

ORIGINAL ARTICLE

Identification of two pistachio allergens, Pis v 1 and Pis v 2, belonging to the 2S albumin and 11S globulin family

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Summary

Background IgE-mediated allergic reactions to pistachio appear to be occurring more frequently; however, little is known about its allergenic proteins.

Objective We attempted to identify pistachio allergens and to clone the encoding genes.

Methods Pistachio proteins were extracted and separated by SDS-PAGE. Immunolabelling was performed with sera from 28 pistachio-allergic individuals. Proteins of interest were further analysed by Edman sequencing and mass spectrometry/mass spectrometry (MS/MS). In parallel, a cDNA library was generated from immature pistachios and screened with primers designed on the basis of internal sequences and peptide spectra. Full-length cDNA clones were isolated from the library and sequenced. Recombinant proteins were expressed and tested with sera from pistachio-allergic patients.

Results Nineteen out of 28 patients (68%) showed IgE binding to a 7 kDa protein fraction, while 14 (50%) showed specific IgE to a 32 kDa protein fraction. Analysis by Edman sequencing and MS/MS revealed that these proteins were homologue to the cashew nut allergens Ana o 3 and Ana o 2, respectively. Screening of the pistachio cDNA library resulted in isolation of novel protein cDNAs. Open-reading frame translation provided the complete amino acid sequences of two new allergenic pistachio proteins. Recombinant proteins were recognized by six out of six selected patients. Therefore, these new allergens were named Pis v 1 and Pis v 2 by the Allergen Nomenclature Subcommittee.

Conclusion Novel allergens in pistachio, Pis v 1 and Pis v 2, which belong to 2S albumin and 11S globulin family, respectively, were isolated and the genes encoding these allergens were identified.

Keywords allergen, pistachio, Pis v 1, Pis v 2

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Introduction

Tree nut allergy is a common disorder with a prevalence rate of about 1% in westernized countries [1, 2]. Similar to peanut allergy, allergies to tree nuts generally begin in childhood and persist throughout life [3, 4]. Peanuts and tree nuts are responsible for more than 90% of fatal anaphylactic reactions to food in the United States [5]. Tree nuts most often reported to cause allergic reactions are walnut (34%), cashew (20%), almond (15%), pecan (9%) and pistachio (7%) [6].

Pistachio (*Pistacia vera*) is one of the tree nuts increasingly attributable to IgE-mediated allergic reactions [7–9].

Food-dependent exercise-induced anaphylaxis [10] and even death after ingestion of pistachio nuts has been reported [5]. Pistachio is often eaten roasted as a snack but may also be added to give colour and taste to ice cream or bakery products.

Pistachios are a member of the *Anacardiaceae* family and show *in vitro* cross-reactivity with cashew and mango, which belong to the same botanical family [7, 11]. Cross-reactivity with nan-gai nut was also reported [12], and an association between Parietaria pollinosis [9] and Artemisia pollinosis [13] has been suggested. Pistachio might also pose a potential hazard to some lipid transfer protein-allergic patients [14]. Previously, four

IgE-binding proteins were identified at molecular weights of 34, 41, 52 and 60 kDa by immunoblotting crude pistachio extract using sera obtained from two pistachio-allergic patients [11]. In another study, pistachio allergens ranging from < 14 to 70 kDa were recognized by sera from three patients [7]. However, only one gene encoding the protein involved in pistachio allergy, Pis v 3, has recently been reported [15].

In this study, we attempted to identify the allergens present in pistachio and to clone genes encoding these allergens. Detection of tree nut allergens is clinically

important for the development of better diagnostic and therapeutic approaches.

Materials and methods

Patient sera

Twenty-eight patients with pistachio allergy were included in the study. Age, pistachio-specific IgE levels, clinical manifestations and coexisting peanut or tree nut allergy are summarized in Table 1. Twelve of the patients

