Identification of the Acidic and Basic Subunit Complexes of Glycinin*

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Paul E. Staswick†, Mark A. Hermodson, and Niels C. Nielsen‡§

From the †United States Department of Agriculture, Science, and Education Administration, and the ‡Departments of Agronomy and Biochemistry, Purdue University, West Lafayette, Indiana 47907

Five complexes consisting of one acidic and one basic subunit that were linked via disulfide bonds were purified from unreduced S-alkylated glycinin. The acidic and basic subunits were identified unambiguously using NH₂-terminal sequence analysis, sodium dodecyl sulfate (SDS)-electrophoresis, and analytical isoelectric focusing. The subunit pairings are A₁B₂, A₁B₁B₂, A₂B₁, A₂B₂, and F₂B₂. Polypeptide A₁ was not linked to a corresponding basic subunit via a disulfide bond. The study shows that pairing between subunits is nonrandom, which is consistent with evidence that glycinin is synthesized as a M₀ = 60,000 precursor that undergoes post-translational modification to form the individual linked subunits.

Numerous attempts have been made to determine the structural relationships between polypeptides contained within the protein complex called glycinin, one of the primary storage proteins in soybean seeds. These studies demonstrated that glycinin consists of two major groups of polypeptides which are designated acidic and basic subunits on the basis of their isoelectric points (1). Resolution of the polypeptides making up each of the two groups has been difficult, however, due to similarities in their size and charge. Recently, the major polypeptides present in each of the two major subunit groups were purified and identified unambiguously on the basis of their NH₂-terminal and partial internal amino acid sequences (2, 3). Six acidic (A₁, A₂, A₃, A₄, A₅, and F₂) and five basic (B₁, B₂, B₃, B₄, and B₅) polypeptides having homologous but distinct sequences were found. These polypeptides could each be the products of more than one gene even though they are homologous in the regions sequenced, since each of the 11 purified acidic and basic subunits exhibited charge heterogeneity upon isoelectric focusing (3).

Evidence has been reported that at least some of the acidic and basic subunits are linked with one another via disulfide bonds (4, 5) to form acidic-basic polypeptide complexes (AB-complexes). Reconstitution studies of reductively denatured glycinin as well as the electrophoretic properties of partially purified complexes suggested that pairing between subunits could be nonrandom (6), although precise identification of the individual polypeptides involved in forming them was not possible with the methods used for those studies.

It is important to determine whether pairing between acidic and basic subunits is specific and, if so, which subunits are involved, since this would not only bear on the assembly process for the glycinin complex, but also on the genetic relationships of the subunits. We report here the use of NH₂-terminal sequence analysis to distinguish between the various polypeptides and identify precisely which subunits are covalently linked.

MATERIALS AND METHODS†

RESULTS AND DISCUSSION

Preparation of Nonreduced Glycinin—Nonreduced glycinin was purified using the procedure of Moreira et al. (2), except that sulfhydryl-reducing agents were removed from the buffers. Sodium dodecyl sulfate-polyacrylamide electrophoresis of purified unreduced glycinin yielded a major band at M₀ = 57,000 and a minor one at about 30,000 (Fig. 1, lane 1). Exposure of the unreduced glycinin to 2-mercaptoethanol prior to electrophoresis resulted in breakdown of the complexes into the component acidic (M₀ = 37,000-42,000) and basic (M₀ = 20,000) subunits (Fig. 1, lane 10). A minor band was present in lane 10 at about 10,000 daltons but was generally lost during destaining of the gels. It was due to acidic polypeptide F₂. These results indicated that the acidic and basic subunits were linked to each other in vivo via one or more disulfide bonds. We refer to the linked subunits as AB-complexes.

Five AB-complexes were purified using anion exchange chromatography and then characterized using NH₂-terminal amino acid sequence analysis, sodium dodecyl sulfate electrophoresis, and isoelectric focusing. Unambiguous identifications of the acidic subunits associated with each complex were made on the basis of the NH₂-terminal sequence data published previously (2, 3), while identification of the basic subunits relied on differences in the isoelectric focusing patterns of the purified proteins (3). The results of these experiments are summarized in Table I, and the procedural details supporting our conclusions are contained in the miniprint supplement.†

The results establish that the acidic and basic subunits of glycinin are nonrandomly associated with each other via disulfide bonding. Each of the acidic and basic polypeptides of
fied AB-complexes. The fractions in lanes 1-5 were unreduced. Those in lanes 7-10 corresponded to the ones in lanes 1-5 following treatment with 2% 2-mercaptoethanol. F1 and F2 had the same mobility as F3, and are not shown. Fraction 7 was also unaffected by treatment with 2-mercaptoethanol.

