



Pin p 1 is a major allergen in pine nut and the first food allergen described in the plant group of gymnosperms



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ABSTRACT

This study aimed to report the complete sequence of a 2S albumin purified from pine nut and to analyze its allergenic properties. Individual recognition of this protein by serum IgE from pine nut-allergic patients was assessed. IgE cross-linking capacity was analyzed in a basophil activation test. Inhibition of IgE-binding and stability to heating was also assessed. The complete nucleotide sequence was obtained and a phylogenetic study was carried out. 2S albumin from pine nut (registered as Pin p 1.0101) was recognized by IgE of 75% of sera. The allergen was heat-stable and had a robust capacity to inhibit IgE-binding to whole pine nut extract. The IgE cross-linking capacity of Pin p 1 on basophils was also demonstrated. Despite the low homology of Pin p 1 sequence with other allergenic 2S albumins from angiosperms, Pin p 1 contains the typical skeleton of 8 cysteine residues, important for its α -helixes enriched structure.

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1. Introduction

Allergy to tree nuts, in comparison with allergy to other foods, has been described as particularly severe. Tree nuts are one of the most frequent cause of fatal anaphylactic reactions (Bock, Muñoz-Furlong, & Sampson, 2007). Pine nuts produced in Europe originate in most cases from *Pinus pinea*, which belongs to the conifers division, the largest group of living gymnosperms. Gymnosperms are evolutionally separated from flowering plants (angiosperms), which produce most of the tree nuts consumed by humans (walnut, hazelnut, cashew, pistachio, Brazil nut, etc). Pine nuts from *Pinus pinea* have been consumed in the Mediterranean region for over 2000 years. Today they are extensively consumed worldwide as an ingredient in different foods, both in raw or roasted form. Pine nuts are a source of valuable nutrients. The fatty acid composition is characterized by a high content of unsaturated fatty acids, such as linoleic acid, which has beneficial effects

on blood lipids, lowers blood pressure and serum cholesterol (Nergiz & Dönmez, 2004). In addition pine nuts contain potassium, phosphorus, magnesium and other minerals, as well as dietary fibres (Nergiz & Dönmez, 2004).

Allergy to pine nuts has been documented in the scientific literature since 1958. Of the 45 cases reported in the literature to date, severe anaphylactic reactions after pine nut consumption accounted for the majority of the described reactions (76%). Moreover, the number of individuals monosensitized to pine nuts compared to tree nuts from species of angiosperms is high. The reported reactions have been described to occur after consumption of pine nuts as part of pesto sauce, salads, meatballs or meat, cakes, candies, cookies as well as after consumption of pine nuts alone (Cabanillas & Novak, 2015).

A few IgE-binding proteins from pine nuts have been studied by means of western blotting so far. However, pine nut allergens have not yet been characterized in detail and official allergen names have not yet been registered by the Allergen Nomenclature Subcommittee from International Union of Immunological Societies (IUIS).

In our previous work, we have purified and partially characterized an IgE-binding protein from pine nuts with a molecular weight around 15–16 kDa under non-reducing conditions and around 6 kDa under reducing conditions (Cabanillas et al., 2012). In the present study, we aimed to analyze the allergenic features

Abbreviations: AP, adapter primer; AUAP, abridged universal amplification primer; HRP, Horseradish peroxidase; IUIS, International Union of Immunological Societies; PBST, PBS plus 0.5% Tween-20; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; TBST, TBS plus 0.5% Tween-20; TMB, tetramethylbenzidine; OD, optical density; RT, room temperature.

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and to describe the complete sequence of this protein, which is the first allergen described and officially registered for pine nuts. We also aimed to analyze the evolutionary differences between this novel allergen and its allergenic homologues from angiosperms.

2. Materials and methods

2.1. Patients and sera

Sera from 8 patients with IgE-mediated clinical allergy to pine nuts, confirmed on the basis of either a history of recent documented severe anaphylaxis after pine nut ingestion or a positive double-blind placebo-controlled food challenge (DBPCFC) with pine nuts, were used in this study (Table 1). Subjects had specific IgE levels to pine nut (quantified by the fluorescent enzyme immunoassay, CAP-FEIA system, Phadia, Uppsala, Sweden) and/or a positive skin prick test response to pine nut performed by the prick-to-prick technique according to standard methods (Heinzerling et al., 2013) (Table 1). Written informed consents were obtained from all patients. The study was approved by the Ethics Committee of the Hospital Universitario 12 de Octubre, Madrid, Spain (Permission No. 0312150129).

2.2. Protein purification and individual recognition by sera's IgE in western blotting

Purification of the IgE-binding protein from pine nuts with a molecular weight of around 6 kDa under reducing conditions and around 15–16 kDa under non-reducing conditions (herein Pin p 1) involved a combination of ammonium sulfate precipitation and ion exchange chromatography as described in Cabanillas et al., 2012. Briefly, protein solubility of pine nut extract was tested at different percentages of ammonium sulfate saturation, and it was found that Pin p 1 precipitated at 100% saturation. The precipitated proteins were collected by centrifugation and the pellet was dissolved in 50 mM Tris buffer (pH 8.4). Solubilized proteins were subjected to ion exchange chromatography. Firstly, the solubilized proteins were loaded on a high Q-support fast flow column (strong anion exchange support, Bio-Rad, Richmond, CA, USA). A linear salt gradient from 0 to 1 M NaCl in 50 mM Tris buffer, pH 8.4, was used to elute bound proteins. The unbound protein fraction, together with the protein fractions eluted with NaCl from 0 to 180 mM,

were pooled and the pH was adjusted to 7.0 with HCl. The pooled fractions were loaded on a high S-support fast flow column (strong cation exchange support, Bio-Rad) and eluted with a 200–400 mM NaCl linear gradient in 50 mM Tris buffer, pH 7.0. Initial characterization, by means of mass spectrometry analysis, showed a high match with the seed storage protein albumin from the species belonging to the *Pinaceae* family (Cabanillas et al., 2012).

