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Effects of Processing on Immunoreactivity of Cashew Nut (*Anacardium occidentale* L.) Seed Flour Proteins

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Cashew nut seeds were subjected to processing including autoclaving (121 °C for 5, 10, 20, and 30 min), blanching (100 °C for 1, 4, 7, and 10 min), microwave heating (1 and 2 min each at 500 and 1000 W), dry roasting (140 °C for 20 and 30 min; 170 °C for 15 and 20 min; and 200 °C for 10 and 15 min), γ-irradiation (1, 5, 10, and 25 kGy), and pH (1, 3, 5, 7, 9, 11, and 13). Proteins from unprocessed and processed cashew nut seeds were probed for stability using anti-Ana o 2 rabbit polyclonal antibodies and mouse monoclonal antibodies directed against Ana o 1, Ana o 2, and Ana o 3 as detection agents. Results indicate that Ana o 1, Ana o 2, and Ana o 3 are stable regardless of the processing method to which the nut seeds are subjected.

KEYWORDS: Cashew nut; seed; proteins; antibodies; processing; tree nut allergy

INTRODUCTION

Tree nuts are one of the "big eight" food groups responsible for food allergies. According to a Food Allergy and Anaphylaxis Network (FAAN) survey, cashew nut allergies are the second most commonly reported tree nut allergies (20% of the total allergies in the survey) in the United States (*1*). A random digital dial telephone survey indicated that up to 41% of individuals with tree nut hypersensitivity are allergic to cashew nuts (*2*). Anaphylaxis upon exposure to cashew nut seeds was more prevalent (74.1%) compared to peanut-induced anaphylaxis (30.5%) in a recent study (*3*). Clark et al. (*4*) compared children suffering from allergies to cashew nut (47 subjects) versus peanut (94 subjects) and found that symptoms caused by cashew nuts were more severe than those caused by peanuts. The subjects in the study were matched for clinical symptoms (except for asthma symptoms). Cashew nuts are widely used in snack foods and as an ingredient in a variety of processed foods such as "butters", bakery, and confectionery products. With their global popularity and increased use, risk of inadvertent exposure to cashew nut seeds is likely to increase.

Currently there are no methods available to cure tree nut allergies. Avoidance of the offending agent is therefore the best way to protect sensitive individuals from unwarranted and unintended exposure. Such avoidance is, however, not always possible for a variety of reasons including cross-contamination, undeclared presence, accidental contamination through improper cleaning or sharing of equipment, or improper handling, storage, and transportation. Developing simple, specific, robust, accurate, and reproducible detection methods is therefore essential to safeguard sensitive individuals from unintended exposure. To this end, we have developed a rabbit polyclonal antibody (pAb) based sensitive sandwich ELISA for the detection of trace amounts of cashew nuts (*5*).

Cashew nut proteins are primarily composed of albumins (45.59%) and globulins (42.37%) with smaller portions of glutelins (11.68%) and prolamins (0.36%) (*6*). The major storage globulin in cashew nut seeds is known as anacardein, cashew major protein (CMP), and Ana o 2. It is mainly composed of 30−32 and 20−22 kDa polypeptides and may account for up to 50% of soluble proteins in the cashew nut seed (*7*). Garcia et al. (*8*) found 15, 30, and 60 kDa proteins reacted strongly when soluble cashew nut seed proteins were tested, using Western blotting, against sera IgE of three patients known to suffer from anaphylactic reactions to cashew nut seed. The authors suggested that the 15 kDa protein was a member of the 2S albumin family, but no confirmatory data were provided. Earlier, we had demonstrated that the major IgE-reactive proteins in aqueous protein extracts prepared from defatted cashew nut flour are legumin-like proteins and 2S albumins as assessed by N-terminal and enzymatic fragment sequencing of native

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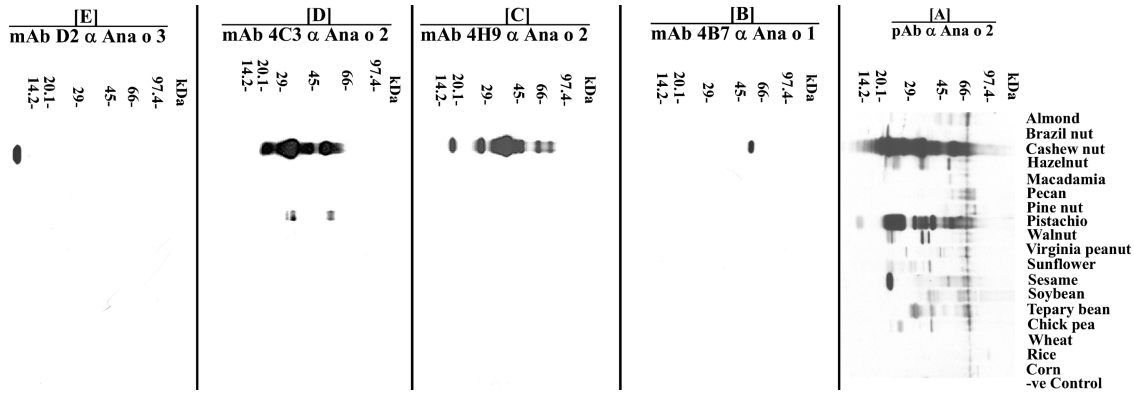


Figure 1. Antibody specificity of select mAbs assessed using Western blotting: (**A**) rabbit pAb (anti-Ana o 2); (**B**) mAb 4B7 (anti-Ana o 1); (**C**) mAb 4H9 (anti-Ana o 2); (**D**) mAb 4C3 (anti-Ana o 2); (**E**) mAb D2 (anti-Ana o 3). Protein load in each lane = 30 μg. Negative control was secondary antibody (goat anti-rabbit IgG for pAb and goat anti-mouse IgG for mAbs).

