A protease responsible for post-translational cleavage of a conserved Asn-Gly linkage in glycinin, the major seed storage protein of soybean

(protein maturation/endopeptidase/purification/*Glycine max*/11S globulins)

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ABSTRACT The assembly of 11S globulin seed storage proteins in plants is regulated in part by the activity of a protease that cleaves between asparagine and glycine residues. Post-translational cleavage of subunit precursors into acidic and basic polypeptides is associated with the ability of subunits in trimers to aggregate into hexamers *in vitro*. An activity is present in extracts from immature soybean seeds that specifically cleaves immature 11S seed storage proteins of soybean and *Vicia faba* into the polypeptides of the mature proteins. Sequence microanalysis has been used to demonstrate that proglycinin and prolegumin are cut at the legitimate site when proteins synthesized *in vitro* are used as substrates. A single amino acid change in the cleavage site renders the substrate uncleavable. The protease responsible for this activity also hydrolyzes a synthetic octapeptide whose sequence reproduces four amino acids on either side of the glycinin subunit G4 cleavage site. This assay permitted the purification and characterization of the protease. It is a glycosylated enzyme with an acidic pH optimum and a molecular mass of about 45 kDa in solution.

Proteolysis is an important mechanism for regulating biological systems. Among the biological systems regulated by proteolysis are blood clotting (1) and the complement system (2). In these cases proteolysis is used to amplify the initial signal and effect a response. Proteolysis is also used to activate enzymes in the proper subcellular location when premature activation may be harmful to the cell. Examples include the activation of mammalian digestive enzymes (3) and peptide hormones (4). We now describe an enzyme that apparently carries out a similar function in plants. The deposition of storage proteins in protein storage vacuoles (PSVs) of legume seeds is regulated, at least in part, by a proteolytic cleavage (5). The cleavage is required for final assembly of oligomers into a more complex structure within the protein bodies and ensures that the deposition and final assembly take place in the proper subcellular organelle.

In soybean (*Glycine max*), the 11S family of storage proteins, trivially named glycinin, makes up a large percentage of the total protein in the seed. This protein serves as a source of reduced nitrogen and carbon for the young seedlings; therefore it is crucial that the proteins be stored in a form that will permit them to survive desiccation of the seed.

Proglycinin is synthesized in cotyledonary parenchyma cells on the rough endoplasmic reticulum (6). After the removal of a signal peptide, proglycinin subunits undergo assembly into trimers that are subsequently transported to the Golgi apparatus (7). Next the trimers move to dense vesicles (6) and are transported to the large central vacuole

of the cell under the direction of a sorting mechanism (8). Once in the large central vacuole, a proteolytic cleavage takes place (6, 9) to form the acidic and basic polypeptides of each subunit. The two polypeptides remain covalently linked by a disulfide bond (10). The mature glycinin subunits are isolated from seeds as hexamers, and they originate from protein bodies that form as buds from the central vacuole as the seed matures (11). It has been shown that the post-translational cleavage step is required for the assembly of trimers into hexamers *in vivo* (5). For this reason, the cleavage is considered a potential regulatory step in the pathway leading to glycinin deposition.

The conserved nature of the area around the cleavage site has been noted (12). The residues on either side of the cleavage [P1 and P1' (13)] are invariably asparagine and glycine, and the fourth residue after the site (P4') is glutamate. Moreover, the physical properties of the amino acids on the COOH-terminal side of the site are conserved through the seventh amino acid. Proteases with this specificity for cleavage have yet to be characterized.

Enzyme activities accomplishing this cleavage have been identified in castor bean (14) and pumpkin (15). In both cases, identification of the activity was based on obtaining proteolytic fragments of the expected size. However, Dickinson *et al.* (5) have shown that proteases such as papain and trypsin are capable of mimicking the effect of the maturation protease by cleaving near the conserved Asn-Gly site. Because the positions of cleavage by papain and trypsin do not correspond to the Asn-Gly bonds cleaved during maturation of proglycinin, it is possible that the proteolytic events described previously could be due to nonspecific cleavage at an exposed loop near the normal processing site. Here we describe the purification of an enzyme from immature soybeans and demonstrate that it cleaves at the legitimate processing site.

