Control of Storage Protein Metabolism in the Cotyledons of Germinating Mung Beans: Role of Endopeptidase 1,2

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ABSTRACT

The autodigestive proteolytic activity of extracts of cotyledons of mung beans (Phaseolus aureus Roxb.) increased 4- to 5-fold during germination. A similar increase was found in the ability of these extracts to digest added casein or mung bean globulins. The increase occurred after a 2-day lag during the next 2 to 3 days of germination and coincided with the period of rapid storage protein breakdown. To understand which enzyme(s) may be responsible for this increase in proteolytic activity, the hydrolytic activity of cotyledon extracts toward a number of synthetic substrates and proteins was measured. Germination was accompanied by a marked decline in leucine aminopeptidase, while carboxypeptidase increased about 50%. There were no dramatic changes in either a-mannosidase or N-acetyl-b-glucosaminidase, enzymes which may be involved in the metabolism of the carbohydrate moieties of the reserve glycoproteins. The increase in general proteolytic activity was closely paralleled by a 10-fold increase in endopeptidase activity. This activity was inhibited by sulphydryl reagents such as N-ethylmaleimide. Studies with inhibitors of proteolytic enzymes showed that reagents which blocked sulphydryl groups also inhibited the rise in general proteolytic activity. Our results suggest that the appearance of a sulphydryl-type endopeptidase activity is a necessary prerequisite for the rapid metabolism of the reserve proteins which accompanies germination.

Leguminous seeds contain large amounts of storage proteins. The characteristics of these storage proteins, their synthesis during seed development, and their metabolism during seed germination have been studied extensively. The storage proteins consist of a 7S component, vicilin, and an 11S component, legumin, both localized in specialized organelles called albumen grains or protein bodies (4, 22). These protein bodies also contain some proteolytic enzymes (8, 17, 20, 24), as well as other hydrolyases (11, 14). During germination the protein bodies enlarge, possibly as a result of their increased osmotic potential due to proteolysis. Germination is accompanied by an increase in the proteolytic activity in the cotyledons of peas (Pisum sativum) (1, 8, 27) and beans (Phaseolus vulgaris) (10, 26), but it is not known what kind of enzymes are responsible for this increase (endo- or exopeptidases), where they are localized (in the protein bodies or in the cytoplasm), and whether this increase is due to enzyme activation, enzyme synthesis, or the disappearance of inhibitors. Legume cotyledons are rich sources of protease inhibitors, but efforts to show that these are involved in the control of storage protein utilization have been inconclusive (8, 15). We have recently begun to investigate the chemistry and metabolism of storage proteins in mung beans (Phaseolus aureus) (3) and the following is an account of our work on measuring the activities of various proteases and peptidases in the cotyledons at different stages of germination.

MATERIALS AND METHODS

Germination. Seeds of mung beans (Phaseolus aureus Roxb., also called Vigna radiata [L.] Wilczek) obtained from a local dealer, were sterilized in 10% commercial bleach for 30 min. The seeds were rinsed five times with sterile H2O and imbibed in sterile H2O for 24 hr with a stream of air bubbling through the water. The imbibed seeds were sterilized again with 1% commercial bleach for 5 min, rinsed with sterile H2O, planted in autoclaved moist vermiculite, and grown at room temperature (18°C) in the dark. Growth was quite uniform. By the 4th day, the cotyledons became slightly pink and started to wrinkle; by the 7th day they were usually completely dried out. Growth under these conditions was somewhat faster than in our earlier experiments (3). The cotyledons were removed at 1-day intervals and stored in lots of 40 in capped vials at −20°C.

Extraction. Lots of 40 cotyledons were homogenized in a cold mortar and pestle in a total volume of 15 ml of 0.1 M borate, pH 8, or 25 mM citrate-phosphate, pH 5, both containing 2 mM 2-mercaptoethanol. The former medium solubilized the storage proteins, the latter did not. The homogenates were filtered through cheesecloth, centrifuged at 12,000g for 20 min, and dialyzed against 25 mM citrate-phosphate, pH 5, with 1 mM 2-mercaptoethanol. The precipitated storage protein was always resuspended before aliquots were removed from the dialyzed solution.

ENZYME ASSAYS

Leucineaminopeptidase. Leucine-paranitroanilide (25 mg) was dissolved in 1 ml of dimethyl sulfoxide and made to a total volume of 50 ml with 50 mM phosphate buffer, pH 6.7. The final concentration was 2 mM. Two ml of this solution were incubated with 0.1 ml of enzyme for 20 min at 30°C. The reaction was stopped by the addition of 1 ml of N per-
chloric acid. If a precipitate formed, it was removed by centrifugation. The absorbance was read against a blank at 410 nm. One unit of activity corresponds to a change of 1 absorbance unit/hr.

