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A cashew specific monoclonal antibody recognizing the small subunit of Ana o 3

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ABSTRACT

Food allergies represent a substantial medical liability and preventing accidental exposure to food allergens requires constant attention. Allergic reaction to cashew nuts is frequently serious, and the small 2S albumin, Ana o 3, is an immuno-dominant cashew allergen. Ana o 3 is composed of five alpha helices, contains 2 subunits linked by cysteine disulfide bonds, and remains soluble even after extensive heating of cashew nuts. The stability and solubility properties of Ana o 3 make it an excellent target for diagnostic and detection methods and tools. In this work, a monoclonal antibody, designated 2H5, aimed at amino acids 39–54 within helices I and II of the small subunit of Ana o 3 was developed that recognizes both recombinant and native Ana o 3 and is cashew specific in ELISA experiments. The K_D against the targeted amino-acid sequence was found to be approximately 7.0×10^{-6} mg/ml (3.3 nM), while the K_D against the native protein was found to be approximately 1.2×10^{-3} mg/ml (92 nM). The 2H5 monoclonal anti-Ana o 3 antibody can distinguish between native and recombinant proteins and represents a useful reagent for the study of antibody cashew-allergen interactions and may enable the development of cashew-specific diagnostic tools that can be used to prevent accidental cashew allergen exposures.

1. Introduction

Food allergies result in Type I hypersensitivity reactions due to Immunoglobulin E (IgE) binding to what are normally benign food proteins. Several reports have indicated the incidence of food allergy has increased over the past two decades [1] and the costs associated with food allergy response and care are substantial [2]. Several factors may affect the onset and progression of food allergy including genetics, diet, food additives such as citric acid and sulfites, as well as environmental [3–6].

Tree nut consumption has been correlated some health advantages [7], but tree nuts are also considered one of eight foods that commonly cause food allergies. Accidental ingestion or contact with tree nuts is common and the frequency of tree nut allergy is growing [8]. Tree nuts are considered potent allergens, and tree nut allergies are usually lifelong allergies that do not improve with age [9]. Cross-reaction among tree nuts has been well documented [10] and due to similar sequence and conformation among conserved tree nut seed storage proteins.

The *Anacardiaceae* family includes cashew nuts, pistachio and mango which may cross-react with cashew nut allergens, and poison ivy and poison oak that may contain skin-irritating saps or oils [11]. Demand for cashew nuts is high in the United States, and cashew nuts are an essential ingredient in many types of foods. IgE mediated cashew nut allergy has been reported towards seed storage proteins including the Ana o 1 [12], Ana o 2 [13], and the Ana o 3 proteins [14], which have homologs in many plant species. Surveys of medical reports indicate that reactions to cashew allergens can often be severe [15–17].

A very small fraction of a single cashew nut can elicit severe reactions, but information that would clearly define threshold doses and be useful for food processors, clinical diagnostic labs, or medical laboratories for labeling transparency have not been adopted by regulatory agencies. Recent research to define a threshold dose for cashew allergens suggests amounts as low as 0.9 mg of cashew nut protein can cause a reaction [18,19]. A survey of cashew cultivars from world regions indicated only very minor variations in allergen content, suggesting no large differences among allergen composition of the nuts. Heating steps performed during cashew nut processing can alter cashew allergen solubility [20,21], and this may complicate diagnostic or clinical testing. Heat-induced chemical alterations on the Ana o 3 cashew allergen have also been documented [22], but the immunological consequence of this

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type of modification has not been defined.

The small 2S albumin proteins are within the prolamin superfamily of proteins and are potent peanut and tree nut allergens [23]. They are often cleaved into large and small subunits and contain conserved cysteine residues that have been demonstrated to contribute to structural stability and resistance of the proteins to peptidases [23]. Several structural studies of 2S albumin proteins from various plant seeds indicate a compact protein containing five alpha-helices connected by short loops [23,24]. A segment of these proteins between helix 3 and 4 has been termed the 'hypervariable region', and dominant linear IgE epitopes have been identified for several of the 2S proteins within this flexible region [23]. In peanuts, the 2S albumins Ara h 2 and Ara h 6, are thought to be responsible for the bulk of allergenic activity in peanut extracts [25,26].

In cashew nuts, the Ana o 3 protein is an important allergen and its sequence has been studied in detail to accurately characterize the boundaries of the small and large subunit within the mature protein and identify IgE cognate epitopes [14,27]. Both the small 6 kDa and large 8 kDa Ana o 3 subunits have been shown to have variation in the ends of their respective sequence [27]. While the Ana o 3 protein has been demonstrated to contain some amino acid sequence variation, as yet no clear differences in immunological properties have been revealed due to these differences. The Ana o 3 protein can withstand several experimental processing methods [28–30], remains soluble even after extensive heating [21], and can survive digestion with pepsin and trypsin [31].

A few methods for cashew allergen detection have been characterized. ELISA for cashew allergens has been described in the past [32] and kits are available from several commercial suppliers. An LC-tandem MS method was developed that can detect tree nuts, including cashew nuts [33], and a Taqman Real-Time PCR Methodology has been described that can detect the presence of cashew nuts in food [34]. Due to its advantageous solubility and stability properties, the Ana o 3 protein presents an excellent diagnostic target. In this work, a monoclonal antibody directed towards a peptide sequence contained within helices I and II of the small Ana o 3 protein subunit that recognizes both native and recombinant Ana o 3 (rAna o 3) has been generated and characterized.

2. Materials and methods

2.1. Materials

Tree nuts and peanuts were bought from NutsOnline (Cranford, NJ, USA). Bovine serum albumin (BSA) fraction V, petroleum ether, One Shot™ BL21(DE3) competent E. coli, and flat-bottom clear-well MaxiSorp 96-well plates were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant Enterokinase was purchased from EMD Millipore (Billerica, MA, USA). The polyclonal rabbit anti-cashew antisera, described previously [35], was generated by Pierce Biotechnology Inc. (Rockford, IL, USA). Secondary antibodies IRDye 680RD labeled donkey anti-mouse IgG and IRDye 800CW labeled donkey anti-rabbit IgG were purchased from LI − COR (Lincoln, NE, USA).

Defatted nut extracts

Ground cashew, pistachio, peanut, walnut, pecan, almond, or heated cashew nuts (mild - 300 °F/149 °C for 12 min, medium - 300 °F/149 °C for 20 min, or dark roasted - 300 °F/149 °C for 24 min) were defatted with petroleum ether using a Kimble Soxhlet extraction device for 24 h as described previously [21]. Defatted nut flours were mixed 1:10 with 100 mM sodium phosphate buffer (pH 8.0) for 1 h, sonicated three times at 4 °C for 15 s using a Sonic Dismembrator (Fisher Scientific Co., Orlando, FL, USA), and centrifuged 30 min (12,000 × g) at 4 °C. Protein concentrations of nut extract samples were determined by absorbance (280 nm) on a Pharmaspec UV-1700 (Shimadzu, Kyoto, Japan). Clarified samples were frozen with liquid nitrogen and kept at − 80 °C during storage.

2.2. Native Ana o 3 purification

Native Ana o 3 was purified from cashew nuts by ion-exchange chromatography as previously described with minor changes [31]. Defatted raw or dark roasted cashew nut extracts were resuspended in Tris buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mm EDTA, and 1 mM PMSF) with stirring at ambient temperature 1 h and then sonicated (3 × 15 s) with a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA, USA). Soluble protein was collected by centrifugation (12,000 x g, 30 min, 4 °C), and sequentially precipitated with 75% and then 100% ammonium sulfate. Protein precipitated by 100% ammonium sulfate was re-solubilized in sodium phosphate buffer (5 mM sodium phosphate pH 6.8), and run through a ceramic hydroxyapatite column (Bio-Rad, Hercules, CA, USA) on a BioLogic LP low-pressure chromatography system (Bio-Rad, Hercules, CA, USA). Protein was eluted using a sodium chloride gradient (5–200 mM) at 3 ml/min. Ana o 3 fractions were pooled and concentrated, and then digested with trypsin and analyzed by mass-spectrometry to confirm identity.

