Sulfhydryl Content of Glycinin: Effect of Reducing Agents

Walter J. Wolf

Biopolymer Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604

Analysis of 15 freeze-dried preparations of glycinin with 5,5'-dithiobis(2-nitrobenzoic acid) gave values of 0.6–2.2 sulfhydryl (SH) groups/mol with a mean value of 1.4 ± 0.5. When reduced with 2-mercaptoethanol (2-ME) in the absence of denaturants and separated from excess 2-ME by gel filtration, the SH content increased and leveled off at 21 SH/mol in the range 0.04–0.1 M 2-ME. With dithiothreitol (DTT), the SH content increased more rapidly with concentration than with 2-ME and leveled off at a maximum of 28 SH/mol at 0.02–0.1 M DTT. Sodium borohydride was less effective at low concentrations but yielded about 18 SH/mol at 0.1 M. Fully reduced glycinin contained 41 SH/mol. Results of SH measurements are compared with the current model for glycinin consisting of six subunits each comprised of an acidic and a basic polypeptide connected by a single disulfide bond. A large discrepancy was noted in the number of SH groups measured vs the number predicted from the model. Reasons for this discrepancy are discussed.

INTRODUCTION

Glycinin is one of the major storage proteins of soybeans. It has a molecular weight of about 320 000 (Badley et al., 1975) and possesses a quaternary structure consisting of six subunits of 54 000–64 000. Each subunit has the generalized structure A–SS–B, where A represents an acidic polypeptide of 34 000–44 000 and B is a basic polypeptide of about 20 000 (Nielsen et al., 1989). The A and B polypeptides are linked by a single disulfide (SS) bond (Staswick et al., 1984a). Sulfhydryl (SH) and SS contents of glycinin have been published, but SH contents vary widely. Reported values range from zero (Draper and Catsimpoolas, 1978; Kella and Kinsella, 1986) to 15 SH groups/mol (10 surface plus 5 buried SH groups; Hoshi and Yamauchi, 1983b).

The SS bond linking the acidic and basic polypeptides is of special interest in the heat denaturation of glycinin. When glycinin is heated at 100 °C for as little as 5 min, about 50% of the protein precipitates and 50% remains soluble as a 3–4 S entity (Wolf and Tamura, 1969; Mori et al., 1982). The precipitated fraction consists of the basic polypeptides, while the acidic polypeptides remain soluble (Mori et al., 1982; German et al., 1982). The rapid separation of the basic chains from the acidic chains of glycinin during heating indicates that the SS bond holding the two chains together is easily cleaved. Because the thermal destruction of cysteine and cystine at 100 °C is much slower than the rate at which the SS bond between the acidic and basic polypeptides is cleaved (Wang and Damodaran, 1990), it appears that SH–SS interchange may be responsible for the rapid cleavage of the interchain SS bond of glycinin. As a first step in examining this hypothesis, I established how many SH groups are present in unheated glycinin and determined how reducing agents affect the number of SH groups found. My findings and their implications for the structure of glycinin are described here.

MATERIALS AND METHODS

Chemicals. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), 2-mercaptoethanol (2-ME), disodium ethylenediaminetetraacetate (EDTA), dithiothreitol (DTT), and iodoacetamide were from Sigma Chemical Co., St. Louis, MO. Sodium borohydride and 4-vinylpyridine were products from Aldrich Chemical Co., Milwaukee, WI, and guanidine hydrochloride (GuHCl) was obtained from Pierce, Rockford, IL.

Purification of Glycinin. Crude glycinin was isolated from hexane-defatted flakes of Raiden cultivar according to the procedure of Thanh and Shibasaki (1976). A pH 8.0, 0.03 M Tris-HCl extract (containing 0.01 M 2-ME) of defatted flakes was adjusted to pH 6.4 with 2 N HCl, cooled to 3–5 °C overnight, and centrifuged at 4 °C. The precipitate was washed with 0.03 M Tris-HCl (pH 6.4), dissolved in standard buffer (pH 7.6, 0.5 ionic strength, 0.033 M K_2HPO_4, 0.0026 M KH_2PO_4, 0.4 M NaCl, 0.02% NaN_3, 0.01 M 2-ME, and 0.001 M EDTA), dialyzed against distilled water in the cold, and freeze-dried to yield crude glycinin. Glycinin was purified by dissolving 2–4 g of the crude protein in 25–50 mL of standard buffer without EDTA, centrifuging, and applying the supernatant to a column (12.5 × 2.6 cm i.d.) of concanavalin A–Sepharose 4B (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) equilibrated with the same buffer. Buffer was passed through the column until absorbance at 280 nm returned to the baseline. The unadsorbed protein fraction was concentrated by pervaporation and applied to two Sepharose 6B or Sephacryl S-300 (Pharmacia LKB Biotechnology) columns (93 × 2.6 cm i.d.) connected in tandem. The columns were equilibrated and eluted with standard buffer described above. Fractions were collected and absorbance measured at 280 nm. Fractions for the front half of the main peak were pooled, analyzed in the ultracentrifuge, dialyzed against distilled water, and freeze-dried to yield purified glycinin.

Ultracentrifugal Analysis. Samples were analyzed in standard buffer in a Beckman Model E analytical ultracentrifuge at 20–25 °C using a 30-mm double-sector cell at 48 000 rpm.

Sulfhydryl Analysis. The procedure of Ellman (1959) was used to measure SH groups in native and denatured glycinin. To 1 mL of protein solution was added 2 mL of buffer plus 0.05 mL of 0.01 M DTNB in buffer. The buffer used was standard buffer (see Purification of Glycinin) without 2-ME. Protein concentrations for assay of freeze-dried glycinin were 15–30 mg/mL. For reduction studies with 2-ME and DTT (see below), initial glycinin concentrations were 3 mg/mL, but passage through a Sephadex G-25 gel filtration column resulted in fractions containing 0.5–2 mg/mL. For borohydride reductions initial glycinin concentration was 5 mg/mL but dropped to 1–3 mg/mL on passage through the Sephadex G-25 column. To determine SH content under denaturing conditions, the 2-mL portion of buffer also contained 6 M GuHCl. The SH content of completely reduced glycinin was determined by using 0.01 M DTT in the presence of 4 or 6 M GuHCl and removing the excess DTT by gel filtration on a Sephadex G-25 (coarse) column (1.5 cm i.d. × 25 cm) previously equilibrated with buffer–GuHCl deaerated with He. Absorbance was measured at 412 nm, and a molar