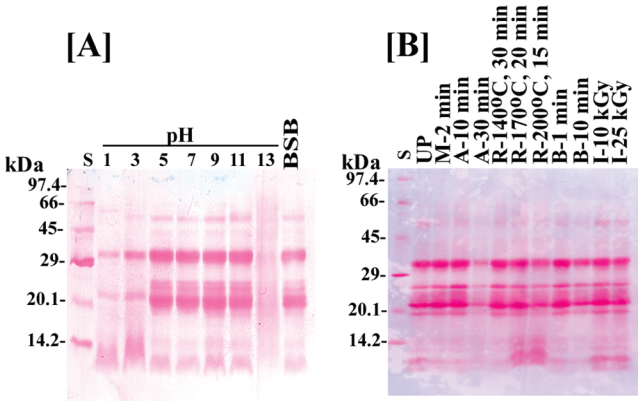


Figure 2. Ponceau S staining of representative of protein extracts prepared from seeds exposed to (**A**) desired pH and (**B**) processing treatment, transferred onto a NC membrane for Western blotting. Protein extracts were prepared from defatted flours of raw and processed cashew nut seeds using 0.1 M BSB (pH 8.45) as the solvent. S, protein standards used as molecular mass markers (molecular mass kDa indicated in the left margin); UP, unprocessed; M, microwaved; A, autoclaved; R, roasted; B, blanched; I, irradiated; as indicated. Protein load in each lane = 30 μg. Note the qualitative stability of polypeptide profile in all, except the proteins exposed to pH 13.

proteins (*9*). Subsequently we have completed cloning, expression, and epitope mapping of three major cashew nut allergens; a 55 kDa legumin protein, Ana o 2 (*10*); a 50 kDa 7S vicilin protein, Ana o 1 (*11*); and, more recently, a 12.6 kDa 2S albumin, Ana o 3 (*12*).

It is generally recognized that individuals allergic to a particular food or food ingredient may respond to one or more allergens present in that food or food ingredient. The strength of such response partly depends on the allergen, the sensitivity of an individual to the specific allergen, and the stability of the targeted allergen. Previous reports have noted such differential responses when aqueous buffer soluble proteins from defatted almond (*13*), cashew nut (*9*), and walnut flours (*14*) were probed (Western blotting) with sera IgE from patients allergic to almonds, cashew nuts, and walnuts, respectively. These observations raise the possibility that within a given food source one may encounter one or more allergens, each of which may elicit different and unequal allergic responses depending on the patient. Depending on the stability of the targeted allergen in a specific food that was previously subjected to processing, potential processing-induced alteration in the immunogenicity of the allergen may compromise detection method(s), data interpretation, or both. For these and several other reasons, it is important to learn about the stability of the targeted allergens in raw and processed foods. Tree nuts are often subjected to a variety of food processing conditions that may cause protein denaturation and aggregation resulting in epitope (linear, conformational, or both) modification, thereby altering their immunoreactivity. Such alterations in a potential allergen, in turn, have practical implications in clinical as well as analytical settings. Earlier, using rabbit polyclonal antibody (pAb) based immunoassays, we demonstrated the stability of proteins from almond, cashew nut, walnuts (*15*), and pecans (*16*) that were subjected to a variety of processing conditions including γ-irradiation alone or in combination with different thermal processing regimens. However, use of rabbit pAbs in the studies did not permit assessment of the stability of specific allergens. Monoclonal antibodies (mAbs) afford investigation of specific allergen as mAbs target a specific epitope on an allergen. We have now developed mouse mAbs specific for the three cashew nut allergens, Ana o 1, Ana o 2, and Ana o 3, and report our findings on the effects of processing on their stability.

MATERIALS AND METHODS

Materials. Cashew nut seeds were purchased from local grocery stores in Sarasota, FL (two samples), and Tallahassee, FL (one sample), flushed with nitrogen, and stored at −20 °C until further use. Sources of chemicals have been reported earlier (*15, 16*).

Methods. *Nut Processing.* Duplicate seed samples, 10−20 g each, were subjected to various processing treatments as described below.

(a) γ-Irradiation. Whole, natural, unprocessed cashew nut seeds were subjected to γ-irradiation at 1, 5, 10, and 25 kGy at Food Technology Service Inc., Mulberry, FL. The effective γ-irradiation doses received by the samples were 1.293, 5.826, 10.842, and 25.941 kGy, respectively.

(b) Thermal Processing. Whole, natural, unprocessed cashew nut seeds were subjected to several thermal processing treatments as described below.

1. Pressure cooking was performed in an autoclave at 121 °C, 15 psi, for 5, 10, 20, and 30 min. Autoclaved samples were air-dried at room temperature (RT, 25 °C) in a fume hood until a constant weight was obtained.

2. Blanching treatment was performed in boiling water (100 °C) for 1, 4, 7, and 10 min. The ratio of nut seeds to water was 1:10 w/v. Samples were gently dried with paper towels and further air-dried in a fume hood at RT until a constant weight was obtained. Blanch water was analyzed for soluble protein using the Bradford assay (*17*) and the immunoreactivity of soluble proteins by Western blotting.

3. Microwave heating was done in a Panasonic microwave oven (Panasonic Co., Secaucus, NJ) at 50% power (500 W) and 100% power (1000 W) for 1 and 2 min each.