**Fig. 1. Sodium dodecyl sulfate-electrophoresis of the purified AB-complexes.** The fractions in lanes 1-5 were unreduced. Those in lanes 7-10 corresponded to the ones in lanes 1-5 following treatment with 2% 2-mercaptoethanol. F1 and F2 had the same mobility as F3, and are not shown. Fraction 7 was also unaffected by treatment with 2-mercaptoethanol.

### Table 1

| Table 1 | Comparison of the methionine content of the acidic and basic subunits and the AB-complexes of glycgin |
|---------|------------------------------------------------------|
| Acidic | Basic | AB-complex | No. Met |
| A1x | 4 | B2 | A1x B2 | 7 |
| A1b | 4 | B1b | A1b B1b | 6 |
| A2 | 6 | B1a | A1a B1a | 8 |
| A3 | 2 | B1 | A1 B1 | 3 |
| F2(2) | 1 | B2 | F2(2) | 1 |
| A4 | 1 | Unknown | Unknown | Unknown |

* Taken from Ref. 2.
* F2(2) is ~ one-fourth the size of other acidics.

glycinin which have been identified thus far on the basis of differences in their primary structures was accounted for in these experiments. With the exception of A4, which is apparently not covalently bound to a basic subunit, each acidic subunit is linked to only one of the basic polypeptides. Given the high degree of sequence homology among the acidic and basic subunits, it is difficult to envision a simple mechanism whereby previously unlinked molecules could associate with one another with such high specificity. We propose that an alternative view accounting for this specificity is that the pairing arises because certain acidic and basic polypeptides are synthesized together from a single gene as a high molecular weight precursor. Evidence supporting this hypothesis is presented in the accompanying paper where it is shown that translating mRNA purified from developing seeds results in the synthesis of a ~60,000 polypeptide which can be selectively immunoprecipitated by antglycinin-IgG (7).

An unanswered question is whether or not A4 has a unique basic polypeptide associated with it as all other acidic polypeptides do. If so, then the two are either not disulfide linked like the other complexes or else its disulfide bonds are particularly sensitive to cleavage. In this regard, it should be noted that several soybean cultivars including Raiden lack F2(2), A4, and B3. Since F2(2) makes up only a small proportion of the total 11 S protein and does not appear to account for all of the B3 which can be purified from glycinin preparations, we propose that A4 is associated with B3 in vivo.

Both F2(2) and A4 exhibited size heterogeneity (Fig. 1). While such heterogeneity had not previously been observed for these polypeptides, A4 had been reported to be heterogeneous in size even though it had a single NH2-terminal sequence. While steps were taken to limit proteolysis during the experiment by addition of diisopropylphosphorofluoridate and phenanthroline, an artifactual origin of the size heterogeneity cannot be ruled out. Other plausible explanations are either that several different gene products have the same NH2-terminal sequence or there is some variability in the posttranslational modification of precursors to the AB-complexes.

Since the soybean storage proteins are nutritionally limiting in methionine and cysteine when fed to monogastric animals, it is important to consider variations in the content of this amino acid among the various complexes which have been identified (Table I). There is an 8-fold difference between the complexes having the highest and lowest methionine content. More importantly, the high methionine acidic and basic subunits pair together, while those low in methionine are also paired. This observation may ultimately be quite important in improving the methionine content through plant breeding, particularly if the ratios of the various glycinin complexes present in the seed can be altered with respect to one another. Since glycinin is the most prevalent protein in soybeans, eliminating the methionine-deficient AB-complexes can be expected to exert marked effect on the nutritional quality of the seed.

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Glycinin Subunit Complexes

Identification of the Acidic and Basic Subunit Complexes of Glycinin
P.I. Staehelin, M.A. Hermanson and R.C. Nielsen

Materials and Methods

Glycinin was purified from soybean cotyledons GM6-1-1-1 [Staehelin and Hermanson (1)] by methods previously described by Staehelin et al. (2). Except that D-terminally labeled material was purified from the buffers. Phenylpropylphosphorofluoridate (PPPF) and 1,2-diaminoethane (DAE) were included in the buffers used to extract protein from freshly-prepared soybean meal. The 2-alkylated complexes were prepared by adding about 50 mg of unalkylated glycinin to 10 ml of 6 M guanidine-HCl in phosphate buffer (pH 7.2) containing NaCl of redistilled 4-vinylpyridine. Other details of the labeling procedure are described elsewhere (3). Following labeling, the sample was dialyzed against Sephadex G-25 column using 10 M formic acid.

SDS-polyacrylamide electrophoresis was performed in 12.5% slab gels (2). Isoelectric focusing was done in a 7.5% glycerol-phosphate slab gel containing 5% urea and 2% ampholyte (pH 3-10) as reported previously (4).

