

SHORT COMMUNICATION

Detection of Brazil Nut Proteins in Foods by Enzyme Immunoassay

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An enzyme immunoassay (EIA) system was developed for the detection of Brazil nut (BN) allergens in foods. The assay utilized a sandwich EIA format with inexpensive chicken egg yolk antibodies (IgY) as a source of immunoreagents for the immuno-specific capture and detection of BN proteins. The assay was capable of detecting less than 1 ppm of BN proteins spiked into various food matrices, and did not cross-react with protein extracts from a variety of seeds known to contain 2S albumins related to the major BN allergen. This simple and inexpensive assay will enable the food industry and regulatory agencies to ascertain the presence of undeclared BN allergens in foods and related products.

Keywords: Brazil nut, allergen, enzyme immunoassay

INTRODUCTION

Tree nuts such as the Brazil nut (*Bertholletia excelsa*) contain allergens capable of causing severe adverse reactions in allergic individuals (Zarkadas *et al.*, 1999). A major allergenic component of the Brazil nut (BN) is the 2S albumin (Bartolome *et al.*, 1997), which is comprised of two polypeptides with molecular weights of ca. 9000 and 3000 (Sun *et al.*, 1987). Because of its high levels of the sulfur-containing amino acids methionine and cysteine (Sun *et al.*, 1987), the BN is considered a rich source of essential amino acids. The nutritional properties of many foods have been enhanced by supplementation with BNs (Antunes and Markakis, 1977) or specific BN proteins produced through recombinant DNA techniques (Nicaud *et al.*, 1994). For these reasons, it is imperative that any foods containing BNs or components thereof be clearly identified so that allergic individuals can avoid exposure. In order to prevent the inadvertent ingestion of foods containing undeclared BN material by allergic individuals, and to follow up incidents of adverse reactions to foods, the

food industry and regulatory agencies will require sensitive detection methods for BN allergens.

The enzyme immunoassay (EIA) technique is a simple and effective tool for the detection of trace quantities of analytes in complex matrices. While detection methods based on EIA have been reported for the assay of a variety of allergens in foods, including peanuts (Yeung & Collins, 1996; Blais & Phillippe, 2000) and hazelnuts (Blais & Phillippe, 2001), there is a paucity of reports in the scientific literature on the development of such methods for detection of BNs in foods. In this report we describe a simple EIA method for the detection of BN proteins in foods based on the use of inexpensive chicken egg yolk antibodies (IgY) as the source of immunoreagents.

MATERIALS AND METHODS

Preparation of BN Antigens

BN antigens were prepared by a modification of the method of Sun *et al.* (1987). BN kernels (50 g) were ground using a mortar and pestle and defatted by soxhlet extraction with hexane as solvent. The powder was air dried overnight, and resuspended in 50 ml of distilled water, and stirred for 60 min at room temperature to extract proteins. The slurry was filtered by passing through four layers of Miracloth™, followed by clarification of the filtrate by centrifugation at $26\,000 \times g$ for 30 min. The resulting supernatant was further clarified by passage through a $5\ \mu\text{m}$ membrane filter, and stored at 4°C until use. This extract contained $28\ \text{mg ml}^{-1}$ of total protein (determined by Bio-Rad Protein assay, Bio-Rad Laboratories Inc., CA, USA), and constituted the BN standard used in all subsequent experiments. The quality of the BN preparation was verified by SDS-PAGE, which revealed the presence of several distinct proteins, of which the major species corresponded in size to the 9 kDa polypeptide associated with the 2 S albumin protein (Sun *et al.*, 1987).

Preparation of Immunoreagents

Anti-BN antibodies (IgY) were produced in chicken egg yolks by contract with Aves Labs Inc. (Tigard, OR, USA). Briefly, the antibody production protocol followed at Aves Labs involved a total of four injections into the pectoral muscles of a hen, starting with the injection of a mixture of antigen with complete Freund's adjuvant (first injection); followed after 3 weeks by injections of antigen in a 1:1 mixture of complete and incomplete Freund's adjuvant at 2 week intervals (second, third and fourth injections). Seven days after the final injection, immune eggs were collected until a total of six eggs were obtained. The antibodies (IgY) were purified from the combined yolks of the immune eggs using a proprietary process involving the following steps: (1) dilution of the yolk with aqueous medium; (2) de-lipidation using an undisclosed organic solvent; (3) salt fractionation and centrifugation; and (4) dialysis against phosphate-buffered saline (PBS). The resulting IgY stock (anti-BN) had a total protein concentration of $20\ \text{mg ml}^{-1}$, and was stored at 4°C until use.