**Carboxypeptidase.** The assay for carboxypeptidase was based on that of Visuri et al. (23). N-Carbenzoxyl-L-phenylalanine-L-alanine (37 mg) was dissolved in 1 ml of warm dimethylsulfoxide and made to a total volume of 50 ml with 25 mM citrate-phosphate, pH 5, containing 0.5 mM EDTA. The reaction was stopped with 1 ml of 15% trichoroacetic acid. The proteins were allowed to precipitate for 15 min in the cold, and the mixture was centrifuged for 3 min at 2000g. The amino acid content of the supernatant was determined using the ninhydrin method of Yemm and Cocking (25). For control samples, substrate and enzyme were incubated separately to allow amino acid release as a result of self-digestion of the enzyme extract. Enzyme activity is expressed as micromoles of amino acid released/hr·bean.

**Caseolytic Activity.** Casein, light white soluble obtained from The British Drug Houses Ltd., was dissolved in 25 mM citrate-phosphate, pH 5.7, at a concentration of 1%. The assay mixture included 1 ml of casein, 0.2 ml of enzyme, and 0.8 ml of H2O, and was incubated for 2 hr at 37 C on a shaking waterbath. The reaction was stopped by the addition of 1 ml of 15% trichoroacetic acid. Proteins were allowed to precipitate in the cold for 15 min, and the precipitated proteins were removed by centrifugation. The amino acid content of the supernatant was determined using ninhydrin as a color reagent (25). Enzyme activity is expressed as micromoles of amino acid released/hr·bean.

**Autodigestive and Globulytic Activity.** Cotyledons were always homogenized in 0.1 M borate, pH 8, to solubilize the globulins. The homogenates were dialyzed (see above) and the pH was adjusted to 5.4. One ml was incubated at 37 C for 2 hr either with 1 ml of H2O (autodigestive activity), or with 1 ml of mung bean globulin (containing 10 mg/ml of protein). At the end of the incubation, proteins were precipitated with 1 ml of 15% trichloroacetic acid and removed by centrifugation. The amino acid content of the supernatant was determined using ninhydrin as a color reagent (25). Enzyme activity was expressed in the same way as caseolytic activity.

**Endopeptidase Activity.** Endopeptidase activity was determined on extracts of cotyledons homogenized in 0.1 M borate pH 8 and dialyzed against 25 mM citrate-phosphate, pH 5. The precipitated storage proteins were removed by centrifugation, and the cleared extracts were used in the assays. Endopeptidase was assayed by viscosimetry according to the method of Sundblom and Mikola (21) using an Ubbelohde capillary viscosimeter and a 5% solution of gelatin (Bacto Gelatin, Difco Laboratories) in 25 mM citrate-phosphate at pH 5.7. The results are expressed as a decrease in specific viscosity/hr·seed. Endopeptidase was also assayed using Azocoll (Calbiochem) as a substrate. Azocoll is a chromogenic substrate consisting of a dye complexed to a protein. Hydrolysis of the protein causes the dye to be released. One ml of extract at pH 4.7 was incubated with 2 ml of H2O and 10 mg of Azocoll for 1 to 3 hr at 37 C. After the incubation, 2 ml of 0.1 N NaOH containing 2% Na2CO3 were added, and the tubes were immediately centrifuged to remove the excess substrate. The absorbance of the released dye was measured at 520 nm and 1 unit of activity represents an increase in 1 absorbance unit/hr.

**Glycosidases.** α-Mannosidase and N-acetyl-β-glucosaminidase were assayed using nitrophenyl derivatives as substrates. Both were assayed at 30 C in 25 mM citrate-phosphate, pH 5. An aliquot of the enzyme (0.1 ml) was added to 2 ml of 2 mM p-nitrophenyl-N-acetyl-β-d-glucosaminide and incubated for 10 min, or 2 ml of 10 mM p-nitrophenyl-α-d-mannoside and incubated for 30 min. The reaction was stopped by the addition of 1 ml of 4 M Na2CO3 and the absorbance was determined at 410 nm. One unit of activity corresponds to a change in absorbance of 1 absorbance unit/hr.

