, an inhibitor species identical to MBTI-F except for the loss of the amino-terminal hexapeptide sequence. The product of this reaction exhibited a slightly lower electrophoretic mobility than authentic MBTI-E.

Dansylation was used to confirm that the product of the action of proteinase F on MBTI-F is indeed MBTI-E. Dansylation of MBTI-F yielded only one α-dansyl-amino acid, dansyl-serine, consistent with our previously published sequence for this inhib-

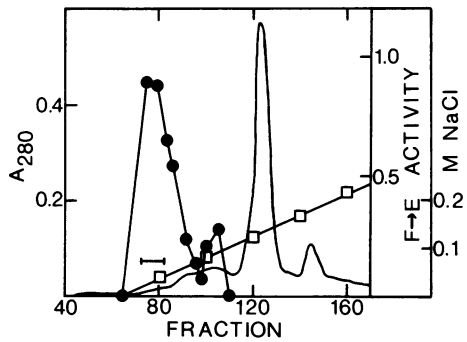

FIG. 3. Ion exchange chromatography of proteinase F on CM-Trisacryl M. The proteinase F pool from DEAE-Trisacryl chromatography (Fig. 2) was dialyzed against 50 mM Na formate (pH 4.0), and applied to a 2.5 × 50 cm column of CM-Trisacryl M equilibrated to the same buffer. The column was eluted at 60 ml/h with a linear gradient of NaCl (0–0.5 M) in 50 mM Na formate (pH 4.0). The total gradient volume was 1200 ml. Fractions of 120 drops were collected. (——), A_{280}; (●), proteinase F; (□), M NaCl. The final preparation of proteinase F (fractions 73–83) was pooled as indicated.

Table I. *Summary of the Proteinase F Purification*

Step	Protein	Proteinase F			
		Total	Specific activity	Yield	Purification
	A_{280} unit[a]	units	units/ A_{280} unit	%	-fold
Crude preparation[b]	3700	135	0.036	100	1
Sephacryl S-200	311	120	0.39	89	11
DEAE-Trisacryl	97	91	0.94	67	26
CM-Trisacryl	0.9	46	51.1	34	1419

[a] One A_{280} unit of protein is defined as giving a solution, when dissolved in 1 ml of solvent, with an A at 280 nm of 1.00 with a 1 cm light path. [b] Crude preparation was derived from a total of 55 g of mung bean meal.

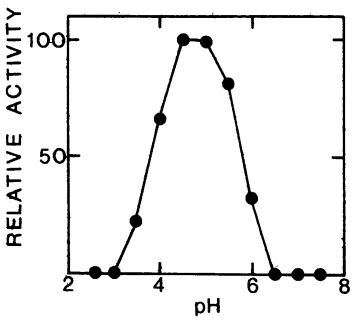

FIG. 4. Variation of proteinase F activity with pH. MBTI-F, 9 μg, and proteinase F, 0.025 units, were incubated in 30 μl citrate/phosphate buffer at the indicated pH values for 24 h at 37°C. The samples were then subjected to PAGE and scanning densitometry to quantitate the conversion of MBTI-F to MBTI-E. Values have been normalized relative to the highest observed activity (equal to 100) at pH 4.5.

itor (28). Dansylation of the reaction mixture containing the putative MBTI-E product revealed two amino-terminal amino acids, dansyl-serine and α,ε-didansyl-lysine. This is consistent with the proteinase cleaving MBTI-F at Asp76-Lys77 to produce MBTI-E, with the release of the carboxyl-terminal four residues as the tetrapeptide Lys-Asp-Asp-Asp. The absence of free aspartate in the reaction, as evidenced by the absence of dansyl-aspartate, rules out the action of proteinase F as a carboxypeptidase. HPLC also indicated that the product of this reaction is identical to MBTI-E. MBTI-F, MBTI-E, MBTI-C, and MBTI-E′ were found to elute from the TSK DEAE-5PW column at Na acetate concentrations of 0.40, 0.33, 0.28, and 0.37 M, respectively, while the reaction product of proteinase F and MBTI-F eluted at 0.33 M Na acetate.

No activity was noted for the proteinase with either mung bean vicilin or azocoll as substrates over the range pH 4.0 to 7.0, nor was any activity found against the oxidized A chain of bovine insulin or the T-IIIA peptide of MBTI.

Activity against Synthetic Substrates. The purified proteinase was found to be inactive against the following synthetic substrates: Z-phenylalanyl-alanine and Z-alanyl-phenylalanine (both at pH 6.0); BAPA (pH 7.3); the Z-L-aminoacyl *p*-nitrophenyl esters of asparagine, glycine, and alanine, and the tBOC-L-aminoacyl *p*-nitrophenyl esters of asparagine and glutamine (all pH 5.0); and leucine *p*- nitroanilide (pH 7.3).