For the preparation of labeled detector antibodies, anti-BN was conjugated with biotin as follows: anti-BN (10 mg) was dialyzed for 16 h at 4°C against three changes of distilled water, and the final concentration was adjusted to $5\ \text{mg ml}^{-1}$ in distilled water. To 1 ml of anti-BN (5 mg) was added 1 ml of 0.2 M-sodium carbonate buffer (pH 9.5) and 0.2 ml of 0.1 M-sodium periodate, followed by mixing and incubation for 30 min at room temperature in the dark. This was followed by the addition of 2 ml of 0.5 M-sodium acetate buffer (pH 5.2), and then a total of $100\ \mu\text{l}$ of biotin (long arm) hydrazide (Vector Laboratories Inc., CA, USA, cat. no. SP-1100) solution ($50\ \text{mg ml}^{-1}$ in dimethylsulfoxide), which was added slowly with constant gentle mixing of the mixture and under subdued lighting. The mixture was incubated for 16 h at room temperature in the dark, and the biotinylated anti-BN was precipitated by addition of an equal volume of saturated ammonium sulfate, followed by centrifugation at $10\,000 \times g$ for 10 min. The pellet was resuspended in distilled water, and

then dialyzed for 40 h at 4°C against three changes of PBS. The protein concentration of the final dialyzate was 1.0 mg mL^{-1} . This stock of biotinylated anti-BN was stored at 4°C until use.

Preparation of Food Samples

Food samples were purchased from local retailers. In preparing the BN-inoculated samples for analysis, the following protocol was used: solid samples such as cookies, granola bars, breakfast cereals, veggie (tofu) dogs and tofu burgers were ground to a granular consistency (or a mash in the case of the veggie dogs and tofu burger) using a mortar and pestle. Cake mix, solid chocolate and ice cream samples were not ground. Samples (1g) were transferred to $18 \times 150 \text{ mm}$ disposable glass test tubes, and then inoculated with $100 \mu\text{l}$ of extraction buffer (PBS) with 0.1% (v/v) Tween 20 and 4% (w/v) skim milk powder containing various quantities of BN protein BN to give a range of concentrations from 0–10 ppm. After 5–10 min to allow for absorption of the inoculum, 5 ml of extraction buffer was added to each tube, followed by vortexing and incubation for 5 min in a 50°C water bath. On removal from the bath each tube was vortexed for 30 s, and then allowed to sit for 30 min at room temperature (with occasional vortexing). A 1 ml portion of suspension was then transferred to a 1.5 ml-capacity microcentrifuge tube, and the solids were sedimented by centrifugation at $10\,000 \times g$ for 5 min. The clarified supernatant was then recovered for use in the assay.

BN EIA Procedure

The wells of a microtiter plate (DiaMed Lab Supplies, Inc., Ontario, Canada, cat. no. 290–8115–01F) were coated with $200 \mu\text{l}$ of anti-BN at $1.5 \mu\text{g mL}^{-1}$ in PBS, and incubated for 16 h at 37°C, followed by washing 8 times with PBS containing 0.05% (v/v) Tween 20 (PBST). All subsequent incubations were carried out at room temperature. The wells were then incubated for 60 min with $100 \mu\text{l}$ of test sample, followed by washing with PBST as above. Bound BN proteins were detected by incubating each well for 30 min with $100 \mu\text{l}$ of biotinylated anti-BN diluted 1:200 in PBST containing 0.5% (w/v) blocking protein (Biorad Laboratories, CA, USA, cat. no. 170–6404) (PBST-B), followed by washing with PBST, and then a further 30 min incubation with streptavidin-peroxidase conjugate (Sigma Chemical Co., MO, USA, cat. no. S5512) diluted 1:1500 in PBST-B. After a final wash with PBST, each well was incubated for 30 min with $100 \mu\text{l}$ of tetramethylbenzidine (TMB) microwell peroxidase substrate (Kirkegaard and Perry Laboratories, MD, USA, cat. no. 50–76–04), and the reaction stopped by the addition of $50 \mu\text{l}$ of $1 \text{ M-H}_2\text{SO}_4$. The absorbance in the wells was read at 450 nm (A_{450}) using a scanning microtiter plate reader.

