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A Sensitive and Robust Competitive Enzyme-Linked Immunosorbent Assay for Brazil Nut (*Bertholletia excelsa* L.) Detection

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Undeclared Brazil nut residue in food products is of great concern because it can trigger life-threatening allergic reactions in sensitive patients. A rabbit polyclonal antibody-based competitive ELISA (IC_{50} = 23.2 ± 9 ng/mL, $n = 76$) with good sensitivity, detection range of 10−90 ng/mL, was developed. The ELISA could detect Brazil nut seed proteins over a pH range of 5−12. The optimal pH range for the detection assay was 7−10. Among the 66 tested foods/ingredients, only cinnamon exhibited statistically significant interference (1.36%, $p = 0.05$). Exposing Brazil nut seeds to processing did not adversely affect the nut seed protein detection using the assay. Brazil nut seed protein recovery from 100 mg of foods spiked with 10 and 1 μg of soluble Brazil nut proteins or 100 and 10 μg of defatted Brazil nut flour exhibited a wide recovery range, 63−315%, indicating protein−food matrix interaction.

KEYWORDS: Brazil nut; protein; ELISA; immunoblotting; allergy; rabbit polyclonal antibody; immunoreactivity

INTRODUCTION

Brazil nut (BN, *Bertholletia excelsa* L., family Lecythidaceae) is a large nut seed compared to most other tree nuts, composed of 66% fat, 14% protein, and 13% carbohydrate (*1*). BN is commonly used as an ingredient in confectionery products in North America and European countries. It ranks third in tree nuts imported in the United States behind cashews and pecans. Among tree nuts, BN is exceptionally high in selenium (2.96 mg/100 g) and sulfur-containing amino acids (∼9% methionine and cysteine) (*2*) and is a good source of polyunsaturated fatty acids (*1, 3*).

In the United States, 4−8% children and 1−3% adults suffer from food allergies and 20% of the population avoids some foods due to perceived allergy-related issues (*4*). A recent study at Isle of Wight (United Kingdom) showed 5−6% of children, under 3 years of age, suffer from clinically determined food hypersensitivity (*5*). Incidents of allergy to peanuts have doubled among children from 1997 to 2002, and more than 3 million U.S. citizens suffer from either or both peanut and tree nut allergy (*6, 7*). Unlike milk and egg allergies, peanut and tree nut allergies are more frequent in adults (*8*) as they are generally not outgrown with age (*9*). Currently there is no cure for food allergy, and therefore avoidance of the offending food is the best defense for allergic patients. One of the early reported case studies of allergic reactions to BN was in children aged 14−38 months in which BN is described as a potent allergen (*10*). BN cross-reactivity with almond, hazelnut, walnut, and peanut (*11, 12*) leading to severe reactions including anaphylaxis in hypersensitive patients has been reported (*13, 14*). Such sensitive patients are at a high risk when the offending allergen is present in the food and not declared on the food label. To help protect sensitive consumers from unintended exposure, the development of rapid, sensitive, and robust detection tests for the targeted allergen is important. Anti-2S albumin rabbit polyclonal antibody (pAb)-based indirect competitive inhibition enzyme-linked immunosorbent assay (ELISA) with 1 part per million (ppm) sensitivity has been developed for BN detection (*15*). Although useful, this assay would not detect other major BN proteins (*16, 17*). For example, Beyer et al. (*18*) recently identified a new BN allergen, Ber e 2, a legumin-like 11S protein. Ber e 2 is a major seed storage protein in BN constituting about 60% of the total seed proteins, with 2S albumin (30%) and 7S vicilin (10%) being the other two major seed storage proteins (*19*). We have therefore developed an anti-BN protein rabbit pAb-based competitive inhibition ELISA with broad specificity and good sensitivity for BN proteins in food matrices.

MATERIALS AND METHODS

Materials. Shelled BNs and foods/ingredients were purchased from a local grocery store. Electrophoresis and immunoblotting supplies were from Hoefer Scientific Co. (San Francisco, CA). Disposable polypropylene columns were from Pierce Inc. (Rockford, IL), and protein G Sepharose 4 Fast Flow beads were from Pharmacia Inc. (Piscataway, NJ). Freund's complete and incomplete adjuvants, alkaline phosphatase-labeled goat anti-rabbit IgG, horseradish peroxidase-labeled goat anti-

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rabbit IgG, Ponceau S, bovine serum albumin, and phosphatase substrate (*p*-nitrophenyl phosphate, disodium) 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 films (BioMax XAR film) were from Eastman Kodak Co. (Rocheser, NY). Ninety-six-well polyvinyl microtiter ELISA plates were from Costar (Cambridge, MA). All other chemicals (ACS grade) and protein markers were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Methods. *Preparation of Protein Extract.* Shelled BNs were ground in an Osterizer blender (speed setting "grind"; Galaxy model 869-18R, Jaden Consumer Solutions, Boca Raton, FL) to homogeneous flour 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 fume hood, it was passed through a 40 mesh sieve and stored in screw-capped plastic vials at −20 °C until further use. Food ingredients used for cross-reactivity studies were prepared as described (*20*). Briefly, heat sensitive foods were freeze-dried (e.g., ice cream, cheese), whereas fresh produce (e.g., fruits and vegetables) and high-sugar dried fruit (e.g., raisins) matrices were oven-dried for 24 h at 50−60 °C and powdered in a blender to obtain uniform powdered (∼40 mesh) samples. The dry matrices containing <1% fat (e.g., wheat flour) were used without any further treatments. Dry food matrices containing >1% fat were defatted using a Soxhlet apparatus as described above, air-dried, and powdered. All dried and powdered matrices were stored at −20 °C in plastic capped vials until further use. Protein extracts from defatted food/ingredient were prepared using borate saline buffer (BSB, 0.1 M H_3BO_3, 0.025 M $Na_2B_4O_7$, 0.075 M NaCl, pH 8.45) (flour/solvent = 1:10 w/v) by continuous vortexing for 1 h at room temperature (RT, ∼25 °C) followed by centrifugation at 16000*g* for 15 min at RT. Aliquots of supernatant were analyzed within 48 h of preparation, and the remainder was stored at −20 °C until further use. The protein content of the supernatant was estimated as per the method of Lowry et al. (*21*) or Bradford (*22*) using bovine serum albumin as the standard protein.