2.3. Mouse monoclonal anti-Ana o 3 antibody production and sequencing

A peptide corresponding to amino acids 39–54 of Ana o 3 (H2N-CQRQFEEQQRFRNCQR − OH), and the 2H5 anti-Ana o 3 peptide antibody were produced by Genscript (Piscataway, NJ, USA). Briefly, the Ana o 3 peptide corresponding to amino acids 39–54 (H2N-CQRQFEEQQRFRNCQR − OH) of Helices I and II within the Ana o 3 protein was synthesized and amino terminally conjugated to keyhole limpet hemocyanin (KLH). The KLH-conjugated peptide was used to immunize 5 Balb/c mice with a traditional immunization strategy. Screening of peptide positive supernatants from collected spleen cell fusions was done by ELISA. Peptide-positive primary hybridoma clones were sub-cloned by limiting dilution. One of the resulting stable hybridoma clones, 2H5, was selected for further analysis based upon preliminary tests using the Ana o 3 protein and a panel of tree nut extracts. Purified 2H5 antibody was collected from culture supernatant with Protein A, concentrated, aliquoted, flash frozen, and stored at − 80 °C.

To obtain the 2H5 antibody variable domain sequence, RNA from 2H5 hybridoma cells was collected using TRIzol® Reagent by following manufacturer instructions. RNA was reverse transcribed using the PrimeScriptTM 1 st Strand cDNA Synthesis Kit (Takara Bio, Mountain View, CA) into cDNA with isotype-specific antisense primers. The variable antibody domains were amplified using rapid amplification of cDNA ends (RACE), and cloned into pGEM-T cloning vectors (Promega Corporation, Madison, WI). Colony PCR confirmed clones with correctly sized inserts, and at least five independent colonies from each domain were sequenced. Insert sequences were aligned and the consensus sequence for each clone was used to predict protein sequence.

2.4. Recombinant Ana o 3 production and purification

A codon optimized Ana o 3 coding sequence was synthesized with a flanking 5′ in-frame BamHI and a 3′ EcoRI restriction site following a stop codon (IDT, Coralville, IA, USA). The synthesized Ana o 3 gene was excised from the production plasmid by digestion, and ligated with pET32 (EMD Millipore, Billerica, MA, USA) to create an in-frame fusion to the thioredoxin protein and a poly-histidine tag resulting in plasmid CPM444. One Shot™ BL21(DE3) chemically competent *E. coli* were transformed with the CPM444 plasmid and cells were grown at 37 °C in Luria Broth (Fisher Scientific, Fairlawn, New Jersey, USA) with 50ug/ml ampicillin to an optical density of 0.4 (OD_{600}). Thioredoxin-poly-histidine-Ana o 3 fusion protein expression was induced by the addition of 1 mM isopropyl-thio-β-galactoside, and cells were allowed to continue growing for 4 h. Cells were harvested by centrifugation,

resuspended in buffer (100 mM Tris pH 8.0, 250 mM NaCl, 5 mM imidazole, 0.1% triton X-100, and 1 mM PMSF), and lysed by sonication. Cell lysates were centrifuged and the recombinant thioredoxin-poly-histidine-Ana o 3 fusion protein was isolated from the clarified lysate with His-Select Nickel Affinity Gel (Sigma Aldrich, St. Louis, MO, USA) and cleaved by digestion with enterokinase according to manufacturers' instructions to remove the thioredoxin-poly-histidine affinity tag. Thioredoxin-poly-histidine protein remaining in the isolated recombinant Ana o 3 (rAna o 3) solution was removed by a final batch purification with His-Select Nickel Affinity Gel. Purified rAna o 3 protein concentration was established by absorbance at 280 nm using a Pharmaspec UV-1700 and purified protein aliquots were frozen in liquid nitrogen and stored at −80 °C.

2.5. Elisa

Comparison of 2H5 antibody-Ana o 3 binding was performed by either direct or competitive ELISA. For direct ELISA, purified native Ana o 3, native Ana o 3 pre-treated with 5 mM DTT for 15 min at 37 °C), or recombinant Ana o 3 (2.5 μg per well for each in 50 μL or half-log dilutions thereof as indicated) in sodium carbonate buffer (0.015 M Na2CO3, 0.035 M NaHCO3, pH 9.6) was used to coat plate wells overnight (ON) at 4 °C. Alternatively, 4 μg of defatted nut extracts made from ready to eat peanut, cashew, pistachio, walnut, pecan, almond, or in the case of cashew nuts, mild (300 °F/149 °C for 12 min), medium (300 °F/149 °C for 20 min), or dark roasted (300 °F/149 °C for 24 min) in 50 μL were used to coat plate wells ON at 4 °C. For non-competitive Ana o 3 dilution ELISA, 1.3 μg of purified Ana o 3 in 50 μL of sodium carbonate buffer, or half-log dilutions thereof, was used to coat plate wells ON at 4 °C. Following 16 h of incubation, plate wells were blocked with phosphate buffered saline (PBS, 10 mM phosphate, 137 mM sodium chloride, pH 7.4) containing 0.1% BSA for one hour at 37 °C. Fifty (50) μL of 2H5 stock antibody (1.729 mg/mL) diluted 1:5000 was added for 1 h at 37 °C, then plate wells were washed, and 1:10,000 dilution of donkey anti-mouse IRDye 680 labeled secondary antibody was added, wells were washed again, and the plate was scanned on a LI−COR Odyssey CLX for signal quantification. The rabbit anti-cashew polyclonal antibody was used at a 1:5000 dilution, and the IRDye 800CW labeled donkey anti-rabbit IgG was used at a 1:10,000 dilution.

For competitive ELISA, plate wells were coated with 2.5 μg of purified native Ana o 3 in 50 μL of sodium carbonate buffer ON at 4 °C, and blocked with BSA the next morning, as described above. For competitive ELISA with the native Ana o 3 protein, 25 μL of the 1:5000 diluted 2H5 antibody was pre-incubated for 1 h at 37 °C with 25 μL of purified Ana o 3 peptide at an initial concentration of 1.3 mg/mL or the indicated half-log dilutions. For competitive ELISA with the Ana o 3 peptide, 25 μL of the 1:5000 diluted 2H5 antibody was pre-incubated for 1 h at 37 °C with 25 μL of the 39–54 residue (H2N-CQRQFEEQQRFRNCQR−OH) Ana o 3 peptide at an initial concentration of 0.1 mg/mL or the indicated half-log dilutions. The 50 μL pre-binding reactions were then added to plate wells containing 20 μL of 10 mM phosphate buffered saline (pH 7.4) and incubated for 1 h at 37 °C. Plate wells were washed as above, a 1:10,000 dilution of donkey anti-mouse IRDye 680 labeled secondary antibody was added, and plates were scanned, as described above, on a LI−COR Odyssey CLX for signal quantification.

2.6. Protein modeling, docking, and epitope prediction

The Ana o 3 peptide and protein (Q8H2B8_ANAOC) were modeled with Molecular Operating Environment software (MOE version 2018.01, Chemical Computing Group, Montreal, QC, Canada) using the *Moringa oleifera* 2S albumin structure as a template [24] and the PAM250 amino acid substitution matrix. MOE was also used for the 2H5 antibody variable fragment model, the in-silico antibody-peptide docking, and epitope prediction.

2.7. Mathematical models for binding

The binding of a fixed concentration 2H5 antibody to varying concentrations of immobilized Ana o 3 protein may be described by the Langmuir isotherm [36,37]:

$$[A{:}\,P_s] = \frac{A_{tot}\,[P_s]}{K_{Ds} + [P_s]} \tag{1}$$

where $[A{:}P_s]$ is the concentration equivalent for the surface density of the antibody-protein complex, A_{tot} is the total concentration of the antibody (free and complexed), $[P_s]$ is the surface concentration of the unbound immobilized protein, and K_{Ds} is the dissociation constant for the surface reaction. When the antibody concentration is well below the value of the dissociation constant, the fraction of the complexed protein will be small, and the total protein concentration P_{tot} may be used in place of $[P_s]$.

In the experiments described here, the solution concentrations of protein used during the immobilization process are used as a proxy for the surface density of the protein. The K_{Ds} derived from these experiments is therefore not the actual dissociation constant, and care must be taken when deriving other quantities using these values.

