

RESEARCH ARTICLE

In vitro digestion of soluble cashew proteins and characterization of surviving IgE-reactive peptides

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Scope: The stability of food allergens to digestion varies. We characterized the stability of cashew allergens to digestion by pepsin and trypsin and identified IgE-binding epitopes that survive digestion.

Methods and results: The ability of pepsin and trypsin to digest cashew allergens was assessed with an in vitro digestion model. Samples were evaluated by SDS-PAGE, MS, ELISA, and immunoblotting to compare IgE binding. Increasing amount of protease resulted in greater degradation of higher molecular weight cashew proteins. Among cashew proteins, the 2S albumin, Ana o 3, was most resistant to digestion by both pepsin and trypsin. MS identified digestion resistant Ana o 3 protein fragments that retained reported IgE-binding epitopes. Pretreatment of extracts or purified Ana o 3 with reducing agent increased the sensitivity of Ana o 3 to protease digestion. Circular dichroism revealed the structure of purified Ana o 3 was largely alpha-helical and was disrupted following reduction. Ana o 3 reduction followed by protease digestion decreased binding of serum IgE from cashew allergic patients. Our results indicate that the Ana o 3 disulfide bond dependent structure protects the protein from proteolysis.

Conclusion: Ana o 3 is the cashew allergen most likely to survive gastrointestinal digestion intact.

Received: April 24, 2013
Revised: September 5, 2013
Accepted: September 9, 2013

Keywords:

Allergen / Cashew / Digestion / Pepsin / Trypsin



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1 Introduction

Food allergies are prevalent and reactions to peanuts and tree nuts are often severe and life threatening [1,2]. The frequency of nut allergy appears to be increasing [3], with an estimated food-related severe allergic reaction occurring every 6 min [4]. Medically related costs associated with food allergy have been estimated to be \$500 million annually [5], and exposure

to cashew nut allergens has been correlated to more severe clinical reactions [6,7].

Cashew nuts are composed of approximately 15–25% protein. Three conserved allergenic seed storage proteins have been characterized in cashew, including the 7S globulin Ana o 1 [8], 11S globulin Ana o 2 [9], and the 2S albumin Ana o 3 [10]. Ana o 1 is a 50 kDa protein in the vicilin family and while it has homology to the peanut Ara h 1 vicilin, there is little overlap in the linear IgE epitopes between the two proteins [9]. Ana o 2 is a legumin composed of a larger acidic (~30 kDa) and a smaller basic subunit (~20 kDa) linked via disulfide bond to form a monomer. Legumin monomers, like those of Ana o 2, associate to form higher molecular weight trimers and hexamers [11]. Ana o 2 accounts for a large portion of soluble cashew protein [12], and the majority of cashew allergic sera recognize the larger Ana o 2 acidic subunit [9,13]. There are seven dominant linear Ana o 2 epitopes [9], and a

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Abbreviations: SGF, simulated gastric fluid; SIF, simulated intestinal fluid

conformational IgE-binding epitope has also been described [14, 15]. Ana o 3, with at least three isoforms observed, has a molecular mass of approximately 13 kDa [10, 13]. Ana o 3 is recognized by a majority of sera from cashew allergic individuals, and at least four linear IgE epitopes have been characterized as strongly reactive [10]. Ana o 3 belongs to the prolamin superfamily and like several other 2S albumins is composed of two subunits joined by disulfide bonds [16].

Cashew allergens have been shown to be stable to several types of processing [17, 18]. In particular, 2S albumins such as Ana o 3 have been considered stable to common food processing methods, at least in part, due to the highly conserved network of cysteine disulfide bonds [16, 19]. 2S albumins from several plant sources have been characterized as stable proteins able to resist digestion and thermal processing [16, 17, 20–24]. Among nut proteins, 2S albumins are potent allergens and peanut 2S albumins have been shown to be important factors in basophil degranulation [16, 25–27]. Disruption of disulfide bonds in the peanut 2S albumins Ara h 2/6, as well as Brazil nut Ber e 1, has been demonstrated to render the proteins more sensitive to digestion with protease [23, 24, 28]. The ability of food proteins to cause an allergic reaction may be affected by the susceptibility of the allergen to digestion by proteases including pepsin and trypsin [29]. To better define the stability of cashew allergens, we characterized the sensitivity of soluble proteins extracted from cashews to digestive proteases. To aid in this analysis, we have purified the cashew 2S albumin Ana o 3 and characterized the effects of reduction and proteolysis on this important allergen. Our results highlight the stability of the Ana o 3 protein to proteolysis by digestive enzymes and reinforce the importance of the 2S albumin family of proteins in food allergy.

2 Materials and methods

2.1 Materials

Ready-to-eat cashew nuts were purchased online from Nuts Online. Trypsin from bovine pancreas (T1426) and pepsin from porcine gastric mucosa (P6887) were purchased from Sigma-Aldrich (St. Louis, MS, USA). SDS-PAGE Any kD Mini-Protean TGX precast Tris-glycine gels were purchased from Bio-Rad (Hercules, CA, USA) and Novex 10–20% Tricine gels were purchased from Life Technologies (Carlsbad, CA, USA). The anti-human IgE antibody was purchased from Southern Biotech (Birmingham, AL, USA).

2.2 Cashew extract preparation

Cashew nuts were purchased online and ground in an electric blender and defatted in a BUCHI B-811 Standard extraction unit (BUCHI Labortechnik AG, Flawil, Switzerland) with petroleum ether. Defatted cashew protein was dried to

remove residual ether in a fume hood, and the material was ground to a fine powder. The defatted cashew flour was resuspended 1:10 w/v in buffer containing 50 mM Tris pH 8.3 and 200 mM NaCl, stirred for 1 h, and sonicated in 15-s intervals for a total of 45 s to solubilize proteins. Following centrifugation (12 000 rpm for 30 min at 4°C) and filtering through cheesecloth to remove insoluble particulates, protein concentrations were determined using a NanoDrop (ThermoFisher, Pittsburgh, PA, USA). Protein samples were aliquoted and frozen at –80°C.

2.3 SDS-PAGE

Proteins were resolved by SDS-PAGE using a Mini Protean system II and prestained Precision Plus molecular weight markers (Bio-Rad). Prior to electrophoresis, 4× NuPAGE LDS sample buffer containing NuPAGE reducing agent was added to the protein samples in a 1:4 ratio and samples were heated at 65°C for 15 min. Protein bands were visualized with Safe Stain (Invitrogen, Grand Island, NY, USA) and images were captured with the 680 nm channel on an Odyssey CLX infrared imaging system (LI-COR, Lincoln, NE, USA).

2.4 LC-MS/MS mass spectrometry

Protein samples were excised as gel slices following SDS-PAGE, and samples were prepared for MS based on Cold Spring Harbor Protocols [30]. Briefly, gel slices were washed sequentially in water and 100 mM ammonium bicarbonate and then dried after rinsing in ACN. Following reduction with DTT, gel slices were alkylated with iodoacetamide and incubated with trypsin (Promega, Madison, WI, USA) overnight. The following day, the supernatant was collected and gel slices were extracted once with 25 mM ammonium bicarbonate and then with 5% formic acid. The supernatants were pooled and dried.

The dried material was resuspended in 5% formic acid and analyzed via LC with MS/MS (LC/MS/MS) using 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). Chromatographic separation was accomplished using a chip consisting of a 160 nL enrichment column and a 150 mm analytical column packed with C18, 5 µm beads with 300 Å pores. One microliter aliquots of the sample were transferred to the enrichment column via the capillary pump operating at a flow rate of 4 µL/min. The nano pump was operated at a flow rate of 600 nL/min. An initial gradient (Solvent A: 100% H₂O, 0.1% formic acid; Solvent B: 90% ACN, 10% H₂O, and 0.1% formic acid) of 97% solvent A was changed to 60% solvent A at 20 min, 20% at 22 min, and held until 25 min. A post-run time of 4 min was employed for column equilibration between samples. The MS source was operated at 300°C with 5 L/min N₂ flow and a fragmentor voltage of 175 V. N₂ was used as the collision gas, and the collision energy varied as a

function of mass and charge using a slope of 3.7 V/100 Da and an offset of 2.5 V. Both the quad and TOF were operated in positive ion mode. Reference compounds of 322.048121 and 1,221.990637 Da were continually leaked into the source for mass calibration. An initial MS scan was performed from m/z 300 to 1600 and up to three multiple charged ions were automatically selected for MS/MS analysis. Following the initial run, a second injection was made excluding ions previously targeted in the MS/MS analysis. LC chromatograms and mass spectra were analyzed using MassHunter software (Version B.0301; Agilent Technologies). Data files were transferred to an Agilent workstation equipped with Spectrum Mill software (Agilent Technologies) for peptide sequencing and protein identification.

