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ARTICLE

Purification and Biochemical Characterization of Brazil Nut (*Bertholletia excelsa* L.) Seed Storage Proteins

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Brazil nut storage proteins, 2S albumin, 7S vicilin, and an 11S legumin, were purified using column chromatography. Analytical ultracentrifugation of the purified albumin, vicilin, and legumin proteins, respectively, registered sedimentation coefficients of 1.8, 7.1, and 11.8 S. Under reducing conditions, the major polypeptide bands in 2S albumin were observed at 6.4, 10−11, and 15.2 kDa. The 7S globulin was composed of one 12.6 kDa, two ∼38−42 kDa, and two ∼54−57 kDa polypeptides, whereas the 11S globulin contained two major classes of polypeptides: ∼30−32 and ∼20−21 kDa. The 7S globulin stained positive when reacted with Schiff reagent, indicating that it is a glycoprotein. The estimated molecular mass and Stokes radius for 2S albumin and 7S and 11S globulins were 19.2 kDa and 20.1 Å, 114.8 kDa and 41.1 Å, and 289.4 kDa and 56.6 Å, respectively. Circular dichroism spectroscopic analysis indicated the secondary structure of the three proteins to be mainly β-sheets and turns. Emission fluorescence spectra of the native proteins registered a $\lambda_{\max}$ at 337, 345, and 328 nm for 2S albumin and 7S and 11S globulins, respectively. When probed with anti-Brazil nut seed protein rabbit polyclonal antibodies, 7S globulin exhibited higher immunoreactivity than 2S albumin and 11S globulin.

KEYWORDS: Brazil nut; storage protein; chromatography; immunoreactivity; rabbit polyclonal antibodies; SDS-PAGE; electrophoresis; ultracentrifugation; sedimentation coefficient

INTRODUCTION

Brazil nut (*Bertholletia excelsa* L.; BN) is a tree nut native to South America and is known for its exceptionally high sulfur-containing amino acid albumin protein (*1*) and selenium content (*2*). BNs are the only internationally traded seed crop that is collected exclusively from natural forests and is harvested across the Brazilian, Bolivian, and Peruvian Amazons (*3*, *4*). BNs are an energy-rich food with 66.7% lipid and 13.9% protein content by weight on an as-is basis (*5*). With the exception of 2S albumin, information on BN proteins is limited. On the basis of ultracentrifugal analysis, seed storage proteins have been reported to be composed of 60% 11S legumin (also known as excelsin), 30% 2S albumin, and 10% 7S vicilin proteins (*1*). The 2S albumin, 7S vicilin, and 11S legumin proteins are referred to here as 2S, 7S, and 11S, respectively. Kamiya et al. (*6*) prepared BN 11S crystals and identified their molecular shape to be a double-layer polygon composed of three subunits each with a hole at the center, which is a characteristic of proteins of the cupin superfamily. The sedimentation coefficient of excelsin has been reported to be ∼11.6−11.8 S (*6*, *7*). Ammonium sulfate fractionation has been used for BN 7S isolation (*8*); however, the 7S has not been characterized any further. Both 7S and 11S belong to the cupin superfamily and are typically oligomeric in nature. The BN 11S is a hexamer, wherein each monomeric polypeptide is composed of a ∼32 kDa acidic and a ∼24 kDa basic subunit linked by disulfide bonds (*1*). The BN 7S is a trimer composed of ∼35−45 kDa polypeptides (*1*, *9*). Beyer et al. (*10*) cloned and expressed BN 11S and found it to be a minor allergen. The 7S molecular characteristics, including allergenic properties, remain to be elucidated. The water-soluble BN 2S is composed of two subunits, of 9 and 3 kDa, linked via disulfide bond(s). Because BN 2S albumin is a high methionine protein, the BN 2S gene has been expressed in canola seeds (*11*) and *Phaseolus* bean, variety Carioca (*12*), to improve sulfur amino acid balance of the proteins in seeds of canola and Carioca bean. In developing transgenic seeds with desired traits, one needs to address the issue of potential allergenicity and allergenic stability of the expressed protein. Failure to address this issue may hinder successful development of improved crops as illustrated by the example of BN 2S maintaining its allergenicity in transgenic soybeans (*13*).

Seed storage proteins, 2S, 7S, and 11S, have been extensively reviewed in relation to food allergies (*14*−*16*). BN has been reported to cause IgE-mediated life-threatening allergic reaction in sensitive individuals (*17*, *18*). Prevention of unintended exposure as a consequence of hidden or undeclared allergens

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present in foods is desirable as currently there is no cure for food allergy. However, such avoidance of the offending agent is not always feasible. Lack of methods to detect the offending agent with adequate sensitivity, specificity, and robustness increase the probability of unintended exposure. To this end, we recently developed a rabbit polyclonal antibody based competitive inhibition ELISA for BN detection (IC_{50} = 23.2 9 ng/mL; detection range of 10−90 ng/mL) (*19*).

Among the number of potential allergenic proteins in BN, only two have been shown to react with patient serum IgE: Ber e 1, a 2S albumin (*20*), and Ber e 2, an 11S legumin (*10*). The BN 2S albumin has been purified and biochemically characterized (*20*−*23*). However, many of the potentially allergenic native proteins have been neither isolated from BN seeds nor tested for their allergenicity. Understanding native seed proteins is crucial in improving our understanding of allergies as the sensitive patients are exposed to seed proteins whether native or denatured. Although recombinant proteins are a convenient source of unambiguous and defined proteins, the recombinant form may not necessarily be equivalent to the native counterpart(s) of the target protein(s) (*24*). Therefore, purification and characterization of targeted native proteins are essential for the purpose of establishing the identity and utility of recombinant counterparts. The purpose of this paper is to report findings on the isolation, purification, and biochemical characterization of BN 2S, 7S, and 11S proteins.

MATERIALS AND METHODS

Materials. Shelled BNs were purchased from a local grocery store. Electrophoresis and immunoblotting supplies were from Hoefer Scientific Co. (San Francisco, CA). Protein G Sepharose 4 Fast Flow beads, chromatography columns, and fraction collectors were from Pharmacia, Inc. (Piscataway, NJ). DEAE DE-53 and PVDF membrane were from Whatman, Inc. (Piscataway, NJ), whereas Sephacryl S200 and S300 HR were from GE Healthcare (Piscataway, NJ). Freund's complete and incomplete adjuvants, horseradish peroxidase labeled goat anti-rabbit IgG, Ponceau S, and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO). Whatman 3MM filter paper and nitrocellulose membrane (0.2 μm) were from Schleicher & Schuell Bioscience, Inc. (Keene, NH). X-ray film (BioMax XAR film) was from Eastman Kodak Co. (Rochester, NY). All other chemicals (ACS grade) and protein markers were purchased from Fisher Scientific Co. (Pittsburgh, PA) or Sigma Chemical Co..

Methods. *Preparation of BN Flour*. Defatted BN flour was prepared as described earlier (*19*). Briefly, shelled BNs were ground and defatted for 8 h using a Soxhlet apparatus and petroleum ether (boiling point range of 38.2−54.3 °C) as extraction solvent. After the powder had been spread in a thin layer and dried overnight in a fume hood, the powder was passed through a 40 mesh sieve and stored in screw-capped plastic vials at −20 °C until further use.