Table 1. Clinical profiles of the patients with pistachio allergy

No.	Sex	Age (years)	Age at first reaction (years)	Pistachio-specific IgE (kU/L)	Clinical manifestations	Coexisting peanut or tree nut allergy
1	F	13	3	39	Hives, vomiting, abdominal pain, nasal congestion, itchy throat, repetitive coughing, wheezing, red or watery eyes, dyspnea	Hazelnut
2	M	11	4	11	Erythema, eczema, vomiting, abdominal pain, itchy throat, wheezing	None
3	M	7	4	13	Hives, abdominal pain, itchy throat, wheezing, red or watery eyes	Cashew nut
4	M	4	2	3	Hives, erythema, lip swelling, repetitive coughing, wheezing, red or watery eyes, dyspnea	None
5	M	15	4	13	Hives, erythema, lip swelling, vomiting, itchy throat, repetitive coughing, dyspnea	None
6	M	5	4	0.4	Hives	Peanut
7	M	3	3	10	Hives, eczema, vomiting, nasal congestion	None
8	F	10	10	4	Lip swelling	Cashew nut
9	F	37	NK	4	Hives, lip swelling, vomiting, abdominal pain, itchy throat, wheezing, red or watery eyes, dyspnea, hypotension	Peanut
10	M	9	7	33	Hives, vomiting, abdominal pain, wheezing	Peanut
11	F	7	2	13	Vomiting	Walnut
12	F	12	2	5	Vomiting, abdominal pain	Peanut, almond, cashew nut
13	M	5	4	2	Hives, nasal congestion	Peanut
14	F	5	4	13	Hives, lip swelling, vomiting, wheezing	Peanut
15	F	9		100	Erythema, abdominal pain, itchy throat, repetitive coughing	Peanut, hazelnut, pecan, walnut
16	M	38	27	100	Erythema, lip swelling, vomiting, diarrhea, abdominal pain	Brazil nut, cashew nut
17	M	8	1	100	Lip swelling, nasal congestion, dyspnea	Cashew nut
18	M	11	10	52	Vomiting, itchy mouth	Hazelnut
19	F	15	4	32	Lip swelling, itchy throat, throat tightness, red or watery eyes	Almond, pecan, cashew nut
20	M	10	1	29	Hives, lip swelling	None
21	F	11	4	29	Repetitive coughing, wheezing	Peanut, hazelnut, walnut
22	F	13	3	25	Hives, vomiting	Hazelnut, Brazil nut, almond, pecan, cashew nut, pine nut, walnut
23	F	7	5	21	Lip swelling	Cashew nut
24	M	11	4	9	Hives, lip swelling, throat tightness, dyspnea, hypotension	Peanut
25	F	2	2	8	Hives, vomiting	Cashew nut
26	M	8	5	3	Itchy throat	Hazelnut
27	F	4	3	2	Hives, erythema, lip swelling, vomiting, throat tightness	None
28	M	38	37	1	Eczema, abdominal pain, itchy throat	Peanut, cashew nut, walnut

NK, not known.

had asthma and 16 had a history of atopic eczema. In 22 patients, allergic reactions to pistachio occurred on their first known exposure. All sera from those 22 patients have specific IgE to cashew. In 18 out of 22, specific IgE to one or more tree nuts other than pistachio and cashew were also detected. Most of the clinical reactions were rapid in onset, occurring within 5 min after ingestion of pistachio. The clinical symptoms varied from skin reactions to anaphylaxis [16]. Coexisting peanut or tree nut allergy was present in 23 (82%) of the 28 patients. In addition, pistachio-allergic patients also had peanut allergy ($n = 10$) and allergic reactions to hazelnut ($n = 6$), walnut ($n = 5$) or almond ($n = 3$). Allergic reactions to cashew were found in 10 patients (Table 1).

Blood samples were obtained and the sera were frozen at -80°C until use. Diagnosis of pistachio allergy was based on a convincing history of allergic reactions to a single ingestion of pistachio and a pistachio-specific IgE > 0.35 kU/L measured with the Phadia CAP-system FEIA (Uppsala, Sweden). Peanut or tree nut allergy was diagnosed using the same criteria as that for pistachio allergy. The study was approved by the local ethical committee and informed consent was obtained from all patients.

Preparation of pistachio protein extract

Pistachio nuts (*P. vera*) were ground in a grinder until a paste was achieved. The paste was defatted by washing with 20 volumes of cold acetone through filtering followed by drying overnight at 4°C . Soluble proteins were extracted from the defatted flour by agitating in phosphate-buffered saline (PBS) containing a protease inhibitor cocktail without ethylenediaminetetraacetic acid (Roche, Penzberg, Germany). After centrifugation at 3000 g, followed by 13 000 g, the supernatant containing the aqueous protein extract was aliquoted and stored at -80°C until use. Protein concentration was determined by the Coomassie Plus Protein Assay (Pierce, Rockford, IL, USA) according to the manufacturer's protocol.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Pistachio extract was mixed with NuPAGE[®] LDS sample buffer (Invitrogen, Carlsbad, CA, USA) with the addition of 0.05 M dithiothreitol (DTT), and heated at 70°C for 10 min. Molecular weight markers (Cat. No. LC5925, Invitrogen) were used to estimate sample MW. Electrophoresis was carried out at 200 V by using NuPAGE[®] Novex Bis-Tris 12 well Gel 4–12% (Invitrogen) with 12.4 μg of total protein from pistachio extract in each well. Proteins were transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA, USA) by electroelution and stained with Imperial[™] Protein Stain (Pierce, Rockford,

IL, USA) to test the quality of transfer and the total protein content.

