Accumulation of β-Conglycinin in Soybean Cotyledon through the Formation of Disulfide Bonds between α′- and α-Subunits[1][W][OA]

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β-Conglycinin, one of the major soybean (Glycine max) seed storage proteins, is folded and assembled into trimers in the endoplasmic reticulum and accumulated into protein storage vacuoles. Prior experiments have used soybean β-conglycinin extracted using a reducing buffer containing a sulfhydryl reductant such as 2-mercaptoethanol, which reduces both intermolecular and intramolecular disulfide bonds within the proteins. In this study, soybean proteins were extracted from the cotyledons of immature seeds or dry beans under nonreducing conditions to prevent the oxidation of thiol groups and the reduction or exchange of disulfide bonds. We found that approximately half of the α′- and α-subunits of β-conglycinin were disulfide linked, together or with P34, prior to amino-terminal propeptide processing. Sedimentation velocity experiments, size-exclusion chromatography, and two-dimensional polyacrylamide gel electrophoresis (PAGE) analysis, with blue native PAGE followed by sodium dodecyl sulfate-PAGE, indicated that the β-conglycinin complexes containing the disulfide-linked α′/α-subunits were complexes of more than 720 kD. The α′- and α-subunits, when disulfide linked with P34, were mostly present in approximately 480-kD complexes (hexamers) at low ionic strength. Our results suggest that disulfide bonds are formed between α′/α-subunits residing in different β-conglycinin hexamers, but the binding of P34 to α′- and α-subunits reduces the linkage between β-conglycinin hexamers. Finally, a subset of glycinin was shown to exist as noncovalently associated complexes larger than hexamers when β-conglycinin was expressed under nonreducing conditions.

The large majority of seed storage proteins from the leguminous species are globulins with sedimentation coefficients of 7 to 8 S (vicilin) and 11 to 12 S (legumin). The corresponding proteins from soybean (Glycine max) are called β-conglycinin and glycinin, respectively (Casey, 1999). The β-conglycinin from soybean seeds is isolated as a trimer with sedimentation coefficients between 7 and 8 S (Thanh and Shibasaki, 1976; Nielsen and Nam, 1999). The size of β-conglycinin complexes depends on the ionic strength of the solution. β-Conglycinin is a trimer at higher than 0.2 M ionic strength, but at neutral pH, it associates into hexamers at decreasing ionic strength (Thanh and Shibasaki, 1979). The β-conglycinin trimers are mixtures of isoforms consisting of different combinations of the three types of subunits, designated α′, α with M_r of 57,000, and β with M_r of 42,000 (Thanh and Shibasaki, 1978; Davies et al., 1985; Nielsen and Nam, 1999). The α′-, α-, and β-subunits are synthesized in the endoplasmic reticulum (ER) of the cotyledon cell as polypeptides with a signal peptide and a propeptide (prepro α′- and prepro α-subunits) or as polypeptides with only a signal peptide (pre-β-subunit; Sengupta et al., 1981; Coates et al., 1985; Harada et al., 1989; Sebastiani et al., 1990; Lelievre et al., 1992). Each signal peptide is cotranslationally cleaved. Two and one N-linked glycans attach to nitrogens of Asn side chains of pro α- and pro α′-subunits and to the β-subunit in the lumen of the ER (Sengupta et al., 1981). These polypeptides are folded in the ER and held together as trimers by noncovalent interactions (Chrispeels et al., 1982; Lelievre et al., 1992). They are then transported via the Golgi and a transport vesicle (dense body) and deposited into the protein storage vacuoles (PSVs). The propeptides of pro α- and pro α′-subunits are cleaved after assembly into trimers (Lelievre et al., 1992). Thus far, the intracellular compartment or the processing enzymes, which cleave the propeptide, have not been identified.

There are five structural genes, G1 to G5, that encode glycinin protomers, and they are divided into two groups, group I (A1aB1b, A2B1a, and A1bB2) and group II (A3B4 and A5A4B3; Marco et al., 1984; Scallon et al., 1985; Cho et al., 1989; Dickinson et al., 1989; Nielsen et al., 1989; Nielsen and Nam, 1999). Each glycinin protomer is synthesized in the rough ER of the cotyledon cell as a single polypeptide composed of an acidic chain and a basic chain. The folding of glycinin protomers occurs with the accompanying formation of intramolecular disulfide bonds (Ereken-Tumer et al., 1982; Nam et al., 1997). In the ER, the folded protomers are held together in trimers by noncovalent interactions. Glycinin trimers are transported via the Golgi and are deposited into the PSV. In the PSV, glycinin protomers are cleaved by PSV-processing enzymes into an acidic chain (M_r generally approximately 35,000) and a basic chain (M_r generally approximately 20,000), which are covalently joined by a disulfide bond near a well-conserved Asn-Gly (Hara-Nishimura and Nishimura, 1987; Scott et al., 1992). After cleavage, a conformational change in the hypervariable region of the acidic chain results in movement away from the association site between the trimers, resulting in glycinin trimer assembly into hexamers (Dickinson et al., 1989; Adachi et al., 2003).

It has been reported that cosuppression of the α'- and α-subunits of β-conglycinin caused alterations in glycinin group I accumulation (Kinney et al., 2001). A number of ER-derived protein bodies have been observed in seeds with reduced α'- and α-subunit levels. Glycinin has been identified as a major component of these ER-derived protein bodies. It has also been reported that the number of ER-derived protein bodies in mutant soybean (α'-, α-, and β-null, α'- and α-null, and group II glycinin protomers-null) was higher than in the wild-type soybean (Mori et al., 2004). Reports suggest that group I-rich glycinin, when insoluble in the ER, causes the formation of ER-derived protein bodies.

Earlier experiments on soybean storage proteins used methods that were established for isolating β-conglycinin and glycinin (Wolf and Sly, 1965; Thanh and Shibasaki, 1976; Moreira et al., 1979). These methods used buffers containing 10 mM 2-mercaptoethanol (2-ME) to prevent the formation of artificial intermolecular disulfide bonds among proteins, which may form during delipidation, heating, and protein extraction procedures. The disulfide bond (Cys-88 and Cys-298) between the acidic chain and the basic chain, and the disulfide bond (Cys-12 and Cys-45) of the native glycinin protomer A1aB1b, are not reduced in this buffer. These disulfide bonds have been identified by analyses of the crystal structures of pro A1aB1b homotrimer (Adachi et al., 2001) prepared with 10 mM 2-ME-containing buffer. In contrast, α'- and α-subunits of mature β-conglycinin have only Cys-69 and Cys-68, located near their N termini. At present, it is assumed that no intermolecular disulfide bond is naturally formed in mature β-conglycinin from soybean. Disulfide-linked α'/α-subunits, together, and disulfide-linked α'- or α-subunit with an allergenic

soybean protein, *Gly m* Bd 30K (P34), have been found in defatted soy milk prepared without sulfhydryl reductant (Samoto et al., 1996). These disulfide-linked polypeptides are thought to be artificial products generated during the process of delipidation and extraction.