MATERIALS AND METHODS

Purification of the Protease. All procedures were carried out at 4°C or in ice where possible. Total protein was determined with the Bio-Rad protein microassay. Crude extracts were prepared by grinding in a chilled mortar 55 g of midmaturation soybeans in 15 ml of 0.7 mM citrate/phosphate (pH 7.0) that contained 0.4 M NaCl, and the slurry was homogenized with a Brinkmann Polytron homogenizer (PT-20 generator, setting 3 on rheostat, 1 min). The extract was centrifuged for 10 min at 15,000 × *g*, then the supernatant was recovered and centrifuged again as before. The super-

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Abbreviations: DTT, dithiothreitol; E64, *N*-[*N*-(1-3-*trans*-carboxy-oxiran-2-carbonyl)-L-leucyl]agmatine; PSV, protein storage vacuole. [‡]To whom reprint requests should be addressed.

nant of the second spin was filtered through Miracloth (Calbiochem) to yield 25 ml of crude extract.

Twenty milliliters of the extract was applied to a 500-ml Sepharose 6B column and eluted with the same buffer used for extraction. Active fractions were pooled, dialyzed into 2.5 mM citrate/phosphate (pH 5.0), and centrifuged 10 min at $15,000 \times g$. The supernatant was applied to a 350-ml SP-Sephadex C-50 column. When unbound protein no longer eluted, the bound protein was eluted with a linear gradient of NaCl from 0 to 0.15 M in 2.5 mM citrate/phosphate (pH 5.0). Active fractions were pooled and adjusted to 50% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ for 30 min. After insoluble protein had been removed by centrifugation for 10 min at $15,000 \times g$, the supernatant was applied to a 5-ml Con A-Sepharose 4B column. Samples were eluted from the column with a linear gradient from 1 mM CaCl_2 /1 mM MnCl_2 and no methyl α -D-mannopyranoside to 0.3 M methyl α -D-mannopyranoside and no CaCl_2 and MnCl_2 . Active fractions were pooled and concentrated with an Amicon Centricon-30 microconcentrator. During the concentration, the buffer was changed to 2.5 mM citrate/phosphate (pH 5.0) that contained phenylmethylsulfonyl fluoride (380 mg/ml), pepstatin (6.8 mg/ml), leupeptin (125 mg/ml), EDTA (12 mM), and dithiothreitol (DTT) (2 mM).

Protease Assays. The peptides ETRNGVEE, ETRN, and GVEE were synthesized using an ABI model 430A peptide synthesizer. Digestion reactions were carried out for 1.5 hr at 30°C in 0.01 M citrate/phosphate (pH 5.0) that contained 2 mM DTT and ETRNGVEE at 0.1 mg/ml. HPLC analysis of reaction products from a 100- μl reaction mixture was performed with an AX-300 anion-exchange column (Syn-Chrome, Lafayette, IN) in 0.01 M Tris-HCl (pH 7.0). The products were eluted from the column with a gradient of NaCl from 0 to 0.5 M. TLC analysis of the reaction products was performed on cellulose TLC plates developed in 50% (vol/vol) 2-propanol and visualized by spraying with ninhydrin; 2–10 μl of sample was spotted on the origin of each lane.

Determination of Molecular Mass. One hundred microliters of purified protease or a 1 mg/ml solution of standard proteins was chromatographed on a Synchropak GPC-300 column (SynChrom) equilibrated in 0.1 M KH_2PO_4 , pH 7.5, at 0.4 ml/min.

Preparation of Proglycinin and Prolegumin Synthesized *in Vitro*. *In vitro* transcription and translation reactions and sucrose gradient fractionation were performed as described by Dickinson *et al.* (16), except the proteins were labeled with both [^3H]leucine and [^{14}C]glutamate. After sucrose gradient fractionation, samples containing the trimers were pooled and concentrated from 1 ml to about 100 μl in an Amicon Centricon-30. During concentration, the buffer was changed to 0.035 M sodium phosphate (pH 7) containing 0.4 M NaCl.