Immunoglobulin E Western immunoblotting and inhibition

Immobilon-P transfer membranes (Millipore) containing blotted protein were cut into strips, and immunolabelled with sera from all 28 pistachio-allergic patients. Serum from an atopic individual who was not allergic to pistachio was used as a negative control. Sera were diluted from 1 : 4 to 1 : 20, depending on the level of pistachio-specific IgE, in PBS-Tween 20 plus 1% bovine serum albumin (BSA) and 10% normal goat serum and incubated for 2 h at room temperature. The strips were then washed with PBS three times for 15 s each and incubated for 1 h at room temperature with ^{125}I -labelled goat anti-human IgE (DiaMed, Windham, ME, USA) diluted in PBS-Tween 20 plus 1% BSA and 10% normal goat serum as per the manufacturer's directions. Blotted membranes were washed with PBS three times as above, exposed to Kodak X-OMAT Imaging Film (Carestream Health Inc., Rochester, NY, USA), and then developed 2–4 days later.

Two-dimensional gel electrophoresis

The crude extract was desalted, concentrated with Perfect Focus (GenoTechnology Inc., St. Louis, MO, USA) and resuspended in rehydration buffer [9.8 mol/L urea, 4% chaps, 2 mmol/L tributylphosphine (TBP), 0.2% ampholytes and 0.001% bromophenol blue; Biorad, Hercules, CA, USA]. For two-dimensional (2D) gel electrophoresis, 50 μg of protein extract was applied to 7 cm immobilized pH-gradient (IPG) strips (Biorad) for rehydration and focusing for 17 h with linear voltage slope up to 20 000 volt-hours followed by 2D gel electrophoresis using Invitrogen Zoom Gels (Invitrogen). Before running the SDS-PAGE, IPG strips were equilibrated with urea, SDS and glycerol-containing Tris-HCl buffer in the presence of 5 mM TBP for 10 min and then 135 mM iodoacetamide for 10 min. The proteins resolved by this technique were subsequently transferred to Immobilon-P membranes (Millipore) and then stained with 2.2% Coomassie blue for total protein analysis. For molecular weight determination MultiMark[™] Multi-Colored Standard (Invitrogen) was used.

Matrix-assisted laser desorption ionization mass spectrometry analysis and Edman sequencing

After 2D gel electrophoresis was completed, the gels were stained with Coomassie blue. The potential allergen at 32 kDa was analysed at the Wistar Institute (Philadelphia, Pa, USA). Briefly, the protein of 32 kDa was excised and digested with trypsin (Promega, Madison, WI, USA). The resulting peptide mixture was separated by means of reverse-phase high-performance liquid chromatography,

followed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis of selected peaks, and Edman sequencing was performed on two peptides with the Applied Biosystems Procise Sequencer (Foster City, CA, USA).

Mass spectrometry/mass spectrometry peptide sequence analysis

Bands of 7 kDa protein were manually excised from gels and digested with trypsin. Bands were concentrated with 100% acetonitrile and dried. Then the samples were reduced with 10 mM DTT and alkylated with iodoacetamide. Finally, samples were digested with sequencing-grade trypsin in 25 mM ammonium bicarbonate (pH 8.5). MALDI time-of-flight (TOF) MS analyses were performed in a Boyage-DESTR instrument (Perseptive Biosystem, Framingham, MA, USA). All mass spectra were calibrated externally using a standard peptide mixture (Sigma-Aldrich, St. Louis, MO, USA). The mass spectrometry/mass spectrometry (MS/MS) sequencing analyses were performed using the MALDI-tandem-TOF MS spectrometer 4700 Proteomics Analyzer (Applied Biosystems).

Construction of pistachio cDNA library

A cDNA library was generated from immature, raw pistachios, harvested approximately 25 weeks after blooming of pistachio trees (before the splitting of the shell). Therefore, pistachio seeds were removed from the shell and immediately frozen in liquid nitrogen. Seeds were later ground with a mortar and pestle and the total RNA was extracted according to the protocol by Chang *et al.* [17], and subsequently mRNA extraction was done by means of Poly(A)Purist™ MAG Kit (Ambion Inc., Austin, TX, USA), according to the manufacturer's protocol. The construction of the cDNA library was performed with the Uni-ZAP XR Gigapack Cloning kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's protocol. The library was amplified in *Escherichia coli* strain XLBlue MRF'. The amplified library was screened with 2S albumin and 11S globulin degenerated primers (5'-CARAAYCTI-GARCARATGGT-3', 5'-GARAACCARTIGAYCARTA-3') designed on the basis of the information obtained by MS/MS or internal sequencing, respectively.

