

# Pistachio vicilin, Pis v 3, is immunoglobulin E-reactive and cross-reacts with the homologous cashew allergen, Ana o 1

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## Clinical and Experimental Allergy

### Summary

**Background** Patients allergic to cashew nuts often report allergy to pistachio, which could be a result of cross-reactivity between the two as both are members of the *Anacardiaceae* family. **Objective** Because cashew 7S globulin (vicilin, Ana o 1) is a recognized major allergen, we cloned the pistachio homologue and assayed it for IgE reactivity and cross-reactivity with Ana o 1.

**Methods** Degenerate primers for 7S globulin were used in PCR to amplify DNA from a pistachio cDNA library. An isolate was sequenced, cloned and expressed in *Escherichia coli*. Reactivity to the allergen was screened by dot blot using 19 pistachio and/or cashew-allergic patients' sera. Cross-reactivity was investigated by inhibition dot- and Western immunoblot assays using pistachio/cashew-allergic patients' sera, and monoclonal antibodies (MAbs) raised against recombinant Ana o 1 (rAna o 1).

**Results** An isolate was found that coded for a 7S vicilin-like protein, designated Pis v 3. IgE reactivity to Pis v 3 was found in the serum of seven of the 19 (37%) patients with histories of allergy to both pistachio and cashew or who were allergic to cashew but had never eaten pistachio. The seven patients with IgE that recognized rPis v 3 also recognized rAna o 1. Six of nine anti-rAna o 1 MAbs also showed reactivity to rPis v 3 on dot blots.

**Conclusion** Of the 37% of pistachio/cashew-allergic patients' sera that recognized the pistachio allergen, rPis v 3, all showed complete cross-reactivity with rAna o 1. The data does not identify the primary sensitizing agent but suggests that IgE reactivity to rPis v 3 and rAna o 1 is focused on the most conserved regions of the proteins. Clinical histories suggest that in some cases, cashew was the sensitizing agent. rPis v 3 is a likely contributor to the observed co-sensitivity to pistachio and cashew in some patients.

**Keywords** cashew, cross-reactivity, food allergy, pistachio, tree nut, vicilin

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### Introduction

Allergic reactions to tree nuts are common and it is estimated that 0.5% of the US population is allergic to one or more tree nuts [1]. Recently, a case-matched comparison of cashew- and peanut-allergic children found that cashew nut caused more severe reactions than peanut in a UK population [2]. The consumption of tree nuts is steadily increasing due to the general perception of their health benefits [3]. According to the USDA (<http://www.ers.usda.gov/publication/FTS/2006/Yearbook/FTS2006.pdf>) in 2005, the per capita tree nut consumption was 2.7 pounds, with cashew nut being the most commonly consumed imported

nut [4]. The consumption of pistachio has been steadily rising over the last few years, which can be attributed to their incorporation into baked goods, ice cream, candies, and other food dishes. In 2003, a random digit dialed phone survey revealed that, of 82 tree nut-allergic individuals, 44% reported allergy to cashew and 22% to pistachio [1].

To date, three major cashew allergens, Ana o 1 (7S vicilin), Ana o 2 (11S globulin), and Ana o 3 (2S albumin), have been identified, all of which are characterized as seed storage proteins [5–7]. Ana o 1, a 7S vicilin, is a homotrimer of 45 kDa subunits, recognized by 10 of the 20 cashew-allergic patients' sera and identified as a major

allergen (i.e.  $\geq 50\%$  reactive) [5]. Epitope mapping performed using synthetic overlapping peptides on this allergen identified 11 epitopes that bind IgE from cashew-allergic individuals [5].