Individual recognition of Pin p 1 by serum IgE was assessed by western blotting. First, SDS-PAGE was performed according to Laemmli's protocol (Laemmli, 1970). Five micrograms of Pin p 1 was mixed with Laemmli sample buffer and β -mercaptoethanol, heated at 95 °C for 10 min, and electrophoresed in a 15% Tris-HCl gel. The protein was visualized with Coomassie Brilliant Blue (Bio-Rad, Hercules, CA, USA). For western blotting, Pin p 1 was transferred to polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). Membranes were cut into 3-mm strips and blocked in Tris-buffered saline (TBS) containing 0.5% Tween-20 (TBST) and 5% non-fat milk for 1 h at room temperature (RT). Membranes were incubated overnight with individual sera from 8 patients with pine nut allergy (1:10 dilution), washed, and then treated with a mouse anti-human IgE monoclonal antibody (clone 1A2, Abbiotec, San Diego, CA, USA) (stock: 1 mg/ml; used at 1:5000) for 3 h. Membranes were washed and incubated with HRP-conjugated goat anti-mouse antibody (stock: 0.4 mg/ml; used at 1:5000) (Santa Cruz Biotechnology, Dallas, Texas, USA) for 1 h. After washing, detection was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA).

2.3. IgE-ELISA and ELISA inhibition

Specific IgE-binding to Pin p 1 was also determined by ELISA using 8 sera from patients with pine nut allergy. Polystyrene 96-well microtiter plates (BD Falcon 353279, Heidelberg, Germany) were coated with 100 μ l/well of Pin p 1 at 8 μ g/ml in PBS and incubated at 4 °C overnight. Wells were washed with PBS containing 0.5% Tween-20 (PBST) and blocked for 1 h with PBS containing 3% non-fat milk plus 0.1% Tween-20 (blocking solution). Plates were incubated with individual sera (1:7 dilution) for 2 h, washed and incubated with a monoclonal mouse anti-human IgE antibody (Abbiotec) (used at 1:1000) for 1 h. Wells were then washed and incubated with HRP-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology) (used at 1:1000) for 1 h. After washing, the peroxidase reaction was developed with tetramethylbenzidine (TMB) substrate (substrate reagent pack, DY999, R&D Systems, Inc., Minneapolis, MN, USA). The reaction was stopped with 2 N H₂SO₄, and the optical density (OD) was measured at 470 nm using a Synergy HT multi-detection microplate reader (BioTek, Bad Friedrichshall, Germany). The following negative controls were used: (i) no Pin p 1 coated-wells: wells coated with non-fat milk (instead of Pin p 1) and incubated with patients' sera and the rest of antibodies following the steps described above; (ii) no serum: wells coated with Pin p 1 and incubated directly with mouse anti-human IgE and HRP-conjugated goat anti-mouse IgG; and (iii) non-allergic sera: wells coated with Pin p 1 and incubated with two sera from healthy subjects, followed by the process described above. All the tests were performed in duplicate. The formula: mean [OD] + 3 $\times$ SD was calculated for every negative control and the highest value was considered as cut-off point of positivity (Cabanillas et al., 2015).

For ELISA inhibition, polystyrene 96-well microtiter plates (BD Falcon 353279) were coated with 100 μ l/well of whole pine nut protein extract at 100 μ g/ml in PBS and incubated overnight at 4 °C. In parallel, a serum pool from 6 pine nut allergic patients (1:10) that recognized Pin p 1 in western blotting and ELISA was pre-incubated with Pin p 1 or whole pine nut protein extract

Table 1
clinical and immunological features of the 8 pine nut-allergic patients whose sera was available to be used in this study.

Patient No./age/sex	SPT (mm)	CAP- FEIA (kU/L)	Symptoms	Diagnostic challenge	Other established food allergies
1/30/F	4.5	0.8	OAS	DBPCFC	Melon
2/38/F	11.5	0.9	U, AE, P, OAS, DS, TS, A, GM	a	...
3/34/M	12	2.27	AE, P, E, OAS, GI, DS, TS, GM	a	...
4/26/M	17.5	48	U	a	...
5/39/F	10	0.02	AE, P, E, OAS, O, TS	a	...
6/35/F	11	7.08	U, P, OAS, GI, GM	a	...
7/17/M	5.5	19.3	OAS	DBPCFC	...
8/18/F	15.5	2.2	AE, DY, DS, OAS, GI	a	...

AE, angioedema; A, asthma; CAP-FEIA, capsulated hydrolytic carrier polymer-fluoro-enzyme immunoassay; DBPCFC, double-blind, placebo-controlled food challenge; DS, difficulty swallowing; DY, dysphonia; E, erythema; GI, gastrointestinal symptoms; GM, general malaise; OAS, oral allergy syndrome; O, ocular symptoms; P, pruritus; SPT, skin prick testing; TS, tongue swelling; U, urticaria. a, Not challenged because of a convincing history of severe reactions to pine nut.

(inhibitors) (final concentrations: 180, 18, 1.8, and 0.18 µg/ml) overnight at 4 °C with stirring. Serum pool pre-incubated with PBS was also included (non-inhibited serum). Wells were washed with PBST and blocked with blocking solution for 1 h at RT. Wells were then incubated for 3 h with the sera preincubated with the two inhibitors, or with serum that was not inhibited. After washing the wells, a mouse anti-human IgE monoclonal antibody (Abbiotec), used at 1:1000 dilution was added and incubated for 1 h. Wells were washed and incubated with a HRP-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology) at 1:1000 dilution for 1 h. After washing, the peroxidase reaction was developed as described above. The percentage of inhibition of the IgE-binding was calculated as previously described (Cabanillas et al., 2015). All tests were performed in duplicate.

2.4. Heat stability of purified Pin p 1

Pin p 1 was boiled for 30, 60, and 120 min at 100 °C and analyzed by IgE-ELISA using a serum pool from 6 patients with pine nut allergy that recognized Pin p 1 in western blotting and ELISA.