Purification of BN Seed Storage Proteins. Defatted Brazil nut flour (2 g) was dispersed in 20 mL of 0.035 M phosphate buffer containing 1 M NaCl, pH 7.5, by continuous mixing for 1 h at room temperature (25 °C) to solubilize flour proteins. The slurry was centrifuged at 27000*g* for 20 min at 4 °C, and the supernatant was loaded on a Sephacryl S200 column (2.6 × 72 cm) previously equilibrated with 0.035 M phosphate buffer, pH 7.5, containing 1 M NaCl. The flow rate of the column was maintained at 24 mL/h, and fractions were collected every 15 min. All protein purification steps were done at 4 °C, and proteins eluted from the columns were monitored by measuring absorbance at 280 nm and electrophoresis of aliquots from the select column fractions.

2S Albumin. The gel filtration peak rich in 2S albumin (tubes 47−55, **Figure 1A**) was pooled and dialyzed against 0.02 M Tris-HCl, pH 8.1, for 48 h with six buffer changes (3 L per change); the dialysate was loaded onto a DEAE DE-53 column (2.6 × 23 cm) equilibrated with 0.02 M Tris-HCl, pH 8.1. The column was flushed with the equilibration buffer until the absorbance at 280 nm returned to baseline. Adsorbed proteins were eluted with a 0−0.5 M NaCl gradient in the equilibration buffer (400 mL each). The column flow rate was 26 mL/h, and fractions were collected every 15 min. The fractions containing 2S were pooled (tubes 89−105, **Figure 1B**), dialyzed against distilled (DI) water for 48 h with six water changes (5 L per change), and lyophilized.

7S Vicilin and 11S Legumin. The gel filtration peak rich in 7S and 11S globulins (tubes 26−40, **Figure 1A**) was pooled, dialyzed against DI water for 48 h with six water changes, and lyophilized. The lyophilized globulin fraction was resuspended in 0.02 M Tris-HCl, pH 8.1, containing 0.1 M NaCl for 1 h and centrifuged at 27000*g* for 20 min to remove insoluble aggregates. The supernatant was loaded onto a DEAE DE-53 column (2.6 × 19 cm) equilibrated with 0.02 M Tris-HCl, pH 8.1, containing 0.1 M NaCl. The column was flushed with the equilibration buffer until the absorbance at 280 nm of the effluent returned to the baseline. The column was subsequently eluted with a 0.1−0.4 M NaCl gradient in the equilibration buffer (250 mL each). The column flow rate was 24 mL/h, and fractions were collected every 15 min. The peaks corresponding to 7S (tubes 12−23, **Figure 1C**) and 11S (tubes 52−66, **Figure 1C**) were pooled separately, dialyzed against DI water for 48 h with six water changes (5 L per change), and lyophilized.

Protein Determination. Soluble protein was determined according to the method of Bradford (*25*). Bovine serum albumin (BSA) in appropriate buffer was used to prepare standard protein curves (0−600 μg/mL) simultaneously.

Electrophoresis. Sodium dodecyl sulfate− polyacrylamide gel electrophoresis (SDS-PAGE) was run as described by Fling and Gregerson (*26*). Appropriate protein samples (typically 10−30 μg of protein) were loaded either on 8−25% gradient or 12% monomer acrylamide separating gel and 4% monomer acrylamide stacking gel. The gel was run, typically at 10 mA/gel, until the tracking dye migrated to the gel edge (typically 20 h) and used either for Coomassie Brilliant Blue R (CBBR) staining or to transfer onto a nitrocellulose membrane. To detect the presence of glycoprotein, gels were stained with periodic acid−Schiff (PAS) stain using the Gelcode Glycoprotein staining (Pierce Chemical Co., Rockford, IL) procedure per the manufacturer's instructions.

2D Gel Electrophoresis (NDND + SDS). Nondenaturing nondissociating (NDND) gel electrophoresis was run according to the method of Andrews (*27*) as described by Sathe (*28*). Briefly, 3−30% linear acrylamide gradient gels of 0.75 mm thickness (acrylamide/bis ratio of 37:1) with 90 mM Tris, 80 mM boric acid, and 2.5 mM Na-EDTA, pH 8.5, and 3% acrylamide stacking gels were used. Running buffer for NDND-PAGE was 90 mM Tris, 80 mM boric acid, and 2.5 mM Na-EDTA, pH 8.4. Samples were mixed with a suitable volume of NDND-PAGE buffer (2 volumes of 0.45 M Tris, 0.4 M boric acid, 12.5 mM Na-EDTA mixed with 1 volume of glycerol) containing 0.001% bromophenol blue as the tracking dye, and 30 μg of protein was loaded on the gel. Gels were typically run at a constant current (10 mA/gel) with tap water cooling. The gels were stained with Coomassie Brilliant Blue R and appropriate lanes were excised for 2D analysis. The excised lanes were soaked in SDS-PAGE sample buffer (0.05 M Tris-HCl, pH 6.8; 0.1% SDS; 0.01% bromophenol blue; 30% glycerol) containing 2% β-mercaptoethanol and heat-denatured in a microwave oven (Kenmore, model 565.68301790, Sears, Hoffman Estates, IL) for 30 s at 1000 W. The strips were then cooled to room temperature, turned 90° counterclockwise, laid on top of the 4% stacking gel with a 8−25% linear monomer acrylamide gradient SDS-PAGE separating gel, and electrophoresed as described under Electrophoresis.

Immunoblotting. Protein samples electrophoresed on SDS-PAGE under reducing condition were transferred onto 0.2 μm nitrocellulose membranes as described by Towbin et al. (*29*). The transferred polypeptides were detected by brief staining, 5 min, with Ponceau S stain and then probed with rabbit anti-BN IgG for Western blotting as described by Sharma et al. (*19*).

N-Terminal Amino Acid Sequencing. SDS-PAGE-separated proteins were transferred to a 0.2 μm PVDF membrane. The N-terminal amino acid sequences of the blotted proteins were determined using an ABI 492 Procise CLC protein sequencer (Applied Biosystems, Inc., Foster City, CA). The sequences were analyzed with BLAST programming (National Center for Biotechonology Information, National Institutes of Health, Bethesda, MD; http://www.ncbi.nlm.nih.gov/BLAST/).