Vicilins are typically homotrimeric proteins with a molecular mass of 150–190 kDa, composed of protomers of 40–80 kDa [8]. Vicilins have previously been identified as allergens in tree nuts including cashew (Ana o 1), walnut (Jug r 2), and hazelnut (Cor a 11), and certain aspects of their cross-reactivity have been investigated [5, 9, 10]. An epitope map comparison revealed that Ana o 1 does not share common linear epitopes with the peanut vicilin, Ara h 1 [5, 11]. Recombinant (r) walnut Jug r 2 was recognized by nine of 15 walnut-allergic patients, and was thus classified as a major walnut allergen. This allergen did not show any cross-reactivity to the homologous pea or peanut vicilins in IgE-binding inhibition experiments [9]. The hazelnut 7S vicilin-like protein (rCor a 11) was recognized by 43% of tested hazelnut-allergic patients. An analysis of the allergen demonstrated that IgE reactivity was not influenced by the presence or absence of glycans. [10]. The authors noted a 67% and 44% amino acid (aa) sequence similarity, between two IgE-binding epitopes identified on Ara h 1 and Cor a 11, respectively, suggesting the possibility of cross-reactivity, however, direct evidence of cross-reactivity was not presented [10]. In a comparative homology modelling study, Barre *et al.* [12] identified structural features associated with the epitopes on the vicilin allergens in peanut, lentil, and pea. Comparison of the epitopes identified on Ara h 1 with the corresponding aa sequence of pea (Pis s 1) and lentil (Len c 1) vicilins revealed a high degree of sequence similarity and three-dimensional conformation, which could account for the observed cross-reactivity between legumes for some patients [12].

There have been several reports of cross-reactivity between pistachio and cashew nut proteins, which is not surprising because both are members of the *Anacardiaceae* family [13–17]. In one study, two pistachio-allergic individuals who had never eaten cashews exhibited IgE specific to both cashew and pistachio nuts using skin prick tests, immunoblotting, and RAST [13]. Western blot assays demonstrated IgE binding to pistachio proteins ranging from 14 to 70 kDa and cashew proteins from 20 to 67 kDa. In another study, ImmunoCAP-inhibition assays were used to demonstrate cross-reactivity between pistachio and cashew using three patients; one allergic to only pistachio and two allergic to pistachio but had never eaten cashew [14]. The results showed that pre-incubation of patients' sera with cashew extract could significantly inhibit IgE binding to pistachio nut on the solid phase [14]. IgE binding to 34, 41, 52, and 60 kDa bands in pistachio nut extract were detected by immunoblotting and, in agreement with Fernandez *et al.* [13] the 34 kDa band exhibited the strongest IgE-binding signal [14].

Also, Goetz *et al.* [15] demonstrated cross-reactivity between cashew and pistachio proteins using rabbit anti-pistachio antisera in a double immunodiffusion assay.

In none of the studies described above were the specific proteins recognized by patient IgE identified or characterized beyond estimates of their molecular masses [13–15]. In this study we (1) report the identification and immunological characterization of a cloned pistachio allergen, a vicilin designated Pis v 3, and (2) show that the cashew and pistachio vicilin homologues are highly cross-reactive when assayed with serum IgE from allergic individuals and with mouse anti-cashew vicilin monoclonal antibodies (MAbs).

## Methods

### *Human sera*

Blood samples were drawn after informed consent from patients to cashew and pistachio nut. The study was approved by the human subjects review committee of the University of California at Davis (Davis, CA, USA). Sera were frozen at  $-70^{\circ}\text{C}$  until use. The presence of pistachio- and cashew-reactive IgE was confirmed by means of Pharmacia ImmunoCAP assay or Western immunoblotting, as described below. Clinical characteristics of the subjects are shown in Table 1. Control sera were obtained from patients with histories of pollinosis to weeds, trees, and/or grasses but who were not food allergic.

### *Cashew and pistachio protein extract*

Cashew and pistachio protein extracts were obtained from defatted cashew or pistachio flour by extraction with buffered saline borate pH 8.2 (0.1 M boric acid, 0.025 M sodium borate, 0.075 M sodium chloride) at room temperature (RT) for 1 h and stored at  $-20^{\circ}\text{C}$  for later analysis as previously described [18]. Protein concentrations were measured using the Bradford protein assay (BioRad Laboratories Inc., Hercules, CA, USA).

### *cDNA production, polymerase chain reaction amplification, and DNA sequencing*

Mature pistachio nuts were frozen in liquid nitrogen and ground with a mortar and pestle. Total RNA was extracted as described earlier [19], using TRIzol (Gibco BRL, New York, NY, USA). mRNA was isolated using a PolyAtract mRNA Isolation Kit (Promega, Madison, WI, USA) as described by the manufacturer. Both 5'- and 3'-RACE were used to generate pistachio cDNA as described in the SMART RACE cDNA Amplification Kit user manual (BD Biosciences Clontech, Palo Alto, CA, USA).