2.5 In vitro simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) assays

Cashew extract samples (25 μ g) or purified Ana o 3 (8 μ g) were subjected to SGF (35 mM NaCl, 84 mM HCl, pH 1.2, [31]) with varying amounts of porcine pepsin (Sigma, P-6887), or SIF (50 mM KH_2PO_4 pH 7.5, [31]) with varying amounts of bovine trypsin (Sigma, T-1426). These proteases were used in a similar analysis of peanut allergens [32]. We sought to identify the most stable cashew allergen(s), the starting point for the assays resulted in a cashew extract (substrate) to protease ratio of 12.5:1 w/w for both pepsin and trypsin. Half-log serial dilutions of the proteases in their respective buffers, starting with the most concentrated protease solutions, were used in the long-term digestion assays. Protease was omitted from zero time point samples, but they were otherwise prepared in a manner identical to the other time points. For assays in which SGF was followed by SIF, the reaction mixture was diluted fourfold and the pH was adjusted to 7.5 with KH_2PO_4 . Proteases were dissolved prior to use in SGF or SIF, and all reagents and reaction tubes were prewarmed to 37°C prior to combining protease and substrate. Protease digestion was stopped by the addition of SDS-PAGE sample buffer and heating. Samples were frozen and stored at -80°C or used immediately for analysis.

2.6 Circular dichroism (CD)

Far-UV circular dichroism (CD) of native and reduced Ana o 3 was measured from 185 to 250 nm on a Jasco-815 spectropolarimeter (JASCO, Oklahoma City, OK, USA) at 25°C using a 2 nm bandwidth and a response time of 1 s. When required reducing agent was removed by filtration, protein samples (1 mL) were prepared in 3 mM sodium phosphate (pH 6.8). CD was obtained at a protein concentration of 0.1 mg/mL with three accumulations per sample in a quartz cuvette with a 1 cm path length. Background measurements were subtracted and mean residue ellipticity was calculated using a mean residue weight of 114.5 g/mole. Sec-

ondary structure was estimated with the K2D3 web server (<http://www.ogic.ca/projects/k2d3/>) [33].

2.7 Human sera and IgE-binding assays

Sera from clinically relevant cashew-allergic individuals (>7 to 55 years of age) 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. Following sequential SGF and SIF in vitro digestion of 25 μ g of cashew extract or 8 μ g of purified Ana o 3 1 mM PMSF was added to inhibit further proteolysis. Digested samples were serially diluted tenfold in PBS with 0.1% Tween-20 (PBST) and diluted samples were combined with a 1:4 dilution of individual cashew-allergic patient serum for 1 h at 25°C. Samples were then added to microtiter plate wells, precoated with 1 μ g of cashew extract or Ana o 3, and allowed to incubate for 1 h at 25°C. After washing, IgE binding was detected by sequential incubation with biotinylated anti-human IgE (Southern Biotech) and IRdye 680 labeled streptavidin (LI-COR) or IRdye 800 labeled streptavidin (LI-COR) separated by washing. After final washing, IgE signal was documented by infrared fluorescence scanning of microtiter plates with an Odyssey CLX imaging system (LI-COR) using the appropriate channel. Relative IRdye values for each individual patient serum were plotted versus the serial cashew extract dilutions.

3 Results and discussion

3.1 In vitro SGF digestion of cashew extracts

To mimic digestion of cashew proteins within the stomach, we incubated cashew extract (25 μ g) with varying amounts of pepsin under acidic conditions in SGF, similar to what has been used to evaluate peanut extracts [32], and we characterized the resulting digestion products. Using a 5-min assay, we evaluated the breakdown of cashew protein with half-log serial dilutions of pepsin (starting activity of 8 U), and the products were visualized by SDS-PAGE (Fig. 1A). Titration of the enzyme demonstrated that the higher molecular weight cashew proteins, greater than 20 kDa, including Ana o 1 (50 kDa) and both of the Ana o 2 bands (30 and 20 kDa), were at least partially digested at the higher enzyme range (0.8–8 U), but at lower pepsin activity (0.08 U), the majority of the proteins were more resistant to digestion. Digestion with the high pepsin activity (2.5 or 8 U) resulted in efficient degradation of the majority of higher molecular weight proteins and yielded a diffuse band migrating near or below the 15 kDa marker.

To better characterize the intermediates during pepsin digestion, we used long-term in vitro digestion assays. We

