

RESEARCH ARTICLE

Pine nut allergy: Clinical features and major allergens characterization

Beatriz Cabanillas¹, Hsiaopo Cheng², Casey C. Grimm², Barry K. Hurlburt², Julia Rodríguez¹, Jesús F. Crespo¹ and Soheila J. Maleki²

¹ Servicio de Alergia, Hospital Universitario 12 de Octubre, Instituto de Investigación Hospital 12 de Octubre (i+12), Madrid, Spain

² U.S. Department of Agriculture, Agricultural Research Service, Southern Regional Research Center, New Orleans, LA, USA

Scope: The aims of this study were to evaluate IgE-mediated hypersensitivity to pine nut with details of clinical reactions and to characterize major pine nut allergens.

Methods and results: The study included ten consecutive teenagers and adults diagnosed with IgE-mediated clinical allergy to pine nut. Two major pine nut allergens were purified and identified and the secondary structures and susceptibility to digestion were characterized. Severe reactions represent 80% of allergic reactions to pine nut in this study. Moreover, 70% of the patients were monosensitized to this nut. Two major allergens with molecular weights of 6 and 50 kDa were purified and identified as albumin and vicilin, respectively. The 6 kDa protein (albumin), rich in α -helix content, was far more stable to peptic and tryptic digestion as compared with 50 kDa protein (vicilin), which was quickly broken down. The secondary structure of the purified 50 kDa protein showed 41% β -sheet, 5% α -helix, and 54% random coil and/or loops.

Conclusion: Eighty percent of allergic reactions to pine nut in the ten patients included in this study were severe. Most patients (70%) were monosensitized to this nut. Two major allergens with molecular weights of 6 and 50 kDa were purified and identified as albumin and vicilin, respectively.

Keywords:

Albumin / Allergens / Anaphylaxis / Pine nut allergy / Vicilin

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1 Introduction

Allergy to tree nuts is one of the leading causes of fatal allergic reactions and its prevalence appears to be increasing [1]. Pine trees belong to the gymnosperm group and they are among the most widely distributed and prominent genera of trees in the world [2]. Pine nuts, the seeds of pine trees, are widely used for human consumption in Europe, America, and Asia [3]. They contain approximately 32% protein, 45% fat and potassium, phosphorus, magnesium, and other minerals as well as dietary fiber [4]. Pine nuts produced in Europe mostly

come from *Pinus pinea* and they are increasingly used in the preparation of pastries, deserts, pasta, salads, and sauces such as pesto [5]. The first description of a hypersensitivity reaction to pine nuts was reported by Fine in 1987 [6]. Since then, individual case reports of severe anaphylactic reactions to this tree nut have been described [5, 7–14]. In most cases, patients were monosensitized to pine nuts [5, 9–11, 13, 14], although cross-reactivity between pine nut and almond has been described [12]. No studies have evaluated IgE-mediated hypersensitivity to pine nut in a large number of patients with details of clinical reactions nor have pine nut allergens been fully characterized. Ibañez et al. [13] and García-Menaya et al. [5] found that sera from three patients allergic to pine nuts strongly recognized a 17 kDa protein and de las Marinas et al. [12] reported an IgE-binding protein from pine nut with a molecular weight of 50 kDa. However, no further experiments were carried out in order to characterize the IgE-binding proteins.

The aims of this study were to identify, purify, and characterize two specific pine nut proteins recognized by sera

Correspondence: Dr. Beatriz Cabanillas, Servicio de Alergia, Hospital Universitario 12 de Octubre, Avenida de Córdoba s/n, 28041, Madrid, Spain

E-mail: bcabanillas.hdoc@salud.madrid.org

Fax: +34-914693470

Abbreviations: CD, circular dichroism; DBPCFC, double-blind placebo-controlled food challenge

from patients with pine nut allergy and to provide a detailed clinical characterization of reactions to pine nut in a case series of ten consecutive patients diagnosed with actual allergy to this food.

2 Materials and methods

2.1 Patient population

The study included ten consecutive teenagers and adults diagnosed with IgE-mediated clinical allergy to pine nut in the Food Allergy Unit of the Hospital Universitario 12 de Octubre (Madrid, Spain). The diagnosis was made from a clinical history and confirmed by positive results of allergy testing by skin prick tests, and/or pine nut-specific serum IgE antibodies, and double-blind, placebo-controlled food challenge (DBPCFC) as described elsewhere [15, 16]. Pine nuts were masked in a vehicle containing a mix of chocolate custard, milk, and sugar. Randomization and preparation of each challenge was performed in the Allergy Laboratory of the Hospital Universitario 12 de Octubre (Madrid, Spain). Subjects were challenged first randomly with either pine nut up to a cumulative dose of pine nuts: 25 g or placebo (vehicle), divided in five doses given every 15 min. The verum and placebo challenges were performed on two separate days. After each challenge, the patient was observed for 3 h. Confirmation by DBPCFC was accepted if the subject had symptoms after provocation with the active substance, and no symptoms after the placebo challenge.

Food challenges were not carried out in patients with a convincing history of recent severe reactions to pine nut. Sera from nine out of ten patients were available to be used in this study after obtaining an informed consent, approved by the Ethics Committee (Permission no. 0312150129).

2.2 Plant material and protein extract

P. pinea seeds were purchased at a local store in Madrid, Spain. The pine nut kernels were ground and the resulting meal was defatted overnight with petroleum ether using soxhlet extraction equipment. Defatted flour was solubilized with extraction buffer (50 mM Tris-Cl, 200 mM NaCl, and 1 mM EDTA, at pH 8.3), followed by sonication and centrifugation at 15 000 rpm for 15 min. The extracted proteins were divided into aliquots and stored at -20°C .

