

RESEARCH LETTER

Seed storage 2S albumins are predictive indicators of exclusive Anacardiaceae cross-reactivity

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Pistachio (*Pistachio vera*) and cashew nut (*Anacardium occidentale*), members of the *Anacardiaceae* family, account for a significant percentage of food allergies worldwide.¹ Nuts are one of the main causes of food anaphylaxis, eliciting a significant number of life-threatening reactions, which are aggravated by the fact that these allergens are often hidden in processed foods. Variability of allergic phenotypes to nut allergens with complex cross-reactivity profiles complicates clinical management of the patient.² In this sense, several allergenic patterns have been defined according to different factors such as geographical area, age, eating habits, pollinosis, and so on.

Most nut allergens belong to a small number of protein families, which homologous members share three-dimensional structure, biologic function and sequence identity, exhibiting inter and intra-species cross-reactivity. In a clinic context, it is important to establish correlations within each food allergen family based on their cross-reactivity and their ability to trigger systemic reactions or anaphylaxis. Nowadays, the availability of few purified and well-characterized allergens makes difficult to predict which of them are included in these particular associations. Different nuts clusters, grouped around one allergen, make it possible to optimize the management of nut allergy.

Previous studies have shown that pistachio and cashew nut presented a high degree of cross-reactivity at the specific IgE (sIgE) level, owing to their botanic proximity.³ Furthermore, at least 86% of patients allergic to nuts are also allergic to multiple species⁴ and for cashew or pistachio allergic subjects a significant proportion are poly-sensitized to different nuts.

Several major allergens have been identified in cashew -Ana o 1 (7S vicilin), Ana o 2 (11S globulin), Ana o 3 (2S albumin),⁵ and in

pistachio -Pis v 1 (2S albumin), Pis v 2 and 5 (11S globulin), Pis v 3 (7S globulin) and Pis v 4.⁶ Except Pis v 4, a manganese superoxide dismutase, all others are seed storage proteins. Prior studies showed that 7S vicilins and 11S globulins from different tree nuts exhibit moderate cross-reactivity at IgE level.⁷ However, cross-reactivity mediated by 2S albumins has not been well explored and appears to be infrequent and, as in *Brassicaceae* seeds, nearly restricted to members of phylogenetically related families.⁸

The aim of the present study was to evaluate a selected group of six patients with allergy to pistachio and/or cashew nut exclusively sensitized to 2S albumins. Results of this study proved that there is IgE cross-reactivity between these two *Anacardiaceae* 2S albumins but they were not cross-reactive to 2S albumins from other sources, which will provide significant data on the clinical management of this subgroup of patients. These patients displayed clinical features described in Table 1. All the patients reported systemic symptoms when eating pistachio and/or cashew nut and showed positive skin test to these nut extracts and sIgE to their 2S albumins, Pis v 1 and Ana o 3, respectively. None of them was sensitized to other nuts or purified 2S albumins (skin test or specific IgE), or allergic to other nuts, which was confirmed by oral challenge test. ImmunoCAP revealed that all patients, with the exception of one patient who have sIgE < 0.1 kU/L against pistachio, were positive to both nuts, pistachio and cashew. Sensitization to pistachio in this patient was confirmed by ELISA and immunoblotting. Controls did not show any positive result. IgE-binding assays showed similar protein pattern recognition to cashew nut and pistachio extracts (Figure 1A). Five out of the six patients (83%) with systemic reactions recognized bands around 14-kDa in both pistachio and cashew nut extracts. IgE-reactive proteins were separated by using a combination of size exclusion chromatography

Informed consent was obtained from the patients to write the manuscript.

in Sephadex G-50 and RP-HPLC. The IgE-recognized proteins were visualized by SDS-PAGE (Figure S2A). The identification of 14-kDa proteins as 2S albumins from cashew nut, and pistachio, already named Ana o 3 and Pis v 1, respectively, was performed by their fingerprint using MALDI-TOF-TOF mass-spectrometry techniques

(Figure S2B), with a 44% and 28% of primary structure coverage of peptides sequenced, respectively. Under reducing conditions, they were split in two subunits of 8 kDa and 4 kDa, linked each other by disulphide bonds due to the presence of a conserved cysteine residues scaffold (Figure S2A).

