JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

A Sensitive Sandwich ELISA for the Detection of Trace Amounts of Cashew (*Anacardium occidentale* L.) Nut in Foods

YANHONG WEI,† SHRIDHAR K. SATHE,‡ SUZANNE S. TEUBER,§ AND KENNETH H. ROUX*,†

Department of Biological Science, and Department of Nutrition, Food and Exercise Sciences, Florida State University, Tallahassee, Florida, and Department of Internal Medicine, School of Medicine, University of California, Davis, California

Trace amounts of cashew nut protein can provoke severe allergic reactions in sensitive patients. Consequently, commercial food processors and regulatory agencies must be vigilant to prevent cashew nut cross-contamination among foods and ensure proper labeling. Toward this end, we have developed a sandwich enzyme-linked immunosorbent (ELISA) to detect the predominant cashew protein fraction (anacardein or cashew major protein, CMP) that can be extracted in aqueous buffer from food matrixes. Protein G-purified goat antiwhole cashew extract IgG and rabbit anti-CMP IgG were used as capture and secondary antibodies, respectively. Immunoadsorption against several nut and seed proteins significantly minimized the inherent cross-reactivity of these reagents. Food samples spiked with cashew flour and CMP were extracted and tested in a sandwich ELISA where standard curves were based on reactivity with CMP. The assay was optimized to detect as little as 20 ng/mL (0.02 ppm) of CMP and was successfully used to quantify CMP, and thus cashew, in various food matrixes.

KEYWORDS: Cashew; *Anacardium occidentale*; CMP; anacardein; globulin; food allergy; allergen; immunoassay; ELISA

INTRODUCTION

Cashew is globally one of the most popular tree nuts and is eaten as a snack or incorporated as an ingredient in a variety of foods. Cashew ranks third in the international tree nut trade with over 20% of the market. Anacardein, the predominant soluble globulin in cashew, also known as CMP, is a multimeric globulin (13S) and constitutes about 40−45% of the total cashew soluble seed proteins (*1*, *2*).

Peanut and/or tree nut allergies affect about 1% of the population (*3*) and are one of the leading causes of fatal and near-fatal food-induced allergic reactions (*4*, *5*). Patients with a severe allergy to peanuts or tree nuts rarely become tolerant of these foods, and these allergies can persist throughout life (*3*, *6*).

Cashew proteins have already been demonstrated to be very potent allergens (*7*−*13*). Six of 30 (20%) patients with type I hypersensitivity to cashew nut were reported to experience an anaphylactic reaction in one study (*10*). Ingestion of chocolate candy containing cashew nut has also been reported to cause an anaphylactic reaction (*8*). In the U.K., recent wide availability of cashew nut butter type spread (*14*) has increased the potential for a higher rate of exposure. It has been reported that 0.08% of British four year olds are allergic to cashew (*11*), whereas 40% of 142 French patients allergic to peanuts were found to be cosensitized to cashew (*15*).

Because treatment of cashew allergy is not yet available, ingestion has to be avoided by the sensitive individual. Problems may thus arise if the presence of the allergen is not discernible due to mislabeling of the products or because of unknown cross-contamination that may occur during industrial food processing.

To protect allergic consumers, sensitive methods are needed to detect and quantify such hidden allergens in complex processed food matrixes to allow more accurate labeling of commercial food products or to facilitate quality control efforts to avoid product cross-contamination. Taylor and Nordlee suggested that immunoassays that can detect food allergens <10 ppm should be adequate (*16*).

Because no suitable method has been developed for cashew nut detection, the aim of this study was to develop a sensitive, specific, and quantitative method for the detection of traces of cashew protein in food matrixes.

MATERIALS AND METHODS

CMP Purification, Cashew Protein Extract Preparation, and Spiking of Commercial Food Products. Cashew nuts (locally purchased) were ground in a Waring blender (~40 mesh flour) and defatted with cold (4 °C) acetone (flour:solvent = 1:5, w/v) with

* To whom correspondence should be addressed. Tel: 850-644-5037. Fax: 850-644-0481. E-mail: roux@bio.fsu.edu.
† Department of Biological Science, Florida State University.
‡ Department of Nutrition, Food and Exercise Sciences, Florida State University.
§ Department of Internal Medicine, University of California.

10.1021/jf025977x CCC: $25.00
Published on Web 04/23/2003

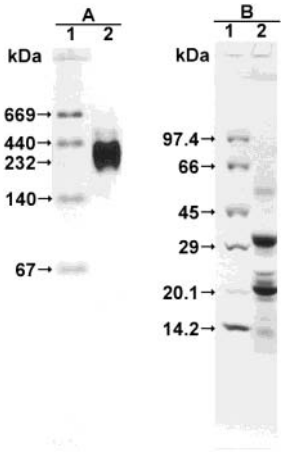

Figure 1. Electrophoretic analysis of CMP under nonreducing, nondenaturing (**A**) and denaturing, reducing (**B**) conditions. (**A**) NDND–PAGE (3–30% linear gradient acrylamide gel, 1.5 mm thick, acrylamide:bis = 37:1 w/w) for purified CMP (lane 2, 50 μg). (**B**) SDS–PAGE (8–25% linear gradient acrylamide gel, 1.5 mm thick, acrylamide:bis = 37:1) for purified CMP (lane 2, 50 μg).

constant magnetic stirring provided. The slurry was filtered through Whatman filter paper no. 4 (vacuum filtration). The residue was reextracted two more times in a similar fashion. The defatted flour was dried in a fume hood, and the dry flour was further ground in a Waring blender to obtain a homogeneous defatted flour. The defatted flour was stored at −20 °C in an airtight plastic bottle until further use.

