

Purification and Characterization of *Anacardium occidentale* (Cashew) Allergens Ana o 1, Ana o 2, and Ana o 3

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S Supporting Information

ABSTRACT: In this study a fast and simple purification procedure for the three known allergens from cashew (7S globulin Ana o 1, 11S globulin Ana o 2, and 2S albumin Ana o 3) is described. The purified allergens are characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot, glycoprotein stain, and protein identification. The purified proteins still bind IgE, and this IgE binding varied between different pools of patient serum. Ana o 1 was found to be a glycoprotein. Ana o 3 has been studied more in detail to identify both the small and large subunits, both displaying microheterogeneity, and epitope mapping of Ana o 3 has been performed.

KEYWORDS: cashew, tree nut allergy, Ana o 1, Ana o 2, Ana o 3

INTRODUCTION

The prevalence of allergy to tree nuts in Europe is 0.5% (based on oral food challenges) to 1.3% (based on self-reported allergy).¹ Within this group, the percentage of cashew (*Anacardium occidentale*) allergy is between 7%² and 55%.³ Individuals suffering from cashew allergy can experience cutaneous, respiratory, and gastrointestinal symptoms and, in the most severe case, anaphylactic shock.^{4–6} Administration of adrenaline is required more often⁷ and the risk of developing an anaphylactic reaction seems higher than in peanut allergic patients.⁴

Currently, three major cashew allergens are known: Ana o 1, Ana o 2, and Ana o 3.⁸ Ana o 1 is a 7S vicilin seed storage protein of 50 kDa⁹ that is expected to form a trimer in the native state.¹⁰ The 11S globulin in cashew, Ana o 2, consists of a 33 kDa large subunit¹¹ and a 20 kDa small subunit, bound together by disulfide bonds¹² forming a 53 kDa protein.¹¹ In native conditions 11S globulins form hexameric structures of ~360 kDa.¹⁰ In literature Ana o 2 is also referred to as anacardein¹³ and cashew major protein.¹⁴

The third major cashew allergen is Ana o 3, a 2S albumin of 12.6 kDa.¹⁵ On the basis of similarity to other 2S albumins, this protein is most probably proteolytically cleaved into an N-terminal small subunit and a C-terminal large subunit that remain associated by four disulfide bonds.^{10,16} Robotham et al. reported denatured and reduced Ana o 3 to contain three isoforms of 6, 8, and 10 kDa. Each isoform had an N-terminal sequence that corresponded to the large subunit, but no small subunit was described.¹⁵

Reports on isolation and characterization of the cashew allergens are scarce. Ana o 3 has been purified before,^{17,18} but neither purity nor yield were mentioned. Occasionally, references to isolated forms of Ana o 2 (anacardein) are

made;^{19,20} however, as already indicated by the authors themselves, these fractions are not “immunochemically pure”.²⁰ When this protocol was repeated in 2010, a purity of >90% was stated, but no protein characterization data like SDS-PAGE or Western blot were shown.¹³

In this article we describe a fast and simple method to obtain Ana o 1, Ana o 2, and Ana o 3 with high purity. While purification of Ana o 1 has never been described, the purification of Ana o 2 has never been fully described or characterized. We describe a simple method that allows simultaneous purification of Ana o 1 and Ana o 3. Biochemical and immunochemical analysis of the isolated proteins revealed that the proteins retain their IgE-binding capacity and showed Ana o 1, but not Ana o 2 or Ana o 3, to be a glycoprotein. Ana o 3 has been examined further to identify the large subunit, as well as the so-far unidentified small subunit. Both the small and large subunits display microheterogeneity.

MATERIALS AND METHODS

Materials and reagents. Blanched cashews (*Anacardium occidentale*) from Brazil were kindly provided by Intersnack (Doetinchem, The Netherlands). Acetone, ammonium sulfate, and glycine were purchased from Merck (Darmstadt, Germany). Filter paper (595 1/2) was purchased from Whatman (Dassel, Germany). Ammonium bicarbonate, NaCl, NaH₂PO₄, β-mercaptoethanol, sodium dodecyl sulfate (SDS), Tween-20, bovine serum albumin (BSA), alkaline-phosphatase conjugated goat antirabbit antibody, Sigmafast 5-bromo-4-

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chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO). Amicon centrifugal filters were purchased from Merck Millipore (Tullagreen, Ireland). Minisart syringe filters (0.45 μm) were purchased from Sartorius Stedim (Goettingen, Germany). Ethylenediaminetetraacetic acid (EDTA), Halt protease inhibitor cocktail (no. 78438), Pierce Coomassie (Bradford) Protein Assay Kit, and Pierce Glycoprotein Staining Kit were purchased from Thermo Scientific (Rockford, IL). NuPAGE lithium dodecyl sulfate (LDS) sample buffer, NuPAGE 1 mm 10% Bis-Tris mini gels, NuPAGE 2-(*N*-morpholino)ethanesulfonic acid (MES) SDS running buffer, and SimplyBlue SafeStain were purchased from Invitrogen (Carlsbad, CA). Protein molecular weight marker and nitrocellulose membrane were purchased from Bio-Rad (Hercules, CA). Tris was purchased from USB (Cleveland, OH). Methanol was purchased from Actua-All (Randmeer, The Netherlands). Rabbit antihuman IgE was purchased from Dako (Glostrup, Denmark). Eight serum samples were purchased from Plasmalab International (Everett, WA). Ana o 3 peptides for epitope mapping were purchased from Synpeptide (Shanghai, China). The polyester backbone was purchased from GL Precision (San Jose, CA). IgE was purchased from Enzo (Farmingdale, NY). Trypsin was purchased from Promega (Madison, WI).

Obtaining defatted cashew and extraction of total soluble protein. Blanched cashews were ground by mortar and pestle. Ground cashews were defatted twice with acetone, 1:5 w/v for 2 h at 4 °C while stirring, followed by filtration using filter paper and drying for 2 h (first defatting step) or overnight (second defatting step).

Defatted cashew was suspended in an ammonium bicarbonate buffer, pH 7.9 (0.1 M ammonium bicarbonate, 0.5 M NaCl), 1:5 w/v and stirred overnight at 4 °C. Subsequently, the samples were centrifuged for 90 min at 4000g at 4 °C, and the obtained supernatant was used for the purification of Ana o 1 and Ana o 3.

Protein precipitation. Proteins were precipitated in a stepwise fashion. In the first step, 15% w/v ammonium sulfate was added and mixed by stirring for 1 h at 4 °C; subsequently this was centrifuged for 1 h at 4 °C in a swinging bucket centrifuge. The process was repeated three more times to yield final ammonium sulfate concentrations of 30%, 45%, and 52.5% w/v. The final supernatant, obtained after the last step of precipitation (52.5%), was diluted four times in Milli-Q water (MQ).

Purification of Ana o 1 using ultrafiltration. Four times 4 mL of the diluted final supernatant of ammonium sulfate precipitation was passed through a 30 kDa Amicon centrifugal filter by centrifugation, followed by two times 5 mL of MQ. Subsequently again four times 4 mL of the diluted final supernatant was passed through the same filter. Five mL of MQ was added to the retentate; this retentate was passed through a new 30 kDa Amicon centrifugal filter and purified in this filter by 10 times 5 min shaking in 5 mL of MQ followed by centrifugation. All centrifugation steps were performed for 5 min at 4000g at 20 °C. The final retentate containing Ana o 1 was suspended in a final volume of 3 mL of MQ, concentrated using a 0.5 mL 3 kDa Amicon centrifugal filter, and stored at −20 °C.

Purification of Ana o 3 using ultrafiltration. The filtrate from Ana o 1 purification was concentrated using a 50 mL 3 kDa Amicon centrifugal filter and stored at −20 °C.

Purification of Ana o 2 using gel filtration chromatography. Ground blanched cashew was stirred in ammonium bicarbonate buffer (1:5 w/v) overnight at 4 °C and centrifuged for 30 min at 4000g at 20 °C. From the supernatant the top white layer and lipid layer were removed and the remaining supernatant was filtered (0.45 μm). Of this filtered supernatant, 300 μL was loaded onto a Superdex 200 column (10/300GL, bed volume 24 mL) on an Äkta purifier (both Amersham Pharmacia Biotech (Uppsala, Sweden)). The proteins were eluted from the column using 15 mL elution buffer (50 mM NaH_2PO_4 , 0.15 M NaCl, pH 7) at 0.5 mL/min; 0.5 mL fractions were collected. The fractions were concentrated using a 0.5 mL 3 kDa Amicon centrifugal filter and stored at −20 °C.