2.5. Basophil activation assay

Basophils from a donor without any clinically relevant allergy to pine nuts and other nuts, but with specific IgE to hazelnut extract (1.33 kU/l) and the recombinant peanut allergen Ara h 8 (1.35 kU/l) were enriched (>90% purity) by depleting non-basophils using the EasySep Human Basophil Enrichment Kit (StemCell Technologies, Grenoble, France). IgE was removed from the surface of the basophils by incubation with lactic acid buffer (10 mM lactic acid, 130 mM NaCl, 5 mM KCl, pH 3.9) for 3.5 min on ice (Cabanillas et al., 2015). A fraction of basophils was kept unstripped and directly subjected to allergen challenge. Stripped basophils were washed and then passively sensitized for 90 min at 37 °C with the sera (dilution 1:3) from four patients with clinically relevant pine nut allergy (patients No. 2, 3, 6 and 8, Table 1). Before allergen challenge, basophils were allowed to recover in HEPES Tyrodes buffer containing 1 mM CaCl₂ for 30 min at 37 °C. Stripped and unstripped basophils were challenged for 20 min with 0.05 mg/ml of Pin p 1. As controls, basophils were incubated with HEPES Tyrodes buffer (negative control) or with a rabbit anti-human IgE polyclonal antibody (50 µg/ml) (Dako, Eching, Germany) (positive control). Cell surface staining of the basophil activation marker CD203c was carried out with anti-human CD203c-PE monoclonal antibodies (clone 97A6, Beckman Coulter, Krefeld, Germany). Cells were measured with the help of a FACSCanto flow cytometer (BD Biosciences, Heidelberg, Germany) and data were analyzed with the BD FACSDiva software (BD Biosciences, Heidelberg, Germany). A percentage of activation higher than 5% was considered as positive.

2.6. PCR

2.6.1. PCR based cloning strategy for the full characterization of Pin p 1 cDNA

Pinus pinea seeds were collected in El Álamo, Madrid (Spain). Total RNA was extracted using Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) and immediately stored at –80 °C to preserve its integrity. 1.8 µg of the total RNA was used for cDNA synthesis according to the 5' RACE system (5' RACE System for Rapid Amplification of cDNA Ends, Invitrogen) using a reverse transcriptase enzyme (Superscript II) and the gene-specific primer GSP1, 5'-ATCATACGATTGAGATTG-3' which was selected on the basis of the described partial cDNA sequence of this allergen (Cabanillas et al., 2012). The cDNA was purified from unincorporated dNTPs and GSP1 using S.N.A.P. columns™ purification. A

poly(C) tail was then added to the 3'-end of the cDNA using TdT and dCTP according to manufacturer's instructions. The dC-tailed cDNA was amplified by PCR using a nested second gene-specific primer GSP2, 5'-GGCTCGGCATCGGCATTGTGGA-3' and the abridged anchor primer provided with the 5' RACE system. The PCR conditions were as follows: 3 min of denaturation at 95 °C, followed by 31 cycles each of 1 min of denaturation at 95 °C, 1 min of annealing at 70 °C, and 37 s of elongation at 72 °C; and a final extension for 10 min at 72 °C. The PCR product was separated on a 1% agarose gel. A unique and well-defined band was obtained. The PCR product was purified and cloned into a pCR4-TOPO vector included in the TOPO TA Cloning Kit for Sequencing (Invitrogen) which is a one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into the plasmid pCR4-TOPO vector for sequencing. The construct was used to transform *Escherichia coli* DH5α competent cells, and the DNAs from several clones were sequenced using M13 forward and reverse sequencing primers (GATC Biotech., Konstanz, Germany). To obtain full-length cDNA, a 3'RACE system was carried out (3' RACE System for Rapid Amplification of cDNA Ends, Invitrogen). 1.5 µg of the total RNA was used for cDNA synthesis using the reverse transcriptase enzyme (Superscript II) and adapter primer (AP, 5'-GGCCACGCGTC GACTAGTACTTTTTTTTTTTTTTTT-3') provided with the system. The cDNA obtained was amplified by means of PCR with a gene-specific primer 5'-GACGTTGAGATGGGTGACTT-3' which was derived from the sequence obtained in the 5'RACE experiments. For the reverse direction, the 3' primer abridged universal amplification primer (AUAP) 5'-GGCCACGCGTCGACTAGTAC-3', complementary to the adapter sequence, was used to prime first strand cDNA synthesis. Amplification was carried out with *Taq* DNA polymerase. The PCR program was: 5 min of denaturation at 95 °C, followed by 31 cycles each of 1 min of denaturation at 95 °C, 1 min of annealing at 60 °C, and 43 s of elongation at 72 °C; and a final extension for 10 min at 72 °C. The obtained PCR product was purified, ligated to pCR4-TOPO and used to transform *Escherichia coli* as described above. DNAs for several clones were sequenced using M13 forward and reverse sequencing primers.

2.6.2. Sequence comparison and phylogenetic studies

Alignment and phylogenetic studies of the amino acid sequence of Pin p 1 with albumins from different species of gymnosperms and angiosperms were carried out with the program CLC Sequence Viewer, version 7.6.1 (CLC bio, Cambridge, MA). The Neighbor-Joining algorithm with 100 replicate bootstraps was used. The selected sequences were: *Pinus strobus* albumin 1 (ID: CAA44298.1), *Pinus strobus* albumin 2 (ID: CAA44299.1), *Pinus strobus* albumin 3 (ID: CAA44300.1), *Pinus strobus* albumin 4 (ID: CAA44301.1), *Picea glauca* albumin-like-protein (ID: AAC34613.1), *Pseudotsuga menziesii* albumin (ID: AAC27000.1), *Anacardium occidentale* 2S albumin (Ana o 3) (ID: AAL91665.1), *Juglans regia* 2S albumin (Jug r 1) (ID: AAB41308.1), *Bertholletia excelsa* 2S albumin (Ber e 1) (ID: AAA33010.1), *Carya illinoensis* 2S albumin (Car i 1) (ID: AAO32314.1), *Juglans nigra* 2S albumin (Jug n 1) (ID: AAM54365.1), *Pistacia vera* 2S albumin (Pis v 1) (ID: ABG73108.1) and *Corylus avellana* 2S albumin (Cor a 14) (ID: AC056333.1).