**Acylamidase Gel Electrophoresis.** Polypeptides were separated by electrophoresis on acrylamide gels (10% acrylamide) containing 0.1% SDS using as a buffer (0.1 M sodium phosphate, pH 7) according to the procedure of Shapiro et al. (18). The protein samples were boiled for a few minutes with 1% 2-mercaptoethanol and 0.2% SDS. The gels were stained with amido-black, destained, and scanned at 610 nm with a Gilson gel scanner.

**Inhibitors.** N-Ethylmaleimide was used at a concentration of 2.5 mM in the presence of 1% 2-mercaptoethanol. It was freshly prepared each time as a 50 mM stock solution. Phenylmethylsulfonyl fluoride was dissolved in ethanol at a concentration of 50 mM and used at a concentration of 1% ethanol (2%) was added to controls whenever appropriate. Enzyme-containing extracts were treated with inhibitors for 12 hr at 0 C.

**RESULTS**

Changes in fresh weight and protein content in different parts of the mung beans grown under our conditions have been summarized in Figure 1, primarily to allow a comparison with the experiments performed in other laboratories and on other legumes. The increase in the fresh weight of the axis follows the familiar sigmoid curve, while the weight of the cotyledons started to decline after 3 days. Protein disappeared at a linear rate after the 2nd day.

The pH optima for the different proteases and peptidases studied are shown in Figure 2. Both autodigestion and caseolytic activity had similar, slightly acidic pH optima of 5.4 and 5.7. The two exopeptidases differed considerably: carboxypeptidase had its optimum at 5, while leucineaminopeptidase's optimum was around 6.7. The two endopeptidase assays used showed quite different pH activity curves. Using the viscosimetric assay the enzyme(s) were active from pH 5 to pH 10, and with Azocoll as a substrate there was a sharp maximum around pH 4.6, but considerable activity was present at higher pH levels up to pH 10. The two glycosidases (α-mannosidase and N-acetyl-β-glucosaminidase) had pH optima between 4.8 and 5 (data not reported).

The changes in the levels of all these enzymes during germination are shown in Figure 3. Autodigestive activity increased 4- to 5-fold after an initial lag, and then declined. Globulytic activity followed a similar pattern, but it remained at a higher level at 5 to 6 days after the start of germination. The proteolytic activity measured with casein as a substrate also followed the same pattern, although the rate at which amino acids were released was much greater. After a 2-day lag caseolytic activity increased about 4- to 5-fold during the next 3 days. Such changes in general proteolytic activity have been documented for other legumes (1, 16, 26, 27), but it has not been shown which enzymes are responsible for this increase.

Neither the glycosidases nor the exopeptidases appear to follow the same pattern. Neither α-mannosidase nor N-acetyl-β-glucosaminidase showed similar large increases in activity during germination (Fig. 3B). Leucine aminopeptidase activity declined gradually while carboxypeptidase activity increased between 50 and 100% after an initial lag (Fig. 3C).
The increase in endopeptidase activity was much greater (10- to 15-fold, see Fig. 3D) and the timing of the increase coincided with the increase in general proteolytic activity. The same pattern was obtained whether gelatin was used as a substrate (at pH 5.7) or Azocoll (at pH 4.6).

The endopeptidase activity was measured on extracts made by homogenizing the cotyledons in 0.1 M borate at pH 8 and then dialyzing against 25 mM citrate-phosphate at pH 5. To eliminate the possibility that the high pH may have inactivated one of the endopeptidases (assuming that more than one was present), cotyledons were also directly extracted at a lower pH with 25 mM citrate-phosphate at pH 5. Such extracts of cotyledons from seeds which had been germinated for 2, 4, and 6 days were assayed for endopeptidase activity with gelatin as substrate, at three different pH values (pH 3.7, 5.7, and 7.5). There was no activity at pH 3.7 and no evidence was obtained that the alkaline extraction inactivated any part of the endopeptidase activity.

Proteases and peptidases are normally classified in at least three groups: those which have a serine residue in their active site, those which have a sulfhydryl group in their active site, and those which have neither. These three classes can be distinguished by the use of inhibitors such as NEM and pCMB for the -SH proteases and DFP and PMSF for the serine proteases. The results in Table I show that carboxypeptidase was inhibited by PMSF but not by NEM, while endopeptidase was inhibited by NEM and pCMB, but not by PMSF. The results suggest that carboxypeptidase is a serine protease while the endopeptidase is a sulfhydryl protease.

The relative contribution of carboxypeptidase and endopeptidase to proteolytic activity in bean extracts was determined by measuring self-digestive activity and caseolytic

**Fig. 1.** Growth kinetics of mung bean seedlings. Left: fresh weight of the axis; right: fresh weight of the cotyledons ( - - - - ) and protein content of the cotyledons ( - - - - ). Plants were grown in the dark at room temperature (about 18°C).

