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# Nitrogen Lowers the Sulfur Amino Acid Content of Soybean (*Glycine max* [L.] Merr.) by Regulating the Accumulation of Bowman−Birk Protease Inhibitor

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Soybeans in general contain 35−40% protein. Efforts are underway to increase further this protein content, thus enhancing their nutritive value. Even though higher protein is a desirable characteristic, whether such an increase will be accompanied by enhanced protein quality is not known. Soybean protein quality could be significantly improved by increasing the concentration of the sulfur-containing amino acids, cysteine and methionine. To ascertain if a correlation existed between protein quantity and quality, a comparison of the amino acids of soybeans differing in protein content was made. Soybeans with higher protein content had a significantly lower percentage of sulfur amino acids, while those with lower protein exhibited a higher content of cysteine and methionine. Nitrogen application elevated the protein content but lowered that of the sulfur amino acids. Transmission electron microscopy examination of thin sections of low protein soybean seeds revealed several protein storage vacuoles that were partially filled with storage proteins. Fluorescence two-dimensional difference gel electrophoresis of soybean seed proteins revealed that nitrogen application favored the accumulation of the $\beta$-subunit of $\beta$-conglycinin while decreasing the accumulation of Bowman−Birk protease inhibitor (BBI), a protein rich in cysteine. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis of 60% 2-propanol-extracted proteins showed a drastic reduction in the accumulation of BBI with increasing protein content. Northern blot analysis indicated that nitrogen had a negative influence on the expression of the BBI gene. Our results indicate that the negative correlation between total protein and sulfur amino acid content is mostly mediated by the differential accumulation of BBI.

**KEYWORDS:** ***Glycine*; Bowman−Birk protease inhibitor; nitrogen; protein quality; sulfur amino acids**

## INTRODUCTION

Soybeans consistently provide a high quality and cost competitive source of protein for the animal industry and human consumption. Protein meal derived from soybeans has high palatability and digestibility and thus is an efficient source of amino acids for supporting growth and development (*1*−*5*). Soybeans contain approximately 35−40% protein, 70% of which are the salt soluble globulins, 7*S* $\beta$-conglycinins and 11*S* glycinins (*6*, *7*). Glycinins are synthesized as precursor proteins and posttranslationally cleaved into 40 kDa acidic and 20 kDa basic subunits. These subunits are then covalently bound by a single interchain disulfide bond. The $\beta$-conglycinins are comprised of the $\alpha'$-, $\alpha$-, and $\beta$-subunits with molecular masses of 76, 72, and 53 kDa, respectively. Even though glycinins are relatively rich in sulfur amino acids in comparison to the glycosylated $\beta$-conglycinins, the overall sulfur amino acid content of these proteins is still not sufficient to meet the nutritional demands of growing poultry and swine. At present, corn−soybean-based rations must be supplemented with synthetic methionine to meet the nutritional requirements of the monogastric diet (*8*).

Cysteine is at the core of sulfur metabolism in plants. This amino acid serves as a precursor for a plethora of chemically active biological compounds including glutathione, biotin, CoA, and enzymes involved in redox reactions (*9*−*12*). Additionally, a sufficient quantity of cysteine must be available for the synthesis of seed storage proteins. The synthesis of cysteine represents a culmination of carbon, nitrogen, and sulfur metabolism and is controlled by coordinated interactions between these respective pathways (*11*−*13*). Cysteine is derived from the amino acid serine, which itself represents a culmination in the coordinated assimilation of carbon and nitrogen (*14*, *15*). The activated form of serine *O*-acetylserine (OAS) reacts with reduced sulfur to form cysteine. OAS is both a rate limiting and a positive regulator in the production of cysteine (*16*, *17*).

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Soybean seed storage protein composition is influenced by plant nutrient availability. The application of nitrogen tends to increase the sulfur poor $\beta$-subunit of $\beta$-conglycinin, while sulfur fertilization enhances accumulation of glycinins (*18*−*22*). Nonnodulating mutants, in contrast to nodulating isolines, accumulate minimal $\beta$-subunits of $\beta$-conglycinins (*23*, *24*). The application of nitrogen can restore the accumulation of the $\beta$-subunit of $\beta$-conglycinin in nonnodulating mutants (*23*, *24*). Similarly, OAS also has been shown to have a positive effect on the accumulation of the $\beta$-subunit of $\beta$-conglycinin (*25*).

One objective as outlined in the United Soybean Board's Better Bean Initiative (http://www.unitedsoybean.org/home.htm) is the development of agronomically viable soybean cultivars, which will contain approximately 44% protein. During the past few decades, soybean breeders have been successful in increasing the protein content (*26*, *27*). However, little is known if this increase is also accompanied by an increase in sulfur amino acid content. Here, we show that a negative correlation exists between total protein and sulfur amino acid content of soybeans. The accumulation of Bowman−Birk protease inhibitor (BBI), a protein rich in cysteine, decreases when the total seed protein increases. We also demonstrate that nitrogen application, which tends to increase the protein content, also decreases BBI accumulation by diminishing the transcription of this gene.

## MATERIALS AND METHODS

**Plant Material and Growth Conditions.** Nodulating (*Rj1Rj1*) and nonnodulating (*rj1rj1*) isolines of Clark soybeans were grown at the Bradford Research and Extension Center near Columbia, Missouri, during 2003 and 2004. To characterize the effect of high levels of exogenous nitrogen on protein accumulation, 45 kg/hectare nitrogen supplied as ammonium nitrate was applied immediately prior to flowering to selected plots. Seed samples were harvested at 20 and 35 days after flowering (DAF), which approximately corresponds to R5 and R6 developmental stages, from the 10th and 11th nodes beginning with the cotyledonary node. After separation according to size, seeds were frozen in liquid nitrogen and stored at −80 °C.