The model for the competition experiments assumes equilibrium between the antibody bound to immobilized protein on the surface and antibody bound to competitor in solution. The competitor in these experiments may be either the Ana o 3 protein, or the peptide fragment. The surface based equilibrium is described by:

$$K_{Ds}\,[A{:}\,P_s] = [A][P_s] \tag{2}$$

where K_{Ds}, $[A{:}P_s]$, and $[P_s]$ are designated as in Eq. 1, and $[A]$ is the concentration of free antibody in solution. The equilibrium between free antibody, competitor, and antibody-competitor complex in solution is described by:

$$K_{DC}\,[A{:}\,C] = [A][C] \tag{3}$$

where K_{DC} is the dissociation constant for the antibody-competitor complex in solution, $[A{:}C]$ is the concentration of the antibody-competitor complex, and $[C]$ is the concentration of free competitor in solution. C therefore designates either the protein or the peptide, so that K_{DC} designates either K_{Dprot}, the dissociation constant for the antibody-protein complex, or K_{Dpept}, the dissociation constant for the antibody-peptide complex. The free surface concentration of the immobilized protein is then described by:

$$[P_s] = P_{tot} - [A{:}\,P_s] \cong P_{tot} \tag{4}$$

where P_{tot} is the total concentration of immobilized protein (free and complexed). Assuming that the antibody concentration is significantly less than K_{Ds} allows for the indicated approximation. The free concentration of competitor is described by:

$$[C] = C_{tot} - [A{:}\,C] \cong C_{tot} \tag{5}$$

where C_{tot} is the total concentration of competitor (free and complexed). Assuming that the antibody concentration is significantly less than K_{DC} allows for the indicated approximation. As the antibody is bound in both surface-protein complexes, and competitor complexes, its free concentration is described by:

$$[A] = A_{tot} - [A{:}\,P_s] - [A{:}\,C] \tag{6}$$

The surface-bound complex $[A{:}P_s]$ is what is detected via a labeled secondary antibody. Solving Eq.s 2–6 for $[A{:}P_s]$ yields:

$$[A{:}\,P_s] = \frac{A_{tot}\,P_{tot}}{K_{Ds} + P_{tot} + \frac{K_{Ds}}{K_{DC}}C_{tot}} \tag{7}$$

It should be noted that both K_{Ds} and P_{tot} are in units of solution concentrations as proxies for surface densities. A_{tot}, K_{DC} and C_{tot} are naturally in units of solution concentration.

2.8. Methods of mathematical analysis

All data was fit to the models using Igor Pro 6.37. Both Eq.s 1 and 7 may be fit to the Hill equation [38,39], which is one of the built-in functions in Igor Pro:

$$y = base + \frac{(\max - base)}{1 + \left(\frac{x_{half}}{x}\right)^{rate}} \tag{8}$$

In all cases, we have fixed *base* at zero, and y is taken to be the measured RFU signal.

To model Eq. 1 with the Hill equation, *rate* is fixed at 1, $x_{half} = K_{Ds}$, $x = [P_s] = P_{tot}$, and $max = R\,A_{tot}$, where R is the conversion between antibody concentration and RFU (generally unknown).

To model Eq. 7 with the Hill equation, *rate* is fixed at -1, and $x = C_{tot}$. The numerator and denominator of Eq. 7 must divided through by $(K_{Ds} + P_{tot})$ to achieve the form of the Hill Equation, so that:

$$\max = \frac{R\,A_{tot}\,P_{tot}}{K_{Ds} + P_{tot}} \tag{9}$$

and

$$x_{\text{half}} = m_C = \frac{K_{Ds} + P_{tot}}{K_{Ds}/K_{DC}} \tag{10}$$

K_{DC} is either the dissociation constant for the protein in solution, K_{Dprot}, if the protein is used as the competitor, or the dissociation constant for the peptide in solution, K_{Dpept}, if the peptide is used as the competitor. The ratio of m_{prot}/m_{pept} is then equal to K_{Dprot}/K_{Dpept}.

2.9. Statistical analysis

Antibody binding (relative fluorescence units, RFU) plots represent the average of at least four samples including ± standard deviation error bars. Experimental data was evaluated using analysis of variance (ANOVA) and a post-hoc Tukey Honest Significant Difference (HSD) Test using an alpha/ p value of < 0.01.

3. Results

3.1. Anti-Ana o 3 monoclonal antibody production

Monoclonal antibody clones directed towards a 16 amino acid peptide corresponding to a segment of the small subunit of Ana o 3 (residues 39-H2N-CQRQFEEQQRFRNCQR – OH-54) were generated in mice. This segment of the protein was chosen because of the large number of charged residues, documented recognition by IgE from cashew allergic patients [14,27], lower likelihood of sequence similarity with other 2S albumins near the carboxy-terminus of the small subunit [23], and the likelihood of solvent exposure based on Ana o 3 modeling (Fig. 1). Several hybridoma cell line supernatants were screened by ELISA for recognition of native Ana o 3, and one clone (2H5) was chosen for further study.

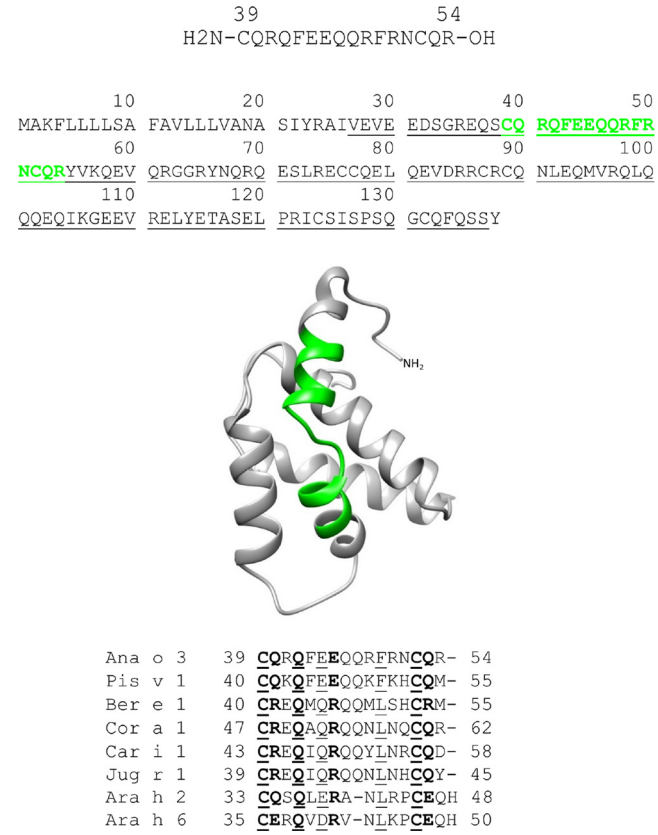

Fig. 1. A peptide corresponding to amino acids 39–54 of Ana o 3 contained within helices I and II of the small Ana o 3 protein subunit was synthesized and used to generate monoclonal antibodies. The peptide is colored green in the Ana o 3 protein sequence and ribbon model. Clustal Omega alignment of the corresponding residues from the pistachio (Pis v 1), Brazil nut (Ber e 1), hazelnut (Cor a 1), pecan (Car i 1), walnut (Jug r 1), and peanut (Ara h 2/6) 2S albumins is shown below. Identical residues are bolded and underlined, strongly similar residues are underlined, and weakly similar residues are bolded.

3.2. Recognition of native Ana o 3 by 2H5 monoclonal antibody

Antibody from 2H5 clone supernatants was purified by protein A affinity and used in direct ELISA with extracts form several nuts to compare the specificity of the antibody. Recognition of cashew nut extract was clearly the dominant signal and the difference in binding compared to the other nut extracts was significant ($p < 0.01$) (Fig. 2). Recognition of pecan nut extract proteins was 15-fold lower and peanut protein recognition was 18-fold lower. Recognition of other tree nuts such as pistachio and walnut (each 24-fold lower) and almond (36-fold lower) were minimal.