The purification and biochemical characterization of CMP have been previously described in detail (*1*). Briefly, crude CMP was extracted from defatted cashew nut flour with 2 M NaCl containing 0.001 M NaN_3 (flour:solvent ratio 1:10 w/v) with constant magnetic stirring for 3 h at 4 °C and centrifuged (12 000*g*, 10 min, 4 °C). The supernatant was filtered through glass wool, and the residue was reextracted with 10 more volumes of the same solvent for one more hour at 4 °C with constant magnetic stirring. The slurry was then centrifuged (12 000*g*, 10 min, 4 °C), and the supernatant was filtered through glass wool. The combined supernatant was dialyzed against distilled water (4 °C, 4−6 changes of water, 36−48 h, dialysis tubing molecular weight cut off 6000−8000) and lyophilized. The lyophilized powder was reconstituted in 0.02 M Tris-HCl buffer (pH 8.1) containing 0.1 M NaCl and 0.001 M NaN_3 in a minimum volume (typically 100 mg/mL buffer) and centrifuged (13 600*g*, 5 min, 25 °C), and the supernatant was loaded onto a Sephacryl S300 HR (Amersham Pharmacia Biotech AB, Uppsala, Sweden) column (1.6 cm × 96.5 cm). The column elution and equilibrium buffer was 0.02 M Tris-HCl (pH 8.1) containing 0.1 M NaCl and 0.001 M NaN_3. The column flow rate was 20 mL/h, and fractions were collected every 15 min. CMP-containing fractions were pooled, dialyzed against distilled water (36−48 h, 4 °C, 4−6 changes), lyophilized, and stored at −20 °C in airtight plastic vials until further use. Purified CMP was dissolved in 0.1 M Tris-HCl, pH 8.1, at 5 mg/mL and stored at −20 °C for later analysis. CMP purity was assessed using nondenaturing gel electrophoresis (NDND−PAGE) and SDS−PAGE. As can be seen from **Figure 1**, NDND−PAGE analysis yields a single band, which upon denaturation and reduction, reveals polypeptide composition (lane 2 in **Figure 1B**) typical of 11S (or legumin type proteins).

Whole cashew nut flour protein extract was prepared by mixing 100 mg of defatted cashew flour with 1 mL of BSB (0.1 M H_3BO_3, 0.025 M $Na_2B_4O_7$, and 0.075 M NaCl, pH 8.2) and stirred until homogenized. The mixture was then stirred (5 rpm by rotation) for 1 h at RT. The aqueous layer was obtained by centrifugation at 11 100*g* for 10 min at RT. Protein concentrations were determined by the method of Lowry et al. (*17*) with BSA as the standard protein. Approximately 40−45% of cashew nut defatted flour protein is solublized by this method, and CMP accounts for about 50% of the total soluble protein.

For denaturation studies, whole cashew nuts were subjected to roasting at 170 °C for 20 min, microwave roasting at 500 W for 2 min, autoclaving at 121 °C/15 psi for 10 min, or blanching at 100 °C for 10 min. Thereafter, the processed cashew nuts were ground into flour and defatted using a Soxhlet apparatus as described below. The defatted flour was stored at −20 °C in an airtight plastic bottle until further use. The defatted flour was extracted in BSB just prior to use as described above. The Bio-Rad protein assay was performed as described by the manufacturer (Bio-Rad Laboratories, Hercules, CA) to determine extractable proteins from the processed and native cashew nut samples.

For protein extracts of nuts, seeds, and other food constituents (sunflower seeds, walnut, almond, Brazil nut, macadamia, soy nut, soybean, pine nut, navy bean, Virginia peanut, Spanish peanut, pecan, sesame seed, pistachio, and green pea) used for cross-reactivity testing and immunoadsorption, 100 mg of defatted flour was mixed with 1 mL of BSB and processed as described for cashew nut flour.

CMP-spiked samples were prepared by adding 200 μL of CMP solution of known concentrations to the defatted ground food, flour, or spice together with 800 μL of BSB. All food samples (including raw and processed cashew nut samples) were ground in a Waring blender (~40 mesh flour) and defatted for 6 h in a Soxhlet apparatus using petroleum ether (boiling point range, 38.2−54.3 °C; flour:solvent ratio 1:10, w/v). The defatted flours were dried in a fume hood, ground in a Waring blender (to obtain homogeneous flour), and stored at −20 °C in airtight plastic bottles until further use. Cashew flour-spiked foods were made by mixing dry defatted cashew flour with food at the appropriate ratios with a mortar and pestle followed by mixing with a Sorvall Omni-Mixer (Ivan Sorvall Inc., Newton, CT) as described previously (*18*). Some commercial cashew nut-containing food samples were obtained from an Asian Indian grocery store in Tallahassee and others from India where cashew is extensively used in many confections and other processed foods. These foods were ground, defatted, and extracted as above. Aliquots of the samples were stored at −20 °C prior to analysis.

Rabbit, Goat, and Human Antisera. Rabbits and goats were immunized with 1 mg of CMP and 2 mg of cashew soluble protein, respectively, using Freund's complete adjuvant (Sigma Chemical Co. St. Louis, MO). After they were boosted with the same amount of antigen in incomplete Freund's adjuvant (Sigma Chemical Co.), the animals were bled and the sera was stored at −20 °C until used. Human antisera from five cashew allergic patients were used to constitute the human serum pool. These subjects had excellent histories of severe, life-threatening reactions to cashew and were positive for cashew specific IgE by Pharmacia ImmunoCAP assay (Pharmacia Biotech Inc., Piscataway, NJ). Food challenges were not performed. Blood samples were drawn after informed consent from patients with allergic reactions to cashew. The study was approved by the Human Subjects Review Committee at the University of California, Davis.