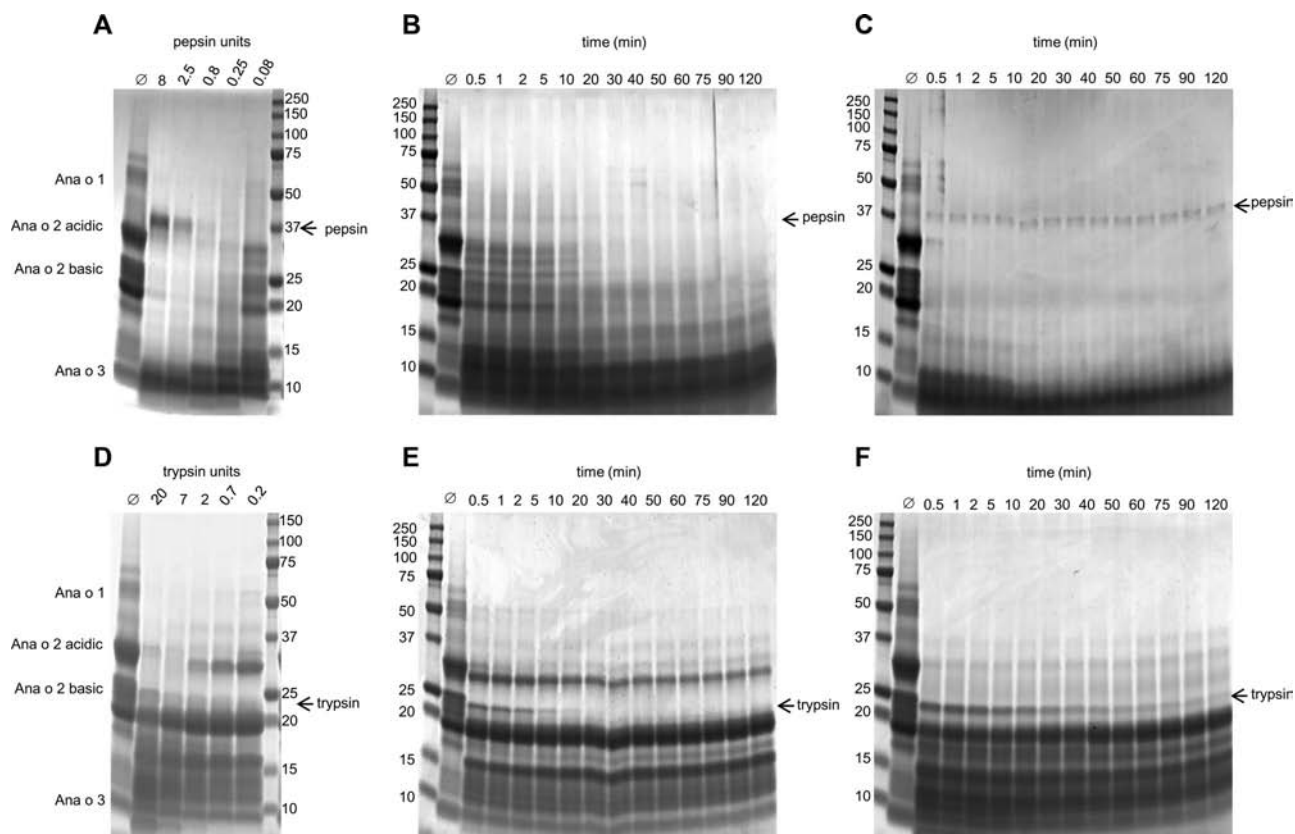


Figure 1. High molecular weight cashew proteins are degraded in simulated gastric and intestinal digestion. Protein samples were subjected to SGF (35 mM NaCl, 84 mM HCl, pH 1.2) with varying amounts of pepsin (Sigma) or SIF (50 mM KH_2PO_4 pH 7.5) with varying amounts of trypsin (Sigma) and analyzed by SDS-PAGE (reduced, Tris-glycine). (A) Analysis of cashew extract (25 μg) incubated for 5 min at 37°C with half-log serial dilutions of porcine pepsin. Diluted enzyme activity is indicated at the top of the gel. (B and C) Time course of cashew extract digested with 0.08 U (B) or 0.8 U (C) of pepsin. (D) Analysis of cashew extract (25 μg) incubated for 5 min at 37°C with half-log serial dilutions of bovine trypsin. (E and F) Time course of cashew extract digested with 2 U (E) or 20 U (F) of trypsin. All reagents and reaction tubes were prewarmed to 37°C, and protease digestion was stopped by the addition of SDS-PAGE sample buffer and heating.

chose to use 0.08 and 0.8 U of pepsin activity for this evaluation. Using 0.08 U of pepsin, we observed a reduction in the amount of protein migrating in the 50 kDa range, which is likely to include Ana o 1 (Figure 1B). Similarly, the intensity of a band corresponding to the larger acidic subunit of Ana o 2 was reduced, concomitant with an accumulation of protein migrating at just under 20 kDa. There was also a reduction in protein bands in the 20–25 kDa range during the 2-h incubation. However, proteins migrating around 10 kDa, or lower, remained at similar levels during the incubation suggesting they were not appreciably proteolyzed. In contrast, incubation of cashew extract with ten times the amount of pepsin activity resulted in a more complete degradation of the proteins. Within 20 min, the majority of higher molecular weight proteins, including Ana o 1 and both Ana o 2 subunits, were not detectable on the gel (Figure 1C). In addition, most of the lower molecular weight proteins were efficiently degraded during the 2-h assay as well. However, a diffuse band migrating at approximately 10 kDa remained for the duration of the assay (Figure 1C).

The size of the remaining band suggested it might include the large subunit of Ana o 3, because the three isoforms of Ana o 3 have been shown to migrate on SDS-PAGE in this range [10, 13]. In addition, digestion of purified Ana o 2 with pepsin has previously been shown to result in a diffuse band migrating around 15 kDa [12]. To determine what protein fragments remained following pepsin digestion (0.8 U), we excised gel slices within the 5–15 kDa size range (Figure 1C) from the time course and analyzed the protein by MS. We identified several peptides from both Ana o 2 and Ana o 3 (Table 1). IgE-reactive peptides from both the acidic and basic subunits of Ana o 2 were observed in our analysis, and we detected several peptides in the basic subunit of Ana o 2 within residues 306–397. Based on these findings, it is likely that fragments of Ana o 2, containing IgE-binding epitopes 16–20 within the basic subunit [9], were migrating in the band around the 10 kDa range (Figure 1C and Table 1) [9, 10]. This is somewhat unexpected since the majority of strongly reacting IgE sera from cashew-allergic sera bind the larger acidic subunit of Ana o 2 [9].

Table 1. Identification of SGF digestion resistant peptides

Protein	Start AA position	Peptide sequence	z	m/z observed	Calculated mass	Observed – calculated	Linear epitope numbers
Ana o 2	48	(E)AWDPNHEQFR(C)	3	433.8626	1299.586	–0.0133	
Ana o 2	96	(G)ISYPGCPETYQAPQQGR(Q)	2	976.4518	1951.897	–0.0004	5
Ana o 2	132	(R)RGDIIPAAGVAHW(C)	3	492.6091	1475.812	0.001	8/9
Ana o 2	306	(L)NSLNLPIIK(W)	2	506.3103	1011.62	–0.0064	16
Ana o 2	315	(K)WLQLSVEK(G)	2	501.7873	1002.562	0.0055	16
Ana o 2	338	(L)NSHSIYGCK(G)	3	393.5267	1178.562	0.0033	17/18
Ana o 2	348	(K)GKGQVQVVDNFGNR(V)	3	506.5977	1517.782	–0.0034	18
Ana o 2	362	(R)VFDGEVR(E)	2	411.2124	821.415	0.0023	
Ana o 2	390	(R)FEWISFK(T)	2	478.742	956.488	–0.0109	19/20
Ana o 3	75	(R)ECCQELQEVDR(R)	2	733.307	1465.605	0.0021	10/11/12
Ana o 3	98	(R)QLQQQEIQK(G)	2	571.8113	1142.616	–0.0011	13/14
Ana o 3	98	(R)QLQQQEIQKGEEVR(E)	2	856.9525	1712.893	0.0051	13/14/15
Ana o 3	112	(R)ELYETASELPR(I)	3	436.5534	1307.648	–0.0021	14/15
Ana o 3	123	(R)ICSISPSQGCQFQS(S)	2	799.8463	1598.694	–0.0084	16

Ana o 2 linear epitope numbers as described in ref. [11]; Ana o 3 linear epitope numbers as described in ref. [12]

Several Ana o 3 peptides were recovered from the SGF digest that accounted for almost the entire large subunit. Ana o 3 sequences containing part, or all, of IgE-binding epitopes 10–16 [10] were identified from the same region of the gel migrating near the 10 kDa marker (Figure 1C and Table 1). Consequently, fragments of Ana o 2 and Ana o 3, large enough to bind and crosslink IgE, remain following digestion under our SGF model conditions. A mouse model has been used to demonstrate that cashew proteins digested with pepsin can be used to elicit a weaker allergenic response and drive tolerance [26]. The assay containing 0.8 U of pepsin (Fig. 1C) resulted in a protein-to-pepsin ratio of 125:1 w/w, similar to what was used by Kulis et al., and our results suggest that a major component of the pepsin digested protein mixture used in that work was comprised of intact Ana o 3. The integration of the cashew work mentioned above [26] with our findings and the identification of peanut 2S albumins, Ara h 2 and 6, as primary elicitors of peanut allergy [25, 34], suggests that Ana o 3 may provide a useful target for cashew allergy diagnostic tools and immunotherapy.

3.2 In vitro SIF digestion of cashew extracts

Cashew extracts (25 µg) were also subjected to SIF to characterize the effects of digestion with trypsin. We evaluated the breakdown of cashew protein in a short-term assay using half-log serial dilutions of trypsin (starting activity of 20 U), and the products were visualized by SDS-PAGE. In contrast to our results with pepsin, several cashew proteins were resistant to digestion with trypsin and large fragments remained in the gel at all concentrations tested (Fig. 1D). At the highest trypsin activity (20 U), we did not observe a band corresponding to Ana o 1 suggesting it was efficiently degraded but were able to identify bands corresponding to the more abundant Ana o 2 and Ana o 3 bands (Fig. 1D).

A band corresponding to the basic subunit of Ana o 2 was clearly visible on the gel, as well as a low-intensity band corresponding to the acidic Ana o 2 subunit. Similarly, a band corresponding to Ana o 3 remained visible at all concentrations tested.