RESULTS AND DISCUSSION

Detection Limit and Specificity of BN EIA

The detection of BN in foods for monitoring purposes or traceback investigations requires a sensitive assay which is specific for the target analyte. The minimum quantity of BN proteins detectable by the BN EIA was determined by subjecting solutions containing different concentrations of BN in extraction buffer to the assay. The amount of color development (A_{450} value) in the microtiter plate wells increased in proportion with the concentration of BN in the test solutions, with the assay producing a positive response (significant color development above background) with as little as $0.015\text{--}0.030 \mu\text{g mL}^{-1}$ (or ca. $1.5\text{--}3.0 \text{ ng}$ per well) (Fig. 1). The specificity of the assay was in part assessed by challenging the BN EIA with protein extracts from a variety of oilseeds containing 2 S albumins analogous to the BN allergen (Sun *et al.*, 1987). None of the oilseed extracts tested produced a significant signal (A_{450} value above the negative control value + 3 standard deviations) in the BN EIA (Table 1), thus confirming the specificity of the assay for the BN allergen. While the minimum dose of BN protein required to provoke an allergic response in sensitive individuals is not known,

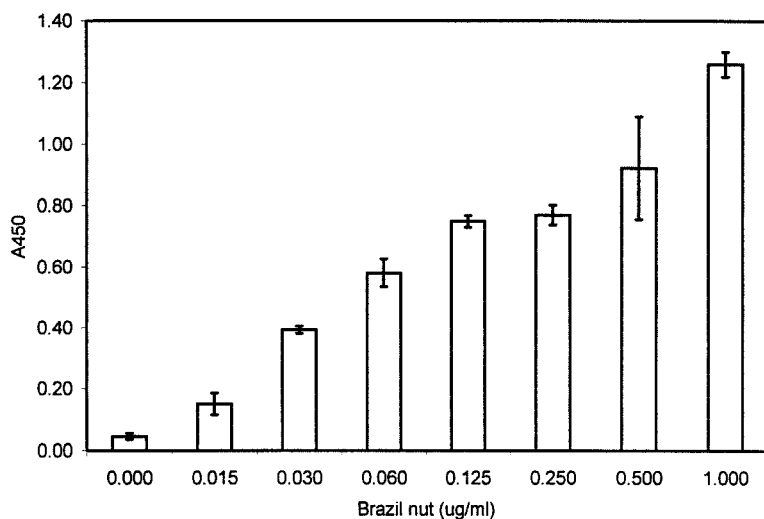


FIG. 1. Effect of varying BN protein concentration on EIA signal. Different concentrations of BN in extraction buffer were subjected to the BN EIA procedure. The results are expressed as mean $A_{450} \pm$ standard deviation ($n = 3$).

the detection limit of the present assay is in the same range as reported for other allergen detection methods (for a review, see Zarkadas *et al.*, 1999). Hence, it may be assumed that the BN EIA is sufficiently sensitive and specific to allow the detection of clinically relevant levels of BN allergens in foods.

Detection of BN Proteins in a Variety of Food Matrices

BN are used in a wide variety of confections and to supplement foods deficient in sulfur-containing amino acids. Foods are fairly heterogeneous with respect to composition, and the food matrix may affect the ability of the assay to detect BN proteins. Therefore, it was

TABLE 1. Specificity of the BN EIA^a

Sample	A_{450}^b
Sunflower seed	0.08 ± 0.02
Cucumber seed	0.06 ± 0.01
Castor bean	0.06 ± 0.01
Hazelnut	0.04 ± 0.01
Peanut	0.05 ± 0.01
Positive control ^c	1.43 ± 0.10
Negative control ^c	0.06 ± 0.01

^a Various oilseeds were ground and proteins extracted following the procedure used in the preparation of food samples, as described in Methods. The extracts were diluted ca. 10–50-fold (depending on the oilseed) with extraction buffer to adjust proteins to a final concentration of 1 mg ml^{-1} . The extracts were then subjected to the BN EIA. Mean absorbance at 450 nm $\pm$ standard deviation ($n = 3$). Positive control was BN at $1.0 \text{ } \mu\text{g ml}^{-1}$ in extraction buffer. Negative control was extraction buffer.