Immunoadsorption of Rabbit and Goat Anticashew Sera. The rabbit and goat antibody-containing immunoglobulin was prepared in a two step process. First, IgG was bound to and eluted from a protein G affinity column using 0.2 M glycine sulfate, pH 2.3, as the elution buffer. The eluate was immediately neutralized with 1.0 M Tris, pH 9.0. The purified IgG was then adsorbed against a nut and seed protein extract column to remove cross-reactive antibodies. The protein extracts covalently bound to the column matrix were from almond, Brazil nut, navy bean, soybean, Virginia peanut, Spanish peanut, pecan, pine nut, pistachio, sesame seed, sunflower seed, and walnut. A 0.8 mg protein aliquot of each was separately coupled to 100 μL of CNBr Sepharose gel (Pharmacia Biotech Inc.) according to the manufacturer's instructions and then pooled. The effluent was collected for further use. The column was regenerated by elution with 0.2 M glycine sulfate, pH 2.3, and washing with BSB.

Electrophoresis and Western Blot. Cashew extract (8 μL, ~300 μg) was mixed with 92 μL of reducing buffer (70 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.05% bromophenol blue) and boiled for 5 min before it was loaded into a 6.7 cm well 12% SDS−PAGE gel. Electrophoresis was conducted (Bio-Rad Mini Protean 3 Electrophoresis Cell System) at 120 V on a Power Pac300 (Bio-Rad Laboratories). Proteins from the gels were transferred

to 0.45 μm pure NC transfer and immobilization paper (Shleicher & Schuell, Keen, NH) for 3 h at 80 mA, using an electrophoresis power supply (Northeastern Science Company, Boston, MA) and a Mini Trans-Blot Transfer Cell (Bio-Rad Laboratories). NC membranes were cut into 4 mm strips, blocked with 0.2% BSA in TBS-T (20 mM Tris, 137 mM NaCl, pH 7.6, with 0.2% Tween 20) for 1 h at RT, and washed.

The strips were then incubated with either a pool of five cashew allergic patients' sera (diluted 1:20 in TBS-T, overnight at 4 °C), adsorbed goat antitotal cashew protein extract IgG (diluted 1:250, 1 h at room temp), or rabbit anti-CMP immunoadsorbed IgG (diluted 1:2000 1 h RT). Strips probed with human antisera were washed two times in TBS-T and a third time in PBS (pH 7.3) for 20 min each wash and incubated overnight at 4 °C with ^{125}I antihuman IgE (Hycor Biomedical Incorporated, Garden Grove, CA) diluted 1:10 in a mixture of PBS, 5% nonfat dry milk, and 0.05% Tween-20. Three final washes in TBS-T were performed, and the strips were exposed to Kodak Biomax X-ray film (Rochester, NY) for 24−96 h. HRP-labeled rabbit antigoat IgG and goat antirabbit IgG were added to the strips containing the corresponding primary antibodies and incubated for 1 h at RT. The strips were washed once for 15 min and three times for 5 min each wash in TBS-T after each antiserum/antibody incubation. The reactive bands were identified using Enhanced Chemiluminescence Plus (ECL+, Amersham Pharmacia) and subsequent exposure to Kodak X-OMAT X-ray film.

ELISA. Ninety-six well round bottom polyvinyl microtiter ELISA plates (Seracluster "U" Vinyl, no. 2797, Costar, Cambridge, MA) were coated with 50 μL/well goat anticashew immunoadsorbed antibodies diluted to 8 μg/mL in coating buffer (48.5 mM citric acid, 103 mM Na_2HPO_4, pH 5.0) and incubated for 1 h at 37 °C. Plates were blocked with 100 μL/well of blocking buffer (5% nonfat dried milk powder, 0.1% Tween 20 in PBS) for 1 h at 37 °C. Emptied plates could either be sealed with Parafilm (American National Can., Chicago, IL) and stored at −20 °C for several months or washed and used immediately. A serial dilution of the CMP standard and the food extract samples in incubation buffer (0.3% BSA, 0.1% Tween 20 in PBS) was performed in the microtiter wells. After a 1 h incubation at 37 °C, 50 μL/well of 8 μg/mL CMP specific immunoadsorbed rabbit antiserum, diluted in incubation buffer, was added to the plates and incubated for 1 h at 37 °C. After a subsequent 1 h incubation at 37 °C with alkaline phosphatase-labeled goat antirabbit IgG (Sigma Chemical Co., no. A-3687), the plates were developed in substrate buffer (50 μL per well of *p*-nitrophenyl phosphate (Sigma Chemical Co.) at 1 mg/mL). Between each step, the plates were washed three times with PBS. To stop the reactions, 15 μL of 3 M NaOH was added to each well. Optical density (OD) was measured in a KC4 v2.5 ELISA reader (Bio-Tek Instruments, Inc, Winooski, VT) at 405 nm wavelength. The amounts of CMP in food samples were quantitatively determined based on the standard curve using KC4 software.

Statistics. All samples were assayed in triplicate, and the assays were repeated 3−4 times on different days. The mean ± standard deviation (SD) is reported.