Digestion and Analysis of Proteins Synthesized *in Vitro*. In a single reaction mixture, 1 μl (7000 cpm) of protein synthesized *in vitro* was incubated with 2 μl of purified protease. The pH of the mixture was adjusted to 5 with 1 μl of 0.02 M citrate. Reaction mixtures were incubated 1.5 hr at 30°C and then components were separated by SDS/PAGE. After electrophoresis, the gels were treated with EN 3 HANCE (New England Nuclear) according to the manufacturer's instructions, dried, and exposed to film.

Plasmid Construction. Plasmids pSP65/248 and pSP65/466 (16) were used to produce G4 proglycinin and G5 proglycinin, respectively. To produce LeB prolegumin, the plasmid pLeMS was constructed by removing the signal sequence coding region from a LeB cDNA (17), using oligonucleotide-directed mutagenesis (18). The processing site mutant, pLeBAN281, was produced from this plasmid by using the same mutagenesis technique to delete the Asn-281 codon.

Microsequencing Digestion Products. G4-proglycinin, 12,000 cpm in 2 μl , was digested in a reaction scaled up from

the one described above. Edman degradation reactions were carried out using an Applied Biosystems gas-phase sequencer model 470A, and the phenylthiohydantoin derivatives of the amino acids were collected. The radioactivity in products from each cycle of the reaction was determined by using a scintillation counter.

Antibody Production and Western Blot Analysis. Mouse polyclonal antibodies were raised against both the 85- and 65-kDa polypeptides isolated from an SDS/PAGE gel (ref. 19, pp. 61–68 and 98–119). Proteins in purified protease preparations were transferred to nitrocellulose membrane by using a Bio-Rad electroblotter. Immunoreactive proteins were detected by using the mouse serum and Bio-Rad's GAM-HRP detection system as described in the manufacturer's protocol.

RESULTS

Octapeptides that Include the G4 Glycinin Cleavage Site Are Cleaved by Plant Extracts. An octapeptide, ETRNGVEE, was synthesized that contained the four amino acids on either side of the Asn-Gly proteolytic cleavage site in G4 glycinin (20). The octapeptide was tested to see if it was cleaved by proteases in extracts from immature seeds. Fig. 1 shows the digestion products separated by TLC. The major products of this reaction have chromatographic mobilities identical to those of the two tetrapeptides expected from the cleavage reaction.

The extent of reaction could be quantified by separating the digestion products by using ion-exchange HPLC and measuring the heights of the resulting peaks. The ratio of the ETRN peak height to the sum of the ETRN and ETRNGVEE peak heights was proportional to the amount of extract added to the reaction mixture. Using this ratio, a unit of activity was defined as the amount of extract required to produce a ratio of 0.01 for ETRN/(ETRN + ETRNGVEE). This assay proved too insensitive to quantify enzyme levels throughout purification. When activity could not be quantified it was measured qualitatively by the TLC assay.

Purification and Characterization of the Protease. Table 1 summarizes the steps undertaken to purify the protease. During the initial step in this scheme, midmaturation soybean seeds were ground with 0.7 mM citrate/phosphate (pH 7.0) that contained 0.4 M NaCl. This step resulted in the coextraction of the major storage proteins with the protease. Coextraction with the storage proteins was necessary for the protease to be removed from the seed efficiently. Gel filtration chromatography was then used to eliminate the bulk of the storage proteins from the extract. Proteolytically active eluate from the column was dialyzed into 2.5 mM citrate/phosphate buffer at pH 5, a step that caused most of the residual storage proteins to precipitate. After dialysis, the solution was cleared by centrifugation and further resolved

FIG. 1. Digestion of the octapeptide ETRNGVEE by the protease. Fractions from the Con A column were incubated with ETRNGVEE and analyzed by TLC. Active fractions (lanes 3 and 4) yield the products ETRN and GVEE in addition to the substrate. The identities of the spots were determined by comparing their mobilities and staining characteristics to those of chemically synthesized peptides.

