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Immunoglobulin E-Reactive Proteins in Cashew (*Anacardium occidentale*) Apple Juice Concentrate

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Cashew apple juice has the potential to be a natural source of vitamin C and sugar in processed foods. The juice of the cashew apple is obtained by pressing the fleshy peduncle or receptacle, which forms a rounded apple that sits above the true fruit, the cashew nut. Cashew nut allergy is the second most commonly reported tree nut allergy in the United States. To determine if cashew apple juice contains cashew nut allergens, immunoblotting was performed using a cashew apple juice 6X concentrate that was extracted and further concentrated through dialysis, lyophilization, and resuspension. Serum IgE of individuals allergic to cashew nut bound proteins in the cashew apple juice concentrate extract. For some serum samples, IgE reactivity could be inhibited by preincubation of the serum with cashew nut extract, suggesting the presence of cashew nut-related allergens. Using monoclonal antibodies specific for cashew nut allergens, the concentrate was found to contain Ana o 1 (vicilin) and Ana o 2 (legumin). Neither IgE from cashew nut allergic sera nor the monoclonal antibodies bound any peptides in 5 kDa filtered cashew apple juice concentrate. The cashew apple juice concentrate used in these studies contains proteins with IgE-reactive epitopes, including cashew nut legumin and vicilin. No IgE-binding peptides remained after 5 kDa filtration of the concentrate.

KEYWORDS: ***Anacardium occidentale*; cashew apple; cashew nut; food allergy; IgE**

INTRODUCTION

Cashew (*Anacardium occidentale*) apple juice is rich in sugars (*1, 2*), antioxidants (*3, 4*), and vitamin C (*1*) and is widely consumed in Brazil (reviewed in ref *5*). Although cashew apple juice is not currently available in the United States, production and distribution of cashew apple juice is increasing. In addition, cashew apple juice has the potential to be a natural source of vitamin C and sugar in processed foods (*6*). However, cashew apple juice may contain cashew nut allergens. Because this juice is obtained by pressing the rounded apple, the peduncle that sits above the cashew nut (**Figure 1**), cashew nut allergens could be in the juice due to contamination by traces of seeds at processing plants. Alternatively, synthesis of these allergenic proteins may occur directly in the apple as mRNA of seed storage proteins have been found in nonseed portions of plants (*7*). Cashew nut allergy is the second most commonly reported

Figure 1. Photo of a cashew apple. Reprinted with permission from The United States Department of Agriculture.

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Table 1. Patient Characteristics

no.	group	gender	age	cashew nut reaction[c]	other nut/seed allergies	cashew nut immunoCAP (kU cashew nut-specific IgE/L blood)	IgE to CAJC[a]
C1	cashew allergic	F	33	V, W, light-headed	walnut, pecan, almond	35.1	++
C2	cashew allergic	F	31	AE, throat-swelling, V, W, abdominal cramps	walnut, sunflower	4.42	+
C3	cashew allergic	F	38	AE, throat-swelling, V, W	peanut, tree nuts	41.9	+++
C4	cashew allergic	F	26	U,V	peanut, walnut, hazelnut	4.04	+
C5	cashew allergic	M	59	AE, throat-swelling, V	peanut, tree nuts	class 4[b]	+
C6	cashew allergic	F	28	AE, light-headed, U, W	peanut, walnut, pistachio	9.51	++
C7	cashew allergic	F	55	nausea, V	peanut, walnut	90.4	++
C8	cashew allergic	F	38	throat swelling, U, V, W, LOC	tree nuts	94.7	+
C9	cashew allergic	F	65	AE, throat swelling, hoarse voice, SOB	pistachio	52.9	++
C10	cashew allergic	M	50	throat swelling, V, SOB	tree nuts	3.18	++
				controls			
O1	peanut allergic	M	16	never eaten	peanut	class 5[b]	+++
P1	pollen allergic	F	48	mouth swelling	pistachio, almond, pecan, walnut	<0.35	+
O2	walnut allergic	F	34	itchy throat	walnut	<0.35	+
O3	walnut allergic	F	34	no reaction	walnut, almond	class 0[b]	+
O4	peanut, walnut allergic	M	43	no reaction	peanut, walnut, pecan	<0.35	+
P2	pollen allergic	F	47	no reaction	latex	5.92	+++
P3	pollen allergic	M	50	no reaction	pollen	0.91	+++