RESULTS

Characterization of the Rabbit and Goat Cashew Specific Antibodies. Rabbits and goats were immunized with CMP and cashew protein extract, respectively, and the antisera were analyzed for reactivity with cashew protein extract and cross-reactivity. Considerable cross-reactivity was observed in both Western blot (data not shown) and ELISA (**Table 1**). Various nuts, seeds, and host foods were included in the cross-reactivity studies including sunflower seeds, walnut, almond, Brazil nut, macadamia, soy nut, soybean, pine nut, navy bean, Virginia peanut, Spanish peanut, pecan, sesame seed, pistachio, and green pea. Each protein extract was tested at dilutions ranging from 1:10 to 1:1 × 10^5 using the original (unadsorbed) rabbit and goat IgG fractions (i.e., from the first purification step). Those extracts that gave signals equivalent to >0.2 μg/100 mg flour (i.e., all except Macadamia nut, soy nut, and green pea) were included in the affinity chromatography protocol (second purification step) to remove cross-reactivity. After the adsorption, the degree of cross-reactivity was greatly reduced (**Table 1**). Only sunflower seeds, pistachio, walnut, and pecan showed some residual cross-reactivity, although the signals were more than 2.5 × 10^4 times lower than for an equivalent amount of CMP. Thus, no substantial cross-reactivities with tested tree nut or seed proteins were observed when using adsorbed IgG fractions. In addition, no cross-reactivity was detected in protein extracts of host foods (whole wheat flour, all purpose wheat flour, rolled oats, milk chocolate, raisin bran cereal, chocolate-filled cookies, and rice cereal) for either the (unadsorbed) IgG fractions or the immunoadsorbed (with tree nut and seed protein extracts) antibodies (data not shown).

Table 1. Effect of Immunoadsorption on Cross-Reactivity Levels of Various Nuts and Seeds in Anti-CMP Sandwich ELISA

	cross-reactivity (μg/100 mg)			cross-reactivity (μg/100 mg)	
sample	before[a]	after[b]	sample	before[a]	after[b]
sunflower seeds	12.5	2.3	navy bean	0.5	<
Brazil nut	1.6	<	pecan	8.1	4.0
macadamia	<	<	green pea	<	<
soynut	<	<	walnut	4.1	1.8
soybean	0.6	<	pistachio	42.3	2.0
pine nut	0.7	<	almond	3.4	<
Spanish peanut	5.2	<	sesame	2.3	<
Virginia peanut	0.3	<			

[a] Cross-reactivity detected by goat and rabbit IgG fractions. Values represent the cashew equivalent signal generated by the cross-reactive substance. [b] Cross-reactivity detected by immunoadsorbed IgG derived by passage of antisera through the nut and seed extract immunoaffinity column. <, denotes cross-reactivity below the detection limit of 0.2 μg/100 mg.

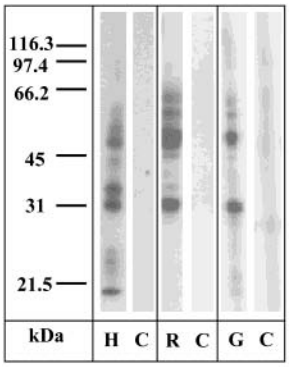

Figure 2. Western blot of cashew nut protein as detected by human IgE (H), rabbit CMP specific IgG (R), and goat cashew specific IgG (G). C, control for nonspecific binding by nonallergic human serum, preimmune rabbit serum, and preimmune goat serum, respectively.

Western blotting was performed to determine if the immunoadsorbed rabbit and goat antibodies would recognize similar polypeptides as does IgE from cashew allergic patients. Cashew nut protein polypeptides were separated by SDS−PAGE under reducing conditions and transferred to NC membranes. The adjacent strips were probed with pooled human antisera and rabbit and goat immunoadsorbed IgG. The results of this comparative Western blot assay revealed a general correspondence between the peptides recognized by human cashew specific IgE and those recognized by the rabbit and goat antibodies, although the relative intensity of the reactions varied (**Figure 2**).

ELISA Standard Curve. A mean standard curve and standard deviation were derived from 26 different experiments performed on different days (**Figure 3**). The IC_{50} of the ELISAs

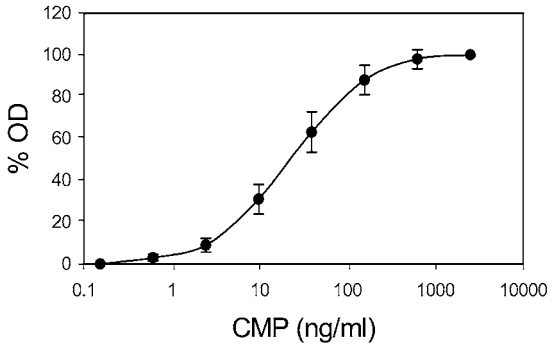

Figure 3. Mean standard curve based on 26 assays performed on different days. The IC_{50} (i.e., the amount of antigen needed to inhibit to 50% of the OD signal) of the ELISAs is about 20 ng/mL, equal to 0.02 ppm of CMP in food matrixes. The detection range was from 10 to 150 ng/mL, demonstrating the sensitivity of this sandwich ELISA.

Table 2. Detection of CMP in Foods Spiked with Cashew Flour

	CMP detected ± SD (μg/100 mg)		
host food	100:1	1000:1	10 000:1
theoretical yield[a] (μg)	200–225	20–22.5	2.0–2.3
whole wheat flour	84 ± 6.7	30.9 ± 3 .4	2.5 ± 0.2
all purpose wheat flour	241 ± 13.3	29.3 ± 1.4	3.0 ± 0.5
rolled oats	233 ± 36.2	21.6 ± 4.5	2.0 ± 0.6
milk chocolate	168 ± 34.9	17.5 ± 4.7	1.3 ± 1.1
raisin bran cereal	133 ± 11.0	9.3 ± 2.2	0.1 ± 0.0
chocolate-filled cookies	339 ± 73.8	25.2 ± 6.2	2.1 ± 0.6
rice cereal	252 ± 47.8	28.6 ± 6.5	3.1 ± 0.4

[a] The theoretical yield of CMP is assumed to be 20–22.5% of the amount of cashew flour added. The theoretical yield is based on the observation that 40–45% of the weight of defatted cashew flour represents aqueous buffer extractable proteins and 50% of this protein is CMP. Thus, a 100:1 mix would have 200–225 μg CMP/100 mg, whereas a 1000:1 and 10000:1 mix would have 10 and 100 times less than that, respectively.

is about 20 ng/mL, equal to 0.02 ppm of CMP in food matrixes. The detection range was from 10 to 150 ng/mL (0.01−0.15 ppm), demonstrating the sensitivity of the sandwich ELISA. For detection of cashew protein in foods, serial dilutions of the aqueous extracts were tested and those that gave an OD value falling closest to the midpoint of the linear portion of the standard curve were used to calculate the CMP concentrations.