In this study, we analyzed β-conglycinin extracted from the cotyledon of both immature bean and dry bean under conditions that prevented the oxidation and reduction of the Cys residues. We found that in the cotyledon cell, intermolecular disulfide bonds were formed between pro α'/pro α-subunits together and between pro α'- or pro α-subunit and P34. A subset of β-conglycinin formed complexes greater than dodecamers, which were linked through intermolecular disulfide bonds. In addition, a subset of glycinin existed as noncovalently associated complexes, greater than hexamers, when β-conglycinin was present under nonreducing conditions. These results suggest that the intracellular delivery and deposition of soybean storage proteins proceeds through covalent and noncovalent interactions between multiple storage proteins in the cotyledon cell.

RESULTS

Disulfide Bonds between Pro α'- and Pro α-Subunits Together or with P34 in Soybean Cotyledon Cells

Pro α'- and pro α-subunits of β-conglycinin contain four Cys residues positioned on the propeptide and one Cys residue positioned on the mature polypeptide (Fig. 1A; Schuler et al., 1982; Shutovet al., 1996). It was unclear if the thiol groups of these Cys residues were biologically oxidized to form intermolecular or intramolecular disulfide bonds. Therefore, proteins were extracted from immature cotyledons using buffer containing N-ethylmaleimide (NEM) without sulfhydryl reductant to block the artificial formation, cleavage, and exchange of disulfide bonds during extraction procedures. The extracted proteins were analyzed by western blot using anti-propeptide antibodies prepared against an undecapeptide amino acid sequence corresponding to the Ser-44 to Cys-54 sequence in the propeptide of the pro α'-subunit. The anti-propeptide antibodies cross-reacted with both pro α'- and pro α-subunits. The 75- and 73-kD bands of pro α'- and pro α-subunits were detected when the proteins were separated by reducing SDS-PAGE (Fig. 1B, lane 2). In contrast, bands were hardly detected when the proteins were separated by nonreducing SDS-PAGE (Fig. 1B, lane 1). The epitope recognized by the anti-propeptide antibodies, Ser-44 to Cys-54, may form disulfide bonds within the propeptide (Fig. 1A), causing a decrease in immunoreactivity with the anti-propeptide antibodies. To further confirm these possibilities, the gel was treated with dithiothreitol (DTT) after nonreducing SDS-PAGE to cleave disulfide bonds within proteins and then analyzed by western blot. Bands migrating at the 100- and 150-kD ranges, and the 75- and 73-kD bands of pro α'- and pro α-subunits, were detected (Fig. 1B, lane 3). Upon two-
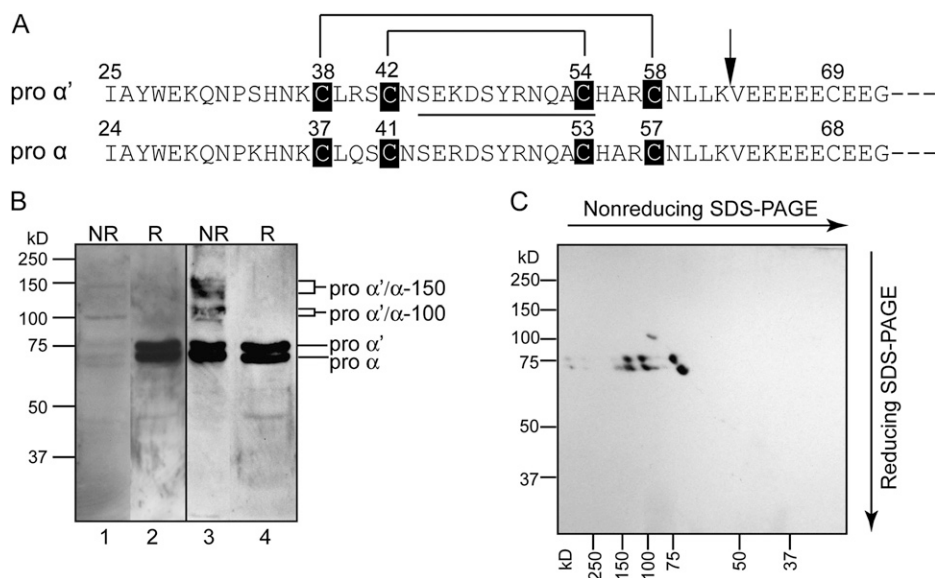
Figure 1. Disulfide-linked complexes of pro α′ and pro α. A, The propeptide sequences of the pro α′- and α-subunits. Solid lines represent putative disulfide bridges. The arrow represents the posttranslational processing site. The underlined sequence corresponds to that used for production of the antibodies against the propeptide of α′. B, Detection of disulfide-linked pro α′ and pro α. Proteins extracted from immature soybean cotyledon were separated by SDS-PAGE under nonreducing conditions (NR; lanes 1 and 3) or reducing conditions (R; lanes 2 and 4). After electrophoresis, the gels were incubated in blotting buffer without (lanes 1and 2) or with (lanes 3 and 4) 50 mM DTT. Proteins were blotted onto a PVDF membrane and detected with anti-propeptide antibodies. C, Proteins were separated by 2D SDS-PAGE under nonreducing conditions for the first dimension and reducing conditions for the second dimension. Separated proteins were blotted onto a PVDF membrane and detected with anti-propeptide antibodies.

dimensional (2D) electrophoresis with nonreducing SDS-PAGE followed by reducing SDS-PAGE, it was confirmed that pro α′- and pro α-subunits were components of the 100- and 150-kD bands (Fig. 1C).

Maintenance of Intermolecular Disulfide Bonds in α′- and α-Subunits after the Processing of Propeptides

After nonreducing SDS-PAGE, the 100- and 150-kD bands and the 75-kD band of the α′-subunit, from immature cotyledon or dry bean cotyledon, were detected by western-blot analysis using the antibodies specific to the α′-subunit (Fig. 2A, lanes 1 and 3). The 100- and 150-kD bands were not detected by reducing SDS-PAGE, suggesting that they are disulfide-linked complexes (Fig. 2A, lanes 2 and 4). 2D electrophoresis with nonreducing SDS-PAGE, followed by reducing SDS-PAGE, demonstrated that the α′-subunit was a component of both the 100- and 150-kD bands (Fig. 2B). The 100- and 150-kD bands were detected in α-subunit-null mutant soybeans (Fig. 2A, lane 5; Supplemental Fig. S1). Neither the 100-kD band nor the 150-kD band was detected in soybeans with α′-subunit-null mutant soybean and knockdown of both α′- and α-subunits (Fig. 2A, lanes 7 and 8). Both the 100- and 150-kD bands were detected in glycinin-null soybean (Fig. 2A, lanes 9 and 10; Supplemental Fig. S1), suggesting that glycinin was not the disulfide-linking partner protein of the α′-subunits. To identify the partner protein that disulfide bonds to the α′-subunit of the 100- and 150-kD