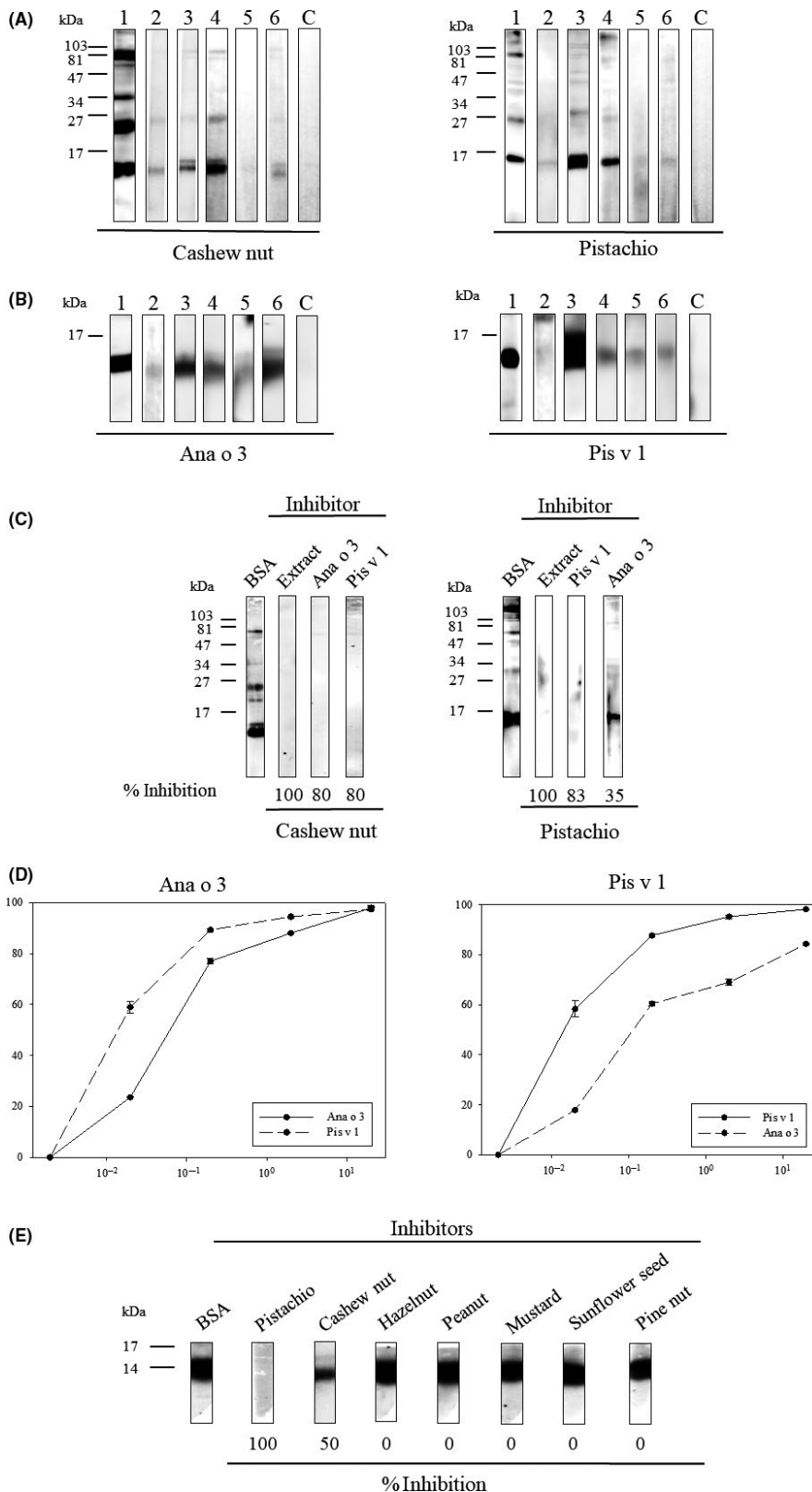


FIGURE 1 Immunoblotting of A, cashew nut and pistachio extracts and purified 2S albumins B, tested with the individual allergic patients sera. N: negative control using a tolerant patient serum. C, Cross-reactivity between Pis v 1 and Ana o 3 2S albumins from pistachio and cashew nut, respectively. Immunoblotting inhibition of the IgE recognition to cashew nut and pistachio extracts using the respective purified 2S albumins. D, ELISA inhibition of IgE recognition between Ana o 3 and Pis v 1. In all cases, the same pool of allergic patients' sera has been used. E, Cross-reactivity among 2S albumins using several tree nuts and seeds extracts as inhibitors of IgE recognition of Pis v 1