Recovery Studies. To test for the ability of the ELISA assay to detect cashew in food and to assess the role that the food matrix may play in detection, seven commercial food samples were spiked with varying amounts of defatted cashew flour. Extractable protein was assayed by ELISA, and the corresponding amount of detected CMP was calculated from the CMP standard curve. Depending on the food matrix, CMP was detected at 84−339 μg per 100 mg of food containing 1% added cashew (theoretical yield = 200−225 μg), 9.3−30 μg from 0.1% added cashew, and 0.1−3.1 μg from 0.01% of added cashew (**Table 2**).

Two hundred microliters of purified CMP at varying concentrations was added to the seven food samples to yield 0.1, 0.01, 0.001, and 0.0001% of CMP in food matrixes. The amount of CMP detected ranged from 50 to 136% of the theoretical yield in different food matrixes over the complete range of spiking levels (**Table 3**). For milk chocolate and raisin bran cereal, the observed values were consistently below the theoretical values at the various spiking levels. CMP-spiked milk chocolate and raisin bran cereal yielded relatively lower values (about 50−77% of the amount added) as compared to other host foods. These results were similar to those seen when cashew flour was the spiking agent (**Table 2**). For CMP-spiked whole wheat flour, all purpose wheat flour, rolled oats, chocolate-filled cookies, and rice cereal, detection was in the 70−136% range.

CMP was also added to spices, sugar, and salt to study their influence on CMP detection (**Table 4**). Ten milligrams, rather than 100 mg, of salt, cinnamon, cardamom, fenugreek seeds, nutmeg, and Spanish saffron were used, as one would not expect these additives to represent more than 10% of the food ingredient. One hundred milligrams of white fine granulated sugar and brown sugar were also spiked with soluble CMP. CMP was detected at 47−164% of the amount actually added (for the 100 and 10 μg CMP samples). The data show that cinnamon and, to a lesser extent, nutmeg have a negative influence ($p < 0.05$) on the CMP detection (<50% of the added amount was detected in most samples), while fenugreek seeds and Spanish saffron enhance the detection level (to ∼140% of the added amount). Spice controls (no CMP added) were also included in this study. Only the cardamom (without CMP) gave background values of 0.02 μg/mL while all of the others were under the detection limit (0.01 μg/mL). Samples spiked with 1.0 μg/mL CMP gave close to quantitative values (0.7−1.3 μg/mL) except for cinnamon and nutmeg, which were lower. However, the 0.1 μg/mL CMP in salt and brown sugar samples shows lower than expected detection values (0.03 and 0.04 μg/mL, respectively).

Investigation of Commercial Food Products. The cashew-containing Indian foods, kaju katli (a thin fudgelike confectionary), kaju wadi (a thick fudgelike confectionary), wadi puri (a ground cashew nut-filled muffin), kaju mix (a snack mix containing puffed rice, toasted and salted legumes, deep fat fried thinly extruded Bengal gram flour, and deep fat fried salted cashew nut pieces), and cashew chikki (a cashew brittle candy), were included in this study. The level of CMP detection varied greatly and ranged from 2.8 to over 12 000 μg/100 mg food (**Table 5**). Cashew chikki contains a minor amount of CMP (2.8 μg/100 mg, correspondence to 28 ppm or 2.8 × 10^{-3}%), whereas wadi puri contains about 12.4% of CMP, which

Table 3. Detection of CMP in Commercial Food Samples That Were Artificially Spiked with Soluble CMP

	CMP detected ± SD (μg/100 mg)			
host food	100[a] (0.1%)	10 (0.01%)	1.0 (0.001%)	0.1 (0.0001%)
whole wheat flour	136 ± 21.4	11.2 ± 2.0	1.0 ± 0.2	0.11 ± 0.04
all purpose wheat flour	105 ± 18.7	13.4 ± 0.5	1.2 ± 0.3	0.13 ± 0.03
rolled oats	122 ± 28.9	12.5 ± 2.2	0.9 ± 0.1	0.08 ± 0.04
milk chocolate	77 ± 10.3	7.2 ± 1.7	0.7 ± 0.2	0.05 ± 0.02
raisin bran cereal	64 ± 24.2	6.6 ± 2.7	0.6 ± 0.1	0.05 ± 0.07
chocolate-filled cookies	120 ± 28.0	9.0 ± 3.9	0.9 ± 0.1	0.07 ± 0.02
rice cereal	110 ± 12.3	13.0 ± 2.1	0.9 ± 0.3	0.08 ± 0.04

[a] Micrograms of added CMP/100 mg food mix (% of added CMP).

