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Heat-induced alterations in cashew allergen solubility and IgE binding

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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].

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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:

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