

Effect of deglycosylation on immunoreactivity and *in vitro* pepsin digestibility of major cashew (*Anacardium occidentale* L.) allergen, Ana o 1

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Abstract: Major cashew allergen, Ana o 1, was purified in its native form from cashew seeds and subjected to enzymatic deglycosylation using PNGase F to assess the potential role of N-glycans in immunoreactivity. Western and dot blotting with pooled human plasma containing anticashew IgE revealed that deglycosylation increased IgE-binding of Ana o 1. Removal of N-glycans may have exposed previously masked Ana o 1 epitopes. Purified glycosylated and deglycosylated Ana o 1 were also subjected to *in vitro* pepsin digestion at pH 3.0 for 2 hr. Both glycosylated and deglycosylated Ana o 1 remained stable and reactive with IgE antibodies following digestion.

Keywords: cashew, glycosylation, *in vitro* digestion, tree nut allergy, 7S vicilin

Practical Application: Understanding the role of glycosylation in Ana o 1 immunoreactivity may provide insight into the potential development of hypoallergenic cashews/cashew products for sensitive individuals in the future.

1. INTRODUCTION

Globally, cashews are the number one produced tree nut (5,932,597 MT in 2018) and the United States is the top importer (153,448 MT in 2017, worth approximately \$1.58 billion) (FAOSTAT, 2020). Per capita consumption of cashew nuts was 106 g/year globally and 445 g/year in the United States (INC, 2017). The high global demand for cashews may be attributed to their desirable sensory properties and nutrient density; cashews are an energy-rich food composed of approximately 47.77% lipid, 16.84% protein, and 30.16% carbohydrate (USDA, 2020). Despite their economic importance and popularity as a food, cashews are known to elicit IgE-mediated food allergy in sensitive individuals (Mendes et al., 2019).

The country with the most reported cashew allergies is the United States (McWilliam et al., 2015), although prevalence data are limited to just Sweden, the Netherlands, France, the United States, and Australia (Mendes et al., 2019). The current estimated prevalence rate of cashew allergy among all U.S. adults is approximately 0.5%; among adults with a convincing cashew allergy, about 57% report physician-diagnosed cashew allergy (Gupta et al., 2019). Currently, three cashew proteins have been identified and are included in the World Health Organization/International Union of Immunological Societies (WHO/IUIS) official list of allergens (Mendes et al., 2019; WHO/IUIS Allergen Nomenclature Sub-Committee, 2020). Two of the identified allergens, Ana o 1 and Ana o 2, are members of the cupin superfamily and the third, Ana o 3, belongs to the prolamin superfamily of proteins.

Ana o 1 is a 7S vicilin-type trimeric protein with two known isoforms, Ana o 1.0101 (538 amino acids) and Ana o 1.0102 (536 amino acids) that differ only by the first two N-terminal amino acid residues, which are present in Ana o 1.0101 but not in Ana o 1.0102 (WHO/IUIS Allergen Nomenclature Sub-Committee, 2020). Recombinant Ana o 1 (rAna o 1) has been expressed in *Escherichia coli* and probed with sera from cashew-allergic patients (Wang et al., 2002). Immunoblotting of rAna o 1 revealed that 50% of the tested sera (10 out of 20) were reactive, suggesting that Ana o 1 is a major cashew allergen. By using rAna o 1 as an inhibitor in Western blotting of cashew total soluble protein extract, it was determined that the native protein precursor corresponded to a band of approximately 50 kDa. Reitsma et al. (2016) presented the only proposed method for purification of native Ana o 1 from cashew seeds. This report was also the first to confirm predicted glycosylation of Ana o 1, a characteristic typical of vicilin-type proteins. Although the site(s) of glycosylation on Ana o 1 have not been determined, one Asn residue falls within the typical consensus sequence (Asn-X-Thr/Ser, where X is any nonproline amino acid) targeted for N-glycosylation (Imperiali, Shannon, Unno, & Rickert, 1992). This residue is within a mapped human IgE-targeted epitope (Guan et al., 2015; Wang et al., 2002); however, the role of glycosylation on Ana o 1 immunoreactivity has yet to be explored.

For the first time, purified native Ana o 1 from cashew seeds was subjected to enzymatic deglycosylation to assess the potential role of attached glycans on allergenic properties. Immunoreactivity of deglycosylated Ana o 1 was compared to its glycosylated counterpart using pooled human plasma containing anticashew IgE. Further, immunoreactivity of purified Ana o 1 after exposure to *in vitro* pepsin digestion was assessed using both the glycosylated and deglycosylated states. Currently, there is no cure for food allergy. Understanding molecular properties of food allergens, such as glycosylation, that contribute to immunorecognition may be used to improve consumer safety.

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Deceased on April 4, 2019.

Table 1—Clinical history of plasma donors.

Donor #	Cashew IgE (kU/L) ^a	Other allergens (positive ImmunoCAP result)
26741-EW	36.667	Peanut, hazelnut, brazil nut, almond, pecan, pistachio, walnut, eggplant, macadamia nut, banana, avocado, <i>Dermatophagoides pteronyssinus</i> , cat dander, birch, CCD MUXF3 from Bromelain
32378-TB	52.8	Codfish, walnut, pistachio, almond, hazelnut, watermelon, celery
32402-JS	77.8	Peanut, soybean, hazelnut, walnut, honeybee venom, cat dander, dog dander, birch
33454-PS	39.8	Egg white, milk, wheat, sesame seed, peanut, soybean, peanut, <i>Alternaria tenuis</i>
20197-BH	81.3	Peanut, pistachio, hazelnut, egg white, lobster, pecan, crab, <i>Cladosporium herbarum</i>

^aMeasured by ImmunoCAP by PlasmaLab International.

2. MATERIALS AND METHODS

2.1 Materials and reagents

Raw cashews were from a local grocery store in Tallahassee, FL, USA. Plasma samples containing anticashew IgE from five individual donors (Table 1) were purchased from PlasmaLab International (Everett, WA, USA) and pooled. Amicon Ultra-15 centrifugal filter units (30 kDa cutoff), porcine pepsin from gastric mucosa, and peroxidase-labeled goat anti-human IgE antibody were from MilliporeSigma (St Louis, MO, USA). A HiPrep 16/60 Sephacryl S-200 HR column was purchased from GE Healthcare Life Sciences (Marlborough, MA, USA). Spectra/Por 1 (6 to 8 kDa cutoff) dialysis tubing was purchased from Spectrum Chemical (New Brunswick, NJ, USA). Criterion TGX Precast Midi 8% to 16% Protein Gels (12 + 2 well, 45 µL), Clarity Western Enhanced Chemiluminescent (ECL) Substrate, and Protein Assay Dye Reagent Concentrate were purchased from Bio-Rad (Hercules, CA, USA). Fisher BioReagents EZ-Run Prestained Rec Protein Ladder and Pierce Glycoprotein Staining Kit were ordered from ThermoFisher Scientific (Waltham, MA, USA). Rapid PN-Gase F was purchased from New England Biolabs (Ipswich, MA, USA). FluoroTrans PVDF transfer membranes were from Pall Life Sciences (0.2 µm pore size) (Port Washington, NY, USA). Ponceau S Staining Solution was from Tocris Bioscience (Bristol, UK). All other chemicals were purchased from MilliporeSigma unless otherwise stated.

2.2 Preparation of cashew flour

Full fat cashew seeds were ground in an Osterizer blender (speed setting “grind”; Galaxy model 869-18R, Jaden Consumer Solutions, Boca Raton, FL, USA) and passed through a 20-mesh sieve. The full fat flour was then defatted for 8 hr using a Soxhlet apparatus with petroleum ether (boiling point range of 38.2 to 54.3 °C) as the solvent (flour-to-solvent ratio of 1:10 w/v). After overnight drying (until constant weight) in a fume hood at room temperature (23 °C), the defatted flour was passed through a 40-mesh sieve and stored at −20 °C until needed.

