

Identification and Characterization of Ana o 3 Modifications on Arginine-111 Residue in Heated Cashew Nuts

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ABSTRACT: Raw and roasted cashew nut extracts were evaluated for protein modifications by mass spectrometry. Independent modifications on the Arg-111 residue of Ana o 3 were observed in roasted but not raw cashew nuts. The mass changes of 72.0064 or 53.9529 Da are consistent with the formation of carboxyethyl and hydroimidazolone modifications at the Arg-111 residue. These same modifications were observed in Ana o 3 purified from roasted but not raw cashew nuts, albeit at a relatively low occurrence. Circular dichroism indicated that Ana o 3 purified from raw and roasted cashew nuts had similar secondary structure, and dynamic light scattering analysis indicated there was no observable difference in particle size. The stability of Ana o 3 purified from raw and roasted cashew nuts to trypsin was similar in the absence of or following treatment with a reducing agent. Only minor differences in IgE binding to Ana o 3 were observed by ELISA among a cohort of cashew-allergic patient sera.

KEYWORDS: food allergy, cashew, Ana o 3, heating, modification

INTRODUCTION

The incidence of self-reported food allergy has increased over the past decades,¹ despite an apparent lack of increase in food-specific IgE.² Several factors, including genetics, environment, diet, microbiota, and food-processing steps, are thought to contribute to the increased incidence of food allergies.³ Food allergies are associated with significant financial, emotional, and social burdens on those afflicted by the disorder as well as on their friends and family.⁴

Food-processing steps are used to increase the sensory, storage, safety, or nutritional qualities of foods. Food processing is also considered a potential factor contributing to the development of food allergies; however, the underlying mechanisms by which food processing can enhance or reduce the ability of food proteins to induce allergic sensitization and/or affect IgE binding are not fully understood.⁵ The steps used to prepare nuts and other foods can precipitate, cross-link, aggregate, denature, or chemically modify proteins, thereby altering their allergenic potential.⁶ For example, heating milk or egg proteins can reduce IgE binding to some allergens,⁷ whereas heating cashew nuts or peanuts can alter allergen solubility.^{8,9} In the presence of glucose or oxidizing agents, chemical modifications may alter the relative abundance of some peptides, as detected by LC-MS/MS, in hazelnut extracts.¹⁰ Similarly, nonenzymatic modification of lysine or arginine residues can destroy protease sites, thereby preventing cleavage by some proteases and potentially increasing the stability of allergens and other proteins.¹¹ In some cases, such as egg and milk allergies, there is clear evidence that heating

these foods in a wheat matrix decreases allergenicity in affected individuals.^{12,13} Efforts have been invested in identifying and characterizing processing-induced modifications in peanut and tree nut allergens and correlating their effect(s) on the immunological properties of seed storage proteins.⁶

Cashew nuts undergo extensive processing prior to consumption to remove harmful oils and acids and improve sensory qualities.¹⁴ There is wide variation in regional cashew nut harvesting and processing steps, and in many instances this work is performed manually on small locally collected batches of nuts. Several steps are needed to remove harmful oils, clean the edible nut, and heat or cool nuts to the appropriate level of moisture.¹⁵ Nuts are heated in-shell to facilitate shell cracking and humidified or steamed to aid in removal of the skin encasing the nut. Isolated nuts can be subjected to another round of heating or flushed with air to dry them to 3–5% moisture content before grading, packaging, and shipping. These processing steps could alter the nutritional, sensory, and immunologic properties of cashew nuts.

Although cashew nuts are considered a good source of beneficial fatty acids, phytochemicals, and other nutrients, they are also included in a group of eight foods that commonly cause food allergies.^{16,17} Studies have suggested that cashew nuts often cause severe allergic reactions,¹⁸ and they are a common

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cause of tree nut allergy in the United States.¹⁹ Cashew nut allergens include the seed storage proteins Ana o 1, Ana o 2, and Ana o 3.^{20–23} Cashew allergens have been shown to be relatively resistant to several types of processing, but heating cashew nuts can alter the solubility of their allergens in aqueous buffer and affect IgE binding.^{9,24} Enzymatic and chemical treatments have been shown to reduce cashew allergen potency in vitro and in a mouse model.^{25–27} Herein, for the first time, heating-induced modifications on the Ana o 3 cashew nut allergen formed during the roasting of cashew nuts have been identified and characterized.

MATERIALS AND METHODS

Materials. Ready-to-eat cashew nuts were purchased from nutsonline.com (<http://www.nuts.com>, Cranford, NJ, USA) and stored at 4 °C. Bovine pancreatic trypsin (Sigma-Aldrich, St. Louis, MO, USA; T-1426), Any kD Mini-Protean TGX precast Tris-glycine gels (Bio-Rad, Hercules, CA, USA), Novex 10–20% Tricine gels (Life Technologies, Carlsbad, CA, USA), and nonsterile Maxisorp 96-well Nunc microtiter plates (Thermo Scientific, Rochester, NY, USA) were used according to the manufacturers' instruction. Anti-human IgE antibody and biotinylated anti-human IgE antibody were from Southern Biotech (Birmingham, AL, USA). IRdye-680-labeled streptavidin and IRdye-800 labeled secondary antibodies were from LI-COR (Lincoln, NE, USA).

Cashew Roasting, Extraction, and Purified Ana o 3 Preparation. Purchased cashew nuts were pooled and thoroughly mixed prior to sample processing to prevent contributions from different lots of nuts. Heated cashew nuts were prepared by roasting as previously described in Mattison et al.⁹ Briefly, 20 g samples of cashew nuts in aluminum trays were heated at either 300 °F/149 °C or 350 °F/177 °C for 12 min (light roast), 20 min (medium roast), or 24 min (dark roast). Cashew extracts were prepared by grinding nuts in a coffee grinder, defatting with petroleum ether, and solubilization at a 1:10 (w/v) ratio for 1 h in a borate-buffered saline (BBS) solution (100 mM H₃BO₃, 25 mM NaB₄O₇, 75 mM NaCl, pH 8.6) with sonication as described in Mattison et al.⁹ Extracts were clarified by centrifugation for 30 min at 14,000 rpm at 4 °C, and protein concentrations from the extracts were determined using a NanoDrop (ThermoFisher, Pittsburgh, PA, USA) device.