Purification of Ana o 3 in the presence of protease inhibitors. Ground blanched cashew was stirred in ammonium bicarbonate buffer (1:5 w/v) with added protease inhibitor cocktail and 5 mM EDTA for 1 h at 4 °C and centrifuged for 90 min at 4000g at 4 °C. Protein precipitation and purification of Ana o 3 was performed as described above with the adaptation of performing all steps at 4 °C.

Protein concentration. Protein concentration was determined using a Coomassie protein assay according to the manufacturer's instructions.

SDS-PAGE. On the basis of a Coomassie protein assay, 20 μg of denatured and reduced samples (10% β -mercaptoethanol in NuPAGE LDS sample buffer, 5 min, 100 °C) were loaded onto NuPAGE 1 mm 10% Bis-Tris mini gels, alongside a molecular weight marker (Precision Plus Protein Dual Xtra Standard). Electrophoresis was performed at 160 V for 55 min in NuPAGE MES SDS running buffer. For SDS-PAGE, the gels were stained with SimplyBlue SafeStain. Imaging and analysis were performed using a Universal Hood III and Image Lab 4.1 software (both Bio-Rad). Nonreducing denaturing SDS-PAGE was performed as described above, but during the sample preparation no β -mercaptoethanol was added.

Western blotting. Transfer of proteins from reducing denaturing SDS-PAGE gel to a nitrocellulose membrane (0.2 μm) was performed using a Criterion blotter (Bio-Rad) in a cold tris-glycine buffer (25 mM tris, 190 mM glycine, 0.1% SDS, 20% methanol) for 36 min at 70 V. Membranes were washed with TBST (50 mM tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4) and blocked with 3% BSA in TBS (TBST without Tween-20) for 1 h at 4 °C. After washing with TBST and TBS, the blots were incubated overnight with a pool (to ascertain sufficient coverage of epitopes) of patient serum (group 1) or plasma (group 2) 1:5 diluted in TBS. Subsequently, the blots were washed with TBST and TBS and incubated with rabbit antihuman IgE diluted 1:15,000 in TBS. Again, the blots were washed with TBST and TBS, incubated with alkaline phosphatase conjugated goat antirabbit diluted 1:20,000 in TBS, followed by washing with TBST and TBS. Blots were stained with Sigmafast BCIP/NBT for 10 min (group 1 and antibody control blot) or 2.5 min (group 2). Imaging and analysis were performed using a Universal Hood III and Image Lab 4.1 software. The antibody control blot was performed as described earlier, but instead of serum/plasma the membrane was incubated overnight with TBS. Inhibition Western blotting was performed as described earlier with the exception that the plasma was incubated with either 1 mg/mL purified Ana o 1 or Ana o 3 for 4 h at room temperature.

Serum and plasma. Serum of Western blot group 1 was obtained from 13 cashew-allergic (double-blind placebo-controlled food challenge (DBPCFC) diagnosed) individuals

Table 1. Clinical Characteristics of Cashew and Tree-Nut Allergic Subjects: Self-Reported Allergies and Cashew-IgE Levels As Measured by Immulite

patient no. serum pool 1	self-reported allergies patients serum pool 1 (blot D1) Dutch children	IgE level cashew (kU/L)	patient no. plasma pool 2	self-reported allergies patients plasma pool 2 (blot D2) American adults	IgE level cashew (kU/L)
1	cashew, ^a pistachio	23.7	1 ^b	cashew, walnut, peanut, tree pollen, cat, dog, horse	100.0
2	cashew ^a	21.8	2 ^b	tree nuts, banana, eggplant, dust	56.9
3	cashew, ^a pistachio	10.9	3 ^b	nuts, avocado, corn, raw vegetables, dog, horse, cow	100.0
4	cashew, ^a chicken egg	11.1	4 ^b	tree nuts, pecan, hazelnut, dust, grass, insect venom, alcohol	19.2
5	cashew, ^a kiwi, shrimp	13.0	5	cashew, brazil nut, almond, hazelnut, peanut, dust	13.2
6	cashew, ^a peanut, hazelnut	38.6	6	cashew, pistachio, pecan, walnut, almond, hazelnut, macadamia, peanut, coconut, cat, dog, dust	59.6
7	cashew, ^a peanut, hazelnut	27.0	7	tree nuts, hazelnut, dust	35.5 ^c
8	cashew, ^a pistachio, citrus fruit	44.7	8	cashew, walnut, peanut, shellfish, fish, scotch bloom	26.2
9	cashew ^a	47.4			
10	cashew, ^a kiwi	51.9			
11	cashew, ^a pistachio, walnut, almond	10.8			
12	cashew, ^a almond, chicken egg, milk	12.7			
13	cashew, ^a cow's milk, chicken egg, pear, peas	48.7			

^aPositive DBPCFC to cashew as described by van der Valk et al., submitted manuscript. ^bSamples 1–4 as used in Ana o 3 epitope mapping experiments. ^cMeasured by ImmunoCAP by Plasmalab International.

out of the multicenter prospective study “Improvement of diagnostic methods for allergy assessment” with cashew allergy in children as a showcase (trial number NTR3572, medical ethical approval number 2012-125, Erasmus Medical Centre Rotterdam). Plasma of group 2 was obtained from PlasmaLab International. Eight patients with self-reported history of cashew or tree nut allergy were selected based on high cashew-IgE titers. Clinical characteristics of the subjects are described in Table 1: self-reported allergies and cashew-IgE levels as determined by Immulite 2000, F202.

Glycoprotein staining. Glycosylation of proteins was checked using a Glycoprotein Staining kit. Samples were run on SDS-PAGE as described above alongside a positive control (horseradish peroxidase, kit content) and a negative control (soybean trypsin inhibitor, kit content). Staining was performed according to the manufacturer's instructions. Imaging and analysis were performed using a Universal Hood III and Image Lab 4.1 software.

Protein identification. Protein identification by MALDI-MS/MS was performed by Alphalyse Denmark. Protein samples for protein identification were excised from denaturing reducing Coomassie-stained SDS-PAGE gels. Trypsin-digested protein bands were analyzed on a Bruker Autoflex Speed MALDI TOF/TOF. The MS data were blasted against the NCBI and UniProt database.

Additional protein identification analysis of the 10 kDa protein band of Ana o 3 was performed by LC-MS/MS analysis. On the basis of the results of Shevchenko et al.,²¹ bands cut from SDS-PAGE gel were washed in 0.2 M NH₄HCO₃ in acetonitrile (1:1) followed by addition of 10 mM DTT in 0.1 M NH₄HCO₃ at 56 °C for 45 min. The gel pieces were incubated in 55 mM iodoacetamide (IAA) in 0.1 M NH₄HCO₃ and washed in 0.1 M NH₄HCO₃ in acetonitrile (1:1), and after drying in a SpeedVac, the gel pieces were digested overnight at 37 °C in 0.1 M NH₄HCO₃ with sequencing grade modified porcine trypsin (Promega). Gel pieces were incubated with 25 mM NH₄HCO₃ for 15 min at 37 °C. After addition of acetonitrile the samples were dried and dissolved in 0.1% formic acid. Trypsin-digested peptides were

separated on a Thermo Scientific EASY column (3-μm bead-packed 10 cm C18 column) connected to an Easy-nLC 1000 ultrahigh-pressure system (Thermo Scientific). Peptides were loaded in solvent A (0.1% formic acid in MQ) onto a precolumn (5-μm bead-packed 2 cm C18 column) prior to separation on the analytical column using a 40 min 5–50% linear gradient of solvent B (100% acetonitrile (ACN) in 0.1% formic acid) at a flow rate of 300 nL/min. During the gradient, online MS analysis of peptides was performed with a Q-Exactive Plus (Thermo Scientific) mass spectrometer using a nanoelectrospray source. Ionization (2.4 kV) was performed using a stainless steel emitter and a heated capillary temperature of 250 °C. Full MS scans were acquired over the *m/z* range 400–1500 with a mass resolution of 70 000 (at *m/z* 200). Full scan target was set at 1 × 10⁶ with a maximum fill time of 100 ms. The five most intense peaks with charge states 1–4 were fragmented in the higher-energy collisional dissociation (HCD) collision cell with a normalized collision energy of 30%. The mass range was set to 200–2000 with a mass resolution of 17 500 (at *m/z* 200). The target value for fragment scans was set at 1 × 10⁵, the intensity threshold was kept at 4 × 10⁴, and the maximum allowed accumulation times were 50 ms. The peptide match was set to preferred, isolation width was set at 4 and isotope exclusion was on, and the dynamic exclusion was set to 30 s. LC-MS/MS data acquired by the Q-Exactive were processed using ProteomeDiscoverer software 1.4 (Thermo Scientific). The fragmentation spectra were searched against an Anacardiaceae database downloaded from Uniprot using Sequest HT with precursor mass tolerance of 10 ppm and fragment mass tolerance of 20 mDa. Minimum peptide length was 6 amino acids, and maximum of miscleavages was set to 2. Carbamidomethylation of cysteine was set as fixed modification, and oxidation of methionine was set as variable modification. The percolator was used for false-discovery rate (FDR) estimation using reversed decoy database filtering on *q*-value below 1% for strict and 5% for relaxed stringency.