TABLE 1 Patients' clinical features

Patient no	Sex/Age (y)	Pistachio/Cashew nut	Symptoms	SPT ^a			CAP ^b			Other food allergies
				Cashew nut	Pistachio	Cashew nut	Pistachio	Ara h 2	Pollen allergy	
1	F/4	Cashew nut	OAS,U,A	Pos	Pos	0.63	0.62	0	Neg	Neg
2	M/7	Pistachio/Cashew nut	AN	Pos	Pos	2.84	2.52	ND	Neg	Neg
3	M/5	Cashew nut	AN, D	Pos	Pos	2.64	8.3	0	Pos	I, f, e
4	M/20	Cashew nut	AN	Pos	Pos	1.79	2.15	0.04	Neg	Neg
5	F/2	Cashew nut	OAS, AE	Pos	Pos	0.2	0.23	0	Neg	Neg
6	M/19	Cashew nut	OAS, U, AE	Pos	Pos	1.31	0.03	0.06	Neg	n

A, asthma; AE, angiodema; AN, anaphylaxis; D, digestive symptoms; e, egg; F, female; f, fish; k, kiwi; l, legumes (lentil, chickpea, bean); M, male; m, melon; n, nuts (including almond); ND, no determined; Neg, negative (<0.100 for ELISA and wheal area < 7 mm² for SPT); OAS, oral allergy syndrome; Pos, positive; U, generalized urticarial.

^aSkin prick test: wheal area for SPT Pos > 7 mm².

^bSpecific IgE determined in CAP (kU/L).

TABLE 2 Purified 2S albumins tested with allergic patients' sera by ELISA

Patient no	ELISA _{492nm} ^a									
	Cor a 14	Ara h 2	Fin p 1	Sin a 1	Tomato seed 2S albumin	Sunflower seed 2S albumin	Ana o 3	Pls v 1		
1	-	-	-	-	-	-	0.56	0.76		
2	0	0.057	0.005	0	0	0	0.325	0.2175		
3	0	0.1145	0	0	0	0	0.1245	0.301		
4	0	0.0925	0	0	0	0	0.155	0.178		
5	0.006	0.084	0.011	0.009	0.019	0.002	0.028	0.0285		
6	0.006	0.099	0.008	0.002	0.014	0.019	0.147	0.1055		

^aSpecific IgE determined in ELISA as OD at 492 nm.

The spectroscopic characterization of these 2S albumins rendered a common five-helix folding motif maintained by disulphide bonds. This pattern, present in other allergens such as LTPs or α -gladins, gives them a high long-term stability, an important requirement to be relevant food allergens.⁹ In fact, the ellipticity of both 2S albumins decreased by thermal treatment, although the helical structure was maintained, which did not recover its initial conformation when the temperature did (Figure S2C). Most of these 2S albumins share a basic pI and high resistance to the thermal denaturation, which is reflected in their CD spectra. However, both cashew nuts and pistachio are unusual 2S albumins because they have an acidic pI and a lower stability than their corresponding relatives.