Degenerate primers were designed based upon conserved homologous sequences found in 7S globulins from

Table 1. Clinical characteristics of pistachio and/or cashew-allergic subjects

No.	Sex/ age	Age on onset of pistachio/ cashew allergy	Pistachio allergic	Cashew allergic	Other atopy history	Food allergy	*ImmunoCap, RAST or positive IgE immunoblot	Positive dot blot to Pis v 3/Ana o 1
1	M/25	3	Yes	Yes	Asthma	Walnut, pecan, hazel	Pistachio 5.65 Cashew 6.95	No/No
3	F/26	2	Yes	Yes	AD, AR, asthma	Peanut, walnut	Pistachio = Class 5 Cashew 9.51	Yes/Yes**
5	F/54	10	Yes	Yes	AR, asthma	Walnut, pecans, hazel	Pistachio 7.24 Cashew 1.62	No/No
7	F/30	10	NE	Yes	AD, AR	Peanut, walnut, hazel	Pistachio 2.80 Cashew 4.04	No/No
9	F/35	2	NE	Yes	AD, AR, asthma	Walnut, pecans, almond	Pistachio = Class 5 Cashew 35.1	Yes/Yes
11	M/50	1	Yes	Yes	AD, AR, asthma	Multiple tree nuts	Pistachio 4.60 Cashew 5.19	No/No
12	F/26	3	NE	Yes	AR, asthma	Multiple tree nuts	Pistachio 2.22 Cashew 2.41	No/No
13	F/39	1	Yes	NE	AD, AR, asthma	Peanut, walnut, hazel, pine nut, brazil	Pistachio 12.5 Cashew 9.53	Yes/Yes
14	F/39	5	Yes	Yes	Asthma	Tree nuts	Pistachio 57.4 Cashew 94.7	Yes/Yes
20	F/48	1	NE	Yes	AD, AR, asthma	Peanut, walnut, hazel	Pistachio 0.56 Cashew + blot	No/No
29	F/49	3	Yes	Yes	AD, AR, asthma	Peanut, sesame, tree nuts	Pistachio 0.38 Cashew 0.52	No/No
30	F/53	15	Yes	Yes	AD, AR, asthma	Tree nuts except almond	Cashew < 0.35 + blot Pistachio + blot	No/No
32	M/38	1	Yes	Yes	AD, asthma	Walnut, pecan, hazel	Pistachio 1.17 Cashew 1.64	No/No
33	F/63	53	NE	Yes	AD, AR, asthma	Peanut, almond, fish, eggs	Pistachio 66.9 Cashew 81.3	Yes/Yes
35	F/54	2	Yes	Yes	AD, AR asthma	Walnut, hazel, pecan, brazil	Pistachio = Class 2 Cashew 0.85	Yes/Yes
46	M/39	18	Yes	No	AR, asthma	Sunflower seed, mango, fruit	Pistachio 2.29 Cashew < 0.35	No/No
47	F/65	Child	Yes	Yes	AR, asthma	Banana, avocado, mango, melon	Pistachio 38.2 Cashew 52.9	No/Yes
48	M/59	4	Yes	Yes	AD, asthma	Peanut, walnut, almond, pecan, hazel, brazil, pine	Pistachio 3.53 Cashew 7.82	Yes/Yes
49	M/35	6	Yes	Yes	AR, asthma	Walnut, pecan, hazel, brazil	Cashew + blot Pistachio + blot	No/No

\*ImmunoCAP results are shown as kU/L, RAST as class.

\*\*Positive results in bold.

