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Heat-induced alterations in cashew allergen solubility and IgE binding

Christopher P. Mattison[a,*], Yvette Bren-Mattison[b], Barry Vant-Hull[c], Aurora M. Vargas[a], Richard L. Wasserman[d], Casey C. Grimm[a]

[a] USDA—Agricultural Research Service, Southern Regional Research Center, New Orleans, LA 70124, USA
[b] BioMed Bridge, LLC., 3700 Quebec Street Suite #100-230, Denver, CO 80207, USA
[c] Prolytix, Inc., 520 Zang Street, Suite 250, Broomfield, CO 80021, USA
[d] Allergy Partners of North Texas Research, Department of Pediatrics, Medical City Children's Hospital, Dallas, TX 75230, USA

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ABSTRACT

Cashew nuts are an increasingly common cause of food allergy. We compare the soluble protein profile of cashew nuts following heating. SDS-PAGE indicate that heating can alter the solubility of cashew nut proteins. The 11S legumin, Ana o 2, dominates the soluble protein content in ready to eat and mildly heated cashew nuts. However, we found that in dark-roasted cashew nuts, the soluble protein profile shifts and the 2S albumin Ana o 3 composes up to 40% of the soluble protein. Analysis of trypsin-treated extracts by LC/MS/MS indicate changes in the relative number and intensity of peptides. The relative cumulative intensity of the 5 most commonly observed Ana o 1 and 2 peptides are altered by heating, while those of the 5 most commonly observed Ana o 3 peptides remaine relatively constant. ELISA experiments indicate that there is a decrease in rabbit IgG and human serum IgE binding to soluble cashew proteins following heating. Our findings indicate that heating can alter the solubility of cashew allergens, resulting in altered IgE binding. Our results support the use of both Ana o 2 and Ana o 3 as potential cashew allergen diagnostic targets.

1. Introduction

Cashew trees (*Anacardium occidentale*) are native to South and Central America, but are now widely grown in several tropical regions including Vietnam, India, Nigeria, Cote d'Ivoire, and Brazil [18,38]. The U.S. is the largest individual importer of shelled cashew nuts [40]. Cashew nuts are in fact seeds, and are harvested after developing along with a brightly colored cashew apple [32]. Cashew nuts, replete with beneficial fatty acids, anti-oxidants, and proteins [1], are consumed in various forms including cashew nut butter, ingredients in bakery products, savory dishes, and as whole nuts. Cashews and other nuts are considered excellent sources of nutrients whose consumption has been linked to numerous health benefits [2,6].

Cashew nut processing involves several steps to shell the edible nut and clear the nut of undesirable solids and oils. Cashew nuts contain anacardic acid and other irritants that must be removed before they can be consumed [12,17]. Under a general protocol of cashew nut processing, the raw nut undergoes several rounds of heating and cooling to facilitate extraction of the nut from the shell and skin. After harvesting and cleaning, nuts are usually dried in the sun or in a roaster to remove excess moisture. Next, in-shell nut roasting or steaming is performed to make the shell brittle and therefore easier to remove along with associated cashew nut shell liquids [38]. The cashew nut shell liquids, their anacardic acid, and other acid compounds are being investigated for use in therapeutic and other applications [21,42]. Once the shell is cut open the nut is removed and humidified, often with steam, in order to loosen and aid in the removal of the skin encasing the nut. Once the skin is peeled away the nut is ready to eat, but the cashew nuts are often heated or flushed with air again to attain an optimal moisture content of 3–5% prior to grading, packaging, and shipping to commercial outlets.

Cashew nuts are considered major food allergens and are included in a list of 8 foods that most commonly cause food allergy. Importantly, the prevalence of allergy to cashew nut appears to be increasing [15,37,45], and reactions to cashew nuts are often severe [14,16]. Characterized cashew allergens include 3 seed storage proteins: Ana o 1, 2, and 3 [31,41,50,51]. The Ana o 2 legumin accounts for approximately 50% of the soluble cashew nut protein [34], while the Ana o 1 and Ana o 3 proteins are less abundant [35].

* Corresponding author at: Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1100 Robert E. Lee Blvd., New Orleans, LA 70124, USA. Fax: +1 504 286 4419.
E-mail address: chris.mattison@ars.usda.gov (C.P. Mattison).

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Food processing steps can alter the nutritional, sensory, and immunological properties of food proteins [24,25,29,33,47]. Although effects vary depending upon conditions, thermal processing of peanuts and tree nuts can alter the profile of extractable proteins and their immunological properties [4,5,9–11,22,23,29,30,36,43,44,48,49]. Previous work has investigated the effects of processing on the stability and IgE binding of cashew nut allergens using several methods including autoclaving, boiling, microwaving, roasting, and irradiation [39,46]. The authors concluded that cashew allergens are generally refractive to denaturation and that there is little change in cashew allergen stability when assessed with antibodies directed towards individual cashew allergens [39,46]. Our studies investigated the utility of enzymatic digestion or chemical treatment to reduce IgE binding to cashew allergens *in vitro* [26,28]. The abundance of Ana o 2, as well as its relative stability during heating and other processing, suggests that the 11S legumin Ana o 2 may serve as a useful protein marker to detect cashew nuts in foods [46].