2.3 SDS-PAGE and Western blot analysis

The proteins extracted were separated by SDS-PAGE. Samples (30 μg /well) were mixed with standard electrophoresis sample buffer, containing 2% SDS and DTT (Invitrogen, Carlsbad, CA, USA), heated at 65°C for 15 min and electrophoresed in 4–20% Tris-Glycine gel (Invitrogen). Proteins were visualized with GelCode Blue Stain Reagent (Thermo

Scientific, Waltham, MA, USA). For Western blot, the proteins were transferred with iBlot™ gel transfer stacks (Invitrogen) to a polyvinylidene difluoride membrane. Membranes were cut in 3-mm strips and were blocked for 30 min at room temperature in 2% blotto (5% dry milk in PBS and 0.05% Tween-20). Membranes were incubated overnight with individual sera from nine patients with pine nut allergy (1:10 dilution), washed, and then treated with horseradish peroxidase-conjugated mouse anti-human IgE (1:5000 dilution for 30 min; Southern Biotech, Birmingham, AL, USA). Detection of IgE-binding proteins was achieved by means of enhanced chemiluminescence using an ECL substrate according to the manufacture's instructions (Amersham Biosciences, Piscataway, NJ, USA). The signal was measured using a CCD camera LAS-1000 system (Fuji Photo Film Co., Ltd., Duluth, GA, USA).

2.4 Protein purification

Two specific pine nut proteins (50 kDa and 6 kDa proteins) recognized by sera from patients with pine nut allergy were purified from pine nut protein extract. The first purification step was carried out by means of differential precipitation with ammonium sulfate. Protein solubility was tested at 25%, 50%, 75%, and 100% saturation. The pellets in each step were collected by centrifugation at 12 000 rpm for 30 min. For the 6 kDa protein purification, protein precipitated with ammonium sulfate at 100% saturation; therefore, the crude lysate was brought to 75% ammonium sulfate and cleared by centrifugation. The supernatant was then brought to 100% ammonium sulfate, centrifuged, and the pellet was dissolved in 50 mM Tris buffer (pH 8.4). This was loaded on 2.5×20 cm high Q-support fast flow column (strong anion exchange support, Bio-Rad, Richmond, CA, USA). The bound proteins were eluted with a linear salt gradient from 0 to 1 M NaCl in 50 mM Tris buffer, pH 8.4. Protein in fractions was detected by absorbance at 280 nm. The unbound protein fraction and the protein fractions eluted with NaCl from 0 to 180 mM were pooled and pH was adjusted to 7.0 with HCl. The pooled fractions were loaded on 2.5×20 cm 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. The 6 kDa protein with high purity was stored at -20°C until used. For the 50 kDa protein purification, protein precipitated with ammonium sulfate at 50% saturation. Therefore, the crude lysate was brought to 25% ammonium sulfate and cleared by centrifugation. The supernatant was then brought to 50% ammonium sulfate and centrifuged. The resultant pellet was dissolved in 50 mM Tris buffer (pH 8.4) and loaded on 2.5×20 cm high Q-support fast flow column. The bound proteins were eluted with a 100–300 mM NaCl linear gradient in 50 mM Tris buffer, pH 8.4. Protein in fractions was detected by absorbance at 280 nm. The protein fractions eluted with NaCl from 240 to 300 mM were pooled and pH was adjusted to 7.0. The pooled fractions

were loaded on 2.5 × 20 cm high S-support fast flow column and protein fractions eluted with NaCl from 100 to 300 mM NaCl linear gradient in 50 mM Tris buffer, pH 7.0. The 50 kDa protein with high purity was stored at −20°C until used.

2.5 Mass spectrometric analysis of the major pine nut allergens

For mass spectrometric analysis, in-solution digestion with pepsin or chymotrypsin was carried out according to the manufacture's instructions (Sigma, The Woodlands, TX, USA). In-gel trypsin digestion was carried out according to Shevchenko et al. [17] in Coomassie blue-stained bands excised from the gel. The digests were analyzed via LC/MS/MS, using an Agilent 1200 LC system, an Agilent Chip-cube interface, and an Agilent 6520 Q-TOF tandem mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was accomplished using a Chip consisting of a 40-nL enrichment column and a 43-mm analytical column packed with C18, 5 µm beads with 300 Å pores. One microliter aliquots of the sample were transferred to the enrichment column via the 1200 capillary pump operating at a flow of 4 µL/min. The 1200 nano pump was operated at a flow rate of 600 nL/min. An initial gradient (Solvent A-100% H₂O, 0.1% formic acid; Solvent B- 90% ACN, 10% H₂O, and 0.1% formic acid) of 97% A was changed to 30% Solvent A at 12 min, 0% at 13 min, 100% at 14 min, 0% at 15 min. A post run time of 4 min was employed for column equilibration. The MS source was operated at 300°C with 5 L/min N₂ flow and a fragmentor voltage of 175 V. N₂ was used as the collision gas with collision energy varied as a function of mass and charge using a slope of 3.7 V/100 Da and an offset of 2.5 V. Both quad and TOF were operated in positive ion mode. Reference compounds of 322.048121 Da and 1221.990637 Da were continually leaked into the source for mass calibration. An initial MS scan was performed from m/z 300 to 1600 and up to three multiply charged ions were selected for MS/MS analysis. Following the initial run, a second injection was made excluding ions previously targeted for MS/MS analysis.

Data files were transferred to an Agilent workstation equipped with Spectrum Mill software (Agilent Technologies). The raw MS/MS data files were extracted, sequenced, and searched against the NCBIInr, TrEMBL, and SwissProt databases.