Analysis of Ana o 3 by UPLC/PDA/ESI-MS. Identification of the heterogeneity of the N- and C-termini was executed by ultra-performance liquid chromatography (UPLC)/photodiode

array (PDA)/electrospray ion source (ESI)-MS. Purified native Ana o 3, denatured and reduced Ana o 3 (10 mM DTT, 1% SDS, 5 min at 100 °C), and proteins eluted from destained SDS-PAGE (50 mM Tris, 0.1 mM EDTA, 0.15 M NaCl, 0.1% SDS, 1 mM DTT, 4 h) were filtered over a 0.5 mL 3 kDa Amicon centrifugal filter and suspended in 200 μ L of eluent A (0.1% trifluoroacetyl (TFA) and 1% ACN in water). The solution was analyzed by LC/PDA/ESI-MS by injecting 2 μ L in an Acquity UPLC separation module equipped with a BEH C4 300 Å column (1.7 μ m, 2.1 $\times$ 100 mm), interfaced with an Acquity PDA detector and a Synapt G2-Si HD mass spectrometer. Gradient elution was performed between eluent A and B. Eluent A was 1% ACN and 0.1% TFA in water; eluent B was 0.1% TFA in ACN. The gradient ramp was 0–2 min isocratic 90% A, 2–12 min linear gradient from 90% A to 25% A, 12–15 min linear gradient from 25% A to 0.1% A, 15–20 min isocratic 0.1% A, plus reconditioning. The flow rate was 0.35 mL/min. PDA detection was performed in the range 200–400 nm, with a resolution of 1.2 nm and a sampling rate of 40 points/s. MS detection was performed in the full scan mode with the following parameters: positive ion mode, capillary voltage 3 kV, source temperature 150 °C, cone voltage 40 V, desolvation temperature 500 °C, cone gas flow (N₂) 200 L/h, desolvation gas flow (N₂) 800 L/h, acquisition range 150–4000 m/z , scan time 0.3 s, and interscan time 0.015 s. The UV chromatogram was obtained by extracting the trace measured at 214 nm from the PDA full scan trace.

Analysis of Ana o 3 tryptic peptides by UPLC/PDA/ESI-MS. Purified Ana o 3 was denatured and reduced (1% SDS, 10 mM DTT, 5 min at 100 °C) and digested with trypsin for 1 h at 37 °C. Samples were analyzed by LC/PDA/ESI-MS by injecting 2 μ L in an Acquity UPLC separation module equipped with a Peptide BEH C18 column, 300 Å (1.7 μ m, 2.1 mm $\times$ 150), interfaced with an Acquity PDA detector and a Synapt G2-Si HD mass spectrometer. Gradient elution was performed between eluent A and B. Eluent A was 1% ACN and 0.1% TFA in water; eluent B was 0.1% TFA in ACN. The gradient ramp was 0–2 min isocratic 97% A, 2–10 min linear gradient from 97% A to 78% A, 12–16 min linear gradient from 78% A to 70% A, 16–19 min from 70% A to 0.1% A, 19–24 min isocratic 0.1% A, plus reconditioning. The flow rate was 0.35 mL/min. PDA detection was performed in the range 200–400 nm, with a resolution of 1.2 nm and a sampling rate of 40 points/s. MS detection was performed in the full scan mode with the following parameters: positive ion mode, capillary voltage 3 kV, source temperature 150 °C, cone voltage 30 V, desolvation temperature 500 °C, cone gas flow (N₂) 200 L/h, desolvation gas flow (N₂) 800 L/h, acquisition range 100–3000 m/z , scan time 0.3 s, and interscan time 0.015 s. Survey MS^c mode was applied with the following parameters: acquisition range 100–3000 m/z , ramp high energy from 20.0 to 30.0, TIC threshold 5.0, survey scan time 0.3 s. Lock spray mass correction was applied by continuously perfusing Angiotensin II as calibrant into the source during the scan. The data were analyzed using BioPharmaLynx 1.3.3 software. Identified peptides were cross-checked manually.

Epitope mapping of Ana o 3. Forty-two peptides of 15 amino acids, with an offset of 3 amino acids, were synthesized by Synpeptide, covering the entire length of Ana o 3 (amino acids 1–138). Peptides were dissolved, and 5 μ mol was spotted in duplicate on nitrocellulose (0.2 μ m, placed on a polyester backbone) alongside two positive controls (0.6 μ g total protein extract and 5 μ g IgE) and a negative solvent control. The same

procedure was followed as explained above for Western blotting with the only difference being that the blots were incubated with plasma from individual patients (group 2) and one blot was incubated with serum from a nonallergic person. The four plasma samples were chosen based on the highest Ana o 3 binding capacity out of the eight plasma samples, as determined by Western blotting (data not shown), for clinical characteristics see Table 1. Staining was performed for 30 min. After washing in TBS, the average pixel intensity of each duplicate spot was calculated using the Image Lab 4.1 software, and the values for the negative serum were deducted per duplicate spot. Strong binding epitopes were defined as the 30% strongest IgE-binding epitopes.

RESULTS

Protein purification. Purification of Ana o 1 and Ana o 3 was achieved by protein extraction from defatted cashew followed by stepwise ammonium sulfate precipitation. The final supernatant, after 52.5% ammonium sulfate precipitation, was used to purify Ana o 1 and Ana o 3. Ana o 2 was purified by gel filtration chromatography of a nondefatted cashew extract. A flow sheet for the purification of Ana o 1, Ana o 2, and Ana o 3 is shown in Figure 1.

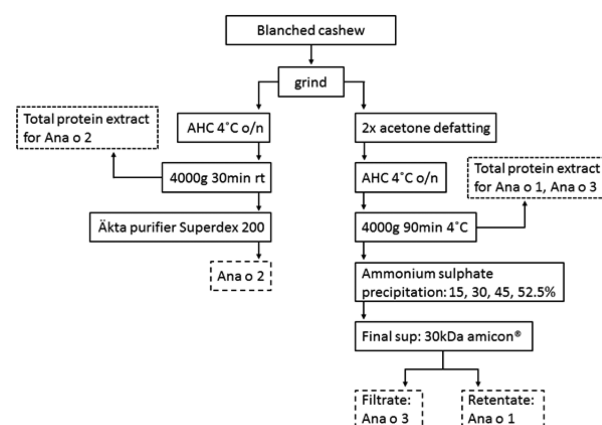


Figure 1. Flow sheet for the purification of Ana o 1, Ana o 2, and Ana o 3. AHC, ammonium bicarbonate buffer; o/n, overnight 16 h; rt, room temperature; final sup, final supernatant.

In Figure 2A, various fractions collected during the purification process of cashew allergens are shown on denaturing and reducing SDS-PAGE gel. The two total protein extracts (defatted and nondefatted) contain several proteins next to Ana o 1, Ana o 2, and Ana o 3, with the 11S globulin Ana o 2 as the most prominent protein. Upon stepwise addition of ammonium sulfate to the total protein extract (defatted), to a final concentration of 15, 30, and 45%, generally Ana o 2 together with various high molecular weight proteins precipitated; see Supporting Information, Figure S-1. After the last step of precipitation (52.5%), the final supernatant contains only Ana o 1 and Ana o 3 (Figure 2A, lane 3); this last fraction was used to purify these two proteins. Omitting the stepwise precipitation steps by directly adding 52.5% ammonium sulfate resulted in a supernatant containing more impurities (data not shown).