[a] Subjectively determined from immunoblot by investigator; (+++), strongly positive; (++), moderately positive; (+), weakly positive. [b] Performed by radioallergosorbent testing prior to our hospital's adoption of ImmunoCAP testing. [c] Abbreviations: AE, angioedema; V, vomiting; U, urticaria; LOC, loss of consciousness; W, wheezing; SOB, shortness of breath.

tree nut allergy in the United States (*8, 9*) and can lead to severe, life-threatening reactions (*10*). A recent report on a British population demonstrated that allergic reactions to cashew may even be more intense than those to peanut (*11*). However, only a single report of a mild reaction to cashew apple juice has been reported (*12*). This is likely because food allergy, in general, is not as common in the countries that dominate cashew production. These countries include Vietnam, Thailand, India, and Brazil, with the latter being the main producer of cashew apple juice. However, before the juice becomes a common ingredient in processed foods in countries where cashew nut allergy is prevalent, it should be determined if cashew apple juice contains cashew nut allergenic proteins.

We performed a series of experiments to determine if a cashew apple juice concentrate (CAJC), obtained from Jusweet Holdings, contains allergens. First, we performed immunoblotting experiments with CAJC extract to determine if IgE in serum samples from cashew nut allergic patients could bind to proteins in CAJC. Since some patients had pollinosis, which could lead to irrelevant IgE cross-reactivity with fruit proteins (*13*), serum samples were preincubated with perennial ryegrass pollen, the predominant grass pollen in Northern California, before immunoblotting against CAJC to determine if IgE-reactivity to CAJC could be inhibited by grass pollen. Serum samples were also preincubated with cashew nut extract and then blotted against CAJC proteins to assess the possible presence of cashew nut allergens in CAJC. To confirm that CAJC contained known cashew nut seed storage protein allergens (*14–16*), we immunoblotted cashew apple proteins with monoclonal antibodies (mAbs) specific for those proteins. Finally, since ultrafiltration is commercially available to juice processors and manufacturers, we filtered the juice concentrate and repeated immunoblotting experiments to determine whether IgE-reactivity could be removed by filtration.

MATERIALS AND METHODS

Human Sera. All participants gave informed consent. The study was approved by the Institutional Review Board of the University of California, Davis. Serum samples were obtained from patients with convincing histories of systemic allergic reactions to cashew nut and positive cashew ImmunoCAP assays (Pharmacia, Uppsula, Sweden). ImmunoCAP assays were performed in our hospital commercial laboratory (University of California, Davis, Medical Center, Sacramento, CA). Total IgE was not measured. Serum samples from patients with histories of pollen, peanut, or walnut allergies were used as controls. Two of these subjects, P1 and O2, reported subjective symptoms upon cashew ingestion; however, ImmunoCAP assays for cashew nut-specific IgE were negative. Thus, these two individuals are included as controls. Patient characteristics can be seen in **Table 1**.

Source of Cashews and Cashew Apple Juice. Mature raw cashew nuts of an unknown cultivar were obtained from a local grocery store (Davis, CA). The cashew apple juice, made from an unknown cashew nut cultivar, was provided as a frozen 6X concentrate (70 Brix) by Valley Processing, Inc. (Sunnyside, Washington) through Jusweet Holdings, LP (Port Washington, NY). The concentrate is not a commercial product in the United States.