Fractionation of BN 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 magnetic stirring at 700 rpm for 1 h at RT. The slurry was centrifuged at 27000*g* for 20 min at 4 °C, and the supernatant was loaded onto a Sephacryl S200 HR column (2.6 × 72 cm) equilibrated with 0.035 M phosphate buffer containing 1 M NaCl, pH 7.5. The column flow rate was maintained at 24 mL/h, and fractions were collected every 15 min. The peaks containing 11S legumin and 2S albumin were pooled and quantified for their protein content.

Thermal Processing of BN. Raw shelled BNs were subjected to different thermal treatments (three to four nuts for each treatment) as previously described by Su et al. (*23*). Briefly, treatments included pressure cooking in an autoclave at 121 °C and 15 psi for 5 and 30 min, blanching in boiling water for 3 and 10 min, frying in vegetable oil at 191 °C for 1 and 2 min, microwave heating at 500 W for 1 and 3 min, and dry roasting at 140 and 160 °C for 30 min each and at 168 and 177 °C for 12 min each. Processed and unprocessed BNs were defatted as described above and stored in screw-capped plastic vials at −20 °C until further use. The soluble proteins from processed and unprocessed BNs were extracted in BSB and analyzed for their protein content.

Effect of pH on Protein Extraction. Defatted BN flour was extracted in distilled (DI) water (flour/water ratio = 1:10 w/v) with final pH of the slurry adjusted from 1−13 with 1.0 N NaOH or 1.0 N HCl with continuous vortexing for 1 h at RT. The pH of the slurry was measured after 15 and 45 min and adjusted to the desired value as needed. The slurry was centrifuged at 20000*g* for 30 min at 4 °C. The protein content of the supernatants was determined, and the supernatants were stored at −20 °C until further use.

Spiking of Food Matrices with BN. The selection of the four food matrices for the spiking studies was partly arbitrary and partly based on the possible presence of BNs in the selected foods. Food matrices were spiked with 10 (1 μg of BN protein extract in 100 mg of food matrix) and 100 ppm (10 μg of BN protein extract in 100 mg of food matrix) of BN protein extract followed by preparation of protein extract using the spiked matrix. In addition, food matrices were also spiked with two different levels of defatted BN flour. A 1:10 (w/w) spiking was done by mixing 50 mg of defatted BN flour with 450 mg of food matrix in a pestle and mortar. Furthermore, 10-fold serial dilutions were prepared by mixing 450 mg of food matrix with 50 mg of previously spiked matrices. Spiking levels of 1:1000 and 1:10000 (w/w) were used to determine the recovery in the present study. The soluble protein content was determined in the spiked and unspiked matrix extracts. Matrices and their protein extracts were stored at −20 °C until further use.

Production of Rabbit Antisera. The rabbit antisera against BSB soluble BN proteins was produced and characterized as described by Acosta et al. (*24*). Rabbits were immunized with defatted BN protein extract intradermally using Freund's complete adjuvant. After four boosters with Freund's incomplete adjuvant, the rabbit was bled, and the serum was collected. An aliquot (2 mL) of the serum was purified using a protein G column as per the manufacturer's protocol, analyzed for soluble protein, and stored at −20 °C until required.

Electrophoresis and Immunoblotting. SDS-PAGE was carried out according to the method of Fling and Gregerson (*25*). Protein samples were boiled in sample buffer [50 mM Tris-HCl, pH 6.8, 1% SDS, 30% glycerol, 0.01% bromophenol blue, 2% v/v β mercaptoethanol (β-ME)] for 10 min, and suitable aliquots normalized to constant protein amount were loaded on the gels. Electrophoresis was carried out either on 8−25% gradient or 12% monomer acrylamide separating gel and 4% monomer acrylamide stacking gel. The gel was run at constant current, typically 10 mA per gel overnight followed by 20 mA per gel until the dye migrated to the gel edge (∼3−5 h) and either stained overnight with 0.25% w/v Coomassie Brilliant Blue R (CBBR) containing 50% v/v methanol and 10% v/v acetic acid or used for transfer onto 0.22 μm nitrocellulose membrane as described by Towbin et al. (*26*). The CBBR stained gels were destained with 50% v/v methanol containing 10% v/v acetic acid until the blue background was removed. The unbound sites on the nitrocellulose membrane were blocked by incubation in Tris-buffered saline (TBS-T; 10 mM Tris, 0.9% w/v NaCl, 0.05% v/v Tween 20, pH 7.6) containing 5% w/v nonfat dried milk (NFDM) for 1 h at RT. The membrane was washed with TBS-T for 5 min and then incubated with protein G-purified rabbit antiserum in TBS-T at 1:10000 v/v dilution overnight at 4 °C. The membrane was rinsed once with TBS-T and then washed thoroughly three times with TBS-T for 15 min each, followed by incubation with horseradish peroxidase-labeled goat anti-rabbit IgG antibody in TBS-T at 1:40000 v/v dilution for 1 h at RT. The sheet was then rinsed with TBS-T and washed three times with TBS-T for 15 min each. The reactive bands were developed by incubating the membrane with a mixture of solution A (100 μL of 250 mM luminol; 44 μL of 90 mM *p*-coumaric acid; 1 mL of 1 M Tris-HCl, pH 8.5; 8.85 mL of DI water) and solution B (6 μL of 30% hydrogen peroxide; 1 mL of 1 M Tris-HCl, pH 8.5; 8.85 mL of DI water) for 5 min and exposing to X-ray film for 30 s to 1 min. For dot-blot assay, 2 μg of protein extract was dotted on the membrane, and the procedure from blocking to development was as described above for Western blotting. A densitometer (Molecular Imager ChemiDoc XRS System, Bio-Rad Laboratories, Hercules, CA) was used to determine the density of the blots.