Comparison of competitive ELISA with either the peptide or the native Ana o 3 protein indicated the peptide was a much better competitor for 2H5 binding. As shown in Fig. 3, the 2H5 antibody was able to detect native Ana o 3 by direct ELISA, fitted to Eq. 1 with a K_D of $1.6 \pm 0.1 \times 10^{-3}$ mg/ml (immobilization concentration).

Competition experiments using either native Ana o 3 or the peptide in solution as competitor against immobilized Ana o 3 for antibody binding are shown in Fig. 4.

Fits to Eq. 7 allowed the determination of the half-max signals as defined in Eq. 10. The half-max signal m_{prot} using native Ana o 3 as competitor was found at $2.8 \pm 0.2 \times 10^{-2}$ mg/ml, while the half-max signal m_{pept} using the peptide as competitor was found at $1.6 \pm 0.1 \times 10^{-4}$ mg/ml. The ratio of m_{prot} to m_{pept} is 175 ± 17, implying that the affinity of the 2H5 antibody is 175-fold better for the peptide than for the native protein (on a mg/ml basis). On a molar basis, this ratio drops to 28-fold. By applying the dissociation constant found by direct ELISA and the immobilized protein concentration of 0.036 mg/ml to Eq. 10, and assuming pipetting errors of 5%, the free-solution dissociation constants may be found for native Ana o 3 and the peptide. These calculated constants are $1.2 \pm 0.1 \times 10^{-3}$ mg/ml (92 ± 8 nM) for Ana o 3, and $7.0 \pm 0.8 \times 10^{-6}$ mg/ml (3.3 ± 0.4 nM) for the peptide.

The 2H5 antibody was used to characterize Ana o 3 in protein extracted from cashew nuts that were heated for various amounts of time at 300 °F/149 °C. A direct ELISA measuring 2H5 recognition of Ana o 3 from 4 µg of cashew extract from unheated or heated cashew nuts indicated an approximately 2.5-fold greater signal from the dark roasted cashew nut extract ($p < 0.01$) (Fig. 5A).

The signals from the other heating treatments were not significantly

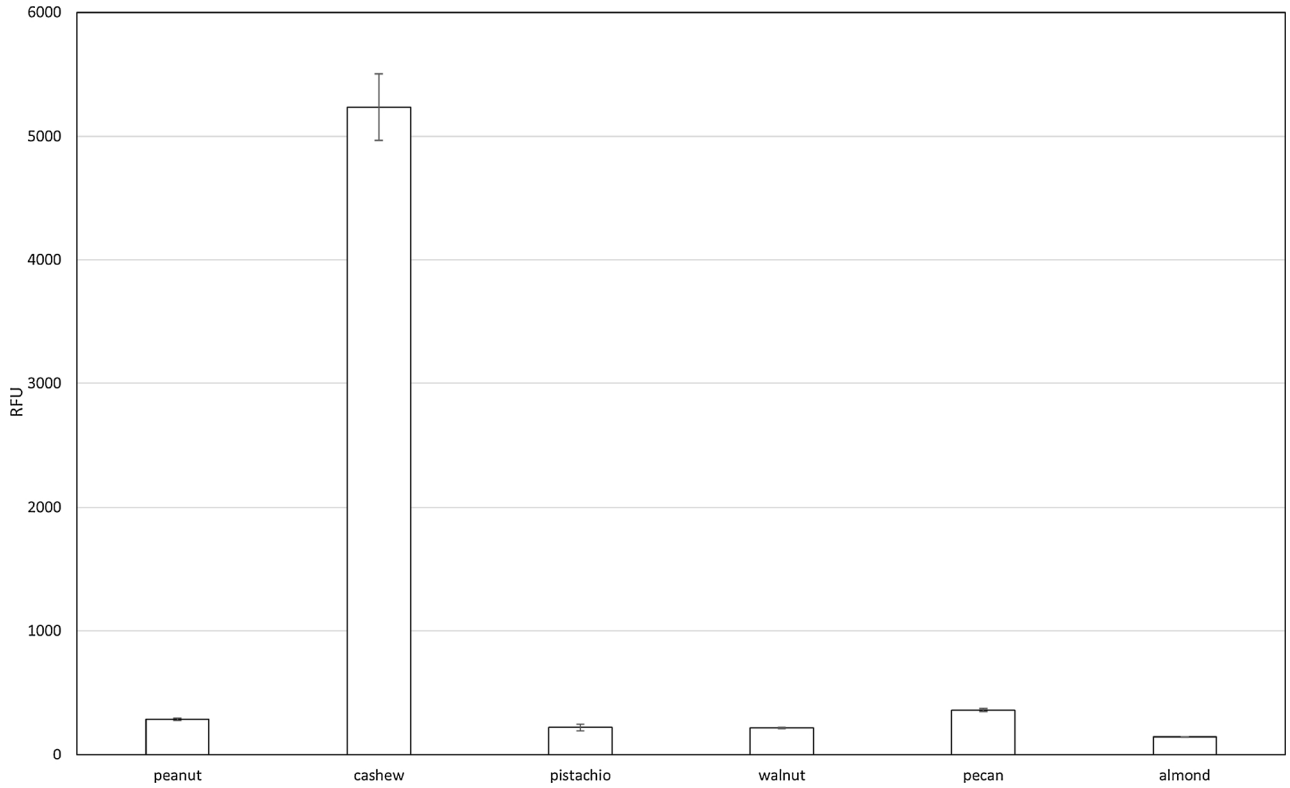

Fig. 2. Direct ELISA comparing 2H5 monoclonal antibody binding to peanut, cashew, pistachio, walnut, pecan, and almond nut extracts. Relative fluorescence units (RFU) are indicated on the y-axis.

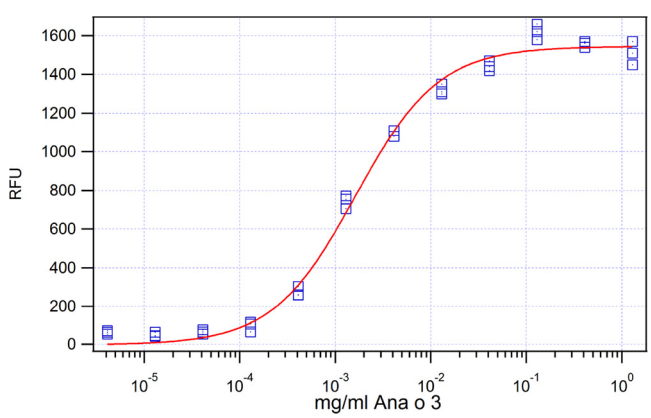

Fig. 3. Detection of native Ana o 3 by direct ELISA with 2H5 antibody. The antibody recognized Ana o 3 with an estimated KD of 1.6 ± 0.1 × 10-3 mg/ml. Ana o 3 concentration (mg/ml) is shown on the x-axis and RFU is shown on the y-axis.

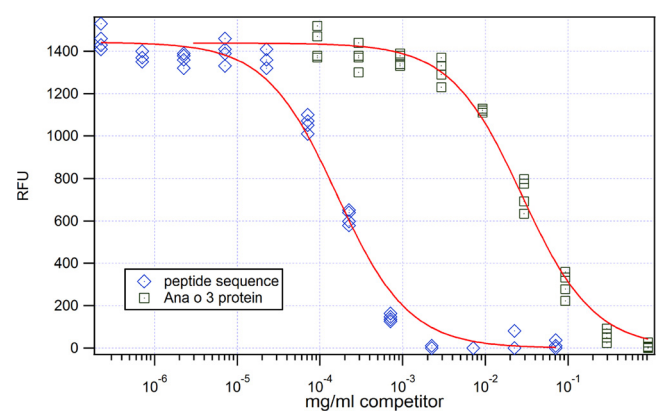

Fig. 4. Competitive ELISA comparing 2H5 binding to Ana o 3 peptide or native protein. Half maximal inhibition with the peptide was determined to be 1.6 ± 0.1 × 10-4 mg/ml while half maximal inhibition for the Ana o 3 protein was 2.8 ± 0.2 × 10-2 mg/ml. The concentration of competitor (peptide or Ana o 3, mg/ml) is shown on the x-axis and RFU are shown on the y-axis.

different ($p > 0.01$). The relatively large difference in signal observed from dark roasted cashew nut extract was likely attributable to a difference in the amount of Ana o 3 in the extract, as previous work has indicated the relative proportion of Ana o 3 increases in extensively heated cashew nut extracts [21]. To determine directly if there was a difference in recognition of Ana o 3 from raw or heated cashew nuts, Ana o 3 was purified from raw or dark roasted cashew nuts and used to coat plate wells. While binding to Ana o 3 purified from dark roasted cashew nuts was slightly reduced, there was not a statistically significant difference in 2H5 binding between Ana o 3 purified from raw versus dark roasted cashews ($p > 0.01$) (Fig. 5B).