Significant cross-reactivity across Ana o 2 to other 11S globulins was previously observed and a linear IgE epitope responsible for this reactivity was identified. Carbohydrates from 7S vicilins including those of *Anacardiaceae* have been pointed as IgE cross-reactivity epitopes.³ However, gradual proteolysis of 2S albumins during seed germination decreases similarity between their amino acid sequences and also the probability of these processes occurring. Moreover, the lack of a panel of purified 2S albumins from tree nuts and seeds has resulted in few comprehensive studies to establish the allergenic profile of these patients, evaluate the cross-reactivity among them or define clusters of nuts associated with these purified allergens. As examples, between Sin a 1 and Bra n 1, or Ara h 2 and Ara h 6.¹⁰ Cross-reactivity between sunflower and mustard seeds 2S albumins was reported in a mustard allergic patient; between sesame and poppy seeds extracts and between mustard, sesame and pine nut seeds.⁷ In this study, we have evaluated the IgE recognition from these patients against a panel of eight 2S albumins, including those from pistachio and cashew nuts and the cross-reactivity degree among them. IgE of all sera recognized both 2S albumins (Figure 1B) but no signal was observed when a pool of sera was used against 2S albumins belong to another phylogenetic families in immunoblotting (Figure S3). Similar results were obtained by ELISA assays when individual sera were tested against the six 2S albumins as shown in Table 2. It is important highlighting that this subset of patients, allergic to *Anacardiaceae*, could eat other nuts without experiencing allergic reactions. They might also eat mango pulp because, although allergy to this *Anacardiaceae* member has been described,¹¹ 2S albumins are restricted to seeds.

Cross-reactivity between these two 2S albumins from *Anacardiaceae* family with a sequence identity degree of 62% and a similarity of 79%, were analysed by immunoblotting and ELISA inhibition assays using a pool of allergic sera. IgE binding to Ana o 3, either to the monomer and aggregated forms of the protein, was nearly completely inhibited after pre-incubation of the sera with Pis v 1 (Figure 1C); but in contrast, pre-incubation of sera with Ana o 3 elicited a partial inhibition of IgE recognition to Pis v 1. These results were confirmed by ELISA inhibition assays (Figure 1D) where Pis v 1 inhibited IgE binding to Ana o 3 at lower amounts of protein than Ana o 3 itself. However, Ana o 3 partially inhibited IgE recognition to Pis v 1, not reaching 100% of inhibition. These results point to Pis v 1 from

pistachio as the primary sensitizer, containing specific epitopes absent in Ana o 3 and support the idea that in these patients, pistachio could be the primary sensitizer and cashew nut sensitization would occur through cross-reactivity between both proteins. Moreover, IgE binding to Pis v 1 was not inhibited with tomato, sunflower, mustard, or nuts as pine seeds, hazelnut or peanut extracts (Figure 1E), confirming that cross-reactivity seems to be restricted to the same phylogenetic family, in this case *Anacardiaceae*. According to the low degree of amino acid sequence identity to other plant species 2S albumins (<40%), it seems unlikely that common regions of IgE recognition could be present in other 2S albumins (Figure S4).

In summary, results of this study proved the existence of pistachio/cashew nut allergic syndrome characterized by symptoms after eating pistachio and/or cashew nut with sensitization to both nuts (skin test and/or specific IgE) through 2S albumins (Pis v 1 and Ana o 3) together with absence of sensitization and tolerance to other nuts. The basis of this pistachio-cashew nut allergic syndrome was the exclusive sensitization to both allergens without cross-reactivity to other 2S albumins. It will have important clinical consequences for both allergists and patients in the clinical practice since the risk of challenge test to other nuts is low, which will contribute to optimize and facilitate the management of nut allergy. The fact that these sensitivities manifest themselves preferentially in children exacerbates what is already a serious health problem that has resulted in significant nutritional deficiencies.

CONFLICTS OF INTEREST

The authors declare that they have no relevant conflicts of interest.

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SUPPORTING INFORMATION

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

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Supporting Information

Materials and Methods

2.1. Patients' sera

Cashew and/or pistachio nut allergic patients were recruited from Allergy Service of Fundación Jiménez Díaz, Madrid (Spain). Clinical data were recorded: age, sex, clinical symptoms, specific IgE levels, and coexistent food allergies (**Table 1 supplementary**). Inclusion criteria were a well-defined clinical history of allergy to pistachio and/or cashew nut and positive specific IgE and/or skin test to pistachio and cashew but negative to other nuts, peanut included. Exclusion criteria those previously reported [22]. We included non-atopic individuals with tolerance to cashew nut and pistachio as negative controls.