2.3 Ammonium sulfate precipitation and ultrafiltration

Crude purification of cashew Ana o 1 was followed according to the method of Reitsma et al. (2016) with modifications. Briefly, 10 g of defatted cashew flour was extracted (1:5 w/v) in ammonium bicarbonate buffer (0.1 M ammonium bicarbonate, 0.5 M NaCl, pH 7.9) overnight at 4 °C with constant stirring. The slurry was then centrifuged at 4,000 g for 90 min using an Eppendorf (Enfield, CT, USA) model 5804R centrifuge and the supernatant was gravity filtered through Whatman filter paper no. 4 (20 to 25 µm pore size) with a thin layer of glass wool.

The filtered supernatant was then subjected to ammonium sulfate precipitation. To the supernatant, 15% w/v ammonium sul-

fate was slowly added, and the mixture was left to stir for 60 min at 4 °C followed by centrifugation at 4,000 g for 60 min at 4 °C. The supernatant was collected, and the ammonium sulfate concentration was increased to 30% w/v followed by 60 min magnetic stirring and 60 min centrifugation under the same conditions. The process was repeated for an increased concentration to 45% and then finally 52.5% w/v ammonium sulfate. The final supernatant was then diluted four times in ultrapure deionized (DI) water (NANOpure DiamondTM, Barnstead International, Dubuque, IA, USA).

The four-times diluted supernatant (herein referred to as “supernatant A”) was then subjected to a series of ultrafiltration steps. Four milliliters of supernatant A was placed in an Amicon Ultra-15 Centrifugal Filter Unit with a 30 kDa molecular weight cutoff and spun for 5 min at 4,000 g at 20 °C. Another 4 mL of supernatant A was added to the retentate and centrifuged under the same conditions. This step was repeated three more times for a total of four times. Five milliliters of DI water was then added to the retentate and the solution was centrifuged under the same conditions. This step was repeated once. Another 4 mL of supernatant A was added to the retentate followed by centrifugation under the same conditions. This was again repeated three more times for a total of four times. Five milliliters of DI water was added to the retentate in the same centrifugal filter unit followed by shaking and then centrifugation for 5 min. The final washing step with DI water was repeated for a total of 10 times and the retentate was further purified using size exclusion chromatography.

2.4 Size exclusion chromatography

Size exclusion chromatography was carried out using an AKTA Pure 25 protein purification system (GE Health Sciences) with a HiPrep 16/60 Sephacryl S-200 HR prepacked column (1.6 × 60 cm, 120 mL column volume, and 0.15 MPa pressure limit). All purification steps took place in a cold room at 4 °C and all buffers were filtered and degassed prior to introduction on the column. The column was equilibrated with 60 mL of DI water at 0.5 mL/min followed by 240 mL of equilibration buffer (50 mM sodium phosphate, 0.15 M NaCl, and pH 7.2) at 1 mL/min. The sample was injected at 0.5 mL/min and proteins were eluted with 180 mL of equilibration buffer at 0.5 mL/min. Elution was monitored by UV absorbance at 214 and 280 nm and gel electrophoresis (described below). Fractions containing Ana o 1 were pooled and dialyzed (6 to 8 kDa MWCO) against DI water for 48 hr with six changes of water at 4 °C. The dialysate was dried in a Savant SpeedVac Concentrator (Thermo Scientific) and stored in an air-tight container at −20 °C.

2.5 Protein solubility

Soluble protein concentration was estimated using the Bradford protein assay (Bradford, 1976). Absorbance at 595 nm was

measured using an Epoch 2 microplate spectrophotometer (Biotek, Winooski, VT, USA). Standard curves for each assay were prepared using bovine serum albumin fraction V in appropriate sample buffer.

2.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using Criterion TGX Precast Midi 8% to 16% Protein Gels. Protein samples were mixed (1:1 v/v) with Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS w/v, 25% glycerol v/v, and 0.01% bromophenol blue v/v). For reducing conditions, 5% v/v β -mercaptoethanol was added before use. Samples were then heated for 10 min using a dry block heater set to 100 °C, cooled to room temperature, and then loaded onto the gel. An EZ-Run Prestained *Rec* Protein Ladder was included in the reference lane for each run. Gels were run (running buffer: 25 mM Tris, 192 mM glycine, 0.1% w/v SDS, pH 8.3) using a Bio-Rad Criterion Vertical Electrophoresis Cell at constant voltage (200 V) until the tracking dye reached the gel edge.

2.7 Coomassie brilliant blue R staining

Gels were immersed in staining solution (0.25% w/v coomassie brilliant blue R (CBBR), 50% v/v methanol, and 10% v/v acetic acid in DI water) overnight at room temperature on a rocker. Gels were then transferred to destaining solution (50% v/v methanol and 10% v/v acetic acid in DI water) and gently mixed on a rocker until background was removed. Gels were imaged using a ChemiDoc Touch Gel Imaging System (Bio-Rad) with epi-white illumination. Image Lab Software (Bio-Rad version 6.1) was used for estimations of molecular mass.

2.8 Protein identification

Ana o 1 was identified by LC-MS/MS in the Translational Science Laboratory at Florida State University (Tallahassee, FL, USA). Twenty micrograms of lyophilized protein was resuspended in 20 μ L of 50 mM ammonium bicarbonate. TCEP (tris(2-carboxyethyl)phosphine, 5mM final concentration) was added for reduction and the mixture was incubated at 55 °C for 20 min. Reduced protein was alkylated by the addition of 10 mM IAA (iodoacetamide) and incubated in the dark at room temperature for 20 min. One microgram of trypsin was added to the mix and incubated at 37 °C for 3 hr. Digested peptides were added to a C18 spin column and eluate from the column was dried using a freeze dryer and resuspended in 0.1% formic acid at 1 μ g/ μ L and loaded onto the LC-MS/MS system.

An externally calibrated Thermo Q Exactive HF (high-resolution electrospray tandem mass spectrometer) was used in conjunction with Dionex UltiMate 3000 RSLCnano System. A 2- μ L sample was aspirated into a 50- μ L loop and loaded onto the trap column (Thermo μ -Precolumn 5 mm, with nanoViper tubing 30- μ m i.d. $\times$ 10 cm). The flow rate was set to 300 nL/min for separation on the analytical column (Acclaim PepMap RSLC 75 μ m $\times$ 15 cm nanoviper). Mobile phase A was composed of 99.9% H₂O (EMD Omni Solvent) and 0.1% formic acid, and mobile phase B was composed of 99.9% ACN and 0.1% formic acid. A 90-min linear gradient from 3% to 45% B was performed. The LC eluate was directly sprayed into the Q Exactive HF mass spectrometer (Thermo Scientific). During the chromatographic separation, the Q Exactive HF was operated in a data-dependent mode and under direct control of the Thermo Excalibur 3.1.66 (Thermo

Scientific) software. The MS data were acquired using the following parameters: 20 data-dependent collision-induced dissociation MS/MS scans per full scan (350 to 1,700 m/z) at 60,000 resolution. MS2 were acquired in centroid mode at 15,000 resolution. Ions with single charge, or charges more than 7 as well as unassigned charge were excluded. A 15-s dynamic exclusion window was used. All measurements were performed at room temperature. Resultant raw files were searched with Proteome Discoverer 2.4 using SequestHT and Mascot as the search engine using Anacardium-specific FASTA database (downloaded from Uniprot).