Table I. Sulfhydryl Content of Glycinin before and after Reduction

		reductant removal		mol of SH/mol of glycinin[a]	
expt	treatment[b]	dialysis[c]	gel filtration[c]	native	denatured[d]
1–7	none	–	–	1.6 ± 0.4	1.7 ± 0.3
8	0.01 M 2-ME	+	–	1.3[e]	2.2[e]
9	0.01 M 2-ME	–	+	9.2 ± 0.7	9.3 ± 0.6
10	0.01 M DTT	+	–	13.8 ± 0.2	16.7 ± 0.2
11	0.01 M DTT	–	+	25.6 ± 1.4	
12	0.015 M $NaBH_4$	–[f]	–[f]	0.9[e]	
13	0.01 M KBH_4	–	+	0.8 ± 0.1	
14	0.01 M DTT + 4 M GuHCl	–	+		41.8 ± 1.1
15	0.01 M DTT + 6 M GuHCl	–	+		40.9 ± 1.0

[a] Assumed molecular weight of 320 000 (Badley et al., 1975). [b] Solvent was standard buffer without 2-ME except as noted otherwise. [c] Carried out under He atmosphere. [d] Denatured with 4 M GuHCl except for experiment 15. [e] Single determination. [f] Excess borohydride was destroyed by lowering pH from ~10 to 7.0.

extinction coefficient of 14 150/cm (Riddles et al., 1979) was used to calculate the concentration of SH. Concentration of glycinin was determined by measuring absorbance at 280 nm using $E^{1\%}_{1cm}$ of 8.4 as determined on a solution dried to constant weight at 100 °C in a vacuum oven after the bulk of the water was evaporated at 80 °C in a forced draft oven. The molar concentration of glycinin was calculated using a molecular weight of 320 000 (Badley et al., 1975).

Reduction of Glycinin. To 5 mL of deaerated (sparged with helium) standard buffer without 2-ME was added 15 mg of glycinin and either 2-ME or DTT in the range 0–0.1 M. The samples were allowed to stand under a blanket of helium for 4 h and were then centrifuged to remove turbidity to avoid erroneous values of absorbance at 280 nm due to light scattering. Excess reducing agent was removed by placing the centrifuged samples on a Sephadex G-25 column (described earlier) previously equilibrated with deaerated standard buffer without 2-ME and eluting with the same deaerated buffer at a flow rate of 1 mL/min. Fractions of 2 mL were collected and assayed immediately with DTNB to determine the SH content, and absorbance at 280 nm was used to determine the glycinin concentration. SH values reported are the means of the assays for three to five fractions from the Sephadex G-25 column.

For reduction with $NaBH_4$, 25 mg of glycinin was dissolved in 5 mL of deaerated buffer and a drop of octyl alcohol was added to minimize foaming. $NaBH_4$ was then added, which increased the pH to about 10. After a 4-h reaction, the pH was lowered to 7.0 with 1 M HCl, and then the sample was centrifuged and passed through the Sephadex G-25 column. Lowering the pH destroyed the excess borohydride. This prevented formation of H_2 bubbles and a disruption of the column bed when reduction mixture encountered the pH 7.6 buffer used to elute the column. Some reductions were also carried out without using the Sephadex G-25 column where the SH assays were conducted immediately after lowering of the pH and centrifugation. When samples were to be alkylated, a 5-fold excess of iodoacetamide over total SH content was added and permitted to react for 1 h in the dark. In the borohydride reaction mixtures, the pH was lowered after the iodoacetamide was added. After this reaction, the excess iodoacetamide and buffer salts were removed by dialysis and the samples were freeze-dried.

Determination of Mixed Disulfides. A modification of the method of Harrap et al. (1973) was used. Glycinin was reduced with 0.1 M $NaBH_4$ for 4 h as described, and a portion of the solution was mixed with an equal volume of 1.6 M trichloroacetic acid to precipitate the protein (Becker et al., 1940). After centrifugation, the supernatant was analyzed according to the Ellman procedure to determine 2-ME released from mixed disulfides by borohydride reduction. Another portion of the reduced protein solution was adjusted to pH 7.0, centrifuged, and assayed for total SH content, i.e., protein SH plus 2-ME released by reduction.

Fractionation of Acidic Polypeptides of Glycinin. The procedure of Staswick and Nielsen (1983) was used with minor modifications. One gram of glycinin was dissolved in 30 mL of 0.13 M Tris, pH 7.6, and 6 M GuHCl containing 0.001 M EDTA (deaerated with He). DTT (450 mg) was added and reduction allowed to proceed for 2 h at 25 °C followed by addition of 900 μL of 4-vinylpyridine and reaction for an additional 90 min. The solution was dialyzed against 400 mL of 0.1 M phosphate buffer, pH 6.6, containing 6 M urea and 0.02 M 2-ME (to react with excess 4-vinylpyridine). It was dialyzed against two changes of buffer–urea without 2-ME. Next, the sample was centrifuged to remove a small amount of turbidity and applied to a DEAE-Sephadex A-25 column (bed 2.6 × 43 cm) equilibrated with 0.1 M phosphate, pH 6.6, and 6 M urea. The column was eluted at 30 mL/h, and 10-mL fractions were collected. After the first two peaks (fractions 5–26) were eluted, the rest of the polypeptides were eluted with a 1.3-L linear NaCl gradient (0–0.4 M) in the same buffer. Absorbance was measured at 280 nm. Tubes containing peaks were carefully pooled and rinsed into SpectraPor 1 dialysis sacs (6000–8000 cutoff) to prevent loss of low molecular weight polypeptides. The polypeptide solutions were dialyzed exhaustively in the cold against distilled water, freeze-dried, and weighed.

Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted in a Hoefer Scientific Instruments (San Francisco, CA) Model SE 600 unit on 1.5-mm gels containing 12.3% acrylamide with an acrylamide/AcrylAide (FMC BioProducts, Rockland, ME) ratio of 36:1.5. Buffers were as in the Fling and Gregerson (1986) modification of the method of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250 (Sigma) as described by Fling and Gregerson (1986). Low molecular weight protein standards (Bio-Rad Laboratories, Richmond, CA) (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme) were run with each gel.

RESULTS

Sulfhydryl Content of Freeze-Dried Glycinin. Analysis of 15 preparations of glycinin gave values that were variable and lower than expected from the current model for the structure of glycinin (see Discussion). Values ranged from 0.6 to 2.2 SH groups/mol with a mean value of 1.4 ± 0.5. Table I (experiments 1–7) shows the mean SH contents for 7 of the 15 preparations along with the mean SH value for the same preparations after denaturation with 4 M GuHCl. Because there were no statistically significant differences in SH content among denatured and nondenatured samples, I concluded there were no buried or internal SH groups in freeze-dried glycinin as I prepared it.