bands, β-conglycinin, prepared from dry bean cotyledons, with the NEM-containing buffer without 2-ME, was separated by 2D nonreducing SDS-PAGE followed by reducing SDS-PAGE (Fig. 2C). Spots corresponding to monomeric α′- and α-subunits and a spot at 34 kD were separated from the 100-kD spot from the first nonreducing SDS-PAGE using a second reducing SDS-PAGE. The N-terminal amino acid sequence of the 34-kD protein was KKMKKEQYS, identical to P34 (Kalinski et al., 1990). The other two spots were confirmed to be α′ or α by N-terminal sequencing. Western-blot analysis of P34-null soybean proteins found no 100-kD band (Fig. 2A, lane 11). From these results, it was concluded that the 100-kD complexes were probably disulfide-linked α′-subunit with P34 and disulfide-linked α-subunit with P34. The 150-kD spot detected in the first nonreducing SDS-PAGE only contained monomeric α′- and α-subunits when separated by the subsequent reducing SDS-PAGE (Fig. 2C). These results suggest that the 150-kD complexes were composed of a combination of α′- and α′-subunits, α′- and α-subunits, or α- and α-subunits. The intermolecular disulfide bonds in the 100-kD complexes and 150-kD complexes could be cleaved by sulfhydryl reductants such as glutathione or Cys (Fig. 2D), suggesting that these disulfide bonds were accessible to an aqueous environment. To determine the amount of disulfide-linked α′-subunit in the total pool of α′-subunit, the intensities of the bands α′, α′-100, and α′-150 were measured (Fig. 2A, lane 3). Approximately 50% each
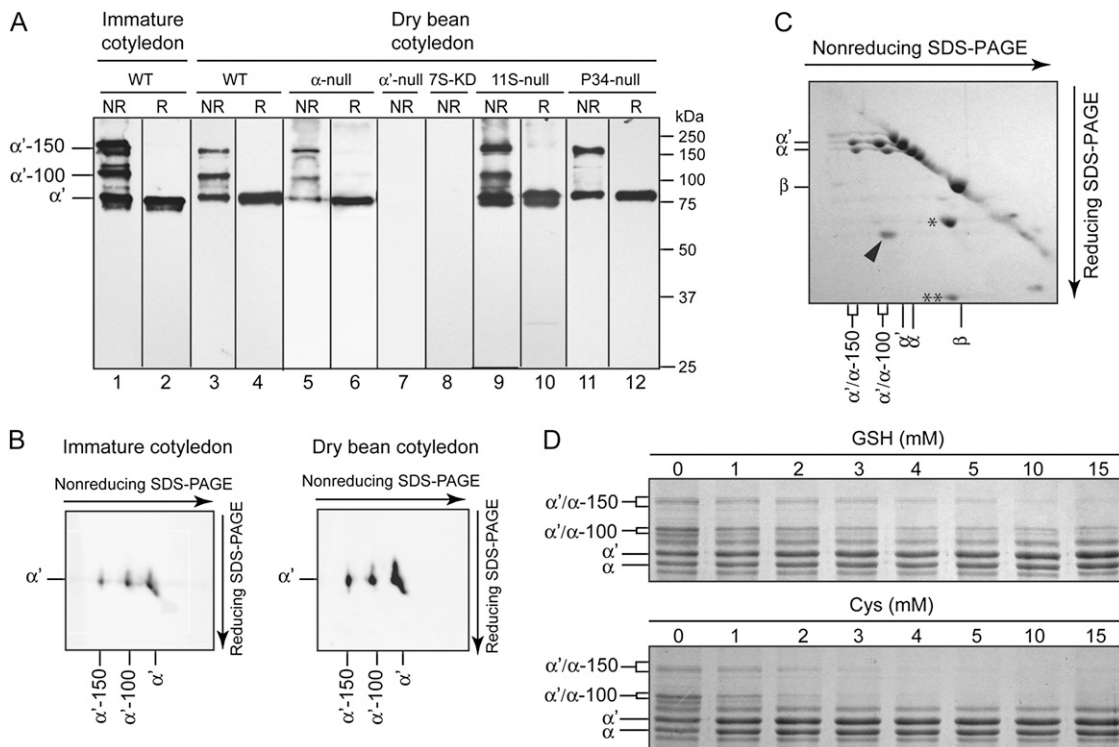
Figure 2. Identification of proteins included in the α'/α-100-kD and α'/α-150-kD complexes. A, Proteins extracted from the immature cotyledons (lanes 1 and 2) or dry bean cotyledons of wild-type soybean (WT; lanes 3 and 4), α-subunit-null mutant soybean (lanes 5 and 6), α'-subunit-null mutant soybean (lane 7), β-conglycinin-knockdown soybean (7S-KD; lane 8), glycinin-null mutant soybean (11S-null; lanes 9 and 10), or P34-null mutant soybean (P34-null; lanes 11 and 12) were separated by SDS-PAGE under nonreducing conditions (NR; lanes 1, 3, 5, 7, 8, 9, and 11) or reducing conditions (R; lanes 2, 4, 6, 10, and 12) and detected by western-blot analyses with anti-α'-subunit antibodies. B, Proteins from immature cotyledons (left panel) or dry bean cotyledons (right panel) of wild-type soybean were separated by 2D SDS-PAGE under nonreducing conditions for the first dimension and reducing conditions for the second dimension. The α'-subunit was detected by western-blot analysis with the anti-α'-subunit antibodies. C, The β-conglycinin (30 μg of protein) prepared from dry bean cotyledon of wild-type soybean, with the NEM-containing buffer without 2-ME, was analyzed by 2D SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The arrowhead indicates a 34-kD spot separated from α'/α-100-kD complexes by the second SDS-PAGE. Because small amounts of glycinin were contaminated in the β-conglycinin preparation, spots of glycinin acidic chain (asterisk) and glycinin basic chain (two asterisks) were detected. D, β-Conglycinin prepared from dry bean cotyledon of wild-type soybean, with the NEM-containing buffer without 2-ME, was incubated in 1 to 15 mM glutathione (GSH) or Cys for 10 min at 25°C. The β-conglycinin was separated by nonreducing SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

of the total α'-subunit was in a disulfide-linked form. There are five Cys residues in the pro α'- and pro α-subunits. Among them, four are present in the propeptide of pro α'- and pro α-subunits and one is present adjacent to the N termini of the mature α'- and mature α-subunits. Hence, we concluded that the intermolecular disulfide bonds were formed using the Cys residue (Cys-69 or Cys-68) of mature α'- or α-subunit.

In the ER lumen, many kinds of protein thiol oxidoreductase-related proteins aid in the folding of nascent polypeptides by inducing disulfide bond formation. One abundant protein thiol oxidoreductase-related protein in the ER of the soybean cotyledon cell is *Glycine max* protein disulfide isomerase L-1 (GmPDIL-1; Kamauchi et al., 2008), which is a universally conserved enzyme in eukaryotes, from yeast to animal species. It has been shown from coimmunoprecipitation experi-

ments that GmPDIL-1 associated with the α'-subunit of β-conglycinin (Kamauchi et al., 2008). Since the intermolecular disulfide bonds of α'- and α-subunits were already formed in the pro α'- and pro α-subunits, we examined the effects of GmPDIL-1 knockdown, by RNA interference (RNAi), on the formation of the 100- and 150-kD disulfide-linked complexes. The expression of small interfering RNA, directed to GmPDIL-1, fully depressed the expression of GmPDIL-1 in the cotyledon (Fig. 3A). A similar amount of α'-subunit accumulated in the dry bean cotyledon in the presence or absence (Fig. 3B) of GmPDIL-1. In addition, the disulfide-linked 100- and 150-kD complexes were formed regardless of the presence or absence of GmPDIL-1, suggesting either that complex formation was catalyzed by an enzyme other than GmPDIL-1 or that the complex was nonenzymatically formed in the cotyledon cell.