Long-term digestions with 2 and 20 U of trypsin activity were performed to better characterize trypsin digestion of cashew proteins. At both the 20 and 2 U trypsin activities, a band corresponding to the 20 kDa basic subunit of Ana o 2 remained throughout the 2-h digestion (Fig. 1E and F). Similarly, a band migrating at the same position as Ana o 3 was present for all time points (Fig. 1E and F). While a band whose migration was consistent with the acidic subunit of Ana o 2 remained throughout the assay with 2 U of trypsin activity, the intensity of this band was drastically reduced at the 20 U trypsin activity assay (Fig. 1E and F). Trypsin was less effective than pepsin, as there were several bands migrating at or below the 20 kDa marker that remained following a 2-h digestion with 20 U of trypsin (Fig. 1F). Proteins migrating in this region include the basic subunit of Ana o 2 [9] and the large subunit of Ana o 3 [10], as well as fragments of other cashew proteins. Our results indicate that several intact proteins and large protein fragments including Ana o 2 and Ana o 3 survive trypsin digestion. However, we observed that trypsin concentrations were reduced during the time course (Fig. 1E and F), suggesting that trypsin activity declined during the assay due to auto-proteolysis.

3.3 Sequential in vitro SGF-SIF cashew extract digestion

Next, we sought to identify cashew proteins that could survive sequential digestion with SGF and SIF, mimicking passage through both the stomach and small intestine. For this assay, we incubated 25 µg of cashew extract first with 0.8 U of pepsin

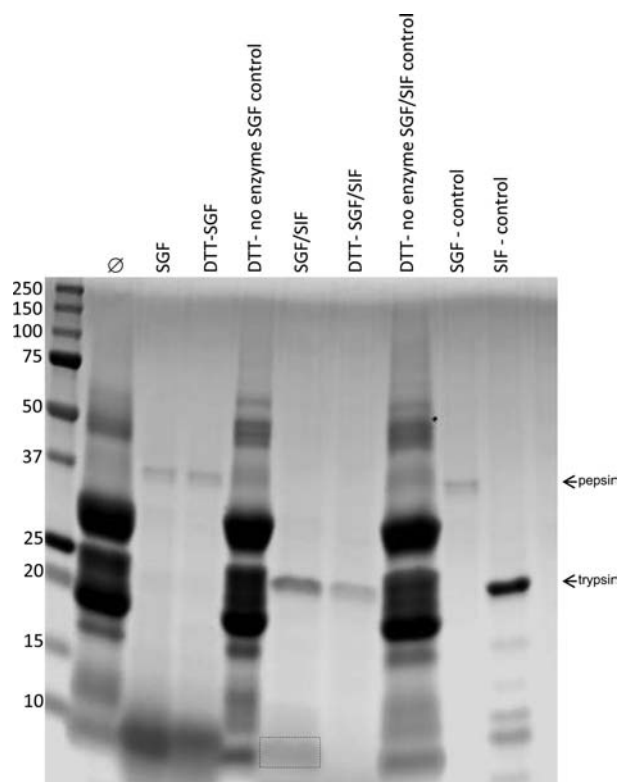


Figure 2. Low molecular weight cashew proteins remain following sequential *in vitro* SGF/SIF digestion of extracts. SDS-PAGE (reduced, Tris-glycine) analysis of *in vitro* digestion resistant cashew protein in the absence of or following prior treatment with DTT. Protein remaining after sequential digestion of cashew extract (25 µg) with pepsin (0.8 U) and trypsin (2 U) was excised from the gel (indicated by hatched box) and analyzed by LC-MS/MS (presented in Table 2).

for 30 min followed by 2 U of trypsin for 30 min in the appropriate buffers. SDS-PAGE sample volumes and pH were adjusted to correct for volume differences following buffer changes to maintain a consistent protein load in the sample. The results of the sequential digestion were consistent with our previous results and indicated that all cashew proteins >15 kDa were extensively digested. A diffuse band migrating

near the 10 kDa marker remained after digestion with SGF, and while a similar band remained following SIF digestion, the intensity of the band was reduced (Fig. 2). The remaining protein generated a band that was more distinct, suggesting there was less diversity within this region of the gel, and the size of the remaining band was consistent with that of Ana o 3.

To determine if Ana o 3 survived the *in vitro* SGF/SIF digestion under these conditions and confirm the identity of the remaining protein(s) (Fig. 2), we excised the band just under 10 kDa and analyzed it by MS. We detected several high-confidence peptides with masses corresponding to those of Ana o 3 (Table 2). We observed 62 of the 70 amino acids contained within the large subunit of Ana o 3 similar to what we observed from the SGF digest (Fig. 1). The peptides we observed started just distal to the proposed amino-terminus of the large subunit [10] and continued to the carboxy-terminal tyrosine of the protein. Consistent with the size of the band on the gel, we did not detect any peptides within the small subunit of Ana o 3. The segment of Ana o 3 recovered in our analysis contained several of the previously mapped Ana o 3 linear IgE epitopes including epitopes 10–16 [10]. We also detected a single peptide from the extracted gel slice with a mass matching that of an Ana o 2 peptide (Table 2) [9]. This peptide lies within the basic subunit of the protein just amino-terminal to linear epitope 16 [9]. Our results indicate that while some portion of the basic subunit of Ana o 2 resists digestion, Ana o 3 is the cashew protein most likely to survive the gastrointestinal tract intact.

The structural integrity and resistance to digestion of 2S albumins has been attributed, at least in part, to the disulfide bonds that link the large and small subunits of these proteins together [16]. Reducing agents, such as DTT, have been used to reduce the cysteine disulfide bonds and alter the conformation of these proteins, as well as their sensitivity to proteolysis [20, 23, 28]. To determine what effect DTT would have on the ability of cashew proteins to be digested *in vitro*, we repeated the sequential digestion of cashew extracts following a pretreatment with DTT. As shown in Fig. 2, cashew extracts exposed to DTT for 15 min at 37°C prior to proteolysis were more thoroughly digested. Digestion with SGF alone following the treatment with DTT reduced the intensity of the band containing Ana o 3, and there were

Table 2. Identification of SGF/SIF resistant cashew peptides

Protein	Start AA position	Peptide sequence	z	m/z observed	Calculated mass	Observed – calculated	Linear epitope numbers
Ana o 2	302	(R)LTTLNLSNLPILK(W)	2	720.4305	1439.883	–0.0295	16
Ana o 3	75	(R)ECCQELQEVD(R)	2	733.3073	1465.605	0.0027	10/11/12
Ana o 3	75	(R)ECCQELQEVD(R)(C)	3	541.2382	1621.706	–0.0057	10/11/12
Ana o 3	98	(R)QLQQQEQIK(G)	2	571.8114	1142.616	–0.0009	13/14
Ana o 3	98	(R)QLQQQEQIKGEEVR(E)	3	571.6292	1712.893	–0.0195	13/14/15
Ana o 3	98	(R)QLQQQEQIKGEEVRELYETASELPR(I)	4	751.1347	3001.523	–0.0055	13/14/15
Ana o 3	112	(R)ELYETASELPR(I)	2	654.3266	1307.648	–0.0018	14/15
Ana o 3	123	(R)ICSISPSQGCQFQSSY(–)	2	924.8919	1848.789	–0.0128	16

Ana o 2 linear epitope numbers as described in ref. [11]; Ana o 3 linear epitope numbers as described in ref. [12]

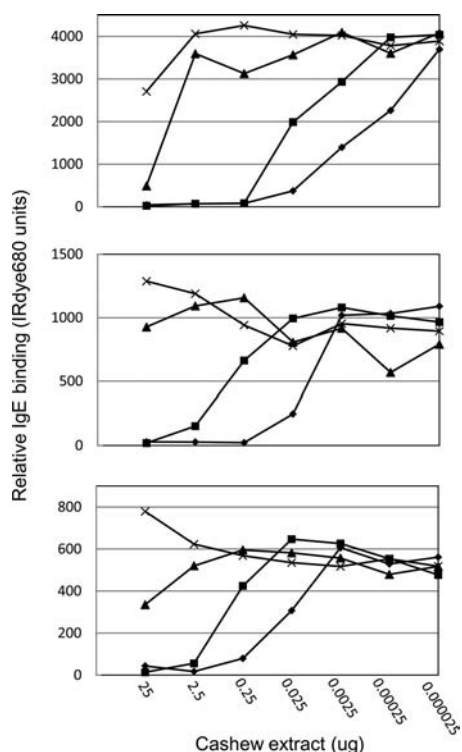


Figure 3. DTT pretreated SGF/SIF digested cashew extract competes weakly for IgE binding. SGF (squares), SGF/SIF (triangles), DTT pretreated SGF/SIF (hatches), or undigested cashew extracts (diamonds) were serially diluted tenfold and tested for IgE binding in a competitive ELISA. Serum samples from three cashew allergic patients were diluted 1:4 and incubated with the indicated cashew extract digestions and IgE binding was evaluated in microtiter plates coated with 1 μ g of undigested cashew extract. Extract dilution is indicated on the x-axis and relative IgE binding in IR-dye 680 units on the y-axis.

no observable bands after digestion with both SGF and SIF following DTT treatment (Fig. 2). Consistent with this, we were unable to detect any cashew allergen fragments in a gel slice corresponding to the same position within the gel from the DTT pretreated SGF/SIF digested sample.