Allergen-specific IgE was measured with the ImmunoCAP System FEIA (ThermoFisher Scientific AB, Uppsala, Sweden) following the manufacturer's recommendations. Skin prick tests were performed with a commercial battery (Laboratorios LETI, S.A; Tres Cantos, Spain) of nut extracts (almond, hazelnut, peanut, chestnut, sunflower seed, pine nut, walnut, pistachio) and prick by prick test with nuts (almond, hazelnut, peanut, chestnut, sunflower seed, pine nut, walnut, pistachio and cashew nut). The ALK-Lancet needle (ALK-Lancet; ALK-Abelló, Horsholm, Denmark) was used for skin tests, which were performed according to EAACI guidelines [23]. Histamine phosphate at 10 mg/ml and normal saline solution were used as positive and negative controls, respectively. A wheal with a diameter at least 3 mm larger than the negative control was considered a positive reaction.

Ethical consent

The Fundación Jiménez Díaz Ethic Committee approved this study and a written informed consent was obtained from all subjects.

2.2. Preparation of protein extracts and purification of 2S albumins

Tree nuts were crushed under liquid nitrogen to obtain a fine flour. Proteins were extracted by homogenization in 0.2 M ammonium bicarbonate buffer, pH 8.0, containing 1 mM PMSF and stirred for one hour at 4 °C. The slurry was centrifuged at 20,000 *g* for 30 min at 4°C, filtered and delipidated in cold acetone. After centrifuging at 9000 *g* for 15 min at 4 °C and repeating this step twice, the organic phase was finally discarded, the sediment dried and resuspended in 0.2 M ammonium bicarbonate buffer, pH 8.0, and stored at -20 °C. The protein concentration was determined by the Lowry method [24].

2S albumins from tree nut extracts were firstly isolated by a size exclusion chromatography in Sephadex G-50 Medium equilibrated with 0.2 M ammonium bicarbonate, pH 8.0. After SDS-PAGE analysis, low molecular-mass protein fractions were pooled and loaded into a High Performance Liquid Chromatography (RP-HPLC), using a C-18 reverse phase column, with an acetonitrile gradient (0-60%). Fractions were analyzed by SDS-PAGE.

Other non-commercially available 2S albumins from tomato, sunflower, pine nut and mustard seeds and hazelnut were purified for this study as described previously (**Figure 1 Supplementary**). Ara h 2 was commercially purchased from Indoor Biotechnologies.

2.3. Identification of 2S albumins by Mass-Spectrometry

Proteins were identified by matrix-assisted laser desorption/ionization (MALDI)-Time of flight (TOF) Mass-Spectrometry in the Proteomics Facility of the Complutense University (Madrid, Spain). For this purpose, SDS-PAGE gel was stained with colloidal Coomassie Blue. Bands of interest were excised from gels to perform peptide mass fingerprinting. Mass spectrometry analysis of purified proteins was performed in a Bruker-Reflex IV MALDI-TOF equipment (Bruker-Franzer Analytic, Bremen, Germany) and in a mass spectrometer 4800 Proteomics Analyzer (AB SCIEX), which allow knowing the molecular masses of proteins and their fingerprint patterns.

2.4. Analytical procedures

Protein purity and apparent molecular mass were analyzed in 17% polyacrylamide gels. Samples were diluted in loading buffer, with/without β -mercaptoethanol (β ME) reducing agent, and denatured for 10 min at 90 °C. Proteins were stained with Coomassie Blue R-250 (Sigma-Aldrich). Molecular mass markers (Fermentas) were used. Protein concentrations were determined by amino acid analysis as described [25].

Circular dichroism (CD) studies were performed on a Jasco J-715 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) equipped with a CDF-426S Peltier temperature-control system interfaced with a NESLAB RTE111 water bath. Far-UV CD spectra were recorded at 20 and 85 °C, at protein concentrations of 0.2 μ g/ μ L 20 mM ammonium bicarbonate, pH 8.0. Spectra were recorded at 20 nm/min, and control buffer baseline was subtracted. Thermal denaturation was monitored by recording the molar ellipticity at 220 nm ($[\theta]^{220}$) while heating or cooling at 0.5 °C/min. The results are expressed as mean residue weight (MRW) molar ellipticity $[\theta]^{MRW} = \theta \times M / (c \times d \times 10)$, where θ is the observed ellipticity, M is the average molecular weight per residue, c is protein concentration (mg/mL) and d optical pathlength (cm).