2.9 Enzymatic deglycosylation

Purified Ana o 1 was deglycosylated using Rapid PNGase F according to the manufacturer's two-step protocol. Briefly, 16 μ L of DI water and 4 μ L of 5X concentrated Rapid PNGase F Buffer were added to 100 μ g of dried Ana o 1 followed by incubation at 80 °C for 2 min. The protein solution was cooled to room temperature followed by the addition of 1 μ L Rapid PNGase F and subsequent incubation at 50 °C for 10 min. Extent of deglycosylation was monitored by glycoprotein staining and mobility shifts on SDS-PAGE gels with CBBR staining. Control experiments were performed where the Ana o 1 protein sample was exposed to the same conditions with 1 μ L of water added in place of Rapid PNGase F.

2.10 Glycoprotein staining

Glycoprotein staining was conducted using a Pierce Glycoprotein Staining Kit according to kit instructions. Briefly, gels were fixed by immersing in 100 mL of 50% v/v methanol for 30 min followed by washing twice (10 min each) in 100 mL of 3% v/v acetic acid. Gels were then transferred to 25 mL of oxidizing solution (periodic acid) and gently mixed for 15 min on a rocker followed by three times washing in 100 mL of 3% v/v acetic acid for 5 min each. Gels were then transferred to 25 mL of glycoprotein stain (Fuchsin Sulfite) and gently mixed for 15 min on a rocker. Gels were then immersed in 25 mL of reducing solution for 5 min followed by washing in 3% v/v acetic acid and DI water. Positive (horseradish peroxidase) and negative (soybean trypsin inhibitor) controls for glycoprotein staining were included on each gel. Gels were imaged using a ChemiDoc Touch Gel Imaging System (Bio-Rad) with epi-white illumination. Image Lab Software (Bio-Rad version 6.1) was used for estimations of molecular mass.

2.11 Western and dot blot

For Western blot, following SDS-PAGE, proteins were transferred onto PVDF membranes using transfer buffer (10% v/v 10X CAPS buffer [pH 11.0], 10% v/v methanol) at room temperature for 90 min at 50 V using a Bio-Rad Criterion Tank Blotter. Following transfer, polypeptide bands were visualized by Ponceau S staining. For dot blot, 1 μ g protein was pipetted onto a PVDF membrane and left to air dry at room temperature (approximately 30 min). All membranes were blocked with 5% (w/v) nonfat dry milk (NFD) in Tris-buffered saline with Tween 20 (TBST) for 60 min at room temperature on a rocker. After blocking, membranes were washed with two changes of TBST for 5 min each and subsequently incubated with pooled human plasma containing anticashew IgE (diluted 1:5 v/v in TBST) overnight at 4 °C on a rocker. Membranes were then washed three times in TBST for 8 min each at room temperature on a shaker. Following washing, membranes were incubated with goat anti-human IgE peroxidase-labeled secondary antibody (diluted 1:10,000 v/v in TBST with

Table 2—Composition of simulated gastric fluid.

Constituent	Final conc. in SGF mixture
KCl	6.9 mM
KH ₂ PO ₄	0.9 mM
NaHCO ₃	25 mM
NaCl	47.2 mM
MgCl ₂ (H ₂ O) ₆	0.1 mM
(NH ₄) ₂ CO ₃	0.5 mM
CaCl ₂ (H ₂ O) ₂	0.075 mM
HCl	150 mM
Pepsin	Approximately 4,000 U/mL

5% NFDM) for 60 min at room temperature on a rocker. Membranes were again washed three times with TBST for 8 min each. Antigens were detected through chemiluminescence using Clarity Western ECL substrate. Membranes were incubated with substrate solution for 5 min on a rocker and imaged using a Bio-Rad ChemiDoc MP Imaging System with chemiluminescence. Band and dot intensities were quantified using Image Lab Software (Bio Rad version 6.1).

2.12 In vitro pepsin digestion

In vitro pepsin digestion was conducted following the protocol described by Minekus et al. (2014) with modifications. Composition of simulated gastric fluid (SGF) is outlined in Table 2. The amount of HCl in the SGF was optimized so that a 1:1 mixture of sample and SGF would be at pH 3.0. SGF and 100 µg aliquots of Ana o 1 in 20 µL 1X PNGase F buffer with or without addition of 1 µL PNGase F were prewarmed separately at 37 °C for 30 min. To start the reaction, samples and SGF were mixed 1:1 v/v and placed in an incubator at 37 °C with shaking (250 rpm). Digestion was carried out for 120 min and the reaction was stopped by neutralization with NaOH and placing on ice. Samples were then mixed 1:1 v/v with SDS-PAGE sample buffer and heated at 100 °C for 10 min. SDS-PAGE, Western, and dot blot were carried out as described in preceding sections. Controls (no pepsin) were included and Ana o 1 samples (glycosylated and deglycosylated) were digested in duplicate. Sample preparation is summarized in Table 3.

2.13 Molecular modeling

Molecular graphics and analyses performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311.

3. RESULTS AND DISCUSSION

3.1 Ana o 1 purification

Purification of native Ana o 1 involved three steps: stepwise ammonium sulfate precipitation, ultrafiltration, and size-exclusion chromatography. Figure 1 shows the SDS-PAGE profiles following purification and corresponding size exclusion chromatogram. As shown in the gel, the starting cashew total soluble protein extract (lane B) contained a series of protein bands ranging from approximately 11 to 59 kDa. To reduce the complexity, ammonium sulfate precipitation followed by ultrafiltration was performed. Three polypeptide bands (approximately 11, 12, and 50 kDa) were observed upon electrophoresis of the final retentate after ultrafiltration (lane C). Based on the observed molecular weight in accordance with previously reported literature (Reitsma et al., 2016), the band near 50 kDa was suspected to be Ana o 1. Due

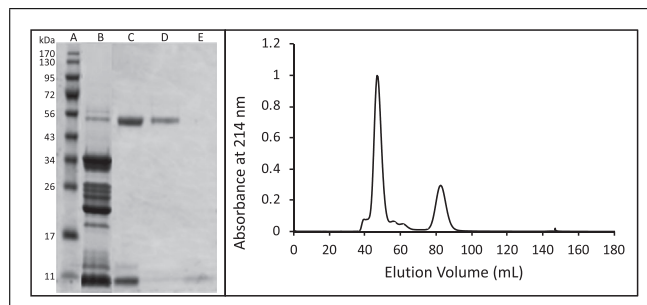


Figure 1—SDS-PAGE profiles following purification of Ana o 1 and corresponding size exclusion chromatogram. SDS-PAGE (left) lane A: molecular weight standards (5 µL), lane B: cashew whole protein extract (10 µg load), lane C: crude-purified cashew protein extract loaded onto S-200 column (5 µg load), lane D: pooled fractions corresponding to 44 to 51 mL eluent (2 µg load), lane E: pooled fractions corresponding to 76 to 89 mL eluent (1 µg load). Gel was run under reducing conditions. Elution off size exclusion column (right) was monitored by UV absorbance at 214 nm. Sample was injected on a HiPrep 16/60 Sephacryl S-200 HR prepacked column (1.6 × 60 cm, 120 mL column volume, 0.15 MPa pressure limit) at 0.5 mL/min and proteins were eluted with 180 mL of equilibration buffer at 0.5 mL/min.

to the size differences, size exclusion chromatography was selected for further purification. Fractions eluting from 44 to 51 mL, corresponding to the first peak in the chromatogram, were collected, pooled, and analyzed by SDS-PAGE (lane D). A single band was observed in the SDS-PAGE gel at approximately 50 kDa. The eluate was also analyzed using LC-MS/MS, and Ana o 1 was confirmed in this fraction although the specific isoform of Ana o 1 was not determined (Figure S-1). The second eluting peak (pooled fractions 76 to 89 mL, lane E) appears to contain the separated impurities. The approximate yield of purified Ana o 1 from 10 g of defatted cashew flour (approximately 34% protein w/w) was 0.13%.