Nut, Pollen, and Cashew Apple Juice Extractions. Aqueous cashew nut extract was prepared as previously described (*17*). Grass pollen extract from *L. perenne* desiccated pollen (Hollister-Stier, Spokane, WA) was prepared as previously described (*18*). Cashew apple juice 6X concentrate (CAJC) was mixed with an equal volume of PBS (pH 7.4) at 4 °C, then dialyzed extensively against ddH_2O at 4 °C. Depending on the experiment, samples were either not filtered or filtered using Amicon Ultra filters with Ultracel low-binding membrane with 5 kDa nominal molecular weight cutoff (Millipore, Billerica, MA). Next, samples were lyophilized and resuspended in PBS (pH 7.4). During this process, 40 mL of 6X concentrate was reduced to a 4 mL sample. Protein concentrations were determined using either Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, Illinois) or microBCA Assay Kit (Pierce) according to the manufacturer's instructions. Purified bovine serum albumin was used as a standard in both assays. Prior to performing protein assays on CAJC samples, Pierce Compat-Able Reagent (Pierce) was used on samples and standards according to the manufacturer's instructions. Use of the Compat-Able reagent was necessary because of the presence of substances that interfered with the protein assays. About 200−400 μg of protein could be recovered from 40 mL of 6X CAJC. All samples were stored at −70 °C until use.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis. Protein samples were boiled for 5 min in either reducing or nonreducing sample buffer (*17*) and loaded at either 10 μg/mm for cashew nut extract or 840 ng/mm for cashew apple juice extract. Low yield of protein (~100 μg/mL for post dialysis/lyophilized/resuspended samples) from extraction of CAJC provided only small amounts of protein available