Several novel approaches for cashew nut detection have been described, including those targeting cashew protein and DNA. For example, sandwich ELISAs using polyclonal antibodies directed against total cashew protein that can detect small amounts of cashew protein in food samples have been developed [19,53]. Similarly, PCR based methods using primers specific for the Ana o 3 cashew allergen gene have been used to detect cashew nut in foods [8], and primers targeting cashew ribosomal sequences have been described for detection of cashew as an adulterant in marzipan [7,20]. Multiplex platforms including thin-film biosensor chips targeting the Ana o 3 gene sequence [52], immuno-magnetic beads [13], and a competitive multi-ELISA format for use on chocolate samples [3] have been developed for the detection of cashew nut as well as other food allergens.

Diagnostic tests for food allergens are an important tool in the food manufacturing and clinical arenas. Several factors, including food manufacturing processes have the potential to change food immunogenicity and allergenicity. Alterations in food allergen secondary or tertiary structure could have detrimental effects on the specificity and sensitivity of cashew allergen detection methods. Here, we characterize changes in cashew allergen solubility and antibody binding following cashew nut roasting. Our findings may enable improvements in cashew allergen detection in the food industry and clinical allergy settings.

2. Materials and methods

2.1. Cashew nut preparation

Ready to eat cashew nuts (designated raw for this study) were purchased from Nutsonline.com. Aluminum trays containing 20 g of raw cashews in a single layer were heated at 300 °F/149 °C (or 350 °F/177 °C) for the following times: 12 min for mild roast, 20 min for medium roast, and 24 min for dark roast. An equal amount of cashew nuts from the same sample was left untreated as a control, unheated sample. After heating, cashew extracts were prepared by grinding nuts in a coffee grinder, followed by defatting with petroleum ether using a BUCHI B-811 Standard Extraction Unit (BUCHI Labortechnik, AG, Flawil, Switzerland). The defatted cashew protein from each sample was dried in a fume hood to completely remove any residual ether residue. The defatted cashew powder was re-ground to fine particles and resuspended at a 1:10 (w/v) ratio for 1 h in borate buffered saline (BBS) solution (100 mM H_3BO_4, 25 mM NaB_4O_7, 75 mM NaCl, pH 8.6) [35] with constant mixing at 4 °C. During this time, each sample was sonicated twice on ice for 15 s using a Sonic Dismembrator (Fisher Scientific Co., Orlando, FL, USA). Clarified cashew extract solutions were prepared by centrifugation for 30 min at 14,000 rpm at 4 °C. Protein solutions from the clarified extracts were collected by pipette and protein concentrations were determined using a NanoDrop (ThermoFisher, Pittsburgh, PA, USA) device. Collected samples were dispensed into 1 ml aliquots and stored at −80 °C prior to use.

2.2. SDS-PAGE

Sample buffer with reducing agent 4X NuPAGE LDS (Life Technologies, Carlsbad, CA, USA) was added to the protein samples in a 1:4 (v/v) ratio, and a Novex Mini Cell gel rig (Life Technologies, Carlsbad, CA, USA) was used for electrophoresis. Pre-stained Precision Plus molecular weight markers (Bio-Rad, Hercules, CA, USA) were used as size indicators. Prior to loading, samples were heated at 65 °C for 15 min, electrophoresed, and protein bands were visualized using Safe Stain (Invitrogen, Grand Island, NY, USA). Gel images were captured and the protein load in each lane was quantified using the 680 nm signal channel of an Odyssey CLx infrared imaging system (LI-COR, Lincoln, NE, USA). Equivalent amounts of protein were empirically pre-determined by normalizing the signal from each lane with the IRDye680 channel on the Odyssey CLx, and load volumes were adjusted accordingly.

2.3. Liquid chromatography–mass-spectrometry (LC–MS/MS)

Cashew extract samples were prepared and characterized by LC–MS/MS in a manner similar to that described in previous work [27]. However, in these experiments equivalent amounts of protein (50 ng) from raw or roasted cashew nuts were digested with 0.2 ng trypsin, and samples were acidified with formic acid before being analyzed with an Agilent 1200 LC system, an Agilent Chip Cube interface, and an Agilent 6520 Q-TOF tandem mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The raw data files were extracted, sequenced, and searched against a custom database containing cashew allergen protein sequences to identify matching peptides using Spectrum Mill software (Agilent Technologies, Santa Clara, CA, USA) and determine relative abundance. Relative quantification of individual peptide intensity was accomplished by integrating the extracted ion chromatogram from the MS data specifically for the respective ion indicated.