Effect of Treatment with Reducing Agents. Hoshi and Yamauchi (1983b) reported 8.8 surface SH groups/mol and 4.1 buried SH groups/mol (both corrected to 14 150 for the molar extinction coefficient of 2-nitro-5-thiobenzoic acid and 320 000 for the molecular weight of glycinin) for a sample which was dissolved in standard buffer and separated from 2-ME by gel filtration. Because these values are considerably higher than the values obtained on the freeze-dried glycinin preparations, I examined the SH content of glycinin after treatment with various reducing agents. When glycinin was dissolved in

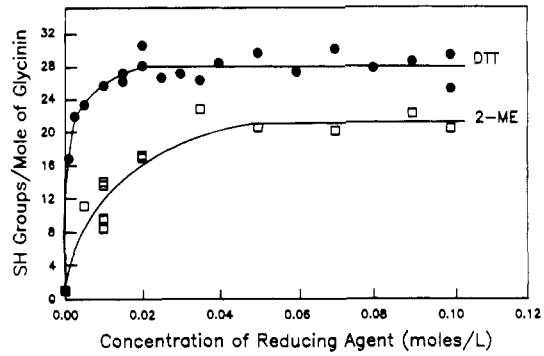

Figure 1. Effect of 2-mercaptoethanol (2-ME) and dithiothreitol (DTT) concentration on the sulfhydryl content of glycinin.

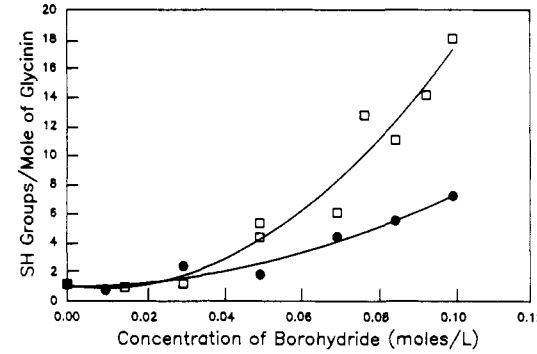

Figure 2. Effect of sodium borohydride concentration on the sulfhydryl content of glycinin. Lower curve was obtained on samples that were adjusted to pH 7, centrifuged, passed through a Sephadex G-25 column, and then assayed. Upper curve is for samples that were adjusted to pH 7 and assayed directly.

standard buffer and then dialyzed (blanketed with He) to remove the 0.01 M 2-ME (Table I), I obtained results in the same range (experiment 8) as those observed for freeze-dried glycinin. Removing the 2-ME by gel filtration, however, gave a value of about 9 SH groups/mol (experiment 9). Again, denaturation with GuHCl revealed no buried SH groups. The use of 0.01 M DTT resulted in a value of 14 SH groups/mol when the reductant was removed by dialysis, and this increased to 17 SH/mol upon GuHCl denaturation. However, the SH content increased to 26/mol when gel filtration was employed (experiment 11). Attempted reduction with borohydride in the same concentration range did not give an increase in the SH content of glycinin (Table I, experiments 12 and 13). The results with 2-ME and DTT clearly show that the excess reducing agent is preferably removed by gel filtration. The low values obtained when dialysis was used indicate that reoxidation or formation of mixed disulfides occurs during dialysis despite maintenance of an inert atmosphere of helium in the sample.

When glycinin was reduced with 0.01 M DTT in the presence of 4 or 6 M GuHCl, the protein was denatured and completely reduced and contained an average of 41.4 SH groups/mol (Table I, experiments 14 and 15).

Effect of 2-Mercaptoethanol Concentration. Because of the differences in the SH content of glycinin when it was reduced with either 0.01 M 2-ME or DTT, I investigated the effect of reductant concentration over the range of 0–0.1 M. Figure 1 shows that as the concentration of 2-ME was increased, the SH content continued to rise but leveled off at about 0.04 M. The mean value in the concentration range 0.035–0.1 M was 21.2 ± 1.6 SH groups/mol of glycinin, which represents 51% of the SH groups in the fully reduced protein.

Effect of Dithiothreitol Concentration. Figure 1 also shows the effect of varying the concentration of DTT. The SH values increased more rapidly with concentration and leveled off at a higher value than in the case of 2-ME. The values were essentially constant from 0.02 to 0.1 M; the mean for this concentration range was 28.1 ± 1.5 SH groups/mol of glycinin and corresponds to 68% of the SH groups in the completely reduced protein.

Effect of Borohydride Concentration. Although ineffective at 0.01 M (Table I), borohydride reduction yielded a gradual increase in SH content of glycinin as its concentration was increased (Figure 2). The lower curve was obtained by reaction with borohydride, followed by lowering the pH to 7, and centrifugation. The resulting supernatant was passed through the Sephadex G-25 column, and the fractions were assayed for SH as they emerged from the column. When the reaction mixture was neutralized, centrifuged, and assayed directly, higher SH values were obtained (Figure 2, upper curve) and approached the values obtained with 2-ME (Figure 1). The shape of both curves in Figure 2, however, differed from those of Figure 1 and exhibited no indication of leveling off, even at 0.1 M. Determination of mixed disulfide content of a sample reduced with 0.1 M borohydride indicated that about 20% of the apparent SH content was accounted for by 2-ME. Such mixed disulfide formation likely occurred during removal of standard buffer by dialysis in the final stage of purification of glycinin.

Table II. Ultracentrifugal Composition of Glycinin before and after Reduction

	composition, %				
treatment	2S	7S	11S	15S	>15S
none[a]		2.9	92.5	4.6	
D, FD[b]	8.5	7.6	58.8	18.6	6.5
D, FD + 0.01 M 2-ME	7.5	5.5	79.8	7.2	
D, FD + 0.1 M 2-Me	9.1	5.8	76.4	8.7	
D, FD + 0.01 M DTT	16.8	9.2	65.7	8.3	
D, FD + 0.1 M DTT	13.5	11.8	67.3	7.4	
D, FD + 0.01 M $NaBH_4$	10.1	7.5	66.4	12.1	3.9
D, FD + 0.1 M $NaBH_4$	16.4	12.2	65.2	6.2	

[a] Analyzed immediately after elution from Sephacryl S-300 column in standard buffer (containing 0.01 M 2-ME). [b] D, FD, dialysis against water followed by freeze-drying.