Susceptibility of Proteinase F to Inhibitory Reagents. The proteinase was strongly inhibited by pCMPS and pHMB, and to somewhat lesser extents by PMSF and chymostatin (Table II). Little or no inhibition was caused by iodoacetate, pepstatin A, leupeptin, antipain, E-64, 1,10-phenanthroline, or the salts $MnCl_2$ and $CuCl_2$. No activation of the proteinase by 0.5 mM DTT was observed. Higher concentrations of DTT resulted in the reduction of the MBTI-F substrate, with no observed proteolysis.

Table II. *Effect of Various Reagents on Proteinase F Activity*

Samples of proteinase F were incubated with the indicated reagents for 3 h at 0°C at pH 4. The proteinase was then assayed for remaining activity. Results are expressed as the percent of the control reaction.

Reagent	Concn	Control
		%
Control		100
PMSF	3 mM	62
pCMPS	5 mM	5
pHMB	3 mM	13
Iodoacetate (Na)	5 mM	93
Pepstatin A	19 μM	96
Leupeptin	19 μM	105
Antipain	19 μM	100
Chymostatin	19 μM	56
E-64	21 μM	99
1,10-Phenanthroline	5 mM	102
$CuCl_2$	2 mM	99
$MnCl_2$	2 mM	102
DTT	0.5 mM	90

Table III. *Subcellular Localization of Proteinase F and MBTI*

Assay	Percent in Fraction[a]		
	I	II	III
Protein	24.4	12.9	62.7
Vicilin	3.0	13.8	83.2
α-Mannosidase	3.9	4.8	91.3
Carboxpeptidase[b]	7.1	21.1	71.8
Leucine aminopeptidase	90.4	3.8	5.8
Dipeptidase[c]	91.4	5.2	3.4
NADH-Cyto c reductase	11.4	4.2	84.4
Proteinase F	1.4	13.7	84.9
MBTI	44.4	0	55.6

[a] Fractions as defined in the text. [b] With Z-alanyl-phenylalanine. [c] With alanyl-glycine.

Subcellular Localization of Proteinase F. The distinctly acidic pH optimum of proteinase F activity suggests that it is localized in the protein bodies of the cotyledon cells. The subcellular fractionation studies further support this contention. Fraction I of the separation scheme (see "Materials and Methods") contained 24% of the total recovered protein and 44% of the MBTI (Table III). It may be identified as the cytosolic fraction by its

content of the majority of the leucine aminopeptidase and dipeptidase (90 and 91%, respectively, of the total recovered activity [20]). Only traces of the markers for protein bodies (23), vicilin, carboxypeptidase I, and α-mannosidase (3, 7, and 4%, respectively, of the total recovered activity) are present, suggesting that relatively little rupture of the protein bodies had occurred. Only traces of proteinase F were found in this fraction.

Fraction III was found to correspond to the protein bodies as evidenced by the contents of vicilin, α-mannosidase, and carboxypeptidase I. This fraction contained nearly all of the proteinase F activity (85%) as well as the majority of the MBTI (56%). It should be noted that the protein body fraction also contained the majority of the NADH-Cyt *c* reductase activity, suggesting the cosedimentation of the ER in this fraction (23). Thus, while the localization of proteinase F in the cytosol is excluded, the presence of some part of this activity in the ER cannot be excluded by these studies.

Attempts were also made to isolate the protein bodies from the cotyledons of mung beans that had been imbibed in water for 12 h using the method of Chappell *et al.* (5). This method did yield a protein body fraction containing the majority of the initial vicilin that was relatively free of NADH-Cyt *c* reductase and leucine aminopeptidase (only 11 and 18% of the total activities applied, respectively), while containing 36% of the applied proteinase F and 9% of the MBTI. However, the results are somewhat difficult to interpret as only 20% and 19.5%, respectively, of the α-mannosidase and carboxypeptidase were recovered in the protein body fraction, indicating the loss of the majority of these enzymes from the protein bodies. The fraction containing the majority (61%) of the NADH Cyt *c* reductase activity was found to contain only 7% of the proteinase F activity and none of the MBTI. Taken in total these two experiments would seem to indicate that the majority of the proteinase F and MBTI are localized in the protein bodies of the ungerminated, or newly rehydrated, mung bean, rather than in the cytosol or endoplasmic reticulum. However, both proteins can be readily lost from the protein bodies under certain conditions of isolation.