While we did not observe protein fragments in the gel from the DTT pretreated SGF/SIF sample, it is possible that small peptide fragments from the sequential digestion retained the ability to be bound by IgE. To test this, we compared the ability of the sequentially digested cashew extracts to compete for binding to IgE from cashew allergic patient sera. Cashew extract digested samples (30 μ g) were serially diluted and tested for IgE binding in a competitive ELISA assay. We found that the DTT pretreated SGF/SIF digested cashew extract was not an effective competitor for IgE binding in any of the three patient sera tested (Fig. 3). Similarly, the SGF/SIF digested extract was not an effective competitor in one of the sera tested, but competed weakly for IgE binding in the other two sera. Conversely, pepsin digested cashew extract was able to compete for binding, but not as effectively as undigested

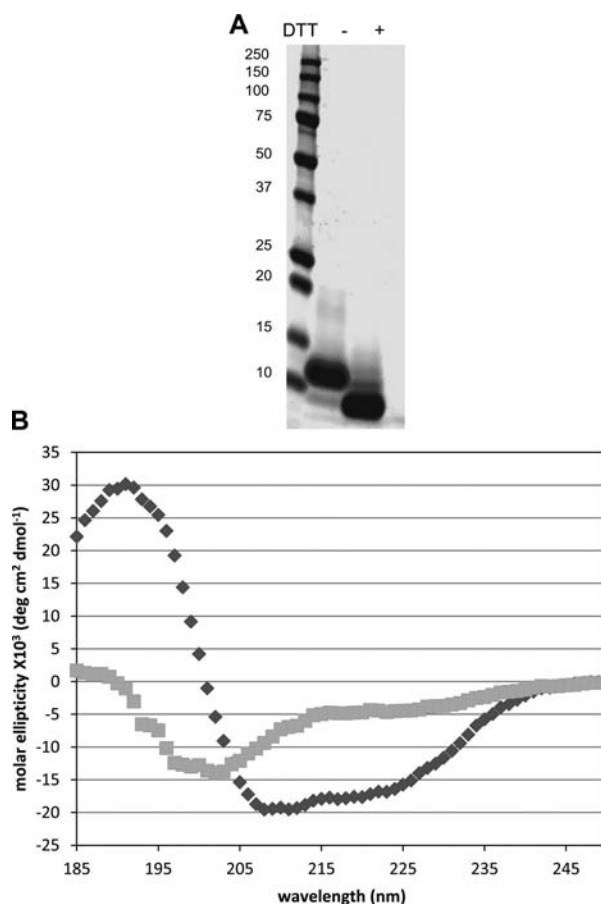


Figure 4. Reduction with DTT disrupts Ana o 3 structure. Purified Ana o 3 was analyzed by SDS-PAGE in the presence or absence of DTT (Tris-glycine gel) (A) and with far-UV circular dichroism from 185–250 nm in native form or after reduction with DTT (B). The dark gray diamonds represent native Ana o 3 and the light gray squares represent Ana o 3 after exposure to DTT.

cashew extract. These results are consistent with the SDS-PAGE results and suggest the most effective reduction in IgE binding occurs in the DTT pretreated SGF/SIF digested extracts.

3.4 In vitro SGF and SIF digestion of purified Ana o 3

To confirm our findings with whole cashew extracts and better characterize Ana o 3, we purified the protein (see Supporting Information). Purified Ana o 3 was subjected to SDS-PAGE under nonreducing or reducing conditions. Under nonreducing conditions on a Tris-glycine buffered gel (Fig. 4A) the protein migrated in a size range consistent with 12 598 Da determined by MS [10]. As expected, migration of the purified protein was altered by the presence of the reducing agent DTT (Fig. 4A). We evaluated the structure of Ana o 3 using far-UV CD. The far-UV CD of Ana o 3 was characteristic of other 2S albumins [23, 24] and had negative extremes at 208

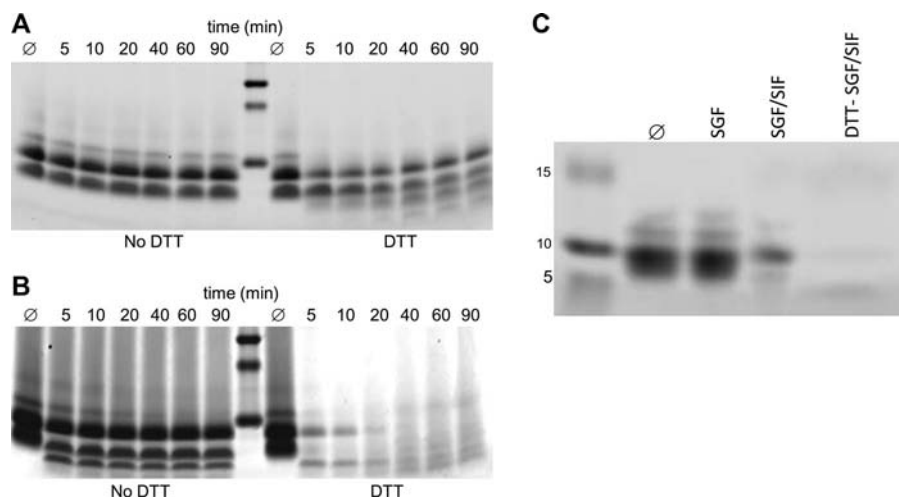


Figure 5. Disruption of Ana o 3 disulfide bonds increases protease sensitivity. Purified Ana o 3 (8 μ g) was either pretreated with DTT or digested directly with SGF (0.8 U pepsin) (A), SIF (2 U trypsin) (B), or sequentially with SGF and SIF (C) and the resulting samples were reduced with DTT and analyzed by SDS-PAGE. Gels in panels A and B are 10–20% tricine gels and panel C is a Tris-glycine gel. Markers shown in the gels from panels A and B correspond to 20, 15, and 10 kDa markers (top to bottom).

and 222 nm and a positive maximum at 191 nm representative of alpha-helical content (Fig. 4) [35]. The native protein was estimated to have 50% alpha-helical structure and 8% β -strand structure using the K2D3 software program [33]. In the presence of DTT, the structure was obviously disrupted (Fig. 4B) and alpha-helical content of Ana o 3 was estimated to be 5% while the β -strand estimation remained relatively unchanged at 10%.