NE, never eaten AD, atopic dermatitis; AR, allergic rhinitis; M, male; F, female.

cashew, hazelnut, sesame, soybean, and fava bean. The degenerate primer, 5'-IGIKATYTTYGTTGCMIKCGA GTTGTA-3', and a universal primer based on the 5'-linker sequence on the 5'-RACE cDNA were used. Sequencing of the PCR products lead to the identification of the pistachio 7S globulin. Gene-specific primers (forward: 5'-TGCTCTA GAAAGACAGACCCAGAGCTGAAAC-3', reverse: 5'-AAACT GCAGTCATTCATCAGCAGCCCTTG-3') were then designed and used to amplify full-length pistachio 7S globulin cDNA which was then TA cloned (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA, USA) and sequenced on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

#### Cloning, expression and purification of cDNA-encoded proteins

As described in detail [for cashew nut [5]], the pistachio cDNA coding sequences were ligated into a modified version of the maltose-binding protein (MBP) fusion expression vector pMAL-c2 (New England BioLabs Inc., Beverly, MA, USA). The modified vector, pMAL-c2-His, contained an eight-residue histidine tag downstream of the *malE* gene and *SacI* restriction site and the factor Xa cleavage site along with the corresponding *XmnI* and *EcoRI* sites were replaced with a thrombin cleavage site.

The cloning, expression, and purification of rAna o 1- and rPis v 3-MBP fusion proteins were carried out as previously described for rAna o 1 [5]. Briefly, cDNA/pMAL-c2-His plasmids encoding rAna o 1 or rPis v 3 were used to transform competent *Escherichia coli* BL21 (DE3) cells (Novagen Inc., Madison, WI, USA). Bacterial colonies were grown at 37 °C with shaking to an OD<sub>600 nm</sub> of 0.5, followed by incubation with 0.3 M isopropyl-D-thiogalactopyranoside. The cells were harvested, resuspended in amylose resin buffer (20 mmol/L Tris-HCl, pH 7.4, 200 mmol/L β-mercaptoethanol, and 1 mmol/L EDTA), lysed with mild sonication, centrifuged at 10 000 g, and the supernatant passed over amylose affinity column. The fusion proteins [rPis v 3-MBP and the previously described rAna o 1-MBP [5]] were eluted with column buffer containing 10 mmol/L maltose and stored at 4 °C until use or for long-term storage, frozen at -80 °C. An alternative purification scheme, in which a nickel affinity column binds the His-tag of the fusion protein, was available but not utilized.

#### *Polyacrylamide gel electrophoresis and protein transfer*

Recombinant proteins (0.5 µg per 4 mm well width) or aqueous total cashew/pistachio extracts (12–14 µg per 4 mm well width) were subjected to SDS-PAGE (12%). Samples were boiled in reducing sample buffer containing β-mercaptoethanol then subjected to electrophoresis and either stained with Coomassie Brilliant Blue R (Sigma-Aldrich, St Louis, MO, USA) or transferred to Nitrocellulose (NC) membranes as previously described [20].

#### *Dot-blot analysis and inhibition*

Recombinant Pis v 3 and rAna o 1 were applied to NC membranes using a 96-well Bio-Dot Microfiltration Apparatus (BioRad Laboratories Inc.) as previously described [21]. Briefly, recombinant proteins (0.5 µg per 2 mm dot) were applied to NC and strips containing dotted rPis v 3 and rAna o 1 were excised and probed as described below. For inhibition dot blots, rPis v 3 and rAna o 1 were used as inhibitors at 100 µg/mL (100–200 µL total volume) and pre-incubated with patients' sera at 1 : 50 dilution (8 µL in 400 µL total volume) overnight (o/n) at 4 °C before incubation with the dotted protein.

#### *Immunoglobulin E immunoblotting and inhibition*

NC strips (4 mm wide) from gel transfers containing 12–14 µg of nut protein extract or 0.5 µg of recombinant Pis v 3 protein per strip were blocked o/n at 4 °C using phosphate-buffered saline-Tween<sup>®</sup> 20 (PBS-T)/5% (v/w) non-fat dry milk. Dotted protein strips were similarly

blocked and NC strips/dots were incubated with sera diluted 1 : 5 or 1 : 50 v/v (for highly reactive sera) o/n at 4 °C. The probed strips/dots were then washed for 90 min in PBS-T at RT, changed three times, before being incubated o/n at 4 °C with <sup>125</sup>I-labelled anti-human IgE (Specific IgE Tracer, Hycor Biomedical Inc., Garden Grove, CA, USA) diluted 1 : 10 in non-fat milk buffer. Membranes were washed again as above and exposed to X-ray film (Kodak X-OMAT, Kodak Molecular Imaging, New Haven, CT, USA).