Soybean cultivar Jefferson was grown in a greenhouse in 25 cm plastic pots containing commercial potting soil. The day and night temperatures in the greenhouse were 26/22 °C with a relative humidity of approximately 60%. Supplemental illumination was provided by high-pressure sodium lamps to maintain a light intensity of 400 $\mu M^{-2}$ $s^{-1}$ for a photoperiod of 14/10 h day/night. To obtain seeds with different protein concentrations, plants were grown at increasing nitrogen levels. Four replications of treatment were arranged in a randomized complete block design. The data were analyzed by the linear model procedure of the SAS statistical software (release 8.02).

**Extraction of Soybean Seed Proteins.** Seeds from each replicate were ground to a fine powder with a commercial blender. A 10 mg aliquot from each of the four replications was extracted with 1 mL of sodium dodecyl sulfate (SDS) sample buffer [60 mM Tris-HCl, pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), and 5% 2-mercaptoethanol] and then boiled for 5 min. The lysate was clarified by centrifugation (15800*g*, 5 min), and aliquots of the supernatant were utilized for SDS−polyacrylamide gel electrophoresis (PAGE) analysis. 2-Propanol soluble proteins were obtained by adding 1 mL of 60% 2-propanol to a 2 mL eppendorf tube containing 100 mg of seed powder (*29*). Extraction was carried out in a 30 °C shaker for 1 h followed by centrifugation (15800*g*, 5 min). A 500 $\mu$L aliquot of the supernatant was mixed with 3 volumes of ice-cold acetone and incubated overnight at −20 °C. Precipitated proteins were recovered by centrifugation, air-dried, and resuspended in 200 $\mu$L of SDS sample buffer.

**SDS−PAGE.** Seed proteins were resolved by SDS−PAGE (*28*) using a Hoefer SE 260 minigel apparatus (Amersham Bioscience, Piscataway, NJ) and visualized by Coomassie brilliant blue staining. Two-dimensional (2D) gel electrophoresis was performed as described below for fluorescence difference gel electrophoresis (DIGE) with the exception of the addition of the dye.

**Amino Acid Analysis.** Dry soybean seed powder was hydrolyzed for 16 h at 155 °C with 6 N HCl under a nitrogen atmosphere. Methionine and cysteine were quantified from duplicate samples that were subjected to oxidation with performic acid prior to acid hydrolysis. Amino acids were separated on a Beckman 6300 Amino Acid Analyzer (Beckman Instruments, Fullerton, CA) equipped with a high performance cation exchange resin column. Amino acid analysis was performed on four independent samples from each treatment. Multiple range tests between the treatments were conducted using Fisher's protected least significant difference test option.

**Fluorescence 2D DIGE.** Finely ground soybean seed was extracted with 2.5 mL of 0.1 M Tris-HCl buffer (pH 8.8) containing 10 mM EDTA, 0.4% 2-mecaptoethanol, and 0.9% sucrose and then gently vortexed. An equal volume of Tris-HCl-buffered phenol (pH 8.8) was added to the lysate, and the mixture was stirred for 30 min at 4 °C. After centrifugation (5000*g*, 10 min, 4 °C), the phenolic phase was reserved and the aqueous phase was reextracted with 2.5 mL of buffered phenol. Proteins were precipitated from the combined phenolic phases by the addition of 5 volumes of chilled 0.1 M ammonium acetate in 100% methanol and stored overnight. Proteins were recovered by centrifugation (20000*g*, 20 min, 4 °C) and then subjected to successive washes in cold 0.1 M ammonium acetate in methanol, ice-cold acetone, and cold 70% ethanol. Prior to isoelectric focusing, the protein pellet was dispersed in 1 mL of buffer {8 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2% Triton X-100, 50 mM dithiothreitol (DTT), and 0.5% ampholytes, pH 3−10}. A 50 $\mu$g aliquot from 31% seed protein was labeled with 200 pmol of Cy3 fluorescent dye, while protein from 37% seed protein was labeled with 200 pmol of Cy5 dye. After it was incubated on ice for 30 min, the nucleophilic reaction was quenched by addition of 10 mM lysine and the fluorescently labeled proteins were combined in a 1:1 (v/v) ratio. The mixture was applied to immobilized pH gradient strips by active rehydration utilizing increasing electric potential, 50 V for 12 h, 500 V for 1 h, 1000 V for 2 h, and 8000 V for 1 h. SDS−PAGE buffer (50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, and 5% SDS) to which 2% DTT had been added was used to equilibrate strips for 2D electrophoresis. The equilibration process was repeated a second time with the addition of 2.5% iodoacetamide to the buffer. After a final rinse in SDS−PAGE running buffer, the strips were placed on an 11−17% acrylamide gradient gel and overlaid with a buffered 0.5% agarose solution (60 mM, Tris-HCl, pH 6.8, 60 mM SDS, and 0.01% bromophenol blue). The FLA-5000 Fluorescent Image Analyzer (Fuji Photo Film Co., Tokyo, Japan) was used to image the electrophoretically separated labeled proteins.