The structure and gel migration of Ana o 3 was altered following reduction of disulfide bonds, and our results with cashew extracts (Fig. 2) as well as research from other groups with 2S albumins [20, 23, 24] strongly suggested that purified Ana o 3 would be more easily digested following reduction of disulfide bonds. To test this, we subjected the purified protein to in vitro digestion using SGF and SIF before and after exposure to DTT. As shown in Fig. 5A, native Ana o 3 was refractory to digestion with SGF (0.8 U pepsin), and there was no detectable difference in the migration pattern or level of protein during the experiment, consistent with our findings with cashew extracts. In contrast, Ana o 3 pretreated with DTT was partially digested by the same pepsin activity resulting in a decrease in the amount of protein remaining and the production of at least two faster migrating protein fragments. We observed similar but more pronounced results following pretreatment with DTT and digestion with SIF (Fig. 5B). Digestion of native Ana o 3 with SIF (2 U trypsin) resulted in a fragment of Ana o 3 that migrated just below the smallest of the Ana o 3 large subunit isoforms. Pretreatment with DTT and SIF digestion resulted in greater fragmentation of the protein and clearly reduced the amount of undigested protein in the gel. We evaluated sequential SGF/SIF digestion of native or reduced Ana o 3 (Fig. 5C). The SGF/SIF sequentially digested protein was reduced in intensity on the gel while digestion with SGF alone had no observable effect. Prior reduction of Ana o 3 and sequential SGF/SIF digestion greatly reduced the amount of protein observed on the gel and we noticed a band migrating below the 5 kDa marker as

well as a faint band migrating at a position consistent with undigested Ana o 3 (Fig. 5C). It is clear that the reduction of Ana o 3 disulfide bonds, like peanut and Brazil nut 2S albumins [20, 23, 24, 28], promotes greater processing by digestive proteases, however, the effect is greater with trypsin.

3.5 IgE binding to Ana o 3

We next examined the effects of digestion on IgE binding to Ana o 3. We compared IgE binding to native Ana o 3, Ana o 3 digested with SGF, SGF/SIF, or reduced Ana o 3 digested with SGF/SIF. Samples from each of the reactions were evaluated for IgE binding in a competitive ELISA, and the three individual sera samples produced similar results (Fig. 6). Consistent with the results of the SDS-PAGE analysis, there was no clear difference in IgE binding to the SGF digested Ana o 3 when compared to the undigested sample. In contrast, the SGF/SIF sequentially digested Ana o 3 was a noticeably weaker competitor for IgE binding. The most obvious effect on IgE binding was observed with Ana o 3 that was first reduced with DTT and then digested with SGF and SIF. The reduced and SGF/SIF digested Ana o 3 was only able to compete for IgE binding at the highest concentrations tested. These results suggest much of the reduced Ana o 3 was destroyed in this sample, however as reflected in the gel, remaining undigested Ana o 3 or Ana o 3 fragments retained some IgE-binding capacity. Immunoblot analysis of the reduced and SIF digested Ana o 3 from Fig. 5B suggests these smaller fragments are not bound well by the membrane (see Supporting Information). Collectively, these results indicate the development of processing methods that incorporate a reduction step prior to proteolysis are likely to reduce the ability of soluble cashew allergens to elicit an allergic response.

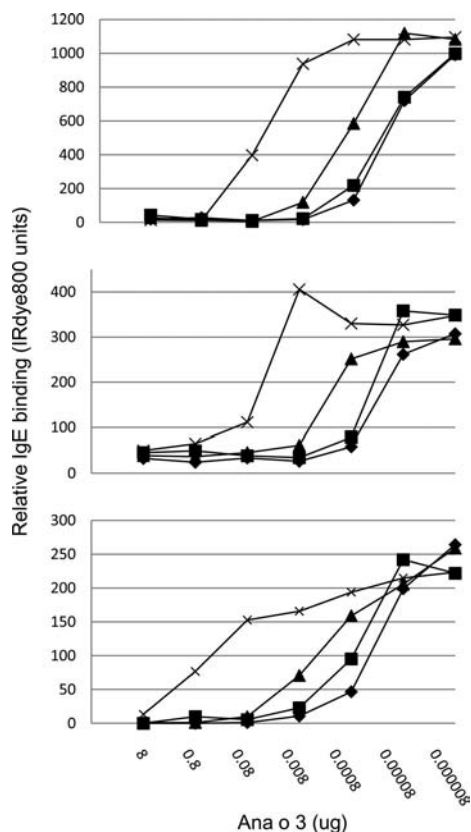


Figure 6. IgE binding to Ana o 3 is reduced following reduction and in vitro digestion. Purified Ana o 3 (8 μ g) was subjected to in vitro digestion and evaluated for binding by IgE in a competitive ELISA format. SGF (squares), SGF/SIF (triangles), DTT pretreated SGF/SIF (hatches), or undigested Ana o 3 (diamonds) were serially diluted tenfold and IgE binding was evaluated. Serum samples from three cashew allergic patients were diluted 1:4 and incubated with the indicated Ana o 3 digestions and IgE binding was evaluated in microtiter plates coated with 1 μ g of native Ana o 3. Ana o 3 dilution is indicated on the x-axis and relative IgE binding in IR-dye 800 units on the y-axis.

4 Concluding remarks

Characterizing the stability of food proteins to digestion can aid our understanding of how they may elicit immune responses leading to food allergy. We used defatted cashew extracts with reduced lipid content and purified proteins lacking lipids that may shield proteins from digestion and affect their survival and absorption as they pass through the digestive tract. Lipid content has been demonstrated to effect reaction severity in response to peanut challenge [36] and is important for sensitization of mice to Brazil nut allergens [37]. Keeping this in mind, our analysis of defatted cashew extracts and purified Ana o 3 protein indicates that Ana o 3 and fragments of Ana o 2 are most likely to resist gastric digestion. The approximate size range of these fragments is consistent with our finding of several IgE-binding epitopes within the extracted protein. Furthermore, Ana o 3 is most likely to survive passage

through both the gastric and intestinal lumen intact, consistent with the stability observed with other 2S albumin family members. Like other 2S albumins, the resistance of Ana o 3 to digestion was compromised after the protein had been exposed to reduction with DTT. Our findings further highlight the importance of the 2S albumin family of proteins in food allergy, support their potential value in immunotherapy, and suggest methods for their attenuation.

We would like to thank Yvette Bren-Mattison for helpful discussion and critical evaluation of the material presented and Wendy Desormeaux for technical support. This research was supported by funds from the U.S. Department of Agriculture–Agricultural Research Service and the Allergy/Immunology Research Center of North Texas. Mention of trade names, commercial products, or companies in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

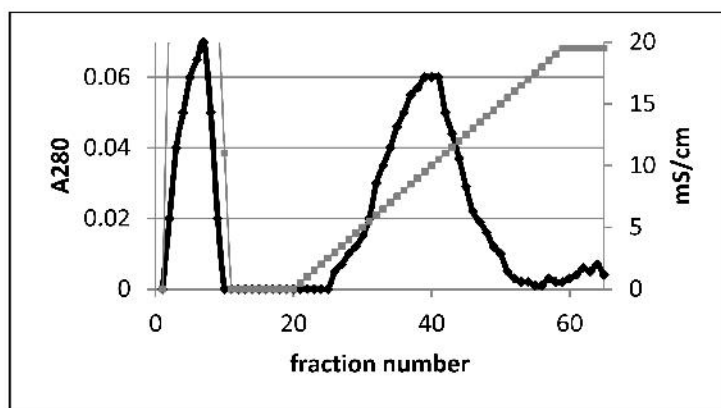
The authors have declared no conflict of interest.