The Formation of Intermolecular Disulfide Bonds in α'- and α-Subunits between Trimers or Hexamers

The size of the β-conglycinin containing the disulfide-linked 100- and 150-kD complexes was measured by sedimentation velocity experiments. The β-conglycinin was prepared from dry bean cotyledons using an NEM-containing buffer without 2-ME and subjected to sedimentation velocity experiments in the presence or absence of 2-ME. The β-conglycinin existed as complexes with sedimentation coefficients of 7 S (Fig. 4A, arrow a) and 10.5 S (Fig. 4A, arrow b) when isolated in 0.4 M NaCl buffer in the absence of 2-ME (Fig. 4A, solid line). The complexes with sedimentation coefficients of 7 and 10.5 S were most likely trimers and hexamers, respectively. Thus, a stable subset of β-conglycinin was present as a hexamer under nonreducing conditions, even when at high ionic strength, when β-conglycinin is present as a trimer. In NaCl-free solution in the absence of 2-ME, β-conglycinin displayed a very broad and nonsymmetrical distribution of sedimentation, ranging from 10.2 to 16.5 S (Fig. 4B, arrow c and solid line), suggesting that the trimers and hexamers, detected in 0.4 M NaCl, may associate at low ionic strength. Cleavage of the intermolecular disulfide bonds of β-conglycinin with 2-ME caused a decrease in the size of β-conglycinin to hexamer. Thus, β-conglycinin existed as a trimer, with a sedimentation coefficient of 6.5 S in 0.4 M NaCl, in the presence of 2-ME (Fig. 4A, dashed line), and as a hexamer, with a sedimentation coefficient of 12 S in NaCl-free solution, in the presence of 2-ME (Fig. 4B, dashed line). These results show that β-conglycinin, which contained the disulfide-linked α'-subunit, α-subunit, and P34, could form larger complexes than those of β-conglycinin containing none of the disulfide-linked subunits.

To examine the relationship between the β-conglycinin complexes and the disulfide-linked α'/α-subunits and P34, β-conglycinin prepared from the cotyledons of dry bean was separated by size-exclusion chromatography and the distributions of α'-, α-, or β-subunit in the effluents were determined by SDS-PAGE. Since β-conglycinin complexes larger than hexamers were eluted into the void volume of a gel filtration column
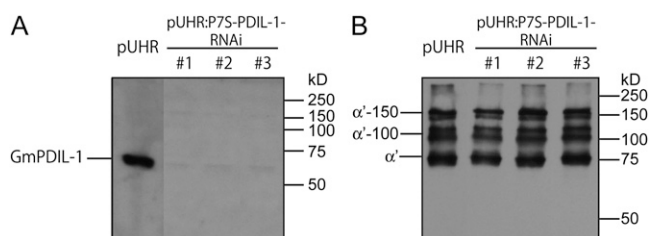

Figure 3. Knockdown of GmPDIL-1 had no effect on the formation of α'/α-100-kD and α'/α-150-kD complexes. Proteins were extracted from dry bean cotyledons of the transformed soybeans with the empty vector pUHR or the RNAi vector pUHR:P7S-PDIL-1-RNAi (clones 1–3) under nonreducing conditions and were analyzed by western blotting with the anti-GmPDIL-1 antibodies (A) or the anti-α' antibodies (B).
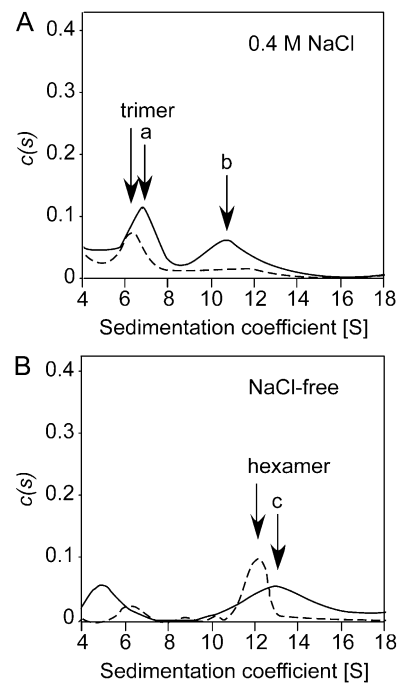
Figure 4. Effects of intermolecular disulfide bonds between α', α-subunits, and P34 on the sedimentation coefficient distribution function [c(s)] of β-conglycinin. β-Conglycinin was extracted with the NEM-containing buffer without 2-ME and fractionated with the buffer without 2-ME. Sedimentation velocity of β-conglycinin was carried out in a buffer without (solid lines) or with (dashed lines) 10 mM 2-ME in the presence of 0.4 M NaCl (A) or in the absence of 0.4 M NaCl (B). Two peaks of sedimentation in the buffer with NaCl in the absence of 2-ME were indicated by arrows a and b. A peak of sedimentation (10.2–16.5 s) in the buffer without NaCl in the absence of 2-ME was indicated by arrow c.