Percentages of amino acid identity or similarity between two sequences were obtained with the NEEDLE program in the EMBOSS package (Rice, Longden, & Bleasby, 2000).

2.7. Identification of potential N-Glycosylation sites in Pin p 1

SDS polyacrylamide gel slices containing Pin p 1 were split and subjected to gel digestion with AspN or GluC (in bicarbonate) (Jeno, Mini, Moes, Hintermann, & Horst, 1995; Rosenfeld, Capdevielle, Guillemot, & Ferrara, 1992). In brief, slices were washed

consecutively with water, 50% acetonitrile (ACN), and 100% ACN. The protein was reduced with 20 mM DTT in 50 mM ammonium bicarbonate and alkylated with 40 mM iodoacetamide (in 50 mM bicarbonate). The slices were washed again and dehydrated with ACN. Dried slices were incubated with 400 ng of sequencing grade protease (Promega, Mannheim, Germany) at 37 °C overnight. The peptide extract was separated and the remaining peptides extracted with 50% ACN. Dried and reconstituted peptides were deglycosylated with PNGase F (New England Biolabs, Frankfurt a. M., Germany) at 37 °C overnight. Peptides were enriched by C18 micropurification (ZipTip, Merck, Darmstadt, Germany).

For LC–MS analysis, 1.5 µl were injected onto a C18 trap column (20 mm length, 100 µm inner diameter) equilibrated with 0.1% TFA. Bound peptides were eluted onto a C18 analytical column (200 mm length, 75 µm inner diameter) equilibrated with 0.1% formic acid (solvent A). Both columns were made in-house with ReproSil-Pur 120 C18-AQ, 5 µm or 1.9 µm, respectively (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Peptides were separated during a linear gradient from 0% to 35% solvent B (80% acetonitrile, 0.1% formic acid) within 60 min at a flow rate of 330 nl/min. The nanoHPLC was coupled online to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptide ions between 330 and 1600 m/z were scanned in the orbitrap detector with a resolution of 30,000 (maximum fill time 400 ms, AGC target 106). The 25 most intense precursor ions (threshold intensity 5000) were subjected to collision induced dissociation fragments analyzed in the linear ion trap. Fragmented peptide ions were excluded from repeat analysis for 15 s.

Raw data processing and analysis of database searches were performed with the Proteome Discoverer software 2.1 (Thermo Fisher Scientific). MS2 data was searched against *Pinacea* sequences from TrEMBL (release 2016_01) with Mascot server version 2.5.1 (Matrix Science Ltd, London, UK). Precursor ion m/z tolerance was 7 ppm, fragment ion tolerance 0.5 Da. b- and y-ion series were included. Peptides with up to two missed cleavages were searched. Carbamidomethylation was set as a static modification of cysteines. Oxidation of methionine and deamidation of asparagine were allowed as a dynamic modification. Spectrum matches were sent to the percolator algorithm (Kall, Storey, MacCoss, & Noble, 2008) version 2.05 as implemented in Proteome Discoverer 2.1.0.81.

3. Results

3.1. Pin p 1 purification, individual recognition by sera's IgE and inhibition assays

The purification of Pin p 1 involved a combination of ammonium sulfate precipitation and ion exchange chromatography. The elution profiles of the different steps in the purification process have been described in our previous work (Cabanillas et al., 2012). The purity of the protein was assessed by SDS-PAGE and by mass spectrometry. Absence of contamination with other proteins was found in the purified sample.

The recognition of Pin p 1 by the serum IgE from 8 patients with pine nut allergy was carried out by means of IgE-western blotting and IgE-ELISA (Figs. 1A and B). The results showed that IgE from 6 out of 8 patients' sera (75%) recognized Pin p 1 in both western blotting and ELISA. ELISA inhibition was performed using a pool of sera from the 6 patients that recognized Pin p 1 in western blotting and ELISA (Fig. 1C). The ELISA inhibition was performed with whole pine nut extract coating the solid phase. The same extract and Pin p 1 were used as inhibitors. Using Pin p 1 as inhibitor, a progressive increase in the percentage of inhibition of IgE-binding to pine nut protein extract up to 83% was observed. A

95% inhibition was reached using whole pine nut extract as inhibitor.

3.2. Stability of Pin p 1 to heating

The stability of Pin p 1 to heating was evaluated as well. Similar OD values in the IgE-ELISA were obtained for every sample subjected to the different boiling times, indicating that the IgE-binding capacity of purified Pin p 1 remained stable after heating (Fig. 1D).

3.3. Basophil activation after challenge with Pin p 1

We next aimed to analyse IgE cross-linking and activation of human basophils upon Pin p 1 exposure by measuring the upregulation of the specific activation marker CD203c (Boumiza et al., 2003). For that purpose, we carried out a stripped basophil activation assay using basophils isolated from a donor without any clinical allergy to nuts. The donor's basophils were subjected to IgE stripping and sensitization, with sera from four pine nut allergic patients (patients Nos. 2, 3, 6, and 8) or they were not stripped (Fig. 2, first column). These basophils, with or without patient sera sensitization, were incubated in the presence of media alone as negative control, Pin p 1 or anti-IgE as positive control. Fig. 2 shows that with this method Pin p 1 was able to induce basophil activation in 75% of the cases (basophils sensitized with sera from patients Nos. 3, 6, and 8), with percentages of activation ranging from 8.1% to 19.7%.