Effect of Reducing Agents on Ultracentrifugal Behavior of Glycinin. Because half to two-thirds of the total SS bonds are reduced by the different reagents, it was of interest to see whether reduction of these SS bonds had an effect on the quaternary structure of glycinin. Accordingly, 0.5% solutions of glycinin in standard buffer without 2-ME were treated with each of the reducing agents at 0.01 and 0.1 M and analyzed in the ultracentrifuge (Table II). Included in Table II are data for the freshly prepared glycinin as it emerged from the Sephacryl S-300 column; it contained about 3% of a 7S contaminant and 5% 15S fraction. The latter is a polymer (dimer?) of glycinin because the bulk of it elutes from the Sephacryl S-300 column just ahead of the glycinin, and on SDS–PAGE analysis (using a reducing gel) this 15S-rich fraction is indistinguishable from glycinin (unpublished results). After dialysis against distilled water and freeze-drying, the glycinin contained 8–9% of both 2S and 7S fractions, which likely are dissociation products (subunits and trimer form of glycinin subunits, respectively). About 20% of the glycinin is converted into 15S and >15S forms, which are SS-linked polymers (possibly dimer and trimer) of the 11S form. When the freeze-dried glycinin was treated with 0.01 or 0.1 M 2-ME, the SS polymers were cleaved and the 11S sedimenting form increased about 20%. DTT

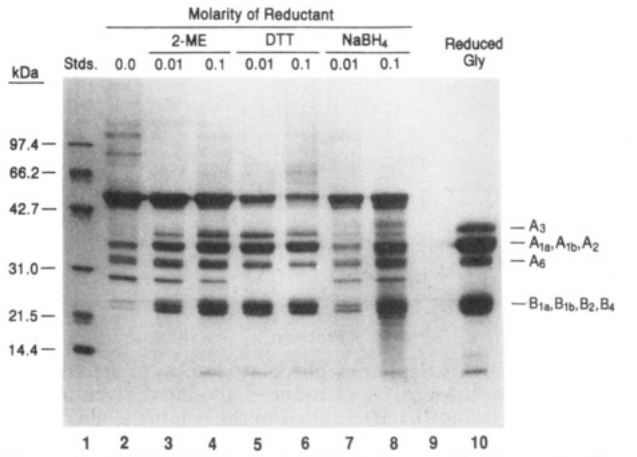

Figure 3. SDS–PAGE of glycinin before and after reduction with 0.01 and 0.1 M 2-mercaptoethanol, dithiothreitol, or sodium borohydride followed by alkylation with iodoacetamide. Lane 1 on far left contains molecular weight standards. Lane 9 was left empty, and lane 10 on far right is fully reduced glycinin (dissolved in sample buffer containing 0.6 M 2-mercaptoethanol and 5 M urea). Only fully reduced glycinin was heated at 100 °C prior to analysis.

also reduced the polymer forms but, in addition, caused more dissociation into the 2S and 7S entities than did 2-ME. Borohydride at 0.01 M caused only partial reduction of the SS polymers (15S + >15S), but with 0.1 M borohydride, the polymers are reduced to the same extent as with 2-ME or DTT. Both concentrations of borohydride caused about the same amount of dissociation into 2S and 7S fractions as noted with DTT.

SDS–PAGE Analysis after Reduction of Glycinin. Although 65–80% of glycinin retains its quaternary structure when it is treated with reducing agents (Table II), it was also of interest to determine whether the reducing agents cleaved the SS bond linking the acidic and basic polypeptide chains. Glycinin was therefore treated with 0.01 and 0.1 M reducing agents and the reduction was quenched by alkylating with iodoacetamide. The alkylated samples were dialyzed against water, freeze-dried, and then analyzed by SDS–PAGE (Figure 3). With the exception of lane 10, analysis of the reduced-alkylated samples was carried out with SDS that lacked 2-ME (lane 9 was not used to prevent diffusion of 2-ME into lane 8). Glycinin, without any reductive treatment (except for exposure to 0.01 M 2-ME during purification), migrated as a major band of 57 000 which consists of the principal subunits resulting from the dissociation of the quaternary structure under the influence of SDS (lane 2). Light bands of 85 000 and >100 000 are also present. Since these bands are absent when glycinin was completely reduced (lane 10, Figure 3), they are assumed to be SS-linked subunits that arose from the dissociation of the SS polymers present in the dialyzed freeze-dried glycinin (Table II). Similar SS polymers were observed in freeze-dried glycinin by Hoshi and Yamauchi (1983a). The untreated glycinin also exhibited bands corresponding to the acidic and basic polypeptides (compare with lane 10 which is fully reduced glycinin). An additional band occurred at 28 000. SDS–PAGE gels were also run in which the untreated glycinin was alkylated with an excess of iodoacetamide or 4-vinylpyridine prior to denaturation with SDS in the presence of the alkylating agents. The resulting SDS–PAGE gels were similar to that in Figure 3.

Glycinin treated with either 0.01 or 0.1 M 2-ME (lanes 3 and 4, Figure 3) exhibited only traces of bands above the 57 000 subunits. This observation further supports the conclusion that the 85 000 to >100 000 bands in untreated glycinin (lane 2) are SS-linked subunits based on the ultracentrifugal data (Table II) showing that both of these concentrations of 2-ME depolymerized most of the polymers of glycinin present after dialysis and freeze-drying. Both concentrations of 2-ME also increased the relative amounts of the acidic and basic polypeptides, decreased the amount of the 28 000 band, and led to the appearance of a band of about 12 000 at the bottom of the gel.

With DTT treatment (lanes 5 and 6, Figure 3), we see further decreases in the amounts of the 57 000 subunits and the 28 000 subunit has almost disappeared (not visible in Figure 3 but detectable on the gel). The highest concentration of DTT also caused some aggregation which was detected as a faint streaking from the top of the gel to the 57 000 subunits. Reduction of the SS bond between the acidic and basic polypeptide chains was extensive with DTT.

Although 0.01 M $NaBH_4$ treatment had no measurable effect on the number of SH groups detected in glycinin (Figure 2), it did eliminate most of the disulfide-linked subunits noted above 57 000 (lane 7, Figure 3) and the SDS–PAGE pattern most nearly resembled that for untreated glycinin (lane 2, Figure 3). Reduction with 0.1 M $NaBH_4$ resulted in more complete cleavage of the interchain SS bonds and an increase in the amounts of the acidic and basic polypeptide chains.