Table 1. Summary of the protease purification

Step	Protein, mg	Activity, units	Specific activity, units/mg	Purification, fold	Yield, %
Extract	2000	3800	1.9	—	100
Sepharose 6B	250	1300	5.2	2.7	34
Dialysis	220	1300	5.9	3.1	34
Cation exchange	2	400	200	105	11
(NH ₄) ₂ SO ₄	1	NQ			
Con A	0.1	NQ			

NQ, not quantified.

by cation-exchange chromatography. The active fractions were pooled and adjusted to 50% saturation with solid (NH₄)₂SO₄. The insoluble protein was removed by centrifugation, and the supernatant was applied to a Con A affinity column. The protease activity bound to the column and was eluted by using a gradient of methyl α -D-mannopyranoside.

As shown in Table 1, the enzyme had been purified about 100-fold after it was eluted from the cation-exchange column. Because of the lack of sensitivity of the enzyme assay and the limited amounts of purified enzyme available, we did not determine specific activity subsequent to purifying it by cation-exchange chromatography. It was clear, however, that the specific activity increased substantially during the last two steps of purification.

The purified enzyme was characterized by gel-filtration chromatography and by electrophoresis. Fig. 2A shows that the proteolytic activity has the retention time in a HPLC size-exclusion column equivalent to that of a globular protein of about 45 kDa. When the preparation was resolved on SDS/PAGE in nonreducing conditions (Fig. 2B), a single band of 85 kDa was observed. When the protein was separated under reducing conditions, however, a band of 65 kDa and two bands of about 23 kDa were seen in addition to the 85-kDa band. We concluded that the smaller polypeptides were disulfide bonded to the 65-kDa polypeptide. Because not all of the 85-kDa polypeptide was eliminated in reducing conditions, it was clear that more than one form of protein was contained within the nonreduced band.

We sought to define the immunological interrelationships among the peptides in the purified protease. For this purpose, the proteins in the 85- and 65-kDa electrophoretic bands were used to raise polyclonal antibodies. The Western blot shown in Fig. 3 indicates that antibody against the 85-kDa protein reacts with the 85-kDa protein and both the 65- and 23-kDa

FIG. 3. Immunodetection of blotted protease preparations. Blots were allowed to react with serum from mice immunized with either the 85-kDa polypeptide (lanes 1 and 2) or the 65-kDa polypeptide (lane 3). Lanes 1 and 3 contain 6 µg of purified protease and lane 2 contains 2 µg of purified protease.

proteins. Conversely, antibody raised against the 65-kDa protein reacted with the 65- and 85-kDa protein bands, but not with 23-kDa protein. These data establish that there is an immunological relationship between proteins in the three major components of the purified protease. Because antibodies raised against the 65-kDa protein did not bind to the 23-kDa bands, we concluded that they originated from the 85-kDa protein.

The effects of DTT, divalent metal ions, pH, and several protease inhibitors on activity of the purified protease were tested. DTT stimulated proteolytic activity 30%, while Hg²⁺ and Cu²⁺ inhibited activity by 90% and 50%, respectively. *N*-[*N*-(1-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine (E-64) inhibited activity 20%, while Ca²⁺, Mn²⁺, Mg²⁺, phenylmethylsulfonyl fluoride, pepstatin, leupeptin, EDTA, and α_2 -macroglobulin each had no measurable effect on activity. Proteolytic activity was maximal at pH 5 and was 30% lower at pH 7 and 4.

Proglycinin and Prolegumin Are Processed Correctly *in Vitro* by the Purified Protease. The specificity of the purified protease was tested by cleavage of proglycinin and prolegumin. For these experiments, radioactively labeled G4 and G5 proglycinin and LeB prolegumin were synthesized from cDNAs and assembled into trimers as described by Dickinson *et al.* (16). The resulting trimers were purified on sucrose gradients and then digested with the purified enzyme. Fig. 4

FIG. 2. (A) Determination of the molecular mass of the protease in solution. Size standards (○) were chromatographed on an HPLC gel filtration column. BAM, β -amylase; ADH, yeast alcohol dehydrogenase; BSA, bovine serum albumin; OVA, hen egg albumin. ●, Elution position of the protease activity. (B) Coomassie blue-stained SDS/polyacrylamide gel showing the polypeptide composition of a purified active protease preparation. Polypeptides in the first two lanes were separated in the absence of reducing agent and samples in the second two lanes contain 10 mM DTT. The two outside lanes were intentionally overloaded to show minor polypeptides. Molecular masses are given on the left in kDa.