2.5. Immunological assays

Proteins were blotted onto nitrocellulose membranes (Amersham Biosciences, Barcelona, Spain) after SDS-PAGE. Immunodetections were performed, by using individual sera or a pool of them (diluted 1:5). Human IgE binding was detected using mouse anti-human IgE monoclonal antibody (diluted 1:5000) kindly provided by ALK-Abelló (Madrid, Spain), followed by incubation with horseradish peroxidase-labelled polyclonal IgG (1:3000 diluted; Pierce, Rockford, Illinois) [26]. The chemiluminiscent signal was developed by ECL-Western Blotting reagent, and detected in a luminescent imager analyzer LAS3000. Quantification of the signal was performed in triplicate using the computer program Multigauge V3.0.

For immunoblotting inhibition assays [27] individual sera or an equivolumetric pool of patients' sera, were diluted (1:5) with 3% skim milk powder (SMP) in PBS-Tween 0.2%, and were pre-incubated with 5 µg of the isolated proteins or 500 µg of the corresponding extracts, at room temperature for 2 h, using BSA as negative control (final dilution of pool or patients' sera was 1:5). The rest of the steps were as described above.

2.6. Indirect and inhibition ELISA

Indirect ELISA was performed in 96-well polystyrene plates (Costar) coated with purified protein (2 µg/mL in phosphate buffered saline, pH 7.6, 100 µL/well) and after incubation overnight at 4 °C. Plates were washed with PBS-T and blocked with 3% SMP in PBS-T. For inhibition ELISA, sera were pre-incubated with purified proteins (0.002-2 µg), tree nut extracts (500 µg), or BSA as negative control at room temperature for 2 h. Inhibition mixtures or whole sera (diluted 1/5 in 3% SMP in PBS-T) were added to the coated plate and incubated at 37 °C for 2 h. Afterwards, plates were incubated with mouse anti-human IgE antibody, followed by HRP-labelled goat anti-mouse IgG antibody, both for 1 h at 37 °C, with PBS-T washes after each incubation. IgE binding was detected using o-phenylenediamine (Sigma) in 0.05M phosphate-citrate buffer (Sigma) and the reaction was stopped with the addition of 4M HCl. The absorbance was measured at 492 nm in an iMark Microplate Absorbance Reader (Bio-Rad) and non-specific binding of antibodies was eliminated by subtracting the absorbance from uncoated controls wells. Positive reactions were considered when the absorbance at 492 nm was greater than three-fold the standard deviations of the mean absorbance obtained with non-atopic donors.

The percentage of inhibition of IgE binding was calculated according to the following formula: inhibition (%) = $[1 - (\text{OD}_{492\text{nm}} \text{ with inhibitor} / \text{OD}_{492\text{nm}} \text{ without inhibitor})] \times 100$.