The 28 000 polypeptide in the untreated control and in partially reduced glycinin exhibited properties reported for A_5B_3, the disulfide-linked portion of glycinin subunit G4 ($A_5A_4B_3$) (Staswick et al., 1981; Nielsen et al., 1989). On reduction, the 28 000 band disappeared presumably by forming B_3 which migrates with the other basic polypeptides plus A_5 which migrated to the bottom of the gel (~12 000, Figure 3, lane 10). Densitometry of the gel indicated that the 28 000 band represented about 10% of the total glycinin.

Isolation of Glycinin in the Absence of Reducing Agent. Purified glycinin normally is isolated in buffers containing 0.01 M 2-ME (Thanh and Shibasaki, 1976), but this procedure appears to cause some reduction of the SS bonds linking the acidic and basic polypeptide chains (Figure 3). It was therefore of interest to attempt the purification without any reducing agent present. The procedure described under Materials and Methods was followed except that no 2-ME was included in any of the purification steps. After passage through concanavalin A–Sepharose followed by gel filtration on Sepharose 6 B, the main peak was divided in half because the contaminants changed from the front to the back of the peak. The solutions of the two fractions were analyzed in the ultracentrifuge in the absence and presence of 0.01 M 2-ME (Table III). In the absence of 2-ME in the ultracentrifugal analysis buffer, the glycinin preparation consisted of only 64–65% 11S component with significant contamination by 2S and 7S components. The 2S plus 7S contamination ranged from 9.5% in the front half of the peak to 25.2% in the back half. Analysis of the samples in the presence of 0.01 M 2-ME resulted in an increase of the 11S

Table III. Ultracentrifugal Analysis of Glycinin Prepared in the Absence of 2-Mercaptoethanol

		composition, %				
analysis buffer[a]	peak fraction	2S	7S	11S	15S	>15S
without 2-ME	front	4.8	4.7	64.8	17.6	8.1
without 2-ME	back	11.4	13.8	63.9	10.9	
with 2-ME	front	6.6	4.8	79.3	9.3	
with 2-ME	back	14.4	11.6	74.0		

[a] Standard buffer without and with 0.01 M 2-ME.

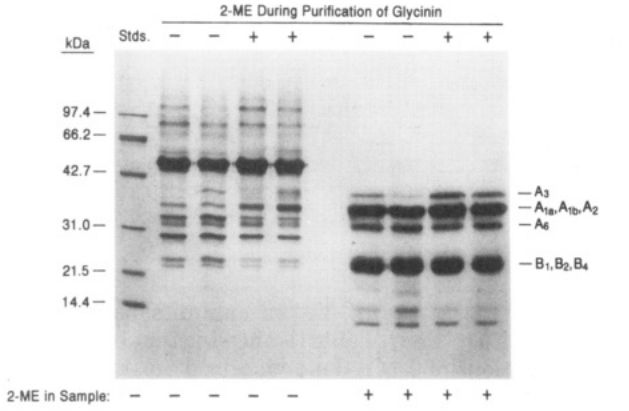

Figure 4. SDS–PAGE of glycinin prepared without and with 2-mercaptoethanol. Samples were analyzed on the gel under nonreducing (left half) and reducing (right half) conditions. Front half (lanes 2, 4, 7, and 9) and back half (lanes 3, 5, 8, and 10) of major peaks from Sephacryl S-300 column are shown. Only the samples in the reducing portion of the gel (lanes 7–10) were heated at 100 °C prior to analysis; reducing agent was 0.6 M 2-mercaptoethanol. Lane numbering is left to right; lane 6 (right of center) was not used.

component accompanied by a decrease in the 15S and >15S fractions, which apparently consisted of SS polymers of 11S. Under these conditions the purity of glycinin in terms of 11S component was only 74–79%. By comparison, mean compositions for seven glycinin preparations prepared and analyzed with 0.01 M 2-ME present were as follows:

	2S	7S	11S	15S
front half of peak	0	0	90.8 ± 4.3	9.2 ± 4.3
back half of peak	1.3 ± 1.8	6.5 ± 2.4	90.3 ± 1.8	1.9 ± 1.9

Typically, the front half of the peak was contaminated only with 15S component, whereas the back half contained primarily 7S contaminant.

After analysis in the ultracentrifuge, the two fractions of glycinin purified in the absence of 2-ME were dialyzed against water and freeze-dried. Analysis of the two fractions for SH groups gave the following results:

fraction	SH/mol
front half	0.5
back half	0.4

These values are a little below the range (0.6–2.2) noted earlier for the 15 samples of glycinin prepared with 0.01 M 2-ME present.

SDS–PAGE of glycinin prepared in the absence and presence of 0.01 M 2-ME (Figure 4) revealed surprisingly few differences between the two glycinin preparations in spite of the fact that the glycinin that had not been exposed to 2-ME was only 74–79% 11S component. Especially noteworthy is the fact that glycinin purified without 2-ME still appeared to contain free acidic and basic polypeptides. In the nonreducing portion of the gel, the back portion of the peak for glycinin prepared without reductant (lane 3) contained a fairly prominent band of about 40 000; its counterpart prepared in the presence of 2-ME (lane 5) contained several minor bands in this region. In the reducing portion of the gel, the back half of the glycinin peak prepared without 2-ME (lane 8) contained only a light A_3 band and exhibited more minor bands in the region below the basic chains as compared to the other samples. It appears that some of the acidic and basic polypeptides of glycinin are not linked by an SS bond in the native state irrespective of whether the glycinin was prepared with or without 2-ME present. The 28 000 subunit noted earlier (Figure 3) was again very obvious (Figure 4) as was the 12 000 band, which appears to arise from this subunit upon reduction.

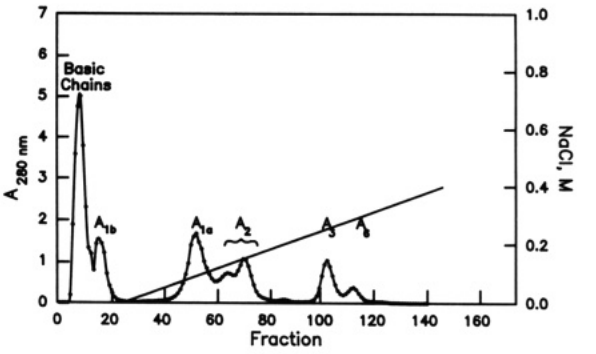

Figure 5. DEAE-Sephadex chromatography to separate the acidic polypeptides of 1 g of reduced-alkylated glycinin. The NaCl gradient (0.0–0.4 M) applied to the column is also shown.