**Electron Microscopy.** Dry soybean seeds were imbibed in water and germinated in a 30 °C incubator for 12 h. Seeds were sliced into 2−4 mm cubes and immediately fixed in 2.5% glutaraldehyde buffered at 7.2 with 50 mM sodium phosphate. Primary fixation was performed for 4 h. Following three washes in phosphate buffer, the tissue was postfixed for 1 h in 1% aqueous osmium tetroxide, dehydrated in a graded acetone series, and infiltrated with Spurr's resin. Thin sections of resin-embedded tissue were cut with a diamond knife and collected on 200 mesh copper grids. After staining with 0.5% uranyl acetate and 0.4% lead citrate, sections were examined at 80 kV under a JEOL 1200 EX (Tokyo, Japan) transmission electron microscope.

**RNA Extraction and Northern Blot Analysis.** Developing soybean seeds (5 g) were ground to a fine powder under liquid nitrogen. Seed powder was extracted with 25 mL of lysis buffer [100 mM Tris-HCl, pH 7.6, containing 1% (w/v) Tris-iso-propyl-naphthalenesulfonic acid, 6% (w/v) *p*-aminosalicyclic acid, 50 mM EDTA, 100 mM NaCl, 1% (w/v) SDS, and 50 mM 2-mercaptoethanol]. An equal volume of phenol: chloroform was added, and the lysate was homogenated. The slurry was centrifuged, and the nucleic acids were precipitated by the addition of 2.5 volumes of ice-cold ethanol and one-tenth volume of 3 M sodium acetate. Total RNA was purified by LiCl precipitation and spectrophotometrically quantified. A 5 $\mu$g aliquot of total RNA was separated on a 1.3% agarose-formaldehyde gel, transferred to a Hybond $N^+$ membrane, and immobilized by UV cross-linking. Prehybridization was performed for 6 h at 65 °C in 7% SDS, 191 mM $Na_2HPO_4$, 58 mM $NaH_2PO_4$, 1% bovine serum albumin (BSA), and 100 $\mu$g/mL denatured salmon sperm DNA. Subsequent hybridization was performed overnight

in the same buffer. Nonspecific binding of the probe was removed by washing the membrane with 0.5% SCC (150 mM NaCl and 15 mM sodium citrate) and 0.1% SDS at 65 °C. Hybridizing RNA transcripts were detected by autoradiography with intensifying screen at −80 °C.

**Statistics.** Analysis of variance was used to assess the effect of the percentage of total seed protein on that of individual amino acids. Seed protein percentages were considered the independent variables while individual amino acids were the dependent variables. In the second trial, the effects of nitrogen application and nodulation were determined. Nitrogen and nodulation were fixed factors, and individual amino acids were the random factors. Differences in the means were considered statistically significant at $P \leq 0.05$. Analyses of variance were performed using the 8.2 version of the General Linear Model SAS software (SAS Institute 2001).

## RESULTS

**Nonnodulating Soybeans Have Higher Sulfur Amino Acid Content.** The protein concentration of nodulating and nonnodulating soybean cv. Clark was determined by near NIR spectroscopy (Infratech 1255 Food and Feed analyzer, Tecator AB, Hoganas, Sweden). Seeds from nodulating soybean contained 38.3 ± 0.6% protein, while those from the nonnodulating isoline were 31.2 ± 1.4% protein. Two-dimensional gel electrophoresis of protein from each isoline provided further evidence of the difference in accumulation (**Figure 1**). Although the number and location of spots on each gel were comparable, indicating analogous protein profiles, spot intensities were greater in the gel containing seed protein from nodulating plants (**Figure 1**). In particular, the spot intensity of the $\beta$-subunit of $\beta$-conglycinin was considerably higher in seed from nodulating as compared to nonnodulating plants (**Figure 1**). To ascertain if a relationship existed between total protein and sulfur amino content, the amino acid profiles of nodulating and nonnodulating soybeans were determined and compared (**Table 1**). Methionine and cysteine comprised 2.95 ± 0.1% of the total of amino acids in nodulating soybean and 3.68 ± 0.1% in nonnodulating soybean. The calculation of sulfur amino acid content per 100 g of dry seed weight substantiated that there was an absolute increase in these two amino acids in nonnodulating soybeans. The application of nitrogen fertilizer reduced the sulfur amino acid content in nodulating isoline by 7% and in nonnodulating isoline by 14% (**Table 1**).

**Inverse Relationship between Protein and Sulfur Amino Acid Content.** Because nonnodulating soybean contained less protein but a higher percentage of sulfur amino acids, we wanted to determine if an inverse relationship existed between total protein and sulfur amino acid content. To examine this possibility, we grew soybean cv. Jefferson under different nitrogen fertilization regimes at different levels of applied nitrogen. Seeds harvested from these plants, when analyzed by NIR spectroscopy, showed that protein concentrations ranged from 29 to 37%. Subsequent analysis by SDS−PAGE showed an incremental increase in seed proteins including 7*S* $\beta$-conglycinins and 11*S* glycinins with application of nitrogen (**Figure 2**). We also determined the amino acid profile of these seeds and found significant differences in the total sulfur amino acid content (**Table 2**). Seeds with the lowest protein concentrations had the highest sulfur amino acid content, while those with high protein concentrations had the lowest sulfur amino acid content (**Table 2**). There was an inverse correlation between sulfur amino acid and protein content.