Supplementary figures and tables

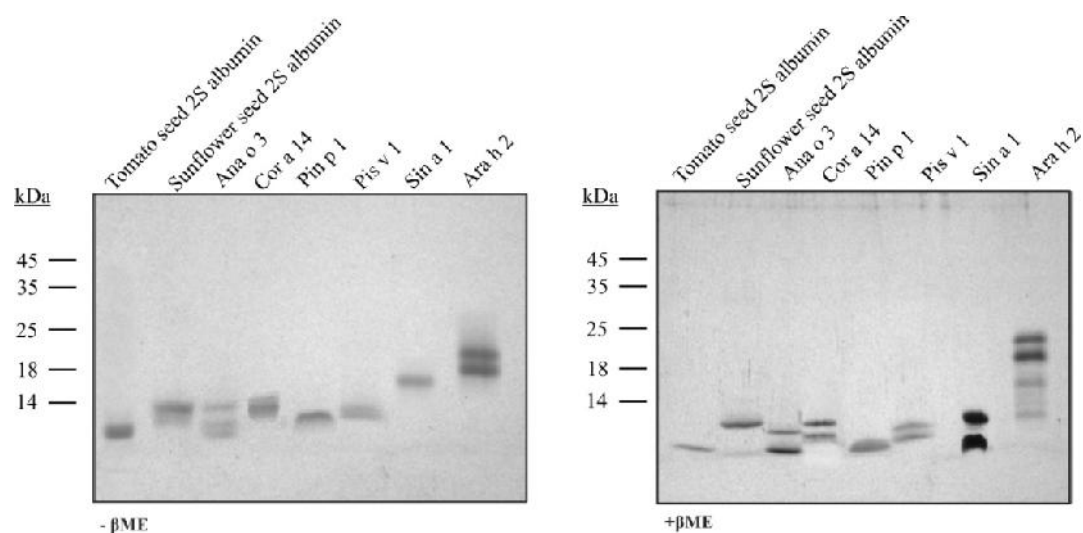


Figure 1 Supplementary. Coomassie blue staining of purified 2S albumins from tomato and sunflower seeds, cashew nut, hazelnut, pine nut, pistachio, mustard seeds and peanut without β -mercaptoethanol (β ME) (A) and with β ME (B).