ELISA. Competitive inhibition ELISA was developed using protein G-purified rabbit anti-BN antibody (3.2 mg/mL). A 96-well polyvinyl microtiter plate was coated with 50 μL of 10 μg/mL of BN protein extract per well (500 ng of BN protein/well) prepared in the coating buffer (48.5 mM citric acid, 103 mM Na_2HPO_4, pH 5.0) and incubated for 1 h at 37 °C. The plate was washed three times with TBS-T, blocked with 5% NFDM in TBS-T for 1 h at 37 °C, and again washed three times with TBS-T. Concurrently, 90 μL of pAb, diluted 1:40000 v/v in TBS-T containing 1% NFDM, was added to the wells of a second uncoated plate. Ten microliters of 1 mg/mL standard protein (unprocessed BN protein extract) or other protein extracts (inhibitor) was added in the first well to achieve a final concentration of 0.1 mg/mL in the well and serially diluted 10 times into the subsequent wells. The plate was incubated at 37 °C for 1 h. Fifty microliters of the content from each well of uncoated plate was transferred to the respective well of the coated plate and incubated for 1 h at 37 °C. The plate was washed three times with TBS-T and incubated with 1:5000 v/v alkaline phosphatase-labeled goat anti-rabbit IgG in TBS-T for 1 h at 37 °C.

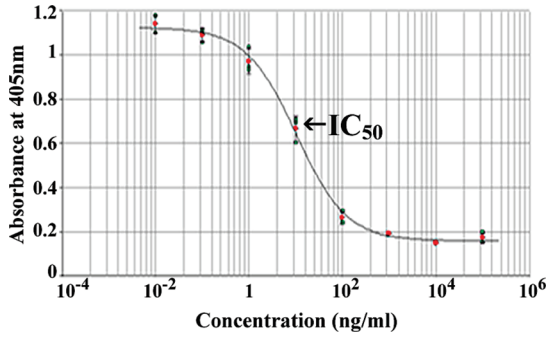


Figure 1. Typical competitive inhibition ELISA standard curve of untreated BN total protein extract using rabbit anti-BN pAbs.

The plates were washed three times with TBS-T and developed by incubation with 50 μL/well of phosphatase substrate [5 mg/mL *p*-nitrophenyl phosphate tablet dissolved in 5 mL of substrate buffer (0.0049% w/v $MgCl_2$, 0.096% v/v diethanolamine, pH 9.8)]. The reaction was stopped by adding 50 μL of 3 M NaOH, and the absorbance in each well was read at 405 nm. The IC_{50} and amount of BN protein in the inhibitor were determined by using the four-parametric curve generated by using known amount of BN protein (standard curve). To determine the percent immunoreactivity, the protein extracted from the food ingredient was used as an inhibitor in the assay at 0.1 mg/mL in the top well followed by 10 times serial dilution in the successive wells. On the same plate BN standard curve was prepared. The reactive protein in the food ingredient sample (C_1) was determined using the sample absorbance closest to the IC_{50} of the BN standard curve. The percent immunoreactivity of the food ingredient (i.e., the sample) was calculated as follows:

$$\% \text{ immunoreactivity of the sample} = [C_1/\text{total protein in the well corresponding to the } C_1] \times 100$$

Statistics. All experiments were carried out at least in duplicate, and data are reported as mean ± standard error of mean (SEM). Data were analyzed for statistical significance by one-way ANOVA using SPSS statistical software (version 15.0; Chicago, IL) and Fisher's least significant difference (LSD) test ($p = 0.05$)

RESULTS AND DISCUSSION

Detection and Quantification of BN. Among four tested buffers (BSB, PBS, citrate, and carbonate), citrate buffer was the best for coating the antigen at 500 ng/well on ELISA plates. For Western blotting, BN protein extracts were prepared in BSB as soluble proteins are efficiently extracted in this buffer (unpublished data). The optimum pAb dilutions for ELISA and Western blot were 1:40000 v/v and 1:10000 v/v, respectively. These dilutions were judged to be optimal based on the BN detection sensitivity without adversely affecting the ELISA IC_{50} curve and identification of major antigenic polypeptides in Western blot and were used for routine analyses. Inhibition ELISA has been successfully used as a tool for identification of traces of almond (*27*), peanut (*28*), and soybean β-conglycinin (*29*). A typical ELISA curve is shown in **Figure 1**. The mean IC_{50} for BN detection was 23.2 ± 9.0 ng/mL (n = 76, 95% confidence interval = 21.1−25.2 ng/mL) with the detection range of 10−90 ng/mL. The major polypeptides (visually judged by the signal intensity and bandwidth) recognized by pAb under reducing (2% v/v β-mercaptoethanol, β-ME) and denaturing conditions were estimated to be 14.4, 19.8, 32.1−38.8, and 49.4 kDa (**Figure 5C**). Interestingly, the 2S albumin polypeptide, ∼12 kDa (**Figure 5C**), did not exhibit high reactivity with the rabbit anti-BN pAb. Although 2S albumin represents 30% of BN seed storage proteins, it may not represent proportional immunoreactivity. There is at least one report showing no immunoreactivity of a Brazil nut allergic patient's IgE toward BN 2S albumin (*30*).

Specificity of pAb. The protein G-purified pAbs were tested for cross-reactivity against 66 different food ingredients using ELISA and Western blot. These ingredients were selected based, in part, on the likehood that the ingredients would be found in association with BN in commonly encountered foods. For example, BN seeds are sometimes a component of "trail mix" snack foods, in which one might also find other tree nut seeds, peanuts, sunflower seeds, dried fruits/vegetables, dairy ingredients, eggs/egg products, sugar, salt, wheat and/or other cereal flour(s), spices, and preservatives (notably antioxidants). The number, types of ingredients, and the proportion of ingredient may vary significantly from product to product. In our competitive ELISAs, the protein-containing inhibitors were used at a standardized protein concentration of 0.1 mg/mL. For those food ingredients with negligible protein content (0−0.1 mg/mL, e.g., spices, salt, sugar), 90 μL of BSB extract was used. Immunoreactivity was measured by using the four-parametric equation of the standard curve obtained by using BN extract as inhibitor on each plate (**Table 1**). There was no significant immunoreactivity observed with different food ingredients. However, cinnamon at 0.1 mg/mL concentration was found to interfere with the reaction giving immunoreactivity equivalent to 1.360%, whereas the rest of the ingredients registered an immunoreactivity value in the range of 0.000−0.030%. Cinnamon did not contain significant amounts of protein (0.4 mg of soluble protein/100 mg of dry powder) and did not exhibit reactive polypeptides in Western blotting, leading us to conclude that the "immunoreactivity" values obtained were the result of interference by this highly colored extract.