(TSK gel G3000SW), chromatography was performed with an equilibration buffer containing 0.4 M NaCl. Distributions of the entire α'-, α-, and β-subunits were analyzed by reducing SDS-PAGE (Fig. 5, A–C; Supplemental Fig. S3). All of the α'-, α-, and β-subunits of β-conglycinin, prepared in the presence of 2-ME, were eluted into fractions 13 to 17, corresponding to the elution volume expected for the trimer (Fig. 5, A–C, white symbols). In addition, α'-, α-, and β-subunits of β-conglycinin, prepared with the NEM-containing buffer without 2-ME, were eluted into a void volume and an elution volume corresponding to that of the trimer (Fig. 5, A–C, black symbols). Most of the disulfide-linked α'/α-subunits (i.e. 150-kD bands) were eluted into the void volume (Fig. 5D). These results suggest that the disulfide bonds were internally formed between the α'/α-subunit in one trimer and the α'/α-subunit in another trimer, rather than between the α'/α-subunits in one trimer. The disulfide-linked α'- and α-subunits with P34 (i.e. 100-kD bands) and the monomeric α' and α-subunits were in the elution volume expected for the trimer (Fig. 5, D and E). A small amount of protein from the 100-kD bands, and the monomeric α'- and α-subunits, were eluted in the void volume.
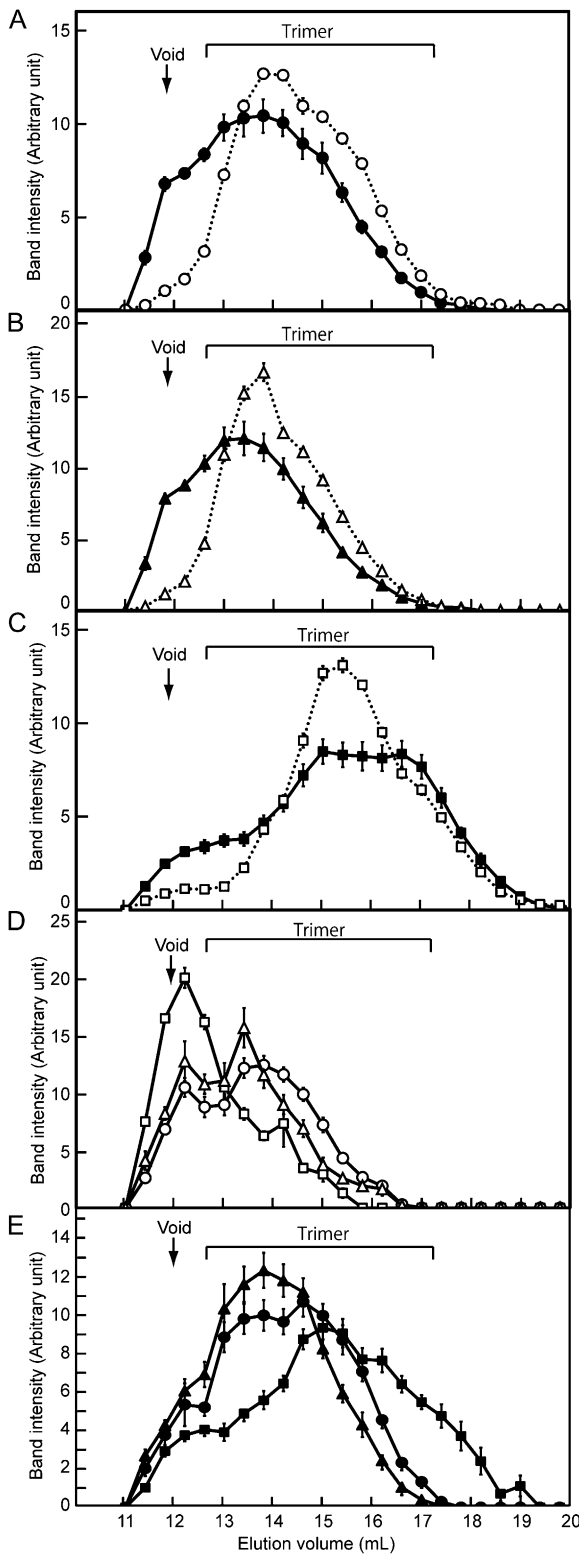
Figure 5. Size distributions of the disulfide-linked α'/α-subunits. A to C, β-Conglycinin prepared from the cotyledons of dry beans with the NEM-containing buffer without 2-ME (black symbols) or with 2-ME (white symbols) was subjected to size-exclusion chromatography. The fractions obtained from the chromatography were subjected to reducing SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue R-250 (Supplemental Fig. S3). Band intensities of α'-subunits (A), α-subunits (B), and β-subunits (C) are plotted. D and E, The fractions obtained from chromatography of β-conglycinin, prepared with NEM-containing buffer without 2-ME, were separated by nonreducing SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (Supplemental Fig. S3). Band intensities of α'-100 kD (D; circles), α-100 kD (D; triangles), and α'/α-150 kD (D; squares), monomeric α' (E; circles), monomeric α (E; triangles), and β (E; squares) are plotted.

To determine the masses of native pro β-conglycinin complexes, proteins from immature cotyledon were separated by 2D-PAGE with blue native (BN)-PAGE, followed by reducing or nonreducing SDS-PAGE, and analyzed by western blot. Pro α'-and pro α-subunits extracted in the presence of 2-ME were detected in the 480- to 720-kD region (Fig. 6A, panel 1). The pro α'- and pro α-subunits, extracted in the absence of 2-ME, migrated in the 480- to 1,048-kD region (Fig. 6A, panels 2 and 3). The mature α'- and α-subunits extracted from immature cotyledons or dry bean cotyledons in the presence of 2-ME were detected as hexamers in the approximately 480-kD region, but mature α'- and α-subunits prepared in the absence of 2-ME were detected in the 480- and greater than 720-kD regions (Fig. 6, B and C, panels 1 and 2). Although the disulfide-linked α'/α-subunits (150-kD spots) were detected mainly at the greater than 720-kD region, most of the disulfide-linked α'/α-subunits with P34 (100-kD spots) were distributed in approximately 480-kD complexes (Fig. 6, B and C, panel 3). Deficiency in P34 had no effect on the formation of the greater than 720-kD β-conglycinin complexes (Fig. 6D). These results suggest that the disulfide bonds were intermolecular between the α'/α-subunits, which were in distinct trimers of β-conglycinin, and that an interdisulfide bond was hardly formed between α'/α-subunits in one trimer.

Assembly of Glycinin into Complexes Larger Than Hexamers in a β-Conglycinin-Dependent Manner

Glycinin is primarily transported to the PSV via Golgi bodies. However, it has been reported that cosuppression of α'- and α-subunits of β-conglycinin caused a decrease in the transport of group I glycinin, via the Golgi, to the PSV (Kinney et al., 2001). From these findings, it is assumed that the glycinin trimer, containing group I protomers, associates with α'- and α-subunits of β-conglycinin complexes and is transported away from the ER to the Golgi. If so, then β-conglycinin complexes, which are more than approximately 720 kD, could be complexes associated with group I glycinin hexamers. However, the β-conglycinin complexes in glycinin-null soybean were larger than approximately 720 kD, suggesting that the existence of glycinin was not essential for the formation of these large β-conglycinin complexes (Fig. 6E, panels 2 and 3).

Group I glycinin complexes, with molecular masses of approximately 500 to 800 kD, were detected in addition to hexamers, with molecular masses of approximately 240 to 480 kD, when the soybean proteins

from the immature cotyledons or the dry bean cotyledons were prepared in the absence of 2-ME, separated by 2D-PAGE with BN-PAGE and reducing SDS-PAGE, and immunostained with the anti-glycinin acidic polypeptide antibodies (Fig. 7, B and E). The approximately 500- to 800-kD glycinin complexes were not detected in β-conglycinin-knockdown soybean, even when the extract was prepared in the absence of 2-ME (Fig. 7F), suggesting that the approximately 500- to 800-kD complexes were aggregates of glycinin and β-conglycinin. Deficiency in P34 had no effect on the formation of the 500- to 800-kD complexes (Fig. 7G). Since these complexes were not detected in the soybean extract prepared in the presence of 2-ME (Fig. 7, A and D), it was expected that the complexes were linked with disulfide bonds. However, western blotting after 2D-PAGE with BN-PAGE and nonreducing SDS-PAGE showed that the 50-kD band of glycinin was found in the approximately 500- to 800-kD complexes, suggesting that the complexes were noncovalently formed (Fig. 7C).