DISCUSSION

We have previously shown that the degradation of MBTI in the germinating bean is initiated by the removal of the last four amino acid residues from the carboxyl-terminus of the native inhibitor (MBTI-F) to produce the first modified form (MBTI-E) of the inhibitor (28). This activity is present in the ungerminated seed (30). Here we have detailed the isolation of this enzyme, proteinase F, from the dry mung bean.

Proteinase F acts as an endopeptidase, cleaving MBTI-F at the Asp76-Lys77 peptide bond, releasing the carboxyl-terminal tetrapeptide Lys-Asp-Asp-Asp. It appears to be highly specific in action. No further degradation of the inhibitor is observed, as evidenced by the inability of proteinase F to further attack MBTI-E or MBTI-C. In addition, the proteinase appears inactive against the native form of the major reserve protein of the mung bean, vicilin. Proteinase F is also inactive towards peptide T-IIIA, which represents residues 61 to 80 of MBTI and thus contains the Asp76-Lys77 peptide bond. This suggests that the conformation of this peptide bond found in the native MBTI molecule may be required for it to serve as a substrate for the proteinase.

It is unclear at present as to which mechanistic class proteinase F belongs. It is strongly inhibited by the mercurials pCMPS and pHMB, suggesting that the enzyme may be a cysteine proteinase. However, proteinase F exhibits little or no inhibition by iodoacetate and the peptide inhibitor E-64, both of which are also generally considered to be inhibitors of cysteine proteinases (12). It is also not affected by leupeptin, an inhibitor of the mung bean cysteine proteinase vicilin peptidohydrolase (1, 2). In addition, there is no evidence that the presence of thiols is required to maintain the enzyme in an active state. No thiol reducing agent such as DTT or mercaptoethanol was used throughout the purification of proteinase F. The final enzyme preparation is remarkably stable without thiols at 4°C under a normal O_2-containing atmosphere, losing only 18% of the initial activity in 10 months. Finally, no activation of proteinase F by DTT was found. It therefore seems unlikely that proteinase F is a cysteine proteinase. Substantial inhibition was noted with two other inhibitors, PMSF and chymostatin. This suggests that proteinase F may be a serine proteinase.

The enzymic properties of proteinase F suggest that it is in fact the enzyme responsible for the initiation of the degradation of MBTI-F in the germinating mung bean. However, for a proteinase and its putative substrate to exist as a true physiological couple they must both be located in, or have access to, the same cellular compartment. For this reason we examined the subcellular localization of the proteinase and MBTI.

There is some question in the literature as to the localization of the Bowman-Birk type trypsin inhibitors in the legume cotyledon parenchyma cell. A number of studies (generally involving subcellular fractionation by centrifugation) have indicated that most or all of the trypsin inhibitor is located in the cytosol (7, 19), while another study using immunochemical EM has demonstrated their presence in the protein bodies (14). Our own studies presented here suggest that over half of the Bowman-Birk type trypsin inhibitor in the mung bean is localized in the protein bodies.

These disparate results may be due to actual differences in the subcellular localization of the inhibitors in different legume species. Alternatively, they may be due to both the nature of the protein body membrane and the small size of the Bowman-Birk type trypsin inhibitors. Protein bodies have proved to be notoriously 'leaky' *in vitro*, readily losing various protein constituents during isolation. It seems likely that if relatively large proteins such as α-mannosidase (mol wt approximately 200,000) are readily lost from the protein body during isolation (17), then the small trypsin inhibitor (mol wt approximately 8,000) would even more readily leak from these organelles.

At present, the only *in vivo* function we can assign to proteinase F is the initiation of MBTI degradation. It is evident that the proteinase is not responsible for the initiation of vicilin degradation, a function that may be ascribed to vicilin peptidohydrolase (2). Nor does it contribute to the further degradation of the partially proteolyzed mung bean trypsin inhibitors and vicilin. That role appears to be played largely by carboxypeptidase II and the vicilin peptidohydrolase.

Given the simultaneous presence of proteinase F and at least a portion of the MBTI-F in the protein body, one must question why MBTI-F, rather than MBTI-E, is the principal inhibitor species in the mature seed. It may be that proteinase F is inactive under the conditions in the protein body during the period of protein accumulation and subsequent desiccation of the seed. This could be due to a pH in the protein body during seed maturation that is sufficiently removed from the pH optimum (pH 4.5) of the proteinase F. During germination and seedling growth the pH within the protein body is presumably titrated to a value consistent with the activity of the hydrolases, including proteinase F, vicilin peptidohydrolase, and carboxypeptidase II.

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