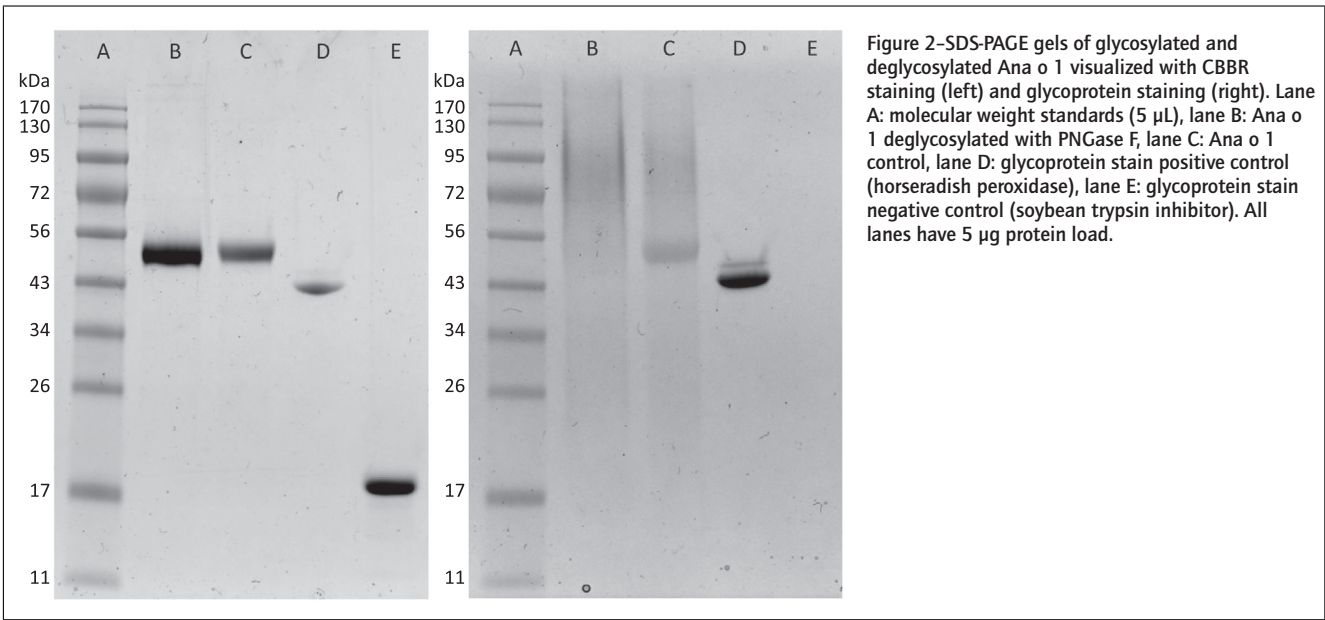
This approach was based on the method of Reitsma et al. (2016) which to our knowledge is the only published method on purification of native Ana o 1. Due to the fact that recombinant expression systems are often unable to replicate post-translational modifications (e.g., glycosylation and disulfide bond formation) that may be important to allergenicity, purification and analysis of the native protein is important (Willison, Sathe, & Roux, 2014). In the original method, the authors were able to purify native Ana o 1 without the additional step of column chromatography. In our attempt to reproduce this method, we were unable to completely remove the low molecular weight peptides by ultrafiltration. The reduced efficiency of ultrafiltration in our hands may be due to the use of a fixed angle rotor instead of a swinging-bucket rotor during centrifugation. This may have resulted in partial clogging of the filter and retention of low molecular weight impurities. Reitsma et al. (2016) also described a shaking step between centrifugations that may have varied from our method in speed and/or time. Nonetheless, ammonium sulfate precipitation and ultrafiltration were valuable preconcentration and purification steps prior to column chromatography.

3.2 Ana o 1 glycosylation

Glycoprotein staining was used to confirm glycosylation of purified Ana o 1 and to ensure deglycosylation after incubation with PNGase F. PNGase F was chosen because of its reported broad specificity (Tarentino, Gomez, & Plummer, 1985); PNGase F cleaves almost all N-linked oligosaccharides, unless there is an

Table 3–Preparation and treatment of samples for *in vitro* pepsin digestion.

Sample	Glycosylated Ana o 1 control	Glycosylated Ana o 1 + pepsin	Deglycosylated Ana o 1 control	DeglycosylatedAna o 1 + pepsin
µg Ana o 1	100	100	100	100
µL H ₂ O	17	17	16	16
µL 5X PNGase F buffer	4	4	4	4
Denaturation at 80 °C	Yes	Yes	Yes	Yes
µL PNGase F	—	—	1	1
Incubation at 50 °C	Yes	Yes	Yes	Yes
µL SGF w/pepsin	—	21	—	21
µL SGF no pepsin	21	—	21	—
Incubation time at 37 °C	120 min	120 min	120 min	120 min
µL neutralizing solution	42	42	42	42
Total volume (µL)	84	84	84	84
Ana o 1 conc. (mg/mL)	1.19	1.19	1.19	1.19



α 1, 3-fucose on the core N-acetyl glucosamine. In Figure 2, the SDS-PAGE profiles of deglycosylated and glycosylated Ana o 1 visualized with CBBR (left) and glycoprotein staining (right) are shown. Lanes B and C contain deglycosylated and glycosylated Ana o 1, respectively. An expected shift in mobility of deglycosylated Ana o 1 (lane B) was observed when stained with CBBR. Based on this shift, the estimated difference in molecular mass between glycosylated (lane C) and deglycosylated Ana o 1 (lane B) was approximately 800 Da. The average molecular mass of an N-glycan on a glycoprotein is approximately 1,000 Da (Schneider & Nagele, 2012), which suggests that Ana o 1 may contain a single glycan. Glycoprotein staining of the same samples in Figure 2 (right) further confirmed deglycosylation of Ana o 1 by PNGase F. The approximately 50 kDa band was not stained in lane B (deglycosylated Ana o 1) as compared to the positive signal observed in lane C. Positive and negative controls for glycoprotein staining were included in lanes D and E, respectively.

Presence of glycoproteins in total soluble cashew protein extract was initially believed to be nonexistent (Sathe et al., 2009). More recently, Reitsma et al. (2016) showed that glycoprotein staining of purified Ana o 1 was positive, as reproduced in this report. In agreement with Reitsma et al. (2016), we hypothesize that the reported negative glycoprotein stain in the total soluble protein extract that

was reported initially (Sathe et al., 2009) was likely due to the low quantity of Ana o 1 in the extract. As corroborated by Figure 1 lane B, the relative abundance of Ana o 1 compared to other proteins in the extract appears to be low. Additionally, the relatively weak glycoprotein staining intensity of Ana o 1 (Figure 2, lane C) compared to the positive glycoprotein control (horseradish peroxidase, Figure 2, lane D) and small migration shift observed with CBBR staining suggest that the extent of Ana o 1 glycosylation is low. Glycoprotein staining may not be sensitive enough to detect Ana o 1 when the whole protein extract is stained.