Ana o 3 from raw and roasted cashew nut extracts was purified according to the method described in Mattison et al.²⁶ Extracts from raw and roasted cashew nuts were precipitated with 75% ammonium sulfate and the supernatant, enriched for Ana o 3, was then precipitated with 100% ammonium sulfate and centrifugation. Ana o 3 was purified from the 100% ammonium sulfate pellet in a 5 mM sodium phosphate (pH 6.8) buffer, using a linear sodium phosphate gradient (5–200 mM), and a ceramic hydroxyapatite column on a BioLogic LP low-pressure chromatography system (Bio-Rad, Hercules, CA, USA) with a flow rate of 3 mL/min.

SDS-PAGE. SDS-PAGE samples were prepared by adding 4× NuPAGE LDS sample buffer with reducing agent (Life Technologies, Carlsbad, CA, USA) to samples with equal amounts of protein (5 µg/lane) in a 1:4 (v/v) ratio and heating at 65 °C for 15 min. A Novex Mini Cell gel rig (Life Technologies) was used for electrophoresis with prestained Precision Plus molecular weight markers (Bio-Rad, Hercules, CA, USA) to mark migration. Electrophoresed proteins were visualized using Safe Stain (Invitrogen, Grand Island, NY, USA), and images were collected using the 680 nm signal channel of an Odyssey CLx infrared imaging system (LI-COR, Lincoln, NE, USA). Protein loading was quantified to ensure equivalent amounts of protein in each lane based on the 680 nm signal.

LC-MS/MS Mass Spectrometry. Soluble cashew extracts and purified Ana o 3 were prepared and analyzed by liquid chromatography coupled with mass spectrometric analysis as described in Mattison et al.²⁶ Following addition of ammonium bicarbonate to a final concentration of 100 mM, protein samples were sequentially reduced with 4 mM dithiothreitol, alkylated with 14 mM

iodoacetamide, and finally quenched by the addition dithiothreitol to 4 mM prior to digestion. Sequencing-grade modified trypsin (0.4 µg) from Promega (Madison, WI, USA) was added to fragment protein extracts and purified Ana o 3 overnight with gentle agitation at 37 °C. Samples were acidified with 1 µL of 95% formic acid prior to LC-MS/MS. Peptide sequencing and BLAST searches were conducted using Agilent Technologies Spectrum Mill MS Proteomics Workbench (Rev B.04.00.127, Palo Alto, CA, USA). Multiply charged peptides from *m/z* 300 to 8000 Da were examined. Precursor mass tolerance was set at ±20 ppm, and product mass tolerance was set at 50 ppm. Peptide sequences were searched against an in-house cashew database. Spectra were autovalidated if the %SPI was >70%; furthermore, key spectra were examined against the proposed sequence. The Spectrum Mill modification library was supplemented with modified amino acids observed and reported in the literature including carboxymethylated lysine and arginine, carboxyethylated lysine and arginine, malondialdehyde arginine, hydroxyproline, deamidated asparagines, lysine guanidination, and pyroglutamic acid.

Circular Dichroism. UV spectra of Ana o 3 purified from raw or roasted cashews were measured on a Cary 400 spectrophotometer (Varian, Santa Clara, CA, USA) at 25 °C and 1 cm path length. The absorbance at 280 nm, an extinction coefficient of 7950 M/cm, and a molecular weight of 16 335 Da were used to compute concentrations of 0.989 and 1.13 mg/mL for raw and roasted Ana o 3 samples, respectively. Stock solutions were diluted 1:5 and 1:10 in 5 mM sodium phosphate buffer (pH 6.8) as described in Mattison et al.²⁷ Data were collected every 1 nm, with 1 nm band-pass at 25 °C on an Aviv model 420 circular dichroism spectrometer (Aviv Biomedical Inc., Lakewood, NJ, USA). Spectral data were corrected by subtraction of a buffer scan. A mean residue weight of 119 Da and a path length of 0.1 cm were used for conversion to molar ellipticity.

Dynamic Light Scattering Analysis. Raw (2 mg/mL) or roasted (5 mg/mL) Ana o 3 protein in 100 mM sodium phosphate buffer was centrifuged at 14,000 rpm for 15 min prior to analysis. Measurements were performed using a Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK). Samples were equilibrated for 120 s before they were analyzed in triplicate with 11 subruns of 10 s in a polystyrene cuvette at 25 °C.

In Vitro Trypsin Digestion. Trypsin digestion of Ana o 3 (20 µg) purified from raw or roasted cashew nuts was performed in a 5 µL reaction volume of potassium phosphate buffer (50 mM, pH 7.5) as described in Mattison et al.²⁶ Reduction of Ana o 3 prior to digestion was done using 1 mM dithiothreitol and incubation at 37 °C for 15 min. Trypsin was omitted from the zero time point and was used at a 1:20 (w/w) ratio with the purified Ana o 3. Samples were incubated at 37 °C for the indicated time points. The assay was terminated by the addition of 4× sample buffer containing reducing agent, and the samples were frozen prior to SDS-PAGE.

Human Serum. Human serum samples from clinically relevant cashew-allergic subjects with a documented history of cashew allergy supported by in vivo (skin test) or in vitro (serum immunoassay) cashew-specific IgE were collected from patients at Dallas Allergy and Immunology (Dallas, TX, USA). The study protocol was approved by the North Texas Institutional Review Board (approval certificate 12.011). Sera were screened for recognition of Ana o 3 by immunoblot analysis, and only Ana o 3-positive sera were used for ELISA experiments (donors 1–6, see Table 2). Plasma samples from patients with a self-reported history of cashew nut allergy that recognize Ana o 3 by ELISA were obtained from PlasmaLab International (donors 7–14, see Table 2) and used in Figure 5C.

Immunoblot and ELISA. Immunoblots to analyze and identify human serum samples with IgE recognizing Ana o 3 were performed as described in Mattison et al.²⁷ Purified Ana o 3 (5 µg) was resolved by SDS-PAGE, transferred to PVDF, blocked for 1 h at room temperature in phosphate-buffered saline (pH 7.4, PBS) containing 0.1% Tween-20 (PBST) and 2% (w/v) nonfat dry milk, and then incubated with individual serum samples diluted 1:5 for 1 h at room temperature. Serum was removed and membranes were washed three times for 5 min with 5 mL of PBST and then incubated for 1 h at room temperature with biotinylated anti-human IgE (Southern