**Fig. 2.** pH profiles of enzymatic activities present in extracts of mung bean cotyledons. Activities are expressed in relative units to simplify the graphs. Extracts of cotyledons from beans germinated for 1 day were used for the carboxypeptidase and the leucine-aminopeptidase. Extracts of cotyledons from beans germinated for 4 days, were used for the other activities. Citrate-phosphate buffer was used for caseolytic activity, autodigestion, carboxypeptidase, and endopeptidase (Azocoll) up to pH 7.5. Phosphate buffer was used for leucine-aminopeptidase and endopeptidase (gelatin) up to pH 8.5. Tris-buffer was used for endopeptidase (gelatin) above pH 8.5.

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*Abbreviations: NEM: N-ethylmaleimide; PMSF: phenylmethyl sulfonylfluoride; pCMB: parachloromercuribenzoic acid; DFP: diisothiouronate.*
activity in the presence of either NEM or PMSF. The increase in proteolytic activity which accompanied germination, was largely inhibited by NEM, but not by PMSF. This was true for both self-digestive and caseolytic activity (Fig. 4).

Electrophoretic separation of the storage proteins on SDS-acrylamide gels was used to determine the role of endopeptidases in the hydrolysis of these proteins. Cotyledons were removed from seeds which had been germinated for 1 or 5 days, homogenized in 0.1 m borate, pH 8, dialyzed against 25 mM citrate phosphate, pH 5, containing 2 mM 2-mercaptoethanol, and the extracts containing both the storage proteins and the enzymes were incubated at 26 °C for 12 hr to allow proteolysis to occur. The extracts were then diluted, suitable aliquots were loaded on SDS-acrylamide gels, and the polypeptides were separated by electrophoresis. In each case similar aliquots taken at the beginning of the incubation and at the end were used (scans of the gels are shown in Fig. 5). The extent of hydrolysis can be assessed by comparing the band pattern before and after incubation. Extracts of cotyledons from seeds germinated for 1 day had five major bands (Fig. 5A), two of large mol wt (around 50,000 daltons), and three of smaller mol wt (around 25,000 daltons). Extracts of cotyle-
dons from seeds germinated for 5 days had only four bands (Fig. 5C). The highest mol wt band was absent and there was relatively much less of the second highest mol wt band in comparison to the three bands of lower mol wt. This suggests that extensive degradation of storage protein has already taken place. Each band may well contain more than one polypeptide. There is little change in the band pattern when the extracts of cotyledons obtained from seeds germinated for 1 day were incubated (compare Fig. 5B with 5A) for 12 hr at 26°C; however, extensive hydrolysis of the largest band occurred when extracts of cotyledons obtained from seeds germinated for 5 days, were incubated in the same way (compare Fig. 5D with 5C). This breakdown was prevented when the extracts containing 2 mM 2-mercaptoethanol were preincubated with 4 mM NEM for 12 hr at 0°C.

Table I. Effect of Protease Inhibitors on Carboxypeptidase and Endopeptidase

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<thead>
<tr>
<th>Treatment</th>
<th>Carboxypeptidase</th>
<th>Endopeptidase</th>
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<td></td>
<td>1-day</td>
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<td>mM</td>
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<td>Control</td>
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<tr>
<td>pCMB 0.1</td>
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DISCUSSION

These results confirm and extend the observations of other workers that seed germination in legumes (1, 16, 26, 27) is accompanied by an increase in proteolytic activity in the storage organs. Our aim has been to identify the enzyme(s) responsible for this increase, by measuring the enzymatic activities of glycosidases, exopeptidases, and endopeptidases.

The storage proteins of mung beans have recently been shown to be glycoproteins containing both mannose and glucosamine (3). This prompted us to measure the activities of glycosidases which could be involved in hydrolyzing the carbohydrate moieties of the storage proteins. No dramatic changes in their activities were observed, suggesting that the level of activity present at the start of germination may be high enough to allow storage protein breakdown (assuming that these enzymes are necessary). The observed decline in leucineaminopeptidase has also been found in the cotyledons of germinating peas (1, 8) and kidney beans (16). The decline suggests that the enzyme may not play an important role in storage protein metabolism.