3.3 Immunoreactivity of glycosylated versus deglycosylated Ana o 1

Glycosylation has the potential to influence immunorecognition of a protein by several means. For example, cotranslational N-glycosylation can influence protein folding and may contribute to the formation (or lack) of conformational epitopes and/or exposure of linear epitopes (Imperiali & O'Connor, 1999). Additionally, the presence of a glycan on the surface of a protein may interfere with immunorecognition by physically masking an epitope, partially or completely (Zhang, Xiao, Zhang, & Zhou, 2019). It is also possible that a glycan can be directly involved in antibody recognition by constituting a portion or entirety of the epitope

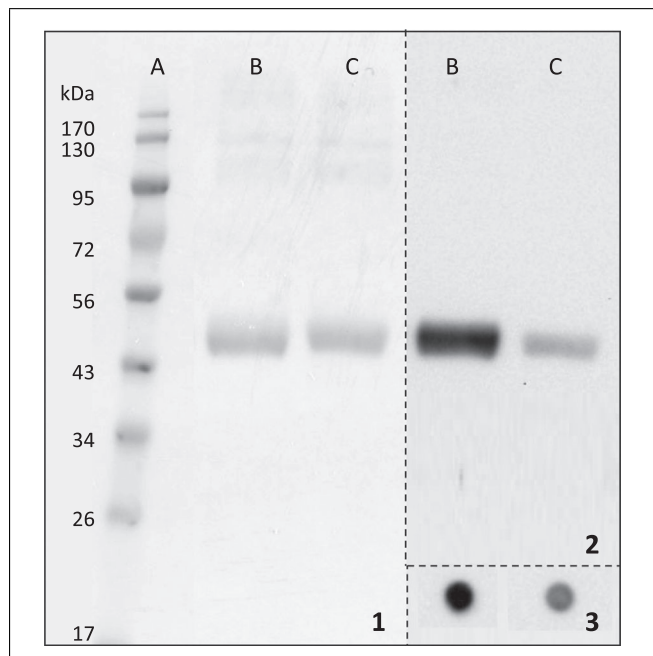


Figure 3—Immunoreactivity of Ana o 1 after deglycosylation. 1: Ponceau S staining after transfer to PVDF membrane (5 µg protein load). 2: Western blot. Lane A: molecular weight standards, lane B: Ana o 1 deglycosylated with PNGase F, lane C: Ana o 1 control. 3: Dot blot with deglycosylated Ana o 1 (left) and Ana o 1 control (right).

(Aalberse, Koshte, & Clemens, 1981; Altmann, 2007). To assess the role of Ana o 1 glycosylation in immunoreactivity, Ana o 1 was deglycosylated using PNGase F and subjected to immunoblotting.

A comparison of glycosylated and deglycosylated Ana o 1 probed with pooled human plasma containing anticashew IgE is shown in Figure 3. Ponceau S staining was used to visualize the transfer of proteins onto the PVDF membrane following electrophoresis (Figure 3-1). Again, a slight shift in electrophoretic migration of deglycosylated Ana o 1 (lane B) compared to its glycosylated counterpart (lane C) was observed. Ponceau S staining also revealed the presence of a small amount of high molecular weight aggregates around 130 kDa in both lanes B and C, likely heat-induced aggregates formed during sample preparation. Figure 3-2 shows the corresponding immunoblot of this membrane probed with pooled human plasma. Interestingly, a stronger signal was observed for deglycosylated Ana o 1 (lane B) compared to glycosylated Ana o 1 (lane C). To corroborate this, the corresponding dot blots for each protein are shown at the bottom in Figure 3-3. Measured band and dot intensity of deglycosylated Ana o 1 were six and three times higher than the glycosylated control, respectively. These results suggest that glycan moieties on Ana o 1 may be physically masking amino acid residues recognized by human IgE used in this study, and their removal thereby increases immunorecognition. It is noted that only a limited number of plasma samples were included in this study. In the future, individual plasma analysis and inclusion of additional plasma samples would improve the clinical significance of these results.

Twelve B-cell epitopes (11 linear (Wang et al., 2002) and 1 conformational (Guan et al., 2015)) have been mapped on Ana o 1 using synthetic peptides and recombinant protein. The Asn residue(s) involved in Ana o 1 glycosylation have not been identified; however, asparagine residues targeted for N-linked glycosylation are typically within the consensus sequence Asn-X-Thr/Ser, where X

can be any nonproline amino acid (Imperiali & O'Connor, 1999; O'Connor & Imperiali, 1996; Schwarz & Aebi, 2011; van Ree, 2002; Wyss & Wagner, 1996). One asparagine residue in the Ana o 1 primary sequence falls within this consensus sequence (position 391) and is also part of an epitope sequence recognized by human IgE (Figure 4). If this predicted N-glycosylation site is glycosylated, then it is likely that the observed increase in immunorecognition after deglycosylation is due to unmasking of epitopes. Identifying site(s) of N-glycosylation on Ana o 1 as well as information on the size and structure of the attached glycans would be useful in the future to support these results. It may also be of interest to explore glycation or neoglycosylation as a potential means to reduce IgE recognition of Ana o 1.

Immunoreactivity following deglycosylation has been investigated for other glycoprotein food allergens as well. Shrimp tropomyosin similarly exhibited increased IgE binding following enzymatic deglycosylation which was also proposed to be due to increased exposure of epitopes previously masked by glycans (Zhang et al., 2019). For several other allergens, such as egg ovalbumin (Park, Yoon, Kim, Han, & Choi, 2017), egg ovomucoid (Benede, Lopez-Fandino, Reche, Molina, & Lopez-Exposito, 2013), soybean β -conglycinin (Amigo-Benavent, Athanasopoulos, Ferranti, Villamiel, & del Castillo, 2009; Yang et al., 2018), and wheat gliadin (Song et al., 2015), immunoreactivity decreased following deglycosylation. Such decreases in IgE binding have been attributed to participation of the carbohydrates in recognition by human IgE. Although the existence of anticarbohydrate IgEs is well established, clinical relevance of carbohydrate epitopes has been a subject of debate (Breiteneder & Mills, 2005; Fotisch & Vieths, 2001; van Ree & Aalberse, 1999). The role of glycosylation in allergenicity appears to be specific to a given protein and depends on a number of factors, including location of the glycans, location of the epitopes, size and number of attached glycans, chemical interactions between the glycan and the protein surface, and immunogenic behavior of the carbohydrate moiety itself.

3.4 *In vitro* pepsin digestion

An important characteristic of food allergens is their resistance toward gastrointestinal digestion (Pekar, Ret, & Untersmayr, 2018). To assess and compare stability of glycosylated and deglycosylated Ana o 1, purified proteins were incubated with a fixed amount of pepsin for 120 min at pH 3.0. The conditions were selected based on the recommendations of Minekus et al. (2014), a consensus report from a network of over 200 scientists from 32 countries proposing a standardized approach for *in vitro* digestion studies. To represent a broad range of meals, 120 min was recommended for gastric digestion time. Although pepsin activity has an optimum range from pH 1.8 to 3.2 (Soybel, 2005; Untersmayr & Jensen-Jarolim, 2006) and is known to vary greatly with pH, a static value of pH 3.0 was recommended to match the 120 min suggested length of gastric simulation and represent average pH with consumption of a meal (Minekus et al., 2014).