FIG. 4. Digestion of 11S subunit precursors with purified protease. Prolegumin or proglycinin subunits were synthesized *in vitro* and digested with the maturation protease. Peptides equivalent in size to the mature acidic and basic chains were generated. SS, size standards (kDa); lane 1, prolegumin; lane 2, G5 proglycinin; lane 3, G4 proglycinin. Note that G4 proglycinin contains additional cleavage sites (21).

shows that cleavage products from both proglycinin and prolegumin were of the same size as the naturally occurring acidic and basic polypeptides. In the case of G4 proglycinin, however, small amounts of other peptides were observed.

To verify that cleavage occurred at the correct site, Edman sequence microanalysis was performed on the reaction products. For these experiments, G4 proglycinin and LeB prolegumin were synthesized with [^3H]isoleucine. After digestion with the protease, the phenylthiohydantoin derivatives of amino acids from the reaction were subjected to scintillation counting to determine the positions of labeled amino acids. Fig. 5 shows that the radioactive residues in both G4 and LeB were in positions expected to be labeled if cleavage took place between the conserved Asn and Gly residues.

A Single Amino Acid Change at the LeB Cleavage Site Abolishes the *in Vitro* Processing Reaction. The specificity of the protease for the conserved Asn-Gly bond was tested by altering an amino acid in the cleavage site of prolegumin. For this purpose, the plasmid pLeBAN281 was produced in which the P1 asparagine codon of the LeB coding region was deleted. This plasmid was used to produce the modified protein LeBAN *in vitro*. Purified LeBAN trimers were used as a substrate for the purified protease. Fig. 6 shows that the modified protein was not cleaved, consistent with the hy-

FIG. 6. Fluorograph showing the effect of a processing site modification on digestion by the protease. *In vitro*-synthesized LeB prolegumin (shown undigested in lane 2) and prolegumin LeBAN (in which the P1 asparagine was deleted) were digested in the same reaction (lane 4). In lane 3, the mutant prolegumin LeBAN is digested alone. LeB α peptide synthesized *in vitro* from the plasmid pGS5 is shown in lane 1 as a size standard.

pothesis that the enzyme has specificity for the Asn-Gly peptide bond.

DISCUSSION

We describe a protease that catalyzes the correct post-translational cleavage of 11S storage proteins of soybean and *Vicia faba*. This protease is not inhibited by inhibitors of acid, metal, or serine proteases, but it is inhibited to some extent by E-64, an inhibitor of several thiol proteases (22). Because the enzyme is also inhibited by HgCl_2 and activated by DTT, we conclude that a free sulfhydryl is involved in conferring activity to the enzyme. However, our data do not permit distinction between an active site sulfhydryl residue and one located elsewhere in the molecule that indirectly affects enzymatic activity. Because none of the inhibitors eliminate enzymatic activity, an indirect effect is considered the more likely possibility.

The protease cleaves a peptide bond between an asparagine and a glycine. This type of bond is found cleaved post-translationally in 11S seed storage proteins and several lectins that are found in PSVs. The protease is of consider-

FIG. 5. Sequence microanalysis of G4 proglycinin (A) and LeB prolegumin (B) after digestion by the protease. Both proteins were synthesized *in vitro* and labeled with [^3H]isoleucine. The acidic polypeptide sequence shown is that of the proprotein synthesized *in vitro*, and the basic polypeptide sequence shown is the sequence expected if the cleavage occurred at the expected site.

able interest because to our knowledge it is the first enzyme purified that specifically cleaves Asn-Gly peptide bonds.