For inhibition immunoblots and dot blots, human sera at 1 : 5 or 1 : 50 dilution (80 or 8 µL in 400 µL total volume) were pre-incubated with 100 µg/mL (100–200 µL total volume) of rAna o 1/rPis v 3 (both with associated MBP) or, as a control, with 7 µg (1.2 µL total volume) of MBP inhibitor o/n at 4 °C or at 37 °C for 1 h and used as described above. Other controls included strips/dots exposed to IgE without inhibitor and strips/dots exposed to serum from an atopic individual without a history of tree nut allergies.

#### *Monoclonal anti-cashew antibodies*

MAbs against rAna o 1 were raised in the Hybridoma Facility at Florida State University using standard techniques [22]. The guidelines for animal care and welfare described in the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animals Resources (National Research Council, National Academy Press, revised 1996) were followed. Briefly, mice were immunized with 40 µg of rAna o 1 in RIBI adjuvant (RIBI ImmunoChem Research Inc., Hamilton, MT, USA), boosted with 20 µg of rAna o 1 in RIBI adjuvant at 3-week intervals, and were given a final injection of 25 µg of rAna o 1 in saline equally split between the intravenous and subcutaneous routes. The resulting hybridomas were screened and assayed for relative strength and specificity by direct-binding ELISA [23].

#### *Monoclonal antibody immuno-dot-blotting*

Dot-containing NC strips were prepared as described above and probed with the rAna o 1-specific MAbs at 1 : 400 or 1 : 500 dilutions in Tris-buffered saline-Tween<sup>®</sup> 20 (TBS-T) at RT for 1 h. Dots were washed three times with TBS-T for 20 min each wash and were then incubated with horseradish peroxidase-labelled goat anti-mouse reagent (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) at a 1 : 3000 v/v dilution in TBS-T for 1 h at RT, and washed as above. Amersham ECL (GE Healthcare, Piscataway, NJ, USA) was used to detect reactivity upon exposure of dot strips to Kodak XAR film (Kodak Molecular Imaging, New Haven, CT, USA).

## Results

### Gene characterization

The 7S globulin gene was amplified from the pistachio 5'-RACE cDNA by means of PCR with a degenerate forward and universal lock dock reverse primer. Subsequently, gene-specific primers were used to clone the full-length gene. The resulting 1560 bp PCR product (GenBank ID EF116865) encodes a 519 aa protein designated Pis v 3 (Fig. 1). The SignalP program (www.expasy.org, Swiss Institute of Bioinformatics, Basel, Switzerland) was used to identify a 26 aa presumptive signal sequence (in red, Fig. 1). Comparison of the aa sequence with the NCBI database using BLAST analysis identified homology with other members of the 7S globulin family of seed storage proteins, several of which are known food allergens (Table 2). In line with the familial relationship between pistachio and cashew, their respective vicilins are 90% aa sequence similar and 80% identical. In contrast, the aa sequence

comparisons with the 14 nut and seed proteins listed in Table 2 revealed only 51–72% similarity and 31–55% identity to pistachio vicilin.

### Protein sequence characterization

The entire Pis v 3 cDNA, beginning at K27 following the presumptive signal peptide was cloned. The DNA segments were ligated into an expression vector designed to yield a MBP fusion protein. The resulting ~102 kDa Pis v 3-MBP fusion protein was affinity purified with the aid of an amylose affinity column, as previously described [5].

### Comparison of the Pis v 3 protein sequence with that of Ana o 1 and its immunoglobulin E-reactive peptides

A sequence alignment of the pistachio vicilin, Pis v 3, and the cashew homologue, Ana o 1, was used to evaluate their structural similarity and compare the aa sequence of



Fig. 1. Nucleotide and derived amino acid sequence of Pis v 3 cDNA. (a) Nucleotide sequence (GenBank accession no. EF116865) and (b) amino acid sequence of the Pis v 3 coding region. The predicted signal peptide is indicated in red.