2.6 cDNA cloning and nucleotide sequencing

Total RNA was extracted from *P. pinea* seeds using Spectrum Plant Total RNA Kit (Sigma). Two microgram total RNA was used for cDNA synthesis using reverse transcriptase enzyme (Superscript II) and adapter primer (AP, 5'-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3') (3' RACE System for Rapid Amplification of cDNA

Ends, Invitrogen). The cDNA obtained was amplified by means of PCR with the oligonucleotide 5' primer 5'-TGCCGCGACTACTTGCA-3', which was designed from a conserved nucleotide sequence at the 5' region of cDNA encoding albumins from *Pinus strobus*, *Picea glauca*, and *Pseudotsuga menziesii*. For the reverse direction, the 3' primer abridged universal amplification primer (AUAP) 5'-GGCCACGCGTCGACTAGTAC-3' (complementary to the adapter sequence used to prime first strand cDNA synthesis) was applied. The amplification was carried out with *Taq* DNA polymerase (New England Biolabs, Ipswich, MA, USA), as recommended by the manufacturer. The PCR program was 5 min of denaturation at 95°C, followed by 30 cycles each of 60 s of denaturation at 95°C, 60 s of annealing at 60°C, and 31 s of elongation at 72°C; and final extension for 10 min at 72°C. The obtained cDNA fragment was purified and cloned into the 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. These constructs were used to transform *Escherichia coli* DH5α-T1R competent cells (Invitrogen), and the DNAs from several clones were sequenced at the DNA sequencing laboratory at the Department of Microbiology and Immunology (University of Arkansas for Medical Sciences, Little Rock, AR, USA) using M13 forward and reverse sequencing primers.

2.7 Analysis of stability of pine nut extract and purified allergens to in vitro digestion

Pine nut protein extract and purified 6 and 50 kDa proteins were incubated in the presence of 0.0336 µM trypsin in a solution of 50 mM Tris, 1 mM EDTA at pH 8.3, at 37°C. Aliquots were taken for SDS-PAGE analysis before (t = 0 min) and after 1, 3, 5, 10, 30 min, 1 h, 2 h, and overnight (15 h) digestion and electrophoresis sample buffer was added.

For pepsin digestion, a simulated gastric fluid was prepared (0.084 M HCl, containing 0.035 M NaCl and 0.006 µM pepsin). Whole pine nut protein extract was incubated in the presence of simulated gastric fluid and aliquots were taken before (t = 0 min) and after 0.5-, 2-, 5-, 10-, 20-, 30-, 60-min digestion. Digestion was stopped by addition of 200 mM NaHCO₃ and electrophoresis sample buffer was added. Proteins were resolved by electrophoresis and visualized with GelCode Blue Stain Reagent.

2.8 Circular dichroism (CD) spectroscopy

Far UV (185–250 nm) CD spectra of the two purified pine nut proteins were obtained. Samples were desalted through use of disposable gel filtration columns (Bio-Rad) into Milli-Q water and immediately used in CD measurements. Protein concentration was 0.1 mg/mL and spectra were obtained

Table 1. Clinical and immunological findings of ten pine nut-allergic patients

Patient Number/ 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	...
3/36/M	8.5	1.82	U, AE, P, OAS, GI, R, TS, A	a	Sunflower seed, Pistachio, Peanut
4/34/M	12	2.27	AE, P, E, OAS, GI, DS, TS, GM	a	...
5/26/M	17.5	48	U	a	...
6/39/F	10	0.02	AE, P, E, OAS, O, TS	a	...
7/35/F	11	7.08	U, P, OAS, GI, GM	a	...
8/17/M	5.5	19.3	OAS	DBPCFC	...
9/18/F	15.5	2.2	AE, DY, DS, OAS, GI	a	...
10/24/M	20	35.3	U, AE, P, E, OAS, O, R, DY, DS, TS	a	Hazelnut, Peanut

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; R = rhinitis; SPT = skin prick testing; TS = tongue swelling; U = urticaria.

a) Not challenged because of a convincing history of severe reactions to pine nut.

at room temperature with a JASCO 815 spectropolarimeter equipped with a Peltier temperature control system (Japan Spectroscopic, Tokyo, Japan). The average of two measurements was obtained using Jasco CD 206 Manager Software. Data were analyzed with CDPro.

3 Results

3.1 Clinical features of pine nut allergy

The median age of the ten patients allergic to pine nut was 32 (range 17–39) and the female/male ratio was 1/1. Seven patients had a convincing history of recent severe systemic anaphylaxis after pine nut ingestion. One patient had a recently disseminated urticarial reaction documented in the ED after isolated pine nut ingestion. Two patients had a positive DBPCFC with pine nut, showing increasing oral symptoms, in the first and second consecutive doses. Subjects had a positive skin prick test response (from 4.5 to 20 mm, median 11.25 mm) and specific IgE level to pine nut ranging from 0.02–48 kU/L (median = 2.2 kU/L). The most frequent symptoms were oral allergy syndrome (9), two of which were without other symptoms, pruritus (6), angioedema (6), urticaria (5), tongue swelling (5), abdominal pain/nausea/vomiting (4), difficulty in swallowing (4), erythema (3), general malaise (3), asthma (2), rhinitis (2), dysphonia (2), and ocular symptoms (2) (Table 1).

Six reactions to other five foods were confirmed in three patients: peanut (2), hazelnut (1), pistachio (1), sunflower seeds (1), and melon (1) (Table 1).