Carboxypeptidase, on the other hand, increased by about 50%. Mikola (private communication) observed that cotyledons of peanuts and peas have low levels of carboxypeptidase and these levels do not change markedly during germination. Germination of cotton, on the other hand, is accompanied by a dramatic increase in carboxypeptidase activity as a result of the de novo synthesis of the enzyme (9). The carboxypeptidase activity of mung bean cotyledons is inhibited by PMSF and the enzyme appears to be a typical plant carboxypeptidase with a serine residue in its active site (9, 19). The inhibition of enzymatic activity by PMSF is more pronounced after 1 day of germination (95% inhibition) than after 5 days (65% inhibition), suggesting that the increase in enzyme activity may be due to the appearance of a different enzyme which reacts with this model substrate for carboxypeptidase, but
The increase in general proteolytic activity observed during germination is largely resistant to PMSF inhibition. This suggests that carboxypeptidase plays only a minor role in the total proteolytic activity of the extracts. Using a similar approach, Mikola et al. (12) found that malt carboxypeptidases play a rather important role in the release of amino acids in the barley mash used for brewing. Carboxypeptidase is found in the protein bodies of mung bean cotyledons (5), but its role in the metabolism of the reserve proteins remains to be elucidated.

Endopeptidase activities have not been previously measured in the cotyledons of germinating legume seeds, except by the use of model substrates such as benzoyl arginine-p-nitroanilide. Others (7, 8) have found, and we have confirmed (unpublished results) that trypsin-like activity, as measured by benzoyl arginine-p-nitroanilide hydrolysis, declines during germination. This activity is normally measured at an alkaline pH (pH 7.5 or above), quite unlike the pH optimum for casein hydrolysis or self-digestion. When endopeptidase activity using gelatin or Azocoll as a substrate was measured at acidic pH (5.7 and 4.6 respectively), we found a dramatic increase in activity. This increase coincided with the disappearance of the storage proteins and the increase in autodigestive and cas elasticity activities. The endopeptidase activity was furthermore inhibited by sulfhydryl inhibitors, but not by PMSF; similarly, we found a strong inhibition of the increase in general proteolytic activity by sulfhydryl inhibitors, but not by PMSF. These results strongly suggest that the enhancement of the endopeptidase activity, which accompanies germination, is a prerequisite for the metabolism of the storage proteins. This conclusion is supported by the observations on the hydrolysis of storage proteins as revealed by acrylamide gels. There is little breakdown when homogenates from cotyledons of 1-day germinated seeds are incubated for 12 hr at 26 C. Breakdown is much more pronounced in the homogenates from cotyledons of 4-day germinated seeds. This breakdown is abolished by prior addition of NEM.

It is not possible to decide whether the increase in endopeptidase activity is due to the appearance of one or more enzymes. The broad pH activity curve suggests the presence of more than one enzyme with endopeptidase activity. Chromatography of an endopeptidase-containing extract on DEAE-cellulose revealed only one peak of endopeptidase activity completely separated from the carboxypeptidase activity.

The first indication that endopeptidases may play an important role in reserve protein metabolism came from the work of Jacobsen and Varner (10), supplemented by that of Sunblom and Mikola (21). The former found that aleurone layers of barley treated with gibberellic acid, synthesize and secrete one or more proteases into the endosperm, while the latter showed that these proteases are primarily sulfhydryl-type endopeptidases. More recently, Harvey and Oaks (6, 7) have shown that the metabolism of storage proteins in corn endosperm is also dependent on the appearance of endopeptidase activity.

A number of important questions remain to be answered. Electron microscopical evidence suggests that the hydrolysis of storage proteins occurs within the intact protein bodies (2, 13). If this is indeed the case, then we would expect the endopeptidase to be located in the protein bodies of those cells in which reserve protein metabolism is taking place (5). Is the increase due to de novo synthesis, to the activation of pre-existing enzyme, or to the disappearance of an inhibitor? Legume cotyledons are rich sources of protease inhibitors, but these are thought to be localized outside the protein bodies (8) and have never been shown to inhibit the endogenous proteases or to be involved in the control of reserve protein metabolism. If the enzyme is synthesized de novo, it needs to be transported to the protein bodies. During germination the protein bodies swell and become large vacuoles. The increase in the total surface area of the limiting membrane implies that new membranous components are added on. This could well be the result of the transport to the protein bodies of enzymes enclosed in small vesicles. Further investigations are needed to determine how storage protein utilization is regulated in leguminous seeds. An elucidation of the mechanism involved in the enhancement of the endopeptidase activity described here will provide an important link in understanding this problem.

LITERATURE CITED


ENDOPEPTIDASE IN MUNG BEAN COTYLEDONS