3.4. Full characterization of Pin p 1 cDNA

The full characterization of the nucleotide sequence of Pin p 1 was carried out in the present study. Total RNA was obtained from *Pinus pinea* seeds. A well-defined, unique amplification product of 5' RACE was obtained using gene specific primers designed from the partial cDNA sequence previously described for this allergen (Cabanillas et al., 2012). Using the information of the sequence derived from the 5'RACE experiment, a gene specific primer was designed in order to characterize the 3' end using the 3'RACE system. The sequences derived from 5' and 3' RACE experiments were aligned using Clustal W, and a full length cDNA of 495 bp, which encoded a protein of 164 amino acids, was obtained (Fig. 3; GenBank Accession No. LN876267.1). These data were submitted to IUIS allergen nomenclature subcommittee, and the official name of Pin p 1.0101 was given to this allergen (<http://www.allergen.org/viewallergen.php?aid=842>).

The amino acid sequence of Pin p 1 was characterized by a skeleton of eight cysteine residues which is well-conserved and characteristic of the members of the prolamin superfamily, to which the 2S albumins belong (Fig. 3). This skeleton structure is found to be conserved in the three-dimensional structure enriched in α -helices of 2S albumins.

3.5. Molecular evolution analysis

Since *Pinus pinea* belongs to the group of gymnosperms, separated in the evolution tree from the species that produce other tree nuts that humans consume (all belonging to the angiosperms group), we focused on the analysis of the specific features of Pin p 1 amino acid sequences compared to other 2S albumins. We first carried out a comparison and a phylogenetic study of Pin p 1 with 2S albumins from different species of gymnosperms and angiosperms. The selected sequences were: *Pinus strobus* albumin 1, *Pinus strobus* albumin 2, *Pinus strobus* albumin 3, *Pinus strobus* albumin 4, *Picea glauca* albumin-like-protein, *Pseudotsuga menziesii* albumin, *Anacardium occidentale* 2S albumin (Ana o 3), *Juglans regia* 2S

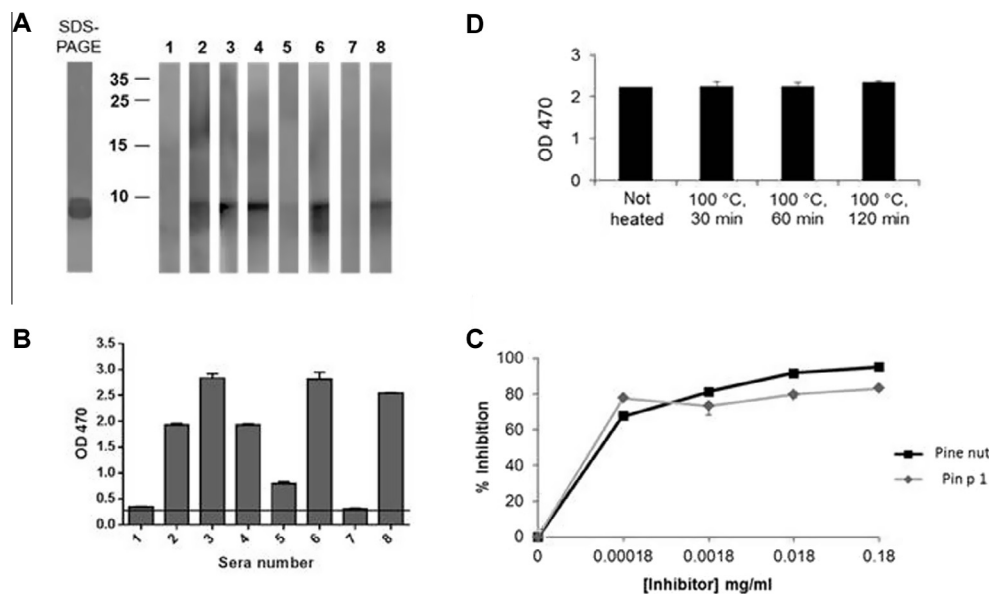


Fig. 1. IgE reactivity, IgE inhibition capacity and thermal resistance of Pin p 1. (A) SDS-PAGE, IgE-western blot and (B) IgE-ELISA analysis of Pin p 1 incubated with sera from patients with clinical allergy to pine nut (1–8). The cut-off point of positivity for the IgE-ELISA is indicated by the horizontal line in the graph (IgE levels > 0.24, calculated as indicated in material and methods). (C) ELISA inhibition with pine nut protein extract in the solid phase and Pin p 1 or pine nut protein extract (indicated in the legend) used as inhibitors at 0, 0.00018, 0.0018, 0.018, and 0.18 mg/ml. A pool of sera from patients allergic to pine nut was used. The percentage of inhibition of the IgE binding was calculated as previously described (Cabanillas et al., 2015). (D) IgE-ELISA analysis of Pin p 1 heated at 100 °C during 30, 60, or 120 min, or kept it unheated. The IgE-ELISA was incubated with sera from patients with clinically relevant allergy to pine nut.