The gel filtration chromatogram from the purification of Ana o 2 showed three peaks (Figure 3): peak 1 contains a mixture of several proteins (among others, Ana o 1, Ana o 2, and Ana o 3),

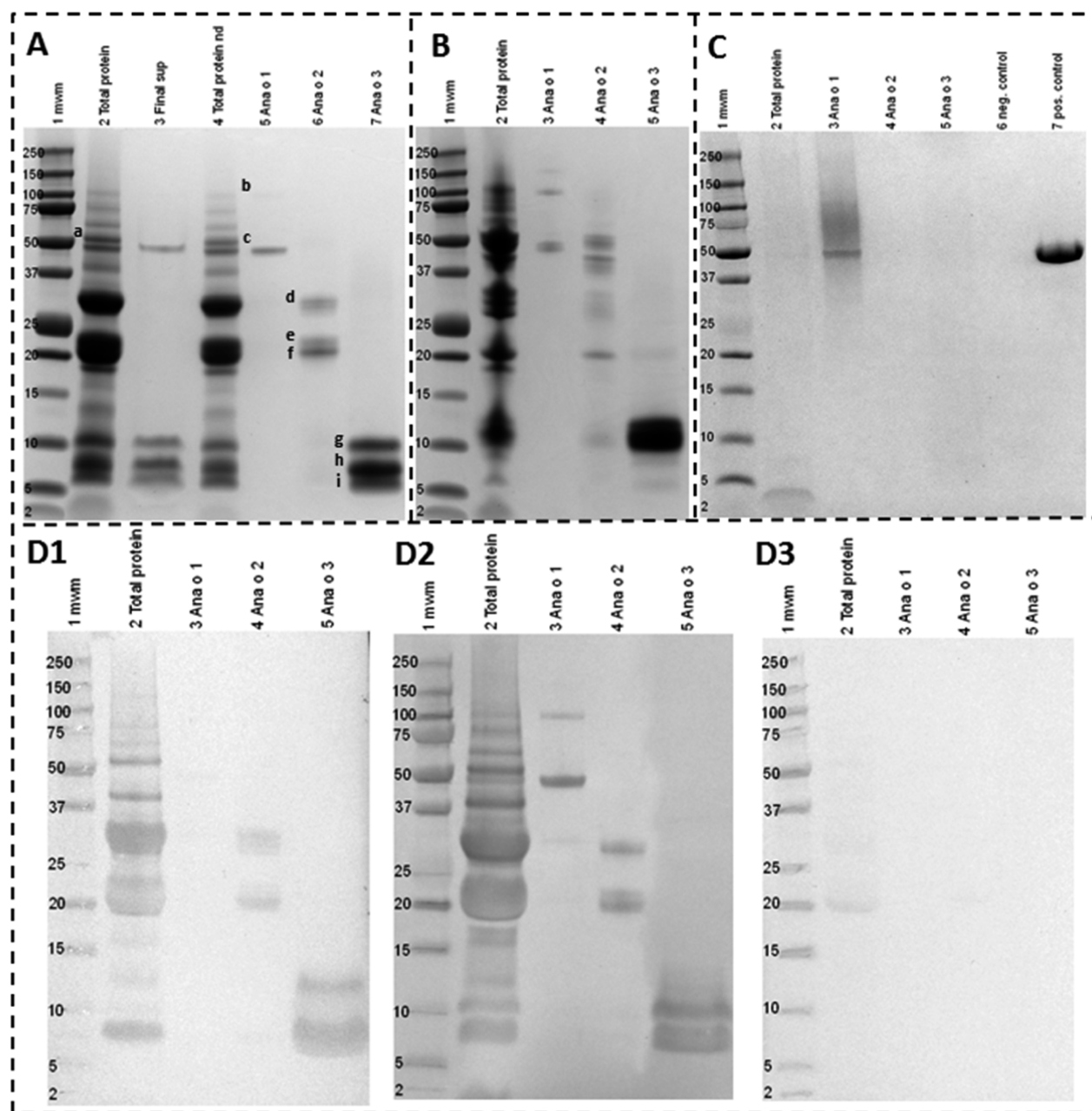


Figure 2. All lanes: mwm, molecular weight marker; Total protein, total protein extract from defatted cashew used for purification of Ana o 1 and Ana o 3; Final sup, final supernatant after 52.5% ammonium sulfate precipitation; Total protein nd, total protein extract from nondefatted cashew used for purification of Ana o 2; neg. control, negative control glycoprotein stain (soybean trypsin inhibitor); pos. control, positive control glycoprotein stain (horseradish peroxidase). (A) Reducing denaturing SDS-PAGE of purified cashew extracts. Letters a–i indicate bands cut for protein identification: (a) 53 kDa Ana o 2, (b) 100 kDa Ana o 1, (c) 50 kDa Ana o 1, (d) 30 kDa Ana o 2, (e) 22 kDa 11S globulin, (f) 21 kDa Ana o 2, (g) 10 kDa Ana o 3, (h) 8 kDa Ana o 3, (i) 6 kDa Ana o 3. (B) Nonreducing denaturing SDS-PAGE of purified cashew extracts, no β -mercaptoethanol added. (C) Glycoprotein stain of purified cashew extracts. (D) Western blots of purified cashew extracts. Blot D1 has been incubated with the serum pool of group 1, blot D2 has been incubated with a plasma pool from group 2, and blot D3 is the antibody control blot that has not been incubated with patient plasma or serum.

peak 2 contains Ana o 1 and Ana o 2, and peak 3 contains the purified Ana o 2 fraction as shown in Figure 2A (lane 6).

Protein identification. As specified in Figure 2A and Figure S-2, on the basis of MALDI-MS/MS, Ana o 1 was confirmed in the 100 kDa band (b) and the 50 kDa band (c). For Ana o 2 the 53 kDa (a), 30 kDa (d), and 21 kDa bands (f) were confirmed as the complete protein, large subunit, and small subunit, respectively. The 22 kDa band (e) was identified as an 11S globulin from pistachio; however, we interpret this to

be Ana o 2 from cashew, as 11S from pistachio and Ana o 2 display 49% sequence identity and 65% sequence similarity (EMBOSS Needle, EMBL-EBI). The three bands in lane 7 (10, 8, 6 kDa; bands g, h, i, respectively) were identified as Ana o 3.

Purity and yield of the proteins. Purities of the Ana o 1, Ana o 2, and Ana o 3 fractions were determined by applying increased protein concentrations (20, 30, and 40 μ g) on SDS-PAGE (data not shown). Purity of these fractions was calculated as the relative percentage of the desired protein

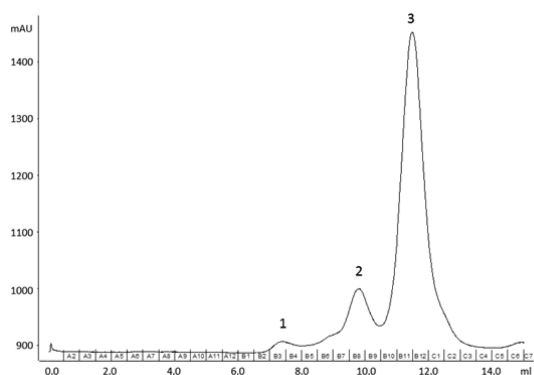


Figure 3. Gel filtration chromatogram of total protein extract of nondefatted cashew used for the purification of Ana o 2.

bands versus visible impurities on SDS-PAGE. Purity of Ana o 1 was $95.6\% \pm 3.3\%$, purity of Ana o 2 was $92.6\% \pm 4.6\%$, and purity of Ana o 3 was $98.5\% \pm 1.3\%$. Protein yield of the purified fractions was calculated based on the Coomassie protein assay and SDS-PAGE analysis of several purified fractions. Overall yield is 1% for Ana o 1 (0.33 mg per run), 34% for Ana o 2 (1.99 mg per run), and 3% for Ana o 3 (5.52 mg per run).