2.4. ELISA

Polyclonal rabbit anti-cashew antisera, used previously in cashew allergen binding studies [26], was purchased from Pierce Biotechnology Inc. (Rockford, IL, USA). Samples containing 250 ng of cashew extract were diluted with half-log serial dilutions in PBS with 0.1% Tween-20 (PBST), and 50 μl was added to microtiter plate wells. After incubating overnight at 4 °C, cashew extract was removed and 50 μl of PBST containing 2% BSA was added for 1 h at room temperature to block remaining binding sites within the wells. After washing 4 times with 200 μl of PBST, rabbit anti-cashew serum (diluted 1:5,000 in PBST) was added to wells and incubated at room temperature for 1 h. Rabbit antisera was removed and wells were washed as above followed by the addition of a secondary anti-rabbit antibody labeled with IRdye-800 (LI-COR, Lincoln, NE, USA) diluted 1:20,000 in PBST. Wells were washed 4 times again with PBST and antibody binding was visualized and quantified using with an Odyssey CLx infrared imaging system (LI-COR, Lincoln, NE, USA). ELISA assays were performed in quadruplicate, and the data for each treatment (raw, medium, and dark) was compared for statistical analysis. The rabbit anti-cashew antibody data was analyzed using a typical saturation kinetics model:

$$S = S_0 + \frac{fPAb}{fP + K_d}$$

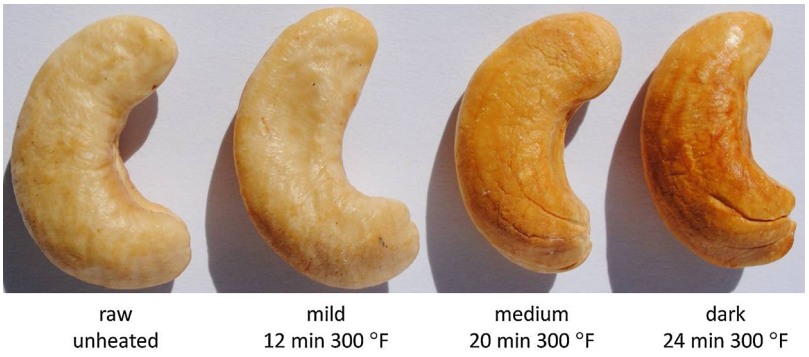

Fig. 1. Representative images of cashew nuts following heating at 300 °F for 12 min (mild), 20 min (medium), 24 min (dark), or un-heated (raw).

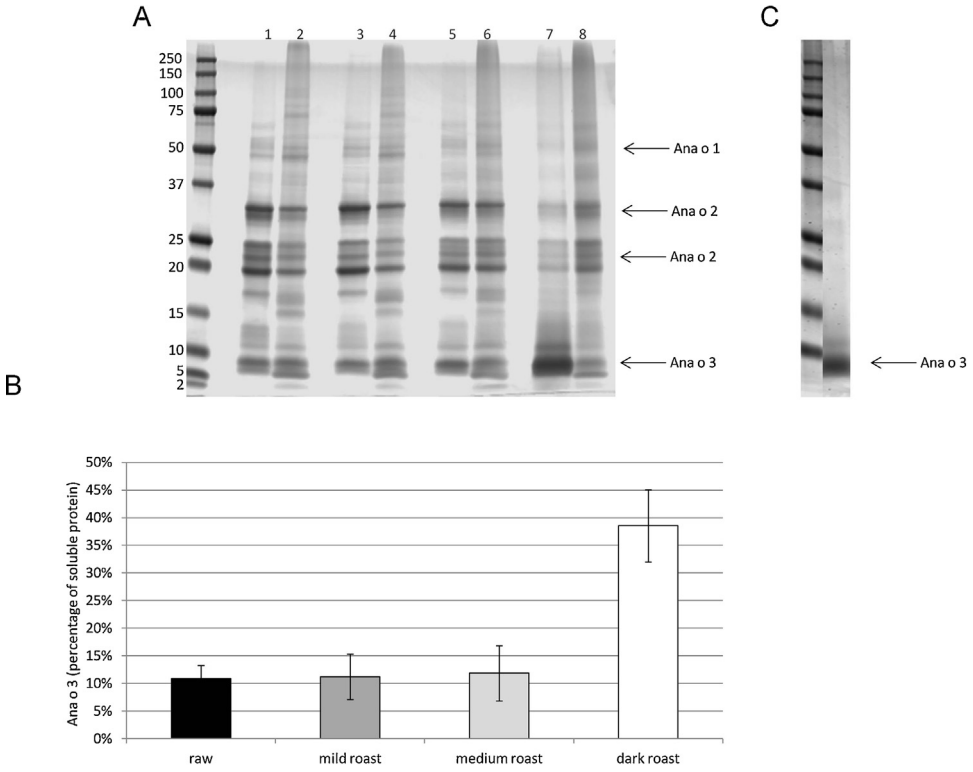

Fig. 2. SDS-PAGE analysis of the soluble and insoluble fractions of raw and heated (300 °F) cashew nut extracts (A) and quantification of soluble Ana o 3 level (B). Lane 1: soluble raw cashew, lane 2: insoluble raw cashew, lane 3: soluble mild roast (12 min) cashew, lane 4: insoluble mild roast cashew, lane 5: soluble medium roast (20 min) cashew, lane 6: insoluble medium roast cashew, lane 7: soluble dark roast (24 min), lane 8: insoluble dark roast cashew. Soluble protein extracted from cashew nuts heated at 350 °F for 24 min (soluble 350 °F dark roast) (C). Molecular weight markers are indicated on the left-most lane of the gels, and the arrows indicate the migration position of the Ana o 1, Ana o 2, and Ana o 3 proteins.