3.2 Allergen pattern recognition

The SDS-PAGE pattern of pine nut extract is presented in Fig. 1. Multiple proteins with an apparent molecular weight

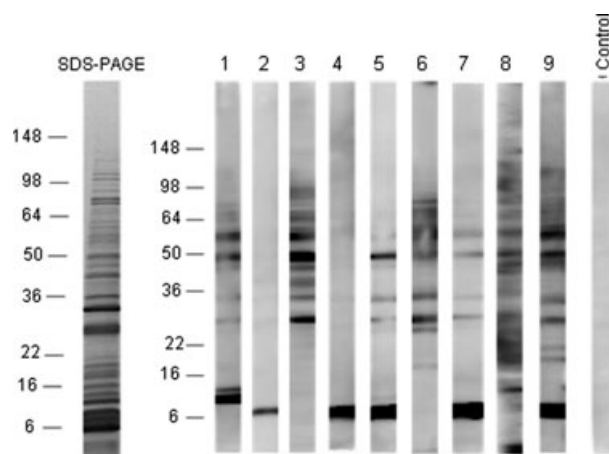


Figure 1. Western blot analysis of pine nut protein extract incubated with sera from patients with clinical pine nut allergy.

ranging from 6 to 120 kDa were obtained. Western blot was performed using individual sera from nine patients with clinical allergy to pine nut (Fig. 1). Five IgE-binding proteins were recognized by more than 50% of patient sera: 6, 30, 35, 50, and 57 kDa. The 6 kDa protein was recognized with the highest intensity. Six and 50 kDa proteins were selected for purification and characterization.

3.3 Protein purification

Both proteins were purified by a combination of ammonium sulfate precipitation and ion exchange chromatography. Figures 2 and 3 show the elution profiles of the 6 kDa (Fig. 2A and B) and 50 kDa (Fig. 3A and B) proteins chromatography on high Q and S support fast flow columns using NaCl linear gradients. SDS-PAGE analyses of the different steps of purification show that both proteins were

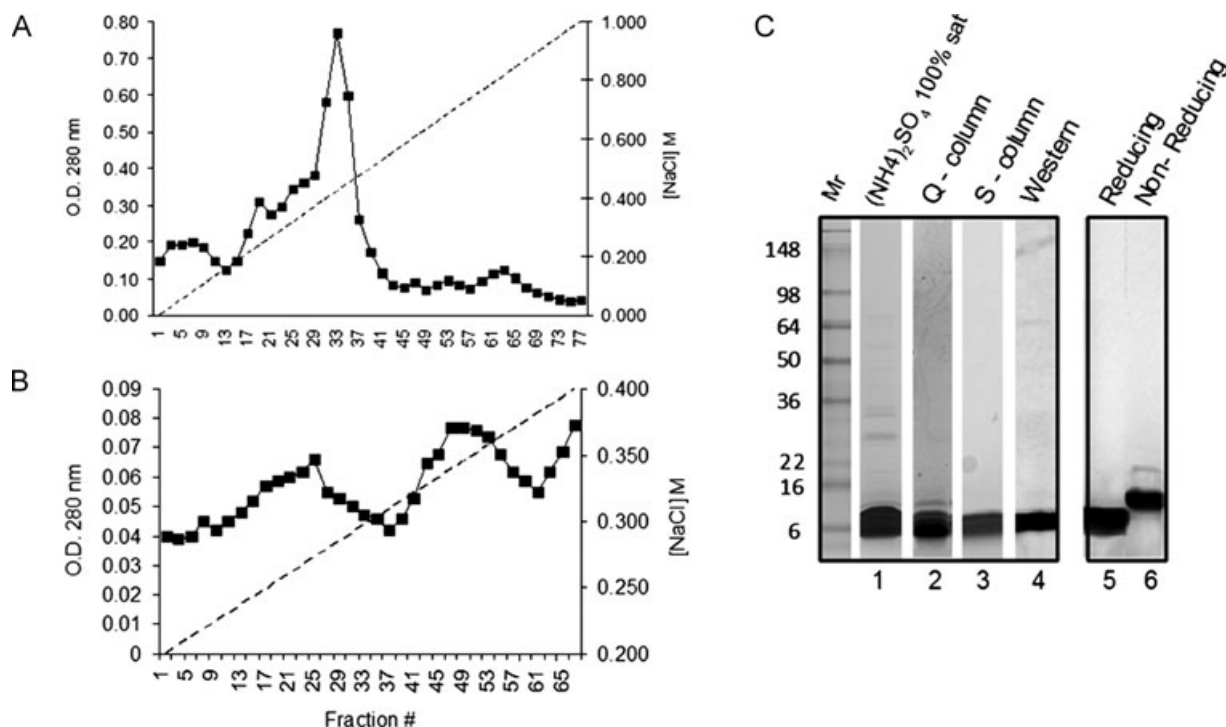


Figure 2. Elution profile of the 6 kDa protein chromatography on high Q-support fast flow column (A) and high S-support fast flow column (B) using NaCl linear gradients. Proteins in fractions were detected by absorbance at 280 nm and plotted versus fraction number. (C) SDS-PAGE analysis of the different steps of purification (1–3) and Western blot of the purified protein incubated with sera from patients with pine nut allergy (4). SDS-PAGE comparison of purified protein under reducing (5) and nonreducing conditions (6) was carried out.

purified to near homogeneity (Fig. 2C and 3C). The IgE-binding capacity of both proteins was further confirmed by Western blot with sera from patient allergic to pine nut (Fig. 2C and 3C). Purified 6 kDa protein was also analyzed under nonreducing conditions and a band of around 16 kDa was found (Fig. 2C).