Table 2. Sequences demonstrating the greatest homology to Pis v 3

Protein description	Organism	Accession no.	% Identity	% Similarity	References	Allergen designation
Vicilin-like protein	<i>Anacardium occidentale</i> (cashew)	AAM73730	80	90	[5]	Ana o 1
48 kDa glycoprotein precursor	<i>Corylus avellana</i> (hazelnut)	AAL86739	55	72	[10]	Cor a 11
7S globulin	<i>Sesamum indicum</i> (sesame seed)	AAK15089	47	65	[24]	Ses i 3
Sucrose-binding protein homologue S-64	<i>Glycine max</i> (soy bean)	AAF05723	46	65	[25]	
Sucrose-binding protein 2	<i>Glycine max</i> (soy bean)	AA048716	46	65	[26]	
7S globulin	<i>Elaeis guineensis</i> (African oil palm)	AAK28402	41	60	[27]	
Vicilin-like protein precursor	<i>Juglans regia</i> (English walnut)	AAF18269	41	65	[9]	Jug r 2
Vicilin seed storage protein	<i>Juglans nigra</i> (black walnut)	AAM54366	40	63	Unpublished	Jug n 2
Vicilin precursor	<i>Macadamia integrifolia</i> (smooth shelled macadamia)	AAD54244	39	60	[28]	
Vicilin	<i>Pisum sativum</i> (pea)	CAF25232	35	52	[29]	Pis s 1
Convivilin	<i>Pisum sativum</i> (pea)	CAB82855	35	52	[29]	Pis s 2
Allergen Len c 1.0102	<i>Lens culinaris</i> (lentil)	CAD87731	34	51	[30]	Len c 1
Allergen Len c 1.0101	<i>Lens culinaris</i> (lentil)	CAD87730	33	51	[30]	Len c 1
Vicilin-like protein	<i>Lupinus albus</i> (white lupine)	CAI84850	32	53	[31]	
7S seed storage protein (vicilin)	<i>Arachis hypogaea</i> (peanut)	AAL27476	31	51	[32]	Ara h 1

the 10 known cashew IgE-binding peptides [5] with the corresponding aa sequence on pistachio (Fig. 2). As described above, the comparison reveals 80% overall aa identity and 90% similarity. Of the two peptide segments previously shown to contain immunodominant epitopes in rAna o 1, peptide #3 had 13 of 15 identical residues and one similar residue, and peptide #10 had eight of the 15 identical residues and four similar residues. All of the variant amino acids were clustered at the C-terminal end of peptide #10 leaving the N-terminal end, which is 100% identical, as a potential source of cross-reactivity. Comparisons of the sequences for the other epitope sites show similar degrees of homology with the exception of peptide #9 where minimal homology is evident. The high degree of sequence homology between the two allergens suggests the likelihood of considerable cross-reactivity and prompted additional studies.

#### Reactivity of recombinant proteins with human immunoglobulin E

Reactivity to rPis v 3 and rAna o 1 was screened using 19 patients' sera: 12 cashew- and pistachio-allergic, five cashew-allergic but who had never eaten pistachios, one

pistachio-allergic but who had never eaten cashew, and one only pistachio-allergic. Of the 14 pistachio-allergic patients (# 1, 3, 5, 11, 13, 14, 29, 30, 32, 35, 46, 47, 48, 49), five (36%) showed IgE reactivity to rPis v 3 by dot blot (Fig. 3a). Interestingly, of the five cashew-allergic patients (# 7, 9, 12, 20, 33) who reported that they had never eaten pistachio, two (40%) showed IgE reactivity to rPis v 3 by dot blot. Each of the seven rPis v 3-reactive sera (patients # 3, 9, 13, 14, 33, 35, 48) also recognized rAna o 1. Only one patient, number 47, was reactive to the cashew vicilin alone and not to the pistachio vicilin (Fig. 3a). Pre-incubation of patient sera with MBP did not inhibit IgE binding to rPis v 3 demonstrating that no MBP-specific antibodies were present in the patients' sera (Fig. 3b). One patient, #33, was not tested for MBP inhibition due to unavailability of serum. Similar results were obtained with MBP inhibition of Ana o 1 reactivity (data not shown).