where S is the signal, S_0 is the background signal, P is the sample (protein) concentration, f is the fraction of active protein (defined by affinity for the antibody), Ab is the effective antibody concentration, and K_d is the dissociation constant. We converted this to a modified Scatchard equation by taking the reciprocal of the above equation:

$$\frac{1}{S - S_0} = \frac{K_d}{fAb}\frac{1}{P} + \frac{1}{Ab}$$

The background S_0 was determined by averaging the signals at the 2 lowest sample concentrations. To avoid further complications of the background, the data point immediately above the 2 background points in each treatment set was excluded from the analysis. Because the equations above are only valid for protein concentrations well above the antibody concentration, the 2 points at the highest concentrations were excluded as well. For each treatment, $1/(S - S0)$ was plotted vs. $1/P$, and the least-squares slope of each plot was determined, corresponding to $Kd/(f\ Ab)$. Although the intercepts appeared to be well behaved in this particular data examination, in general this determination is more subject to error than the determination of slope for a Scatchard analysis, and therefore they were not included in the analysis. IgG binding data was evaluated using a one-way ANOVA analysis comparing 3 of the treatments ("Raw," "Medium" and "Dark").

For ELISA using human sera samples, microplate wells were coated with 1 μg of raw, mild, medium, or dark roast cashew extract and left at 4 °C overnight. The following morning, PBST containing 2% BSA was added to the well and incubated for 1 h at room temperature. Wells were washed 4 times with 200 μl of PBST and pooled human serum samples from cashew allergic patients previously

characterized [26,28] were diluted 1:5 with PBST and added to microplate wells. After 1 h at room temperature they were washed 4 times with 200 μl of PBST, and secondary anti-human IgE antibody labeled with IRdye-800 (diluted 1:5,000 in PBST) was added for 1 h at room temperature. Following incubation of the membranes for 1 h at room temperature, the membranes were washed and visualized as above using an Odyssey CLx infrared imaging system (LI-COR, Lincoln, NE, USA). The average of 4 human sera IgE binding experiments for each treatment was used for analysis. The average of IgE binding to raw cashews was set to 100% and the average of the other treatments was converted as a percentage of the raw cashew signal using the formula $(B/A) \times 100$, where B is the average of the treatment in question and A is the average of the raw treatment antibody binding value. The average percent binding for each of the samples was plotted with standard deviation indicated in the error bars.

3. Results

3.1. Solubility profile of heated cashews

Food processing and preparation steps are meant to enhance flavor, but they can alter several other food characteristics. To determine the effect that dry heat has on cashew nuts, we began by characterizing the solubility profile of heated cashew nut extracts using SDS-PAGE. Protein extracts were made from untreated cashew nuts (raw) or cashews heated in a single layer at 300 °F/149 °C for the following times: 12 min for mild roast, 20 min for medium roast, and 24 min for dark roast (Fig. 1). Normalized soluble and insoluble protein loading was empirically determined by adjusting loading volume based upon previous analysis of Coumassie stained SDS-PAGE and quantification using the IRDye 680 channel of a Odyssey CLx instrument. Equivalent amounts of protein (25 μg) from the soluble and insoluble portions of ready to eat (raw) or heated cashew were then electrophoresed in SDS-PAGE, stained with Coumassie, and the protein content in whole lanes was again quantified using the IRDye 680 channel of a Odyssey CLx instrument to ensure equivalent loading. As shown in Fig. 2A, heating for either 12 (mild roast) or 20 (medium roast) min at 300 °F did not appreciably alter the profile of soluble and insoluble protein compared to that of raw cashews. In contrast, heating cashews for 24 min (dark roast) resulted in a visible change in the extraction profile (Fig. 2A, lanes 7, 8). Most notably, we observed an increase in the relative amount of Ana o 3 in the soluble protein fraction of the dark roast sample. Quantification of the Ana o 3 band from each individual lane indicated that the level of Ana o 3 in the soluble fraction from the dark roast sample was increased by approximately 40%, and that there was a corresponding decrease in the insoluble Ana o 3 fraction (Fig. 2A and B). Conversely, soluble levels of both Ana o 1 and Ana o 2 were decreased, respectively, in the dark roast cashew nuts heated for 24 min (Fig. 2). More intense heating of cashew nuts at 350 °F (177 °C) lead to an even greater proportion of Ana o 3 in the soluble extract fraction. Heating cashew nuts for greater than 22 min at 350 °F resulted in Ana o 3 being essentially the only protein extracted from the heated nuts (Fig. 2C).

3.2. Liquid chromatography mass–spectrometry of raw and heated cashew extracts

To further characterize the changes in soluble protein profiles, we assessed the raw and heated cashew samples by mass-spectrometric analysis. Following treatment of samples with trypsin and LC/MS/MS, we detected a qualitative shift in the relative number and intensity of peptides surveyed by the mass-spectrometric analysis (Fig. 3A). For example, the number of Ana o