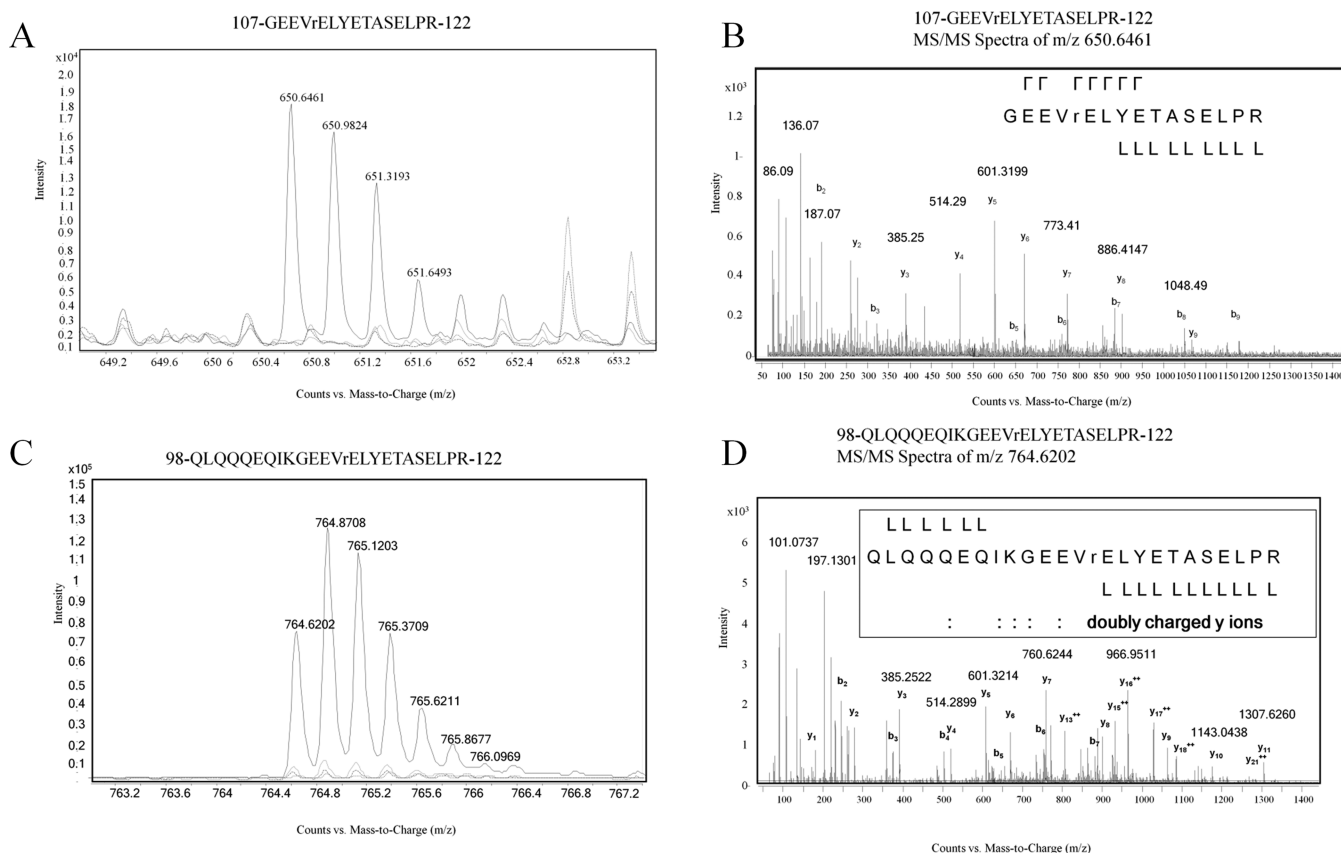


Figure 1. Ana o 3 modification observed in dark-roasted cashew nut extracts. (A) Mass spectra of the m/z 650.65 ion matched to the Ana o 3 107-GEEVrELYTASELP-122 peptide from dark-roasted cashew nuts (solid line). The mass of the 107-GEEVrELYTASELP-122 peptide is increased by 72 Da, consistent with carboxyethylation, where “r” is the modified arginine. The corresponding MS spectra of trypsinized extracts from raw (short dashes), light-roasted (long dashes), and medium-roasted (dotted line) cashew nuts are also shown. (B) MS/MS fragmentation spectra of the m/z 650.65 ion are consistent with carboxyethylation on Ana o 3 arginine-111 residue. Four fragment ions, including b_5 , b_6 , b_7 , b_8 , and b_9 , in the MS/MS spectra have an increased mass of 72 Da, supporting carboxyethylation on the arginine-111 residue. (C) Mass spectra of the m/z 764.62 ion matched to the Ana o 3 98-QLQQEQIKGEEVrELYTASELP-122 peptide from dark-roasted cashew nuts (solid line). The mass of the 98-QLQQEQIKGEEVrELYTASELP-122 peptide is increased by 53.9529 Da, consistent with a hydroimidazolone modification, where “r” is the modified arginine as shown. The corresponding MS spectra of trypsinized extracts from raw (short dashes), light-roasted (long dashes), and medium-roasted (dotted line) cashew nuts are also shown. (D) MS/MS fragmentation spectra of the m/z 764.6202 ion are consistent with hydroimidazolone-modified arginine on Ana o 3 residue-111. The partial y^{+2} fragment ion series shows y_{13}^{+2} , y_{15}^{+2} , y_{16}^{+2} , y_{17}^{+2} , y_{18}^{+2} , and y_{21}^{+2} ions with a mass increase of 53.9529 Da, supporting the hydroimidazolone modification of the arginine residue at amino acid 111.

Biotech, Birmingham, AL, USA) diluted 1:1000 in PBST. Membranes were then washed three times as above and incubated for 30 min at room temperature with IRdye-800-labeled streptavidin (1:5000 in PBST). IRdye-800 signal was visualized with an Odyssey CLx 195 instrument (LI-COR, Lincoln, NE, USA).

Ana o 3 ELISA with select serum samples was performed as described in Mattison et al.,²⁷ with the exception that microtiter plates were coated with 1 μ g per well of Ana o 3 purified from either raw or roasted cashew nuts in coating buffer (15 mM Na_2CO_3 , 35 mM NaHCO_3 , pH 9.6) overnight at 4 °C, and a direct ELISA binding assay was performed. Ana o 3 was removed from the plate wells by aspiration, and wells were blocked with PBST containing 1% bovine serum albumin (w/v) at room temperature for 1 h. Individual serum samples were diluted 1:5 with PBST, and 50 μ L was added to each well. Plates were incubated at 37 °C for 1 h. Serum samples were removed, and wells were washed three times with 100 μ L of PBST. IgE binding to Ana o 3 was detected by sequential incubation with 50 μ L of biotinylated anti-human IgE (Southern Biotech) diluted 1:1000 and IRdye 800-labeled streptavidin (LI-COR) diluted 1:5000 separated by washing steps as described above. After the final wash, the IgE signal was documented by scanning the plates with the Odyssey CLx imaging system (LI-COR). Plotted data represent the average of at least three experimental repetitions with standard deviation as error bars.