Sequencing and corresponding analysis of selected genes

Phages containing the cDNA were prepared for *in vivo* excision of single clone. The phage stock was combined with XL1-Blue MRF' cells and excised by using the ExAssist helper phage with SOLR strain (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. Plasmid DNA was isolated from the mixture of excised phagemid and SOLR strain and sequenced at the Mount

Sinai Core Facility. Analysis of DNA sequences and deduced amino acid sequences were performed using Vector NTI software program (Invitrogen).

Production of recombinant protein

The protein coding regions of the 2S and 11S globulin were amplified by means of PCR with the specific primers, which allowed the introduction of unique restriction sites suitable for ligation into the expression pET24b(+) (Novagen, Madison, WI, USA) vector. The specific primers used were as follows: 5'-GTCCATCTACCGTGCTACTGT-3' and 5'-ATAAGACCAGTATGGTGAACCTA-3' for Pis v 1, 5'-GCAGATAGAACAGGTTGTAACTC-3' and 5'-GTGTATCTCCCTCTCTCCC-3' for Pis v 2.0101, 5'-GCAGATAGAACAGGTTGTAACTC-3' and 5'-GTGTATCTCCCTCTCTCCC-3' for Pis v 2.0201. The ligated insert-vector constructs were introduced into *E. coli* XL1-Blue strain by means of chemical transformation, following a protocol provided by the cell manufacturer (Stratagene). The plasmids were purified, and the inserts orientation and nucleotide sequence were confirmed. Subsequently, each individual expression plasmid was introduced into the BL21 expression *E. coli* strain. The recombinant protein was expressed upon induction of the bacterial culture at 0.4–1.0 optical density at 600 nm with 1 mM of isopropyl β -D-1-thiogalactopyranoside for 16 h at 30 °C. The recombinant protein expression was detected by probing the Western blot with the anti-His-tag antibody. His-tag (6 $\times$ His) is at the C-terminal end of the protein. In addition, the recombinant protein has N-terminal T7-tag, which adds extra 11 amino acids. The recombinant proteins were purified using a His-bind Ni²⁺-chelating NTA-matrix resin (Novagen) according to the manufacturer's protocol. The purity and the size of the purified recombinant proteins were confirmed by immunolabelling with monoclonal antibodies against N-terminal T7-tag and C-terminal His-tag.

Screening of recombinant protein with patient sera

Finally, the recombinant proteins were screened with individual patient sera from six out of 28 pistachio-allergic patients from whom sufficient quantities of sera were available for testing. Sera from one atopic and one non-atopic person were used as negative controls. The recombinant proteins were separated on SDS-PAGE (Nu-Page MES Bis-Tris, Invitrogen), according to the manufacturer's protocol. Two micrograms of each protein was loaded into each well. The proteins were subsequently transferred to Immobilon-P membrane (Millipore) and then stained with 2.2% Coomassie blue for total protein analysis. For molecular weight determination SeeBlue® Plus2 Pre-stained Standard (Invitrogen) was used. Patient and control sera were diluted from 1 : 4 to 1 : 20 (depending on the level of pistachio-specific IgE) in PBS-Tween 20 plus 1% BSA and 10% normal goat serum and

incubated with immunoblots for 2 h with gentle agitation at room temperature. After 2 h, membranes were briefly rinsed with PBS and then incubated with ^{125}I -labelled anti-human IgE (DiaMed) that was diluted as per the manufacturer's protocol. After agitation for 1 h at room temperature and washing with PBS, immunoblots were dried and exposed to Kodak Biomax MS (Carestream Health Inc.) imaging film for 7–14 days.

Results

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting analysis

SDS-PAGE and immunoblot results for 28 pistachio-allergic patients and a control subject are shown in Fig. 1. IgE-binding proteins were identified at 62, 51, 41, 36, 32, 28, 23, 18, 15, 10 and 7 kDa. Proteins at 7 and at 32 kDa were bound by IgE from 19 (#1, #2, #3, #5, #7, #8, #10, #15, #16, #17, #18, #20, #21, #22, #23, #24, #25, #26 and #27) and 14 patients (#1, #2, #3, #5, #8, #10, #11, #13, #14, #15, #19, #22, #24 and #26), respectively. These proteins were considered potential major allergens, because they reacted with more than 50% of the patients, and therefore chosen for further analysis.