3.3. Expression and purification of recombinant ana o 3

To characterize the 2H5 antibody further and compare recognition of native and recombinant Ana o 3, a synthetic Ana o 3 gene was synthesized and sub-cloned into a pET32 expression vector, creating an amino-terminal fusion to thioredoxin and 6 histidine repeats with a predicted molecular weight of 32 kDa. The plasmid carrying Ana o 3 was transformed into *E. coli* to characterize the recombinant fusion protein. Induction of the transformed *E. coli* strain harboring the Ana o 3 expressing plasmid with IPTG demonstrated the presence of an inducible band migrating at the expected 32 kDa size (between the 37 and 25 kDa markers) after 6 h of induction (Fig. 6A).

The protein was expressed well, but was not entirely soluble. The

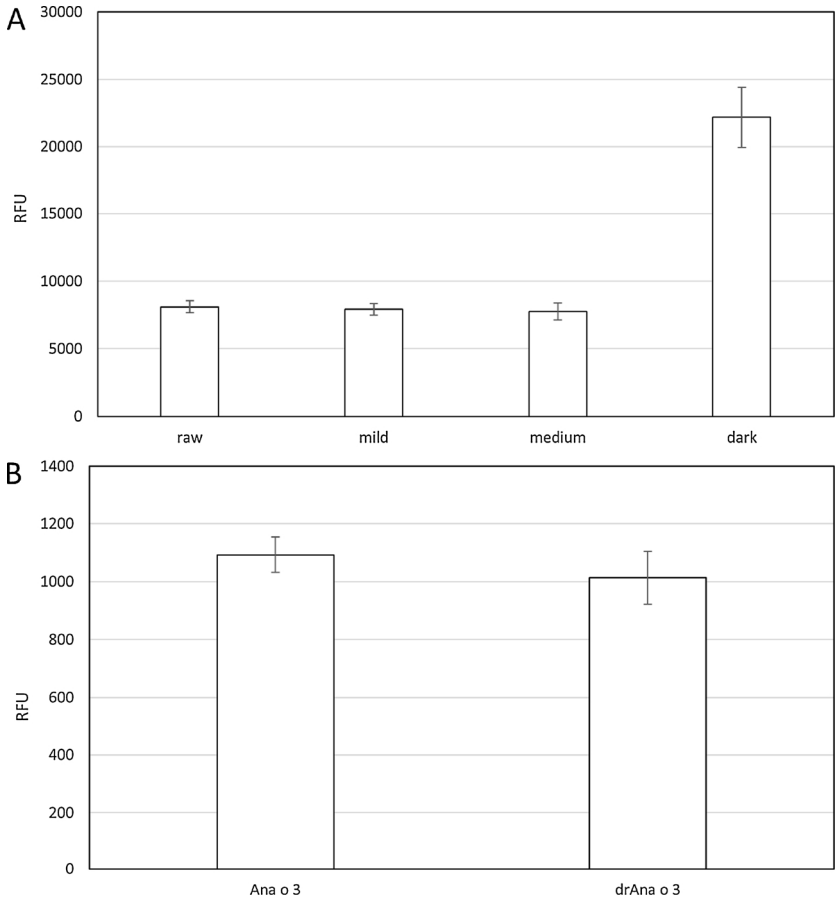

Fig. 5. Direct ELISA comparison of 2H5 binding to extracts from raw (ready to eat), mild, medium, or dark roasted cashew nuts (A). Comparison of 2H5-Ana o 3 binding using protein purified from unheated or dark roasted (drAna o 3) cashew nuts (B). RFU are indicated on the y-axis.

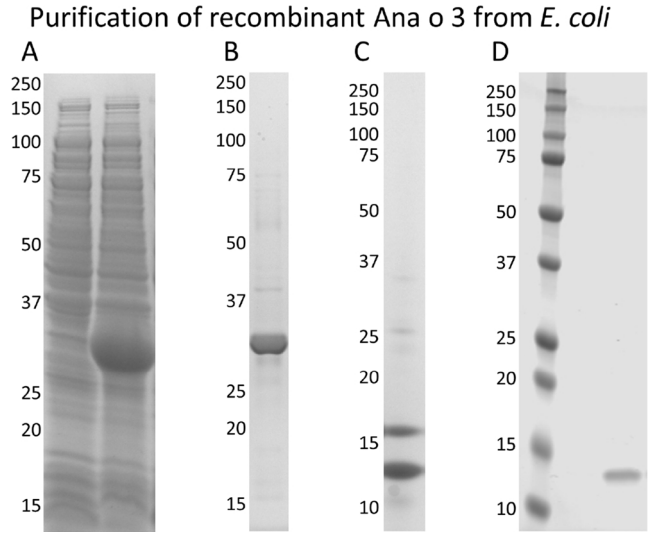

Fig. 6. Expression and purification of recombinant Ana o 3 from *E. coli*. SDS-PAGE of extracts from un-induced or induced *E. coli* expressing a thioredoxin-poly-histidine tagged recombinant Ana o 3 protein (trxA-6xHis-rAna o 3) (A). TrxA-6xHis-rAna o 3 fusion protein purified by nickel affinity gel (B). TrxA-6xHis-rAna o 3 fusion protein cleaved by enterokinase (C), and isolated rAna o 3 protein (D), with molecular weight standards on the left of each panel.

soluble portion was purified via nickel affinity gel and was digested with enterokinase while attached to a solid support affinity column resulting in 2 smaller bands migrating at 18 (thioredoxin) and 13 kDa (rAna o 3) (Fig. 6C). Mass-spectrometric analysis of the purified recombinant protein identified 5 peptides, matching those of the Ana o 3 protein and representing 34% of the protein.

3.4. Recognition of recombinant Ana o 3 by 2H5 monoclonal antibody

A direct ELISA was used to compare recognition of the native and purified recombinant Ana o 3 proteins by the 2H5 antibody. The 2H5 antibody recognized the recombinant Ana o 3 protein more robustly (nearly 4-fold greater, $p < 0.01$) than the native protein (Fig. 7A).

When the native Ana o 3 was pre-treated with a reducing agent (DTT) to alter its structure, and used to coat plate wells, there was a slight but noticeable increase in recognition of the denatured protein compared to the properly folded protein (Fig. 7A). The relatively slight discrimination between 2H5 recognition of native Ana o 3 pre-treated compared to untreated native Ana o 3 with reducing agent was significant ($p < 0.01$). In comparison, a rabbit polyclonal sera to whole cashew extract that has been previously demonstrated to recognize native Ana o 3 generated only slightly different signals for the native and recombinant Ana o 3 proteins that were not significantly different ($p > 0.01$) (Fig. 7B). However, the same rabbit anti-cashew sera recognized the DTT pre-treated native Ana o 3 poorly, over 30-fold less, compared to either the native or recombinant proteins ($p < 0.01$) (Fig. 7B).

3.5. Ana o 3 peptide and 2H5 monoclonal antibody docking

The peptide-antibody interaction was modeled to highlight residues important for the interaction between the molecules. The antibody variable fragments were modeled and docked with the peptide and they appeared to create a pocket that enveloped the peptide (Fig. 8, A–C).