Analytical Ultracentrifugation. Analytical ultracentrifugation experiments were performed in a Beckman XL-I centrifuge (Beckman Coulter,

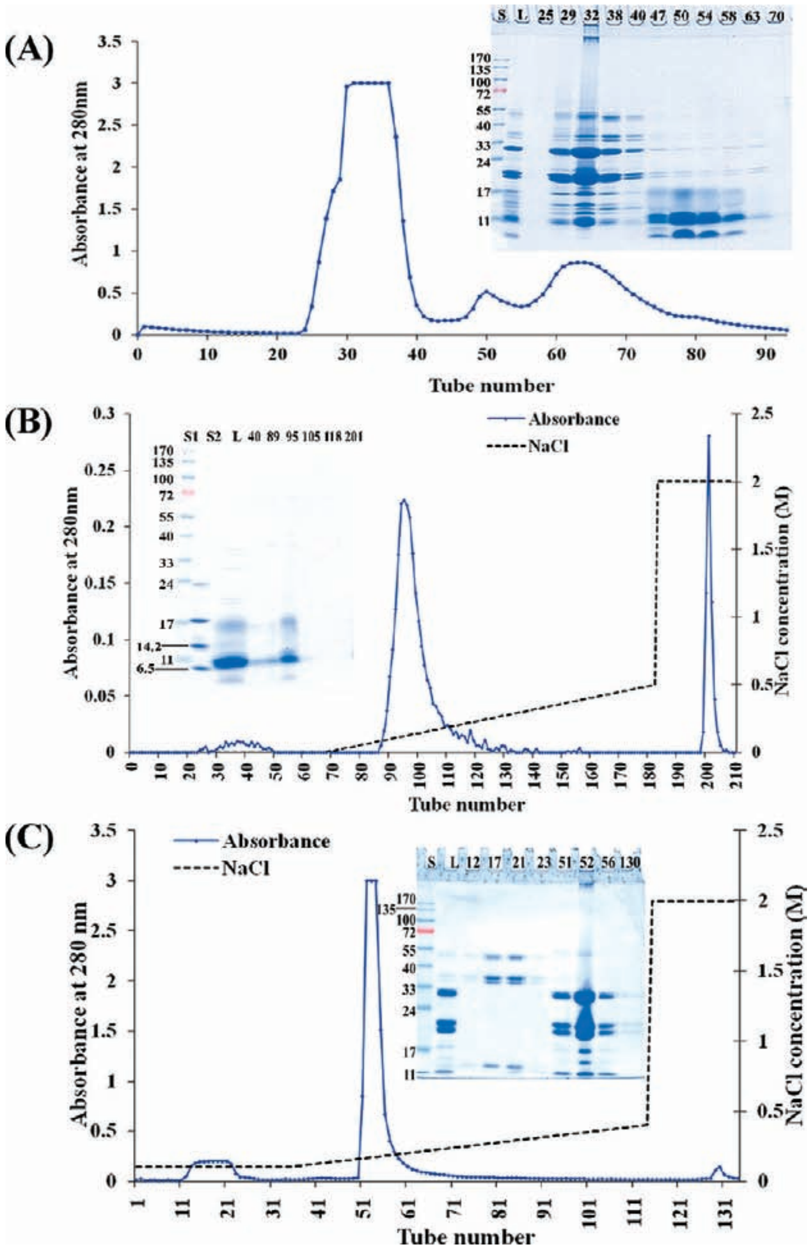


Figure 1. (**A**) Elution profile of Brazil nut extract (L) off a Sephacryl S200 column. Fractions containing globulins (tubes 26−40, 88 mL) and 2S albumin (tubes 47−55, 53 mL) were pooled separately. (**B**) Elution profile of 2S albumin obtained from (**A**) off a DEAE DE-53 anion exchange column. Tubes 89−105 (136 mL) were pooled to yield 2S albumin. (**C**) Elution profile of globulins obtained from (**A**) off a DEAE DE-53 anion exchange column. Tubes 12−23 (71 mL) and 52−66 (90 mL) were pooled to yield 7S vicilin and 11S legumin, respectively. (Insets) SDS-PAGE (reducing condition) analysis of fractions eluted off the corresponding column indicated by number on top of the gel lane. L, protein loaded on to the column; S, S1, S2, protein markers. The molecular mass of each standard is indicated in the left margin of each inset.

Inc., Fullerton, CA) using absorbance optics by measuring intensity scans at 280 nm. The experiments were performed at 20 °C in two-channel Epon centerpieces with an AN60 Ti rotor at 55000, 40000, and 30000 rpm for the 2S, 7S, and 11S proteins, respectively. BSB was the solvent used for protein solubilization. Data were analyzed using the UltraScan II version 9.9 software suite (*30*). Data were first analyzed with the two-dimensional spectrum analysis (*31*) with simultaneous time invariant noise subtraction according to the method of Schuck and Demeler (*32*). After noise subtraction, the data were examined for heterogeneity with the enhanced van Holde−Weischet analysis (*33*). The partial specific volumes at 20 °C of 2S (0.714 cm^3/g), 7S (0.717 cm^3/g), and 11S (0.720 cm^3/g) were estimated from their peptide sequence as described by Durchschlag (*34*). Because the BN 7S peptide sequence is not known, and the fact that sesame 11S exhibited highest similarity with the BN 11S sequence, we selected the sesame 7S sequence (accession no. AAK15089) to estimate the partial specific volume for BN 7S. All computations were performed on the TIGRE cluster at the University of Texas Health Science Center at San Antonio and the Texas Advanced Computing Center at the University of Texas in Austin.

Molecular Mass and Stokes Radius. A calibrated Sephacryl S300 HR column (1.6 × 76.5 cm) equilibrated with 0.02 M Tris-HCl, pH 8.1, containing 0.1 M NaCl was used to estimate the molecular mass and Stokes radius of BN proteins. Fractions were collected every 15 min, and protein elution was monitored by UV absorbance at 280 nm. The column was calibrated using high and low molecular weight standard protein kits (Amersham Biosciences, Piscataway, NJ), and the Stokes radius was calculated as per the manufacturer's instruction. Each standard protein was eluted at least twice to calibrate the column. Each BN protein sample was run at least in duplicate at column flow rate maintained at 14 mL/h. The Stokes radii were confirmed by using the equation $f/f_o = r/[3\nu M/4\pi N]^{1/3}$, where ν = partial specific volume (0.75 cm^3/g), M = molecular mass, and N = Avogadro's number (6.023×10^{23}) (*35*, *36*). The accessible

Table 1. Summary of BN Protein Purification[a]

purification step	total protein (mg)
extract loaded on S200 column	480
globulins off S200 (peak 1)	360
2S albumin off S200 (peak 2)	51
2S albumin off DEAE DE-53	46
globulins loaded on DEAE DE-53	270
7S vicilin off DEAE DE-53 (peak 1)	20[b]
11S legumin off DEAE DE-53 (peak 2)	182[b]

[a] Data are for typical preparation starting with 2 g of defatted BN flour extracted with 20 mL of 0.035 M phosphate buffer containing 1 M NaCl, pH 7.5. [b] Total protein based on the weight of lyophilized proteins.

surface areas (A_s) for these proteins were calculated using the equation $A_s = 5.3M^{0.76}$ (*37*).

Ultraviolet Spectrum. Ultraviolet spectra were determined using an Ultrospec 2100 pro spectrophotometer (GE Healthcare). BN proteins were dissolved in 6 M guanidine hydrochloride for 1 h at room temperature and centrifuged at 16000*g* for 15 min to remove insoluble aggregates. The protein concentration of the supernatant was standardized to 2 mg/mL prior to scanning. Scans were obtained over the 240−350 nm wavelength range at 0.5 nm increments. Protein concentrations of 0.5, 0.75, 1, and 2 mg/mL were read at 280 nm, and their average value was used to calculate $A^{1\%}_{280\ \mathrm{nm}}$.