Evidence was presented that deletion of asparagine from prolegumin results in a substrate that is not cleaved by the purified protease. We considered it unlikely that this lack of cleavage is due to a conformational change in the protein that prevents interaction of the protease with the cleavage site, although this possibility cannot be formally eliminated. However, unpublished results indicate that deletions of other amino acids in the immediate vicinity of the Asn-Gly bond do not prevent the cleavage from taking place. Systematic studies will be required to characterize the substrate requirements of the enzyme more fully.

We have been unable to determine accurately the specific activity of the purified enzyme. The enzyme emerges from the cation-exchange column about 100-fold purified compared with the original extract, and several observations suggest that a considerable improvement in purity is realized during the final steps in the purification. Ammonium sulfate precipitation results in the removal of about half of the protein and no detectable activity is recovered in the precipitate. About 90% of the protein remaining after ammonium sulfate fractionation fails to bind the Con A-Sepharose column and contains no detectable activity. Consequently, at least a 1000-fold purification compared to the original extract may have been achieved. The data shown in Fig. 2 support this possibility. A single major component is visible when SDS/PAGE is carried out under nonreducing conditions. Although three major components are visible in gels after reduction of disulfide bonds (Fig. 2), immunological evidence shown in Fig. 3 establishes that the three components are related to one another. These arguments, and the fact that the same three bands copurify with the protease activity through four other steps of purification, convince us that other proteins not visible on the SDS/PAGE gels are unlikely to account for the protease activity.

The relationship between the 85-, 65-, and 23-kDa peptides contained in the purified preparation of the protease remains of interest. Although reduction of sulfhydryls in the purified enzyme preparation results in a partial conversion of 85-kDa protein to 65- and 23-kDa peptides (Fig. 2), the question arises as to the identity of 85-kDa protein that remains unchanged after reduction of disulfides. The results suggest that part of the protease was cleaved during purification while the remainder was not. Alternatively, the preparations could contain more than one 85-kDa protein.

The Western blot analyses shown in Fig. 3 indicate that all three peptides are related immunologically and suggest that the 23-kDa proteins originate from one end of the 85-kDa protein. In support of this conclusion, Edman analysis of the 85- and 65-kDa proteins purified from SDS/PAGE gels by electroblotting has revealed that their NH₂-terminal amino acid sequences (FYP; unpublished results) appear to be the same. We therefore consider that the 23-kDa proteins originate from the COOH-terminus of the 85-kDa polypeptide.

A disparity exists between the molecular mass of the protease in solution and its molecular mass as determined by SDS/PAGE. Although the structural basis for this inconsistency has not been explored, glycans attached to the polypeptide chain undoubtedly contribute to the difference in molecular masses observed. Glycans tend to interact with gel filtration media to retard their elution and result in a lower molecular mass estimate than expected. Conversely, glycoproteins migrate more slowly than predicted during SDS/PAGE because the carbohydrates add to the bulk of the SDS-coated protein in the gel. It seems unlikely, however, that glycans are completely responsible for the differences observed, so other structural features are probably involved as well.

The contents of PSVs are thought to be organized in a microcrystalline structure (23), and this high level of organization suggests that the assembly of storage proteins is a carefully controlled event rather than a random process. During PSV formation, the reserve proteins are initially deposited at the inner surface of the membrane that delimits the large central vacuole in the cell. The PSVs form around these protein deposits as they bud away from the large central vacuole (24). While the molecular details of this process are not well understood, hexamers of mature 11S subunits can be purified from PSVs of dry seeds. These hexamers obviously originate from a more complex oligomeric structure in the vacuole. In this regard, Dickinson *et al.* (5) report that the post-translational cleavage of proglycinin subunits into acidic and basic peptide chains is a prerequisite to their assembly into hexamers *in vitro*. If hexamer formation is required for the assembly of a more complex oligomeric structure, the protease that carries out this post-translational cleavage would control the initial deposition of protein in the central vacuoles. It remains to be shown, however, that the protease we purified carries out this particular modification *in vivo*. Genetic evidence establishing that the enzyme must be present for cleavage to occur, or immunocytological evidence that the enzyme resides within PSVs in developing seeds, would help resolve this issue.

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