4. Dry roasting was performed at 140 °C for 20 and 30 min, at 170 °C for 15 and 20 min, and at 200 °C for 10 and 15 min each. Samples

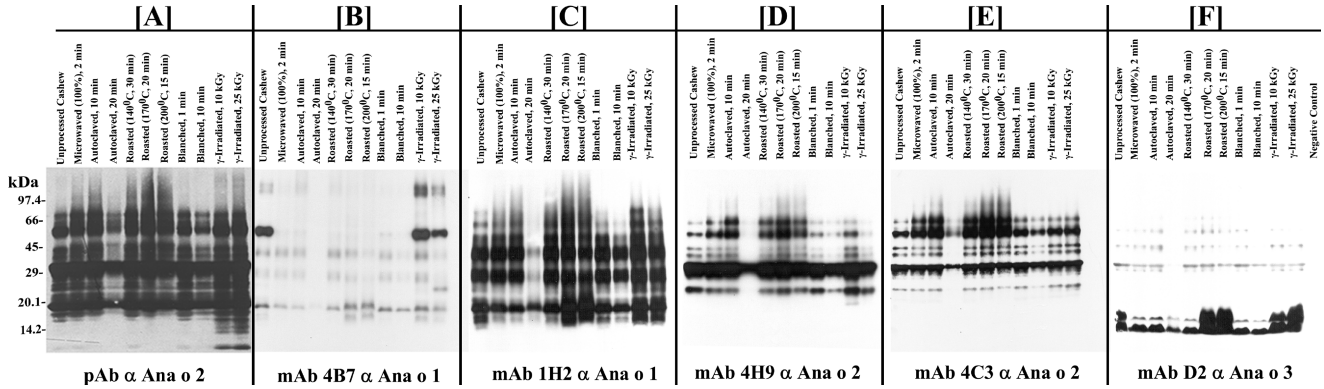


Figure 3. Effects of processing on cashew nut immunoreactivity assessed by Western blotting: (**A**) rabbit pAb (anti-Ana o 2), included for comparative purposes; (**B**) mAb 4B7 (anti-Ana o 1); (**C**) mAb 1H2 (anti-Ana o 1); (**D**) mAb 4H9 (anti-Ana o 2); (**E**) mAb 4C3 (anti-Ana o 2; (**F**) mAb D2 (anti-Ana o 3). Protein load in each lane = 30 μg. Note the overall stability of polypeptide profiles except when probed with 4B7.

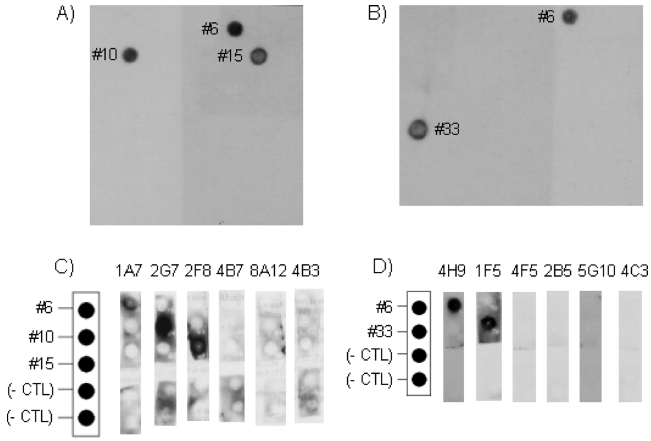


Figure 4. Immunoblots of Ana o 1 and Ana o 2 SPOTs membranes with pooled mAbs: (**A**) 3 of the 69 overlapping Ana o1 specific peptides (no. 6, 10, and 15) showed mAb reactivity; (**B**) 2 of the 58 overlapping Ana o 2 specific peptides (no. 6 and 33) showed mAb reactivity; (**C**) mAb 1A7 was solely responsible for binding to the Ana o 1 peptide (no. 6), mAb 2G7 for the binding of no. 10, and mAb 2F8 for the binding of no. 15; (**D**) mAbs 4H9 and 1F5, respectively, bound to Ana o 2 peptides 6 and 33.

were placed in ceramic bowls and subjected to roasting in an oven previously set at the desired temperature (Thermolyne Corp., Subsidiary of Sybron Corp., Dubuque, IA).

(c) pH Exposure. Defatted cashew nut seed flours (not subjected to γ-irradiation or thermal processing treatments) were dispersed in distilled deionized water (flour-to-water ratio of 1:10 w/v), the pH was adjusted to the desired value with 1.0 M HCl and/or NaOH, and the mixture was magnetically stirred for 3 h at RT, neutralized to pH 7.0, and centrifuged (16100*g*, RT, 15 min); the supernatants were collected and stored at 4 °C in airtight containers until further use. Exposure time of 3 h to the desired pH was chosen arbitrarily. Supernatants were analyzed within 120 h.

Unprocessed nut seeds (no γ-irradiation or any thermal treatment) were used as controls.

Preparation of Cashew Nut Seed Flours, Protein Extracts, and Reference Proteins. All seed samples, controls and processed, were powdered manually using a mortar and pestle to about 40 mesh, and the resultant flours were defatted for 6 h using a Soxhlet apparatus (Fisher Scientific Co., Orlando, FL) with petroleum ether as a solvent (flour-to-solvent ratio of 1:10 w/v, boiling point range of 38.2−54.3 °C). Defatted flours were air-dried in a fume hood, powdered again using a mortar and pestle (to obtain a homogeneous sample of ∼40 mesh), and stored in screw-capped plastic vials at −20 °C until further use.

Borate saline buffer (BSB, 0.1 M H_3BO_3, 0.025 M $Na_2B_4O_7$, 0.075 M NaCl, pH 8.45) was used for routine protein extractions. Typically, defatted flours were extracted with BSB buffer (flour-to-buffer ratio of 1:10 w/v) at RT for 1 h with vortex mixing (Vortex Genie 2, Scientific Industries Inc., Bohemia, NY). Samples were centrifuged (10 min, 16100*g*, RT) in an Eppendorf tabletop microcentrifuge (model 5415D, Brinkmann Instruments, Inc., Westbury, NY), and supernatants were stored at −20 °C until further use.

Rabbit pAb, Goat pAb, and Mouse mAb Production. Rabbit and goat pAb production has been described earlier (*5*). Mouse mAbs were produced using standard procedures (*18*). Briefly, pairs of mice (BALB/c) were immunized with 40 μg of native cashew nut seed protein extracts in RIBI adjuvant (RIBI ImmunoChem Research Inc., Hamilton, MT), boosted with 20 μg of protein in RIBI adjuvant at 3 week intervals, and given a final injection of 25 μg of protein in saline equally split between the intravenous and subcutaneous routes. Following fusion, the resultant hybridomas were screened for relative strength of reaction to cashew nut seed protein extract by direct binding enzyme-linked immunosorbent assay (ELISA) and immunoblotting (*19*). Appropriate clones recognizing the targeted allergen were selected and further screened for specificity by Western blotting.