Cross-reactivity assessed by Western blot indicates identification of different polypeptides with various degrees of reactivity in most, but not all, tested food ingredients (**Figure 2**). Although BN polypeptides exhibit a distinct profile when probed with rabbit pAbs, cross-reactivity of polypeptides in several food ingredients precludes use of Western blot as a reliable tool for BN detection. Cross-reactivity was dependent on immunoassay format. For example, cinnamon was highly cross-reactive in ELISA and Western blot, whereas sesame seed proteins were strongly reactive in Western blot but not in ELISA. The reasons for format-dependent differential cross-reactivity in an immunoassay may be a result of differences in (a) epitope accessibility, (b) epitope stability, (c) antibody−antigen interactions that are independent of immuno recognition (i.e., immunologically irrelevant protein−protein interactions), or (d) a combination of a−c. For example, protein is denatured by SDS prior to transfer on a nitrocellulose membrane in Western blotting, which has been reported to increase buried epitope accessibility (*31, 32*).

pAb Reactivity to BN Proteins. Gel filtration chromatography resolved BN proteins in three peaks (**Figure 3**). SDS-PAGE analysis of the fractions indicated that the first (fractions 26−40) and second peak (fractions 47−55) are rich in 11S legumin (18−22 and 30−32 kDa) and 2S albumin (12 kDa), respectively (**Figure 3** inset). The third peak (fractions 60−70) displayed significant A_{280nm}, but exhibited minimal polypeptide staining. Sun et al. (*19*) using Sephadex G-150 gel filtration chromatography reported a BN profile consisting of three peaks. The first peak contained 11S globulin and 7S vicilin-like proteins, whereas the second peak was mainly composed of 2S albumin proteins. Similar to the findings of the current investigation, Sun and co-workers noted the third peak was

Table 1. Cross-Reactivity of Rabbit Anti-BN pAb with Different Food Ingredients As Measured by Competitive ELISA[a]

ingredient	% immunoreactivity	ingredient	% immunoreactivity	ingredient	% immunoreactivity
tree nuts		**legumes**		**fruits**	
almond	0.005 ± 0.003	black bean	0.003 ± 0.003	banana	0.008 ± 0.005
cashew nut	0.005 ± 0.002	black-eye pea	0.002 ± 0.001	cherry	0.024 ± 0.003
hazelnut	0.006 ± 0.002	black gram	0.001 ± 0.001	pineapple	0.005 ± 0.003
macadamia	0.006 ± 0.002	chick pea	0.002 ± 0.001	raisin	0.006 ± 0.001
pecan	0.007 ± 0.003	cow pea	0.002 ± 0.001	**vegetables**	
pine nut	0.000 ± 0.000	fava bean	0.003 ± 0.002	carrot	0.004 ± 0.003
pistachio	0.000 ± 0.000	green lentil	0.002 ± 0.001	cauliflower	0.004 ± 0.002
walnut	0.003 ± 0.003	green pea	0.003 ± 0.002	mushroom	0.007 ± 0.001
seeds		horse bean	0.006 ± 0.003	red potato	0.015 ± 0.009
poppy	0.015 ± 0.002	Inca peanut	0.000 ± 0.000	spinach	0.004 ± 0.002
sesame	0.013 ± 0.002	kidney bean	0.004 ± 0.001	turnip	0.019 ± 0.002
sunflower	0.003 ± 0.002	lima bean	0.003 ± 0.002	**spices**	
cereals		lupine	0.004 ± 0.002	black pepper	0.008 ± 0.004
amaranth	0.005 ± 0.001	moth	0.007 ± 0.003	cardamom	0.009 ± 0.006
barley	0.000 ± 0.000	mung bean	0.002 ± 0.001	cinnamon	1.360 ± 0.954
corn	0.001 ± 0.001	navy bean	0.001 ± 0.001	nutmeg	0.007 ± 0.003
oat bran	0.003 ± 0.002	pigeon	0.003 ± 0.002	**others**	
rice	0.002 ± 0.001	pinto	0.003 ± 0.001	baking powder	0.012 ± 0.005
rye	0.009 ± 0.003	Spanish peanut	0.000 ± 0.000	cocoa	0.002 ± 0.001
whole wheat	0.005 ± 0.002	soybean	0.003 ± 0.001	egg white	0.008 ± 0.003
dairy		tepary	0.005 ± 0.003	egg yolk	0.005 ± 0.003
NFDM	0.006 ± 0.003	Val	0.011 ± 0.001	salt	0.030 ± 0.008
Swiss cheese	0.015 ± 0.001	Virginia peanut	0.000 ± 0.000	sugar	0.005 ± 0.003
vanilla ice cream	0.012 ± 0.002	winged bean	0.001 ± 0.001	tapioca	0.005 ± 0.003

[a] Data are expressed as mean ± SEM. LSD = 0.081 ($p = 0.05$, $n = 4$). Differences between two means exceeding LSD value are significant.