Subunit Composition of Glycinin. Relative amounts of the different subunits in glycinin isolated from Raiden soybeans were estimated by DEAE-Sephadex chromatography of the reduced-alkylated protein (Figure 5). The elution diagram is in good agreement with the results of Staswick and Nielsen (1983), but they did not quantitate their separation. The two peaks eluting in fractions 61–82 were both designated polypeptide A_2 as per Staswick and Nielsen (1983). These two fractions are homologous in the their N-terminal sequences (Moreira et al., 1979) but demonstrate sequence heterogeneity farther down the chain (Staswick et al., 1984b). Relative amounts of the acidic polypeptides were estimated by weighing the materials recovered on exhaustive dialysis against water and freeze-drying (Table IV). Overall recovery of all of the fractions by weighing was 80% of the 1.0 g of sample placed on the column.

These percentages reflect the distribution of subunits in Raiden glycinin assuming that, prior to reduction, each major acidic chain is attached to a basic chain and all of the basic chains are about the same size (ca. 20 000). It is apparent that the major subunits present are $A_{1a}B_2$ (38%) and A_2B_{1a} (34%); an average of two of each of these subunits is present per mole (Table IV). SDS–PAGE analysis of the separated subunits (Figure 6) showed that the 12 000 protein eluted with the basic polypeptides, although traces of it also occurred in the adjacent fraction, A_{1b}. The other major fractions appear similar to those reported by Staswick and Nielsen (1983). Fractions 83–94, not reported by Staswick and Nielsen, consisted of a major band with a M_r of 24 000 plus minor bands of 53 000, 34 000, and 35 000; they represented only 1.4% of the recovered protein. Another previously uncharacterized fraction eluted in tubes 121–130. This very minor fraction consisted of a major band of 20 000 plus five less prominent bands in the range 28 000–39 000 (Figure 6 and Table IV).

DISCUSSION

Table V compares SH contents of glycinin as reported in the literature with the values obtained in this study. Except for the preparations of Hoshi and Yamauchi (1983b), the samples all appear to have been dialyzed to remove 2-ME and then analyzed or they were dialyzed, freeze-dried, and analyzed as done in this study. Excluding the results of Hoshi and Yamauchi, the literature values for surface SH groups (measured in the absence of denaturant) tend to be low and variable (0–2 SH/mol). My values for glycinin likewise are low and variable and fall into nearly the same range (0.6–2.2 SH/mol) with a

Table IV. Distribution of Acidic Polypeptides and Subunits in Raiden Glycinin Based on DEAE-Sephadex Chromatography

column fraction[a]	recovery,[b] mg	distribution of acidic chains by wt, %	M_r of major fractions	corresponding subunit[c]	no. of subunits/mol[d]	predicted SH groups/mol[e]
basic chains	353		25 000, 23 000, 11 000			
A_{1b}	33	7.5	34 000	$A_{1b}B_{1b}$(G3)	0.45	0.9
A_{1a}	167	38.0	35 000	$A_{1a}B_2$(G1)	2.28	4.6
A_2(61–67)[f]	45	10.3	34 000	A_2B_{1a}(G2)	2.06	4.1
A_2(68–82)[f]	105	24.0	34 000			
83–94[f]	11		24 000			
A_3	63	14.4	38 000	A_3B_4(G5)	0.86	1.7
A_6	26	5.9	32 000	$A_6B_?$	0.35	
121–130[f]	<1		20 000			
total	803	100.1			6.0	11.3

[a] Listed in order of elution (Figure 5); fraction designations as per Staswick and Nielsen (1983). [b] Based on 1 g of glycinin. [c] See Nielsen et al. (1989) for nomenclature used to designate subunits. [d] Calculated as % by wt × 6 × 10^{-2}. [e] Assuming minimum of two SH groups per subunit predicted from model (Nielsen et al., 1989) as per Discussion. [f] Numbers refer to column fractions (Figure 5).

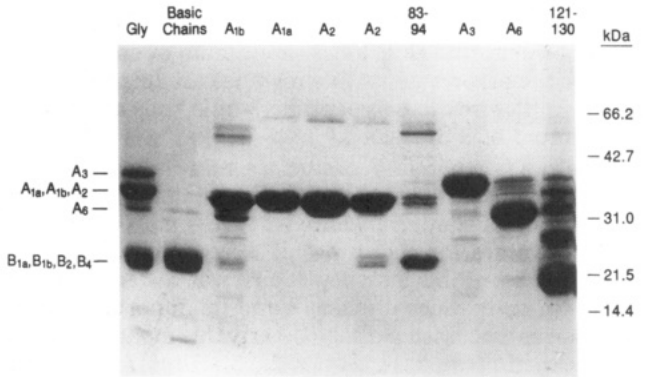

Figure 6. SDS–PAGE of the basic and acidic polypeptides separated by ion-exchange chromatography (Figure 5) from reduced-alkylated glycinin.

Table V. Sulfhydryl and Half-Cystine Content of Glycinin from the Literature and This Study[a]

mol of SH/mol[b]	half-cystine/mol[b]	ref
0	33	Kella and Kinsella (1986)
0	40[c]	Catsimpoolas et al. (1971)
	42[c]	Badley et al. (1975)
	32[c]	Fukushima (1968)
0 (surface) + 1.7 (internal)	40	Draper and Catsimpoolas (1978)
0.46		Fuke et al. (1985)
0.4–1.3 (surface) + 1.2–1.4 (internal)		Nakamura et al. (1984)
1.0 (surface) + 3.9 (internal)	37	Simard and Boulet (1978)
1.0		Iwabuchi and Yamauchi (1987)
2		Saio et al. (1971)
4.3 (surface)[d] + 3.2 (internal)[d]	42.9	Hoshi and Yamauchi (1983b)
8.8 (surface)[e] + 4.1 (internal)[e]		Hoshi and Yamauchi (1983b)
1.4 ± 0.5	41.4[f]	this study

[a] All data for freeze-dried preparations unless noted otherwise. [b] Based on MW of 320 000 (Badley et al., 1975) and molar extinction coefficient of 14 150 (13 700 in 6 M GuHCl) for 2-nitro-5-thiobenzoic acid (Riddles et al., 1979) when determined by Ellman's reagent. [c] Based on amino acid analysis. [d] Prepared in the presence of 0.01 M 2-ME, separated from 2-ME by gel filtration, and freeze-dried. [e] Same as described in footnote d but not freeze-dried. [f] Mean of values in experiments 14 and 15, Table I.

mean of 1.4 ± 0.5. Although varietal differences (e.g., subunit heterogeneity) may account for some of the variability in literature values, my results for 15 preparations from a single cultivar (Raiden) indicate that much of this variability is inherent in the method of preparation.