ELISA. For the detection of total cashew proteins, sandwich ELISAs using goat pAbs (raised against total cashew proteins) and anti-Ana o 2 rabbit pAbs were done as previously described by Wei et al. (*5*). For the specific detection of Ana o 2 and Ana o 3, suitable mAbs (4C3 for Ana o 2 and D2 for Ana o 3) were used for the development of sandwich ELISAs. The optimized assay conditions were as follows. Microtiter plates (96 wells, polyvinyl, Fisher Scientific Co., Philadelphia, PA) were coated with 200 ng/well of immunosorbed (protein G) goat anti-whole cashew pAbs (diluted in coating buffer: 48.5 mM citric acid, 103 mM Na_2HPO_4, pH 5) as the capture antibody and then blocked as previously described (*5*). Protein extracts and appropriate controls (unprocessed cashew protein) in 1% (w/v) nonfat dry milk (NFDM) in TBS-T (10 mM Tris, 0.9% w/v NaCl, 0.05% v/v Tween 20, pH 7.6) were added to the top row of the coated plate at a concentration of 250 μg/mL and serially diluted (10-fold) in the next six rows. Aliquots (50 μL) of the suitably diluted mAb were used for the detection and quantification of the targeted allergen. Alkaline phosphatase (AP) labeled secondary antibody (anti-mouse rabbit pAb, 1:5000 v/v dilution) was used for the detection of bound mAbs. Color was developed by adding 50 μL of substrate *p*-nitrophenyl phosphate [(1 PNPP tablet per 5 mL of AP buffer (pH 9.8)] to each well. Color development was stopped by the addition of 50 μL of 3.0 M NaOH to each well. Immunoreactivity of processed sample was expressed as percent reactivity of the corresponding unprocessed control.

Electrophoresis, Western Blotting, and Dot Blotting. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting experiments were performed as previously described (*20*). Dot blotting assays were done as follows: protein extract (1 μg/dot) was placed on a nitrocellulose (NC) paper (0.2 μm pore size) and allowed to dry (37 °C, 10 min). The NC paper containing the dried protein dots was rehydrated with TBS-T, blocked with 5% (w/v) NFDM in TBS-T at RT for 1 h with rocking, washed four times with TBS-T (once for 15 min and three times for 5 min each), and incubated

Table 1. Stability of Cashew Nut Allergens Using mAb-Based Dot Blotting Assays[a]

	% immunoreactivity relative to unprocessed cashew extract				
	Ana o 1		Ana o 2		Ana o 3
processing treatment	1H2	4B7	4C3	4H9	D2
unprocessed (control)	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
autoclaving (121 °C)					
10 min	93.7 ± 4.8	62.5 ± 5.1	99.9 ± 9.0	37.7 ± 22.5	73.5 ± 9.1
30 min	69.87 ± 1.1	29.1 ± 7.2	85.1 ± 3.4	50.8 ± 34.9	26.5 ± 4.9
blanching (100 °C)					
1 min	64.92 ± 11.2	32.5 ± 5.2	99.7 ± 5.6	92.7 ± 26.8	74.6 ± 2.5
10 min	64.1 ± 3.2	34.2 ± 3.6	99.3 ± 7.6	86.2 ± 29.8	71.7 ± 10.2
microwaving, 100%, 2 min	99.0 ± 2.1	68.2 ± 3.6	99.6 ± 9.0	93.2 ± 23.6	81.9 ± 5.7
roasting					
140 °C, 30 min	83.3 ± 6.6	58.5 ± 2.1	90.0 ± 4.2	93.0 ± 34.9	28.0 ± 2.5
170 °C, 20 min	74.0 ± 5.8	53.5 ± 7.9	94.8 ± 1.7	104.8 ± 52.0	51.0 ± 2.3
200 °C, 15 min	74.6 ± 5.8	53.2 ± 7.5	108.1 ± 1.9	158.5 ± 71.3	88.9 ± 4.2
γ-irradiation					
10 kGy	93.5 ± 0.5	76.4 ± 5.3	130.1 ± 9.5	112.3 ± 16.5	109.8 ± 11.1
25 kGy	141.7 ± 3.4	73.8 ± 1.3	122.0 ± 10.0	151.9 ± 12.4	98.2 ± 7.8
R	0.98	0.99	0.97	0.93	0.95
$y = mx + c$	$550.7x + 317.2$	$674.3x + 172.9$	$602.3x + 802.4$	$281.1x + 192.6$	$501.9x + 465.6$
LSD	11.60	11.53	15.07	75.04	14.73

[a] Data are expressed as mean ± standard error of mean (SEM, σ_{n-1}), $n = 3$. Differences between means exceeding the corresponding LSD value (in the same column) are significant ($p = 0.05$). Percent immunoreactivity relative to defatted (unprocessed) cashew nut seed flour extract calculated as (average dot intensity from processed samples/average dot intensity of unprocessed sample) ×100. $y = mx + c$ is the respective linear regression equation for the corresponding standard curve. R = correlation coefficient.