Pepsin-induced proteolysis of Ana o 1 visualized with CBBR staining is shown in Figure 5-1. After *in vitro* gastric digestion, both deglycosylated (lane B) and glycosylated Ana o 1 (lane D) exhibited some degree of degradation compared to their respective controls (no pepsin, lanes C and E). Deglycosylated Ana o 1 appeared to undergo more proteolysis, as indicated by the diminished 50 kDa band in lane B (deglycosylated Ana o 1) with a concurrent increased detection of low molecular weight bands

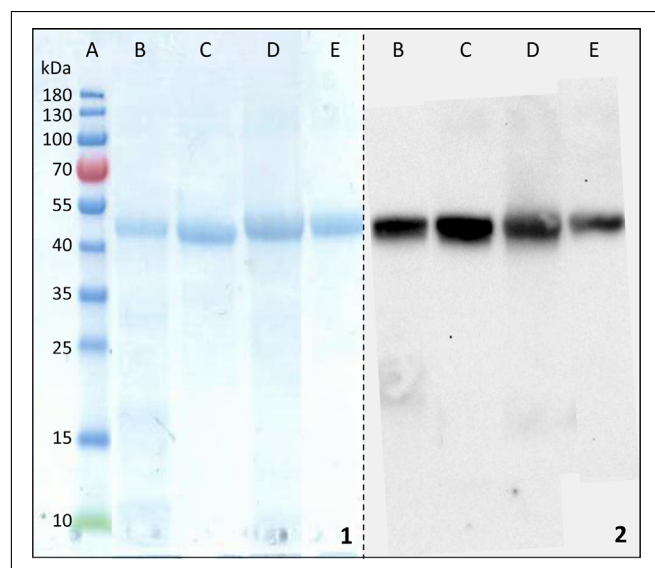
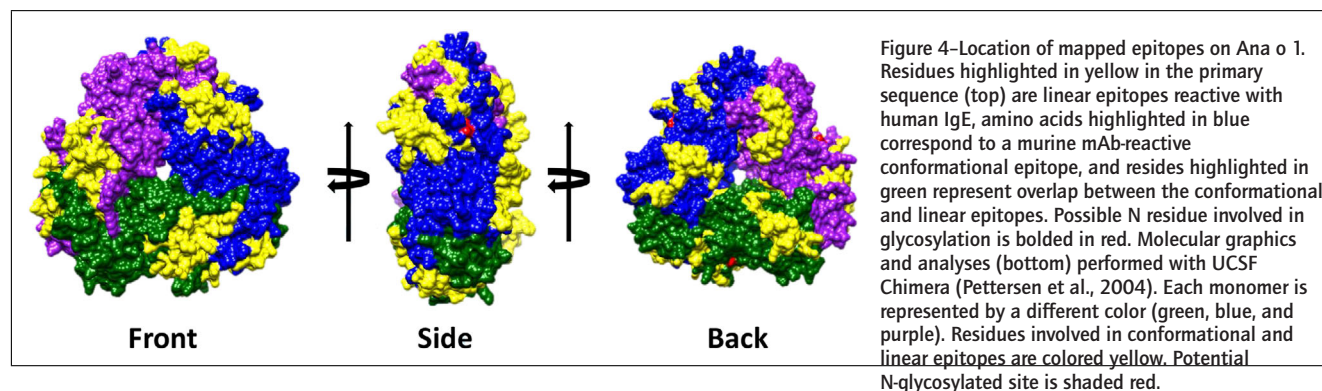


Figure 5-*In vitro* pepsin digestion of Ana o 1. 1: SDS-PAGE with CBBR Staining (10 µg protein load). 2: Western blot with pooled human plasma (5 µg protein load). Lane A: molecular weight standards (5 µL), lane B: deglycosylated Ana o 1 + 120 min pepsin digestion, lane C: deglycosylated Ana o 1, lane D: Ana o 1 control + 120 min pepsin digestion, lane E: Ana o 1 control.

around 30, 17, and 11 kDa, compared to lane C (glycosylated Ana o 1).

Western blotting was used to assess immunoreactivity following *in vitro* pepsin digestion (Figure 5-2). Compared to the deglycosylated Ana o 1 control (no pepsin, lane C), an anticipated reduction in immunoreactivity of deglycosylated Ana o 1 following exposure to pepsin (lane B) was observed, associated with the breakdown of the 50 kDa Ana o 1 band. It also appears that some of the degradation products from Ana o 1 proteolysis may still retain IgE-reactive epitopes as indicated by the weak signals observed in the low molecular weight regions of both lanes B and D. Contrastingly, immunoreactivity of glycosylated Ana o 1 (lane D) increased after pepsin exposure compared to the glycosylated control (no pepsin, lane E). It is possible that partial cleavage by pepsin of glycosylated Ana o 1 resulted in structural changes that increased exposure of epitopes; however, the attached glycans may have interfered with more severe proteolysis observed in the deglycosylated pepsin-digested protein. It is further noted that increasing the quantity of protein in the Western blot may reveal additional IgE-reactive peptides that may be present in low amounts following pepsin digestion. In this report, protein load was limited by the quantity of native Ana o 1 obtained after purification.

Pepsin is a nonspecific endopeptidase that preferentially cleaves the peptide bond on the C-terminal side of aromatic amino acids (Phe, Tyr, and Trp) (Hamuro, Coales, Molnar, Tuske, & Morrow, 2008). It is likely that deglycosylation exposed more pepsin cleavage sites resulting in the observed increased proteolysis of the deglycosylated protein. Similar results were reported for soybean allergen, Gly m 5, in which *in vitro* gastrointestinal digestibility was enhanced after deglycosylation with PN-Gase F. The authors similarly suggested that attached glycans may limit the accessibility of pepsin to cleavage sites on the protein (Amigo-Benavent, Clemente, Ferranti, Caira, & del Castillo, 2011).

This report is the first investigation of *in vitro* gastric digestion of purified Ana o 1; however, Mattison, Grimm, and Wasserman (2014) assessed *in vitro* digestion of total soluble cashew protein extract. The authors found that at 0.8 U of pepsin, bands corresponding to Ana o 1 were undetectable in their gels after 20 min digestion. These results are difficult to compare to ours because in their study, digestion was conducted at pH 1.2, which is more representative of a fasted state pH. As previously mentioned, pepsin activity is highly dependent on pH and complete proteolysis of Ana o 1 is not unexpected at a lower pH. Additionally, the low quantity of Ana o 1 in the whole protein extract makes it difficult to determine if bands have diminished completely or are present in amounts too low to detect by staining.

Stability of Ana o 1 toward proteolysis may be a result of its structural characteristics. As previously mentioned, Ana o 1 is a member of the cupin superfamily, named after the Latin word for barrel, “cupa,” due to the conserved β -barrel structural core domain shared across members (Dunwell, 1998). Like other globulins, Ana o 1 is a bicupin, containing two conserved β -barrel domains (Breiteneder & Radauer, 2004). The β -barrels are composed of two walls of antiparallel β -sheets with an associated loop domain and are connected through an α -helix linker region (Barre et al., 2008). The double-stranded region of the cupin fold is noted to be a very stable structural motif that is resistant toward thermal denaturation and proteolysis and likely plays a role in the survival of immunologically active fragments following digestion (Mills et al., 2002). Structural stability following the digestion of similar vicilin allergens, including peanut Ara h 1 (Maleki et al., 2000) and soybean Gly m 5 (Zheng, Qin, Tian, & Zhang, 2017), has been reported.

Overall, glycosylation may play a minor role in the stability of Ana o 1 toward pepsin digestion because both forms of the protein remained stable and reactive with human IgE following simulated gastric digestion. Subsequent exposure of Ana o 1 to

pancreatic enzymes (e.g., trypsin, chymotrypsin, and carboxypeptidase) encountered in the small intestine should be investigated in the future.