3.4 Identification of proteins

The purified IgE-binding protein of 6 kDa was digested with trypsin in order to carry out the analysis by LC/MS/MS. Two peptides (GREEEEEEVERA and EELPDRCNVR, LS-DCRDYLQR) were matched to albumin from *P. strobus* (GI:550396) giving a coverage of 20%. A homology search allowing the substitution of a single amino acid (AA) per peptide yielded a third peptide (CCEELRR) and raised coverage to approximately 25%. This is strong evidence that the 6 kDa protein is a homolog of the albumin from *P. strobus*. A sequence comparison was undertaken by Clustal W. between the peptides found in the 6 kDa protein and other albumins from gymnosperm and angiosperm species. The peptides found in the 6 kDa protein were also homologous to albumins from other species belonging to the *Pinaceae* family such as *P. glauca* and *P. menziesii*. In contrast, the sequences showed lower homology to 2S albu-

mins from angiosperm plants such as peanut, walnut, or sunflower.

Purified 50 kDa protein was independently digested with trypsin, chymotrypsin, and pepsin and by means of LC/MS/MS analysis, three internal peptides were obtained: ASSEAGEIR, QFLAGK, and FGVPSGHTFY. The peptides matched a vicilin-like storage protein from *P. glauca* (Pinaceae family) (GI:75102412) giving a coverage of approximately 6%. A homology search of the vicilin-like storage protein allowing the substitution of a single AA per peptide increased the coverage to approximately 40% (Fig. 4). This is again strong evidence that the 50 kDa protein is a homolog of the vicilin-like protein from *P. glauca*. When the peptides found were compared against known allergens described in angiosperm plants corresponding to vicilin, the identity was lower than 40%.

3.5 Initial sequence characterization

To obtain data on the nucleotide sequence of albumin from *P. pinea*, total RNA was obtained from *P. pinea* seeds and the single-stranded cDNA was synthesized. Specific cDNA was then amplified by PCR using the AUAP 3' primer and a gene-specific 5' primer that anneals to a conserved region of known exon sequences of albumins from species of the *Pinaceae*

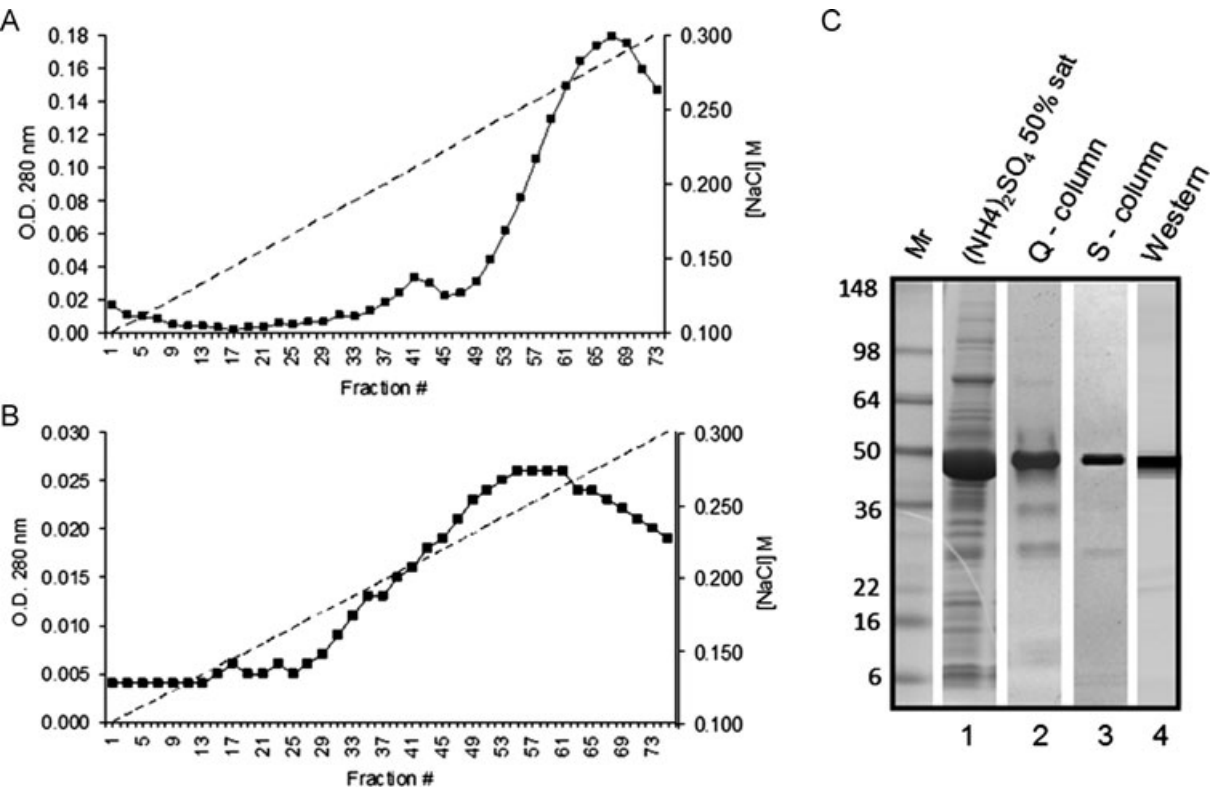


Figure 3. Elution profile of the 50 kDa protein chromatography on high Q-support fast flow column (A) and high S-support fast flow column (B) using NaCl linear gradients. Proteins in fractions were detected by absorbance at 280 nm and plotted versus fraction number. (C) SDS-PAGE analysis of the different steps of purification (1–3) and Western blot of the purified protein incubated with sera from patients with pine nut allergy (4).

family. The obtained band was 573 bp in size and the nucleotide sequence of this fragment showed an open-reading frame for 110 amino acid residues. Figure 5A shows the nucleotide and deduced amino acid sequences. The comparison of the amino acid sequence with those from albumins of *Pinaceae* family present in database banks confirmed that the characterized fragment was highly homologous to albumin from *P. strobus* (81% identity), *P. glauca* (68%), and *P. menziesii* (69%). However, a low identity with 2S albumin from peanut (28%) was obtained. Protein domain sequence analysis (Pfam database) revealed that the characterized albumin fragment from *P. pinea* contains the trypsin/ α -amylase inhibitor family domain (Tryp_alpha_amyl, PF00234) (Fig. 5B).