5 References

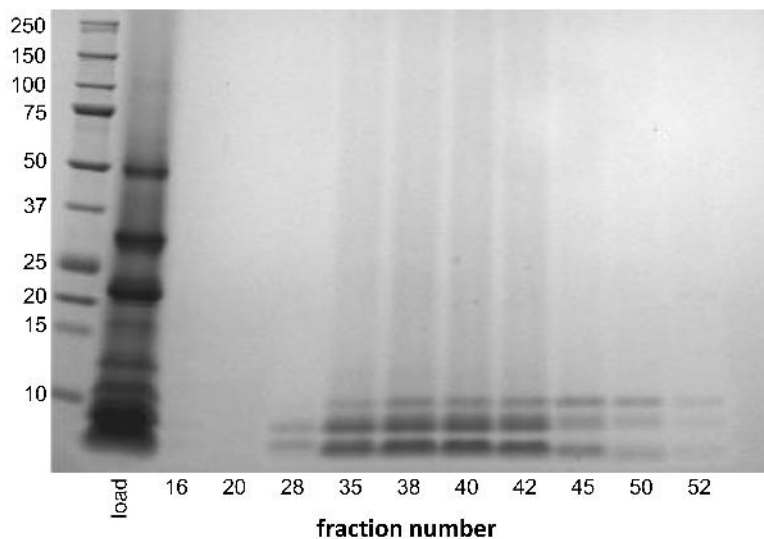
- [1] Gupta, R. S., Springston, E. E., Warrier, M. R., Smith, B. et al., The prevalence, severity, and distribution of childhood food allergy in the United States. *Pediatrics* 2011, 128, e9–e17.
- [2] Bock, S. A., Munoz-Furlong, A., Sampson, H. A., Further fatalities caused by anaphylactic reactions to food, 2001–2006. *J. Allergy Clin. Immunol.* 2007, 119, 1016–1018.
- [3] Sicherer, S. H., Munoz-Furlong, A., Godbold, J. H., Sampson, H. A., US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J. Allergy Clin. Immunol.* 2010, 125, 1322–1326.
- [4] Clark, S., Espinola, J., Rudders, S. A., Banerji, A. et al., Frequency of US emergency department visits for food-related acute allergic reactions. *J. Allergy Clin. Immunol.* 2011, 127, 682–683.
- [5] Patel, D. A., Holdford, D. A., Edwards, E., Carroll, N. V., Estimating the economic burden of food-induced allergic reactions and anaphylaxis in the United States. *J. Allergy Clin. Immunol.* 2011, 128, 110–115.
- [6] Davoren, M., Peake, J., Cashew nut allergy is associated with a high risk of anaphylaxis. *Arch. Dis. Child* 2005, 90, 1084–1085.
- [7] Clark, A. T., Anagnostou, K., Ewan, P. W., Cashew nut causes more severe reactions than peanut: case-matched comparison in 141 children. *Allergy* 2007, 62, 913–916.
- [8] Wang, F., Robotham, J. M., Teuber, S. S., Tawde, P. et al., Ana o 1, a cashew (*Anacardium occidentale*) allergen of the vicilin seed storage protein family. *J. Allergy Clin. Immunol.* 2002, 110, 160–166.
- [9] Wang, F., Robotham, J. M., Teuber, S. S., Sathe, S. K. et al., Ana o 2, a major cashew (*Anacardium occidentale* L.) nut allergen of the legumin family. *Int. Arch. Allergy Immunol.* 2003, 132, 27–39.

- [10] Robotham, J. M., Wang, F., Seamon, V., Teuber, S. S. et al., Ana o 3, an important cashew nut (*Anacardium occidentale* L.) allergen of the 2S albumin family. *J. Allergy Clin. Immunol.* 2005, **115**, 1284–1290.
- [11] Radauer, C., Breiteneder, H., Evolutionary biology of plant food allergens. *J. Allergy Clin. Immunol.* 2007, **120**, 518–525.
- [12] Sathe, S. K., Sze-Tao, K. W. C., Wolf, W. J., Hamaker, B. R., Biochemical characterization and in vitro digestibility of the major globulin in cashew nut (*Anacardium occidentale*). *J. Agric. Food Chem.* 1997, **45**, 2854–2860.
- [13] Teuber, S. S., Sathe, S. K., Peterson, W. R., Roux, K. H., Characterization of the soluble allergenic proteins of cashew nut (*Anacardium occidentale* L.). *J. Agric. Food Chem.* 2002, **50**, 6543–6549.
- [14] Robotham, J. M., Xia, L., Willison, L. N., Teuber, S. S. et al., Characterization of a cashew allergen, 11S globulin (Ana o 2), conformational epitope. *Mol. Immunol.* 2010, **47**, 1830–1838.
- [15] Xia, L., Willison, L. N., Porter, L., Robotham, J. M. et al., Mapping of a conformational epitope on the cashew allergen Ana o 2: a discontinuous large subunit epitope dependent upon homologous or heterologous small subunit association. *Mol. Immunol.* 2010, **47**, 1808–1816.
- [16] Moreno, F. J., Clemente, A., 2S albumin storage proteins: what makes them food allergens? *Open Biochem. J.* 2008, **2**, 16–28.
- [17] Venkatachalam, M., Monaghan, E. K., Kshirsagar, H. H., Robotham, J. M. et al., Effects of processing on immunoreactivity of cashew nut (*Anacardium occidentale* L.) seed flour proteins. *J. Agric. Food Chem.* 2008, **56**, 8998–9005.
- [18] Su, M., Venkatachalam, M., Teuber, S. S., Roux, K. H. et al., Impact of g-irradiation and thermal processing on the antigenicity of almond, cashew nut, and walnut proteins. *J. Sci. Food Agric.* 2004, **84**, 1119–1125.
- [19] Mills, E. N., Sancho, A. I., Rigby, N. M., Jenkins, J. A. et al., Impact of food processing on the structural and allergenic properties of food allergens. *Mol. Nutr. Food Res.* 2009, **53**, 963–969.
- [20] Moreno, F. J., Mellon, F. A., Wickham, M. S., Bottrill, A. R. et al., Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant in vitro gastrointestinal digestion. *FEBS J.* 2005, **272**, 341–352.
- [21] Moreno, F. J., Maldonado, B. M., Wellner, N., Mills, E. N., Thermostability and in vitro digestibility of a purified major allergen 2S albumin (Ses i 1) from white sesame seeds (*Sesamum indicum* L.). *Biochim. Biophys. Acta* 2005, **1752**, 142–153.
- [22] Alcocer, M. J., Murtagh, G. J., Bailey, K., Dumoulin, M. et al., The disulphide mapping, folding and characterisation of recombinant Ber e 1, an allergenic protein, and SFA8, two sulphur-rich 2S plant albumins. *J. Mol. Biol.* 2002, **324**, 165–175.
- [23] Koppelman, S. J., Nieuwenhuizen, W. F., Gaspari, M., Knipfels, L. M. et al., Reversible denaturation of Brazil nut 2S albumin (Ber e1) and implication of structural destabilization on digestion by pepsin. *J. Agric. Food Chem.* 2005, **53**, 123–131.
- [24] Sen, M., Kopper, R., Pons, L., Abraham, E. C. et al., Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *J. Immunol.* 2002, **169**, 882–887.
- [25] Porterfield, H. S., Murray, K. S., Schlichting, D. G., Chen, X. et al., Effector activity of peanut allergens: a critical role for Ara h 2, Ara h 6, and their variants. *Clin. Exp. Allergy* 2009, **39**, 1099–1108.
- [26] Kulis, M., Macqueen, I., Li, Y., Guo, R. et al., Pepsinized cashew proteins are hypoallergenic and immunogenic and provide effective immunotherapy in mice with cashew allergy. *J. Allergy Clin. Immunol.* 2012, **130**, 716–723.
- [27] Zhuang, Y., Dreskin, S. C., Redefining the major peanut allergens. *Immunol. Res.* 2012, **55**, 125–134.
- [28] Hazebrouck, S., Guillon, B., Drumare, M. F., Paty, E. et al., Trypsin resistance of the major peanut allergen Ara h 6 and allergenicity of the digestion products are abolished after selective disruption of disulfide bonds. *Mol. Nutr. Food Res.* 2012, **56**, 548–557.
- [29] Astwood, J. D., Leach, J. N., Fuchs, R. L., Stability of food allergens to digestion in vitro. *Nat. Biotechnol.* 1996, **14**, 1269–1273.
- [30] Niessen, S., McLeod, I., Yates, J. R., 3rd, Silver staining, digestion, and extraction of peptides from an acrylamide gel for MS analysis. *CSH Protoc.* 2006, **2006**, 1–5.
- [31] The United States Pharmacopeial Convention 1990, pp. 1788–1789.
- [32] Koppelman, S. J., Hefle, S. L., Taylor, S. L., de Jong, G. A., Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: a comparative in vitro study and partial characterization of digestion-resistant peptides. *Mol. Nutr. Food Res.* 2010, **54**, 1711–1721.
- [33] Louis-Jeune, C., Andrade-Navarro, M. A., Perez-Iratxeta, C., Prediction of protein secondary structure from circular dichroism using theoretically derived spectra. *Proteins* 2012, **80**, 374–381.
- [34] Kulis, M., Chen, X., Lew, J., Wang, Q. et al., The 2S albumin allergens of *Arachis hypogaea*, Ara h 2 and Ara h 6, are the major elicitors of anaphylaxis and can effectively desensitize peanut-allergic mice. *Clin. Exp. Allergy* 2012, **42**, 326–336.
- [35] Kelly, S. M., Jess, T. J., Price, N. C., How to study proteins by circular dichroism. *Biochim. Biophys. Acta* 2005, **1751**, 119–139.
- [36] Grimshaw, K. E., King, R. M., Nordlee, J. A., Hefle, S. L. et al., Presentation of allergen in different food preparations affects the nature of the allergic reaction—a case series. *Clin. Exp. Allergy* 2003, **33**, 1581–1585.
- [37] Mirotti, L., Florsheim, E., Rundqvist, L., Larsson, G. et al., Lipids are required for the development of Brazil nut allergy: the role of mouse and human iNKT cells. *Allergy* 2013, **68**, 74–83.