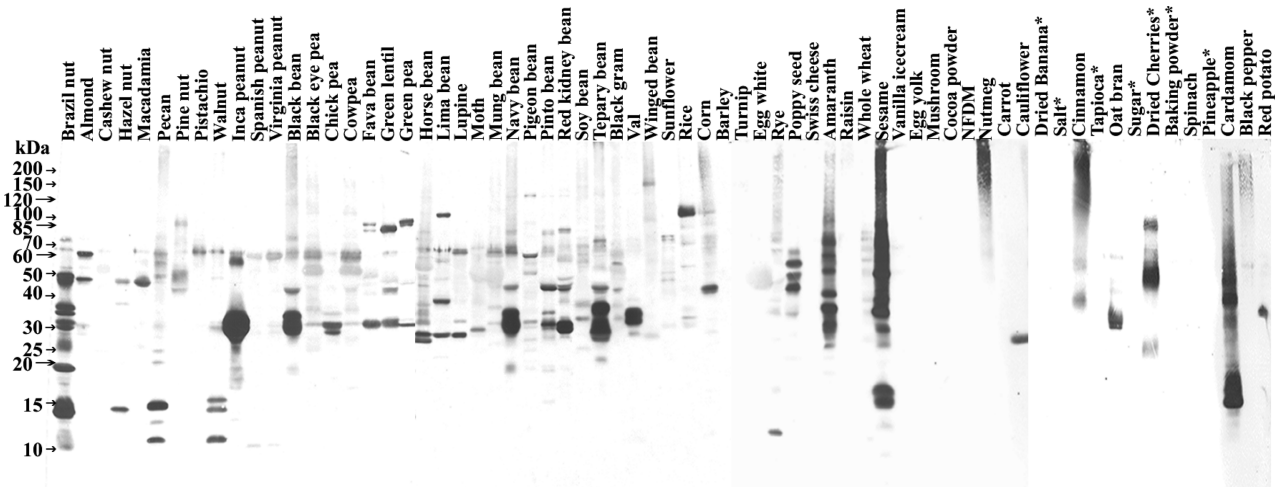


Figure 2. Cross-reactivity of rabbit anti-BN (1:10000 dilution, v/v) with different food ingredients. Protein load: 30 μg for extracts with protein content >0.1 mg/mL and 100 μL for extracts with protein content ≤0.1 mg/mL (indicated by *).

composed of UV-absorbing nonprotein materials. Our fractions containing 11S globulin and 2S albumin were pooled and analyzed for immunoreactivity with anti-BN pAbs. Western blotting results of the BN fractions under reducing (**Figure 4A**) and nonreducing conditions (**Figure 4B**) revealed that the 11S globulin-rich Sephacryl S200 HR fraction pool (fractions 26−40) exhibited immunoreactivity similar to that of the BN BSB extract. However, the 2S albumin-rich Sephacryl S200 HR fraction showed little immunoreactivity. The major polypeptides of the BN proteins observed under nonreducing conditions had molecular masses of about 50−55, 38, 28−30, and 10−12 kDa (marked by * in **Figure 4B**). Because 11S globulin is an hexameric protein composed of six acidic and basic chains linked by disulfide bonds, the SDS-PAGE under nonreducing conditions shows the major polypeptides in the 50−55 kDa region (32−34 kDa acidic + 19−22 kDa basic subunit). Western blotting under nonreducing conditions showed immunoreactivity of the major polypeptides in the 11S globulin fraction (50−55 kDa) and slight immunoreactivity (based on visual intensity of the bands) of the 2S albumin fraction (∼12 kDa), suggesting that the maintenance of disulfide bonds may be important to the integrity of 2S albumin epitopes. Competitive ELISA using standard assay conditions and inhibitor concentration of 0.1 μg/mL were also conducted to assess the immunoreactivity of the separated 11S globulin and 2S albumin fractions (**Figure 4C**). The results of IC_{50} determinations for BSB extract, 11S globulin, and 2S albumin suggested the 2S albumin fraction is immunogenically the least potent. If BN 2S albumin is less immunogenic than 11S globulin, immunoassays targeting 2S albumin as the antigen of choice may not be effective in detecting BN. Moreover, 11S globulin reportedly accounts for ∼60% of total proteins in BN seeds (*19*). The results of the current investigation suggest the 11S globulin is a better immunogenic protein than the 2S albumin for BN detection.

Effects of Processing on BN Protein Immunoreactivity. BNs are often heat treated prior to or during their use in food preparations (e.g., BN in frozen dessert, mixed trails, and

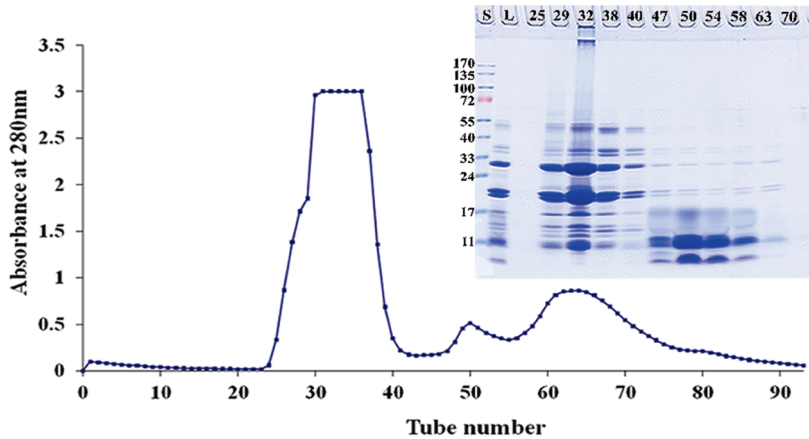
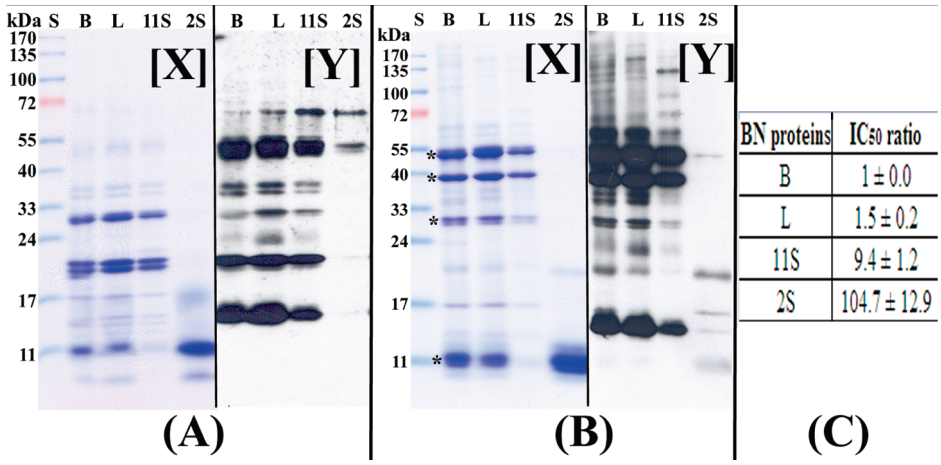


Figure 3. Elution profile of BN extract (OE) off Sephacryl S200 HR column. Fractions containing 11S legumin (tubes 26−40, 88 mL) and 2S albumin (tubes 47−55, 53 mL) were pooled. Inset: SDS-PAGE analysis of fractions eluting off the column are indicated by the number on top of the gel lane. S, protein standards; L, protein loaded on to the column.