**Accumulation of BBI Decreases with Increasing Protein Concentration.** To determine if the sulfur amino acid content correlates with that of Bowman−Birk inhibitor, we isolated 2-propanol soluble proteins from soybean seeds containing a range of protein concentrations. The 2-propanol-extracted proteins when resolved by SDS−PAGE and visualized by Coomassie blue stain (**Figure 3**) contained a prominent low molecular protein previously identified as BBI (*29*). Accumulation of BBI was inversely related to the protein concentration (**Figure 3**). A densitometer scan of this gel showed that seeds with 29% protein had 5.2-fold higher content of BBI than that from seeds with 37% seed protein. In contrast, the accumulation of a 20 kDa protein, which migrates as a diffuse band under the experimental condition used in this study, was not significantly affected in seeds with varying protein concentrations (**Figure 3**).

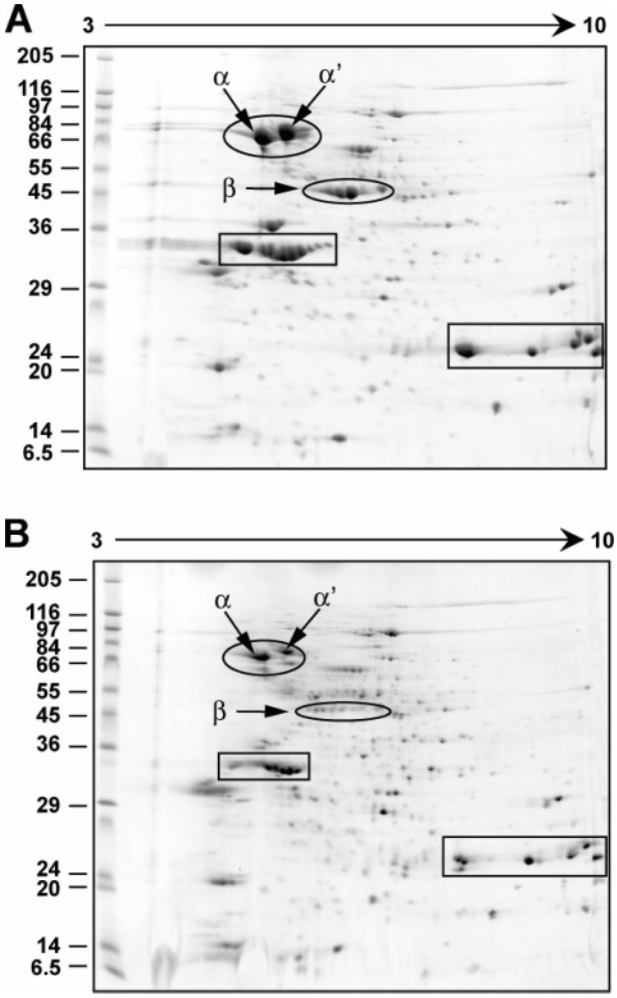


**Figure 1.** Two-dimensional gel separation of soybean seed proteins. Proteins obtained on the basis of equal seed dry weight from nodulating (**A**) and nonnodulating (**B**) soybeans were separated in the first dimension on an IPG strip (pH 3–10) and in the second dimension on a 13% SDS-polyacrylamide gel. The gel was stained with colloidal Coomassie blue. The α′-, α-, and $\beta$-subunits of $\beta$-conglycinins are enclosed by an oval outline while the acidic and basic subunits of glycinins are enclosed by rectangles. Sizes of the molecular weight markers are also shown at the sides of each figure.

**Nitrogen Lowers the Accumulation of BBI.** We compared seed protein profiles exhibited by control and nitrogen-fertilized plants using DIGE. Control plants yielded seeds containing 31% protein, which was labeled with Cy3 dye while plants receiving exogenous nitrogen produced seeds containing 37% protein, which was labeled with Cy5 dye. Relative accumulation of individual proteins was determined by visualization of spot color of Cy3 and Cy5 superimposed images (**Figure 4**). A yellow-colored spot indicates that the particular protein accumulated

**Table 1.** Amino Acid Composition (w/w %) of Nodulating and Nonnodulating Clark Soybean Seeds[a]

| | *Glycine max* cv. Clark | | | |
|---|---|---|---|---|
| amino acid | nodulating | nonnodulating | nodulating + nitrogen | nonnodulating + nitrogen |
| aspartic acid | 11.2 ± 0.2 | 11.30 ± 0.0 | 11.5 ± 0.4 | 11.4 ± 0.3 |
| threonine | 3.7 ± 0.1 | 4.0 ± 0.0 | 3.5 ± 0.2 | 3.70 ± 0.1 |
| serine | 4.3 ± 0.1 | 4.5 ± 0.1 | 4.1 ± 0.3 | 4.1 ± 0.3 |
| glutamic acid | 16.5 ± 0.2 | 16.3 ± 0.3 | 17.5 ± 0.5 | 17.3 ± 0.5 |
| proline | 7.7 ± 0.6 | 6.9 ± 0.1 | 8.2 ± 0.8 | 7.7 ± 0.6 |
| glycine | 4.2 ± 0.1 | 4.4 ± 0.0 | 4.2 ± 0.2 | 4.4 ± 0.2 |
| alanine | 4.7 ± 0.2 | 5.0 ± 0.1 | 4.5 ± 0.3 | 4.7 ± 0.2 |
| valine | 4.9 ± 0.1 | 5.0 ± 0.1 | 3.7 ± 1.3 | 3.7 ± 1.3 |
| isoleucine | 4.7 ± 0.1 | 4.8 ± 0.1 | 4.8 ± 0.2 | 4.8 ± 0.2 |
| leucine | 9.4 ±0.9 | 8.1 ± 0.2 | 9.9 ± 1.0 | 9.3 ± 0.8 |
| tyrosine | 3.2 ± 0.1 | 3.3 ± 0.1 | 3.0 ± 0.1 | 3.1 ± 0.1 |
| phenylanine | 5.1 ± 0.1 | 5.0 ± 0.1 | 5.1 ± 0.2 | 5.1 ± 0.2 |
| histidine | 2.6 ± 0.1 | 2.8 ± 0.0 | 2.6 ± 0.1 | 2.7 ± 0.1 |
| lysine | 6.5 ± 0.2 | 7.1 ± 0.0 | 6.4 ± 0.2 | 6.7 ± 0.2 |
| arginine | 7.3 ± 0.2 | 6.9 ± 0.0 | 7.4 ± 0.2 | 7.2 ± 0.2 |
| cysteine | 1.6 ± 0.0 | 2.1 ± 0.0 | 1.5 ± 0.1 | 1.8 ± 0.1 |
| methionine | 1.3 ± 0.0 | 1.6 ± 0.1 | 1.3 ± 0.1 | 1.4 ± 0.1 |