3.6 Secondary structure analysis by CD spectroscopy

The secondary structure of 6 and 50 kDa proteins purified from pine nut protein extract was analyzed by CD spectroscopy and the far-UV spectra are shown in Fig. 6. The percentages of α -helix, β -sheet, and random coil and/or loops are shown in the table annexed to Fig. 6. The CD spectrum of the 50 kDa protein rendered 41% β -sheet, 5% α -helix, and 54% random coil and/or loops. In contrast, the secondary structure for the 6 kDa protein was formed by α -helix (67%) and random coil and/or loops (23%) without β -sheet structure.

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1 MALASLLIIL LAISSSSAAL TEPLASTANP EVFPEYLGRG RGRREEEREE NPYVFHSDSF
61 RTRASSEAGE IRALPNFGEV SELLEGIRKF RVTCIEMKPN TVMLPHYIDA TWILYVTRGR
121 GYIAYVHQNE LVKRLKEEGD VFGVPSGHTF YLVNDDHNT LRILASLVRPV STVRGEYQPF
181 YVAGGRNPQT VYSAFSDDLV EAAFNTNVQQ LERIFGGHKS GVIIHANEEQ IREMMRKRGF
241 SAGSMSAPEH PKPFNLNRQK PDFENENGRF TIAGPKNYPF LDALDVSGL ADLNPGSMTA
301 PSLNSKSTSI GIVTNGEGRI EMACPHLGQH GWSSPRERGD QDITYQRVWA KLRTGSVYIV
361 PAGHPITEIA STNSRLQILW FDLNTRGNER QFLAGKNNVL NTLEREIRQL SFNVPRGEEI
421 EEVLQAQKDQ VILRGPRRRS RDEARSSS
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Figure 4. Vicilin-like storage protein amino acid sequence from *P. glauca*. Peptides found in 50 kDa protein are highlighted.

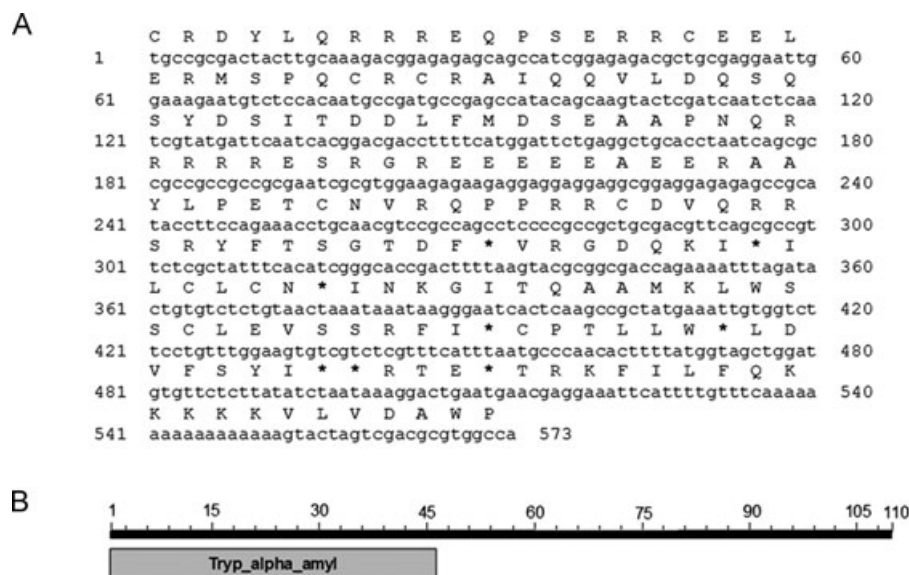


Figure 5. (A) Nucleotide sequence of the cDNA fragment of albumin from *Pinus pinea* and the deduced amino acid sequence. (B) Pfam search results: the gray box indicates the trypsin/ α -amylase inhibitor family domain location in the amino acid sequence.

3.7 Susceptibility of pine nut proteins to pepsin and trypsin digestion

In the first experiment, whole pine nut protein was digested with pepsin. Figure 7A shows the time course of the digestion as visualized by SDS-PAGE under reducing conditions. At time point 0, before adding pepsin, a characteristic pine nut extract pattern was found. By 0.5 min, substantial proteolysis of the pine nut extract was obtained. Protein bands with molecular weights higher than 22 kDa disappeared rapidly, whereas the 6 kDa protein together with proteins with a molecular weight around 10 and 20 kDa were resistant to digestion after 1 h.

Trypsin was also used to study the digestion effects on whole pine nut extract and purified 6 and 50 kDa proteins (Fig. 7B). Pine nut proteins in the molecular weight region of 30–6 kDa were resistant to digestion with trypsin and most of them remained up to an incubation time of 16 h. In contrast to the relative stability of the protein bands at approximately 30–6 kDa, the protein bands at higher molecular weights disappeared rapidly. Digestions of purified proteins confirmed the data obtained with whole pine nut extract. The 50 kDa protein was digested rapidly and proteolytic breakdown products were obtained during the first hour of digestion (Fig. 7B3). The protein was completely digested after overnight digestion. In contrast, 6 kDa protein was stable to trypsin digestion (Fig. 7B2).