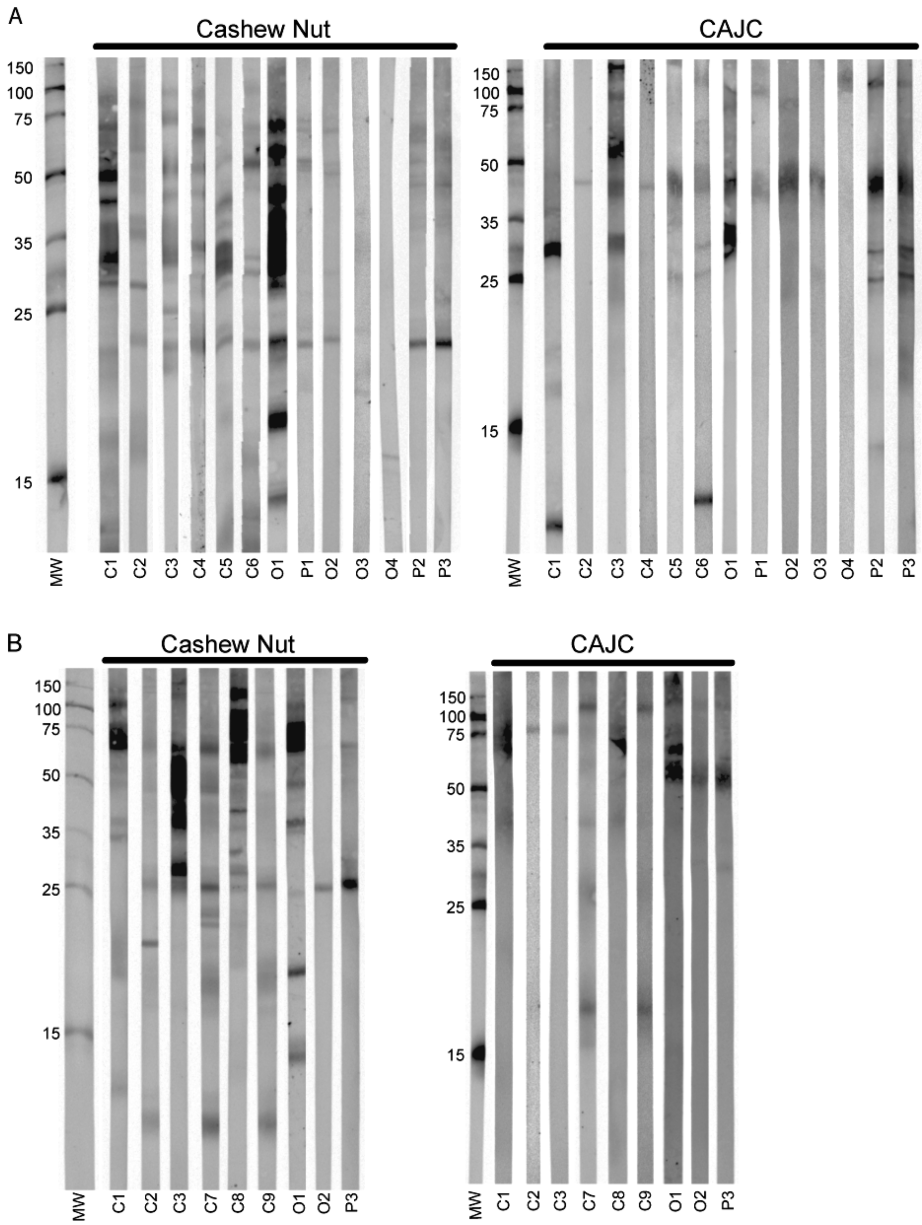

Figure 2. IgE from cashew nut allergic individuals reacts with proteins in cashew apple juice concentrate. Immunoblot of cashew nut and CAJC extract run on 14% gels with samples under reducing (**A**) or nonreducing (**B**) conditions.

for study. All samples were run on 14% gels. Gels were run on a Hoefer SE 600 vertical electrophoresis unit (Amersham Biosciences, Piscataway, NJ) at a constant current of 30 mA per gel.

Immunoblotting. Proteins were transferred to 0.22 μm nitrocellulose membranes (Osmonics Inc., Minnetonka, MN) overnight at 30 V using a Hoefer TE 42 Tank Transfer Unit (Amersham) at room temperature. The blots were cut into strips, 3 mm in width, and blocked in PBS with 5% nonfat dry milk for 60 min at room temperature and then incubated overnight at 4 °C in sera diluted 1:10 or mAbs (generated in the Hybridoma Laboratory, Department of Biological Science, Florida State University) diluted 1:350 in PBS/5% milk/0.1% Tween 20. Strips were then rinsed, washed for 15 min, and washed for 5 min three times in PBS/0.1% Tween 20 at room temperature. Strips were then incubated at room temperature for 90 min with anti-human IgE-HRP (Southern Biotechnology Associates, Inc., Birmingham, AL) diluted 1:6000 or anti-mouse IgG-HRP (Pierce) diluted 1:8000 in PBS/5% milk/0.1% Tween 20. Strips were washed as before and incubated with ECL Plus (Amersham) for 5 min at room temperature. Strips were then scanned with the Storm 860 (Molecular Dynamics, Sunnyvale, CA) set to blue fluorescence/chemifluorescence, 100 μm, with a PMT setting of 1000 V.

Inhibition Immunoblotting. Serum samples, diluted 1:10 in PBS/5% nonfat dry milk/0.1% Tween 20, were incubated overnight at 4 °C with chicken egg ovalbumin (Sigma, St. Louis, MO; 1 mg/mL), grass pollen extract (1 mg/mL), cashew nut extract (1 mg/mL), or diluent alone and then used in immunoblotting as described above.

RESULTS

Cashew apple juice contains proteins that are recognized by serum IgE from cashew nut (CN) allergic individuals. IgE binding to CN and cashew apple juice concentrate (CAJC) under reducing (**Figure 2A**) and nonreducing (**Figure 2B**) conditions can be seen in **Figure 2**. Several of the CAJC proteins recognized by IgE from CN allergic individuals were also recognized by IgE from individuals allergic to other nuts or pollens.

To determine if processing could decrease IgE reactivity with CAJC, the extract of the concentrate was passed through a 5 kDa filter. Immunoblotting demonstrated that no IgE-reactive proteins remained after 5 kDa filtration of the CAJC (**Figure 3**).

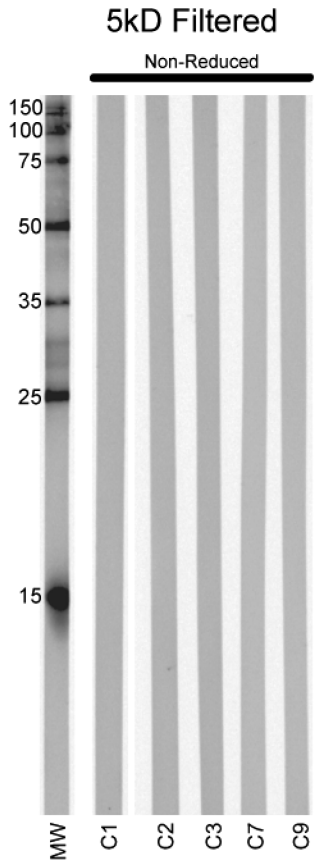

Figure 3. 5 kDa filtration removes IgE-reactive proteins from cashew apple juice concentrate. Immunoblot of 5 kDa filtered CAJC extract. Gels were 14% nonreducing SDS−PAGE.