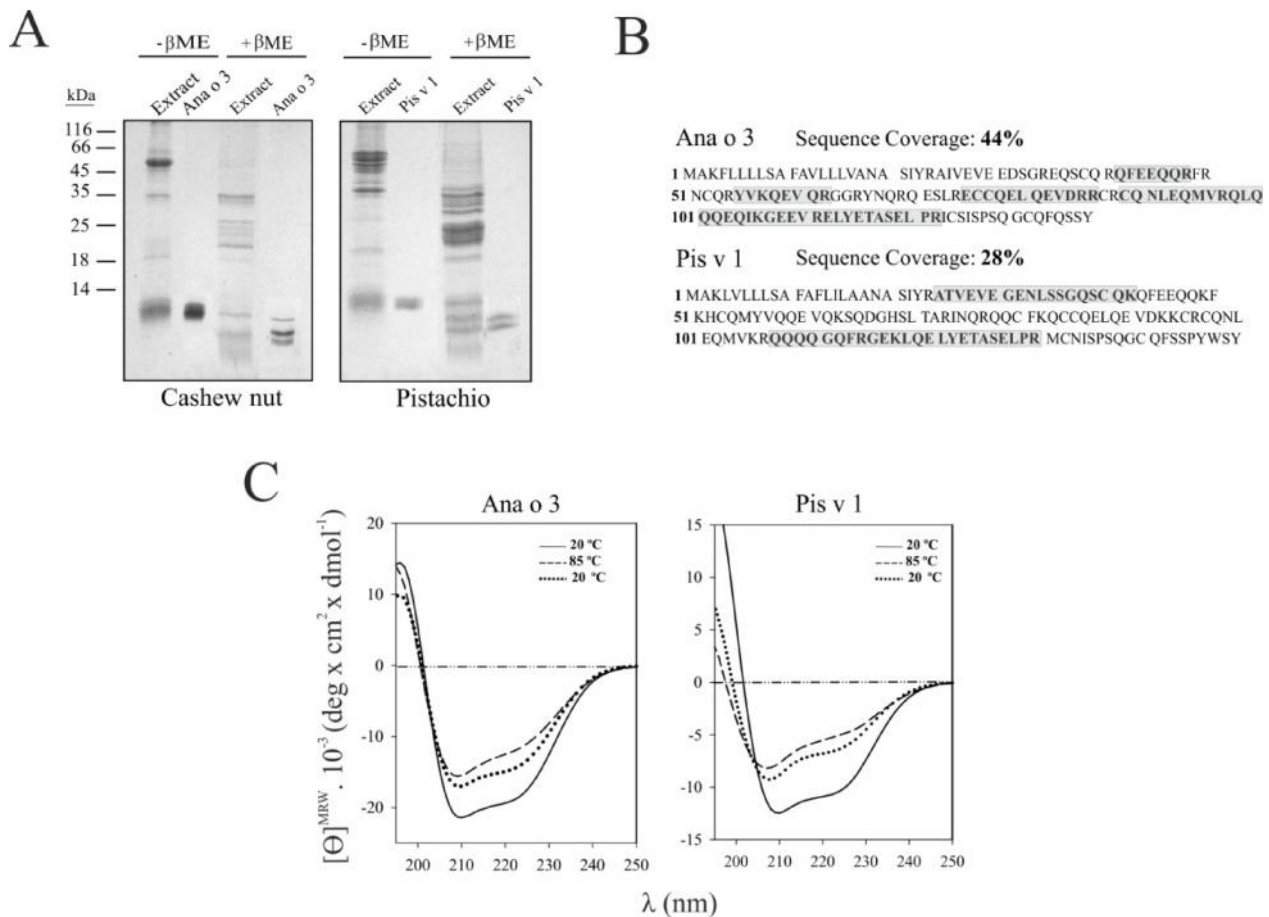


Figure 2 Supplementary. Structural characterization of purified Pis v 1 and Ana o 3. (A) Coomassie blue stained 17% SDS-PAGE of tree nuts extracts and purified 2S albumins in the absence or presence of β ME. (B) Matched peptides (bold) obtained by Mass Spectrometry. (C) CD spectra of purified Ana o 3 and Pis v 1 in the far-UV at 20°C (—), at 85°C (----), and cooling down again at 20°C (····).

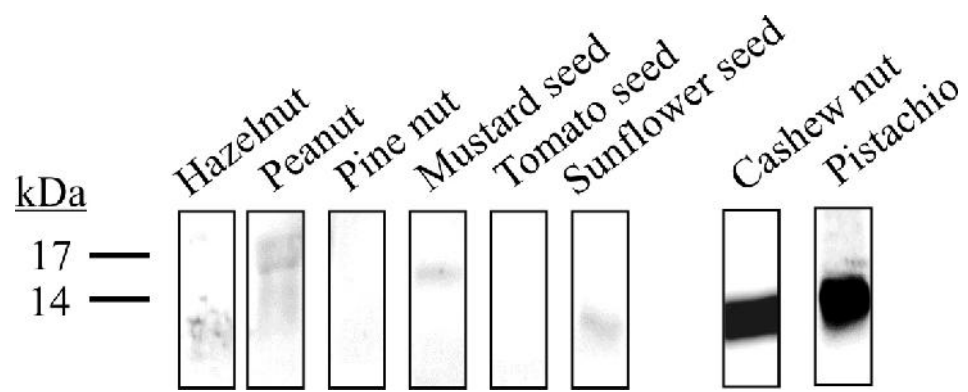


Figure 3 Supplementary. Immunoblotting of 2S albumins (Cor a 8, Ara h 2, Pin s 1, Sin a 1, tomato seeds, sunflower seeds) and, cashew nut and pistachio, tested with a pool of patients' sera allergic to cashew nut and pistachio.

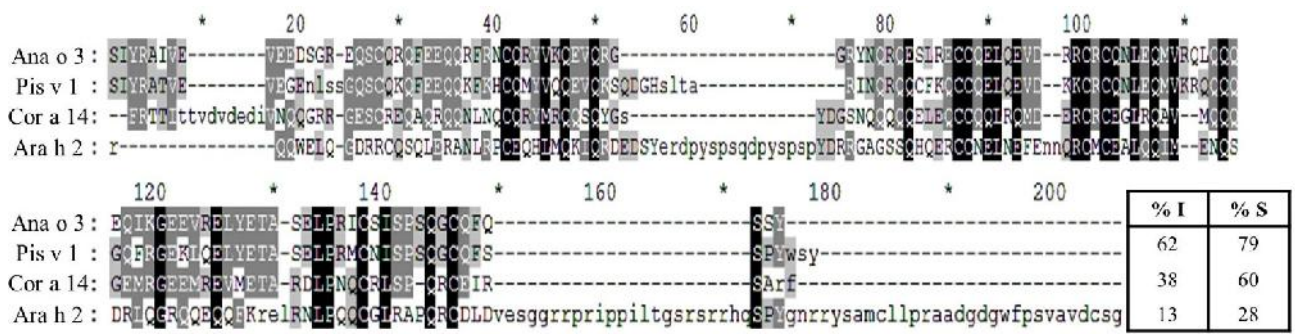


Figure 4 Supplementary. Cashew nut, pistachio, hazelnut and peanut 2S albumin sequences alignment, using Ana o 3 amino acid sequence as model, indicating identity (I) and similarity (S) percentages.