Western blot. In Figure 2D, two Western blots performed with two different pools of patient serum/plasma are shown; blot D1 was performed using 13 sera from cashew-allergic children as determined by DBPCFC, while blot D2 was performed using 8 plasma samples from cashew- or tree-nut-allergic adults. Less strong IgE-binding to cashew allergens is observed using serum from group 1 (blot D1), compared to

plasma from group 2 (blot D2). Blot D1 shows binding of IgE to Ana o 2 (lane 4; 30, 22, and 21 kDa), Ana o 3 (lane 5; 10 and 8 kDa, not 6 kDa), and slightly to Ana o 1 (lane 3; 50 kDa). In the total protein extract, the same proteins as well as additional proteins of 80, 70, 53 (full length Ana o 2), 40, and 16 kDa appear IgE-reactive. In blot D2 binding of IgE to Ana o 1 (lane 3; 100 and 50 kDa), Ana o 2 (lane 4; 30, 22, and 21 kDa), and Ana o 3 (lane 5; 10 and 8 kDa, not 6 kDa) is visible. In the total protein extract, the same proteins as well as additional proteins of 80, 65, 53 (Ana o 2), 40, 16, and 12 kDa bind IgE. In blot D2, lane 3, a small impurity of 30 kDa (Ana o 2) can be observed in the purified Ana o 1 extract. In the third blot, D3, the antibody control blot is shown, demonstrating marginal nonspecific binding of the antibodies to the 22–21 kDa subunits of Ana o 2. These Western blots indicate that all three purified protein fractions are able to bind IgE.

Inhibition blotting, presented in Figure S-3, shows that purified Ana o 1 completely inhibited binding to Ana o 1, and Ana o 3 completely inhibited binding to Ana o 3 in the total protein extract. Besides inhibition of binding to Ana o 1, purified Ana o 1 also diminished IgE binding to Ana o 2 and Ana o 3.

Nonreducing denaturing SDS-PAGE. To check the presence of disulfide bridges in the purified allergen fractions, a nonreducing denaturing SDS-PAGE, in the absence of β -mercaptoethanol, was performed. In the Ana o 1 fraction (Figure 2B, lane 3) protein bands of 160, 100, and 50 kDa can be observed. In lane 4, containing Ana o 2, diffuse bands of 53, 42, 39, 30, 20, and 10 kDa are present. Ana o 3 (lane 5) shows an abundant protein band of 12 kDa and minor bands of 20 and 5 kDa.

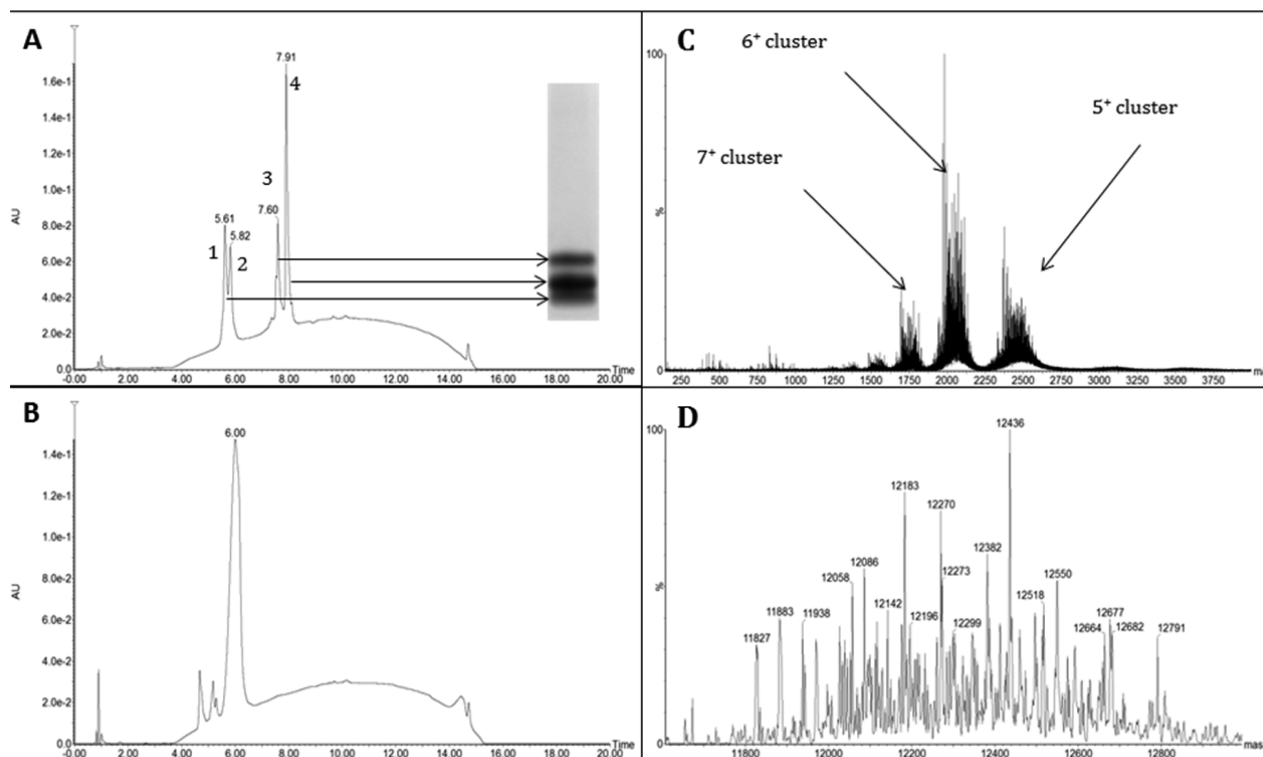


Figure 4. Chromatographic analysis (UV trace at 214 nm) of denatured (A) and native (B) Ana o 3. MS spectrum associated with the major peak in native Ana o 3 (full spectrum from 200 to 4000 m/z) showing the clusters corresponding to the 7⁺-charged, 6⁺-charged, and 5⁺-charged ions (C), and charge-state deconvolution of this spectrum shown in (C), performed by the MaxEnt software, indicating the actual molecular masses of the main isoforms present (D).

Table 2. Molecular Masses of Denatured Ana o 3 Derived from UPLC/PDA/ESI-MS Analysis and Their Tentative Sequence Identification

	tentative sequence annotation within the range 0.5 Da from the theoretical calculated mass	experimental molecular mass (Da)	leader peptide ^b	small subunit ^b	large subunit ^b
leader peptide	1–20 ^a 1–20/28 ^b				
small subunit	21–65 ^a 29–65 ^b				
large subunit	66–138 ^a 69–137 ^b				
chromatographic peak 1 (Rt = 5.61)	34–62/35–63	3742.7		X	
	34–63/35–64/36–65	3799.7		X	
	33–63	3886.8		X	
	33–65	4099.9		X	
chromatographic peak 2 (Rt = 5.82)	37–64	3514.6		X	
	37–65	3670.7		X	
	22–53	3959.8		X	
	39–69	4016.8		X	X
	33–66	4263.1		X	
	31–66	4507.1		X	
chromatographic peak 3 (Rt = 7.60)	2–70 or 56–122 or unknown isoform	8161.7	X	X	X
	1–71 or unknown isoform	8421.8	X	X	X
chromatographic peak 4 (Rt = 7.91)	69–136	8084.7			X
	69–137	8171.7			X

^aAccording to Moreno and Clemente.¹⁶ ^bAccording to our data.

Glycosylation of cashew proteins. On the basis of prediction models (NetNGlyc 1.0, Denmark, <http://www.cbs.dtu.dk/services/NetNGlyc/>), the amino acid sequences of Ana o 1 (amino acid 391, asparagine) and Ana o 3 (amino acid 19, asparagine), but not that of Ana o 2, contain putative N-glycosylation motifs. The predicted glycosylation of Ana o 1 was confirmed by glycoprotein staining (see Figure 2C). Both Ana o 2 and Ana o 3 do not seem to be glycosylated, neither in the purified nor in the total protein fraction. In addition an unknown protein of 3 kDa, present in the full protein extract, also seems to be glycosylated.

Analysis of Ana o 3. Protein characterization and protein identification of Ana o 3 revealed some discrepancies with data from literature. It was previously reported that all three denatured protein bands from SDS-PAGE represented the large subunit of Ana o 3.¹⁵ However, our results identify one of the bands as the small subunit from Ana o 3. Besides, the IgE binding on Western blot to two out of three bands of denatured Ana o 3 indicated the possibility of one of these bands possibly being the small subunit of Ana o 3. To analyze this possibility, both denatured reduced and native Ana o 3 samples were analyzed for peptide mass, purity, and annotation of protein sequences using UPLC/PDA/ESI-MS.