The similarity in signal intensity between the two probed allergens for any given serum sample suggested the likelihood of considerable cross-reactivity. To investigate this potential cross-reactive relationship, SDS-PAGE of cashew and pistachio extract followed by Coomassie Brilliant Blue staining indicated the presence of similar molecular mass proteins in both nut extracts (Fig. 4a) as



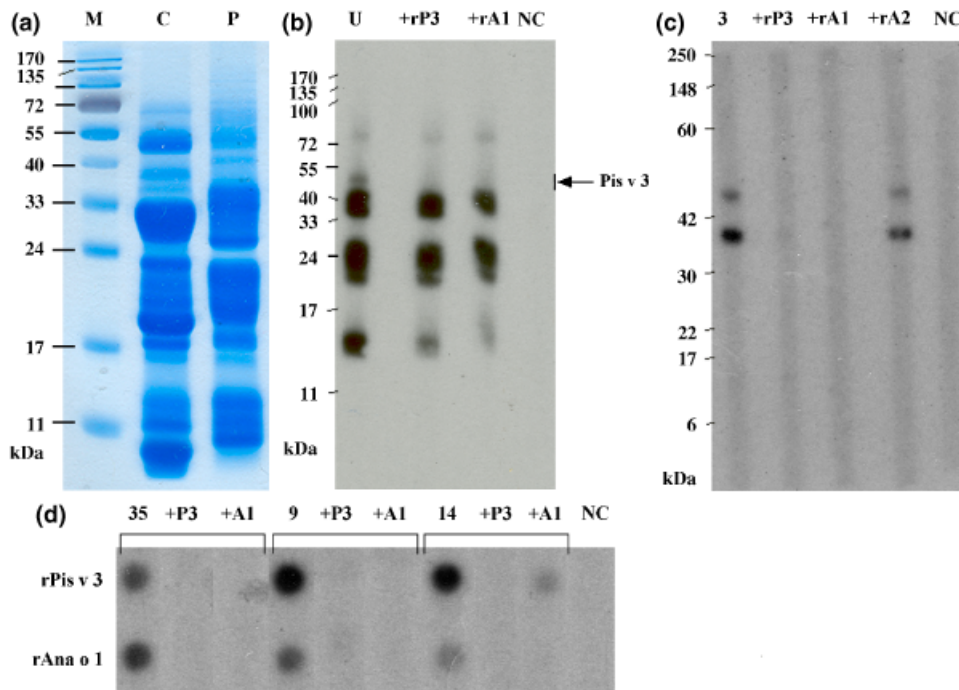


Fig. 4. Coomassie Blue stain and inhibition blots. (a) SDS-PAGE and Coomassie Blue stain of cashew (c) and pistachio (p) protein extract. M, molecular weight marker. (b) Inhibition immunoblot of pistachio protein extract probed with a pool of patient sera (# 3, 9, 14) either unabsorbed (U), pre-absorbed with rPis v 3 (+rP3), or pre-absorbed with rAna o 1 (+rA1). NC, negative control. Putative Pis v 3 band indicated by arrow. (c) Inhibition immunoblot of pistachio extract probed with serum from patient #3 either unadsorbed (3) or pre-incubated with recombinant allergen inhibitors, rPis 3 (+rP3), rAna o 1 (+rA1) [5], or rAna o 2 (+rA2) [6], NC, atopic serum negative control. (d) Inhibition dot blot with recombinant allergens in which patients' sera #35 (35), #9 (9), and #14 (14) were pre-incubated with the indicated recombinant allergens.

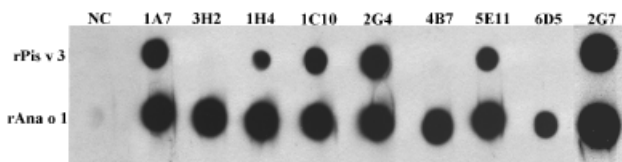


Fig. 5. Dot blot containing rPis v 3 and rAna o 1 probed with cashew monoclonal antibodies raised against rAna o 1. The negative control (NC) contained no primary antibody.