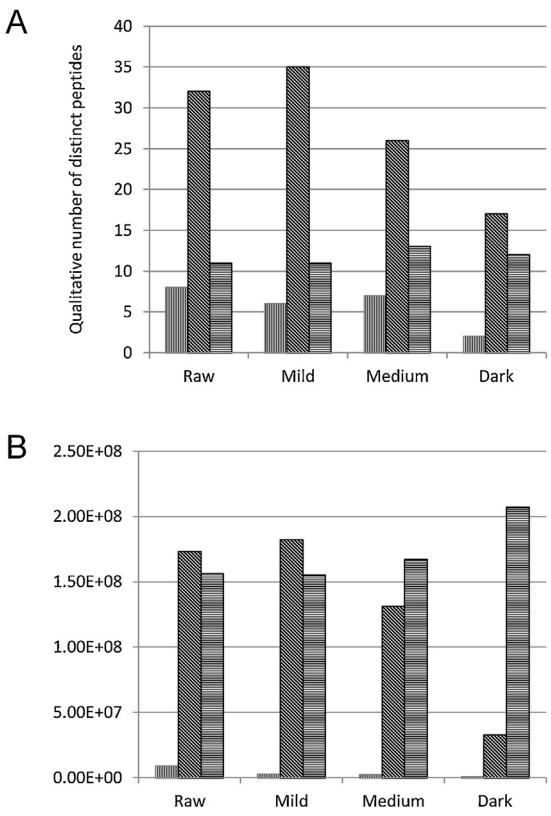

Fig. 3. Qualitative mass-spectrometric analysis of observed peptides from trypsin-digested soluble extracts of raw and heated cashew nuts. Qualitative number of distinct peptides (A) and Qualitative intensity of observed peptides (B) from Ana o 1 (vertical bars), Ana o 2 (diagonal bars), and Ana o 3 (horizontal bars).

1 and 2 peptides observed in the soluble fraction from dark roast cashew nuts was reduced compared to the raw and mildly roasted samples. Further, there was a reduction in the qualitative intensity of Ana o 1 and Ana o 2 peptides detected in the dark roast sample compared with the raw and mildly roasted samples (Fig. 3B). Conversely, the qualitative number and intensity of Ana o 3 peptides was relatively constant among the various samples (Fig. 3B). While these results are qualitative, they are consistent with our observations from our SDS-PAGE analysis.

To further characterize the changes in the observed mass-spectrometry signals, we assembled the 5 most commonly observed peptides for each of the cashew allergens and integrated the respective peak areas for each of these peptides. We noticed changes in the intensity of peptides detected as cashew nut heating progressed (see Tables 1–3). The most commonly observed Ana o 1 and Ana o 2 peptides varied as heating progressed; however, the same 5 most common Ana o 3 peptides were observed independent of heating duration. Further, there was an increase in the signal intensity of the Ana o 3 peptides observed in the dark roast sample. These data further illustrate the differences in soluble proteins and peptide profiles following cashew nut heating.

3.3. IgG and IgE binding to raw and heated cashew extracts

To evaluate the immunological significance of the changes in solubility we observed, we tested the binding of rabbit anti-cashew polyclonal antisera to the cashew samples. Because the SDS-PAGE and mass-spectrometry results were so similar between the raw and mild samples, we did not analyze the mild roasted samples using the polyclonal sera. Antibody binding to serial half-log dilutions of the samples starting with a concentration of 0.25 μg/ml was

Table 1
The sequence and integrated peak areas of the 5 most commonly observed Ana o 1 peptides from raw and roasted cashew nuts by LC/MS analysis. Note that the Ana o 1 peptide 342-IWPFTEESTGSFK-354 was present in the raw, mild, and medium samples at a relatively high level, but was not among the most commonly observed peptides in the dark roast sample. Conversely, the Ana o 1 peptide 28-IDPELK-33 was observed in the medium and dark roast samples.

Ana o1	Peptide sequence	AA Start	RT (min)	Precursor *m/z*	Mass $[MH]^+$	Peak area
raw	(K)IWPFTEESTGSFK(L)	342	7.546	764.867	1528.73	3,77,89,982
raw	(R)AFSWEILEAALK(T)	293	18.561	689.371	1377.74	4,31,72,441
raw	(R)IDYPPLEK(L)	375	10.786	487.763	974.519	4,40,20,857
raw	(K)QDEEFFFQGPEWR(K)	517	16.058	857.88	1714.75	2,09,49,306
raw	(R)QGDIVSISSGTPFYIANNDENEK(L)	238	14.711	833.391	2498.168	2,25,95,486
mild	(K)IWPFTEESTGSFK(L)	342	7.719	764.866	1528.73	3,09,02,244
mild	(R)EREHEEEEEEWGTGGVDEPSTHEPAEK(H)	68	9.658	625.268	3122.31	3,17,53,066
mild	(R)AFSWEILEAALK(T)	171	18.422	689.371	1377.74	3,07,82,590
mild	(K)YGQLFAER(I)	366	10.768	556.77	1112.54	2,16,28,229
mild	(R)IDYPPLEK(L)	375	10.733	487.237	974.519	2,16,28,229
med	(k)IDPELK(Q)	28	7.98	357.705	714.403	2,50,10,289
med	(K)IWPFTEESTGSFK(L)	342	7.453	764.868	1528.73	3,19,13,750
med	(R)AFSWEILEAALK(T)	293	18.546	689.365	1377.74	3,48,39,007
med	(K)QDEEFFFQGPEWR(K)	517	15.979	857.878	1714.75	1,90,94,254
med	(K)YGQLFEAER(I)	366	10.77	556.77	1112.54	2,26,51,363
dark	(k)IDPELK(Q)	28	7.98	357.705	714.403	20,93,985
dark	(R)QYDEQQKEQCVK(E)	44	14.549	791.862	1582.72	27,14,972
dark	(R)AFSWEILEAALK(T)	293	12.022	689.375	1377.74	29,36,063
dark	(K)HLSQCMR(Q)	95	7.138	466.217	931.424	15,53,769
dark	(K)QDEEFFFQGPEWR(K)	517	16.179	857.866	1714.75	5,60,779