Pistachio allergens of 2S albumin family (Pis v 1)

For the protein at 7 kDa, MS/MS peptide sequence analysis was performed after in-gel digestion with trypsin, and the peptides were compared with known proteins in the database. Three peptide spectra (ECCQELQEVD, CQNLEQMVR and ELYETASELPR) were found to be homologous to the sequences of Ana o 3, a cashew nut allergen of the 2S albumin family. In parallel, RNA isolated from pistachio was used to construct a cDNA library. An oligonucleotide (5'-CARAACTIGARCARATGGT-3') was designed on the basis of MS/MS analysis data and used to isolate a novel protein cDNA from this cDNA library. An isolated full-length clone was sequenced and showed 767 bp cDNA and 450 bp of coding sequence. The nucleotide sequence was submitted to the GenBank Database under accession number DQ631675. Open-reading frame translation of this novel protein revealed that this clone had 149 amino acids and 64% identity and 74% similarity to Ana o 3, a cashew nut allergen of the 2S albumin family (Fig. 2). The Allergen Nomenclature Subcommittee has designated the 2S albumin of pistachio (*P. vera*) as Pis v 1. Finally, the recombinant protein of Pis v 1 (rPis v 1) was screened with individual patient sera from six pistachio-allergic patients (#2, #5, #10, #15, #22 and #24) (Fig. 3). rPis v 1 has multiple bands that represents oligomers of the same protein. This was confirmed by immunolabelling



Fig. 1. SDS-PAGE analysis for pistachio protein extracts (P) and immunolabelling with sera from 28 patients with pistachio allergy (#1–#28). Lower and upper marks indicate Pis v 1 at 7 kDa and Pis v 2 at 32 kDa, respectively. M, molecular markers; P, pistachio protein; C, negative control.

	1	50
Pis v 1	(1) MAKLVLLLSAFAFLILAANASIYRATVEVEGENLSSGQSCQKQFEEQQKF	
Ana o 3	(1) MAKFLLLLSAFAVLLLVANASIYRAIVEVEEDSGRE-QSCQRQFEEQQRF	
	51	100
Pis v 1	(51) KHCQMIVVQQEVQKSQDGHSLTARINQRQCFKQCCQELQEVDKKCRQCQL	
Ana o 3	(50) RNCQRVVKQEVQRG-----GRYNQRQESLRECCQELQEVDKRCRCQNL	
	101	150
Pis v 1	(101) EQMVKRRQQQGGFGEKLELYETASELPRMCNISPSQGCQFSSPYWSY-	
Ana o 3	(93) EQMVRQLQQQEIQKGEEVRELYETASELPRICISPSQGCQFQSSY----	

Fig. 2. The amino acid sequence of the nPis v 1 and the comparison with Ana o 3 of cashew nut (GenBank accession no. AY081853). Red letters indicate the identical amino acids between nPis v 1 and Ana o 3. Green letters represent the amino acid with similar property (e.g. hydrophobicity).

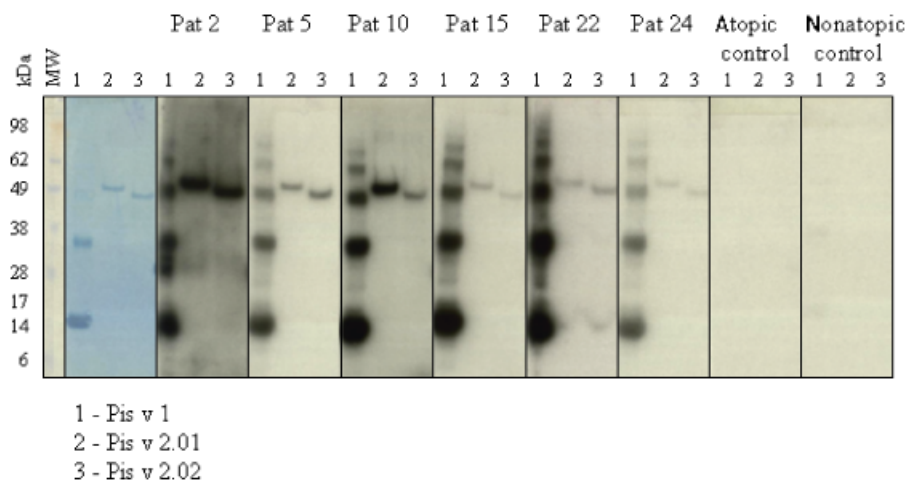


Fig. 3. SDS-PAGE analysis for rPis v 1 and rPis v 2. Shown are the labelling with Coomassie blue for total protein analysis and immunolabelling with sera from six pistachio-allergic patients, a non-atopic and an atopic control.