DISCUSSION

Soybean β-conglycinin is believed to accumulate in the PSV in the form of a noncovalently associated trimer, based on experiments performed in the presence of salt such as NaCl and sulfhydryl reductant such as 2-ME (Fig. 8A; Nielsen and Nam, 1999). In this study, we analyzed the molecular association of β-conglycinin under conditions that would maintain native disulfide bonds and prevent the artificial formation and exchange of disulfide bonds. As a result, it was found that around half of the β-conglycinin α'/α-subunits were intermolecularly disulfide bonded together or with P34 prior to the removal of their N-terminal propeptides. The intracellular compartment, and a catalytic system responsible for the formation of the intermolecular disulfide bonds between the α'/α-subunits and the α'- or α-subunit with P34, remain to be determined.

The ionic strength of buffers generally used in experiments on soybean globulin proteins is mostly higher than the physiological ionic strength (0.1–0.2 M). At less than 0.2 M of ionic strength, the majority of β-conglycinin was present as hexamers in vitro (Thanh and Shibasaki, 1979). Thus, β-conglycinin may be present as hexamers in the cotyledon cell. We find that the disulfide-linked α'/α-subunits were mostly incorporated into greater than 720-kD complexes but not into approximately 480-kD complexes (hexamers). Hence, we assume that the intermolecular disulfide bonds were formed between α'/α-subunits present in distinct hexamers (Fig. 8B). A portion of the monomeric α'-, α-, and β-subunits

Figure 6. Effects of intermolecular disulfide bonds on the sizes of the pro β-conglycinin or β-conglycinin complexes. Proteins were extracted from immature cotyledons of wild-type soybean (A and B) or dry bean cotyledons of wild-type soybean (C), P34-null mutant soybean (D), or glycinin-null mutant soybean (E) with 2-ME-containing buffer (panel 1) or with the NEM-containing buffer without 2-ME (panels 2 and 3). The extracted proteins were separated by 2D electrophoresis with BN-PAGE

and reducing SDS-PAGE (panels 1 and 2) or BN-PAGE and nonreducing SDS-PAGE (panel 3). Pro α'- and pro α-subunits (A) and α'-subunit (B–E) were detected by western-blot analyses with the anti-propeptide antibodies and the anti-α'-subunit antibodies, respectively. Asterisks in A show the spots of the unknown proteins detected by western blotting.
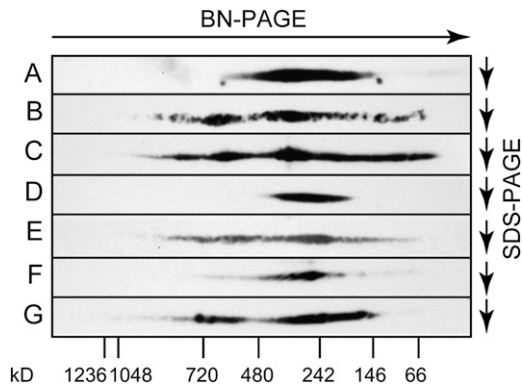
Figure 7. Glycinin forms complexes that are greater in size than the predicted hexamers and depend on the presence of β-conglycinin. Proteins were extracted from immature cotyledons of wild-type soybean (A–C) or dry bean cotyledons of wild-type soybean (D and E), β-conglycinin-knockdown soybean (F), or P34-null mutant soybean (G) with the buffer including 2-ME (A and D) or with the NEM-containing buffer without 2-ME (B, C, and E–G). The extracted proteins were separated by 2D electrophoresis with BN-PAGE and reducing SDS-PAGE (A, B, D–G) or BN-PAGE and nonreducing SDS-PAGE (C). Glycinin was detected by western-blot analysis with the anti-glycinin acidic chain antibodies. In A, B, and D to G, the 30-kD spots of the acidic chain of glycinin are shown; in C, the 50-kD spots of the disulfide-linked acidic and basic chains are shown.

was also present in greater than 720-kD complexes. This implied that substantial amounts of β-conglycinin α'-, α-, and β-subunits were present as dodecamers in cells. Generally, proteins that are highly polymerized by intermolecular disulfide bonds tend to be insoluble. As the size of β-conglycinin complexes becomes larger, the solubility of the complexes should decrease. This could contribute to their accumulation into the PSV. We analyzed the β-conglycinin complexes extractable from the cotyledon under conditions in which the native structures of the proteins were maintained. Insoluble protein fractions were not analyzed, but this will be done in the future.

A portion of the α'- and α-subunits was disulfide linked with P34 and was mostly present in approximately 480-kD complexes. This suggests that the binding of P34 to α'- or α-subunits by disulfide bonds may reduce the bonds between the β-conglycinin hexamers. P34 is homologous to a Cys proteinase, papain (Kalinski et al., 1990). However, P34 has no proteolytic activity due to replacement of the conserved catalytic Cys residue with a Gly (Kalinski et al., 1992). During seed maturation, P34 is synthesized in the ER of the cotyledon cell as a 47-kD glycoprotein precursor and transported to the PSV via Golgi bodies (Kalinski et al., 1992). Pro P34 is posttranslationally processed to mature P34 on the carboxyl side of Asp-122. Mature P34 then accumulates in the PSV. Mature P34 has seven Cys residues that are highly conserved in many plant species. These Cys residues are also conserved in *Pachyrhizus erosus*, an ortholog of P34 (SPE31). Its crystal structure has been determined (Zhang et al., 2006). Among seven Cys residues of SPE31, the first Cys from the N terminus is the only free Cys residue. The other six Cys residues form intramolecular disulfide bonds. Therefore, it is predicted that Cys-132 of P34, corresponding to the free Cys residue of SPE31, will form a disulfide bond with Cys-69 of the α'-subunit or Cys-68 of the α-subunit. The structure of the core region of β-conglycinin α'-trimer has been demonstrated (Maruyama et al., 2004). However, the structure of the extension region of β-conglycinin, in which Cys-69 is located, is unclear and is assumed to be disordered. SPE31 and perhaps P34 both retain the papain fold, which is composed of two domains with a cleft between them. It has been shown that peptides bind to the cleft of SPE31 at the surface through hydrogen bonding. We speculate that P34 may
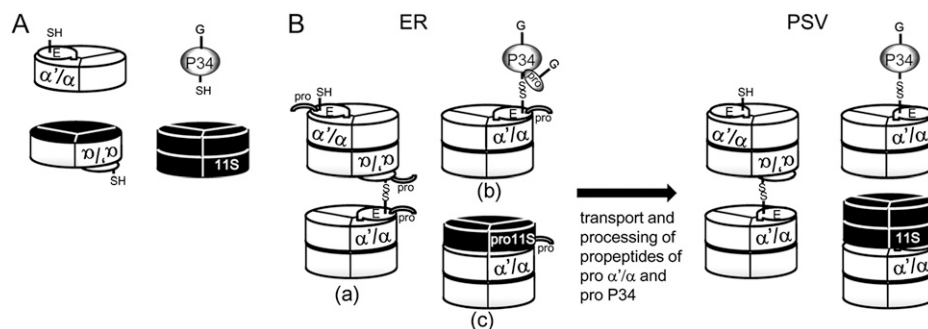