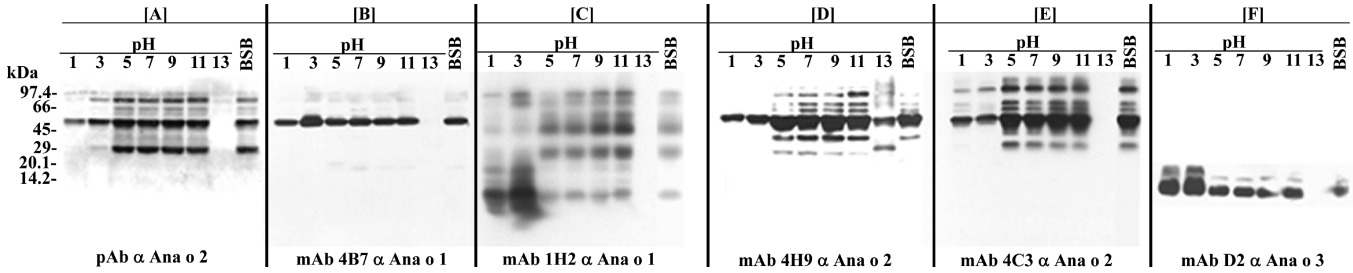


Figure 5. Effects of pH on cashew nut protein immunoreactivity assessed using Western blotting: (**A**) rabbit pAb (anti-Ana o 2); (**B**) mAb 4B7 (anti-Ana o 1); (**C**) mAb 1H2 (anti-Ana o 1); (**D**) mAb 4H9 (anti-Ana o 2); (**E**) mAb 4C3 (anti-Ana o 2); (**F**) mAb D2 (anti-Ana o 3). Protein load in each lane = 30 μg. With the exception of pH 13 exposure, note the stability of polypeptide profiles.

overnight at 4 °C with the suitably diluted (in TBS-T) primary antibody (1:10000 for pAb; 1:1000 for mAbs 4B7, 1H2, 4H9, and 4C3; and 1:250 for mAb D2; all v/v). NC strips were washed as described above and incubated with suitably diluted appropriate secondary antibody [1:10000 v/v goat anti-mouse and 1:40000 v/v goat anti-rabbit horseradish peroxidase (HRP) labeled] for 1 h at RT with rocking and then washed again as described above. Reactive dots were developed using the luminol/*p*-coumaric acid substrate system and exposed to X-ray film (Kodak X-OMAT, Rochester, NY).

Solid-Phase Peptide (SPOTs) Synthesis and Blotting. SPOTs membranes for Ana o 1, Ana o 2, and Ana o 3 were synthesized as previously described (*10*−*12*). Western blotting of Ana o 1 and Ana o 2 membranes after overnight blocking in the blocking buffer, supplied with the manufacturers' SPOTs kit, was carried out with pooled mAbs (pools consisted of six mAbs, each showing specific reactivity to the recombinant protein in dot blotting assays) at a dilution of 1:100 (v/v) for each mAb. For Ana o 3, immunoblotting was only performed with mAb D2. Primary antibody incubation was continued overnight at 4 °C and washed with TBS-T followed by incubation with HRP-labeled goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 h at RT. After washing of the SPOTs membranes, reactive SPOTs were detected using ECL chemiluminescent substrate (Amersham Pharmacia, Piscataway, NJ) and subsequent exposure to X-ray film (Kodak X-OMAT). Those SPOTs that were identified as reactive, after probing with pooled mAbs, were synthesized along with negative controls on individual strips for both Ana o 1 and Ana o 2 to test for the reactivity of each individual mAb (**Figure 4C**,**D**).

Protein Determination. Soluble protein content was determined according to the method of Lowry et al. (*21*) and/or the Bradford assay (*17*). BSA standard curves were prepared (for each assay) in appropriate buffer, and suitable blanks were used in all assays.

Statistical Analyses. Appropriate data were analyzed for statistical significance using ANOVA (SPSS for Windows 2003, Microsoft Corp., version 13.0, Chicago, IL) and Fisher's least significant difference (LSD, $p = 0.05$) test.

RESULTS AND DISCUSSION

Antibody Specificity. Although sera IgE from allergic patients can be used to directly detect allergenic proteins, variable reactivity profiles between patients, the very low level of specific IgE, and the several issues relating to acquisition and use of human serum preclude its use for screening purposes. A more practical approach is to use pAbs (usually IgG) generated in animals (e.g., rabbit, goat, sheep) for routine analysis and testing. However, lot-to-lot variability and the mix of affinities and specificities can make standardization difficult. We have previously developed pAbs for Ana o 2 detection and found them to be sensitive (detection level was 20 ng/mL) (*22*). However, even after extensive adsorption, the pAbs exhibited cross-reactivity with several nontargeted proteins including hazelnut, pistachio, walnut, and sesame exhibited stronger cross-reactivity compared to others (**Figure 1A**). Cross-reactivity of

Table 2. Stability of Cashew Nut Allergens Using Sandwich ELISA[a]

processing treatment	% recovery, mean ± SEM		
	rabbit pAb	mAb	
	α Ana o 2	D2	4C3
unprocessed (control)	100.00 ± 5.18	100.00 ± 0.00	100.00 ± 0.00
autoclaving (121 °C)			
5 min	76.50 ± 2.63	nd	nd
10 min	80.17 ± 3.60	43.06 ± 5.16	51.14 ± 3.01
20 min	85.67 ± 6.52	nd	nd
30 min	84.33 ± 3.61	71.06 ± 4.49	19.03 ± 0.97
blanching (100 °C)			
1 min	57.67 ± 2.99	117.57 ± 33.93	86.22 ± 7.84
4 min	64.00 ± 5.27	nd	nd
7 min	68.17 ± 2.39	nd	nd
10 min	75.17 ± 3.30	44.27 ± 0.05	55.31 ± 1.80
microwaving			
100% power, 1 min	183.33 ± 45.68	nd	nd
100% power, 2 min	186.50 ± 44.62	75.79 ± 5.82	90.21 ± 7.30
50% power, 1 min	65.17 ± 2.47	nd	nd
50% power, 2 min	75.17 ± 1.54	nd	nd
roasting[b]			
140 °C, 20 min	89.00 ± 4.96	nd	nd
140 °C, 30 min	107.50 ± 3.89	51.85 ± 14.24	123.01 ± 30.50
170 °C, 15 min	71.17 ± 5.70	nd	nd
170 °C, 20 min	71.00 ± 2.49	34.01 ± 4.80	24.01 ± 3.73
200 °C, 10 min	86.17 ± 6.11	nd	nd
200 °C, 15 min	87.00 ± 7.30	nd	7.13 ± 1.10
γ-irradiation			
1 kGy	73.50 ± 1.69	nd	nd
5 kGy	80.50 ± 1.91	nd	nd
10 kGy	84.67 ± 4.24	74.27 ± 2.44	84.67 ± 10.67
25 kGy	85.67 ± 3.55	82.65 ± 5.58	79.51 ± 17.73
pH			
1	0.97 ± 0.10	0.01 ± 0.00	0.05 ± 0.01
3	133.33 ± 9.48	0.03 ± 0.02	21.06 ± 1.87
5	84.18 ± 3.37	118.31 ± 21.64	162.01 ± 22.11
7	81.33 ± 3.93	164.09 ± 36.82	154.06 ± 34.97
9	131.29 ± 13.5	61.92 ± 9.51	198.76 ± 36.18
11	157.45 ± 21.9	93.97 ± 14.04	192.78 ± 33.04
13	0.07 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
LSD	30.52	36.29	34.27