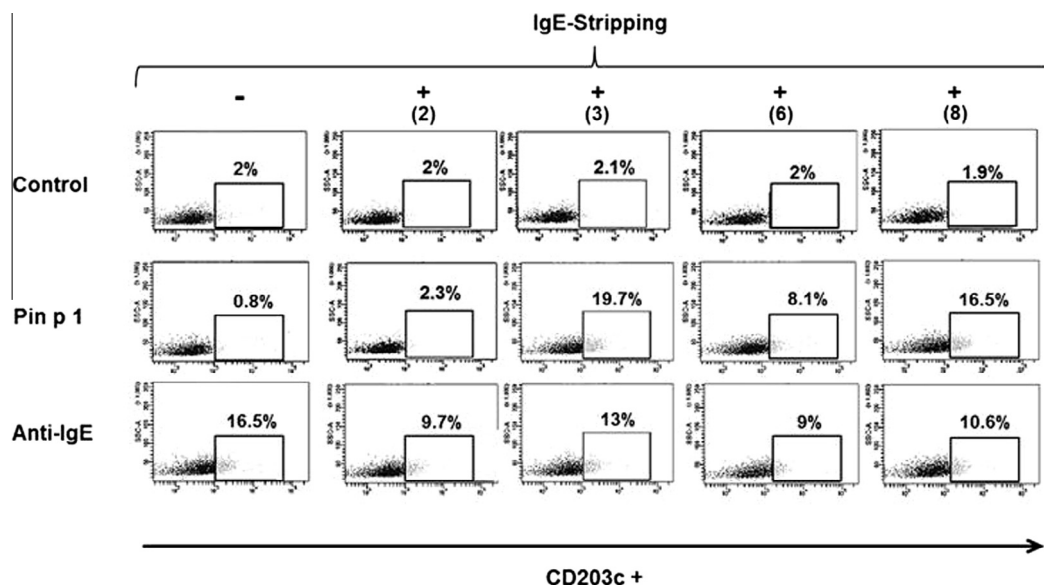


Fig. 2. Stripped basophil activation assay. Percentages of activated basophils (CD203c+) (unstripped (–) or stripped (+) and sensitized with sera from patients with pine nut allergy (Nos. 2, 3, 6, and 8) measured by flow cytometry after stimulation with medium alone (negative control), Pin p 1 or a anti-human IgE polyclonal antibody (positive control).

albumin (Jug r 1), *Bertholletia excelsa* 2S albumin (Ber e 1), *Carya illinoensis* 2S albumin (Car i 1), *Juglans nigra* 2S albumin (Jug n 1), *Pistacia vera* 2S albumin (Pis v 1) and *Corylus avellana* 2S albumin (Cor a 14). In order to visually represent the evolutionary clusters of the sequences, a phylogenetic tree was constructed using the Neighbor-joining method. The phylogenetic tree in Fig. 4A shows that 2S albumins from the angiosperms species cluster separately from the 2S albumins from species of gymnosperms, including Pin p 1, highlighting the evolutionary differences between the sequences of 2S albumins in both groups of plants. Then we were interested in the analysis of the percentages of

similarity and identity of the amino acid sequence of Pin p 1 with allergenic 2S albumins from tree nuts belonging to the angiosperm group. Results are shown in Fig. 4B. We found that Pin p 1 had the lowest percentage of similarity and identity with allergenic 2S albumins from tree nuts, with percentages of similarity ranging between 36% and 46% and percentages of identity between 22% and 29%.

Interestingly, in spite of the low homology between Pin p 1 and the allergenic 2S albumins from tree nuts from angiosperm species, a multiple amino acid sequence alignment of Pin p 1 with seven allergenic 2S albumins from angiosperms (Fig. 5), revealed that Pin

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1  M G V F S S P M S T L R W V T L F A A L
1  ATGGGTGTGTTCTCCTCTCCAATGTCGACGTTGAGATGGGTGACTTTATTCGCGGCCCTT
21  L S L L E W G T A H E D I V M D G D Q V
61  CTGTCGCTCCTTGAGTGGGGCACTGCTCATGAAGACATCGTCATGGATGGAGATCAGSTA
41  V Q Q Q G R S [C] D P Q R L S A [C] R D Y L
121  GTGCAACAGCAAGGACGGTCTGCGACCCGAGAGATTGCTGCTTGTCTGGGACTACTTG
61  Q R R R E Q P S E R [C] [C] E E L Q R M S P
181  CAGCGCCGGAGAGAACAGCCATCGGAAAGATGTTGCGAGGAAGTCAAGAAGATGTCTCCA
81  H [C] R [C] R A I E R A L D Q S Q S Y D S S
241  CACTGCCGATGCCGAGCCATAGAGCGGGCACTAGATCAATCTCAATCGTATGATTTCATCG
101  T D S D S Q D G A P L N Q R R R R R G E
301  ACGGATTCCGATTCTCAGGATGGTGCACCACTTAATCAACGCCGTCGACGCCGCGCGAA
121  G R G R E E E E A V E R A E E L P N R [C]
361  GGGCGCGGAAGGGAGGAAGAGGAGGCGGTGGAGAGAGCCGAAGAGCTTCCGAATAGATGC
141  N L R E S P R R [C] D I R R H S R Y S I I
421  AACCTCCGCGAGTCACCCCGCGCTGCGATATTGACGCCATTCTCGCTATTCTATTATC
161  G G S D *
481  GGCGGTAGCGACTGA

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Fig. 3. Nucleotide sequence of the complete cDNA of Pin p 1 and the deduced amino acid sequence. The conserved 8 cysteines are highlighted with squares.