Circular Dichroism (CD) Spectrum. Optically clear BN protein solutions in 20 mM phosphate buffer (0.25 mg/mL) were used to record CD spectra (195−260 nm) in a 1 mm quartz cuvette (Fisher Scientific, Atlanta, GA) with an AVIV CD spectrometer (Aviv Biomedical, Lakewood, NJ). Three spectra of each sample were averaged and used for analysis. The molar ellipticity per amino acid residue was calculated from raw data after correction for buffer by using the formula $[\theta] = (\theta \times 100 \times \mathrm{MW})/(c \times l \times n_A)$, where $[\theta]$ = mean residue ellipticity, θ = experimental ellipticity in millidegrees, MW = molecular weight of the protein in kDa, c = protein concentration in mg/mL, l = cuvette path length in cm, and n_A = number of amino acids in the protein. Secondary structure was interpreted by visual assessment of the spectra and using the computer program CDPro (http://lamar.colostate.edu/~sreeram/CDPro/main.html).

Fluorescence Spectrum. Protein solutions (200 μg/mL for 2S; 50 μg/mL for 7S and 11S) prepared in 20 mM sodium phosphate, pH 7.5, were used to obtain fluorescence spectra. The proteins were excited at 280 nm, and emission wavelength spectra were from 300 to 450 nm at constant temperature (25 °C) in Varian Cary Eclipse Fluorometer (Varian, Inc., Walnut Creek, CA). Excitation and emission slits were set at 5 nm each. The protein solution was incubated overnight at room temperature with 6 M urea to study the shift in fluorescent spectra. Fluorescent spectra for appropriate blanks were run simultaneously. All blank spectra registered a fluorescence intensity of < 10, indicative of no interference in protein fluorescence spectra.

RESULTS AND DISCUSSION

BN Storage Protein Purification. Typical gel filtration and anion exchange column profiles for purification of 2S, 7S, and 11S are shown in **Figure 1**. Defatted BN flour protein extract prepared in 0.035 M phosphate buffer containing 1 M NaCl, pH 7.5, was resolved in three major peaks by Sephacryl S200 gel filtration (**Figure 1A**). The fraction rich in low molecular weight polypeptides (tubes 47−55 in **Figure 1A**) resulted in a single peak when further purified by the DEAE DE-53 anion exchange column (**Figure 1B**). This fraction was 2S albumin, and it typically eluted off the DEAE DE-53 column at a NaCl concentration range of 70−180 mM. The first peak obtained from the S200 column (tubes 26−40 in **Figure 1A**) was passed through a DEAE DE-53 column equilibrated at 100 mM NaCl concentration in the equilibrium buffer, and the proteins were eluted using a NaCl linear gradient (100−400 mM). The 7S eluted off the column before the start of the NaCl gradient (i.e., ≤100 mM NaCl concentration), whereas the 11S eluted off the column at 150−190 mM NaCl concentration. Typically, we obtained ~51, 20, and 182 mg of 2S, 7S, and 11S proteins, respectively, from 2 g of defatted BN flour (**Table 1**). Sun et al. (*1*) reported the ratio of 2S/7S/11S to be 30:10:60 when phosphate buffer soluble BN proteins were fractionated by sucrose gradient centrifugation. Our column chromatography fractionations suggest the proportion of 2S/7S/11S to be ~19:8:73, which is comparable with the ultracentrifugal fractionation reported by Sun et al. (*1*).

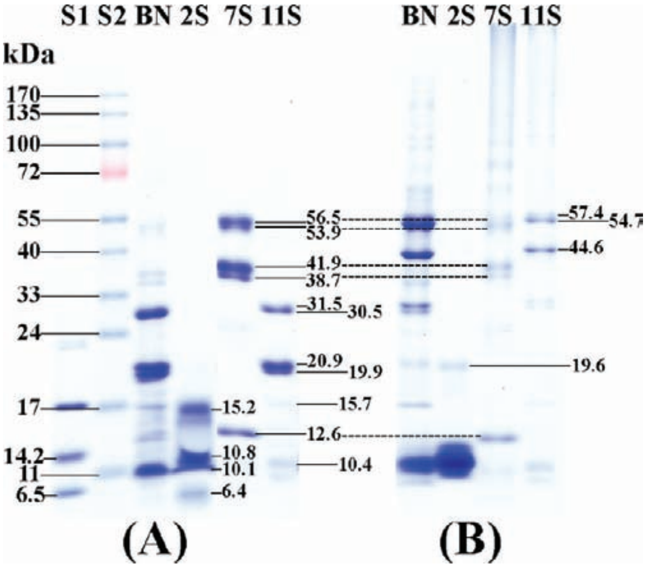


Figure 2. SDS-PAGE of BN proteins in the presence (**A**) and absence (**B**) of reducing agent. Protein load for BN extract was 30 μg, whereas that for purified proteins (2S, 7S, and 11S) was 20 μg. S1 and S2 are protein markers. The equation used for calculating molecular mass was $y = -1.8651x + 2.376$ ($R^2 = 0.979$).

Electrophoresis of BN Proteins. *SDS-PAGE.* The BN 2S, 7S, and 11S were electrophoresed by SDS-PAGE in the presence (**A**) and absence (**B**) of a reducing agent (β-mercaptoethanol) as shown in **Figure 2**. In the absence of reducing agent, 2S was characterized by several bands in the region of ~8−12 kDa and by a minor band at 19.6 kDa. When the 2S was reduced, major bands appeared at 6.4, 10−11, and 15.2 kDa. Under reducing as well as nonreducing conditions major bands in 7S appeared at three different regions: one at 12.6 kDa, two at ~38−42 kDa, and two at ~54−57 kDa. The 11S consisted of two distinct regions of polypeptides under nonreducing conditions: ~44.6 and 54−57 kDa. SDS-PAGE in the presence of reducing agent revealed BN-11S to be composed of two major classes of polypeptides: a 30−32 kDa (acidic) subunit and a 20−21 kDa (basic) subunit. Some minor bands at ~10 kDa were visible in 11S under both reducing and nonreducing conditions. The polypeptide composition and the estimated molecular masses of 2S, 7S, and 11S polypeptides are consistent with earlier findings on BN proteins (*1*, *9*, *21*).

2D PAGE. To determine which polypeptides may be involved in disulfide bond formation, 2D SDS-PAGE was used in which BN proteins were electrophoresed in the first dimension without reduction followed by reduction, heat denaturation, and SDS-PAGE in the second dimension (**Figure 3**). The 19.6 kDa minor band observed in 2S under nonreducing condition, upon reduction, migrated as a single polypeptide at ~11 kDa (marked by the circle in the 2S panel of **Figure 3**), indicating 19.6 kDa 2S may be a dimer.