4 Discussion

In this study, an extensive analysis of pine nut allergy, including clinical features and allergen characterization was carried out. The clinical study showed that severe reactions represented 80% of allergic reactions to pine nut in ten consecu-

tive patients included in this study. Most patients (70%) were monosensitized to pine nut. Two patients had allergy to other nuts and seeds (peanut, hazelnut, pistachio, and sunflower seeds) and one patient was allergic to melon. In previous studies reporting allergenic reactions to pine nut, most of the patients had a history of severe anaphylaxis with this nut and usually they were monosensitized [5, 9–11, 13, 14].

At a molecular level, two major pine nut allergens were purified, identified, and their secondary structure and stability to digestion were analyzed. The first characterized allergen, a protein with a molecular weight of 6 kDa, was strongly recognized by sera from 55% patients in this study. The internal sequences obtained by LC/MS/MS analysis showed a high match with the seed storage protein albumin from species belonging to the *Pinaceae* family (gymnosperms). However, a low match was found with albumins from angiosperm species such as peanut, walnut, or sunflower. CD analysis demonstrated that purified 6 kDa protein was rich in α -helix content and did not have β -sheet structure. According to numerous studies, 2S albumins are characterized by a similar three-dimensional structure enriched in α -helix structure [18]. The 2S albumins are compact molecules because of the presence of four conserved disulfide bonds that exhibit high thermostability and resistance to extreme pH and proteolysis [19].

In this study, an initial nucleotide sequence characterization was carried out for albumin from *P. pinea*. A conserved nucleotide sequence at the 5' region of cDNA encoding albumins from *P. strobus*, *P. glauca*, and *P. menziesii* was chosen for 5' primer design. The 573 bp fragment obtained showed an open-reading frame for 110 amino acid residues. The amino acid sequence had high homology with albumin from *P. strobus*, *P. glauca*, and *P. menziesii* and a low homology with 2S albumin from peanut. Albumins have been described as major food allergens in angiosperm species,

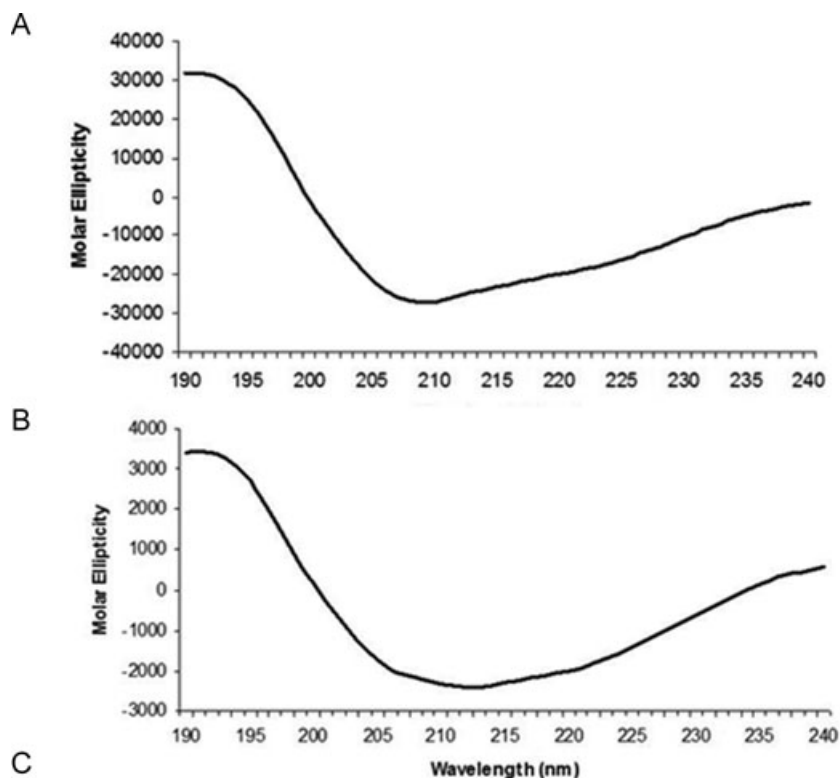


Figure 6. CD spectrum of 6 kDa (A) and 50 kDa (B) protein. Percentages of α -helix, β -sheet, and random coil and/or loops are shown in the table annexed (C).

like peanuts (Ara h 2, Ara h 6, and Ara h 7), cashew (Ana o 3), walnut (Jug r 1), almond (Pru du 2S), etc. [20]. However, so far 2S albumins have not been described as allergens in gymnosperm species. The low matches with albumins from angiosperms found in this study could be explained by the evolutionary differences between gymnosperms and angiosperms. The conifers, a division to which the pines belong, separated from flowering plants (angiosperms) about 100 million years ago and by most measures have evolved very slowly and conservatively [3]. These facts could support, to some extent, the clinical data described here, where 70% of the patients were monosensitized to pine nut and only 20% were allergic to other nuts and seeds such as peanut, hazelnut, and pistachio. Some authors have demonstrated immunological cross-reactivity among nuts from gymnosperms and a lack or a low cross-reactivity between nuts from angiosperms and gymnosperms. Jansen et al. [9] showed with RAST inhibition using serum from a patient with clinical allergy to pine nuts from *P. pinea*, that IgE binding to seeds from *P. pinea* was completely inhibited by *Pinus cembra*, whereas peanut or hazelnut did not show any inhibitory capacity. De las Marinas et al. [12] demonstrated by the means of CAP inhibition using

sera from a patient allergic to pine nut and almond, a 37% IgE-binding inhibition of pine nut over almond.