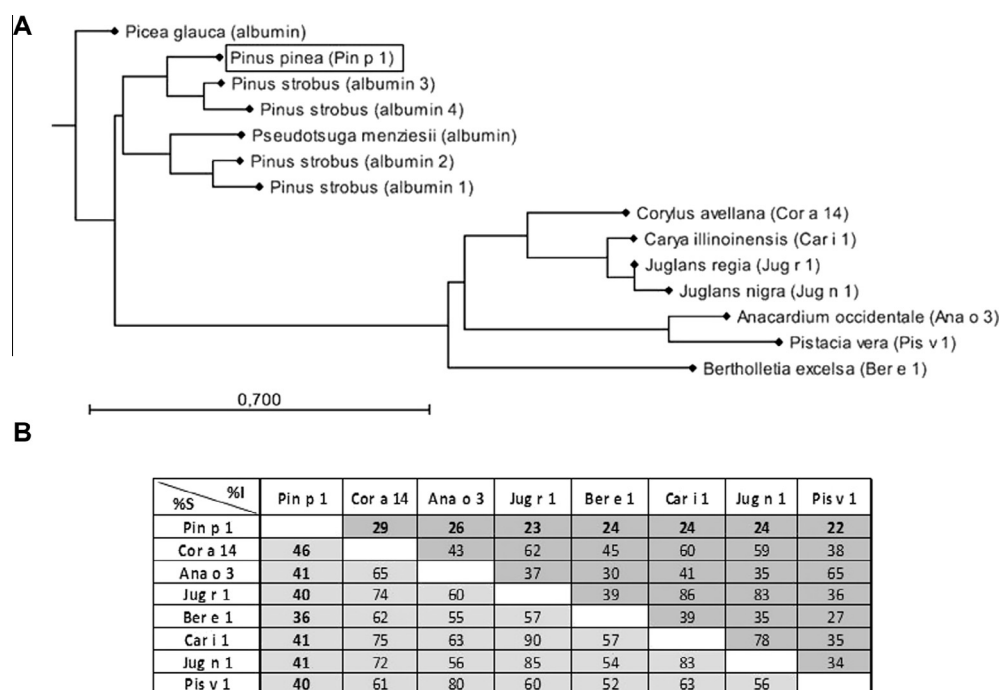


Fig. 4. (A) Phylogenetic analysis of the amino acid sequences of 2S albumins from different species of gymnosperms and angiosperms. The Neighbor-Joining algorithm with 100 replicate bootstraps was used (performed with CLC sequence viewer program). (B) Percentages of identity (%I) and similarity (%S) of amino acid sequences of Pin p 1 and allergenic 2S albumins from angiosperm species (performed with NEEDLE program in the EMBOSS package).

p 1 shares the placement of 8 cysteine residues at similar positions seen in the other sequences. The conserved 8 cysteines in 2S albumins are considered important for the α -bundle structure that is typical of these proteins (Fig. 5).

3.6. First evidence of a N-glycosylation site in Pin p 1

In order to identify N-glycosylation sites, Pin p 1 was digested by proteinases and then deglycosylated by PNGase F, the digests were analyzed by LC-MS. PNGase F cleaves N-linked oligosaccharides from glycoproteins resulting in the conversion of initially glycosylated asparagine to aspartic acid. The resulting mass change of the peptide can be analyzed in order to identify N-glycosylation sites. Our results showed the first evidence of a potential N-glycosylation site at N¹³⁸ in Pin p 1, in accordance to the ASN-X-Cys motif (Fig. 1, Supplementary material).

4. Discussion

In the present study we have analyzed the allergenic features and characterized the complete sequence of the first food allergen described from gymnosperms, the pine nut 2S albumin Pin p 1. A high percentage (75%) of recognition of Pin p 1 by individual sera from patients with pine nut allergy was found. This indicates that Pin p 1 is most likely one of the major allergens of pine nut recognized by our study population. Moreover, a common characteristic of the patients whose sera recognized Pin p 1 was a history of severe anaphylaxis after pine nut consumption. ELISA inhibition showed that Pin p 1 had a robust capacity to inhibit IgE-binding to whole pine nut extract, reaching a percentage of inhibition of 83% compared to 95% of inhibition reached by the whole pine nut extract. IgE-cross linking capacity of Pin p 1 in basophils as effector cells of allergic reactions has been also demonstrated.

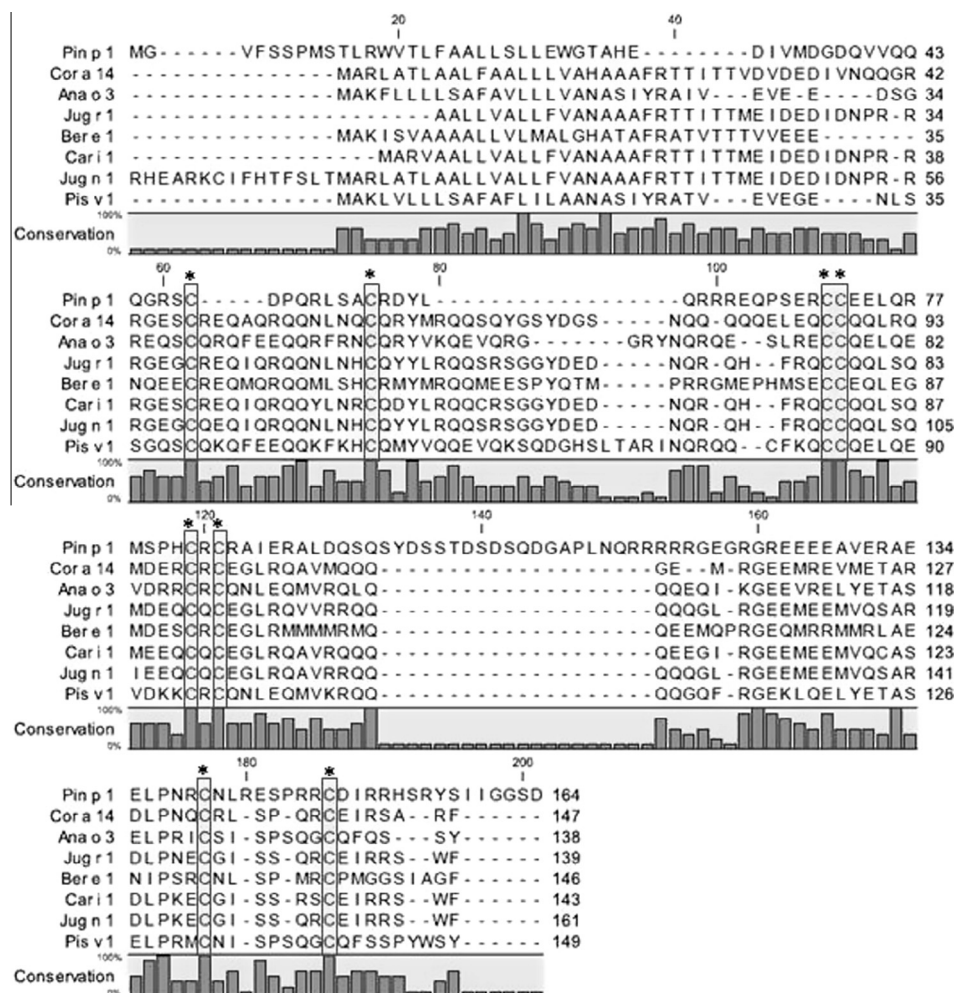


Fig. 5. Alignment of the amino acid sequences of Pin p 1 with allergenic 2S albumins from angiosperm species: *Corylus avellana* 2S albumin (Cor a 14), *Anacardium occidentale* 2S albumin (Ana o 3), *Juglans regia* 2S albumin (Jug r 1), *Bertholletia excelsa* 2S albumin (Ber e 1), *Carya illinoensis* 2S albumin (Car i 1), *Juglans nigra* 2S albumin (Jug n 1), and *Pistacia vera* 2S albumin (Pis v 1). The level of conservation at each position in the alignment is shown by the grey bars at the bottom of the alignment. The conserved skeleton of eight cysteines is marked with rectangles and asterisks.