#### Reactivity of cashew monoclonal antibodies with recombinant proteins

The above-described sequence alignment and specific IgE-binding data revealed a high degree of homology between the cashew and pistachio vicilin. To further assess the nature of the cross-reactivity between cashew and pistachio vicilin, a panel of murine IgG MAbs, previously generated against cashew rAna o 1, was assayed. Of the nine MAbs tested, six (67%) also recognized rPis v 3 to varying degrees on dot blots (Fig. 5) indicating considerable epitope homology between rPis v 3 and rAna o 1.

#### Discussion

In recent years a number of allergens have been identified in a variety of foods and plants. Not only has this

information defined the proteins that are directly responsible for food allergies but it has also revealed structural relationships between allergens including, in some cases, the structural basis for allergen cross-reactivity [8, 33–37].

It is not uncommon for food-allergic individuals to react to more than one allergen, a pattern which can be a result of several independent sensitization events. Another potential factor contributing to complex allergen sensitivity patterns is allergen cross-reactivity [36]. Cross-reactivity between allergens occurs when IgE originally raised against one allergen recognizes and binds to a structurally similar protein from a different source even in the absence of prior exposure to the cross-reacting agent [33, 36, 38]. Such situations may or may not be apparent through examination of clinical histories. The term 'co-recognition', which includes cross-reactivity, has been used to define an alternative situation wherein possible co-exposure to two or more agents that contain homologous (and likely cross-reactive) molecules masks the identity of the primary sensitizing agent [35]. From an immunological perspective, the degree of epitope sharing may be sufficiently greater in such co-recognition that the cross-reactivity may be described as symmetric. In such cases the epitope reactivity profiles induced by sensitization to any one of the two or more cross-reactive allergens

are essentially equivalent [38]. This situation contrasts with the more typical asymmetric cross-reactivity which, when assayed *in vitro*, shows that one allergen inhibits IgE binding to a second allergen better than the second allergen inhibits binding to the first [38].

Patients allergic to cashew often report allergy to pistachio as well, which is likely a result of cross-reactivity between the two closely related tree nuts [13–17]. In this study the pistachio 7S vicilin-like protein was identified as an allergen (37% of patients' serum were reactive) and designated Pis v 3. A sequence alignment of Pis v 3 with the vicilin-like allergen, Ana o 1, from the closely related cashew revealed a high degree of homology (80% identity and 90% similarity) between the two proteins. This finding, coupled with considerable similarity between the two nut aa sequences in the regions corresponding to the previously identified linear epitopes of cashew vicilin [5], is a strong predictor of cross-reactivity. The results obtained from the IgE-binding studies provide further support for this supposition because all but one of the tested pistachio and/or cashew-allergic patients' sera that recognized the vicilin from one nut, also reacted with the other in an IgE dot-blot assay. Included in this population are patients who reported previous exposure to only one of the two nuts. Inhibition dot- and Western immunoblots, wherein IgE binding to either allergen could be completely prevented by pre-incubation of sera with either allergen demonstrate not only cross-reactivity but also that the cross-reactivity is symmetric in these patients, at least with respect to this IgE-binding protein. Cross-reactivity is not limited to patient IgE as six of nine randomly selected murine IgG anti-rAna o 1 MAbs also bound to Pis v 3. Together, these data suggest that antibody recognition of these proteins, whether by patient IgE in a natural allergenic situation or in an artificial murine immunization/hybridoma situation, is focused on the most conserved regions of the proteins.

If allergy to pistachio follows a pattern similar to that for cashew, the recognition of several different allergenic proteins by patient IgE can be expected. To date three major cashew allergens, a 7S vicilin (Ana o 1), an 11S legumin (Ana o 2), and 2S albumin (Ana o 3), have been identified which are characterized as seed storage proteins [5–7]. For each, their recombinant molecules have been cloned, sequenced, expressed and their linear epitopes mapped. Several studies have demonstrated that multiple proteins in pistachio extract exhibit IgE binding [13, 14]. On the basis of these previously published immunoblots and comparison with similar blots for cashew extract [13, 14], it could be surmised that the 45 kDa pistachio vicilin-like protein (here identified as Pis v 3) and a legumin-like protein, 33 kDa in size, are important allergens and that other allergenic pistachio proteins remain to be characterized. Indeed, we have recently cloned an IgE-binding pistachio legumin (unreported data).

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