Ana o 3 Modeling. The full-length Ana o 3 protein sequence (UniProt Q8H2B8) was submitted to the SWISS-MODEL online web server (<https://swissmodel.expasy.org/>) for model prediction.^{28,29} Twenty-three templates were identified by alignment, and of these, a single Ana o 3 model based upon the crystal structure of the *Moringa oleifera* seed 2S albumin was chosen (PDB 5DOM).³⁰ The model was generated without ligands, as a monomer, and resulted in prediction quality scores of 0.51 for GMQE, −2.74 for QMEAN4, and −1.37 for C β interaction, an all atom value of −0.1, a solvation score of −0.56, and a torsion value of −3.04. Visualization and highlighting of the Ana o 3 structural model was done using Chimera software.³¹

RESULTS

Ana o 3 Modification in Roasted Cashew Nut Extracts.

In a previous paper, it was demonstrated that heating cashew nut samples for various times can alter cashew allergen solubility in aqueous buffer.⁹ Herein, cashew nut samples heated for various times were evaluated to uncover evidence of thermally induced allergen modifications. No heating-induced modifications on Ana o 1 or Ana o 2, including carboxymethylation of lysine and arginine, were detected from cashew nut extracts, but LC-MS/MS analysis of the

Table 1. Integrated Peak Areas of Modified Ana o 3 Peptides from Raw and Roasted Cashew Nut Extracts

AA start	m/z peptide		raw	light	medium	dark
107	650.6483 GEEVrELYETASELPR	average	0	0	134,731	1,421,436
		RSD ^a (%)			74.0	147.5
98	764.6243 QLQQQEIQKGEEVrELYETASELPR	average	146,746	205,992	957,955	3,998,476
		RSD (%)	65.8	46.1	29.0	15.5
98	571.6352 QLQQQEIQKGEEVR	average	351,733,556	365,076,588	499,380,988	588,658,073
		RSD (%)	18.2	27.0	27.0	20.3
112	436.5542112 ELYETASELPR	average	118,694,523	136,603,920	166,525,132	228,236,576
		RSD (%)	20.5	20.0	28.1	30.2

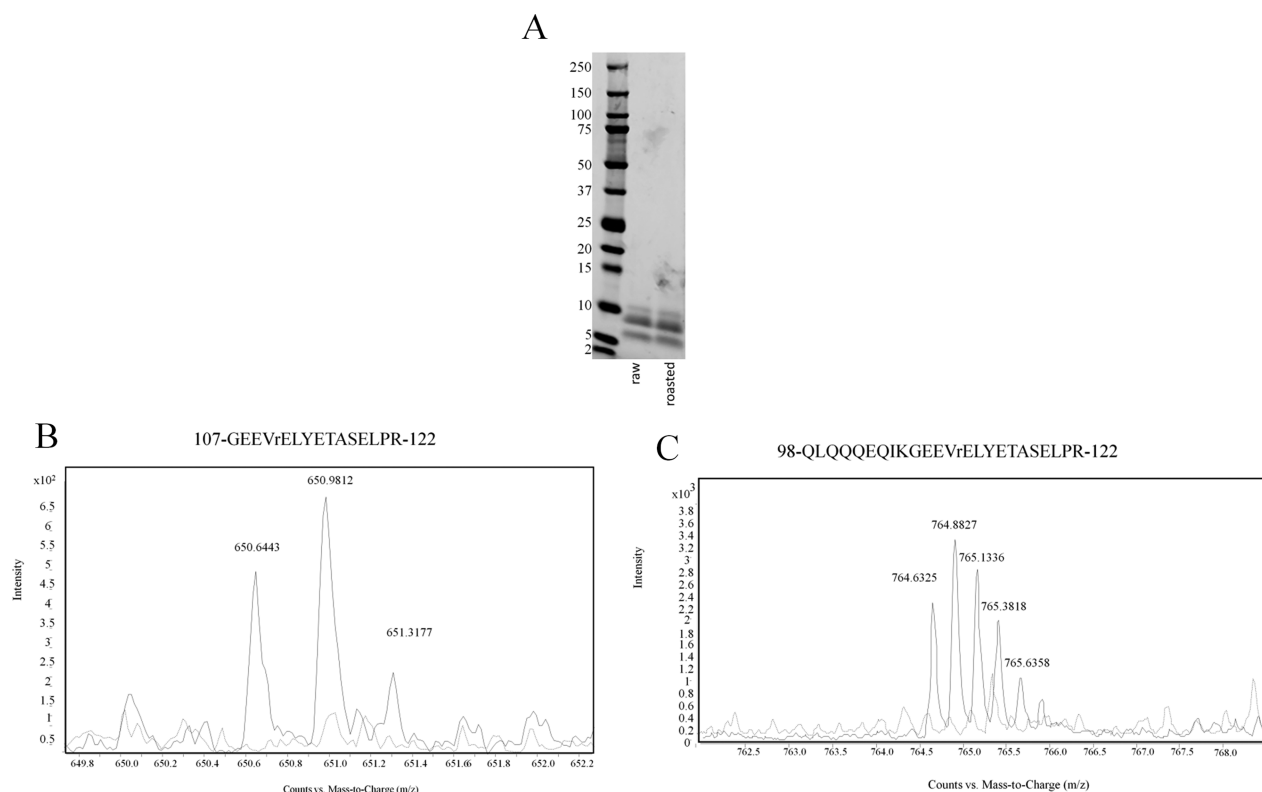
^aRSD, relative standard deviation.

Figure 2. Modification of purified Ana o 3 from dark-roasted cashew nut extracts. (A) Ana o 3 purified from dark-roasted cashew nuts is modified on arginine-111. SDS-PAGE of Ana o 3 purified by ion-exchange chromatography from raw and roasted cashew nuts. (B) Spectrum of a triply charged ion at m/z 650.6443 from Ana o 3 purified from dark-roasted cashew nuts (solid line) and Ana o 3 purified from raw cashew nuts (short dashes). The mass difference in the dark-roast Ana o 3 sample matches the predicted carboxyethylation modification of arginine-111 residue in the 107-GEEVrELYETASELPR-122 peptide. (C) Mass spectra for a quadruply charged ion at m/z 764.6325 indicate the presence of the 98-QLQQQEIQKGEEVrELYETASELPR-122 modified peptide and a predicted hydroimidazolone modification on arginine-111 residue in Ana o 3 purified from dark-roasted (solid line) but not raw (short dashes) cashew nuts.

samples identified an arginine residue, Arg-111, within the Ana o 3 2S albumin as a site that was modified in a heat-dependent manner.