The 7S exhibited a ~13 kDa band and multiple bands in the range of 35−200 kDa under nonreducing condition. Under reducing condition, 7S revealed several polypeptides in three regions as described earlier. The high molecular mass polypeptides

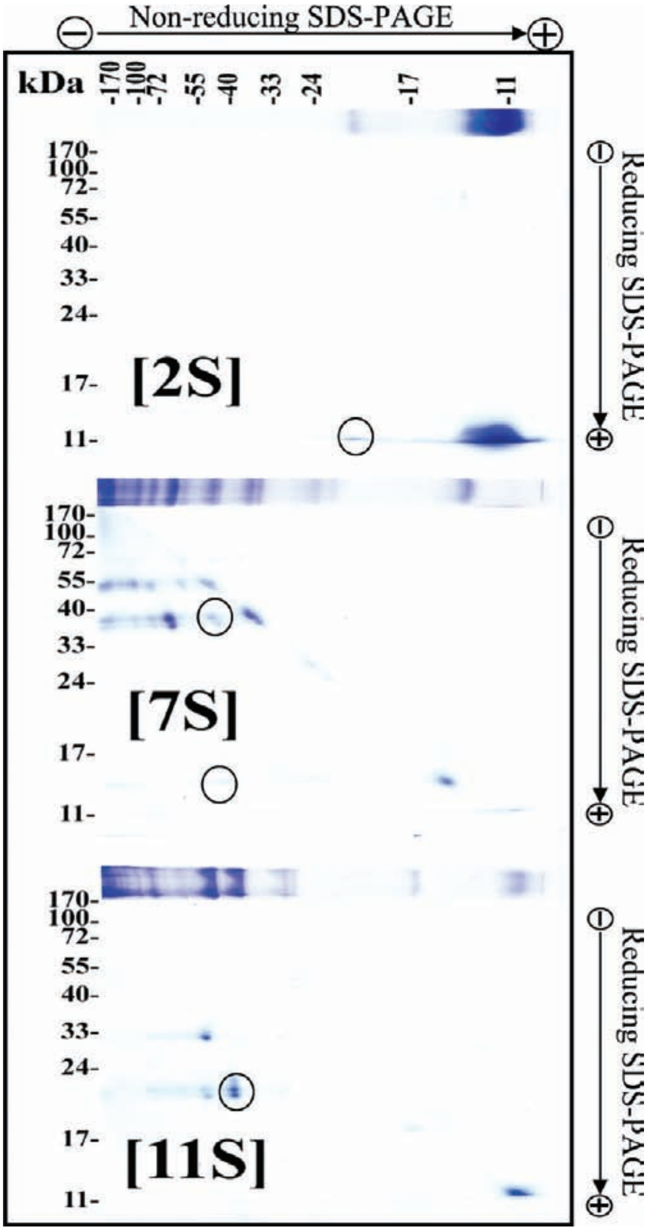


Figure 3. Two-dimensional (nonreducing + reducing) PAGE of BN proteins. SDS-PAGE without reducing agent was used in the first dimension and SDS-PAGE with reducing agent in the second dimension. Protein load in the first dimension was 30 μg.

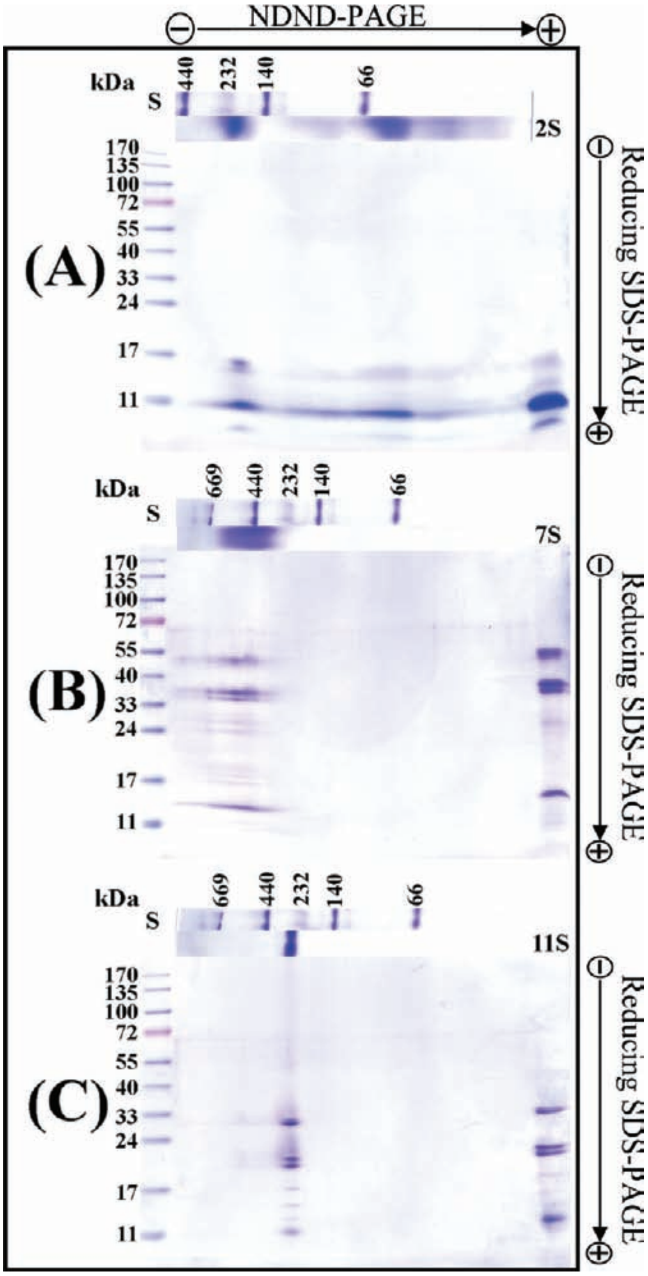


Figure 4. Two-dimensional (NDND + SDS) PAGE of BN proteins: (**A**) 2S albumin; (**B**) 7S vicilin; (**C**) 11S legumin. NDND-PAGE was used in the first dimension and SDS-PAGE in the second dimension. Protein load in the first dimension was 30 μg. S, 2S, 7S, and 11S are protein standard, 2S albumin (10 μg), 7S vicilin (10 μg), and 11S legumin (10 μg), respectively, loaded directly on the SDS-PAGE.

under nonreducing condition could be a result of either aggregate formation or oligomeric nature of 7S linked by disulfide bonds. The appearance of 38−42 and 13 kDa bands upon reduction of 7S 55 kDa polypeptide (marked by the circle in the 7S panel of **Figure 3**) suggests that the 7S 55 kDa band is composed of 38−42 and 13 kDa polypeptides. To date, there appear to be no reports in the literature indicating the presence of disulfide-linked subunits constituting 7S globulins. Whether the occurrence of disulfide-linked 7S globulin is unique to BN remains to be determined.

The 11S 55 kDa band observed in the first dimension under nonreducing conditions was composed of two polypeptides with estimated molecular masses of 20−22 and 30−32 kDa. Interestingly, the 45 kDa band observed in the first dimension was composed of only 20−22 kDa subunits (marked by the circle in the 11S panel of **Figure 3**), suggesting the disulfide-linked dimerization of the polypeptide. Whether such an occurrence of a 45 kDa polypeptide is unique to BN storage proteins remains to be determined.

The charge heterogeneity of undenatured BN storage proteins was investigated using NDND-PAGE in the first dimension followed by SDS-PAGE in the second dimension (**Figure 4**). Note the molecular mass standards used in NDND-PAGE are not true indicators of the molecular mass, as the proteins are separated on the basis of their net electrical charge, which may not necessarily be proportional to the mass of the protein. The 2S exhibited multiple bands in NDND-PAGE (at least six bands), indicating the presence of several charged species (isoforms). The second dimension run of 2S indicated that these isoforms have similar molecular masses, indicating the importance of the electrical charge in the generation of the isoforms. These observations are consistent with the findings of Moreno et al. (*21*), who demonstrated the presence of at least seven isoforms of 2S of varying pI values. The 7S appeared as a broad band on NDND-PAGE that, upon reduction, denaturation, and SDS-PAGE, separated into several subunit polypeptides in the range of 13−56 kDa, a behavior similar to that of vicilin in amaranth (*38*), pea (*39*), and sesame (*40*). The 11S appeared as a single band on NDND-PAGE, dissociating into its respective subunits when subjected to SDS-PAGE.