[a] Values are the averages of four replications with standard errors.

**Table 2.** Amino Acid Composition (w/w %) of Soybean Seeds with Increasing Protein Content[a]

| | *Glycine max* cv. Jefferson with different protein content | | | | |
|---|---|---|---|---|---|
| amino acid | 37% | 36% | 34% | 31% | 29% |
| aspartic acid | 10.9 ± 1.5 | 11.6 ± 0.5 | 11.9 ± 0.5 | 11.6 ± 0.1 | 11.2 ± 0.1 |
| threonine | 3.5 ± 0.4 | 3.6 ± 0.2 | 3.6 ± 0.2 | 3.8 ± 0.2 | 4.2 ± 0.0 |
| serine | 4.2 ± 0.8 | 4.1 ± 0.4 | 4.0 ± 0.5 | 4.3 ± 0.5 | 4.8 ± 0.3 |
| glutamic acid | 16.8 ± 2.8 | 17.7 ± 0.8 | 17.8 ± 0.7 | 16.8 ± 0.6 | 15.8 ± 0.5 |
| proline | 7.3 ± 1.0 | 7.7 ± 0.9 | 7.3 ± 0.5 | 6.8 ± 0.2 | 7.1 ± 0.3 |
| glycine | 4.0 ± 0.5 | 4.3 ± 0.2 | 4.4 ± 0.2 | 4.35 ± 0.1 | 4.40 ± 0.1 |
| alanine | 4.3 ± 0.4 | 4.6 ± 0.3 | 4.8 ± 0.3 | 4.8 ± 0.2 | 5.1 ± 0.3 |
| valine | 4.6 ± 0.7 | 3.6 ± 2.4 | 3.7 ± 2.5 | 5.0 ± 0.3 | 4.9 ± 0.2 |
| isoleucine | 4.4 ± 0.6 | 4.7 ± 0.3 | 4.9 ± 0.3 | 4.8 ± 0.2 | 4.7 ± 0.2 |
| leucine | 8.4 ± 1.0 | 8.9 ± 1.1 | 8.6 ± 0.5 | 7.7 ± 0.1 | 7.6 ± 0.2 |
| tyrosine | 2.9 ± 0.3 | 3.0 ± 0.2 | 3.1 ± 0.2 | 3.1 ± 0.2 | 3.2 ± 0.1 |
| phenylanine | 4.8 ± 0.5 | 5.1 ± 0.3 | 5.2 ± 0.3 | 5.0 ± 0.1 | 4.8 ± 0.2 |
| histidine | 2.5 ± 0.3 | 2.7 ± 0.1 | 2.7 ± 0.1 | 2.7 ± 0.0 | 3.1 ± 0.0 |
| lysine | 6.2 ± 0.8 | 6.6 ± 0.3 | 6.8 ± 0.3 | 7.0 ± 0.1 | 7.5 ± 0.1 |
| arginine | 6.9 ± 0.9 | 8.3 ± 0.5 | 7.3 ± 0.3 | 6.9 ± 0.1 | 6.3 ± 0.1 |
| cysteine | 1.6 ± 0.2 | 1.7± 0.1 | 1.8 ± 0.1 | 2.2 ± 0.1 | 2.8 ± 0.1 |
| methionine | 1.4 ± 0.1 | 1.4 ± 0.0 | 1.4 ± 0.1 | 1.6 ± 0.0 | 1.8 ± 0.0 |

[a] Values are the averages of four replications with standard errors.

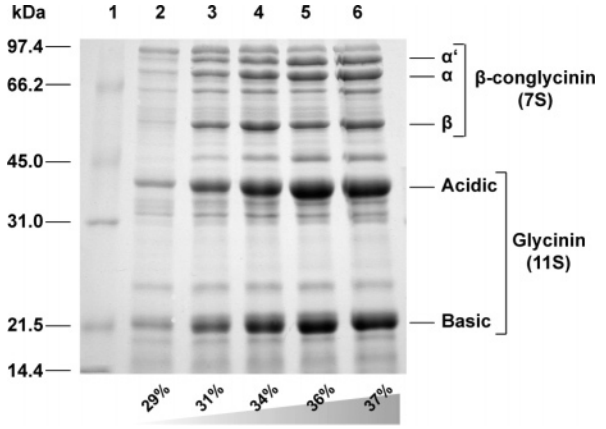


**Figure 2.** SDS–PAGE analysis of soybean seed proteins. Total proteins obtained on the basis of equal seed dry weight were separated on a 13% SDS–PAGE. Resolved proteins were stained with Coomassie Brilliant Blue. Sizes of protein standards are shown in kDa. The number on the bottom of the figure indicates the concentration of seed protein in percentage.