BN proteins	IC_{50} ratio
B	1 ± 0.0
L	1.5 ± 0.2
11S	9.4 ± 1.2
2S	104.7 ± 12.9

Figure 4. Coomassie stain (8−25% gradient gel) [X] and Western blotting [Y] of BN fractions under reducing (**A**) and nonreducing conditions (**B**). Protein load in each lane was 20 μg except 2S was 10 μg. S, protein standards; B, BSB extracted BN proteins; L, BN protein loaded on the S200 column; 11S, first peak off column; 2S, second peak off column. (**C**) IC_{50} ratio of BN protein fractions to BSB extract expressed as mean ± SEM (n = 6; LSD = 27.1, p = 0.05).

Table 2. Effect of Thermal Processing on Immunogenicity of BN Proteins Assessed by Competitive ELISA and Dot Blot[a]

thermal processing	ELISA	dot blot
unprocessed control	100 ± 0	100 ± 0
autoclaving at 121 °C for 5 min	83 ± 5	100 ± 2
autoclaving at 121 °C for 30 min	96 ± 10	99 ± 3
blanching at 100 °C for 3 min	64 ± 8	99 ± 1
blanching at 100 °C for 10 min	69 ± 15	99 ± 1
frying at 191 °C for 1 min	74 ± 10	97 ± 2
frying at 191 °C for 2 min	90 ± 12	97 ± 1
microwaving at 500 W for 1 min	94 ± 13	98 ± 2
microwaving at 500 W for 3 min	132 ± 9	97 ± 2
roasting at 140 °C for 30 min	117 ± 19	100 ± 2
roasting at 160 °C for 30 min	89 ± 27	98 ± 3
roasting at 168 °C for 12 min	77 ± 24	99 ± 2
roasting at 172 °C for 12 min	99 ± 30	99 ± 2
LSD (p = 0.05)	26	2.9

[a] Data are expressed as percent immunoreactivity as compared to corresponding unprocessed controls, mean ± SEM (n = 4). Differences between two means within the same column exceeding corresponding LSD value are significant.

cookies). The effect of some of the most commonly employed thermal processing methods (autoclaving, blanching, frying, microwave heating, and roasting) on the immunoreactivity of BN was therefore assessed. Protein extracts of unprocessed and processed BNs were normalized to 1 mg/mL in BSB to compensate for the loss of protein solubility due to processing treatments before using the protein extracts for IC_{50} determination. A comparison of the IC_{50} ratio of processed to unprocessed BN seed protein extracts by ELISA and dot blotting (**Table 2**) suggests no significant loss of immunoreactivity upon processing. However, the stability and immunoreactivity of individual polypeptides cannot be judged by ELISA or dot blot. Hence, the processed BN samples were analyzed by SDS-PAGE under denaturing and reducing conditions (CBBR staining, **Figure 5A**; or Ponceau S staining, **Figure 5B**). We observed a decrease in the intensity of the 36−39 kDa polypeptides (indicated by an arrow in **Figure 5A**) for those BNs samples processed for longer time/higher temperature combinations: autoclaving (121 °C for 30 min), frying (191 °C for 2 min), microwave heating (500 W for 3 min), and roasting (140 and 160 °C for 30 min; 168 and 172 °C for 12 min) (e.g., compare lanes A1 and A2). A similar decrease in intensity has also been observed for processed almond (*27, 33*), cashew (*23*), pecan (*34*), and walnut (*23*) proteins. Blanching at 100 °C for 3 and 10 min reduced the immunoreactivity of BN proteins when measured by ELISA, but did not seem to affect the intensity of the polypeptide bands when assessed by western blot. Blanching has been shown to cause a reduction in immunoreactivity of pecan and walnut (*23, 34*) but does not seem to affect almond immunoreactivity (*27, 33*). Interestingly, prolonged roasting for