[a] Data are expressed as immunoreactivity percent recovery, mean ± SEM ($n = 6$ for pAb, $n = 2$ for D2, and $n = 3$ for 4C3). nd, not determined. Difference between two means exceeding the LSD value is significant ($p = 0.05$). [b] Cashew samples heated at 200 °C for 15 min were also tested using mAb D2 with ELISA and dot blots. However, the results were significantly different from those of the rest of the processing treatments and hence were not included for statistical analysis.

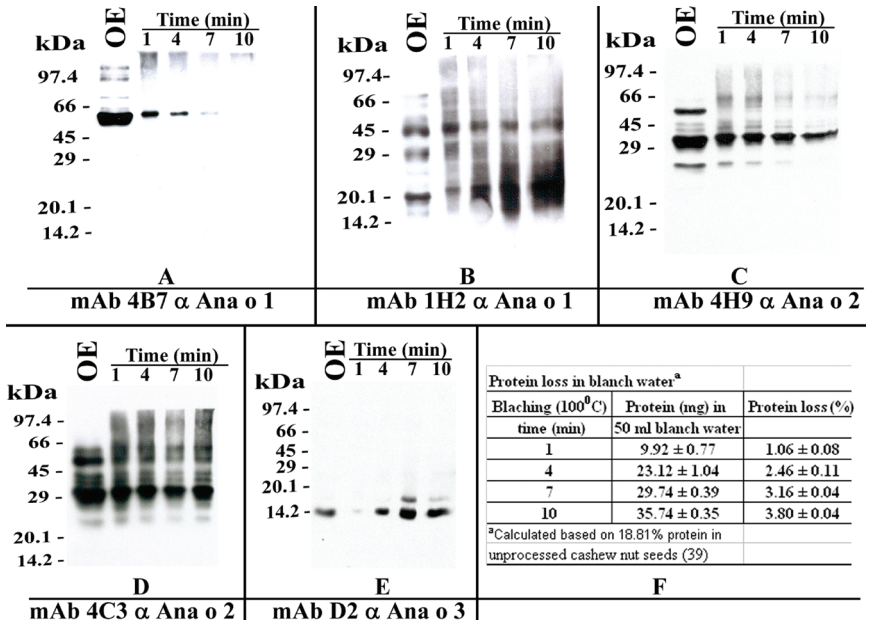
anti-Ana o 2 rabbit pAbs in the current investigation is consistent with the pAb and IgE cross-reactivity between cashew, pistachio, and walnut proteins reported earlier (*22−25*).

In contrast, the selected mAbs were specific for their targeted antigens (**Figure 1B−E**). The only exception was mAb 4C3 (**Figure 1D**), which exhibited cross-reactivity with certain polypeptides of pistachio proteins. Cross-reactivity of anti-Ana o 2 rabbit pAbs and mAb 4C3 with pistachio polypeptides (range = 20−55 kDa; **Figure 1A**,**D**) was not surprising as both cashew and pistachio belong to the Anacardeceae family. Tawde (*25*) investigated the cross-reactivity between cashew and pistachio proteins using immunoblotting. Total pistachio protein extract was probed with rabbit anti-CMP antisera and pooled cashew and tree nut allergic patient sera. The immunoreactivity of pistachio proteins analyzed with rabbit anti-CMP antisera demonstrated cross-reactivity between cashew and pistachio protein bands at 31−35 and 55 kDa. The pooled cashew and tree nut allergic patient sera showed IgE reactivity to pistachio protein bands at 9−12, 25, 31−35, and 45 kDa.

Stability. With the availability of mAbs targeting Ana o 1, Ana o 2, and Ana o 3 one could investigate the stability of purified allergens (e.g., Ana o 2) after subjecting them to the desired processing conditions. The targeted protein used for stability studies may be native (i.e., isolated and purified from mature seeds), recombinant, or both. Although useful, such investigations do not assess the influence of interaction between targeted allergen and food matrix on immunoreactivity. Understanding such interactions is important as allergens are most often ingested as a component of unprocessed or processed food matrix (*44−47*).