4. CONCLUSION

As previously stated, there is no cure for food allergy. In this report, deglycosylation was explored as a potential means to reduce immunoreactivity of major cashew allergen, Ana o 1. The results of this investigation indicate that deglycosylation increased recognition of Ana o 1 epitopes targeted by the pooled human plasma used in this study. Immunoreactivity of deglycosylated Ana o 1 was higher than the glycosylated control as assessed by Western and dot blot. Based on these results and due to the proximity of a predicted N-glycosylation site to mapped epitopes on Ana o 1, we hypothesize that the observed increase in immunorecognition after deglycosylation is due to unmasking of epitopes. Although deglycosylation may have slightly increased the susceptibility of Ana o 1 toward pepsin proteolysis, both forms of the protein were recognized by human IgE following *in vitro* digestion under the tested conditions. More work is needed to understand the role of glycosylation in gastrointestinal digestibility of Ana o 1. Further, it is suggested that protein glycation be explored as a possible alternative method to reduce immunoreactivity.

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AUTHOR CONTRIBUTIONS

S.K. Sathe conceived the idea. V.D. Zaffran conducted the laboratory work. M.G. Roper supervised all the laboratory work. V.D. Zaffran, M.G. Roper, and S. Gupta designed the study, analyzed the results, and contributed to manuscript writing.

CONFLICTS OF INTEREST

There are no conflicts of interest.

REFERENCES

- Aalberse, R. C., Koshte, V., & Clemens, J. G. J. (1981). Immunoglobulin-E antibodies that cross-react with vegetable foods, pollen, and Hymenoptera venom. *Journal of Allergy and Clinical Immunology*, 68(5), 356–364. [https://doi.org/10.1016/0091-6749\(81\)90133-0](https://doi.org/10.1016/0091-6749(81)90133-0)
- Altmann, F. (2007). The role of protein glycosylation in allergy. *International Archives of Allergy and Immunology*, 142(2), 99–115. <https://doi.org/10.1159/000096114>
- Amigo-Benavent, M., Athanasiou, V. I., Ferranti, P., Villamil, M., & del Castillo, M. D. (2009). Carbohydrate moieties on the *in vitro* immunoreactivity of soy beta-conglycinin. *Food Research International*, 42(7), 819–825. <https://doi.org/10.1016/j.foodres.2009.03.003>
- Amigo-Benavent, M., Clemente, A., Ferranti, P., Cairas, S., & del Castillo, M. D. (2011). Digestibility and immunoreactivity of soybean beta-conglycinin and its deglycosylated form. *Food Chemistry*, 129(4), 1598–1605. <https://doi.org/10.1016/j.foodchem.2011.06.015>
- Barre, A., Sordet, C., Culerrier, R., Rance, F., Didier, A., & Rouge, P. (2008). Vicilin allergens of peanut and tree nuts (walnut, hazelnut and cashew nut) share structurally related IgE-binding epitopes. *Molecular Immunology*, 45(5), 1231–1240. <https://doi.org/10.1016/j.molimm.2007.09.014>
- Benede, S., Lopez-Fandino, R., Reche, M., Molina, E., & Lopez-Exposito, I. (2013). Influence of the carbohydrate moieties on the immunoreactivity and digestibility of the egg allergen ovomucoid. *PLoS One*, 8(11). <https://doi.org/10.1371/journal.pone.0080810>
- Bradford, M. M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry*, 72(1–2), 248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
- Breiteneder, H., & Mills, E. N. C. (2005). Molecular properties of food allergens. *Journal of Allergy and Clinical Immunology*, 115(1), 14–23. <https://doi.org/10.1016/j.jaci.2004.10.022>
- Breiteneder, H., & Radauer, C. (2004). A classification of plant food allergens. *Journal of Allergy and Clinical Immunology*, 113(5), 821–830. <https://doi.org/10.1016/j.jaci.2004.01.079>
- Dunwell, J. M. (1998). Cupins: A new superfamily of functionally diverse proteins that include germins and plant storage proteins. *Biotechnology and Genetic Engineering Reviews*, 15, 1–32.