Glycoprotein Staining. Glycosylation of protein may be important for IgE binding and therefore facilitating allergic reactions (*41*). The BN proteins were separated by SDS-PAGE and stained for the presence of sugar moieties. As can be seen from **Figure 5**, 7S is a glycoprotein, whereas 2S and 11S are not. The type and number of sugar residues, although not a part of the current investigation, need to be determined.

N-Terminal Sequencing and Identification of BN Peptides. The electrophoresed BN proteins were transferred onto PVDF membrane, and select polypeptides were subjected to N-terminal amino acid sequencing (**Figure 2**; **Table 2**). Three polypeptides (15.2, 10.8, and 10.1 kDa) exhibited 100% identity with the large subunit of BN 2S albumin. On the basis of the amino acid sequence, the theoretical molecular mass of the 2S large subunit is 9 kDa, which is similar to the lower two bands (**Figure 2**). The identity of the 15.2 kDa 2S polypeptide remains to be ascertained. Attempts to sequence the 7S polypeptides were not successful. The theoretical molecular masses of BN acidic and basic subunits, based on the cDNA derived amino acid sequence, are 29.5 and 20.5 kDa, respectively. One each of the acidic (31.5 kDa) and basic (20.9 kDa) subunits of 11S in the current investigation had 100% identity with the known cDNA derived BN 11S sequence (accession no. AAO38859) residues 21−29 and 280−291, respectively. This suggests the first 20 amino acids of the BN 11S proprotein are cleaved as a part of signal peptide during post-translational modification. Signal peptide prediction software, SignalP, available at ExPASy Proteomics Server (http://ca.expasy.org/), also predicts the cleavage of the first 20 amino acids to yield a mature protein. The 15.7 kDa polypeptide in BN 11S exhibited high homology with almond 11S residues 140−151 and sesame 11S residues 140−155 (**Table 2**). However, this polypeptide amino acid sequence did not exhibit identity with the known sequence of BN 11S, pointing toward the possible presence of BN 11S isoforms.

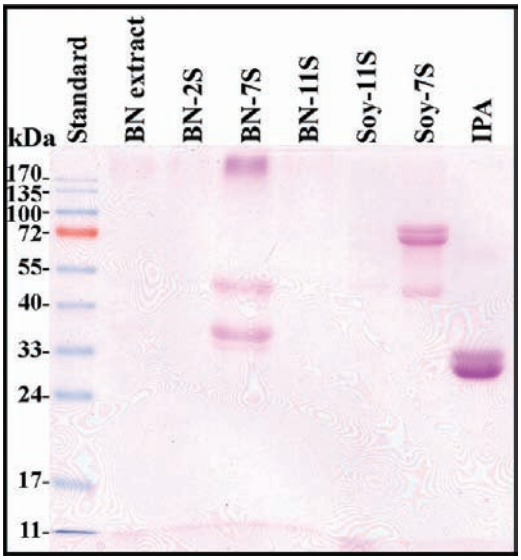


Figure 5. Glycoprotein stain of BN protein. Soy 11S was used as a negative control, and soy 7S and IPA (Inca peanut albumin) were used as positive controls. Protein loaded in each lane was 30 μg.

Hydrodynamic Properties. *Ultracentrifuge Analysis*. Distribution plots after analysis of the boundaries according to the method of Demeler and van Holde (*33*) and their associated histogram envelopes are shown in **Figure 6**. The S values for BN 2S, 7S, and 11S were 1.8, 7.1 and 11.8 S, respectively (**Figure 6**). The BN-7S and -11S samples exhibit a homogeneous composition with $>80\%$ of the sample comprising the 7S and 11S fractions, respectively. The BN 2S sample, however, revealed heterogeneity with sedimentation coefficients ranging from 1 to 2.3 S. The minor ∼3S (∼10%) component observed in the 11S ultracentrifuge analysis could represent a monomeric form of the 11S hexamer. Schwenke et al. (*42*) reported the succinylated subunit of pea legumin to have a sedimentation coefficient of 3.2 S. Analysis at different concentrations (∼0.3, 0.5, and 0.7 sample optical density) revealed no concentration-dependent shift in S values. The frictional ratios (f/f_o) of the 2S, 7S, and 11S proteins were ∼1−1.2, 1.25, and 1.3, respectively, suggesting molecular symmetry.

Stokes Radius and Molecular Mass. The purified BN proteins were passed through the calibrated Sephacryl S300 HR gel filtration column to determine their apparent molecular mass and Stokes radius. The standard curves (**Figure 7**) obtained using proteins of known molecular mass and Stokes radii were used for the calculations. All three proteins (2S, 7S, and 11S) eluted off the

Table 2. N-Terminal Amino Acid Sequences of BN Polypeptides Obtained from SDS-PAGE Separated Proteins (**Figure 2**)

band (kDa)	N-terminal	similar to	% identity	% similarity
15.2	PRRGMEPHM	BN 2S albumin (P04403; ACI70207; ACI70206; BBA96554)	100	100
10.8	PRRGMEPHMSE	BN 2S albumin (P04403; ACI70207; ACI70206; BBA96554)	100	100
10.1	(M)PRRGMEPHMSE	BN 2S albumin (P04403; ACI70207; ACI70206; BBA96554)	100	100
15.7	TLRRQDRHQKLRQIRQ	almond 11S globulin (CAA55010)	75	91
		sesame 11S globulin (AAK15087)	68	81
		Ficus pumila 11S globulin (ABK80751; ABK80752)	64	78
20.9	GLEETICSATFI	BN 11S globulin (AAO38859)	100	100
		Chenopodium quinoa 11S globulin (ABI94735; ABI94736; AAS67037; AAS67036)	100	100
		Vicia sativa legumin (CAA83674)	100	100
		Pisum sativum legumin (CAA47809)	100	100
31.5	IEYEQEELY	BN 11S globulin (AAO38859)	100	100

column as single major peaks. On the basis of their elution volume (V_e, mL), the estimated molecular masses (M) of the proteins were 2S, 19.2 ± 3.5 kDa; 7S, 114 ± 20.6 kDa; and 11S, 289 ± 18.6 kDa. It should be noted that the gel filtration column separates proteins on the basis of their hydrodynamic radii. Assuming 7S is a trimer and 11S is a hexamer (*43*), the molecular masses of the monomeric 7S and 11S would be ~38 and 48 kDa, respectively. The Stokes radii (r) of 2S, 7S, and 11S are estimated to be 20.1 ± 2.0, 41.1 ± 2.5, and 56.6 ± 1.4 Å, respectively. The Stokes radii obtained using the equation $f/f_o = r/[3\nu M/4\pi N]^{1/3}$ for 2S (19.7 Å), 7S (40.5 Å), and 11S (57.4 Å) and f/f_o values from ultracentrifuge runs were similar to those obtained using calibrated gel filtration columns. On the basis of the molecular masses of BN proteins obtained from the calibrated gel filtration column, the oligomer accessible surface areas of 2S, 7S, and 11S were 9540.8, 36942.3, and 74913.2 Å^2, respectively.