ELISAs permit protein immunoreactivity assessment when the target protein is in solution. Western blotting typically uses SDS-PAGE to separate proteins and polypeptides. The proteins may be reduced and denatured or denatured in the absence of a reducing agent prior to SDS-PAGE. Western blotting may therefore furnish information on accessible epitopes on targeted proteins in denatured or reduced and denatured forms. Dot blotting, on the other hand, allows assessment of immunoreactvity of proteins immobilized on a solid support such as NC membrane under the desired experimental conditions. In the current investigation all three immunoassay forms were used to assess stability in the desired sample. Protein extracts prepared from defatted flours of unprocessed and variously processed cashew nut seeds were probed with select mAbs targeted against Ana o 1, Ana o 2, and Ana o 3. One concern in assessing immunoreactivity of extracted proteins from processed foods is the possible loss of protein solubility resulting from processing-induced changes in the targeted proteins. Such changes may include protein denaturation, protein aggregation, or both. The primary reasons for the concern are loss of conformational epitopes as a result of protein denaturation/aggregation, and epitopes that were otherwise stable and accessible may become inaccessible due to steric hindrance generated as a consequence of processing-induced protein unfolding/refolding/aggregation. Alternatively, one may find increased assay signal due to improved accessibility of buried epitopes due to processing induced protein unfolding leading to improved exposure of the previously inaccessible epitopes in the native protein and possible formation of processing-induced new epitopes (i.e., neogen formation). As processing-induced alterations in epitope accessibility were of concern, we routinely checked Western blotting transfers with Ponceau S staining to qualitatively assess the stability of targeted protein profile. Ponceau S staining is reportedly as sensitive as Coomassie Brilliant Blue R with detection limits of 1−2 and 1.5 μg, respectively (Hoefer Protein Electrophoresis Application Guide 1994, Hoefer Scientific Instruments, San Francisco, CA; p 91). A representative scan of a NC paper containing the transferred proteins stained with Ponceau S (**Figure 2**) demonstrated the protein polypeptide profile to be qualitatively stable for all of the tested samples with some variations in staining intensity for certain polypeptides (e.g., 30 min of autoclaving and pH 1 and 3 exposure). At pH 13, the polypeptide profile was quite diffuse, suggesting major disruption in protein structure.

Ana o 1. Ana o 1 is a vicilin (*11*). Stability of Ana o 1 was evaluated using mAbs 4B7 and 1H2. Western blotting of the processed cashew proteins (**Figure 3B**) probed with mAb 4B7 indicates that the thermal processing treatments made Ana o 1 undetectable (loss of ∼50 kDa band). Interestingly, exposure to γ-irradiation did not cause such a loss in immunorecognition. However, when probed with mAb 1H2 (**Figure 3C**), Ana o 1 polypeptides were detectable even after seeds had been subjected to thermal processing, with a notable decrease in signal intensity for only the 20 min autoclaved sample. Together, these results



Protein loss in blanch water[a]		
Blaching (100°C) time (min)	Protein (mg) in 50 ml blanch water	Protein loss (%)
1	9.92 ± 0.77	1.06 ± 0.08
4	23.12 ± 1.04	2.46 ± 0.11
7	29.74 ± 0.39	3.16 ± 0.04
10	35.74 ± 0.35	3.80 ± 0.04

[a]Calculated based on 18.81% protein in unprocessed cashew nut seeds (*39*)

Figure 6. Effects of blanching on protein loss and immunoreactivity: (**A**) mAb 4B7 (anti-Ana o 1); (**B**) mAb 1H2 (anti-Ana o 1); (**C**) mAb 4H9 (anti-Ana o 2); (**D**) mAb 4C3 (anti-Ana o 2); (**E**) mAb D2 (anti-Ana o 3). Protein load in each lane = 15 μg.

suggest that 4B7 and 1H2 targeted different epitopes of Ana o 1. In addition, the results also indicate that 4B7 is directed against a thermally unstable epitope. SPOTs assays (**Figure 4C**) demonstrated that 4B7 did not recognize any of the previously identified linear epitopes of Ana o 1 (*11*). The ability of 4B7 to detect Ana o 1 previously exposed to SDS, β-ME, and γ-irradiation indicates the epitope is not stabilized by either hydrogen bonds, ionic interactions (that would be disrupted by SDS), or disulfide bond(s). Vicilins typically lack cysteine residues and are therefore usually not stabilized by disulfide bonds (*26*). The ability of dot blotting assays (**Table 1**) to detect Ana o 1 using 4B7 in processed cashew nut seed samples suggests possible refolding of the protein on the NC paper. Protein refolding on solid supports, such as NC paper, has been reported for human C-reactive protein (*27*), grass pollen allergens (*28*), and recombinant c-Ha-ras (mammalian ras protooncogene group member) (*29*).

Except for pH 13, Ana o 1 was stable over the tested pH range (**Figure 5B**,**C**). The significance of instability at pH 13 is probably minimal as pH 13 is seldom encountered in cashew nut seed processing. The lower signal at pH 1 and 3 in Western blotting experiments (**Figure 5**) is not surprising as cashew nut seed proteins exhibit low solubility in the pH range of 1−5 compared to the pH range of 6−12. As can be seen from this figure, Ponceau S staining (**Figure 2A**) indicated lower protein solubility at pH 1 and 3 compared to at pH 5, 7, 9, and 11.

Ana o 2. Ana o 2 is a legumin (*10*). Consistent with our earlier findings (*15*), anti-Ana o 2 rabbit pAb-based sandwich ELISA data (**Table 2**) suggest the protein is stable. Additional assessments using mAb 4C3 as the detection antibody in ELISAs (**Table 2**) and mAbs 4H9 and 4C3 for detection in dot blotting (**Table 1**) confirmed the stability of Ana o 2. SPOTs assays (**Figure 4D**) indicated that mAb 4H9 was directed against the linear epitope YEAGTVAWDPNHEQ of Ana o 2. The failure of mAb 4C3 to bind with any of the tested epitopes (**Figure 4D**) suggests that 4C3 may recognize a conformational epitope. It was therefore not surprising to note a statistically significant loss in signal when mAb 4C3 was used as the detection antibody in ELISA (**Table 2**). With the exception of exposure to pH 1 and 13 that resulted in almost complete loss of immunoreactivity, blanching treatment typically caused a greater decrease in immunoreactivity when compared with the processing treatments investigated. Removal of water soluble proteins in the blanch water was the primary reason for loss of immunoreactivity when nut seeds were subjected to blanching treatment. Soluble protein loss and Western blotting analyses of the blanch water (**Figure 6F**) confirmed leaching losses of soluble proteins recognized by mAbs directed against Ana o 1 (1H2, 4B7), Ana o 2 (4H9, 4C3), and Ana o 3 (D2). Mondoulet et al. (*30*) reported a decrease in peanut protein reactivity against patient sera IgE as a result of proteins leaching out in the cook water. Extreme heat treatments such as autoclaving for 30 min and roasting at 200 °C for 15 min resulted in loss of Ana o 2 detection. Western blotting (**Figure 3D**,**E**) of samples autoclaved for 30 min probed with mAb 4H9 and 4C3 exhibited less intensity for polypeptides in the molecular mass range of 30−66 kDa. Ana o 2 was stable regardless of the assay format and detection antibody used. Only extreme processing (e.g., pH 1, autoclaving for 30 min) conditions seemed to cause some decrease in antigen detection in pAb-based ELISA. However, Western blotting of the same samples revealed the presence of stable polypeptides. Similar stability of extracted proteins from pecans (cultivar, Desirable) subjected to different thermal treatments has been recently reported (*16*).