Table 4. Detection of CMP in Salt, Sugars, and Spices Artificially Spiked with Soluble CMP

		CMP detected ± SD (μg CMP used for spiking)			
host food	amount[a] (mg)	100	10	1.0	0.1
salt (NaCl)	10	127 ± 16.2	11.6 ± 0.7	0.7 ± 0.1	0.03 ± 0.01
cinnamon	10	48 ± 7.4	4.7 ± 0.7	0.2 ± 0.1	0.03 ± 0.00
cardamom	10	119 ± 16.1	7.2 ± 1.9	0.7 ± 0.1	0.07 ± 0.02
fenugreek seeds	10	164 ± 37.3	13.5 ± 1.0	1.3 ± 0.3	0.08 ± 0.04
nutmeg	10	92 ± 14.2	4.9 ± 1.1	0.3 ± 0.0	0.05 ± 0.01
Spanish saffron	10	146 ± 19.9	14.3 ± 2.3	0.8 ± 0.2	0.10 ± 0.01
fine granulated sugar	100	129 ± 12.2	10.6 ± 0.7	1.0 ± 0.1	0.06 ± 0.01
brown sugar	100	142 ± 30.9	11.0 ± 3.5	0.7 ± 0.1	0.04 ± 0.01

[a] Amounts of spiked samples extracted with 1.0 mL of buffer.

Table 5. Detection of CMP Levels in Commercial Foods[a]

commercial food	CMP level (μg/100 mg)
kaju katli	7380 ± 735
kaju wadi	996 ± 150
wadi puri	12 400 ± 1200
kaju mix	2.9 ± 0.4
cashew chikki	2.8 ± 49

[a] All samples were defatted prior to protein extraction.

corresponds to a defatted cashew nut composition of 55−62% (CMP = 20−22.5% of the defatted cashew). These data suggest that the assay is flexible and can accommodate foods with a large range of cashew content.

Effect of Processing on CMP Detection. As cashew is usually subjected to processing before or after addition to food matrixes, we evaluated the effect of oven roasting, microwave roasting, autoclaving, and blanching on the detection of CMP by the rabbit and goat anti-CMP antisera and cashew allergic human serum. Protein extracts of processed and unprocessed cashew nut defatted flour were initially normalized for protein content as the amount of extractable protein varied considerably depending on the heat treatment applied (**Table 6**). Four micrograms of each protein extract was loaded per 3 mm well and subjected to Western blotting (**Figure 4**). Overall, the blots probed with rabbit and goat IgG and human IgE show a similar pattern, although, upon heat processing, one band at ~53 kDa was diminished in relative intensity as compared to the native protein extracted from unprocessed cashew flour.

The processed cashew samples were also evaluated for effects of processing on antigenicity using the sandwich ELISA. One hundred milligrams of defatted processed and unprocessed cashew samples was extracted and diluted at 1:2000 (v/v) and subjected to the ELISA. For this assay, no adjustments were made for differences in protein solubility. The amounts of extractable protein and the CMP levels detected in the variously processed samples are shown in **Table 6**. Some diminution of reactivity was found in all processed samples but was most pronounced in the oven roasted sample (170 °C, 20 min) where

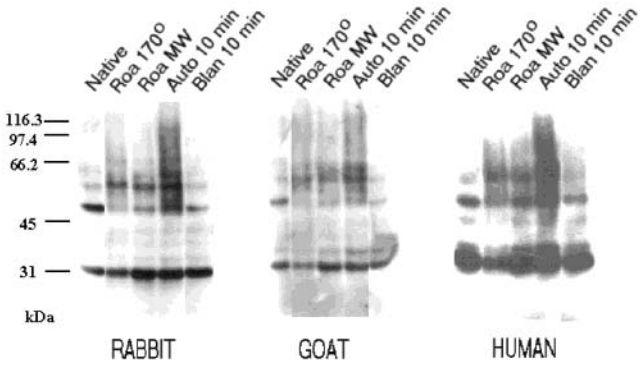

Figure 4. Western blot of reduced native and processed cashew protein extracts probed with rabbit anti-CMP, goat anticashew, and human cashew allergic sera. Roa 170°, roasted at 170 °C for 20 min; roa MW, microwave roasted; auto, autoclaved; blan, blanched.

only 18% of the expected signal was detected. The lower values for detected CMP parallel the general reduction in total protein solubility following heat treatment. Note that the proportion of CMP detected in all extracts was fairly uniform at between 40 and 57% regardless of the processing technique.

DISCUSSION

Food allergic individuals can react adversely to the ingestion of sometimes minute quantities of allergenic foods, including trace residues contaminating otherwise nonallergenic foods. The threshold dose needed to trigger an allergic reaction has not been defined for tree nuts and almost certainly varies from food to food and from one individual to another. The improper labeling by the supplier/manufacturer and the lack of knowledge of food components on the part of the consumer are contributing factors in some cases of severe reactions to foods (*19*).

Several new methods based on ELISA have been developed for the detection of trace amounts of allergens in foods. Assays for peanut (*20*), almond (*18*, *21*), and hazelnut (*22*) have been described. These assays have the most immediate application in the food industry and regulatory agencies where raw ingre-

Table 6. Effect of Processing[a] on CMP Detection (1:2000 v/v Dilution of the Whole Extract)

processing method	total extractable protein from 100 mg flour (mg)	extractable protein as % of unprocessed cashew sample	CMP detected in extract from 100 mg flour (mg)	CMP as % of unprocessed cashew sample	CMP as % of total soluble protein
unprocessed (control)	19	100	9	100	47
roasted (170 °C, 20 min)	4	21	1.6	18	40
microwave roasted (500 W, 2 min)	14	74	8	89	57
autoclaved (121 °C, 15 psi, 10 min)	9.3	49	4	44	43
blanching (100 °C, 10 min)	9.2	48	5.1	57	55

[a] All samples were defatted prior to protein extraction.

dients, processing equipment, and final products can be checked for contamination to protect the food allergic consumers more efficiently.