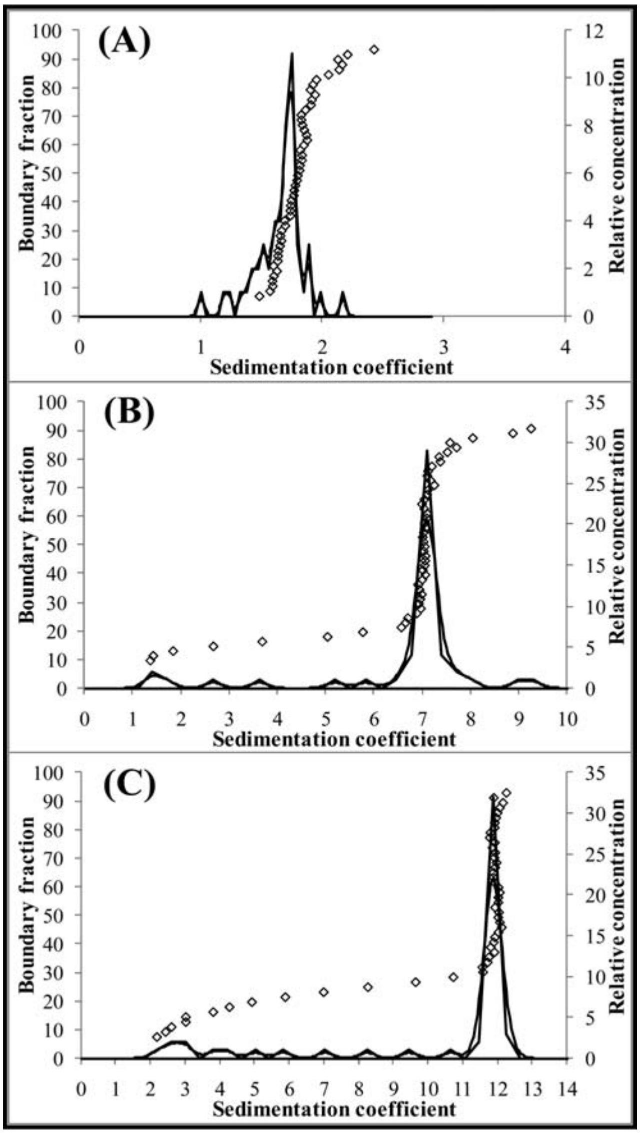


Figure 6. van Holde−Weischet analysis of BN 2S (**A**), 7S (**B**), and 11S (**C**). Integral distributions are represented by the open squares, and the percent boundary fraction is represented on the left *Y*-axis. The solid line represents the relative concentration with values on the right *Y*-axis.

Spectroscopy ($A^{1\%}_{280\ nm}$, CD, Fluorescence). The ultraviolet spectrum of BN proteins (2 mg/mL) is shown in **Figure 8**. The highest absorbance at 280 nm was observed in 11S, whereas 2S exhibited the lowest absorbance. The absorbance values of 1% solutions at 280 nm ($A^{1\%}_{280\ nm}$) were 2S, 1.486; 7S, 3.149; and 11S, 9.023 (**Table 3**). The data suggest 11S has the highest amount of tryptophan residues.

The secondary structure of the three proteins was determined using far-UV CD spectroscopy. The characteristic maximal negative mean residual ellipticities [θ] for 2S, 7S, and 11S were observed at 221, 213, and 210 nm, respectively, whereas there was a common crossover point (201−202 nm) for the three proteins

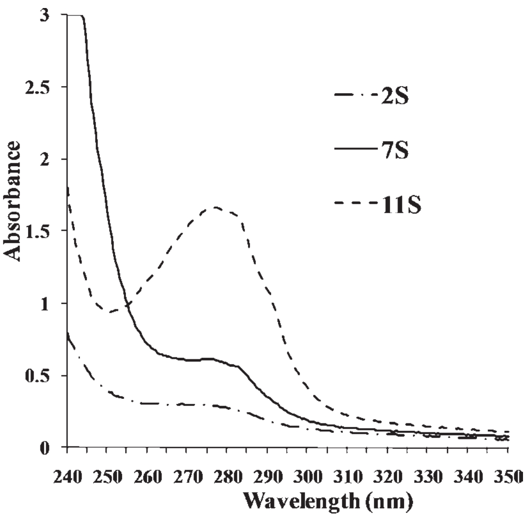
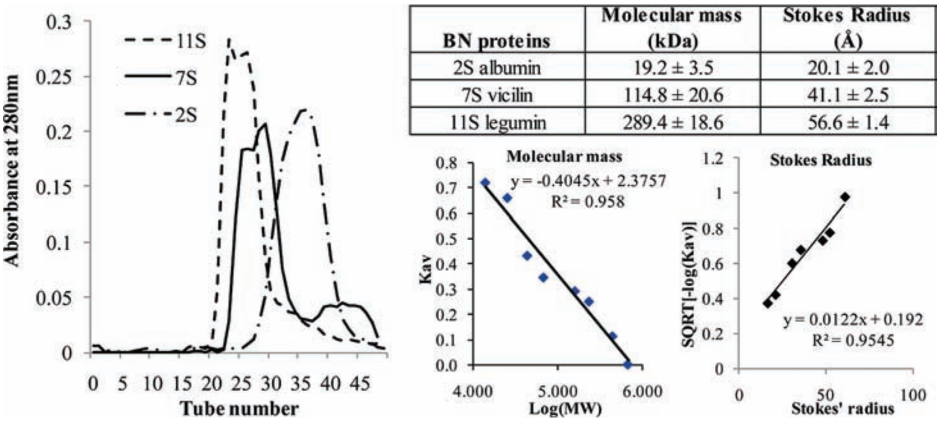


Figure 8. UV spectra of BN proteins (2 mg/mL).



BN proteins	Molecular mass (kDa)	Stokes Radius (Å)
2S albumin	19.2 ± 3.5	20.1 ± 2.0
7S vicilin	114.8 ± 20.6	41.1 ± 2.5
11S legumin	289.4 ± 18.6	56.6 ± 1.4

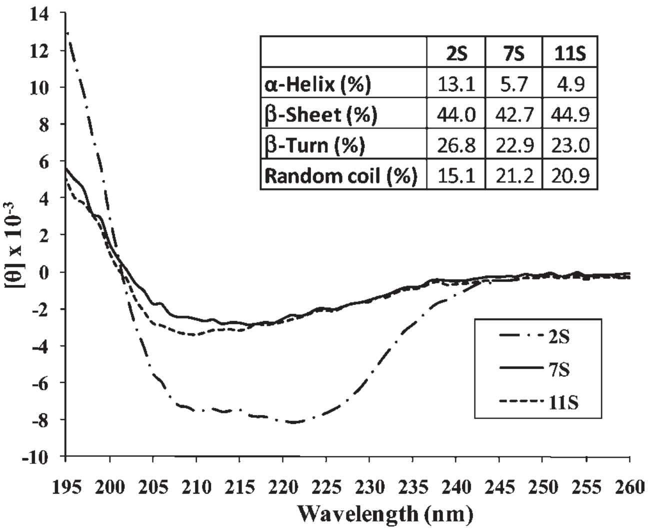
Figure 7. Molecular masses and Stokes radii of BN proteins ($n = 2$): elution profiles of BN proteins off calibrated Sephacryl S300 HR columns (left of the table) and calibration curves used for calculation of molecular masses and Stokes radii (below the table).