The same residue within Ana o 3, Arg-111, was altered by two different modifications after heating. The triply charged ion at m/z 650.6461 from the dark-roasted cashew nut sample (solid line) yields the mass spectrum shown in Figure 1A and matches the 107-GEEVrELYETASELPR-122 peptide, where “r” is a modified arginine. The $[MH]^+$ ion of 1949.9303 Da is 72.0064 Da higher than the unmodified peptide calculated $[MH]^+$ of 1877.9239 Da. The corresponding MS spectra from

the raw (short dashes), light-roasted (long dashes), and medium-roasted (dotted line) cashew nuts are also shown; however, if present, the triply charged ion at m/z 650.6461 is at a much lower intensity. The b_5 , b_6 , b_7 , b_8 , and b_9 fragment ions shown in the MS/MS spectra have an increased mass of 72 Da (Figure 1B). The exact mass of the additional group indicates the addition of a C_3H_4O moiety and is consistent with carboxyethylation of the arginine at amino acid 111. The carbonyl end represented by the y ions shows that the additional mass is not present on any of the amino acids on the latter part of the peptide. The MS/MS spectrum is consistent

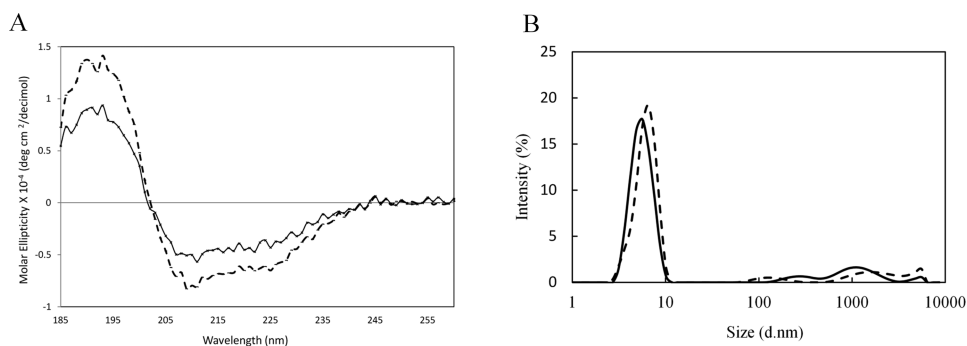


Figure 3. Biophysical characteristics of purified Ana o 3. (A) Secondary structure of Ana o 3 purified from raw or roasted cashews. Far-UV circular dichroism (from 185 to 260 nm) was used to evaluate the secondary structure of Ana o 3 purified from raw (short dashes) or roasted (solid line) cashew nuts. (B) Dynamic light scattering analysis of Ana o 3 particle size. Size distribution of Ana o 3 purified from raw (short dashes) or dark-roasted (solid line) cashew nuts. Results are shown as intensity on the Y-axis by average size in nanometers on the X-axis. The average size of Ana o 3 purified from raw cashew nuts is 6.2 ± 1.6 nm and is 5.6 ± 1.5 nm for Ana o 3 purified from dark-roasted cashew nuts.

with a modified arginine at amino acid 111 on the 107-GEEVrELYETASELPR-122 peptide. The unmodified peptide (107-GEEVRELYETASELPR-122) is not observed, although the 107-GEEVR-111 and 112-ELYETASELPR-122 fragments are readily observed in the tryptic digest (Table 1). These observations suggest that the presence of the modified guanidinium side chain on arginine hinders cleavage by trypsin at that site, similar to what has been reported for a modified lysine site in a chemically synthesized Ara h 1 peptide.¹¹

A second modification of arginine at amino acid 111 from the dark-roasted cashew nut sample was observed in a peptide with an additional missed cleavage starting at amino acid 98. Spectrum Mill software identified the peptide as the 98-QLQQQEQIKGEEVrELYETASELPR-122 with an $[MH]^+$ of 3055.4745 (Figure 1C, solid line). Again, the corresponding MS spectra from the raw (short dashes), light-roasted (long dashes), and medium-roasted (dotted line) cashew nuts are shown. In this case, the quadruply charged ion at m/z 764.6202 may be easier to discern in the medium-roasted sample (dotted line) and other samples, but is again at much lower intensity. The identified peptide has a mass of 53.9529 Da above the expected weight of 3001.5224 Da. The exact mass indicates a C_3H_2O modification and is consistent with the addition of methylglyoxal to the guanidinium side chain to form a hydroimidazolone-modified arginine.³² The MS/MS fragmentation spectrum of this ion is shown in Figure 1D. The lysine site at amino acid 106 and the arginine site at amino acid 111 are the most probable modification sites. Neither the b nor the y series of progeny ions is of sufficient length to show where the modification occurs. However, the partial y^{+2} series provides y_{13}^{+2} , y_{15}^{+2} , y_{16}^{+2} , y_{17}^{+2} , y_{18}^{+2} , and y_{21}^{+2} ions that show a mass increase consistent with the modification occurring on the arginine residue at amino acid 111. The y_{13}^{+2} , y_{15}^{+2} , and y_{16}^{+2} ions show the expected mass change for the modification on Arg-111 prior to the lysine at y_{17}^{+2} . For example, the y_{16}^{+2} ion has an m/z of 966.9511 Da, whereas the calculated value for the double-charged unmodified y_{16}^{+2} would be 939.46559.

The spectra shown in Figure 1 were taken at the apex of the chromatographic peak for each of the four treatments (raw and light, medium, and dark roast). The integrated peak areas for the m/z 764.6 ± 0.1 modified ion increase nearly 30-fold with increased roasting time, providing a correlation between roasting time and the presence of the modified arginine residue. Integrated peak areas for the respective ions of the peptides are given in Table 1. A general increase in these

peptides is observed with increased roasting. The modified peptides increase at a greater rate than the unmodified peptides. Whereas the 107-GEEVrELYETASELPR-122 peptide appears only when the modification is present, the unmodified 98-QLQQQEQIKGEEVRELYETASELPR-122 peptide is present in all of the samples.

Arg-111 Modification of Purified Ana o 3 Is Detected Only from Dark-Roasted Cashew Nuts.