A



B

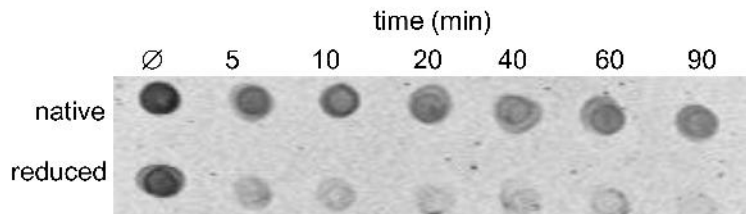


C

Ana o 3 peptides observed by mass-spectrometry after reduction and trypsin digestion

21 SIYRAIVEVEEDSGREQSCQRQFEEQQRFRNCQRVYVKQEVQRGGRYNQRQESLRECCQEL 80
 81 QEVDRRRCRCQNLEQMVRQLQQQEIQKGEEVRELYETASELPRICSISPSQGCQFQSSY 138

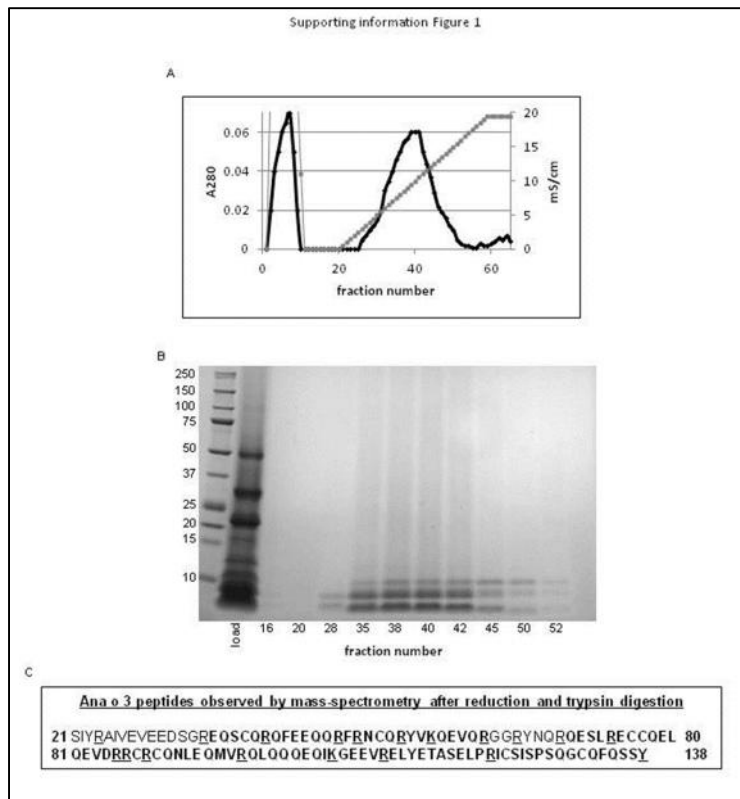
Supporting information Figure 2



Supporting information

Ana o 3 purification

Ana o 3 was purified from cashew extracts using a sodium phosphate gradient on a ceramic hydroxyapatite column (supporting information Figure 1A). Defatted cashew flour was resuspended (1:10 w/v) in buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, and 1 mM PMSF) by stirring at room temperature for 1 hour and sonication (3 x 15 seconds) using a 60 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA, USA). The resulting slurry was centrifuged at 12,000g for 30 minutes at 4°C to remove insoluble particulates. To enrich for Ana o 3, soluble protein was precipitated first with 75% ammonium sulfate and centrifugation. The supernatant was retained and Ana o 3 was precipitated by addition of ammonium sulfate to 100% followed by centrifugation. Precipitated protein from the 100% ammonium sulfate pellet was re-solubilized in buffer (5 mM sodium phosphate pH 6.8), and Ana o 3 was purified by ion-exchange chromatography with ceramic hydroxyapatite resin (Bio-Rad) and sodium phosphate gradient (5–200mM) on a BioLogic LP low-pressure chromatography system (Bio-Rad) using a flow rate of 3 ml/minute. Ana o 3 identity was confirmed by liquid chromatography coupled mass spectrometry. As shown in supporting information Figure 1B, the isolated Ana o 3 consisted of at least three separate isoforms, consistent with previous reports [12], which were separated well on gels buffered with tricine. Column fractions containing Ana o 3 were pooled, digested, and analyzed by mass-spectrometry. We observed 14 Ana o 3 peptides representing more than 80% of the protein confirming that we had purified Ana o 3 (supporting information Figure 1C).

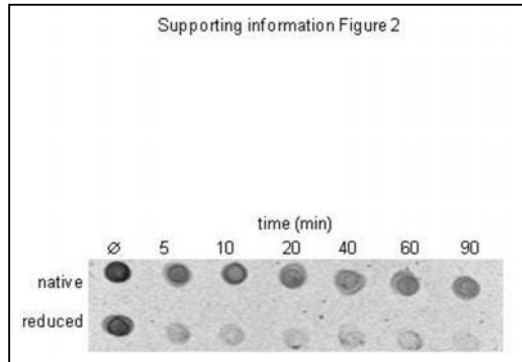


Supporting information Figure 1. Purification of Ana o 3 from cashew extracts. Ion-exchange chromatogram profile of protein concentration (A280) in black and specific conductance (mS/cm) in grey (A) and SDS-PAGE (tricine) of fractions containing purified Ana o 3 (B). Fractions containing Ana o 3 were pooled and the identity of Ana o 3 was confirmed by LC/MS and observed peptides are shown in bold with trypsin cleavage sites underlined (C).

SIF digested Ana o 3 immunoblot

Following digestion in SIF, Ana o 3 (8ug) samples were spotted onto PVDF membrane and blocked in tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% non-fat dry milk for 1 hour. Human sera samples from 9 individuals were pooled and added at a 1:10 dilution to membranes spotted with Ana o 3 digestion samples and incubated overnight at 4°C. Membranes were washed with TBST and incubated with mouse anti-human IgE secondary antibody (Southern Biotech) conjugated to IR-Dye 800 (1:5000). Membranes were subjected to a final washing and signal was documented by infrared fluorescence with an Odyssey CLX imaging system (LI-COR) using the 800nm channel. Following reduction and SIF digestion, IgE binding to Ana o 3 was appreciably decreased, even after just 5 minutes of proteolysis.

The IgE signal correlated with the amount of Ana o 3 remaining in the samples analyzed by SDS-PAGE (see Figure 5B in main text).



Supporting information Figure 2. Effect of SIF on IgE binding to purified Ana o 3.

Purified native or reduced Ana o 3 (8ug) was digested with SIF for the indicated times, samples were spotted onto nitrocellulose, allowed to dry, and serum IgE binding from a pool of cashew allergic individuals was evaluated. Although there was a decrease, IgE binding to native Ana o 3 digested with SIF was relatively unchanged. In contrast, the IgE signal of Ana o 3 that was first reduced and then digested with SIF was appreciably decreased.