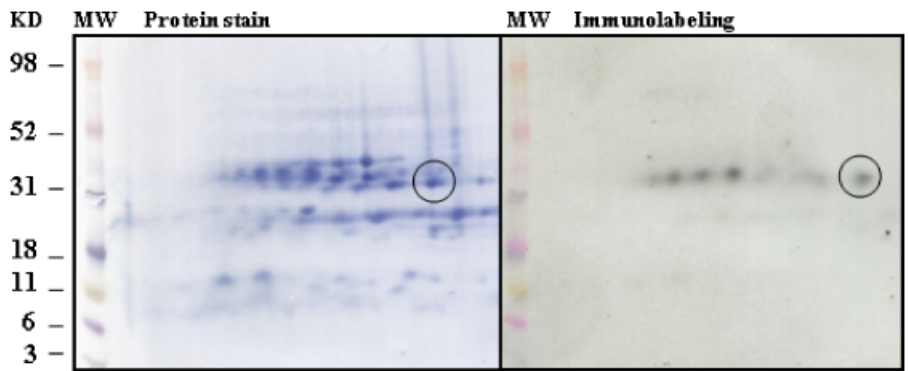


Fig. 4. Two-dimensional proteomic map for pistachio proteins. Shown is the labelling with Coomassie blue for total protein analysis and the immunolabelling of one representative patient. The control sera did not show any labelling. Molecular weight standards (MW) are shown on the left-hand side. The circle indicates the protein for which Edman sequencing was performed.

with specific monoclonal antibodies against the His-tag. Sera from one atopic and one non-atopic person were used as negative controls. All six pistachio-allergic patients showed IgE binding to the expressed rPis v 1.

Pistachio allergens of 11s globulin family (Pis v 2)

For further analysis of the protein at 32 kDa, pistachio proteins were separated by 2D PAGE. Sera from 12 patients (#1–#11, #15) out of 28 were used for immunolabelling and Fig. 4 shows one representative data (patient #6). Edman sequencing was performed for the protein spot that reacted with the majority of patient sera. The internal sequences obtained for this protein was FQTQCQIQNLNALE and LVLVALADVGNSENQLDQY, respectively. An oligonucleotide (5'-GARAACCARCTIGAYCARTA-3') was de-

signed on the basis of this information and used for screening the cDNA library resulting in the isolation of a novel protein cDNA. Ultimately we obtained two different isoforms with cDNA of 1736 and 1628 bp, respectively. The nucleotide sequences were submitted to the GenBank Database under accession number DQ631676 and DQ631677, respectively. DQ631676 had 1491 bp of coding sequence and the isoform DQ631677 had 1419 bp starting with methionine residue 19 and ending with an asparagine residue 1434.

Open-reading frame translation revealed two proteins that showed 48% and 46% homology to the 11S globulin Ana o 2, a major cashew nut allergen (Fig. 5). The Allergen Nomenclature Subcommittee has designated these 11S globulins of pistachio (*P. vera*) as Pis v 2.0101 and Pis v 2.0201, respectively. The isoelectric points (pI) of these isoforms were 7.3 and 6.85. Pis v 2.0101 and its isoform had 496 and 472 amino acids, respectively. They had