Table 2
The sequence and integrated peak areas of the 5 most commonly observed Ana o 2 peptides from raw and roasted cashew nuts by LC/MS analysis. Note that the Ana o 2 peptide 292-ADIYTPEVGR-301 was among the most commonly observed peptides in the raw, mild, and medium samples but not the dark roast. The Ana o 2 39-VEYEAGTVEAWDPNHEQFR-57, 198-NLFSGFDTELLAEAFQVDER-217, and 390-FEWISFK-396 peptides were observed in the dark roast sample, but were not among those most commonly observed in the raw, mild, and medium samples.

Ana o2	Peptide sequence	AA Start	RT (min)	Precursor *m/z*	Mass $[MH]^+$	Peak area
raw	(R)LDALEPDNRVEYEAGTVEAWDPNHEQFR(C)	30	14.25	825.886	3300.519	37,56,82,411
raw	(R)ADIYTPEVGR(L)	292	10.344	560.786	1120.563	28,81,83,427
raw	(R)LKENINDPAR(A)	282	7.851	390.548	1169.627	51,56,99,539
raw	(R)EGQMLVVPQNFAVVK(R)	369	15.35	829.956	1658.893	23,01,82,192
raw	(R)KFHLAGNPK(D)	175	7.58	337.863	1011.573	42,99,69,213
mild	(R)LDALEPDNRVEYEAGTVEAWDPNHEQFR(C)	30	14.179	825.883	3300.519	60,47,44,767
mild	(R)LKENINDPAR(A)	282	7.773	390.548	1169.627	82,32,15,133
mild	(R)ADIYTPEVGR(L)	292	10.282	560.783	1120.563	51,51,30,530
mild	(R)KFHLAGNPK(D)	175	7.46	337.864	1011.573	57,39,18,793
mild	(R)EGQMLVVPQNFAVVK(R)	369	15.151	829.956	1658.893	34,84,03,379
med	(R)LDALEPDNRVEYEAGTVEAWDPNHEQFR(C)	30	14.099	825.878	3300.519	41,07,69,443
med	(R)LKENINDPAR(A)	282	7.717	390.548	1169.627	59,26,46,710
med	(R)ADIYTPEVGR(L)	292	10.218	560.783	1120.563	33,88,91,251
med	(R)KFHLAGNPK(D)	175	7.47	337.866	1011.573	37,01,33,067
med	(R)EGQMLVVPQNFAVVK(R)	369	15.177	829.956	1658.893	24,87,50,019
dark	(R)LDALEPDNRVEYEAGTVEAWDPNHEQFR(C)	30	14.324	825.878	3300.519	5,78,65,998
dark	(R)LKENINDPAR(A)	282	7.6	390.548	1169.627	4,70,39,354
dark	(R)NLFSGFDTELLAEAFQVDER(L)	198	20.489	767.697	2301.103	9,83,21,604
dark	(R)FEWISFK(T)	390	15.487	478.474	956.488	3,41,28,938
dark	(R)VEYEAGTVEAWDPNHEQFR(C)	39	2.937	759.674	2277.021	2,90,55,243

analyzed by ELISA. IgG binding to the dark roast cashew extract had lower initial and final values compared to the raw and medium roast samples. We evaluated the data using a one-way ANOVA analysis and found that there was significant difference between the 3 treatments ("Raw," "Medium", and "Dark") with a confidence level greater than 99.9% ($F(2, 5) = 41.3$). A representative plot of the slopes for each treatment is shown in Fig. 4A. Assuming the effective antibody concentration and the dissociation constant to be stable between treatments, the reciprocal of the slope is proportional to the active (antibody binding) fraction of protein within each sample. Normalizing to the raw samples, we show that the fraction of active protein drops more than 5-fold going from raw to medium, and drops another 50% going from medium to dark roast (Fig. 4B).

ELISA assays using a pool of 6 cashew-allergic patient sera were also used to characterize the antigenic differences between the raw and roasted cashew extracts. Pooled sera were diluted 1:4 with PBS and added to wells containing 1 μg of soluble protein from the raw, mild, medium, or dark roasted cashew nuts. There was reduced IgE binding to 1 μg of protein made from heated cashew extracts. While IgE binding to the mild and medium roasted cashews was reduced to 88% and 84%, respectively, compared with the raw extract, IgE binding to the dark roast cashew extract was reduced to less than 60% of the raw extract (Fig. 5). These data are consistent with the reduced solubility of the higher molecular weight bands observed in the SDS-PAGE gel and suggest a reduction in IgE binding epitopes within the roasted samples.

Table 3
The sequence and integrated peak areas of the 5 most commonly observed Ana o 3 peptides from raw and roasted cashew nuts by LC/MS analysis. Note that the same 5 most common Ana o 3 peptides were observed irrespective of heating duration.