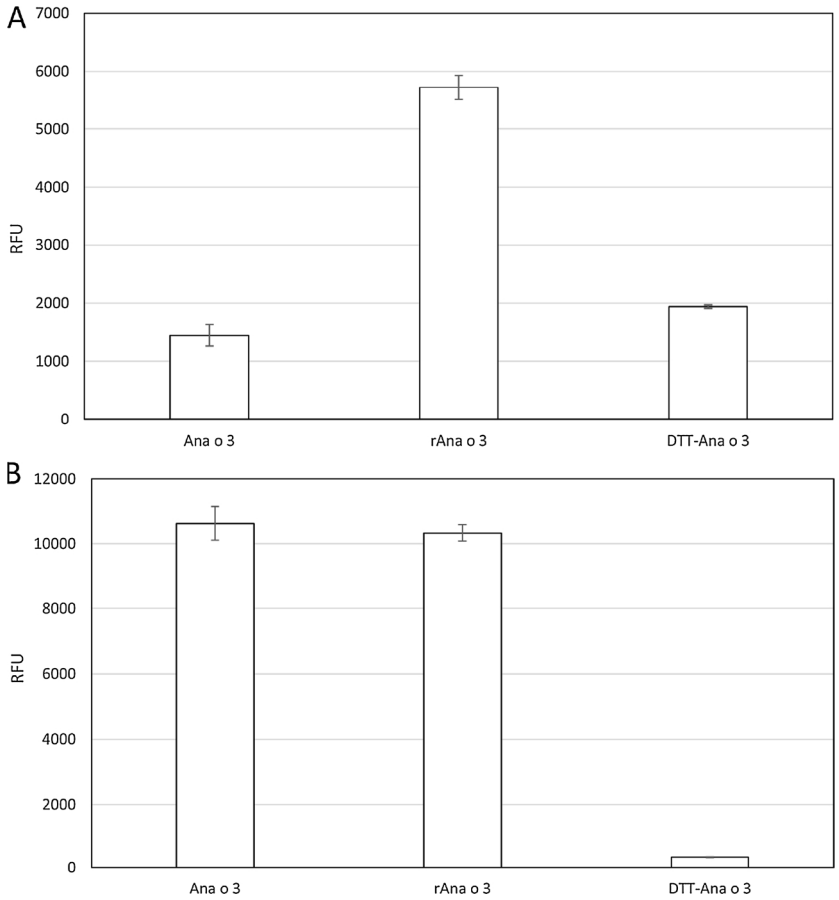

Fig. 7. Direct ELISA comparing 2H5 monoclonal (A) and rabbit polyclonal anti-cashew antibody (B) binding to native Anao3, rAna o 3, or native Ana o 3 pre-treated with 5 mM reducing agent (DTT-Ana o 3) to disrupt protein structure. RFU are shown on the y-axis.

Within this pocket there were at least 3 relatively strong interactions that were likely important for peptide binding. These include the Asp121 residue within the heavy chain complimentary determining region 3 (CDR3) interacting with peptide residue Arg-16, the heavy chain CDR2 Asp71 - peptide Arg10 interaction, and the light chain CDR2 Lys74 - peptide Arg-16 interaction (Fig. 8, D–F).

4. Discussion

The 2H5 antibody is a cashew specific monoclonal antibody targeted towards the small subunit of the Ana o 3 allergen. This antibody provides a new tool for laboratory and structural studies aimed at characterizing the Ana o 3 2S albumin cashew allergen and may be useful in the creation of improved diagnostic tests for food safety applications. The antibody recognized both native and *E. coli* produced recombinant Ana o 3 protein, although recognition of the recombinant protein appeared to be more robust. This is in contrast to the rabbit polyclonal sera towards whole cashew extract. The rabbit polyclonal sera recognizes native and recombinant Ana o 3 at a roughly equivalent level, but is very sensitive to changes in native Ana o 3 structure following treatment with a reducing agent, suggesting the dominant epitope(s) within the population is primarily conformational. Consistent with recognition of a linear epitope, the 2H5 antibody recognized denatured native Ana o 3 slightly more robustly, likely due to the loss of structure and possibly increased access to the linear epitope it was targeted towards. Further, the native protein was not recognized by the 2H5 antibody as well as the recombinant protein, suggesting that the epitope recognized by 2H5 may be partially hidden by folding in the native protein.

No statistically significant difference in 2H5 binding between Ana o 3 purified from unheated or heated cashews ($p > 0.01$) was observed, and the lowered recognition of Ana o 3 purified from heated cashew nuts was relatively minor (Fig. 5B). The very slight difference observed in the ELISA likely reflects some small inaccuracy in protein concentration determination, rather than reflecting actual differences in antibody binding. Previous work with Ana o 3 purified from heated cashew nuts has provided evidence of heating-induced modifications of the protein, and hints that there may be loss or alteration of aromatic amino acids [22]. Perhaps these or other alterations contribute to difficulties in accurately quantifying protein concentration when comparing Ana o 3 samples purified from unheated or heated cashew nuts.

The small chain of 2S albumin proteins is thought to be composed of 2 helices connected by a short flexible linker. Structural and modeling studies have indicated that the helices within the 2S albumin allergens generally adopt the same formation, but that the length and spacing of the helices may differ among 2S albumin allergens [23,40]. This region of 2S albumin proteins has proven to be important for recognition by IgE. For example, the first and second helices of the Ara h 2 peanut allergen contain important IgE epitopes that when fused to an amino-terminal fusion protein or mutated by alanine scanning can reduce IgE binding [40]. The small chain of Ana o 3 was recently found to migrate at an observed mass of 6 kDa and contains sequence heterogeneity at both the amino and carboxy termini [20], but it is not clear if this is a result of natural post-translational processing, differences in sample storage, or other factors.

Based upon NMR spectroscopy studies of Ara h 6 it is thought that helices 2, 4, and 5 are involved in forming the hydrophobic core of the protein [41]; therefore, some portion of the 2H5 epitope within helix 2 may be hidden in the native Ana o 3 conformation. This observation also suggests that the recombinant protein is not entirely folded