Figure 8. Hypothetical model of β-conglycinin complexes. A, β-Conglycinin and 11S extracted with 2-ME-containing buffer under an ionic strength of more than 0.2. β-Conglycinins are present as trimers. There is no intermolecular disulfide bond in the β-conglycinin trimer or in P34. B, Pro β-conglycinin hexamers associate into dodecamers via intermolecular disulfide bonds between Cys-69 of the α'-subunit or Cys-68 of the α-subunit in the ER (a). Cys-69 of the α'-subunit or Cys-68 of the α-subunit in some pro β-conglycinin hexamers is disulfide linked with Cys-132 of pro P34 (b). Part of the β-conglycinin hexamers associate with the proglycinin (pro 11S) trimer in the ER (c). They are transported into the PSV via the Golgi. Propeptides of α'/α and P34 are cleaved by proteases. Subunits of the pro 11S trimer are also enzymatically processed, and the processed 11S trimers associate into hexamers. E is the extension region of α'/α, pro is the propeptide of α'/α and P34, G is N-glycan, SH is sulfhydryl, and S-S is disulfide bond.

specifically bind to an unidentified region of the pro α′- or pro α-subunit at the cleft surface and that the disulfide bond between P34 and the α′/α-subunit is formed. Kinney et al. (2001) have demonstrated that P34 was deposited in novel protein bodies that were derived from the ER of transgenic seeds with reduced β-conglycinin levels. That report together with our data suggest that the disulfide linkage of P34 with the pro α′- or pro α-subunit is formed in the ER and may play an important role in the transport of P34 to the PSV via Golgi bodies. If so, then pro P34, prior to processing to mature P34, may form disulfide bonds with the pro α′- or pro α-subunit (Fig. 8B). Western-blot analyses detected a minor band above the 100-kD band of α′ linked with P34 in samples from immature cotyledon but not in dry bean samples (Fig. 2A, lane 1). Since P34 is a relatively insoluble protein, binding of P34 to α′/α-subunits may contribute to β-conglycinin deposition into the PSV. There are two putative N-glycosylation sites in pro P34 (NP 001238219) and SPE31. Glycosylation of Asn-159 of SPE31 corresponding to Asn-292 of P34 has been reported (Zhang et al., 2006). Among the two putative glycosylation sites of pro P34, Asn-70 and Asn-292, at least one Asn residue has been shown to be glycosylated (Kalinski et al., 1992). Since the N-terminal propeptide (M^1-Asn-122) was cleaved from P34 by proteolytic processing, N-glycan bound to Asn-70 is lost from the mature P34. The N-glycan bound to Asn-292 was predicted from the crystal structure of SPE31 to be located on the opposite side of Cys-132 of mature P34 (Fig. 8).

Vicilin and vicilin-like proteins in the seeds of many plant species have a Cys-rich hydrophilic region proximal to their N-terminal signal peptide sequence (Marcus et al., 1999). In soybean, the propeptide of β-conglycinin α′- and α-subunits corresponds to the same region. The proximal N-terminal Cys-rich hydrophilic region of vicilins from many plant species contains multiple segments with a characteristic four-Cys motif of C-X-X-X-C-(10-12X)-C-X-X-X-C (Marcus et al., 1999). The propeptides of β-conglycinin α′- and α-subunits also contain this C-X-X-X-C-(11X)-C-X-X-X-C motif. The four-Cys motif of buckwheat (*Fagopyrum esculentum*) trypsin inhibitor BWI-2b, which may have diverged in the buckwheat vicilin family, has been shown to form two intramolecular disulfide bonds, resulting in a hairpin structure (Park et al., 1997). In this study, we observed increases in the immunoreactivity of α′- and α-subunits with anti-propeptide antibodies after the reduction of disulfide bonds (Fig. 1B), suggesting that the Cys residues of the propeptide of pro α′- and α-subunits might form intramolecular disulfide bonds similar to those of BWI-2b (Fig. 1A). In the case of BWI-2b, the intramolecular disulfide bonds may be important for stabilization of the peptide. In addition, BWI-2b and a similar pumpkin (*Cucurbita* sp.) peptide, C2 peptide, have trypsin inhibitory activity (Yamada et al., 1999). Arg-19 between the two C-X-X-X-C sequences of BWI-2b is the reactive site for trypsin. Arg-44 is present at

an analogous position between the two C-X-X-X-C motifs of the propeptide of the pro α-subunit of β-conglycinin. It is unclear if the cleaved propeptides of α′- and α-subunits, with disulfide bonds, accumulate in seeds and have trypsin inhibitory activity.

We detected glycinin not only as hexamers (approximately 240–480 kD) but also as the larger complexes (approximately 500–800 kD). Since the larger complexes were not detected in the β-conglycinin-knockdown soybean, the glycinin hexamers may be associated with β-conglycinin. The associated β-conglycinin is predicted to be hexamers rather than dodecamers based on the molecular sizes of the larger complexes. The larger complexes were noncovalently associated. Nevertheless, the addition of 10 mM 2-ME caused dissociation of the larger complexes. The disulfide-linked α′/α-subunits were hardly detected in the β-conglycinin hexamers. The formation of the larger complexes was P34 independent. Taken together, it is assumed that a reduction of disulfide bonds between some Cys residues of glycinin, but not Cys residues of β-conglycinin and P34, by 2-ME may alter the affinity of glycinin for β-conglycinin. It has been known that two intramolecular disulfide bonds (Cys-12-Cys-45 and Cys-88-Cys-298) of the prevalent glycinin subclass, A1aB1b, were not reduced by 10 mM 2-ME. However, there is no information about the redox state of the three other Cys residues found in A1aB1b. Further studies on the Cys residues of glycinin need to be done.

CONCLUSION

Approximately half of the α′- and α-subunits of soybean β-conglycinin were disulfide linked, together or with P34. Disulfide bonds are formed between α′/α-subunits residing in different β-conglycinin hexamers. The binding of P34 to α′- and α-subunits reduces the linkage between β-conglycinin hexamers. In addition, a subset of glycinin is accumulated as noncovalently associated complexes with β-conglycinin in the PSVs of soybean cotyledon cells.

MATERIALS AND METHODS

Materials

A protein assay kit (RC DC protein assay) and polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad Laboratories. Native PAGE, Novex 3% to 12% bis-Tris gels, and native PAGE sample buffer were obtained from Invitrogen. Horseradish peroxidase-conjugated anti-rabbit IgG goat serum was obtained from Promega. Antibodies against the propeptide of pro α′-subunit, α′-subunit, glycinin-acidic chain, and GmPDIL-1 were prepared as described previously (Wadahama et al., 2007; Kamauchi et al., 2008). The Western Lightning Chemiluminescence Reagent was purchased from Perkin-Elmer Life Science. All other reagents were of analytical grade from Wako Pure Chemical Industries.