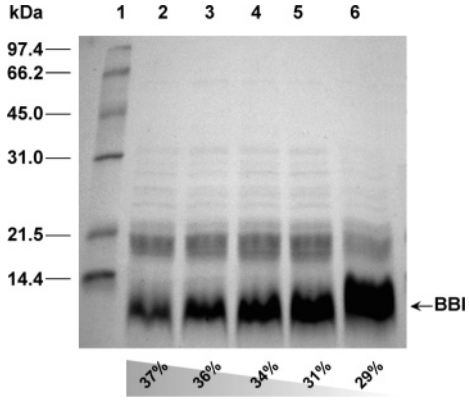


**Figure 3.** SDS–PAGE analysis of 60% 2-propanol-extracted proteins from soybean. Equal weights of dry soybean seed powder were extracted with 60% 2-propanol, and the recovered proteins were resolved on a 13% SDS–PAGE gel. Proteins were visualized by staining with Coomassie Brilliant Blue. The number on the bottom of the figure indicates the concentration of seed protein in percentage. Lane 1 contains protein standards.

equally between treatments. Red spots signify that the specific proteins accumulated predominantly in seeds, which contained 37% total protein, while green spots reveal that the particular protein was more prevalent in seeds with 31% protein content. When the image was analyzed with FLA-5000 laser analyzer, red spots corresponding to the $\beta$-subunit of $\beta$-conglycinin were seen denoting that these proteins were abundant in seeds with 37% protein (**Figure 4**). Several low molecular weight green spots with acidic isoelectric points were also observed suggesting that these proteins were more prevalent in the seed containing the lower total protein (31%). Some of these protein spots have been earlier identified as BBIs (*29*). Most other abundant soybean seed protein spots including the $\alpha'$, $\alpha$, and the acidic and basic subunits of glycinins revealed a yellow color indicating that the accumulation of these proteins is not significantly affected in soybean seeds differing in protein content (**Figure 4**).

Earlier, we demonstrated that the 60% 2-propanol-extracted protein fraction is enriched in Kuntz trypsin and BBIs (*29*). Because BBI accumulation is inversely related to the protein concentration (**Figure 3**), we sought to ascertain whether application of nitrogen, which increases total protein content, has an effect on the accumulation of the BBI. SDS−PAGE analysis of the 60% 2-propanol-extracted protein fraction revealed that application of nitrogen had a negative effect on the accumulation of BBI (**Figure 5**).

**Nitrogen Lowers the Expression of BBI mRNA.** The low accumulation of BBI in soybean seeds grown in the presence of nitrogen led us to examine the corresponding mRNA levels in developing soybean seeds. We isolated total RNA from soybean seeds at 20 and 35 DAF from plants grown either with or without exogenous nitrogen and performed Northern blot analysis. $\beta$-Conglycinin and BBI probes hybridized to 1.6 kB and 0.5 kB RNA transcripts, respectively (**Figure 6**). The abundance of the $\beta$-subunit of $\beta$-conglycinin and BBI mRNA was much greater at 35 DAF than at 20 DAF. Clearly, nitrogen enhanced the expression of the $\beta$-subunit of $\beta$-conglycinin. In contrast, the accumulation of BBI RNA transcript was drastically reduced in nitrogen-fertilized plants (**Figure 6**).

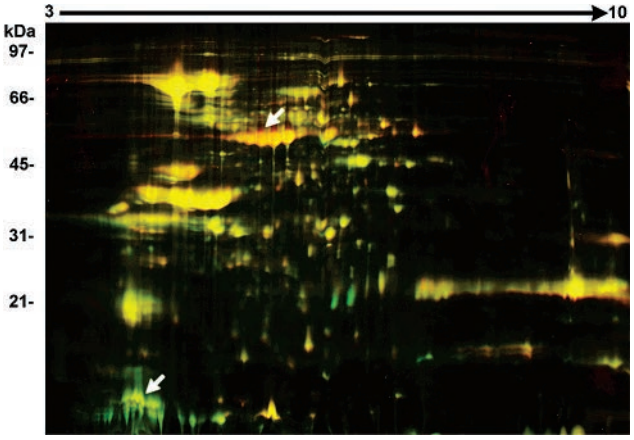


**Figure 4.** Comparison of protein profile of soybean seeds differing in protein concentration by DIGE. Equal amount of proteins from soybean seeds with 31 and 37% were labeled with Cy3 and Cy5 dyes and analyzed by 2D DIGE. The gel was scanned at emission wavelengths specific for Cy3 and Cy5, and the resulting image was overlaid and visualized with a FLA-500 laser analyzer. The arrows point to the $\beta$-subunits of $\beta$-conglycinin and the BBI, respectively. Sizes of molecular mass markers in kDa are also shown.