Denatured and reduced Ana o 3 shows four peaks (Figure 4A). Peaks 1 and 2, corresponding to the 6 kDa protein band on SDS-PAGE, were compatible with the small subunit of Ana o 3 with molecular weights of 3.7–4.5 kDa. Peak 3 (10 kDa band) and peak 4 (8 kDa band) were both compatible with the large subunit of Ana o 3 with molecular weights of 8.1–8.4 kDa. Using both trypsin-digested and nondigested denatured reduced Ana o 3, the peptide sequences from the four peaks were annotated (see Table 2). The range of protein molecular weights in the four peaks was found to originate from N- and C-terminal microheterogeneity, also called N- or C-terminal clipping (see Table 3 and Figure 5). Tryptic peptides clearly showed that the small subunit displayed both N- and C-terminal microheterogeneity, with its N-terminus starting at

Table 3. Sequence Annotation of Ana o 3 Tryptic Peptides Including Termini Based on Molecular Mass (<10 ppm Error) and Fragment Identification

peptide <i>m/z</i> (charge state)		identified peptide sequence
565.9 (3+)	36–48	<i>N</i> -termini small subunit until trypsin cleavage site (amino acid 48)
618.0 (3+)	35–48	
637.0 (3+)	34–48	
666.0 (3+)	33–48	
704.3 (3+)	32–48	
747.3 (3+)	31–48	
790.3 (3+)	30–48	
823.4 (3+)	29–48	
660.4 (2+)	55–65	trypsin cleavage site (amino acid 55) until <i>C</i> -termini small subunit
582.3 (2+)	55–64	
553.8 (2+)	55–63	
759.7 (3+)	69–86	<i>N</i> -terminus large subunit until trypsin cleavage site (amino acid 86)
786.4 (2+)	123–137	trypsin cleavage site (amino acid 123) until <i>C</i> -termini large subunit
742.8 (2+)	123–136	

MAKFLLLSAFAVLLLVANASIYRAIVEVEEDSGREOSCORO
FEEQORFRNCORYVKQEVQGGRYNOROESLRECCQELQ
EVDRCRCRCONLEQMVROLOOQEQIKGEVRELYETASELP
RICSISPSQGCQFOSSY

Figure 5. Amino acid sequence (coding region) of Ana o 3, with the small and large subunits indicated as based on UPLC/PDA/ESI-MS analysis. The leader peptide is underlined, the black bars indicate the constant regions, and the light gray bars indicate regions of microheterogeneity. A dotted box is placed around the small subunit (first box) and the large subunit (second box) of Ana o 3.

amino acid 29–36 and its C-terminus terminating at amino acid 63–65. The large subunit displayed only minimal C-terminal microheterogeneity, starting at amino acid 69 and ending at amino acid 136–137. When Ana o 3 was purified using

Table 4. Linear Epitope Mapping of Ana o 3 Using Plasma of Four Individuals (no. 1–4) from Group 2; Bold Peptide Numbers Indicate a Strong IgE-Binding Epitope, Bold Amino Acid Numbers Indicate an IgE-Binding Peptide Region; Underlined Amino Acid Sequences Indicate the Small Subunit of Ana o 3 (Amino Acids 29–65), between the Leader Sequence (1–20) and the Large Subunit (69–137) of Ana o 3

peptide	amino acid no.	amino acid sequence	IgE-binding intensity			
			1	2	3	4
1	1–15	MAKFLLLLSAFVLL				
2	4–18	FLLLLSAFVLLVA				
3	7–21	LLSAFVLLLVANAS				
4	10–24	AFVLLLVANASIYR	+	+	+	++
5	13–27	VLLLVANASIYRAIV	+	+		+
6	16–30	LVANASIYRAIVE VE	+	+		+
7	19–33	NASIYRAIVE VEEDS				
8	22–36	IYRAIVE VEEDSGRE				
9	25–39	AIVE VEEDSGREQSC				
10	28–42	EVEEDSGREQSCQRQ				
11	31–45	EDSGREQSCQRQFEE				
12	34–48	GREQSCQRQFEEQQR				
13	37–51	QSCQRQFEEQQRFRN				
14	40–54	QRQFEEQQRFRNCQR	++			
15	43–57	FEEQQRFRNCQRYVK				
16	46–60	QQRFRNCQRYVKQEV				
17	49–63	FRNCQRYVKQEVQRG	+	++		
18	52–66	CQRYVKQEVQRGGRY				
19	55–69	YVKQEVQRGGRYNQR	++	+++		+
20	58–72	QEVQRGGRYNQRQES				
21	61–75	QRGGRYNQRQESLRE				
22	64–78	GRYNQRQESLRECCQ				
23	67–81	NQRQESLRECCQELQ				
24	70–84	QESLRECCQELQEV				
25	73–87	LRECCQELQEVDRRC				
26	76–90	CCQELQEVDRRCRCQ	+		++	+
27	79–93	ELQEVDRRCRCQNLE				
28	82–96	EVDRCRCQNLEQMV				
29	85–99	RRRCQNLEQMVRQL	++	+++	+	+
30	88–102	RCQNLEQMVRQLQQQ	++	++	++	++
31	91–105	NLEQMVRQLQQEQI				
32	94–108	QMVRQLQQEQIQKGE	++	++	++	+++
33	97–111	RQLQQEQIQKGEEVR				
34	100–114	QQEQIQKGEEVRELY				
35	103–117	EQIKGEEVRELYETA				
36	106–120	KGEEVRELYETASEL				
37	109–123	EVRELYETASELPRI				
38	112–126	ELYETASELPRICSI	+		+	
39	115–129	ETASELPRICISISPS				
40	118–132	SELPRICISISPSQGC				
41	121–135	PRICISISPSQGCQFQ	+++	+++	+	+
42	124–138	CSISPSQGCQFQSSY				
total cashew positive control			+++	+++	+++	+++

protease inhibitors, the same degree of microheterogeneity was obtained (data not shown).

Native Ana o 3 eluted as one major peak of 11.8–12.8 kDa (Figure 4C and D) with a purity of 88% (Figure 4B, purity calculated by UV trace at 214 nm). The observed masses fitted with the molecular masses of the small subunit (peaks 1 and 2) linked to the large subunit (peak 4) by two disulfide bridges. Together with the two other predicted disulfide bridges within the large subunit,¹⁶ a total of 4 disulfide bridges can be found within native Ana o 3. Besides demonstrating microheterogeneity in Ana o 3, the obtained data also indicate the presence of isoforms of the large subunit.

Epitope mapping of Ana o 3. Using 42 overlapping linear peptides, 12 IgE-binding peptides were detected in Ana o 3 (see Table 4). Two IgE-binding peptide regions were identified, with the first consisting of amino acids 10–30 and the second, more strongly IgE-bound region consisting of amino acids 85–108. Besides 4 mildly binding epitopes described in Table 4, 2 strongly binding peptides were identified at amino acids 55–69 and 121–135. All strongly binding epitopes were detected in the peptides of the large subunit or containing part of the large subunit.

DISCUSSION

In this study we have purified and characterized the three major cashew allergens Ana o 1, Ana o 2, and Ana o 3. Our method enables the simultaneous purification of Ana o 1 and Ana o 3.

Using precipitation, ultrafiltration, and gel filtration chromatography, we have isolated three cashew allergens with high purity. The purities of the single allergen fractions are approximately 96% for Ana o 1, 93% for Ana o 2, and 99% for Ana o 3 as based on SDS-PAGE and 88% for Ana o 3 based on high-performance liquid chromatography (HPLC) analysis. The only IgE-reactive impurity we observed, as established by Western blotting, was a protein of 30 kDa in the Ana o 1 fraction (Figure 2, D1). A substantial difference in IgE-binding to Ana o 1 is apparent between the two patient groups (blots D1 and D2), which might be explained by a population difference of the sera. Group 1 (blot D1) consists of Dutch children, and group 2 (blot D2) consists of American adults. Geographical and age differences have been described before to explain differences in allergen binding.^{22,23}

For Ana o 3 we observed only IgE binding to the 10 and 8 kDa proteins and not to the 6 kDa protein. This is consistent with other Western blots performed by us using both various patient pools and individual patient sera. Teuber et al., however, did detect IgE binding to the 6 kDa protein.²⁰ Our analyses show that the 6 kDa protein is the small subunit of Ana o 3 while both the 10 and 8 kDa proteins represent the large subunit. Epitope mapping of Ana o 3 by Robotham et al.¹⁵ showed more strongly binding epitopes on the small subunit compared to our data. Table 4 shows that our patient IgE binds strongest to the peptides of the large subunit. One mildly IgE-binding peptide region was identified between the presumptive leader sequence¹⁵ and the start of the small subunit as determined by UPLC/PDA/ESI-MS analysis. It is unknown if this peptide is present in cashew when eaten; however, IgE binding to leader peptides that are not attached to the mature allergen has been observed before for Ana o 1⁹ and Ana o 2.¹¹

On Western blot, besides binding of IgE to Ana o 1, Ana o 2, and Ana o 3, several other proteins were bound in the total protein extract (Figure 2, D1 and D2). These allergens of 12, 16, 40, 65, 70, and 80 kDa have been observed before^{20,24} but have not been identified.