Ana o 3 was purified from raw and dark-roasted cashew nuts to determine whether these modifications could be detected on the purified protein (Figure 2A). Figure 2B shows the mass spectra for the range containing the triply charged ion at m/z 650.6443 ion as a function of increased roasting time, with the modified 107-GEEVrELYETASELPR-122 peptide appearing only in the purified Ana o 3 sample from dark-roasted cashews (solid line), and not in the purified Ana o 3 sample from raw cashews (short-dashed line). Figure 2C shows the mass spectra for the range containing the quadruply charged ion at m/z 764.6325, indicating the presence of the 98-QLQQQEQIKGEEVrELYETASELPR-122 modified peptide. Both of the Arg-111-modified peptides were observed only in samples originating from dark-roasted cashew nuts, and they were present at approximately 100-fold lower signal intensity relative to the corresponding unmodified peptides in the same sample.

Secondary Structure of Ana o 3 Purified from Raw and Dark-Roasted Cashew Nuts.

Far-UV circular dichroism spectra are used to monitor and evaluate changes in secondary structure. Figure 3A shows spectra for Ana o 3 purified from raw and dark-roasted cashews. Both protein samples exhibited similar spectra that were consistent with previous studies.^{26,27} The minima at ~ 209 and 220 nm and maxima at ~ 193 nm are indicative of α -helical content³³ (Figure 3A). Whereas the spectra in Figure 3 have different magnitudes, the shapes are very similar and are superimposable if the magnitudes at either 220 or 210 nm are normalized to -1 . This suggests that the proteins have the same secondary structure, but the A_{280} measurement or calculations of protein quantification may be flawed. This could be due to changes in the extinction coefficient, which would alter the results of protein concentration calculations, and it is interesting that there is a tyrosine in the vicinity of the modified Arginine-111 residue.

Particle Size of Ana o 3 Purified from Raw and Dark-Roasted Cashew Nuts. Ana o 3 purified from raw and dark-roasted cashew nuts was analyzed by dynamic light scattering to identify any differences in protein particle size following

heating. Heating has been demonstrated to cause protein aggregation, and this could affect IgE binding.⁵ The size distributions of the two protein preparations were similar. The majority of the scattering intensity for Ana o 3 from raw cashew nuts had an average diameter of 6.2 ($\pm$ 1.6) nm, and Ana o 3 purified from dark-roasted cashew nuts was of similar size at 5.6 ($\pm$ 1.5) nm (Figure 3B). The minor peaks observed around and above 100 nm were similar in size and intensity and were likely due to the presence of protein aggregates.

Stability of Ana o 3 Purified from Raw and Dark-Roasted Cashew Nuts to Digestion with Trypsin. The *in vitro* stability of purified Ana o 3 protein samples from raw and dark-roasted cashew nuts was compared following trypsin digestion. The modification should inhibit trypsin cleavage at the Arg-111 site. However, no appreciable difference in trypsin sensitivity between the two purified Ana o 3 samples (Figure 4A) was observed even after 2 h of incubation. The trypsin

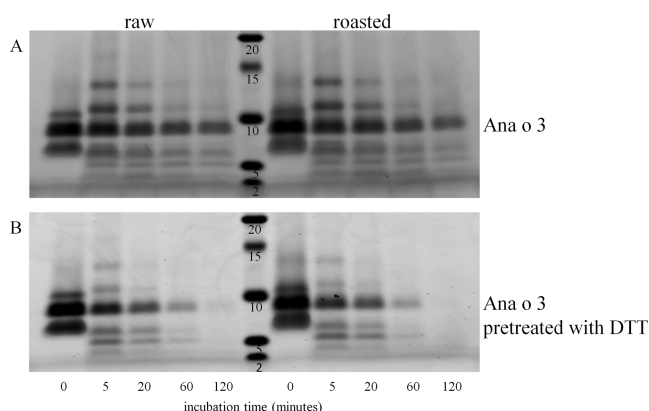


Figure 4. Stability of Ana o 3 purified from raw or dark-roasted cashews following trypsin digestion *in vitro*. Ana o 3 (20 μ g) from raw or dark-roasted cashews was incubated with bovine trypsin (1 U) prior to (A) or after (B) exposure to dithiothreitol. Trypsin is not present at the zero time point in the assay. Molecular weight markers are indicated in the middle of gels, and incubation times for each lane are indicated at the bottom of the gels. The bands at approximately 13 kDa and just above 11 kDa correspond to trypsin autolysis fragments.

digestion was also performed after the proteins had been treated with the reducing agent dithiothreitol. Although the digestion of Ana o 3 proceeded more quickly, no differences in digestion pattern were seen when the two samples were compared after treatment with dithiothreitol (Figure 4B). In both cases after pretreatment with the reducing agent, a majority of the Ana o 3 was cleaved into smaller fragments by the 2 h time point.

IgE Binding to Ana o 3 Purified from Raw and Dark-Roasted Cashew Nuts. To determine whether there were differences in binding to the Ana o 3 purified from dark-roasted cashew nuts, IgE binding was evaluated by ELISA. Six serum samples were selected from a cohort of 10 patients with cashew allergy whose IgEs were shown to bind Ana o 3 by immunoblot and ELISA (Figure 5A and Table 2). IgE binding of these serum samples to the Ana o 3 purified from either raw or dark-roasted cashews was evaluated by ELISA (Figure 5B). In one of the samples the IgE binding to Ana o 3 from dark-roasted cashews was slightly reduced (donor 2). For three of the samples, the IgE binding was essentially equivalent (donors 1, 3, and 6). In two of the samples, the IgE binding of Ana o 3

from dark-roasted cashews was slightly elevated (donors 4 and 5).

Eight plasma samples selected for recognition of Ana o 3 by ELISA, which likely recognize conformational epitope(s) because they did not recognize Ana o 3 by immunoblot, were also evaluated for differences in IgE binding. A noticeable reduction in IgE binding to the Ana o 3 purified from dark-roasted cashew nuts was observed for one of the samples (donor 8, Figure 5C). In this case, IgE binding from the donor 8 sample to Ana o 3 purified from dark-roasted cashews was reduced by 33% compared to Ana o 3 from raw cashew nuts. For all other plasma samples (donors 7 and 9–14), there was no observable change in binding. The very slight differences in binding that were observed for the majority of the samples are likely due to experimental variability, but for donor 8 there may be an alternative explanation.