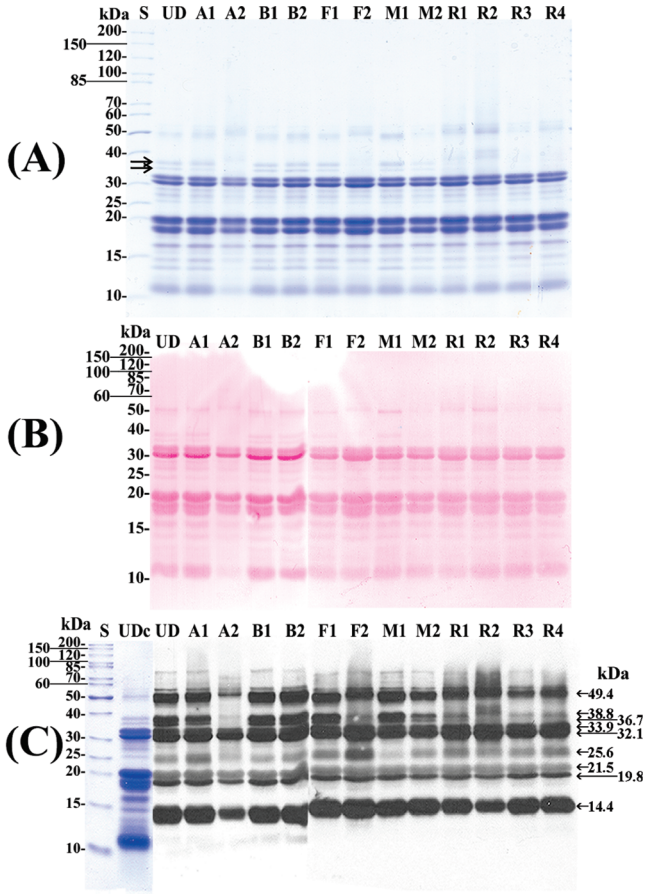


Figure 5. Effect of processing on BN immunoreactivity (**A**) Coomassie stain (8−25% gradient gel), (**B**) Ponceau S stain, and (**C**) Western blot probed with rabbit anti-BN pAb: S, protein standard; UDc, Coomassie stain of unprocessed defatted BN extract; UD, unprocessed defatted; A1, A2, autoclaving at 121 °C for 5 and 30 min; B1, B2, blanching at 100 °C for 3 and 10 min; F1, F2, frying at 191 °C for 1 and 2 min; M1, M2, microwave heating at 500 W for 1 and 3 min; R1, R2, roasting at 140 and 160 °C for 30 min; R3, R4, roasting at 168 and 172 °C for 12 min. Protein load in each lane = 30 μg.

Table 3. Effect of Extraction pH on the Detection of BN by ELISA[a]

pH of extraction	IC_{50}
1	NI
2	NI
3	NI
4	NI
5	1476.3 ± 91.6
6	289.8 ± 12.1
7	49.9 ± 3.4
8	54.2 ± 0.9
9	53.2 ± 4.0
10	67.5 ± 2.7
11	134.2 ± 12.5
12	1219.8 ± 28.4
13	NI
BSB	28.8 ± 1.5
LSD ($p = 0.05$)	66.8

[a] Data expressed as mean ± SEM ($n = 4$). NI, no inhibition. Differences between two means within the same column exceeding corresponding LSD value are significant.

30 min at 160 °C resulted in the appearance of new polypeptides of molecular mass around 40 kDa. Roux et al. (*27*) also observed new high molecular mass polypeptides (75−84 kDa) in dry-roasted almond samples. These results were further confirmed

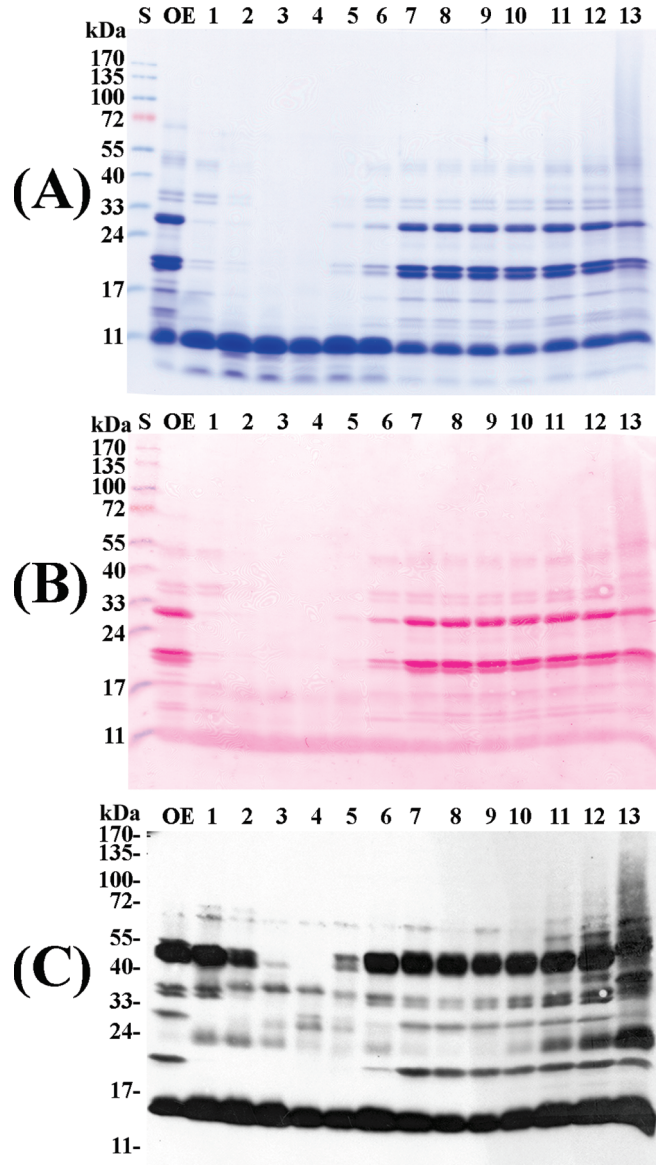


Figure 6. Effect of pH on BN immunoreactivity (**A**) Coomassie stain (8−25% gradient gel), (**B**) Ponceau S stain, and (**C**) Western blotting. S, protein standard; OE, BN extract in BSB. Protein load in each lane = 30 μg.

by Western blot when probed with rabbit anti-BN pAb (**Figure 5C**). Moreover, the intensity of 49.4 kDa polypeptide, as assessed by Western blot, was reduced in BNs autoclaved at 121 °C for 30 min. Overall, the results demonstrate that the immunogenic stability of BN was maintained irrespective of the type of heat treatment. There are several studies on the effect of temperature on the stability of the major allergen, Ber e 1, in BN. The 2S albumin of BN have a high denaturation temperature at pH 7 (>110 °C), which may play an important role in maintaining the allergenicity by retaining the native structure of Ber e 1 in thermally processed BN (*35, 36*). Beyer et al. (*37*) reported high allergenicity of peanut allergens Ara h 1, Ara h 2, and Ara h 3 when peanuts were roasted but reduced IgE binding after frying or boiling.

Effect of pH. Food protein solubility in the extraction buffer is a critical step in immunoassay applications. Because the pH of food varies depending on the processing parameters and consumer preferences, we tested the effect of extraction at different pH values on the detection of BN by rabbit antisera. The protein extracts were tested by ELISA and Western blotting.