Because CAJC-specific IgE was present in sera from CN-allergic and CN-tolerant individuals, inhibition immunoblots of CN and CAJC were done to determine whether the IgE-reactive peptides were pollen-related or specific to CN (**Figure 4**). Preincubations with the nonspecific control protein, chicken egg ovalbumin, were run for each serum under each condition. Immunoblots using sera samples preincubated with ovalbumin were identical to those done with sera alone (data not shown). For CN-allergic individuals, the nut extract almost completely inhibited IgE binding to CN. The nut extract also inhibited IgE binding to CN for all of the pollen-allergic controls. Pollen was unable to inhibit IgE binding to CN for CN-allergic individuals but completely inhibited IgE binding to CN for pollen-allergic controls. For two CN-allergic individuals (C1 and C9) CN, but not pollen, also inhibited IgE binding to CAJC. However, pollen, but not CN, inhibited IgE−CAJC interaction in the serum of one CN-allergic individual (C3). In the case of the individual who had never eaten cashew nut but had a high cashew nut specific-IgE titer (O1), neither pollen nor CN could completely inhibit IgE binding to either CN or CAJC, though CN was a better inhibitor of IgE binding to both CN and CAJC.

Since CN extract could inhibit the binding of IgE to proteins in CAJC, mAbs to two of the major CN allergens (*19*) were used to confirm that the IgE-reactive proteins could be cashew nut allergens (**Figure 5**). The mAbs bound nonreduced proteins in CAJC as well as in CN. However, no binding was seen when mAbs were blotted against nonreduced 5 kDa filtered CAJC. The linear epitope with which the mAb against Ana o 2 reacts has been mapped to the acidic subunit and is known to consist of a linear polypeptide segment: YEAGTVEAWDPNHEQ (Robotham, J., and Roux, K., unpublished data). Since this mAb targets a legumin group protein, it is expected to bind several polypeptides in a nonreduced sample. Legumins are hexameric proteins in which each of the six pairs consists of an acidic and basic subunit. Each acidic (30−40 kD)/basic (20 kDa) subunit pair is generated from a single precursor protein (50−60 kDa). The acidic and basic subunits exist in several isoforms and thus combine in sets with varying MWs.

DISCUSSION

In the age of fortification, supplementation, and super foods, cross-contamination with and mislabeling of food allergens in the food supply is an increasing problem (*20, 21*). This creates a difficult situation for all individuals with food allergies and a dangerous situation for individuals with severe food allergies. The only treatment for food allergy thus far is avoidance, a relatively easy proposition in the case of whole foods but a more challenging task in the face of manufactured foods for which the individual must rely on proper ingredient labeling. Herein, we show that a potential natural sweetener for foods, cashew apple juice concentrate, could contaminate portions of the food supply with cashew nut allergens. Notably, cashew apple concentrate from only a single supplier was examined. Fresh cashew apples themselves were not studied. Thus, further studies will be needed to determine if our results are applicable to cashew apple products in general.

We have shown that serum IgE from cashew nut allergic individuals binds proteins in an extract of cashew apple juice concentrate. In some of the serum samples that we assayed, the IgE reactivity could only be inhibited by cashew nut extract and not by an extract from the predominant grass pollen in Northern California. This suggests that the IgE was to nut-specific polypeptides rather than specific for pollen proteins with fruit homologues or specific for cross-reactive carbohydrate determinants common to many types of pollen. This presumption was further supported by the data showing that mAbs to cashew nut allergens also bound polypeptides in the CAJC extract. Moreover, the peptides in cashew apple and nut extracts recognized by the patients' IgE and the anti-cashew allergen mAbs showed similarity in MW indicating that they were probably recognizing the same polypeptides. Thus, it appears possible that concentrated cashew apple juice could cause IgE-mediated allergic reactions in cashew allergic individuals.