Highlighting the Arg-111 Site of Modification in an Ana o 3 Model. The Ana o 3 Arg-111 residue lies within the large subunit of the protein in a relatively polar and hydrophilic segment.^{20,21,34,35} Arg-111 lies within an α -helical segment of the protein that is predicted to be relatively flexible.³⁶ One study has indicated that the Arg-111 residue lies within a major IgE epitope, and a synthetic peptide (QEIKKGEEVREL) containing the Arg-111 residue is considered a strongly reactive IgE epitope.²¹ However, a similar study did not identify this segment of the protein as a noteworthy region for IgE binding.²⁰

The Ana o 3 protein structure was modeled using the SWISS-MODEL online server to highlight the location of the arginine-111 site within the predicted protein conformation.^{28,29} The Ana o 3 model is based upon the crystal structure of the *Moringa oleifera* seed 2S albumin, mMo-CBP3-1 (PDB 5DOM)³⁰ and includes Ana o 3 residues Ser-38–Gln-135 (Figure 6). The mMo-CBP3-1 model had a sequence identity of 40% to Ana o 3 and a sequence similarity value of 42%. The Ana o 3 Arg-111 residue is positioned within helix five as shown in the modeled protein (Figure 6). The surface depictions indicate that the Arg-111 residue lies on the surface of the model, and the predicted location is consistent with this residue residing within a previously mapped IgE epitope and being available for modification during heating.

DISCUSSION

The relative soluble proportion of Ana o 3 has been demonstrated to increase compared to other proteins in heated cashew nuts.⁹ For the first time a modification on the 2S albumin Ana o 3 allergen was observed specifically from heated cashew nuts. The exact nature of the modifications observed here is currently unknown, but the altered molecular masses are consistent with arginine-111 carboxyethylation and a hydroimidazolone-modified arginine at residue 111. Hydroimidazolone adducts are common in nature, may be an early indicator of diabetes, and are found in foods such as baked goods and coffee.^{37,38} It is worth pointing out that both the hydroimidazolone and carboxyethylation modifications occur at the same site (Arg-111) within Ana o 3, indicating that the conformation of the protein may make this residue readily accessible for modification. An Ana o 3 structural model was generated (Figure 6), and in this model the Arg-111 residue lies in a surface-exposed region of the protein, and it is included in a previously mapped IgE-binding epitope. An alignment of several other tree nut and peanut 2S albumins (data not shown) indicates that the Arg-111 site is not highly conserved,

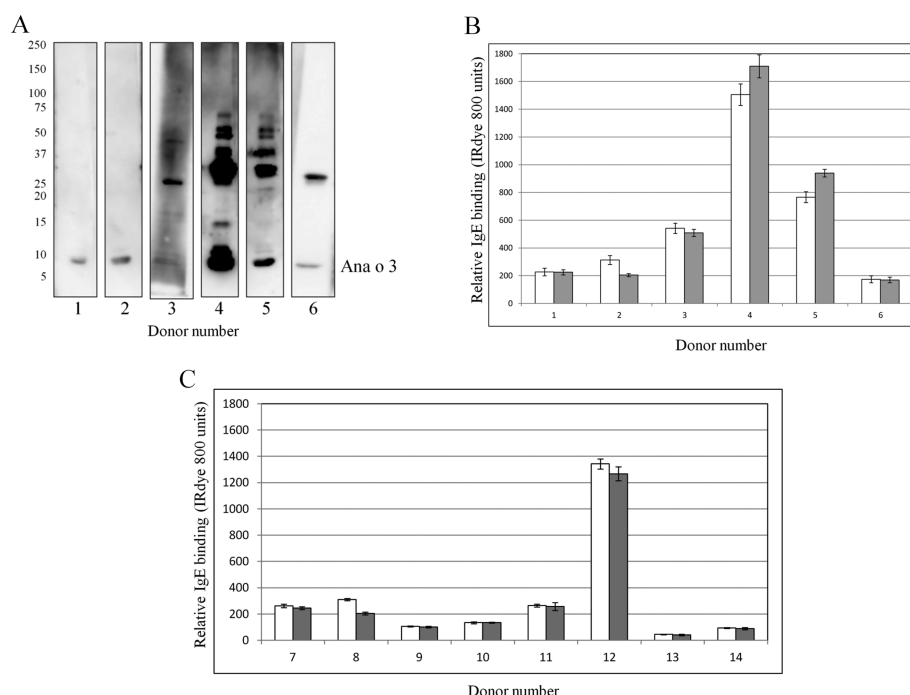


Figure 5. IgE binding to Ana o 3 purified from raw or roasted cashew nuts. (A) Immunoblot analyses of six individual cashew-allergic donor sera recognizing Ana o 3 in soluble raw cashew nut extracts. (B) ELISA of Ana o 3 purified from raw (white) or dark-roasted (gray) cashew nuts using six cashew-allergic sera shown in panel A and eight additional cashew-allergic plasma samples.

Table 2. Ana o 3 Cognate Donor Samples

donor	sex	age	initial reaction	test/CAP kU/l	other allergies
1	F	12	urticaria	3.52	egg, soy, peanut
2	M	11	oral allergy syndrome	SPT = 4 mm	banana, peanut, walnut, almond
3	F	11	anaphylaxis	24.4	peanut
4	F	9	anaphylaxis	SPT = 18 mm	hazelnut, fish, shrimp
5	M	9	anaphylaxis	13.57	pistachio
6	F	8	urticaria	1.77	pistachio
7	F	24	anaphylaxis	23.07	tree nuts, peanut, coconut
8	F	30	anaphylaxis	35.2	tree nuts, dust mite, grass
9	M	58	wheezing, swollen throat	16.6	tree nuts, peanut, shellfish
10	F	33	anaphylaxis	36.7	tree nuts, banana, egg plant
11	F	42	urticaria	12.89	almond, walnut, pecan
12	M	46	urticaria	65.4	raw vegetables, avocado, tree nuts
13	M	26	anaphylaxis	10.1	tree nuts, peanut
14	M	30	anaphylaxis	33.6	peanut, almond, walnut, pecan, latex

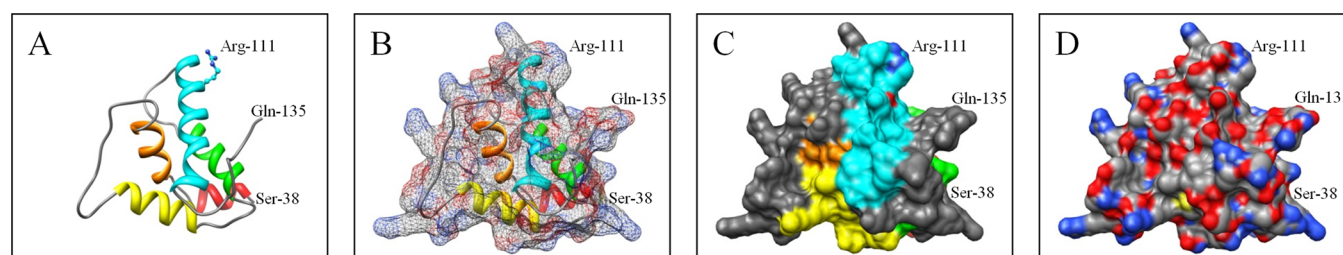


Figure 6. Ana o 3 model highlighting the arginine-111 residue. The model is based on the crystal structure of the *Moringa oleifera* seed 2S albumin, mMo-CBP3-1 (PDB 5DOM) and was generated using SWISS-MODEL and visualized using Chimera software. The model includes Ana o 3 residues Ser-38–Gln-135 and shows ribbon (A), surface mesh (B), solid surface (C), and surface element (D) depictions of the model. In the ribbon diagram helices 1–5 are colored red, orange, yellow, green, and aqua, respectively.