Although no official allergen names for pine nut have been registered by Allergen Nomenclature Subcommittee so far, some IgE-binding proteins from pine nuts have been detected with western blotting in previous studies. A protein from pine nuts with a molecular weight of 15–17 kDa under non reducing conditions and around 7 kDa under reducing conditions has been described in two studies (García-Menaya et al., 2000; Ibáñez, Lombardero, San Ireneo, & Muñoz, 2003). This protein was recognized by the serum IgE of 3 patients with severe allergic symptoms after pine nut consumption. In both studies, authors hypothesized that the strong recognition of this single IgE-binding protein correlated with the severity of symptoms after pine nut intake. However, no further analysis besides the IgE-western blotting analysis was undertaken (García-Menaya et al., 2000; Ibáñez et al., 2003). Since Pin p 1 has a molecular weight of 15–16 kDa under non reducing conditions and ~6 kDa under reducing conditions (Cabanillas et al., 2012), the IgE-binding protein pointed out in the two mentioned studies might be Pin p 1.

Several 2S albumins have been characterized as major food allergens in tree nuts, such as cashew (Ana o 3), walnut (Jug r 1), Brazil nut (Ber e 1), and have been suggested to be involved in severe allergic reactions. The allergen Pin p 1 also seems to be implicated in the severe allergic reactions in patients with pine nut allergy. The highly conserved and disulfide-linked compact

structure of 2S albumins seems to play an important role in keeping the protein intact until they reach the effector cells of our immune system. Upon ingestion, these allergic proteins can resist and survive harsh conditions and can be absorbed in the gut effecting immunological responses (Moreno & Clemente, 2008).

The nucleotide and amino acid sequence of Pin p 1 was found to share high homology with 2S albumins from species of gymnosperms and low homology with 2S albumins from angiosperms. Phylogenetic studies carried out in this study confirmed these findings. The group of gymnosperms, to which *Pinus pinea* belongs, are evolutionally separated from flowering plants (angiosperms) which produce most of the tree nuts consumed by human beings (walnut, hazelnut, cashew, pistachio, etc). This evolutionary difference seems to be important, and has an impact in the clinical findings concerning pine nut allergy, in which a high rate of individuals monosensitized to pine nuts compared to other tree nuts is found (Cabanillas & Novak, 2015). The scientific literature confirms the high immunological cross-reactivity among nuts from species of gymnosperms, but a low or even missing cross-reactivity between nuts from gymnosperms and angiosperms (de las Marinas, Vila, & Sanz, 1998; Jansen, Vermeulen, Dieges, & van Toorenbergen, 1996). The unique characteristics of pine nut allergens may have implications in the detection of pine nuts in foodstuff. A recent study has selected marker peptides for the detection of nuts in a

variety of food products using enzymatic digestion LC-MS/MS. The selection criteria were based on representative marker peptides that uniquely represent the specific nut. In the case of pine nuts, two peptides from pine globulin-1 were selected (Sealey-Voyksner, Zweigenbaum, & Voyksner, 2016). The characteristic low homology of Pin p 1 with other allergenic 2S albumins from angiosperms may also indicate the potential to specifically detect the presence of pine nuts in foodstuff at hidden and trace levels.

Interestingly, despite low sequence homologies, Pin p 1 shares with other allergenic 2S albumins described in angiosperms the typical skeleton of 8 cysteine residues at a similar position of the sequence. These cysteines are involved in the formation of four intra-chain disulphide bonds. The pattern of 8 cysteines in specific order appears to be a structural scaffold of conserved helical regions that forms a network of disulfide bridges necessary for the maintenance of the tertiary structure (Moreno & Clemente, 2008). Therefore, in spite of the low homology of Pin p 1 with the sequences of 2S albumins from angiosperms, such as Cor a 14 from hazelnut, Jug r 1 from walnut or Ana o 3 from cashew, it seems that they share a common and compact three-dimensional structure enriched by α -helices, as observed by circular dichroism analysis for Pin p 1 in our previous studies (Cabanillas et al., 2012). The highly conserved and compact structure of 2S albumins has been shown to be responsible for thermostability, resistance to extreme pH and proteolysis. These features have also been observed in Pin p 1, which is characterized by resistance to high temperatures and resistance to proteolytic treatments (Cabanillas et al., 2012).

To the best of our knowledge, Pin p 1 is the first food allergen that has been described in the group of gymnosperms. Although flowering plants (angiosperms) produce most of the seeds consumed by human beings, the seeds of certain gymnosperms are also edible, such as pine nuts or ginkgo nuts (Cheng, Abdullah, Mathai, & Yunus, 2013; Rosengarten, 2004).

Further studies will be necessary to obtain recombinant Pin p 1 for immunological comparability with natural Pin p 1 and to assess its thermal stability in a food matrix. Additionally, the identification of the immunodominant areas containing IgE-binding epitopes in this allergenic protein will allow us to analyze the differences with the IgE epitopes that have been already described for allergenic 2S albumins in angiosperms species.

In conclusion, we have analyzed allergenic features and described the complete sequence of the first food allergen described in pine nuts and in the group of gymnosperms, the 2S albumin Pin p 1. We have shown that the sequence of Pin p 1 has common and differential characteristics with other allergenic 2S albumins from angiosperms, a fact that might impact on the singular features of pine nut allergy, which is characterized by a high monosensitization rate and severe allergic symptoms.

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.04.068>.

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