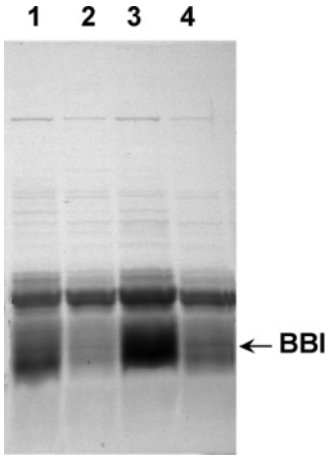


**Figure 5.** Nitrogen lowers the accumulation of BBI. Soybean seeds were harvested from control (lanes 1 and 3) or nitrogen-supplemented plants (lanes 2 and 4) at 20 (lanes 1 and 2) and 35 DAF (lanes 3 and 4). Equal weights of dry soybean seed powder were extracted using 60% 2-propanol and were resolved on a 13% SDS–PAGE gel and visualized by staining with Coomassie Brilliant Blue. The arrow points to BBI.

**Electron Microscope Observation of Protein Storage Vacuoles.** The storage proteins of soybean accumulate within specialized protein storage vacuoles (PSVs) in the cotyledons (*30*). Recent studies have demonstrated that in addition to PSVs, soybean seed proteins can also accumulate in protein bodies both under normal growing conditions and in soybean mutants that fail to accumulate a certain class of storage proteins (*31*, *32*). To determine if there are any alterations in the PSVs of soybeans containing different concentrations of protein, we performed ultrastructural analysis of soybeans with 29 and 37% protein. Electron microscopy of thin sections of mature soybeans with 37% protein revealed that the storage parenchyma cells contained several large PSVs (**Figure 7**). These PSVs were filled completely with proteinacious material. The rest of the cell's volume was primarily occupied by lipid bodies (**Figure 7**). Similar ultrastructural features were seen in dry seeds with 29% protein. However, in some cells, the PSVs were not completely filled with proteincious material (**Figure 7**).

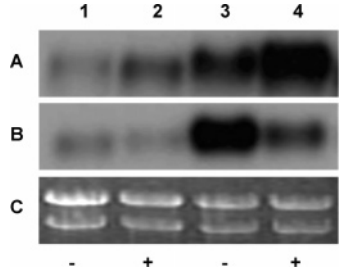


**Figure 6.** Effect of nitrogen on soybean seed protein mRNA accumulation. Total RNA was isolated from soybean seeds at 20 (lanes 1 and 2) and 35 DAF (lanes 3 and 4), resolved on a formaldehyde-agarose gel, and transferred to a nitrocellulose membrane. The blots were hybridized with $^{32}$P-labeled soybean cDNAs encoding the $\beta$-subunit of $\beta$-conglycinin (**A**) and BBI (**B**), washed, and subjected to autoradiography. Panel **C** is an ethidium bromide-stained gel showing uniform loading and integrity of RNA samples. The − and + signs at the bottom of the figure indicates that the plants were grown in the absence or presence of supplemental $NH_4NO_3$.

## DISCUSSION

In this study, we demonstrate an inverse relationship between total soybean seed protein and sulfur amino acid content. It has been established that the nitrogen and sulfur ratio affect the seed protein profile of soybean (*19*, *22*, *23*). The relative availability of nitrogen and sulfur anions to the plant influences the uptake and assimilation of the other (*33*, *34*). Nitrate uptake and transport, from the external medium to the root and from the root to the leaves, have been shown to be inhibited under sulfur limiting conditions (*33*). Storage protein synthesis during seed fill imposes a large demand for nitrogen and sulfur (*35*, *36*). A preponderance of either nutrient shifts the relative accumulation of the major seed storage proteins. A high nitrogen-to-sulfur ratio favors 7*S* $\beta$-conglycinins, which are poor in sulfur amino acids, while a low nitrogen-to-sulfur ratio leads to an increase in accumulation of the 11*S* glycinins (*21*, *22*, *37*). In this study, we demonstrate that nitrogen supplementation not only elevates the accumulation of the $\beta$-subunit of $\beta$-conglycinin but also reduces the accumulation of BBI, a protein rich in the sulfur amino acids.

OAS is a positive regulator of sulfur metabolism (*25*, *38*). The application of OAS upregulates APS reductase gene expression (*39*), elevates sulfate uptake (*40*), and increases the activities of APS reductase and cysteine synthase [*O*-acetylserine(thiol)lyase] (*41*). Kim et al. (*25*) correlated nitrogen/sulfur ratio with the accumulation of OAS and the relative expression of storage proteins. The application of OAS increased the level of sulfur poor $\beta$-subunit of $\beta$ conglycinin and decreased that of glycinin. Additionally, the presence of this metabolite in media elevated the accumulation of mRNA encoding $\beta$-subunit of $\beta$ conglycinin (*25*). In this study, we found that nitrogen decreased the expression of BBI gene resulting in reduced accumulation of this protein. The lack of an adequate amount of cysteine for incorporation into proteins rich in this amino acid such as BBI could result in a reduction in their synthesis as observed in this study. Currently, we do not know how or if OAS specifically controls the transcription of BBI gene.