It is worth noting that the yield of proteins from extraction of the juice was small and difficult to quantify because of the high concentration of polyphenolic compounds also present. Additionally, the amount of protein in the concentrate is higher than that of pure juice, and as such, pure cashew apple juice may present less of a risk to cashew nut allergic patients. However, each liter of unconcentrated cashew apple juice contains 80 g of fructose and glucose (*1*), and one can of cola contains 39 g of sugars. If cashew apple juice concentrate were used as the source of sugars for a can of cola, each can of cola would contain the equivalent of ∼83 mL of 6X cashew apple concentrate. Therefore each can of cola would potentially contain 400 μg−800 μg of cashew apple protein. Trace amounts of protein allergens can cause reactions in highly sensitive individuals (*22–25*). Thus, the addition of concentrated cashew apple juice to food has the potential to contaminate processed foods with proteins that share homology with cashew nut seed storage protein allergens, as identified using monoclonal antibodies, posing a possible risk to cashew nut-sensitized individuals.

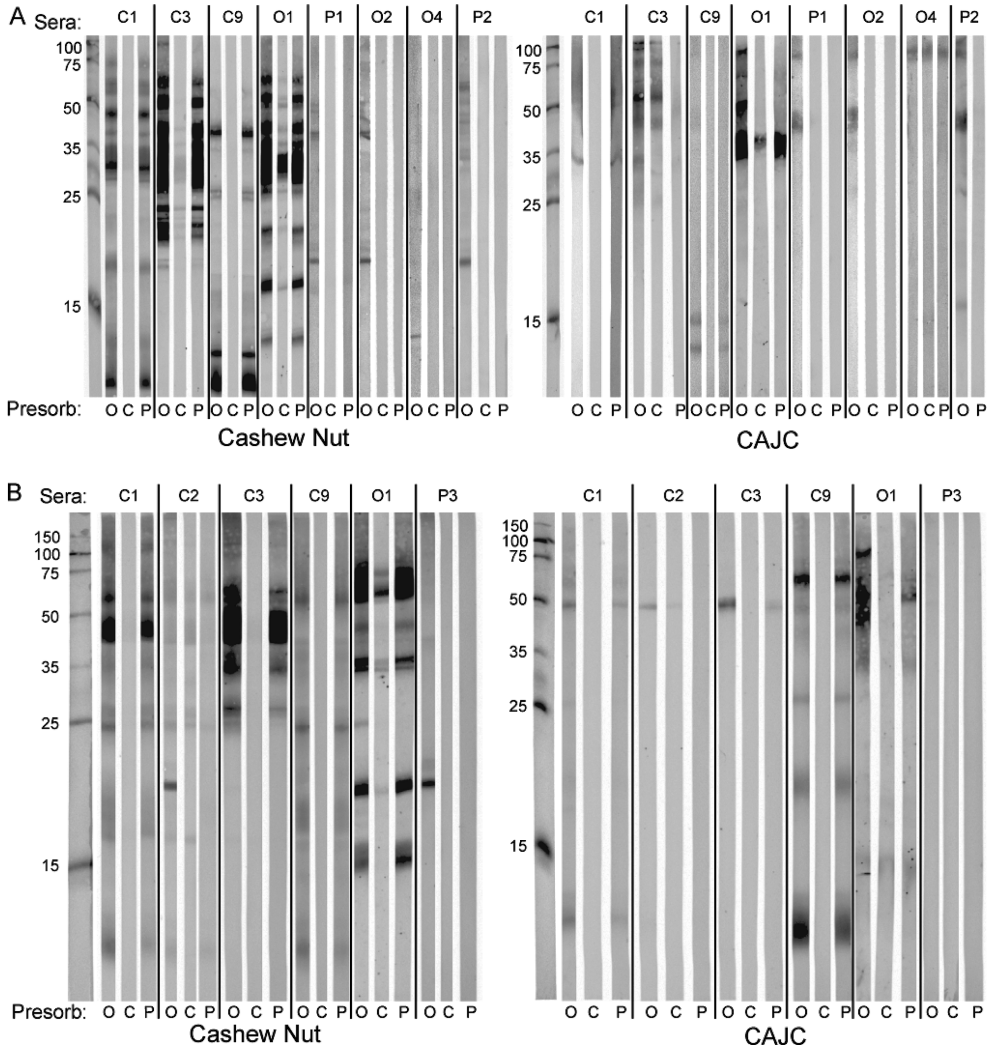

Figure 4. Some IgE reactivity to CAJC can be inhibited by preincubation with cashew nut extract. Immunoblot of cashew nut and CAJC extract. Sera were preincubated with nothing (O), cashew nut extract (C, 1 mg/mL), or grass pollen extract (P, 1 mg/mL). Gels were 14% reducing (**A**) or nonreducing (**B**) SDS−PAGE.

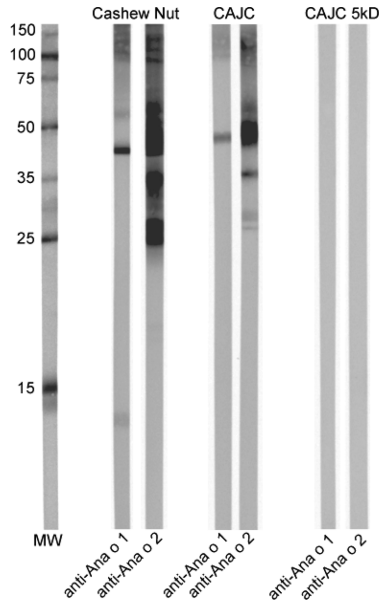

Figure 5. Assessment of cashew allergens in cashew nut extract, CAJC, and CAJC 5 kDa filtrate using cashew allergen-specific mAbs (anti-Ana o 1, anti-Ana o 2). Gels were 14% nonreducing SDS−PAGE.

We have also shown that 5 kDa filtration of the concentrate eliminated IgE binding as well as mAb binding to the cashew apple extract. Using a 5 kDa filtration method of processing may render CAJC safe for consumption by cashew nut allergic individuals. Others have shown that 10 kDa ultrafiltration can remove allergens from peach juice (*26*). However, as with peach, for CAJC processed using a commercially applicable 5 kDa ultrafiltration system to be considered generally recognized as safe for consumption by nut allergic individuals, double-blind placebo-controlled food challenges would need to be performed in individuals with documented CN allergy.