The higher values of Hoshi and Yamauchi (1983b) were obtained for glycinin separated from 0.01 M 2-ME by gel filtration and analyzed directly or freeze-dried, dispersed in buffer, and then analyzed for SH content. My results are consistent with theirs. I obtained values of 8–14 SH/mol when glycinin was treated with 0.01 M 2-ME and the excess 2-ME was removed by gel filtration (Figure 1). However, when the 2-ME was removed by dialysis in a helium atmosphere, the SH per mole was only about 1 (experiment 8, Table I). Glycinin SH groups apparently oxidize readily and/or form mixed disulfides with the reductant unless the reducing agent is removed rapidly by gel filtration. Reduction of glycinin with DTT gave significantly higher SH values than 2-ME but, again, removing the excess reductant by dialysis gave values only about half those obtained when the DTT was separated by gel filtration (experiments 10 and 11, Table I).

Literature values for SH content of fully reduced glycinin vary considerably (Table V), but five of them are in the range 37–43 with a mean of 41. My value of 41.4 is in good agreement with this mean.

Of particular interest are the effects of reducing agent concentration on the SH content of the protein (Figures 1 and 2). When 2-ME is used, some variability in SH contents can be expected in the range 0–0.03 M because the number of SH groups increases rapidly through this concentration range and then levels off at about 21 SH/mol. Values of 8–14 SH/mol at 0.01 M 2-ME are of historical interest because this concentration of 2-ME has been used extensively in many laboratories since its use was introduced by Briggs and Wolf (1957).

The greater efficacy of DTT as compared to 2-ME in reducing SS bonds is well-known (Cleland, 1964) and is demonstrated in two ways in Figure 1. First, the number of SS groups reduced increases very rapidly as DTT is increased. Second, more SS bonds are reduced by DTT and about 28 SH/mol are formed with 0.02–0.1 M DTT. The difference in the maximum number of SH per mole formed by DTT vs 2-ME suggests that there are an average of about 3.5 SS bonds that are resistant to reduction by 0.1 M 2-ME but readily reduced by DTT.

The shape of the curve relating SH per mole and concentration of $NaBH_4$ (Figure 2) differs from that of the thiol compounds, and considerably higher concentrations of borohydride are required to effect the same extent of reduction obtained with 2-ME and especially DTT. Borohydride does have the advantage that the excess reagent is readily destroyed by lowering the pH.

Although glycinin will contain about 12 SH/mol when it is in the presence of 2-ME (Figure 1), it was surprising that only 0.6–2.2 SH/mol was found in the dialyzed, freeze-dried protein. Apparently the SH groups of glycinin are

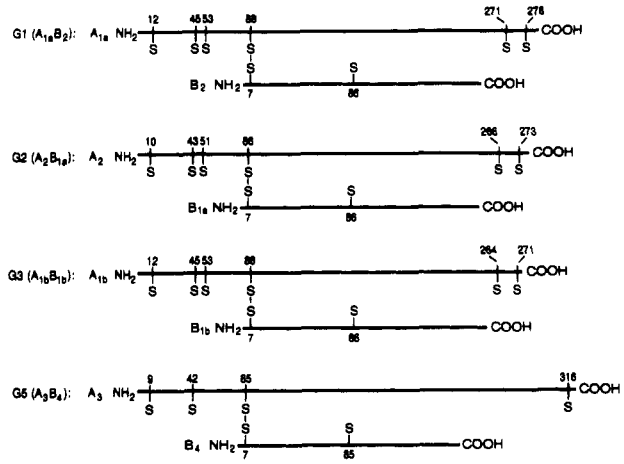

Figure 7. Schematic structures of subunits of Raiden glycinin based on sequences reported by Nielsen et al. (1989). Half-cystine residues are designated by S.

very prone to oxidation. The low concentrations of 2-ME that occur as the 2-ME is removed during dialysis in the presence of oxygen (no attempt was made to exclude oxygen during the normal preparation of glycinin) may serve as a catalyst to promote re-formation of SS bonds as noted in regeneration of reduced lysozyme (Saxena and Wetlaufer, 1970). Nonetheless, excluding oxygen during dialysis to remove 2-ME (experiment 8, Table I) did not have much effect on the SH content of the glycinin. However, excluding oxygen during dialysis to remove DTT resulted in much higher SH contents (experiment 10, Table I), although they were only about half of the expected value predicted from Figure 1. Figure 1 also suggests that 0.001 M DTT would give about the same extent of reduction as 0.01 M 2-ME. It may be desirable to substitute the more stable and less odorous DTT for 2-ME in the purification of glycinin.

The ability of glycinin to form SS-linked polymers has been known for many years (Briggs and Wolf, 1957) but is not unique to this protein. This characteristic is shared with a variety of other proteins including triadin (Caswell et al., 1991), κ-casein (Rasmussen et al., 1992), human von Willebrand factor (Chopek et al., 1986), multimerin (Hayward et al., 1991), and fibronectin (Skorstengaard et al., 1986).

The currently accepted model of glycinin is that of a hexamer where the monomeric subunits have the generalized structure AB, where A represents an acidic polypeptide of 34 000–44 000 and B is a basic polypeptide of 20 000. The A and B chains are linked by a single SS bond (Staswick et al., 1984a). Five major subunits have been characterized (Nielsen et al., 1989):

subunit	subunit structure
G1	$A_{1a}B_2$
G2	A_2B_{1a}
G3	$A_{1b}B_{1b}$
G4	$A_5A_4B_3$
G5	A_3B_4

Raiden cultivar does not appear to contain G4 but instead contains an acidic polypeptide designated A_6 (Staswick and Nielsen, 1983; Figure 5). The subunits occurring in Raiden cultivar are represented schematically in Figure 7 on the basis of DNA sequences derived from genes cloned from Forrest and Dare cultivars (Nielsen et al., 1989). A subunit containing acidic chain A_6 has not been included because A_6 is a minor fraction (Table IV) and its sequence is unknown. Moreover, a basic polypeptide partner for A_6 has not yet been identified (Staswick and Nielsen, 1983), and 2-dimensional SDS–PAGE suggests that there may not be an SS-linked basic chain associated with it (Kagawa and Hirano, 1988).