		1		70
Pis v 2.0101	(1)	MGYSLLSFSLGFLLLFHC	FAIEQVNSQQRQQQRFQTCQIQNINALEPKRRIS	EAGVTEFWDQN
Pis v 2.0201	(1)	MGYSLLSFSLGFLLLFHC	FAIEQVNSQQRQQQRFQTCQIQNINALEPKRRIS	EAGVTEFWDQN
Ana o 2	(1)	-----LSVCFLLFHLGCLAS-----	-----RQEWQQQDECCQIDRLDALEPDNRVEYEAGTVEAWDIN	
		71		140
Pis v 2.0101	(71)	EEQLQCANVAVFRHTIQSRGLLVPSYD	NAPELVYVVGSGINGAVFPGCPET	FQEESSQSRSSRSRSE---
Pis v 2.0201	(71)	EEQLQCANVAVFRHTIQSRGLLVPSYD	NAPELVYVVGSGINGAVFPGCPET	FQEESSQSRSSRSRSE---
Ana o 2	(53)	HEQFRCAQVALVRHTIQENGLLLPOYSNAPOLIVVVGSGMTGISY	PGCPETYQAPQOQGRQOQSGR---	
		141		210
Pis v 2.0101	(138)	---QSGEQHQKVRPIQEGDVI	ALPAGVAHWIYNNQSSKLVLVALADVGNSENQLDQYLKRFVLGSSPQOEI	
Pis v 2.0201	(141)	SQSGEQHQKVRHIREGDI	IALPAGVAHWIYNNQSSKLVLVALADVGNSENQLDQYLKRFVLGSSPQOEI	
Ana o 2	(120)	---FQDRHOKIRRFRRGDI	IAIPAGVAHWIYNNQSSKLVLVALADVGNSENQLDQYLKRFVLGSSPQOEI	
		211		280
Pis v 2.0101	(206)	QSGSQSRSSRSQSSRRGQ	QGGQSSNNILSAFDEEILAQSFNIDTQLARRLQKEKRGQIIVRVQEDLEV	
Pis v 2.0201	(211)	QGGQSSWSQSSRS---	RKGGQSSNNILSAFDEEILAQSFNIDTQLVKKLQREKRGQIIVRVQEDLEV	
Ana o 2	(187)	QQ---CQOHQSRG-----	---RNLFSGFDTLLAFAQVDERLILQLKSEDNRGIGIVKVDDELVI	
		281		350
Pis v 2.0101	(276)	SPHRQRE-----Q---	EYBERERRQRNGLEETFCMTLKYININDPSRADVYNPRGGRVSSVNAINLEI	
Pis v 2.0201	(276)	SPQRQKE-----Y---	SDNGLEETFCMTLKYININDPSRADVYNPRGGRVSSVNAINLEI	
Ana o 2	(243)	RPSRSQSEERSESEES	EDKRRWGQRDNGLEETFCMTLKYININDPSRADVYNPRGGRVSSVNAINLEI	
		351		420
Pis v 2.0101	(338)	LRFLQLSAKKGVLHRDAIL	APHNNVNAHSIVYITRNGRIQIVSENGESVDFEIRGQLVVPQNFVAV	
Pis v 2.0201	(329)	LRFLQLSVEKGVLYQNAI	APHNNVNAHSIVYITRNGRIQIVSENGESVDFEIRGQLVVPQNFVAV	
Ana o 2	(313)	LKWLQLSVEKGVLYKNAI	LVPHNNVNAHSIVYITRNGRIQIVSENGESVDFEIRGQLVVPQNFVAV	
		421		490
Pis v 2.0101	(408)	KRASDKFEWVSFKTNGLS	QTSQLAGRVSVFRALPLDVINKNSFDISREDARLKESRSETTIFAPGSSSQ	
Pis v 2.0201	(399)	KRASDKFEWVSFKTNGLA	KISQLAGRVSVFRALPLDVINKNSFDISREDARLKESRSETTIFAPGSSSQ	
Ana o 2	(383)	KRAERFEWISFKTNDRAM	TSPLAGRTSVLGGMPEEVLANAFQISREDARKIKFNNGQTTLS-GESSH	
		491		
Pis v 2.0101	(478)	RKSQSERERQRREBREI	H---	
Pis v 2.0201	(469)	RQRN-----		
Ana o 2	(453)	HMRDDA-----		

Fig. 5. The amino acid sequence of nPis v 2.0101 and its isoform nPis v 2.0201 and the comparison with Ana o 2 of cashew nut (GenBank accession no. AF453947). Red letters indicate the identical amino acids among the three allergens, while blue letters are shown when only two of them show identical amino acids. Green letters represent the amino acid with similar property (e.g. hydrophobicity).

83.5% identical residues and 86.7% similarity (Fig. 5). Finally, the recombinant protein of Pis v 2 (rPis v 2) was screened with individual patient sera from six pistachio-allergic patients (Fig. 3). Human sera from one atopic and one non-atopic person were used as negative controls. All six pistachio-allergic patients showed IgE binding to the expressed rPis v 2.

Discussion

In our study, 28 patients with pistachio allergy were enrolled based on convincing histories of allergic reactions following an isolated ingestion of pistachio and elevated serum pistachio-specific IgE. Twenty-two out of 28 manifested clinical reactions to the first known exposure to pistachio, which suggests either prior sensitization to an unknown exposure, or possibly cross-reactivity due to prior sensitization to a highly cross-reactive protein source. Actually 82% of the patients had coexisting peanut or tree nut allergy. In the present study, cashew was the most common offending tree nut with 10 (36%) of the pistachio-allergic patients reporting allergic reactions following cashew ingestion. However, the true prevalence is likely greater because seven patients with cashew-specific IgE out of the remaining 18 patients had never eaten cashew after their initial tree nut allergy was discovered. This high rate of coexisting pistachio and cashew allergy can be explained by the fact that both nuts belong to the family *Anacardiaceae*, which suggests their structural similarity. *In vitro* cross-reactivity between

pistachio and cashew has been shown by inhibition assays [7, 11, 18]. By contrast, other tree nuts belong to different orders. For example, pistachio and cashew belong to the order *Sapindales*, whereas walnut, hazelnut, pecan and chestnut are included in the order *Fagales*. Almond, Brazil nut and Macadamia nut belongs to the order *Rosales*, order *Ericales* and order *Proteales*, respectively. The less close botanical relationship is likely to account for the decreased prevalence of coexisting allergies. However, it has not yet been determined whether these concomitant tree nut allergies other than cashew occur because of multiple independent sensitizations of each individual allergen or by cross-reactivity.