Because reactions to cashew are reported to be as severe as those to peanut (*14*), it is important to develop a method to detect trace amounts of cashew in food. Our aim was to develop a cashew specific detection system for both screening and quantification purposes. The capture ELISA was able to quantify extractable CMP from cashew-spiked food with reasonable accuracy in most cases (**Tables 2−4**). The presence of cashew nut was also detected in commercial food samples known to contain cashew nuts (**Table 5**). Because the rabbit anti-CMP and goat antiwhole cashew nut protein extract reagents both recognized a similar set of bands on Western blot and reacted similarly when used as either the capture antibody or the reporter antibody in the ELISA, it is likely that the CMP complex contains most of the antigens recognized by both species. Thus, the CMP purification step may be unnecessary. The immunoadsorption step was essential in improving the specificity of the assay by greatly reducing the level of cross-reactivity. Confidence in the value of these reagents is increased by the observation that they recognized many of the same proteins as did human allergic IgE (**Figure 2**).

The stability of cashew nut under processing conditions frequently encountered in food processing was also evaluated. The ELISA and Western blot data both show that roasting, autoclaving, and blanching somewhat reduced CMP detectability. This decrease in CMP signal in the ELISA appears due primarily to a heat-induced reduction in protein solubility, although Western blot analysis showed that certain bands were disproportionately affected by the heat treatments suggesting possible destruction (or partial destruction) of certain epitopes.

Because the rabbit, goat, and human antibodies seem to be recognizing a similar subset of extractable proteins in both the unprocessed and heat-treated samples (Western blot, **Figure 4**) and the Western and ELISA data are similarly affected by specific heat processing methods, the ELISA assay appears likely to be recognizing the same proteins and in roughly the same proportion as does the patients' IgE. It is not unreasonable to assume that patient reactivity to the processed nuts will be similarly graded depending upon the heat treatment process. However, there are as yet no clinical data comparing patients' reactivity to native vs variously processed cashew nuts nor is it known what fraction of heat-processed protein or CMP is ultimately available for interaction with IgE in vivo. Should such studies demonstrate that significantly more allergenic cashew nut protein is released in vivo than is detected by our extraction/ELISA assay procedure, then alternative extraction methods will need to be explored.

The antisera used in the ELISA had high specificity for cashew nut protein due to its immunoadsorption against cross-reactive proteins. It has been previously reported that there are cross-reactions among cashew, pistachio, sunflower seed, walnut, peanut, almond, and chestnut in some allergic patients (*23*−*28*). These various cross-reactive proteins are likely to share some epitopes and should be further investigated. This would be especially true of pistachio, which is a member of the same plant family (*Anacardaceae*). Moreover, patients allergic to one or the other of these nuts frequently show reactivity to both (*27*, *28*). With the exception of chestnut, we have verified that adsorption of the antibodies with proteins extracted from these various nuts and seeds significantly reduced or eliminated cross-reactivity. Because the immunoadsorptions were not fully successful in eliminating all signal generation in the ELISA by the noncashew protein extracts, despite the large excess of nut and seed protein on the columns, we suggest that some of the signal generated may have a nonimmunological basis. Nevertheless, the minimal apparent cross-reactivity should not be detrimental to the performance of the ELISA for its intended applications (i.e., detection of cashew nut in foods).

The ELISA was developed using CMP as the standard since CMP is a major component of extractable cashew protein and can thus serve as a stand-in for total protein extract. Interestingly, although the relative intensities vary, the goat IgG antibody (which was raised against a total extract of cashew and was used as the capture antibody), the rabbit IgG antibody (which was raised against CMP and was the detection antibody), and the human IgE antibody (presumably stimulated naturally, following food ingestion) all reacted with similar bands on Western blots. This suggests that CMP is not only a major protein in the extract by percentage but that it includes the immunodominant antigens present in the total extractable cashew proteins. Thus, we have three different species sensitized in different ways and with different cashew protein fractions yet all reacting to cashew proteins similarly. We have observed a similar phenomenon with goat, rabbit, and human polyclonal and mouse monoclonal antibodies in reaction with AMP, the almond major protein (*29*).

In summary, the ELISA successfully detected residues of CMP at concentrations below the reported 10 ppm threshold of concern to allergic individuals (*16*). Specifically, the assay is capable of detecting pure CMP at concentrations of as low as 0.02 ppm (0.000002%). When mixed with food, CMP is detectable at 1 ppm (0.1 μg/100 mg food) and has been applied to cashew nut detection in wheat flours, cereals, spices, chocolate, and snack foods. This sandwich ELISA should therefore be of value to the food industry, regulatory agencies, and concerned consumers for the detection of "hidden" cashew allergens.

ABBREVIATIONS USED

CMP, cashew major protein; ELISA, enzyme-linked immunosorbent assay; BSB, buffered saline borate; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; NC, nitrocellulose; TBS-T, Tris-buffered saline with 0.2% Tween-20; PBS, phosphate-buffered saline; RT, room temperature (∼25 °C).

LITERATURE CITED

(1) Sathe, S. K. Solubilization and electrophoretic characterization of cashew nut (Anacardium occidentale) proteins. *Food Chem.* **1994**, *51*, 319−324.

(2) Sathe, S. K.; Sze-Tao, K. W. C.; Wolf, W. J.; Hamaker, B. R. Biochemical characterization and in vitro digestibility of the major globulin in cashew nut (Anacardium occidentale). *J. Agric. Food Chem.* **1997**, *45*, 2854−2860.

(3) Sicherer, S. H.; Munoz-Furlong, A.; Burks, A. W.; Sampson, H. A. Prevalence of peanut and tree nut allergy in the US determined by a random digit dial telephone survey. *J. Allergy Clin. Immunol.* **1999**, *103*, 559−562.

(4) Sampson, H. A.; Mendelson, L. M.; Rosen, J. P. Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *N. Engl. J. Med.* **1992**, *327*, 380−384.