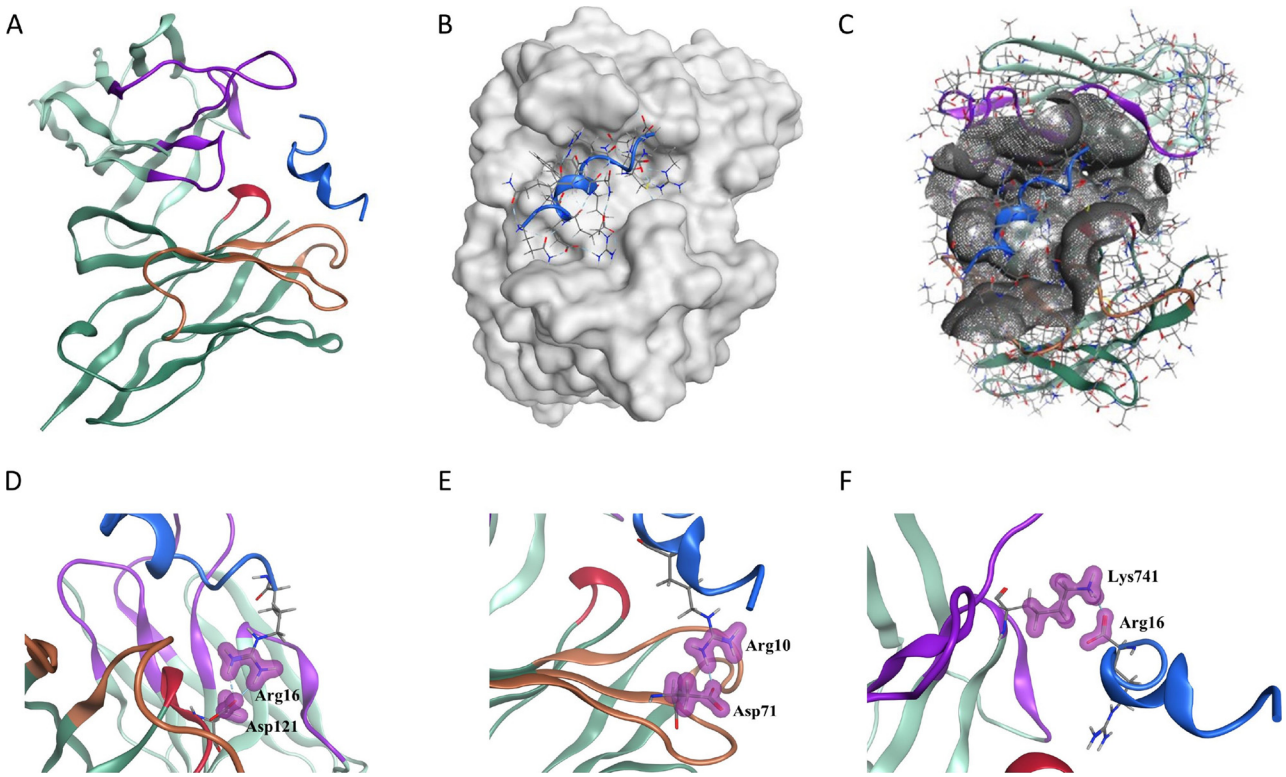

Fig. 8. In-silico modeling of Ana o 3 peptide-antibody interaction. Ribbon diagram of 2H5 antibody variable domain and Ana o 3 peptide docking site (A), surface model (B), and highlighted interaction pocket (C). Predicted strong interactions between antibody and peptide based upon epitope analysis include heavy chain CDR3 Asp121 - peptide Arg-16 (D), heavy chain CDR2 Asp71 - peptide Arg10 (E), and light chain CDR2 Lys74 – peptide Arg-16 (F) are highlighted in purple. The Ana o 3 peptide is dark blue, and the 2H5 antibody light chain variable fragment CDR1-3 in purple, heavy chain CDR1 and 2 in brown, and heavy chain CDR3 in red.

correctly, and that the peptide sequence contained within helices I and II may be more accessible than in the native protein. The small subunit in the native protein likely adopts a conformation that partially hides the linear epitope recognized by the 2H5 antibody, and this could explain the reduced recognition of the native protein compared to the recombinant. This is not surprising, given that in some cases other 2S albumin allergens that have been recombinantly produced in *E. coli* have not been accurately proteolytically processed, may not contain properly formed cysteine disulfide bonds, and lack proper folding.

The 2H5 antibody recognized the Ana o 3 protein in cashew extracts and although the sequence of the peptide antigen was very similar to sequences found within other peanut or tree nut 2S albumins, including Pis v 1 from pistachio nuts [42], it did not observably cross-react in experiments with other nut extracts. This antibody was targeted towards a linear peptide epitope that is detectable at some level in the native properly folded Ana o 3 protein, although it is likely not entirely accessible due to the conformation of the protein. For some targets, conformational epitope studies may be more useful. For example, a conformational epitope has been described for the Ana o 2 legumin allergen that is recognized by human IgE Ana o 2 antibodies [43,44], although it is readily destroyed by treating the protein with denaturants. Using phage display libraries to identify IgE epitopes of peanut 2S albumins [45] maybe advantageous as 2S albumins are resistant to digestive enzymes [23,31] and may remain intact in the gut; potentially providing more opportunity for clonal selection of conformational epitopes.

Allergen content within a given nut or seed may vary season-to-season. For example, genomic analysis in rice has been used to predict possible rice allergens and 2-D gel analysis can be used to evaluate allergen content in genetically modified soy beans [46,47]. The 2H5 antibody represents a new tool in the detection and characterization of Ana o 3, and could be used for similar studies of allergen content variability for a given source of cashew nuts. The 2H5 antibody recognized denatured Ana o 3 that was pretreated with a reducing agent, and may have an advantage for applications testing samples that require denaturing processing steps. The results generated with the 2H5 antibody thus far provide a small window into the complex interplay of linear and discontinuous molecular interactions between antibodies and allergenic 2S albumins. Linear IgE epitope mapping studies can be very useful, but usually tell only part of the story. IgE epitopes are often discontinuous and improved characterization of those types of interactions is essential to understanding IgE-allergen interactions. The Ana o 3-2H5 antibody interaction could serve as a proxy for IgE-allergen interactions. In-silico modeling of the 2H5 and the Ana o 3 peptide immunogen highlighted potentially important interaction residues thought to contribute to the binding interaction. Thoughtful engineering of the 2H5 antibody variable domain may allow improved antibodies directed towards Ana o 3, or other tree nut allergens, and may provide detection reagents with superior specificity and avidity. Continued study of the 2H5 antibody and similar antibodies may help create new tree-nut allergen specific reagents that could be used in food origin identification, allergen component analysis, toxicity, and other food safety related applications.

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Declaration of competing interest

The authors declare no competing financial interest.