Ana o3	Peptide sequence	AA Start	RT (min)	Precursor *m/z*	Mass $[MH]^+$	Peak area
raw	(R)ELYETASELPR(I)	112	11.189	654.321	1306.628	1,31,29,09,658
raw	(R)CQNLEQMVR(Q	89	10.263	589.277	1176.539	1,11,34,56,233
raw	(R)ECCQELQEVDRR(C)	75	8.518	541.24	540.233	99,79,74,248
raw	(R)QLQQQEQIKGEEVR(E)	98	8.344	856.948	1711.882	9,04,92,853
raw	(R)ICSISPSQGCQFQSSY(−)	123	11.942	924.893	1847.783	1,41,52,444
med	(R)ELYETASELPR(I)	112	11.057	654.33	1306.645	1,63,77,45,040
med	(R)CQNLEQMVR(Q	89	10.147	589.274	1176.534	1,29,45,05,423
med	(R)ECCQELQEVDRR(C)	75	8.591	541.236	540.229	1,26,10,18,230
med	(R)QLQQQEQIKGEEVR(E)	98	8.234	856.949	1711.884	13,14,47,629
med	(R)ICSISPSQGCQFQSSY(−)	123	11.912	924.901	1847.783	3,07,14,199
mild	(R)ELYETASELPR(I)	112	11.137	654.326	1306.638	1,82,76,46,498
mild	(R)CQNLEQMVR(Q	89	10.254	589.277	1176.539	1,29,52,54,642
mild	(R)ECCQELQEVDRR(C)	75	8.632	541.24	540.233	1,44,99,74,404
mild	(R)QLQQQEQIKGEEVR(E)	98	8.27	856.95	1711.885	21,28,84,939
mild	(R)ICSISPSQGCQFQSSY(−)	123	11.898	924.899	1847.783	1,39,84,077
dark	(R)ELYETASELPR(I)	112	10.876	654.331	1306.647	2,45,13,14,832
dark	(R)CQNLEQMVR(Q	89	9.844	589.269	1176.524	1,50,65,80,462
dark	(R)ECCQELQEVDRR(C)	75	8.518	541.24	540.233	1,81,38,01,039
dark	(R)QLQQQEQIKGEEVR(E)	98	8.038	856.953	1711.891	30,48,64,976
dark	(R)ICSISPSQGCQFQSSY(−)	123	11.919	924.897	1847.783	5,42,88,703

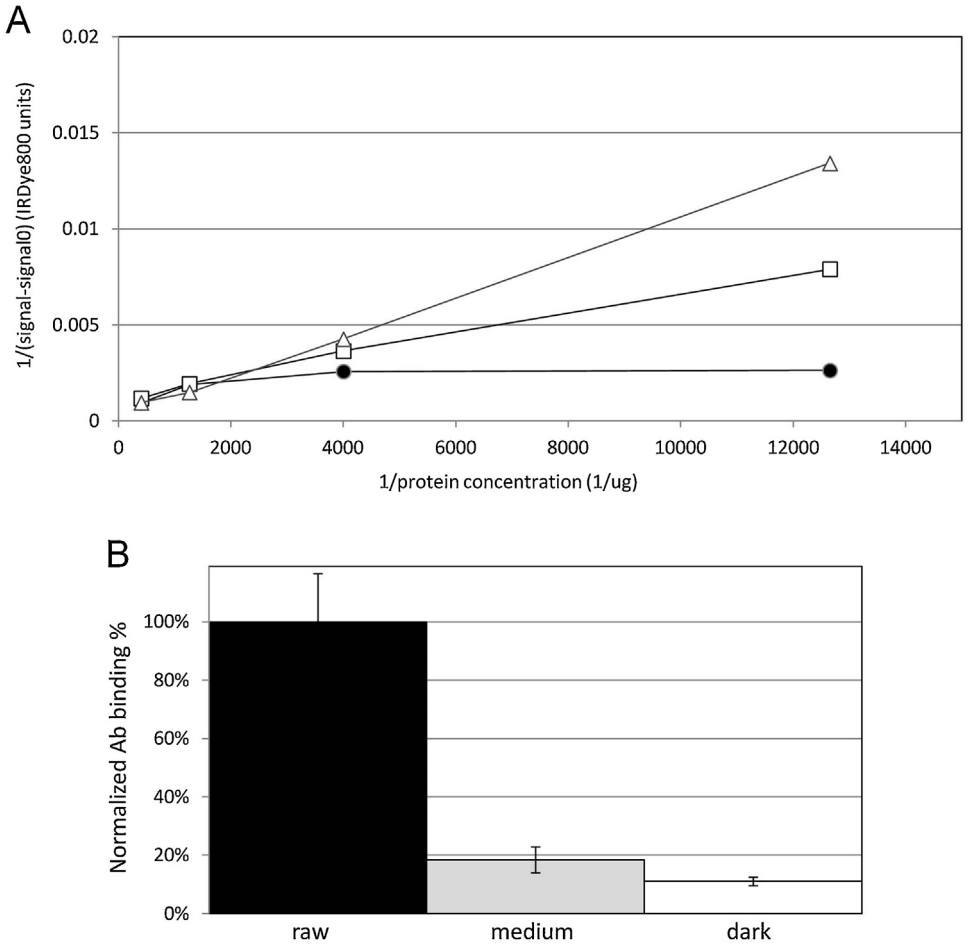

Fig. 4. Direct IgG ELISA using serial half-log dilutions of soluble extract from raw cashew (dark circles), medium roast cashew (open squares), dark roast cashew (open triangles); and rabbit anti-cashew polyclonal sera. Plot of antibody binding to extracts from each treatment (A), where the inverse of protein concentration is indicated on the *x*-axis and the *y* axis is 1/(signal—background). Determination of percentage of active antibody binding protein from each sample (B) with raw (dark bar), medium (dark grey bar), and dark roast extract (white bar).