Table 4. Recovery of BN Protein Spiked in Different Food Matrices

	BN detected in the 100 mg matrices using competitive ELISA				
		spiked with BN protein		spiked with defatted BN flour[a]	
matrix	not spiked (control), μg	10 μg	1 μg	100 μg	10 μg
wheat flour	0.13 ± 0.04	14.99 ± 3.56	1.89 ± 0.36	25.56 ± 4.56	8.19 ± 1.28
dark chocolate	0.10 ± 0.04	9.04 ± 0.93	0.95 ± 0.27	16.26 ± 4.61	1.92 ± 0.73
oat cereal	0.01 ± 0.00	11.91 ± 0.7	1.05 ± 0.12	17.66 ± 4.52	3.07 ± 1.22
shortbread cookies	0.02 ± 0.00	12.27 ± 1.08	1.30 ± 0.23	26.47 ± 7.64	4.51 ± 1.23
LSD ($p = 0.05$)	0.089	6.36	0.84	17.93	3.71

[a] The theoretical yield of BN protein is assumed to be 26% of the amount of defatted BN flour added (defatted flour contains ~40% protein and ~60−65% is soluble in BSB extract). Thus, 100 μg of defatted BN flour would have 26 μg of soluble protein. Data expressed as mean ± SEM ($n = 3$). Differences between two means within the same column exceeding corresponding LSD value are significant.

The pH of the solution influences the solubility and integrity of the protein by changing the charge on their surface. Proteins have minimum solubility at their isoelectric pH and hence tend to aggregate. The solubility of BN proteins varied considerably over the pH range of 1−13. The protein solubility was low in the pH range of 1−6, with the lowest at pH 3 and increasing gradually thereafter. High protein solubility was observed under alkaline condition (pH 8−13). Similar results have been reported by Poms et al. (*38*) for peanut proteins extracted in various buffers with different pH values. The proteins extracted at different pH values also showed qualitative differences when SDS-PAGE gels were stained with Commassie (**Figure 6A**) and Ponceau S (**Figure 6B**). Defatted BN flour extracted at low pH (1–4) mostly yielded low molecular mass polypeptides (<12 kDa). These polypeptides were also stable toward thermal processing, but were not identified by rabbit anti-BN pAbs. Lowering the pH to 2 has been shown to reduce the denaturation temperature of Ber e 1 to ~82 °C as compared to >110 °C at neutral pH (*35*). The combined effect of thermal processing and extraction pH of BN proteins remains to be elucidated. There was no inhibition observed by ELISA with the BN proteins extracted at pH 1−4 and 13. In contrast, the IC_{50} values of the proteins extracted at pH 7−10 were similar to those of BSB extracted BN proteins (**Table 3**). Hence, the extraction pH of 7−10 was found to be optimal for ELISA. Western blot of BN proteins extracted at different pH values with rabbit antisera was done to visualize the immunoreactive polypeptides (**Figure 6C**). The protein extracts were normalized to constant protein concentration before loading on the gel. It should be noted that reduced concentration of a polypeptide on the gel may be due to formation of aggregates, and these aggregates could retain their immunoreactivity, which will not be visible in Western blot. The polypeptides identified by rabbit anti-BN pAbs in the pH range of 7−10 were similar to those of BSB-extracted BN polypeptides. There were wide variations in pAb identified polypeptides (range = 20−55 kDa) in the pH range of 1−5. For example, the 20 and 32 kDa bands were visible at pH ≥6 and ≥7, respectively. Interestingly, a band below 32 kDa was observed in the pH range of 3−5 but not in the BSB extract. Moreover, the 49.4 kDa polypeptide was strongly recognized at pH 1, decreasing gradually until pH 3, not observed at pH 4, increasing gradually until pH 6, and then constant until pH 12. Similarly, the 25.6 kDa polypeptide was strongly recognized at extreme pH conditions (pH 1−3 and 11−13). Some new high molecular mass polypeptides (40−70 kDa) were visible in the pH 13 extract when probed with rabbit anti-BN. Overall, the solubility of proteins is markedly affected by the pH conditions, thereby greatly influencing the immunoassay.

Recovery of BN-Spiked Matrices. Several food matrices were spiked with BN protein extract and defatted BN flour to check whether food matrices had any interference with the antigen−antibody reaction. The food matrices were selected on the basis of those that may be used in conjunction with BN. The recovery of BN proteins varied depending on the spiking level and food matrices (**Table 4**). The recoveries of soluble BN proteins in 100 mg matrices spiked with 1 and 10 μg of BN extract were 95−189 and 91−150%, respectively. The matrices were also spiked with defatted BN flour to ascertain the effect of other constituents in BN on the recovery of proteins. BNs typically contain 14% protein and 66% fat, which makes ~41% protein in the defatted flour. Considering 60−70% of the protein is extractable, the theoretical yield of soluble BN protein in the samples spiked with 100 μg of defatted BN flour will be ~26 μg. The matrices spiked with 100 μg of BN flour showed 16.26−26.47 μg of soluble BN protein (63−102% recovery), whereas those spiked with 10 μg yielded 1.92−8.19 μg of soluble BN protein (74−315% recovery). Among four different food matrices tested, wheat flour showed highest recovery (except at 100 μg of defatted BN flour spiking), whereas the lowest recovery was observed in the dark chocolate irrespective of the level and type of spiking. These results suggest that food matrices may affect the antigen quantification by (1) increasing or decreasing the extraction efficiency of the antigen in the presence of food matrices and (2) inhibiting/promoting the antigen−antibody interaction via protein−protein interaction of food matrices and antigen. The reduced recovery in dark chocolate matrix may be partly due to the formation of BN protein−dark chocolate polyphenol insoluble complexes, resulting in low protein extraction efficiency.

A rabbit pAb-based sensitive, robust, and specific immunoassay was developed for BN detection. The results of immunoassays revealed the BN 11S globulin to be a stronger immunogen as compared to the 2S albumin for the purpose of rabbit pAb production. ELISA results indicated that food matrix did influence BN recovery from spiked samples. The assay was successfully applied for BN detection and quantification from select foods.

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