TABLE 2. Effect of food matrix on the detection of Brazil nut proteins^a

Sample	Brazil nut (ppm) ^b						
	0.00	0.13	0.25	0.50	1.00	5.00	10.00
Milk chocolate	0.04 ± 0.01	0.07 ± 0.01	0.10 ± 0.01	0.21 ± 0.02	0.24 ± 0.03	0.32 ± 0.04	0.52 ± 0.05
Almond granola bar	0.02 ± 0.00	0.08 ± 0.01	0.10 ± 0.02	0.11 ± 0.03	0.02 ± 0.04	0.21 ± 0.04	0.35 ± 0.06
Chocolate ice cream	0.03 ± 0.00	0.09 ± 0.02	0.11 ± 0.02	0.15 ± 0.02	0.22 ± 0.02	0.43 ± 0.04	0.91 ± 0.09
Honey nut breakfast cereal	0.09 ± 0.03	0.10 ± 0.02	0.17 ± 0.02	0.21 ± 0.02	0.32 ± 0.03	0.50 ± 0.05	0.89 ± 0.04
Cake mix	0.03 ± 0.00	0.10 ± 0.01	0.20 ± 0.01	0.29 ± 0.02	0.38 ± 0.03	0.68 ± 0.03	1.10 ± 0.07
Apple crisp granola bar	0.03 ± 0.00	0.13 ± 0.02	0.17 ± 0.02	0.25 ± 0.04	0.35 ± 0.06	0.39 ± 0.06	0.42 ± 0.04
Muesli breakfast cereal	0.02 ± 0.00	0.14 ± 0.02	0.20 ± 0.03	0.21 ± 0.04	0.32 ± 0.05	0.36 ± 0.06	0.51 ± 0.04
Oatmeal cookies	0.03 ± 0.00	0.09 ± 0.02	0.11 ± 0.02	0.16 ± 0.02	0.20 ± 0.03	0.30 ± 0.05	0.66 ± 0.03
Veggie dog	0.00 ± 0.00	0.16 ± 0.02	0.20 ± 0.02	0.25 ± 0.02	0.43 ± 0.03	0.90 ± 0.04	1.28 ± 0.15
Tofu burger	0.03 ± 0.00	0.09 ± 0.02	0.10 ± 0.02	0.11 ± 0.02	0.20 ± 0.01	0.25 ± 0.02	0.72 ± 0.18

^a Various foods were spiked with different concentrations of BN, and then subjected to the sample preparation and BN EIA procedure. The concentrations of spiked BN are expressed as parts per million (ppm) in the starting food sample.

^b Mean absorbance at 450 nm. Standard deviation (*n* = 3) is given in parentheses.

necessary to ascertain the performance of the BN EIA in the assay of BN proteins inoculated into different food matrices. The detection limit of the assay varied in the presence of different types of foods (Table 2). Using an A_{450} value of 0.2 as the cut-off for positive signals, the assay exhibited a limit of detection of 0.25 ppm in the presence of cake mix, soy-based veggie dog and muesli breakfast cereal; 0.5 ppm in milk chocolate, honey nut breakfast cereal and apple crisp granola bar; and 1.0 ppm in chocolate ice cream, almond granola bar, oatmeal cookies and tofu burger. The signals obtained with the (unspiked) food samples devoid of BN proteins were generally negligible, though in some instances (e.g. honey nut breakfast cereal) higher background values were observed. None of the matrices gave values exceeding the cut-off for positives ($A_{450} = 0.2$).

These studies demonstrate the applicability of the BN EIA as a sensitive and specific assay for BN proteins in a variety of foods. In its present form the assay is intended to be qualitative, due to the variability of the dose-response characteristics observed with different food matrices, which make the BN EIA unreliable as a quantitative assay. This should not detract from the usefulness of the assay, since at present there are no criteria established for minimum quantities of BN proteins provoking an allergic reaction (i.e. any amount of undeclared BN proteins in foods should be considered significant). The use of IgY as immunoreagents in the assay has several advantages, including the low cost of producing antibodies in chickens, the avoidance of discomfort to the animals in harvesting the antibodies from eggs, and the high antibody yields obtained from a single round of immunizations. Furthermore, the antibodies used in these experiments were obtained by contract with a commercial producer, obviating the need for animal facilities at the laboratory. These attributes place this assay within easy reach of most laboratories wishing to test for BN proteins in foods.

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