A processing method that could render CAJC cashew nut allergen free is promising because the cashew apple has many valuable properties as an additive to functional foods. These properties include a higher vitamin C content than the same weight of orange juice (*1*), antimicrobial activity (*27*), and a high concentration of sugars such as glucose and fructose, which are commonly used as sweeteners (*1, 2*). Additionally, the cashew apple is generally a waste byproduct of cashew nut production, and use of this portion of the cashew nut tree could economically benefit the geographic regions where cashew nut trees grow. As a drawback, the proposed filtration methods would remove pulp and could potentially remove other desired molecules, thus diminishing the marketability of the product for some applications.

In summary, the cashew apple juice concentrate we tested contains peptides reactive with mAbs to the cashew allergens, Ana o 1 (vicilin) and Ana o 2 (legumin), and therefore has

the potential to elicit allergic reactions in cashew nut allergic patients. After low MW-cutoff filtration, no detectable cashew allergens remained in the extract. Oral food challenges are needed to determine whether the amount of allergens in cashew apple juice concentrate, juice, or filtered juice can cause reactions in cashew nut allergic patients. Although studies with fresh cashew apples and other cashew apple juice concentrates are needed to confirm our findings, for now, it would be wise for clinicians to advise cashew nut allergic patients to avoid cashew apple juice and products containing cashew apple juice until manufacturing processes to remove cashew nut allergens from the juice have been developed and standardized.

ABBREVIATIONS USED

CN, cashew nut; mAb monoclonal antibody; CAJC, cashew apple juice concentrate; MW, molecular weight.

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