but there are nearby arginine and lysine residues within the linear sequence of the peanut Ara h 2 and Ara h 6 proteins. The

walnut Jug r 1 and pecan Car I 1 2S albumin proteins do not have arginine or lysine residues in the nearby linear sequence.

The modified Ana o 3 protein is of relatively low abundance compared to the unmodified protein, and this likely complicated evaluation of the effect that the modifications may have had on the structure, stability, and IgE binding to the protein. Recent findings from van der Valk et al. indicate that skin prick tests with extract and/or component resolved diagnosis with purified Ana o 1, 2, and 3 cashew allergens from blanched cashew nuts correlate well with food challenge,³⁹ and the present findings do not indicate that the observed Ana o 3 modifications would complicate such diagnostic or skin prick testing. IgE binding to the Ana o 3 protein purified from raw or dark-roasted cashews was essentially equivalent, although one sample with clearly reduced binding to Ana o 3 purified from dark-roasted cashew nuts was identified. It is not clear why the donor 8 sample had reduced binding to Ana o 3 purified from dark-roasted cashew nuts, but further testing with this and additional serum samples from cashew allergic patients may be informative.

Differences in the magnitudes of secondary structure analysis using circular dichroism were observed in the comparison of Ana o 3 purified from raw or roasted cashews, despite the careful analysis and normalization of protein concentration between the two samples. Aromatic rings are primarily responsible for the absorbance peak at 280 nm, and it is interesting to note that there is a tyrosine (Tyr-114) residue close to the Arg-111 modification site. The differences in magnitude of molar ellipticity for the Ana o 3 samples could be due to changes in the apparent extinction coefficient, which would alter the results of protein concentration calculations. The data suggest there may be changes in sequence or biophysical characteristics of amino acids, such as loss or modification of a tyrosine or other aromatic residue, contributing to the measurement of Ana o 3 protein concentration. Perhaps there are as yet unidentified alterations in aromatic acids within Ana o 3 that can help to explain the magnitude difference between samples purified from raw and roasted cashew nuts. In a related observation, it was previously noted that some isoforms of Ara h 2 differ by several amino acids and can lack carboxy-terminal residues, including tyrosines that could affect measurement of protein concentration when using the absorbance peak at 280 nm.⁴⁰

The similar size distributions of Ana o 3 purified from raw or dark-roasted cashew nuts using dynamic light scattering are in line with previous results, in which the two proteins showed comparable migration following SDS-PAGE analysis, demonstrating similar molecular weights. In addition, liquid chromatography mass-spectrometry of these two samples indicate that the five most commonly observed Ana o 3 peptides remain unchanged before and after heat treatment.⁹

The Arg-111 modified Ana o 3 protein was a relatively small proportion of the soluble unmodified Ana o 3 protein from dark-roasted cashew nuts. The regional variability in cashew nut processing steps may in some cases lead to protein modifications that alter the immunologic properties of cashew nuts and complicate the analysis of this factor. Although the results identify and characterize a thermally induced protein modification that occurs within heated cashew nuts, support for the hypothesis that this type of modification may directly affect the allergenicity of Ana o 3 is lacking. For example, if these modifications prevent digestion by digestive or antigen-processing proteases (through alteration or destruction of arginine, lysine, or other protease cleavage site), they could result in a disproportionate amount of the modified protein

surviving digestion in relation to the unmodified protein and thereby act as a "more stable" antigen, potentially leading to a more robust humoral response to the modified protein. Well-characterized examples of increased specific binding activity of antibodies toward modified allergen sites would help to support this hypothesis. On the other hand, according to the National Peanut Board roasted peanuts have been consumed in the United States since the late 1800s, but only more recently have reports of the increased incidence of peanut and other food allergies emerged. It is difficult to reconcile this latter observation with the idea that roasting peanuts and any resulting heating-induced allergen modifications have contributed only recently to the reported increase in peanut allergy incidence.

It may be that food processing affects food allergy indirectly through the alteration of normal gut flora due to differences in diet. There is evidence supporting the idea that diet affects the diversity of the gut flora and the incidence of human disease.⁴¹ There are several lines of evidence supporting the concept that commensal bacteria can influence the incidence of food allergy⁴² and that the use of antibiotics, common modern diet changes, and the addition of food additives may alter gut microbiota.^{43,44} A recent paper has demonstrated a link between the metabolite 12,13-DiHOME, CD4+ T cell dysfunction, and the presence of specific gut flora in infants.⁴⁵ A diverse gut flora could be reflected in a varied, well-balanced diet with little processed food. Processed foods may favor a different or possibly less diverse population of gut bacteria, whereas a varied diet may enable a more diverse population of gut flora.

Although several factors likely influence the apparent increase in food allergy prevalence, improved therapies are being developed. Peanut extracts are being used with some success in immunotherapies such as oral immunotherapy and sublingual immunotherapy, and the early application of oral immunotherapy with lightly roasted peanut extracts in young children has shown promise in suppressing allergic response to peanut allergens.^{46,47} Similarly, a processed peanut butter-flavored snack consumed as part of experimental dietary modification in young children has been strongly correlated with peanut allergy prevention.^{48,49} Furthermore, updated interim guidance has been redefined to recommend the inclusion of peanut consumption in young children's diets to reduce the risk of developing peanut allergy.⁵⁰

In summary, a heating-induced modification of the Ana o 3 cashew nut allergen was detected, but the relative amount of the modified protein is small in relation to the unmodified protein. The evidence supports the Arg-111 residue as a site of modification, but no clear consequence for this modification using *in vitro* assays of the stability, structure, and IgE binding of the protein was demonstrated. More concrete examples of this type of modification from *in situ* samples followed by clinical testing could be used to demonstrate their significance.

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Notes

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