The possibility exists that sequences for glycinin from other cultivars may differ slightly because of random genetic drift even though the same genes are involved. However, if it is assumed that such genetic drift does not involve the half-cystine residues of Raiden glycinin, two major conclusions can be drawn from the model structures in terms of their SH contents:

(1) All of the basic chains contain only one half-cystine in position 85 or 86 which is not covalently linked to the acidic polypeptide chains. Consequently, this SH group should contribute 1 SH/basic chain or a total of 6 SH/mol of glycinin.

(2) All of the acidic polypeptides contain an odd number of half-cystine residues exclusive of the half-cystine connected to residue 7 of the basic chain. Each acidic polypeptide would thus have a minimum of one free SH if all of the other half-cystine residues are intrachain SS bonded. The acidic polypeptides would thus contribute a minimum of 6 SH/mol.

This model therefore predicts a minimum of 2 SH/subunit or 12 SH/mol of glycinin. The minimum predicted SH content for Raiden glycinin is 11.3 SH mol (Table IV). The SH values for each subunit were weighted for the subunit content, but polypeptide A_6 was disregarded for this purpose because it is small and its importance is in question as discussed earlier. Clearly this model does not fit the experimental results of 1.4 ± 0.5 SH/mol for dialyzed and freeze-dried glycinin. When the 2-ME used during purification of glycinin is removed by dialysis, the SH groups apparently are readily reoxidized. Ultracentrifugal analysis (Table II) indicates that some SS polymerization of glycinin occurred as a result of dialysis and freeze-drying, yet about 60% of glycinin was still in the unpolymerized form (11S). Consequently, mixed disulfide formation with 2-ME and interchain SS formation must account for much of the decrease from about 12 to 1–2 SH/mol.

A value of 12 SH/mol is approximated by the SH content of glycinin reduced with 0.01 M 2-ME (Figure 1). However, the results for glycinin isolated in the absence of 2-ME suggest that such a high SH content is not typical of the native protein as it exists in the defatted meal (assuming that the isolation procedure does not lead to alteration of SH groups). Glycinin isolated under these conditions is also highly oxidized, i.e., less than 0.5 SH/mol was detected. If a low SH content is characteristic of the native state, then there may be a strong propensity to return to it when the protein is partially reduced and the reductant is removed as in the present method for purification of glycinin (Thanh and Shibasaki, 1976).

The identity of the half-cystine residues in the acidic polypeptides that exist in the SH form in the 12 SH/mol model is unknown. Likely candidates are positions 53 in G1 and G3, 51 in G2, and 316 in G5 (Figure 7) because the first two half-cystines from the N-terminal end of each acidic chain (i.e., positions 12 and 45 in G1) are strictly conserved (Nielsen et al., 1989), which suggests that they are involved in intrachain SS bonds (Thornton, 1981) and are important in stabilizing the native conformation of glycinin.

Because the subunits are nonidentical, it is difficult to speculate which SS bonds are cleaved as 2-ME concentrations are increased to 0.03 M and higher, where the maximum number of SH per mole is 21 (Figure 1). The situation is further complicated by the fact that the SS bond between the acidic and basic chains is also cleaved

to some extent at 0.1 M 2-ME (Figure 3). DTT at 0.01 and 0.1 M likewise appears to cleave some of the interchain SS bonds (Figure 3). Yet, with many of the interchain SS bonds apparently broken, about two-thirds of the glycinin has retained its quaternary structure as indicated by sedimenting as an 11S component (Table II). Reduction of 13–14 SS bonds of the total of about 20 by 0.1 M DTT in the absence of a denaturant indicates that these SS bonds are readily accessible to the reducing agent. The remaining 6–7 SS bonds apparently are more shielded and require that a denaturant such as 4–6 M GuHCl be present for their reduction (Table I). Although the number of these shielded SS bonds agrees closely with the six theoretical SS linkages expected for the cross-links between the acidic and basic polypeptides, Figure 3 indicates that some of these interchain bonds were broken by reduction with 0.1 M DTT. Consequently, the residual SS bonds appear to consist of interchain plus intrachain SS bonds.

Existence in glycinin of acidic and basic polypeptides not connected by a disulfide bond has been observed by others (Nakamura et al., 1985; Rothenbuhler and Kinsella, 1986). The presence of free acidic and basic polypeptides in glycinin prepared with 0.01 M 2-ME was therefore not surprising, but their existence in glycinin prepared without 2-ME was unexpected. The significance of these findings is unknown. Staswick et al. (1981) reported that the intrachain SS bonds of glycinin subunits isolated from cultivar CX635-1-1-1 were unstable in SDS but found they could stabilize them by alkylation of free SH groups with 4-vinylpyridine in 6 M GuHCl. I observed free acidic and basic polypeptides in Raiden glycinin when either iodoacetamide or 4-vinylpyridine was present prior to addition of SDS (Figure 3). This suggests that some of the acidic and basic polypeptides were free in the native protein unless their appearance was an artifact resulting from an SH–SS interchange that was more rapid than the alkylation reaction in SDS solution.

The finding of a 28 000 polypeptide in unreduced and partially reduced glycinin (Figures 3 and 4) and its behavior on reduction (formation of a basic polypeptide plus a polypeptide of ~12 000) suggest that it is the 30 000 disulfide-linked portion, A_5B_3, of glycinin G4 ($A_5A_4B_3$) previously isolated from cultivar CX635-1-1-1 by Staswick and Nielsen (1983). Observation of this polypeptide was unexpected because Raiden cultivar contains a null allele for the Gy_4 gene; hence, glycinin subunit G4 ($A_5A_4B_3$) is essentially absent from this cultivar (Scallon et al., 1987). Earlier, Staswick and Nielsen (1983) concluded that polypeptide A_4 from this subunit may be present in Raiden at <10% of the level found in cultivar CX635-1-1-1. I found no evidence for polypeptide A_4 on SDS–PAGE of unreduced glycinin (Figures 3 and 4), although it may have been lost from the gels during destaining because of its low M_r. I did, however, observe a band that may be A_5 and which is also of low M_r. The DEAE-Sephadex chromatogram of the acidic glycinin polypeptides (Figure 5) closely resembled that of Staswick and Nielsen (1983), and I likewise found polypeptide A_6 instead of A_4. Traces of A_4 may have been present in the A_6 preparation as revealed by SDS–PAGE (Figure 6).

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