Plants

P34-null mutant soybean (*Glycine max*; PI 567476; Joseph et al., 2006) was obtained from the Soybean Germplasm Collection of the Agricultural Re-

search Service, U.S. Department of Agriculture. Wild-type soybeans (cv Jack), α-null mutant soybean, α′-null mutant soybean, the transgenic soybeans with β-conglycinin-RNAi, and glycinin-null mutant soybeans were described previously (Takahashi et al., 2003; Mori et al., 2004; Nishizawa et al., 2010).. Soybeans were planted in 5-L pots and grown in a controlled-environment chamber at 25°C under a 16-h-day/8-h-night cycle.

Extraction of Soybean Proteins

Immature cotyledons from 80- to 100-mg bean or cotyledons from dry seeds were frozen under liquid nitrogen and then ground into a fine powder with a micropestle (SK-100; Tokken). The ground cotyledons (100 mg) were dispersed in 75 μL of 63 mM Tris-HCl buffer, pH 7.0, with 20 mM NEM and incubated for 5 min on ice. The suspension with NEM was diluted with a 9-fold volume of 63 mM Tris-HCl buffer, pH 7.8, incubated for 1 h at 4°C, and centrifuged at 18,000g for 15 min at 4°C on a RA-200J rotor using a Kubota 1710 centrifuge. In other cases, proteins were extracted with 63 mM Tris-HCl buffer, pH 7.8, with 10 mM 2-ME. Protein concentrations of the resulting supernatants were measured with the RC DC protein assay kit (Bio-Rad).

For the isolation of β-conglycinin, the protein extract from dry seeds was adjusted to pH 6.6 with 0.1 M HCl, dialyzed against 63 mM Tris-HCl buffer, pH 6.6, for 3 h at 4°C, and centrifuged at 10,000g for 20 min at 4°C. The pH of the resulting supernatant was adjusted to 4.8 with 0.1 M HCl. The precipitated protein (β-conglycinin fraction) was collected by centrifugation at 10,000g for 20 min at 4°C, and pellets were dissolved in 35 mM phosphate buffer, pH 7.6, containing 0.4 M NaCl with or without 10 mM 2-ME. The protein concentration of each solution was measured with the RC DC protein assay kit (Bio-Rad) using γ-globulin as a standard.

Western-Blot Analysis

Western-blot analysis was performed as described previously (Wadahama et al., 2007). Briefly, proteins were boiled in SDS sample buffer (Laemmli, 1970) with 5% 2-ME for 5 min, for reducing SDS-PAGE, or incubated in SDS sample buffer without 2-ME at 37°C for 2 h, for nonreducing SDS-PAGE. For the analysis of pro α′- and pro α-subunits separated by nonreducing SDS-PAGE, disulfide bonds of the proteins were reduced on the gel by incubation in blotting buffer containing 50 mM DTT for 30 min at room temperature and then transferred onto a PVDF membrane.

For 2D SDS-PAGE, proteins treated with SDS-sample buffer without reducing reagent were separated by SDS-PAGE. Lanes cut from the first SDS-PAGE gel were incubated in 20 mM Tris-HCl buffer, pH 7.8, containing 50 mM DTT, for 30 min at 25°C, followed by incubation in SDS-sample buffer with 5% 2-ME, for 30 min at 25°C. The gel slices were then subjected to a second SDS-PAGE and separated proteins were blotted onto a PVDF membrane.

For 2D electrophoresis with BN-PAGE (Wittig et al., 2006) and SDS-PAGE, proteins were extracted with 50 mM bis-Tris, pH 7.2, 150 mM NaCl, 1% digitonin, 0.001% Ponceau S, and 1% protease inhibitor (Invitrogen). Coomassie Brilliant Blue G-250 was added to the protein solution at a final concentration of 0.25%, and the protein solution was subjected to native PAGE with a 3% to 12% polyacrylamide gradient gel according to the manufacturer's protocol for the Native PAGE Novex Bis-Tris Gel System (Invitrogen). Lanes cut from the BN-PAGE gels were then subjected to reducing or nonreducing SDS-PAGE. The separated proteins were blotted onto a PVDF membrane. The membranes were probed with specific antibodies first, followed by treatment with a horseradish peroxidase-conjugated IgG secondary antibody, using a Western Lightning Chemiluminescence Reagent.

RNAi of GmPDIL-1

pUHR:P7S-IR was previously constructed as a Gateway-compatible RNAi vector for particle bombardment-mediated transformation (Takagi et al., 2011). The promoter sequence of the soybean pUHR:P7S-IR corresponds to the gene for the α′-subunit of β-conglycinin. For construction of the RNAi vector targeting GmPDIL-1 (AB182628), a 214-bp cDNA fragment, which corresponds to a part of GmPDIL-1 (Supplemental Fig. S2), was amplified by PCR using soybean (cv Jack) cDNA. Transfer of the respective RNAi target sequences to pUHR:P7S-IR was performed to yield pUHR:P7S-PDIL-1-RNAi (Supplemental Fig. S2), as described previously (Nishizawa et al., 2010).

Transformation of soybean by particle bombardment and subsequent plant regeneration were performed as described previously (Khalafalla et al., 2005).

The plants regenerated from somatic embryos made tolerant to hygromycin B (Roche Diagnostics) were monitored with a fluorescence stereomicroscope (Leica), and those expressing the red fluorescent protein DsRed2 were grown under greenhouse conditions. A transgenic line transformed with the empty vector, pUHR (Nishizawa et al., 2006a, 2006b), was used as a control.

Sedimentation Velocity

β-Conglycinin fractions prepared from dry seed in the absence of 2-ME were dialyzed overnight against 35 mM phosphate buffer, pH 7.6, with or without 10 mM 2-ME in the presence or absence of 0.4 M NaCl. Sedimentation velocity experiments were performed with an Optima XL-I (Beckman Coulter) using an An50Ti rotor at 20°C. Dialysis buffer used for the preparation of β-conglycinin fractions was used as a reference solution. All data were acquired without time intervals between successive scans. The sedimentation coefficient distribution function was obtained using the SEDFIT program (Schuck, 2000; Schuck et al., 2002). The partial specific volume of the protein from the amino acid sequence, buffer density, and viscosity were calculated by using the Sednterp program (Laue et al., 1992).

Size-Exclusion Chromatography

The β-conglycinin fraction obtained from dry seeds was applied to a 7.5-mm × 60-cm TSK gel G3000SW column (Tosoh) equilibrated with 35 mM phosphate buffer, pH 7.6, containing 0.4 M NaCl. Eluted fractions (0.4 mL each) were collected and subjected to reducing or nonreducing SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue R-250. Band intensities of α′, α, β, α′-100, α-100, and α′/α-150 were quantified by ImageJ (National Institutes of Health). The relative percentage of a band intensity detected in each fraction, when compared with the sum of the total band intensities detected in all fractions, was calculated as the mean ± SE of three experiments.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. SDS-PAGE of proteins extracted from dry bean cotyledons of wild-type and mutant soybeans.

Supplemental Figure S2. Construction of RNAi vectors for the manipulation of PDIL-1 gene expression.

Supplemental Figure S3. SDS-PAGE of β-conglycinin fractionated by size-exclusion chromatography with a TSK gel G3000SW column.

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