(**Figure 9**). Similar secondary structure profiles have been reported for soy 7S and 11S (*44*) and BN 2S (*21*). The secondary structure was calculated using three different programs (Selcon3, Continll, Cdsstr), and their averages are reported in **Figure 9**. Continll yielded higher β sheets and β turns and lower α helices and random coils compared to the other two programs for BN 2S. All three proteins were primarily composed of β sheets with small amounts of α helices. The β turns and random coils were equally distributed in 7S and 11S, whereas slightly higher β turns were observed in 2S. Sze et al. (*44*) have shown soy globulins to contain higher β sheets, followed by random coils, β turns, and α helices.

Table 3. $A^{1\%}_{280\ \mathrm{nm}}$ of BN Proteins (n = 4)

	$A^{1\%}_{280\ \mathrm{nm}}$	
BN protein	experimental	theoretical[a]
2S	1.49 ± 0.09	1.76[b]
7S	3.15 ± 0.39	na[c]
11S	9.02 ± 1.04	8.20[b]

[a] Based on the ProtParam software available at ExPASy server (http://ca.expasy.org) and assuming no Cys residues appear as half-cystines. [b] Protein accession numbers used for 2S and 11S were ACI70207 and AAO38859, respectively. [c] na, not available.



	2S	7S	11S
α-Helix (%)	13.1	5.7	4.9
β-Sheet (%)	44.0	42.7	44.9
β-Turn (%)	26.8	22.9	23.0
Random coil (%)	15.1	21.2	20.9

Figure 9. CD spectra of BN proteins. On the basis of the molecular weight and number of amino acids of 7S proteins, those of BN 7S were assumed to be 60 kDa and 520 amino acids.

Moreno et al. (*21*) showed BN 2S isoforms to be primarily composed of α helices (31−47%). The larger extreme at 222 nm compared to that at 208 nm in the 2S CD spectrum indicates higher concentration of β strands. The helical content of proteins can be estimated by the equation given by Chen et al. (*45*): $f_H = -([\theta]_{222} + 2340)/30300$, where f_H = fraction of helix and $[\theta]_{222}$ = mean residual ellipticity at 222 nm. On the basis of this formula, helical content of BN 2S was estimated to be 19%, which was comparable to the experimental value of 13%. The CD data suggest the BN 2S has more ordered structure than either 7S or 11S.

Fluorescence emission spectra of BN proteins were recorded by exciting the intrinsic chromophore (tryptophan) at 280 nm (**Figure 10**). The emission λ_{max} values for undenatured 2S, 7S, and 11S in 20 mM phosphate buffer were 337, 345, and 328 nm, respectively. Upon 6 M urea denaturation, a red shift was observed in all three proteins. The λ_{max} values of denatured proteins were 2S, 351 nm; 7S, 350 nm; and 11S, 353 nm. The highest red shift was observed in 11S (25 nm), suggesting a significant gain in tryptophan solvent accessibility upon denaturation. Sze et al. (*44*) reported a higher red shift in 11S (λ_{max} shifted from 344 to 353 nm) compared to 7S (λ_{max} shifted from 344 to 350 nm) when soybean globulins were exposed to 6 M urea (2 h) at room temperature. Arntfield et al. (*46*) similarly found a

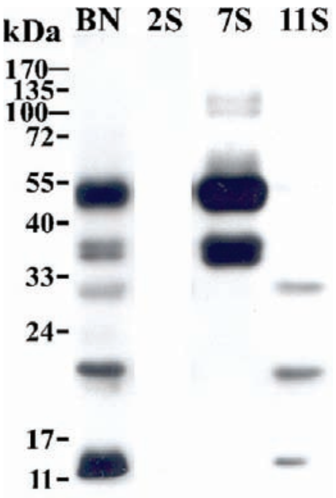


Figure 11. Western blot of BN proteins using rabbit anti-BN polyclonal antibodies. Protein load was 20 μg of BN extract and 10 μg of purified proteins.

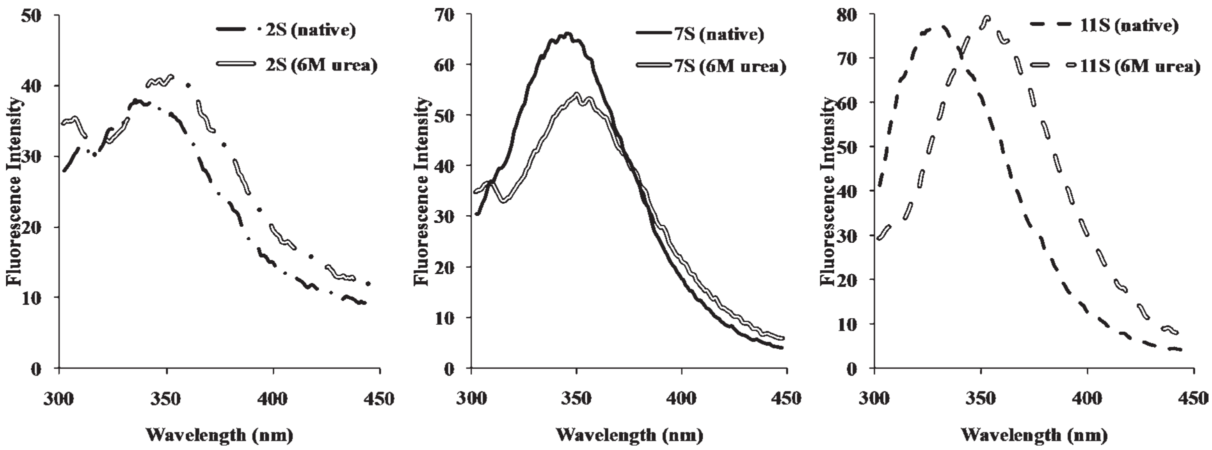


Figure 10. Fluorescence spectra of BN proteins under native and denatured condition.

6 nm red shift (from 347 to 353 nm) for fava bean vicilin subjected to 3 M urea denaturation.

Immunoreactivity of BN Proteins. With the exception of BN 2S, much remains unknown about the BN storage proteins' immunoreactivity. Immunoreactivity of purified proteins using rabbit anti-BN sera in a Western blot format reveals the following rank order: 2S $<$ 11S $<$ 7S (**Figure 11**). The reactive polypeptides in 7S were 38 and 54 kDa, whereas the lower band (∼13 kDa) did not exhibit recordable reactivity. The acidic and basic subunits of 11S and an ∼11 kDa band were reactive when probed with rabbit polyclonal antibodies (pAbs). 2S did not exhibit reactivity when probed with rabbit pAbs, an observation consistent with our previous finding (*19*). Although the 7S globular proteins have been reported to be major allergens in almond (*47*), cashew (*48*), pistachio (*49*), and walnut (*50*), BN 7S remains to be identified as an allergen. Identification of the gene(s) encoding BN 7S will be helpful in determining the amino acid sequence of the protein. A recombinant 7S with known amino acid sequence, along with its native counterpart, can be used for investigating immunochemical and biochemical properties of BN 7S. Ongoing investigations in our laboratories indicate that polypeptides constituting native BN 7S (molecular mass ranges of 38−42 and 53−57 kDa) are reactive with BN-allergic patients' serum IgE. Additional experiments are underway to fully define the BN 7S immunoreactivity.

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