Micellar-terminal sequence analysis was performed using a Beckman 8904 Sequencer as described by Hermanson et al. (5). Identification of the acidic and basic subunits was accomplished by high performance liquid chromatography. A modification of the procedure of Hermanson et al. (6) was used to confirm the identification of certain tryptic and aliphatic residues (6).

Results

Preparation of non-reduced Glycinin: The A/B-complexes were not soluble under denaturing conditions. Heating the non-reduced glycinin samples in SDS-phenol sample buffer prior to the addition of SDS and reducing reagent caused the subunits to aggregate. In the absence of reducing agents were excluded from the buffer (Fig. 2, miniprint, lanes A and B). Complexes isolated under identical conditions, but in the absence of SDS, were still more stable, although considerable breakdown of the complexes still occurred. Presumably the denaturing effect of SDS and elevated temperature promotes exchange of intermolecular disulfide bonds for intramolecular ones involving free sulfhydryls.

Purification and Identification of Subunits: Anion exchange chromatography of reduced intact glycinin on a DEAE-cm column (5) with linear NaCl gradient was used to separate the A and B subunits. The 20,000 molecular weight band eluted at 0.6 M NaCl from the DEAE-cm column. The subunit A2B2 present in the sample was recovered in the absence of NaCl. The 20,000 molecular weight band was rechromatographed that they had complexed with the A2B2 (Table 1, miniprint).

The peak F3 was identified as the matrix A2B2 (Table 1, miniprint). The two AN terminal sequences of non-reduced glycinin and the A2B2 was identified as the complexing A2B2. The sequence analysis of F6 confirmed this identification and showed the A2B2 pairing (Table 2, miniprint).

The peak F2 from the DEAE-cm column resulted in several bands following SDS-electrophoresis and had molecular weights ranging from 20,000 to 30,000 (Fig. 1). These mobilities did not change when the sample was treated with D-terminally labeled material. Despite the slower migration, one peptide was evident in samples containing these bands were analyzed (Table 2, miniprint).

Clear identification of each acidic polypeptide, as well as A2 and B2, was possible based on examination of SDS-electrophoresis and amino acid analysis. Three free polypeptides sequenced of the acidic subunits, while 20,000 molecular weight bands containing the A2B2 subunits were identified by its complex elution profile from SDS-electrophoresis. It was not examined further. For this F6 contained A2B2 (Table 2, miniprint).

The peak F2 from the DEAE-cm column resulted in several bands following SDS-electrophoresis and had molecular weights ranging from 20,000 to 30,000 (Fig. 1). These mobilities did not change when the sample was treated with D-terminally labeled material. Despite the slower migration, one peptide was evident in samples containing these bands were analyzed (Table 2, miniprint).

A high degree of sequence homology between A2 and B2, as well as their spectral properties, indicated a subunit. This was examined further by sequence analysis. A high degree of sequence homology between A2 and B2, as well as their spectral properties, indicated a subunit. This was examined further by sequence analysis. A high degree of sequence homology between A2 and B2, as well as their spectral properties, indicated a subunit. This was examined further by sequence analysis.
Table 1:  
Automated Sequencer Determination of the NH₂-terminal Primary Structure of the Purified 85-kDa Glycinin Subunit Complexes

| Fraction | P1(a) | P1(b) | P1(c) | P1(d) | P2(a) | P2(b) |
|----------|-------|-------|-------|-------|-------|-------|
| Amount degraded (mg) |       |       |       |       |       |       |
| 1         | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |
| 2         | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |
| 3         | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |
| 4         | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |
| 5         | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |
| 6         | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |
| 7         | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |
| 8         | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |
| 9         | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |
| 10        | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |
| 11        | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |
| 12        | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |
| 13        | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   |

Figure 4: Separation of 65,000 dalton complexes from their basis on Sephacryl 50-100. 30 ml of protein from P1 were applied to the column (2.5 x 100 cm) that had been equilibrated with 0.1 M phosphate (pH 6.0) containing 6 M urea.

Figure 5: Isoelectric focusing analysis of the isolated components. All samples were reduced with 100 mM 2-mercaptoethanol and applied using 4% gels containing 7% gel during prior to application on the gel. The gel contained 25 amperograms spanning pH 3-11.

Figure 3: Separation of 65,000 dalton complexes on Sephacryl S-500. 1.2 g of unreduced 5-alkylated glycinin were eluted from a Superose 6 column (2.5 x 100 cm) previously equilibrated with 0.1 M phosphate (pH 6.0) containing 0.5 M urea. Fractions P1-P2 were eluted using the same buffer, then the remaining fractions were removed using a 3.5-liter linear gradient from 0.1 M NaCl. The elution profile of P1 after it was rechromatographed is shown in the inset.