The yield of purification for Ana o 1 is 1%, for Ana o 2 is 34%, and for Ana o 3 is 3%. The low yield can be explained by the precipitation steps of the total protein extract where Ana o 1 and Ana o 3 are discarded along with the undesirable proteins. However, as the protein content of cashew is 18.8%,²⁵ the amount of cashew needed is relatively low. No isoform selection is expected as inhibition blotting clearly shows complete inhibition of IgE binding to Ana o 1 and Ana o 3 in the total protein extract upon inhibition with, respectively, purified Ana o 1 or Ana o 3. Besides inhibition of IgE binding to Ana o 1, purified Ana o 1 also inhibited binding of IgE to Ana o 3 and partially to Ana o 2. Cross-reactivity between Ana o 1 and Ana o 3 was unexpected, although some cross-reactivity between recombinant Ana o 1 and Ana o 2 has been shown before.¹¹ Cross-reactivity between 7S globulins, 11S globulins, and 2S albumins has been observed before for peanut.²⁶

Glycoprotein staining of the cashew protein fractions showed Ana o 1 and an unknown protein of 3 kDa to be glycosylated. Both Ana o 2 and Ana o 3 are not glycosylated. Glycosylation of cashew proteins has been studied once before²⁷ where only one glycosylated protein of ~3 kDa was indicated. In their

study Sathe et al. used a total protein extract that, as can also be observed from our glycoprotein stained gel, possibly did not contain enough Ana o 1 to show a clear band in glycoprotein staining. Another explanation could be a difference in cashew variety used.

Under nonreducing denaturing conditions, Ana o 1 is present as both a dimer of 100 kDa and a single protein of 50 kDa. Nonreduced denatured Ana o 2 displays some protein bands that are also visible in reducing denaturing SDS-PAGE: the 53 kDa full protein and 30 and 21 kDa subunits. Ana o 3 seems to form one complex of ~12 kDa linked by disulfide bridges, which was confirmed by LC/ESI-MS.

Purified Ana o 3 manifested as a triplet of 10, 8, and 6 kDa proteins on SDS-PAGE under denaturing reducing conditions. Native 2S-albumins are post-translationally processed into an N-terminal small subunit of ~5.5 kDa and a C-terminal large subunit of ~8.8 kDa, held together via cysteine linkages.¹⁶ In an attempt to further identify the protein triplet, we subjected both native and denatured reduced Ana o 3 to LC-MS analysis. This indicated the 10 and 8 kDa proteins to be the large subunit of 8.2–8.4 and 8.1–8.2 kDa, respectively, and the 6 kDa protein to be the small subunit of 3.7–4.5 kDa. Tryptic digestion of Ana o 3 indicated the small subunit to span amino acids 29–36 until 63–65, including N- and C-terminal microheterogeneity, and the large subunit to span amino acid 69 until 136–137, including C-terminal microheterogeneity, differing slightly from the proposed amino acid stretches proposed by Moreno et al.¹⁶ The same degree of microheterogeneity was observed when Ana o 3 was purified in the presence of protease inhibitors, indicating this microheterogeneity is not an artifact from the method of extraction but is due to proteolytic processes, the presence of different precursors, or the shift in cleavage sites in the cashew itself.¹⁶ Microheterogeneity of 2S albumins has been described before for castor bean,²⁸ Brazil nut,²⁹ and sesame.³⁰

This data differs from the data reported by Robotham et al., who described all three protein bands to be isoforms of the large subunit of Ana o 3 with very similar N-terminal sequences, differing in only 1–2 amino acids out of 10.¹⁵ An explanation for this discrepancy could lay in a difference in cashew variety or storage conditions.

As food allergy is highly prevalent and the incidence of cashew allergy seems to be rising,³¹ it is relevant to study which proteins are involved. Ana o 1, Ana o 2, and Ana o 3 are the three major allergens in cashew. Using pure allergen fractions allows for determining to which specific cashew allergen a patient reacts, and they can be used in cross-reactivity studies, where pure allergens can be used for inhibition blotting. Besides, these allergen fractions can also be used for analysis of allergen structure, comparing cashew varieties, and cashew protein processing stability.

The advantage of using purified allergens over the use of recombinant allergens is clear for Ana o 1 and Ana o 3. Recombinant expression of a glycosylated allergen like Ana o 1 is difficult as both prokaryotic (e.g., *E. coli*) and eukaryotic (e.g., *P. Pastoris*) expression systems may not be capable of reproducing the natural glycation pattern as in cashew.^{32,33} Considering Ana o 3, recombinant expression will not properly display the microheterogeneity of the native protein; however, recombinant proteins are required to compare microheterogenic variations of Ana o 3. Besides, other post-translational processing modifications such as disulfide bridges (Ana o 2, Ana o 3) might prove more difficult in *E. coli* expression systems.³²

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b04401.

Pellets of protein precipitation obtained during the purification of Ana o 1 and Ana o 3; protein identification results from MALDI-MS/MS analysis and LC-MS/MS analysis; inhibition Western blots of Ana o 1 and Ana o 3 (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

final sup, final supernatant; MQ, Milli-Q water; mwm, molecular weight marker; neg. control, negative control; pos. control, positive control; TBS, tris-buffered saline

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Supporting information

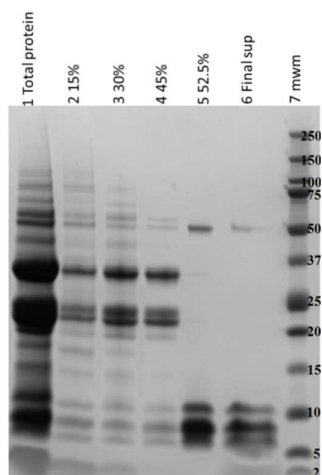


Figure S-1: Reducing denaturing SDS-PAGE of ammonium sulphate precipitation. Depicted are (left to right): total protein extract from defatted cashew used for purification of Ana o 1 and Ana o 3, 15%, 30%, 45%, and 52.5% pellet of ammonium sulphate precipitation, final supernatant after 52.5% ammonium sulphate precipitation, molecular weight marker.

100kDa protein band:

Identified as, Ana o 1, vicilin-like protein (*Anacardium occidentale*), GenBank AAM73729.1

14% sequence coverage, Mascot score 179

PPTKFSFSLFLVSVLVLCLGFALAKIDPELKQCKHQCKVQRQYDEQQKEQCVKECEKYYK
EKKGREHEHEEEEEWGTGGVDEPSTHEPAEKHLSQCMRQCERQEGGQQKQLCRFCQER
YKKEGQHNYKREDEDEDEDEAEEDENPYVFEDEFTTKVKTEQGGVLLPKFTQKSK
LLHALEKYRLAVLVANPQAFVVPVSHMDADSIFFVSWGRGTITIKLENKRESINVRQGDIV
SISSGTPFYIANNDENKLYLVQFLRPVNLPGHFEVFHGPGENPESFYRAFWEILEAA
LKTSKDTLEKLFKQDQGTIMKASKEQVRAMSRREGPKIWPFTTEESTGSFKLFKKDPSQ
SNKYGQLFEAERIDYPPLEKLDVVSYANITKGGMSVPFYNSRATKIAIVSGEGCIEA
CPHLSSSKSSHPYKRLRARIRKDTVFIVPAGHPFATVASGNENLEIVCFVNAEGNIRY
TLAGKKNIIKVMKEAKELAFKMEGEEVDKVFQKQDEEFFQGPWEWRKEGRADE

50kDa protein band:

Identified as Ana o 1, vicilin-like protein (*Anacardium occidentale*), GenBank AAM73729.1

36% sequence coverage, Mascot score 360.