When probed with mAbs 4H9 and 4C3 (**Figure 5D**,**E**) Ana o 2 was detectable over the tested pH range except mAb 4C3 was unable to detect the pH 13 sample. Also of interest was the qualitative decrease in band intensity at pH 1, 3, and 13 (mAb 4H9) and pH 1 and 3 (4C3). Sandwich ELISA results using mAb 4C3 (**Table 2**) also indicated Ana o 2 was undetectable at pH 1 and 13. Loss of immunoreactivity at pH 1 and 13 is perhaps of limited value in food processing as cashew nut processing typically does not involve exposure of seeds to such pH extremes. However, from a physiological point of view, loss of immunoreactivity at pH 1 is of interest as the pH in the stomach of healthy individuals is usually acidic, pH 1−1.2 (*40*). Although the optimum pH for human pepsin is not precisely defined, it is active in the acid pH range (pH 1−2), a pH commonly encountered in the human stomach (*41*−*43*). Human gastric juice contains pepsin isozymes 1, 2, 3, and 5 (gastricsin). Each of these isozymes exhibits a different rate of catalytic activity (optimum pH range of 1.5−4) as a function

of pH. Pepsin catalytic activity is partly dependent on the substrate used (*43*). Understanding the in vivo stability of cashew nut proteins when subjected to pepsin at low pH conditions is therefore important and needs to be investigated.

The remarkable stability of Ana o 2 toward a variety of processing conditions combined with our previous reports on Ana o 2 being the major storage protein and a major allergen in cashew nut seeds (*6, 7, 9, 10, 22*) suggests Ana o 2 to be an excellent marker protein for the detection of raw and processed cashew nut regardless of the type of detection immunoassay (pAb- and/or mAb-based-ELISA, Western, dot blotting) used.

Ana o 3. Ana o 3 is a 2S albumin allergen (*9, 12*). Anti-Ana o 3 mAb D2 did not bind to any of the previously identified linear epitopes (data not shown) and therefore is presumed to recognize a conformational epitope or a linear motif not yet identified to be reactive with patient sera IgE. Seeds subjected to 10 min of autoclaving, blanching, or roasting registered a significant decrease in immunoreactivity when soluble proteins were assessed by sandwich ELISA using mAb D2 (**Table 1**). Western (**Figure 3F**) and dot blotting results (**Table 1**) using mAb D2 also confirmed the decrease in immunoreactivity upon autoclaving and roasting treatments.

The 2S albumin protein family is known to be highly stable to thermal denaturation and is characterized by their conserved cysteine skeletons. Many of the 2S heterodimeric proteins generally contain eight or more disulfide-bridged cysteine residues, which hold the two subunits (large and small) together and thus contribute to their stability and compactness (see refs *31* and *32* and several references therein).

The effects of pH exposure on Ana o 3 using Western blotting (**Figure 5F**) indicate Ana o 3 is stable over the pH range of 1−11 but undetectable at pH 13 when probed with mAb D2. Sandwich ELISA results using the same mAb (**Table 2**) also show that Ana o 3 was not detected when the soluble seed proteins were exposed to pH 13. Sandwich ELISA was also unable to detect any signal at pH 1 and 3. These results are comparable to published reports on the stability of 2S albumins to heat and extreme pH treatments. For example, a recombinant 2S albumin from rapeseed (rproBnIb) (*33, 34*) and the 2S albumin from Brazil nut (Ber e 1) (*35*) were shown to unfold at elevated temperatures (80−85 °C). Upon cooling, the proteins recovered their initial structure, indicating the changes induced by heat treatment were reversible. In another study, both Ber e 1 and rBer e 1 showed no unfolding at temperatures lower than 75 °C, and both retained most of their secondary structure at 95 °C (*36*). Peanut allergens Ara h 2 and Ara h 6, both 2S albumins, contain cores that are highly resistant to proteolytic digestion and to temperatures of up to 100 °C (*35*). Similarly, Vereijken et al. (*37*) found the 2S albumins from sunflower (SFAs) to be stable against pH changes (pH 3.0 to 9.0) and heat treatment (>100 °C).

An increase in polypeptide band intensity, indicative of increased immunoreactivity, was observed when samples subjected to roasting at 170 °C for 20 min (**Figure 3**) were probed with rabbit pAbs and mAbs (excluding mAb 4B7) for Western blotting. Although the observed qualitative increase in immunoreactivity indicated by Western blotting does not necessarily equate to increased allergenicity, such an increase is of concern as, at least in the case of peanuts, roasting has been reported to increase allergenicity (*38, 39*). Interestingly, Mondoulet et al. (*30*) found no differences in reactivity of raw unprocessed and roasted whole peanuts when tested against 21 patient sera IgE used in the EAST inhibition assay.

The results of the current investigation suggest that simple food-processing treatments may not be sufficient to eliminate or substantially reduce cashew nut allergenicity as all three major allergens seem to survive processing conditions. The results do suggest that suitable processing may help to inactivate certain susceptible epitopes.

For a sensitive individual to react to ingested raw or processed cashew nut, epitope recognition by patient IgE is essential. Such epitope recognition may take place immediately after contact in the mouth or by skin contact or at the gastrointestinal tract level, where food digestion and absorption take place. For these reasons, the evaluation of allergen stability toward proteolysis is important and warranted.

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