SDS-PAGE of a pistachio nut extract followed by immunoblotting analysis identified 12 antigenic fractions with molecular weights ranging from 7 to 70 kDa. In one case report, four antigenic fractions with molecular weights of 34, 41, 52 and 60 kDa were identified by sera from three patients with pistachio allergy [11], while six IgE bands with molecular weights between <14.4 and 70.9 kDa were detected in two patients in another case report [7]. When compared with these previous data [7, 11], detection of more IgE-binding protein fractions on immunoblots in the present study might be attributed to the larger study population, and suggests significant heterogeneity of recognition patterns between the patients. Our result demonstrates that in addition to the 7 and 32 kDa proteins, patients often recognized proteins with molecular weight of 23 and 36 kDa, respectively, which still remain to be elucidated.

We identified and sequenced pistachio allergens that belong to the 2S albumin (Pis v 1) and 11S globulin (Pis v 2) families. Considering most tree nut allergens identified to date are vicilins (7S globulins), legumins (11S globulins) and 2S albumins [19], it is not surprising that the 2S albumin and 11S globulin identified are major allergens of pistachio. Pistachio allergens showed structural similarities to cashew allergens when sequences were compared. Deduced amino acid sequences of Pis v 1 and Pis v 2 had 64% sequence identity to Ana o 3 (accession no. AY081853) [20] and 48% to Ana o 2 (accession no. AF453947) [21], respectively. This rate of sequence identity explains their potential cross-reactivity. A BLAST alignment of Pis v 1 and 2S albumins of Black walnut (Jug n 1, accession no. AY102930), English walnut (Jug r 1, accession no. U66866) [22] and Brazil nut (Ber e 1, accession no. M17146) [23] demonstrates 39%, 36% and 33% of amino acid sequence identity, respectively. This indicates that sequential structures of 2S albumins of other tree nuts are less similar to Pis v 1 compared with cashew. In contrast, Pis v 2 has similar sequence homology with Jug r 4 of English walnut (accession no. AY692446) (50%), Cor a 9 of hazelnut (accession no. AF449424) (47%) and Ber e 2 of Brazil nut (accession no. AY221641) (46%) as well as with Ana o 2 of cashew [24, 25]. Based on the guidelines for assessment of genetically modified crops that suggest a sequence identity of 35% as a cut-off for potential cross-reactivity [26], there is a possibility of cross-reactivity between tree nut allergens of the 11S globulin family. In terms of peanut allergy, Pis v 1 showed 25% sequence identity to Ara h 2 (accession no. AY007229) or Ara h 6 (accession no. AF092846), peanut allergens of the 2S albumin [27]. The sequence alignment of Pis v 2 with legumin-type allergens of peanut, Ara h 3 (accession no. AF093541) or Ara h 4 (accession no. AF086821), revealed 35–38% amino acid sequence identity [27, 28]. The low degree of sequence homology between pistachio and peanut allergens suggests that cross-reactivity is less likely.

There are some limitations in our study. The recombinant allergens were analysed with only six out of 28 sera from pistachio-allergic subjects due to insufficient serum volume in the majority of patients. Although six sera showed consistent reactions with rPis v 1 and rPis v 2, they may not be representative of all 28 sera. In addition, we did not perform *in vivo* or *ex vivo* test with recombinant allergens to demonstrate biologic activity, which will need to be performed in future studies.

With recent advances in recombinant DNA technology, diagnosis of food allergy can be improved through the identification of individual allergen components [29, 30]. This concept of 'component-resolved diagnostics' may prove more useful to predict the outcome of food allergy [31, 32], to find the cross-reactivity against other food or inhalant allergens [33] and to under-

stand the clinical relevance of severe food-allergic reactions [34]. In this regard, food allergens, especially those allergens responsible for the majority of the allergic reactions or those eliciting severe anaphylaxis, need to be characterized. However, this will be difficult because there are many potential allergens, and several isoforms of individual allergens as shown in Pis v 2.0101 and Pis v 2.0201.

In conclusion, novel allergens in pistachio, Pis v 1 and Pis v 2, which belong to the 2S albumin and 11S globulin families, were identified as major pistachio allergens. The characterization of pistachio allergens helps to explain the possible cross-reactivity between tree nut allergens and is the first step towards better diagnostic and therapeutic approaches.

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