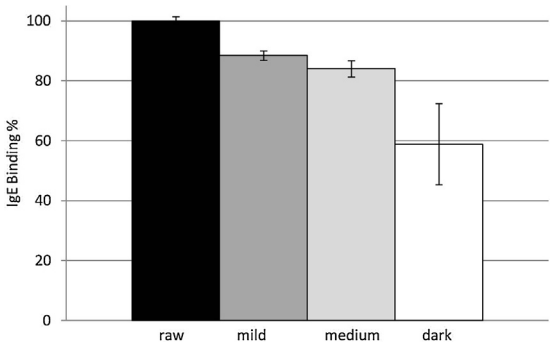

Fig. 5. Direct IgE ELISA with 1 μg of soluble protein extract from raw cashew (dark bar), mild roast cashew (dark grey bar), medium roast cashew (light grey bar), dark roast cashew (white bar); and a pool of 6 human sera from patients with clinically defined cashew allergy. Cashew sample is indicated on the *X*-axis, percent of IgE binding relative to that of raw cashew nut is indicated on the *Y*-axis, and standard deviation is used for errors.

4. Discussion

In the present study, we describe changes in the soluble protein profile of heated cashew nuts, and we propose that these results are likely to have food allergen detection and immunological consequences. While Ana o 2 makes up roughly 50% of the soluble cashew protein in raw or ready-to-eat nuts, our SDS-PAGE results indicate that heating can alter the relative amount of soluble immunoreactive Ana o 2. This reduction in protein solubility leads to a greater relative percentage of the Ana o 3 allergen. This is consistent with previously published analysis of extracts from cashew nuts heated for 20 min at 170 °C or 15 min at 200 °C in which SDS-PAGE analysis indicated a change in relative cashew allergen solubility [46]. Our results support targeting both Ana o 2 and Ana o 3 for cashew allergen detection-methods development. Ana o 1 makes up a smaller percentage of soluble cashew protein, and we found that its level was reduced in heated cashews, making it less useful as a target for allergen detection in processed foods.

We report that among the cashew allergen peptides detected using LC/MS/MS analysis, the Ana o 3 peptides are relatively abundant and intense. In fact, the list of 5 most common Ana o 3 peptides was unaltered in the raw and heated cashew extracts. Furthermore, the overall accumulated intensity of these peptides was increased in heated cashew samples. The most commonly observed Ana o 3 peptides we found included amino acid sequences from a previously mapped strongly reacting Ana o 3 IgE epitope, epitope 10-72-SLRECCQELQEV-83 [31]. Although Ana o 2 peptides were relatively well conserved among the samples, the overall intensity was decreased, consistent with the SDS-PAGE results. In contrast, the 5 most commonly observed Ana o 1 peptides changed in heated cashews and the overall relative intensity decreased.

There is a large body of evidence indicating that peanut and tree protein solubility and immunological characteristics are altered following thermal processing [4,5,9–11,22,29,30,36,43,44,48,49]. Similarly, our findings suggest that heating can alter the solubility of cashew allergens and antibody binding. Our findings are consistent with previous studies showing that monoclonal antibodies to Ana o 1 and Ana o 2 exhibited reduced binding to heated cashew extracts relative to unheated cashew extracts [46]. The results for each cashew allergen from the Venkatachalam et al. [46] study varied depending upon the monoclonal antibody used and the test being performed, and this may reflect changes in allergen structure or heating-induced modification. Our finding that the relative amount of Ana o 3 is increased after heating is also consistent with the Venkatachalam et al. [46] study that reported an increase in monoclonal antibody binding to Ana o 3 by immunoblot in extracts made from cashews heated for 20 min at 170 °C or 15 min at 200 °C [46]. Although boiling or blanching has been shown to reduce soluble Ana o 2 and Ana o 3 levels [46], collectively these findings suggest that diagnostic approaches targeting both Ana o 2 and Ana o 3 may be advantageous.

Our findings indicate that heating can alter the solubility of cashew allergens and thus change the relative amount of cashew allergens such as Ana o 2 and Ana o 3 present in the extract. Cashew nuts are processed in several steps using processing systems and machinery that can vary regionally before they reach consumers. The methods and equipment used could introduce variation in the amount of heating that cashew nuts are exposed to resulting in variations in allergen solubility. Further studies characterizing different processing steps and methods from various regions may identify differences that affect allergen solubility. Similarly, cashew nuts are used in various foods from stir-fry to confections and heating steps are likely to alter the soluble allergen profile. Our findings could be applied to the conception and design of improved diagnostic tools and methods for detection of cashew nut allergens in mislabeled or contaminated foods.

Transparency document

The Transparency document associated with this article can be found in the online version.

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