PPTKFSFSLFLVSVLVLCLGFALAKIDPELKQCKHQCKVQRQYDEQQKEQCVKECEKYYK
EKKGREHEHEEEEEWGTGGVDEPSTHEPAEKHLSQCMRQCERQEGGQQKQLCRFCQER
YKKEGQHNYKREDEDEDEDEAEEDENPYVFEDEFTTKVKTEQGGVLLPKFTQKSK
LLHALEKYRLAVLVANPQAFVVPVSHMDADSIFFVSWGRGTITIKLENKRESINVRQGDIV
SISSGTPFYIANNDENKLYLVQFLRPVNLPGHFEVFHGPGENPESFYRAFWEILEAA
LKTSKDTLEKLFKQDQGTIMKASKEQVRAMSRREGPKIWPFTTEESTGSFKLFKKDPSQ
SNKYGQLFEAERIDYPPLEKLDVVSYANITKGGMSVPFYNSRATKIAIVSGEGCIEA
CPHLSSSKSSHPYKRLRARIRKDTVFIVPAGHPFATVASGNENLEIVCFVNAEGNIRY
TLAGKKNIIKVMKEAKELAFKMEGEEVDKVFQKQDEEFFQGPWEWRKEGRADE

53kDa protein band:

Identified as, Ana o 2 (*Anacardium occidentale*), GenBank AAN76862.1

27% sequence coverage, Mascot score 156

LSVCFILFHGCLASRQEWQQQDECQIDRLDALEPDNRVEYEAGTVEAWDPNHEQFRCAG
VALVRHTIQPNGLLPQYSNAPQLIYVVQGEGMTGISYPGCPETYQAPQQGRQQQSGRF
QDRHQKIRRRFRGDIIAIPAGVAHWYCYNENSPVTVTLTLDVSNQNLDRTPRKFLHLAG
NPKDVFQQQQQHQSRRNLFSGFDELLAEAFQVDERLIKQKSEDNRGGIVKVKDELRL
VIRPSRSQSERGSESEEESEDEKRRWQQRDNGIETICTMRLKENINDPARADIYTPVVG
RLTTLNSLNLPILKWLQLSVEKGVLYKNALVLPWNLNSHSIYGCKGKGQVQVVDNFGN
RVFDGEVREGQMLVVPQNFVVKRAREERFEWISFKNDRAMTSLAGRTSVLGGMPPEEV
LANAFQISREDARKIKFNNQTTLTSGESSHHMRDDA

30kDa protein band:

Identified as, Ana o 2 (*Anacardium occidentale*), GenBank AAN76862.1

35% sequence coverage, Mascot score 306

LSVCFILFHGCLASRQEWQQQDECQIDRLDALEPDNRVEYEAGTVEAWDPNHEQFRCAG
VALVRHTIQPNGLLPQYSNAPQLIYVVQGEGMTGISYPGCPETYQAPQQGRQQQSGRF
QDRHQKIRRRFRGDIIAIPAGVAHWYCYNENSPVTVTLTLDVSNQNLDRTPRKFLHLAG
NPKDVFQQQQQHQSRRNLFSGFDELLAEAFQVDERLIKQKSEDNRGGIVKVKDELRL
VIRPSRSQSERGSESEEESEDEKRRWQQRDNGIETICTMRLKENINDPARADIYTPVVG
RLTTLNSLNLPILKWLQLSVEKGVLYKNALVLPWNLNSHSIYGCKGKGQVQVVDNFGN
RVFDGEVREGQMLVVPQNFVVKRAREERFEWISFKNDRAMTSLAGRTSVLGGMPPEEV
LANAFQISREDARKIKFNNQTTLTSGESSHHMRDDA

22kDa protein band:

Identified as, 11S globulin (*Pistacia vera*), GenBank ABU42022.1

13% sequence coverage, Mascot score 138

MGYSLLSFSGLFLLFHCSFAQIEQVVNSQQRQQQRFQTQCQIQNLNALEPKRRIESE
AGVTEFWQDQNEEQQLQCANVAVFRHTIQSRGLLVPSYNNAPELVYVVQSGSIHGAVFPGCP
ETFQEESQSQRSQHSRSERSQQSGEQHQKVRHIREGDIIALPAGVAHWIYNNQSKLVL
VALADVGENSENLQDQYLRKFLVGGSPQQEIQGGGQSWQSRSRKGQQSNILSAFDEEI
LAQSLNIDTQLVKKLQREEKQRGIIVRVKEDLQVLSPQRQEKEYSDNGLEETFCTMTLKL
NINDPSRADVYNPRGGRVTSINALNLPILRFLQLSVEKGVLYQNAIMAPHWNMNAHSIVY
ITRGNRMQIVSENGESVFDEEIREGQLVVPQNFVAVKRASSDGFVWVSFKTNGLAKIS
QLAGRISVMRGLPLDVIQNSFDISREDAWNLIKESREMTIFAPGSRSQRRN

21kDa protein band:

Identified as, Ana o 2 (*Anacardium occidentale*), GenBank AAN76862.1

30% sequence coverage, Mascot score 400

LSVCFLLFHGCLASRQEWQQQDECQIDRLDALEPDNRVEYEAGTVEAWDPNHEQFRCAG
VALVRHTIQPNGLLPQYSNAPQLIYVVQGEGMTGISYPCPETYQAPQQGRQQQSGRF
QDRHQKIRRRFGDIIAIPAGVAHWYCNENSPVTVTLTLDVSNQNLDRTPRKFHLAG
NPKDVFQQQQHQSRGRNLFSGFDELLAEAFQVDERLIKQLKSEDNRGGIVKVKDELRL
VIRPSRSQSERGSESEEESEDEKRRWGQRDN**GIEETICTMRLKENINDPARADIYTPEVG**
RLTTLNSLNLPILKWLQLSVEKGVLYKNALVLPHWNLNSHSIYGCKGKGQVQVVDNFGN
RVFDEGEVREGQMLVVPQNFVAVKRAREERFEWVSFKTNDRAMTSLAGRTSVLGGMPPEV
LANAFQISREDARKIKFNNQQTTLTSGESSHMRDDA

10kDa protein band: (LC-MS/MS analysis)

Identified as, Ana o 3, 2s Albumin (*Anacardium occidentale*), GenBank AAL91665.1

33% sequence coverage

MAKFLLLSAFAVLLLVANASIYRAIVEVEEDSGREQSCQRQFEEQQRFRNCQRYVKQEV
QRGGRYNQ**RQESLRECCQELQEVDRRCRCQNLEQMVRQLQQQEQIKGEEVRELYETASEL**
PRICISPSQGCQFQSSY

8kDa protein band:

Identified as, Ana o 3, 2s Albumin (*Anacardium occidentale*), GenBank AAL91665.1

54% sequence coverage, Mascot score 407

MAKFLLLSAFAVLLLVANASIYRAIVEVEEDSGREQSCQRQFEEQQRFRNCQRYVKQEV
QRGGRYNQRQESLRECCQELQEVDRRCRCQNLEQMVRQLQQQEQIKGEEVRELYETASEL****
PRICISPSQGCQFQSSY

6kDa protein band:

Identified as, Ana o 3, 2s Albumin (*Anacardium occidentale*), GenBank AAL91665.1

52% sequence coverage, Mascot score 336

MAKFLLLSAFAVLLLVANASIYRAIVE**VEEDSGREQSCQRQFEEQQRFRNCQRYVKQEV**
QRGGRYNQRQESLRECCQELQEVDRRCRCQNLEQMVRQLQQQEQIKGEEVRELYETASEL****
PRICISPSQGCQFQSSY

Figure S-2: Protein identification, analysis of protein bands from SDS-PAGE indicated in Figure 2. Results based on MALDI-MS/MS analysis unless stated otherwise. Indicated are the identified protein, GenBank number, sequence coverage, Mascot score, and the protein sequence with matched peptides underlined. For the proteins identified as Ana o 3 the large subunit (10 and 8kDa protein band) or the small subunit (6kDa protein band) as based on Figure 5 are indicated in bold. For the proteins identified as Ana o 2 the large subunit¹¹ (30kDa protein band) or the small subunit¹¹ (21kDa protein band) are indicated in bold.

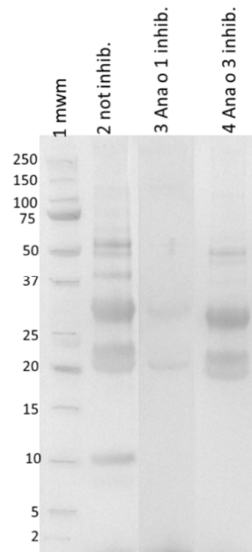


Figure S-3: inhibition western blot of Ana o 1 and Ana o 3, using plasma pool of group 2. Molecular weight marker followed by 3 lanes containing a total protein extract from defatted cashew used for purification of Ana o 1 and Ana o 3. Lane 2 is not inhibited, the plasma used in lane 3 was inhibited with 1mg/ml purified Ana o 1, the plasma used in lane 4 is inhibited with 1mg/ml purified Ana o 3.