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References

[1] R.S. Gupta, E.E. Springston, M.R. Warrier, B. Smith, R. Kumar, J. Pongracic, J.L. Holl, The prevalence, severity, and distribution of childhood food allergy in the United States, Pediatrics 128 (1) (2011) e9–17.
[2] R. Gupta, D. Holdford, L. Bilaver, A. Dyer, J.L. Holl, D. Meltzer, The economic impact of childhood food allergy in the United States, JAMA Pediatr. 167 (11) (2013) 1026–1031.
[3] J.E. Yu, A. Mallapaty, R.L. Miller, It's not just the food you eat: environmental factors in the development of food allergies, Environ. Res. 165 (2018) 118–124.
[4] M.C. Berin, H.A. Sampson, Food allergy: an enigmatic epidemic, Trends Immunol. 34 (8) (2013) 390–397.
[5] K.W. Lien, D.P.H. Hsieh, H.Y. Huang, C.H. Wu, S.P. Ni, M.P. Ling, Food safety risk assessment for estimating dietary intake of sulfites in the Taiwanese population, Toxicol. Rep. 3 (2016) 544–551.
[6] I.E. Sweis, B.C. Cressey, Potential role of the common food additive manufactured citric acid in eliciting significant inflammatory reactions contributing to serious disease states: a series of four case reports, Toxicol. Rep. 5 (2018) 808–812.
[7] C. Alasalvar, B.W. Bolling, Review of nut phytochemicals, fat-soluble bioactives, antioxidant components and health effects, Br. J. Nutr. 113 (Suppl 2) (2015) S68–78.
[8] T. Weinberger, S. Sicherer, Current perspectives on tree nut allergy: a review, J. Asthma Allergy 11 (2018) 41–51.
[9] D.M. Fleischer, The natural history of peanut and tree nut allergy, Curr. Allergy Asthma Rep. 7 (3) (2007) 175–181.
[10] J.M. Smeekens, K. Bagley, M. Kulis, Tree nut allergies: allergen homology, cross-reactivity, and implications for therapy, Clin. Exp. Allergy (2018).
[11] T. Yi, J. Wen, A. Golan-Goldhirsh, D.E. Parfitt, Phylogenetics and reticulate evolution in Pistacia (Anacardiaceae), Am. J. Bot. 95 (2) (2008) 241–251.
[12] F. Wang, J.M. Robotham, S.S. Teuber, P. Tawde, S.K. Sathe, K.H. Roux, Ana o 1, a cashew (Anacardium occidental) allergen of the vicilin seed storage protein family, J. Allergy Clin. Immunol. 110 (1) (2002) 160–166.
[13] F. Wang, J.M. Robotham, S.S. Teuber, S.K. Sathe, K.H. Roux, Ana o 2, a major cashew (Anacardium occidentale L.) nut allergen of the legumin family, Int. Arch. Allergy Immunol. 132 (1) (2003) 27–39.
[14] J.M. Robotham, F. Wang, V. Seamon, S.S. Teuber, S.K. Sathe, H.A. Sampson, K. Beyer, M. Seavy, K.H. Roux, Ana o 3, an important cashew nut (Anacardium occidentale L.) allergen of the 2S albumin family, J. Allergy Clin. Immunol. 115 (6) (2005) 1284–1290.
[15] A.T. Clark, K. Anagnostou, P.W. Ewan, Cashew nut causes more severe reactions than peanut: case-matched comparison in 141 children, Allergy 62 (8) (2007) 913–916.
[16] M. Davoren, J. Peake, Cashew nut allergy is associated with a high risk of anaphylaxis, Arch. Dis. Child. 90 (10) (2005) 1084–1085.
[17] A. Grigg, C. Hanson, C.M. Davis, Cashew allergy compared to peanut allergy in a US tertiary care center, Pedriatic Asthma Allergy and Immunology 22 (2009) 101–104.
[18] A. Divaret-Chauveau, I. Clerc-Urmes, J.M. Renaudin, V. Cordebar, P. Dumond, C. Schweitzer, Threshold dose distribution for cashew nut allergy in children, Clin. Exp. Allergy (2018).
[19] J.P. van der Valk, R. Gerth van Wijk, J.L. Baumert, J.A. Nordlee, B.J. Vlieg-Boerstra, H. de Groot, A.E. Dubois, N.W. de Jong, Threshold dose distribution and eliciting dose of cashew nut allergy, Ann. Allergy Asthma Immunol. (2016).
[20] M. Reitsma, S. Bastiaan-Net, L. Sijbrandij, E. de Weert, S. Sforza, R. Gerth van Wijk, H.F.J. Savelkoul, N.W. de Jong, H.J. Wichers, Origin and processing methods slightly affect allergenic characteristics of cashew nuts (Anacardium occidentale), J. Food Sci. 83 (4) (2018) 1153–1164.
[21] C.P. Mattison, Y. Bren-Mattison, B. Vant-Hull, A.M. Vargas, R.L. Wasserman, C.C. Grimm, Heat-induced alterations in cashew allergen solubility and IgE binding, Toxicol. Rep. 3 (2016) 244–251.
[22] C.P. Mattison, C.C. Grimm, Y. Li, H.J. Chial, D.R. McCaslin, S.Y. Chung, Y. Bren-Mattison, R.L. Wasserman, Identification and characterization of Ana o 3 modifications on Arginine-111 residue in heated cashew nuts, J. Agric. Food Chem. 65 (2) (2017) 411–420.
[23] F.J. Moreno, A. Clemente, 2S Albumin Storage Proteins: What Makes them Food Allergens? Open Biochem. J. 2 (2008) 16–28.
[24] A. Ullah, R.B. Mariutti, R. Masood, I.P. Caruso, G.H. Costa, C. Millena de Freita, C.R. Santos, L.M. Zanphorlin, M.J. Rossini Mutton, M.T. Murakami, R.K. Arni, Crystal structure of mature 2S albumin from Moringa oleifera seeds, Biochem. Biophys. Res. Commun. 468 (1-2) (2015) 365–371.
[25] X. Chen, Q. Wang, R. El-Mezayen, Y. Zhuang, S.C. Dreskin, Ara h 2 and Ara h 6 have similar allergenic activity and are substantially redundant, Int. Arch. Allergy Immunol. 160 (3) (2012) 251–258.
[26] M. Kulis, X. Chen, J. Lew, Q. Wang, O.P. Patel, Y. Zhuang, K.S. Murray, M.W. Duncan, H.S. Porterfield, A, W. B, S.C. Dreskin, 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, Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology 42 (2) (2012) 326–336.
[27] M. Reitsma, S. Bastiaan-Net, S. Sforza, J.P. van der Valk, R. van Gerth van Wijk, H.F. Savelkoul, N.W. de Jong, H.J. Wichers, Purification and Characterization of Anacardium occidentale (Cashew) Allergens Ana o 1, Ana o 2, and Ana o 3, J. Agric. Food Chem. 64 (5) (2016) 1191–1201.
[28] M. Venkatachalam, E.K. Monaghan, H.H. Kshirsagar, J.M. Robotham, S.E. O'Donnell, M.S. Gerber, K.H. Roux, S.K. Sathe, Effects of processing on immunoreactivity of cashew nut (Anacardium occidentale L.) seed flour proteins, J. Agric. Food Chem. 56 (19) (2008) 8998–9005.
[29] C. Cuadrado, H. Cheng, A. Sanchiz, I. Ballesteros, M. Easson, C.C. Grimm, M.C. Dieguez, R. Linacero, C. Burbano, S.J. Maleki, Influence of enzymatic hydrolysis on the allergenic reactivity of processed cashew and pistachio, Food Chem. 241 (2018) 372–379.
[30] A. Sanchiz, C. Cuadrado, M.C. Dieguez, I. Ballesteros, J. Rodriguez, J.F. Crespo, N.L. Cuevas, J. Rueda, R. Linacero, B. Cabanillas, N. Novak, Thermal processing effects on the IgE-reactivity of cashew and pistachio, Food Chem. 245 (2018) 595–602.
[31] C.P. Mattison, C.C. Grimm, R.L. Wasserman, In vitro digestion of soluble cashew proteins and characterization of surviving IgE-reactive peptides, Mol. Nutr. Food Res. 58 (4) (2014) 884–893.
[32] F.E. Gaskin, S.L. Taylor, Sandwich enzyme-linked immunosorbent assay (ELISA) for detection of cashew nut in foods, J. Food Sci. 76 (9) (2011) T218–26.
[33] L.S. New, A. Schreiber, J. Stahl-Zeng, H.F. Liu, Simultaneous analysis of multiple allergens in food products by LC-MS/MS, J. AOAC Int. 101 (1) (2018) 132–145.
[34] A. Garcia, R. Madrid, T. Garcia, R. Martin, I. Gonzalez, Detection of food allergens by Taqman real-time PCR methodology, Methods Mol. Biol. 1592 (2017) 95–108.
[35] C.P. Mattison, W.A. Desormeaux, R.L. Wasserman, M. Yoshioka-Tarver, B. Condon, C.C. Grimm, Decreased immunoglobulin E (IgE) binding to cashew allergens following sodium sulfite treatment and heating, J. Agric. Food Chem. 62 (28) (2014) 6746–6755.
[36] I. Langmuir, The adsorption of gases on plane surfaces of glass, Mica And platinum, J. Am. Chem. Soc. 40 (9) (1918) 1361–1403.
[37] P.W. Stevens, D.M. Kelso, Estimation of the protein-binding capacity of microplate wells using sequential ELISAs, J. Immunol. Methods 178 (1) (1995) 59–70.
[38] A.V. Hill, The possible effects of the aggregation of the molecules of hæmoglobin on its dissociation curves, J. Physiol. (Lond.) 40 (1910) 4–7.
[39] J.N. Weiss, The Hill equation revisited: uses and misuses, FASEB J. 11 (11) (1997) 835–841.
[40] G.A. Mueller, R.A. Gosavi, A. Pomes, S. Wunschmann, A.F. Moon, R.E. London, L.C. Pedersen, Ara h 2: crystal structure and IgE binding distinguish two subpopulations of peanut allergic patients by epitope diversity, Allergy 66 (7) (2011) 878–885.
[41] K. Lehmann, K. Schweimer, G. Reese, S. Randow, M. Suhr, W.M. Becker, S. Vieths, P. Rosch, Structure and stability of 2S albumin-type peanut allergens: implications for the severity of peanut allergic reactions, Biochem. J. 395 (3) (2006) 463–472.
[42] K. Ahn, L. Bardina, G. Grishina, K. Beyer, H.A. Sampson, Identification of two pistachio allergens, Pis v 1 and Pis v 2, belonging to the 2S albumin and 11S globulin family, Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology 39 (6) (2009) 926–934.
[43] J.M. Robotham, L. Xia, L.N. Willison, S.S. Teuber, S.K. Sathe, K.H. Roux, Characterization of a cashew allergen, 11S globulin (Ana o 2), conformational epitope, Mol. Immunol. 47 (9) (2010) 1830–1838.
[44] L. Xia, L.N. Willison, L. Porter, J.M. Robotham, S.S. Teuber, S.K. Sathe, K.H. Roux, 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. 47 (9) (2010) 1808–1816.
[45] X. Chen, S.S. Negi, S. Liao, V. Gao, W. Braun, S.C. Dreskin, Conformational IgE epitopes of peanut allergens Ara h 2 and Ara h 6, Clin. Exp. Allergy 46 (8) (2016) 1120–1128.
[46] C. Pallen, C. Friry-Santini, C. Herouet-Guicheney, A. Capt, Technical variability of 2D gel electrophoresis - Application to soybean allergens, Toxicol. Rep. 1 (2014) 734–742.
[47] F.C. Zhu, R.Z. Jia, L. Xu, H. Kong, Y.L. Guo, Q.X. Huang, Y.J. Zhu, A.P. Guo, Genome-wide analysis of potential cross-reactive endogenous allergens in rice (Oryza sativa L.), Toxicol. Rep. 2 (2015) 1233–1245.