- FAOSTAT. (2020). Crops. <http://www.fao.org/faostat/en/#data/QC>
- Fotisch, K., & Vieths, S. (2001). N- and O-linked oligosaccharides of allergenic glycoproteins. *Glycoconjugate Journal*, 18(5), 373–390. <https://doi.org/10.1023/A:1014860030380>
- Guan, X., Noble, K. A., Tao, Y., Roux, K. H., Sathe, S. K., Young, N. L., & Marshall, A. G. (2015). Epitope mapping of 7S cashew antigen in complex with antibody by solution-phase H/D exchange monitored by FT-ICR mass spectrometry. *Journal of Mass Spectrometry*, 50(6), 812–819. <https://doi.org/10.1002/jms.3589>
- Gupta, R. S., Warren, C. M., Smith, B. M., Jiang, J., Blumenstock, J. A., Davis, M. M., ... Nadeau, K. C. (2019). Prevalence and severity of food allergies among US adults. *JAMA Netw Open*, 2(1), e185630. <https://doi.org/10.1001/jamanetworkopen.2018.5630>
- Hamuro, Y., Coates, S. J., Molnar, K. S., Tuske, S. J., & Morrow, J. A. (2008). Specificity of immobilized porcine pepsin in H/D exchange compatible conditions. *Rapid Communications in Mass Spectrometry*, 22(7), 1041–1046. <https://doi.org/10.1002/rcm.3467>
- Imperiali, B., & O'Connor, S. E. (1999). Effect of N-linked glycosylation on glycopeptide and glycoprotein structure. *Current Opinion in Chemical Biology*, 3(6), 643–649. <http://www.ncbi.nlm.nih.gov/pubmed/10600722>
- Imperiali, B., Shannon, K. L., Unno, M., & Rickert, K. W. (1992). A mechanistic proposal for asparagine-linked glycosylation. *Journal of the American Chemical Society*, 114(20), 7944–7945. <https://doi.org/10.1021/ja00046a069>
- INC. (2017). Nut & dried fruits statistical yearbook.
- Maleki, S. J., Kopper, R. A., Shin, D. S., Park, C. W., Compadre, C. M., Sampson, H., ... Bannon, G. A. (2000). Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *Journal of Immunology*, 164(11), 5844–5849. <http://www.ncbi.nlm.nih.gov/pubmed/10820263>
- Mattison, C. P., Grimm, C. C., & Wasserman, R. L. (2014). *In vitro* digestion of soluble cashew proteins and characterization of surviving IgE-reactive peptides. *Molecular Nutrition & Food Research*, 58(4), 884–893. <https://doi.org/10.1002/mnfr.201300299>
- McWilliam, V., Koplin, J., Lodge, C., Tang, M., Dharmage, S., & Allen, K. (2015). The prevalence of tree nut allergy: A systematic review. *Current Allergy and Asthma Reports*, 15(9), 54. <https://doi.org/10.1007/s11882-015-0555-8>
- Mendes, C., Costa, J., Vicente, A. A., Oliveira, M., & Mafra, I. (2019). Cashew nut allergy: Clinical relevance and allergen characterisation. *Clinical Reviews in Allergy & Immunology*, 57(1), 1–22. <https://doi.org/10.1007/s12016-016-8580-5>
- Mills, E. N. C., Jenkins, J., Marigheto, N., Belton, P. S., Gunning, A. P., & Morris, V. J. (2002). Allergens of the cupin superfamily. *Biochemical Society Transactions*, 30, 925–929.
- Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., ... Brodtkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food — An international consensus. *Food & Function*, 5(6), 1113–1124. <https://doi.org/10.1039/c3fo60702j>
- O'Connor, S. E., & Imperiali, B. (1996). Modulation of protein structure and function by asparagine-linked glycosylation. *Chemistry & Biology*, 3(10), 803–812. <http://www.ncbi.nlm.nih.gov/pubmed/8939697>
- Park, H. Y., Yoon, T. J., Kim, H. H., Han, Y. S., & Choi, H. D. (2017). Changes in the antigenicity and allergenicity of ovalbumin in chicken egg white by N-acetylglucosaminidase. *Food Chemistry*, 217, 342–345. <https://doi.org/10.1016/j.foodchem.2016.08.112>
- Pekar, J., Ret, D., & Untersmayr, E. (2018). Stability of allergens. *Molecular Immunology*, 100, 14–20. <https://doi.org/10.1016/j.molimm.2018.03.017>
- Petersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., & Ferrin, T. E. (2004). UCSF Chimera—A visualization system for exploratory research and analysis. *Journal of Computational Chemistry*, 25(13), 1605–1612. <https://doi.org/10.1002/jcc.20084>
- Reitsma, M., Bastiaan-Net, S., Sforza, S., van der Valk, J. P., van Gerth van Wijk, R., Savelkoul, H. F., ... Wichers, H. J. (2016). Purification and characterization of Anacardium occidentale (cashew) allergens Ana o 1, Ana o 2, and Ana o 3. *Journal of Agricultural and Food Chemistry*, 64(5), 1191–1201. <https://doi.org/10.1021/acs.jafc.5b04401>
- Sathe, S. K., Venkatchalam, M., Sharma, G. M., Kshirsagar, H. H., Teuber, S. S., & Roux, K. H. (2009). Solubilization and electrophoretic characterization of select edible nut seed proteins. *Journal of Agricultural and Food Chemistry*, 57(17), 7846–7856. <https://doi.org/10.1021/jf9016338>
- Schneider, S., & Nagele, E. (2012). N-glycan analysis of monoclonal antibodies. *Innovations in Pharmaceutical Technology*(42), 22–26. <http://www.iptonline.com/articles/public/Agilent.pdf>
- Schwarz, F., & Aebi, M. (2011). Mechanisms and principles of N-linked protein glycosylation. *Current Opinion in Structural Biology*, 21(5), 576–582. <https://doi.org/10.1016/j.sbi.2011.08.005>
- Song, T. W., Hong, J. Y., Lee, K. E., Kim, M. N., Kim, Y. H., Lee, S. Y., ... Kim, K. E. (2015). IgE reactivity to carbohydrate moieties of glycoproteins in wheat allergy. *Allergy and Asthma Proceedings*, 36(3), 192–199. <https://doi.org/10.2500/aap.2015.36.3815>
- Soybel, D. I. (2005). Anatomy and physiology of the stomach. *Surgical Clinics of North America*, 85(5), 875–894. <https://doi.org/10.1016/j.suc.2005.05.009>
- Tarentino, A. L., Gomez, C. M., & Plummer, T. H. Jr. (1985). Deglycosylation of asparagine-linked glycans by peptide-N-glycosidase F. *Biochemistry*, 24(17), 4665–4671. <https://doi.org/10.1021/bi00338a028>
- Untersmayr, E., & Jensen-Jarolim, E. (2006). The effect of gastric digestion on food allergy. *Current Opinion in Allergy and Clinical Immunology*, 6(3), 214–219. <https://doi.org/10.1097/01.all.0000225163.06016.93>
- USDA. (2020). FoodData Central. <https://fdc.nal.usda.gov/>
- van Ree, R. (2002). Carbohydrate epitopes and their relevance for the diagnosis and treatment of allergic diseases. *International Archives of Allergy and Immunology*, 129(3), 189–197. <https://doi.org/10.1159/000066770>
- van Ree, R., & Aalberse, R. C. (1999). Specific IgE without clinical allergy. *Journal of Allergy and Clinical Immunology*, 103(6), 1000–1001. [https://doi.org/10.1016/S0091-6749\(99\)70169-7](https://doi.org/10.1016/S0091-6749(99)70169-7)
- Wang, F., Robotham, J. M., Teuber, S. S., Tawde, P., Sathe, S. K., & Roux, K. H. (2002). Ana o 1, a cashew (Anacardium occidentale) allergen of the vicilin seed storage protein family. *Journal of Allergy and Clinical Immunology*, 110(1), 160–166. <https://doi.org/10.1067/mai.2002.125208>
- WHO/IUIS Allergen Nomenclature Sub-Committee. (2020). Allergen nomenclature. <http://www.allergen.org/>
- Willison, L. N., Sathe, S. K., & Roux, K. H. (2014). Production and analysis of recombinant tree nut allergens. *Methods (San Diego, Calif.)*, 66(1), 34–43. <https://doi.org/10.1016/j.jymeth.2013.07.033>

- Wyss, D. F., & Wagner, G. (1996). The structural role of sugars in glycoproteins. *Current Opinion in Biotechnology*, 7(4), 409–416. [https://doi.org/10.1016/S0958-1669\(96\)80116-9](https://doi.org/10.1016/S0958-1669(96)80116-9)
- Yang, A., Deng, H., Zu, Q., Lu, J., Wu, Z., Li, X., ... Chen, H. (2018). Structure characterization and IgE-binding of soybean 7S globulin after enzymatic deglycosylation. *International Journal of Food Properties*, 21(1), 171–182. <https://doi.org/10.1080/10942912.2018.1437628>
- Zhang, Z., Xiao, H., Zhang, X., & Zhou, P. (2019). Insight into the effects of deglycosylation and glycation of shrimp tropomyosin on in vivo allergenicity and mast cell function. *Food & Function*, 10(7), 3934–3941. <https://doi.org/10.1039/c9fo00699k>
- Zheng, S., Qin, G., Tian, H., & Zhang, F. (2017). Three-dimensional structure of Gly m 5 (beta-conglycinin) plays an important role in its stability and overall allergenicity. *Food Chemistry*, 234, 381–388. <https://doi.org/10.1016/j.foodchem.2017.05.020>

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S-1. Ana o 1 identification by LC-MS/MS. Matched peptides by SequestHT are bolded and underlined.

Supplemental material

Identified as, Ana o 1, vicilin-like protein (*Anacardium occidentale*), Uniprot Q8L5L5.

SequestHT Results: 75 % sequence coverage, score: 44892

Mascot Results: 68 % sequence coverage, score: 44401

MGPPTKFSFSLFLVSVLVLC LGFALAKIDPELKQCKHQCKVQRQYDEQQKEQCVKECEKY
YKEKKGR**EREHEEEEEEWGTGGVDEPSTHEPAEK**HLSQCMRQCERQEGGQQKQLCRFRCQ
ERYKKERGQHNYKREDDDEDEDEAEEDENPYVFEDEDFTTKVKTEQGKVLLPKFTQK
SKLLHALEKYRLAVLVANPQAFVVP SHMDADSIFFVSWGRGTITKILENKRESINVRQGD
IVSISSGT PFYIANNDENEKLYLVQFLRPVNLPGHFEVFHGPGENPESFYRAFSWEILE
AALKTSKDTLEKLF EKQDQGTIMKASKEQIRAMSRRGEGPKIWPFTTEESTGSFKLFKKDP
SQSNKYGQLFEAERIDYPPLEKLD MVVSYANITKGGMSVFFYNSRATKIAIVVS GEGCVE
IACPHLSSSKSSHPSYKKLRARIRKDTVFI VPAGHPFATVASGNENLEIVCFEVNAEGNI
RYTLAGKKNIIKVMEKEAKELAFKMEGEEVDKVFGKQDEEFFFGPEWRKEKEGRAD E

Figure S-1. Ana o 1 identification by LC-MS/MS. Matched peptides by SequestHT are bolded and underlined. Due to the similarity in the two isoforms of Ana o 1 and the lack of N-terminal peptides detection, the specific isoform (Ana o1.0101 vs. Ana o1.0102) of Ana o 1 was not determined.