(5) Yunginger, J. W.; Sweeney, K. G.; Sturner, W. Q.; Giannandra, L. A.; Teigland, J. D.; Bray, M.; Benson, P. A.; York, J. A.; Biedrzycki, L.; Squillace, D. L.; et al. Fatal food-induced anaphylaxis. *JAMA, J. Am. Med. Assoc.* **1988**, *260*, 1450−1452.

(6) Bock, S. A.; Atkins, F. M. The natural history of peanut allergy. *J. Allergy Clin. Immunol.* **1989**, *83*, 900−904.

(7) Hamilton, T. K.; Zug, K. A. Systemic contact dermatitis to raw cashew nuts in a pesto sauce. *Am. J. Contact Dermatitis* **1998**, *9*, 51−54.
(8) Rasanen, L.; Makinen-Kiljunen, S.; Harvima, R. J. Pectin and cashew nut allergy: cross-reacting allergens? *Allergy* **1998**, *53*, 626−628.
(9) Quercia, O.; Rafanelli, S.; Marsigli, L.; Foschi, F. G.; Stefanini, G. F. Unexpected anaphylaxis to cashew nut. *Allergy* **1999**, *54*, 895−897.
(10) Li, H.; Zhang, H. Hypersensitivity of cashew nut. *Chin. Med. Sci. J.* **1997**, *12*, 189−192.
(11) Tariq, S. M.; Stevens, M.; Matthews, S.; Ridout, S.; Twiselton, R.; Hide, D. W. Cohort study of peanut and tree nut sensitization by age of 4 years. *BMJ* **1996**, *313*, 514−517.
(12) Burks, A. W.; James, J. M.; Hiegel, A.; Wilson, G.; Wheeler, J. G.; Jones, S. M.; Zuerlein, N. Atopic dermatitis and food hypersensitivity reactions. *J. Pediatr.* **1998**, *132*, 132−136.
(13) Stricker, W. E.; Anorve-Lopez, E.; Reed, C. E. Food skin testing in patients with idiopathic anaphylaxis. *J. Allergy Clin. Immunol.* **1986**, *77*, 516−519.
(14) Hourihane, J. O.; Harris, H.; Langton-Hewer, S.; Kilburn, S. A.; Warner, J. O. Clinical features of cashew allergy. *Allergy* **2001**, *56*, 252−253.
(15) Moneret-Vautrin. D.; Rance, F.; Kanny, G.; et al. Food allergy to peanuts in France-evaluation of 142 observations. *Clin. Exp. Allergy* **1998**, *28*, 1113−1119.
(16) Taylor, S. L.; Nordlee, J. A. Detection of food allergens. *Food Technol.* **1996**, *50* (5), 231−234, 238.
(17) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.
(18) Roux, K. H.; Teuber, S. S.; Robotham, J. M.; Sathe, S. K. Detection and stability of the major almond allergen in foods. *J. Agric. Food Chem.* **2001**, *49*, 2131−2136.
(19) Hefle, S. L. Hidden food allergens. *Curr. Opin. Allergy Clin. Immunol.* **2001**, *1*, 269−271.
(20) Holzhauser, T.; Vieths, S. Indirect competitive ELISA for determination of traces of peanut (Arachis hypogaea L.) protein in complex food matrixes. *J. Agric. Food Chem.* **1999**, *47*, 603−611.
(21) Hlywka, J. J.; Hefle, S. L.; Taylor, S. L. A sandwich enzyme-linked immunosorbent assay for the detection of almonds in foods. *J. Food Prot.* **2000**, *63*, 252−257.
(22) Holzhauser, T.; Vieths, S. Quantitative sandwich ELISA for determination of traces of hazelnut (Corylus avellana) protein in complex food matrixes. *J. Agric. Food Chem.* **1999**, *47*, 4209−4218.
(23) Hoffman, D. R.; Haddad, Z. H. Diagnosis of IgE-mediated reaction to food antigen by RAST. *J. Allergy Clin. Immunol.* **1974**, *54*, 165−168.
(24) Malish, D.; Glovsky, M. M.; Hoffman, D. R.; et al. Anaphylaxis after sesame seed ingestion. *J. Allergy Clin. Immunol.* **1981**, *67*, 35−38.
(25) Kaga, M. K.; Wuthrich, B. Falafel burger anaphylaxis due to sesame seed allergy. *Ann. Allergy* **1993**, *71*, 127−129.
(26) Zhang, H. Y. Evaluation of pharmacia CAP system for specific IgE (sIgE) determination. *Chin. J. Microbiol. Immunol.* **1996**, *1* (Suppl.), 6−12.
(27) Garcia, F.; Moneo, I.; Fernandez, B.; Garcia-Menaya, J. M.; Blanco, J.; Juste, S.; Gonzalo, J. Allergy to Anacardiaceae: Description of cashew and pistachio nut allergens. *J. Invest. Allergol. Clin. Immunol.* **2000**, *10*, 173−177.
(28) Fernandez, C.; Fiandor, A.; Martinez-Garate, A.; Martinez Quesada, J. Allergy to pistachio: crossreactivity between pistachio nut and other *Anacardiaceae*. *Clin. Exp. Allergy* **1995**, *25*, 1254−1259.
(29) Sathe, S. K.; Teuber, S. S.; Gradziel, T. M.; Roux, K. H. Electrophoretic and immunological analyses of almond (*Prunus dulcis* L.) genotypes and hybrids. *J. Agric. Food Chem.* **2001**, *49*, 2043−2052.

Received for review September 23, 2002. Revised manuscript received February 28, 2003. Accepted March 27, 2003. This research was partly supported by USDA NRICGP Grant No. 9901530, the College of Human Sciences (CHS Research Initiation Awards Program, S.K.S.), and the Council for Faculty Research (COFRs, S.K.S.), Florida State University.

JF025977X