Even though nitrogen fertilization increased the percentage of total protein, it also lowered the sulfur amino acids content. The percentage of arginine, cysteine, histidine, lysine, methionine, and threonine varied significantly with the total protein content of the seed ($P \leq 0.05$). With the exception of arginine, each of these amino acids showed the highest percentage accumulation in the seed containing the lowest percentage of

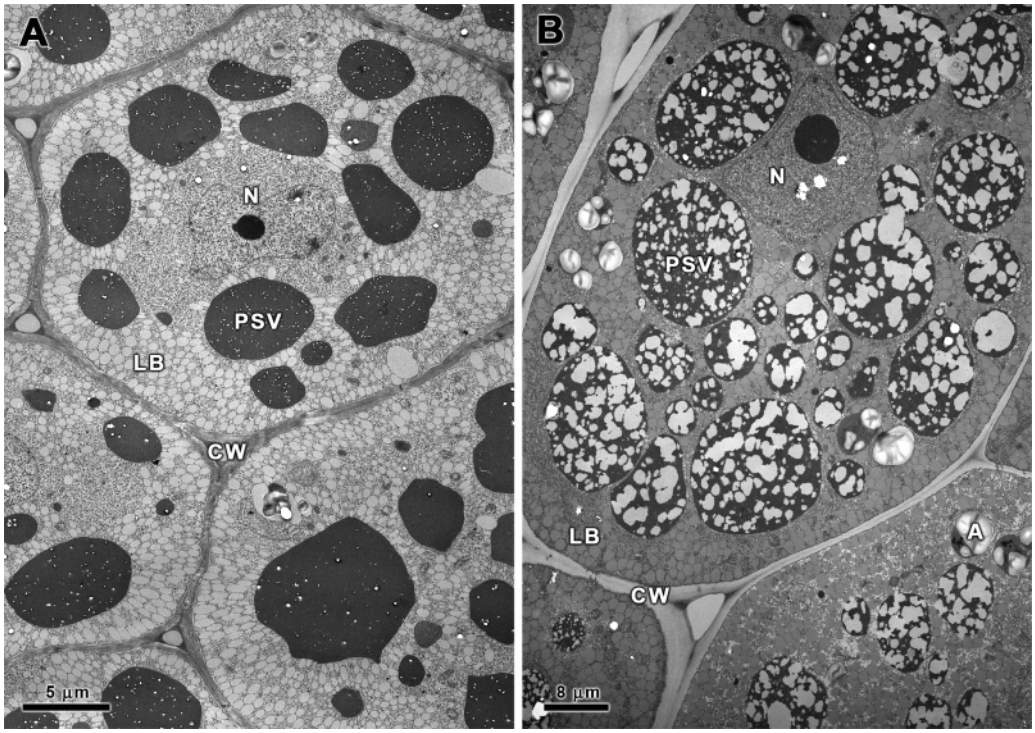


**Figure 7.** Transmission electron micrographs of soybeans differing in protein concentration. Soybean seeds with 37% protein contain several protein storage vacuoles (PSVs) that are completely filled with storage proteins (**A**). Seeds with 29% protein also contained several PSVs; however, in some instances, they were only partially filled with storage proteins (**B**). LB, lipid bodies; N, nucleus; CW, cell wall; and A, amyloplast.

protein. Analysis of variance showed significant effects of nodulation or nonnodulation and nitrogen application with respect to methionine, cysteine, and threonine accumulation ($P \leq 0.05$). No significant interaction between nitrogen and cultivar type on the accumulation of these amino acids was observed. The nonnodulating mutant contained a significantly higher amount of these amino acids than did the wild type ($P \leq 0.05$). The application of nitrogen to the mutants diminished the accumulation of these amino acids ($P \leq 0.05$).

Evidence shows that fixed sulfur in the leaf tissue is not mobilized during the high demand of pod fill nor does the soluble fraction of sulfur in the vacuoles of leaves contribute significantly indicating that sulfur for seed protein involves a net uptake from the growth medium (*35*, *36*). Immediately prior to onset of seed enlargement, a pool of sulfur is seen to accumulate in the pod walls (*35*, *36*). This diminishes rapidly as pod fill begins with a concomitant increase in homoglutathione followed by an increase in this metabolite in the developing seed (*42*). High levels of nitrogen have been found to inhibit the transfer of sulfur from mature to developing leaves (*35*, *36*). Should the same principle apply to movement of sulfur from leaves to seeds, then elevated levels of nitrogen could diminish the sulfur to developing seed. High nitrogen levels thus would favor accumulation of the $\beta$-subunit and diminish the availability of sulfur for the synthesis of the sulfur rich BBI.

To obtain soybean cultivars containing both high protein and elevated sulfur amino acid content, it will be necessary to break the negative correlation with protein content and sulfur amino acids. During the last few decades, the concerted efforts of soybean breeders have enabled them to break the negative correlation between protein and oil content (*43*). A similar approach may be required to overcome the inverse relationship between high protein and sulfur amino acid content. Protease inhibitors, Kunitz and Bowman−Birk proteins comprise approximately 6% of the total seed protein and are the major contributors of sulfur amino acids of soybean (*44*). Accumulation of these proteins is thus dependent upon sulfur assimilation and cysteine availability (*45*). Because BBI represents a small portion of the total seed protein but is rich in the sulfur amino acids, increasing its expression by genetic engineering presents a possible avenue for the improvement of the sulfur amino acid content. Thus far, biotechnological methods to improve the sulfur amino acid content of soybean have met with limited success (*46*−*48*). Interestingly, the accumulation of heterologous sulfur-rich proteins in soybeans appears to occur at the expense of endogenous sulfur-rich proteins, which alludes to a limitation in the sulfur amino acids availability. Clearly, enhancing the sulfur amino acid content of soybean while maintaining agronomic viability may require subtle and multiple adjustments in the regulation and expression of genes involved in sulfur uptake, translocation, and assimilation.

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