Two previous studies reported a predominant protein in pine nut strongly recognized by sera from three patients with severe clinical symptoms to pine nut. The protein had a molecular weight of 17 kDa under nonreducing conditions, and 7 kDa under reducing conditions. Authors hypothesized the recognition of this protein could be correlated with the patients' severe symptoms with pine nut [5, 13]. In the present study, the purified 6 kDa protein was also analyzed under nonreducing conditions and a band around 16 kDa was found. Moreover, most of the patients with a history of severe anaphylaxis to pine nut recognized this protein. These results seem to indicate that the albumin identified in this study could correspond with the 17 kDa protein found under nonreducing conditions in the mentioned studies [5, 13].

Second, a 50 kDa protein was also identified as a major allergen in pine nut seeds. By the means of mass spectrometry analysis, it was demonstrated that the 50 kDa protein showed a high match with vicilin from *P. glauca*. However, matches lower than 40% were found with vicilins from angiosperm species. The secondary structure of the purified

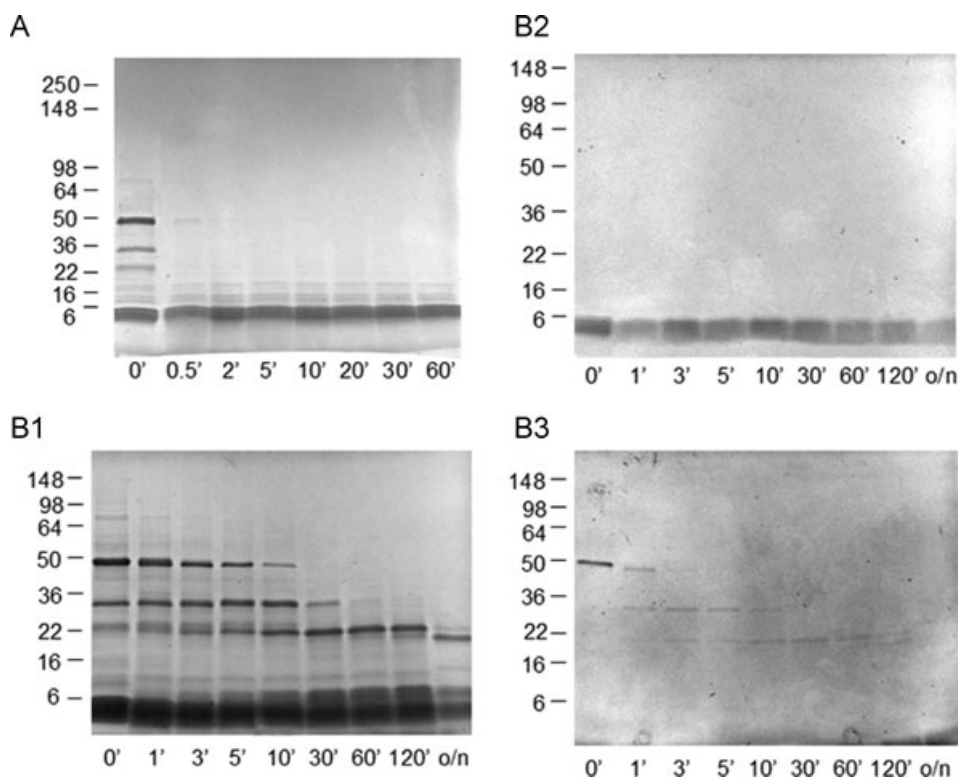


Figure 7. SDS-PAGE analysis of the digestion with pepsin (A) and trypsin (B1, B2, and B3) of whole pine nut protein extract (A, B1) and purified 6 kDa (B2) and 50 kDa (B3) proteins. Digestion times are indicated below each figure.

protein showed 41% β -sheet, 5% α -helix, and 54% random coil and/or loops. Vicilins (7S globulins) are seed storage proteins from the cupin family and they are characterized by a common β -barrel structure. Vicilins have been described as allergens in angiosperms species such as peanut (Ara h 1), walnut (Jug r 4), pistachio (Pis v 3), etc. [21].

By evaluating the potential digestibility of purified pine nut allergens in our study, it was demonstrated that the 6 kDa protein (albumin) was far more stable to peptic and tryptic digestion as compared with 50 kDa protein (vicilin), which is quickly broken down. Similar results were obtained by Koppelman et al. [22] in peanut, evaluating the potential gastric digestion of its purified major allergens. They concluded that Ara h 2 and Ara h 6 (2S albumins) were more stable to peptic digestion than Ara h 1 (vicilin) and that may explain why Ara h 2 and Ara h 6 allergens are more often recognized by IgE from the sera of peanut-allergic patients [22]. It has been demonstrated that the highly conserved and compact structure of 2S albumins plays an important role in the ability of these proteins to reach the immune system intact. These proteins can resist and survive, at least to some extent, the harsh conditions (acid pH, denaturing effects of surfactants, and proteolytic activities of digestive enzymes) and they could be absorbed in immunologically active forms by the gut, facilitating the presentation of these allergens to the immune system [20]. Moreover, in the present study, protein domain sequence analysis revealed that the characterized albumin fragment from *P. pinea* contains the trypsin/ α -amylase inhibitor family domain, suggesting a role for the

albumin in trypsin resistance. Several authors have described the high resistance of 2S albumins to digestive enzymes [23–26].

In conclusion, the results indicate severe reactions represent 80% of allergic reactions to pine nut in ten consecutive patients included this study. Moreover, 70% of the patients were monosensitized to this nut. Two major allergens with molecular weights of 6 and 50 kDa were purified and identified as albumin and vicilin, respectively. Their secondary structures, as well as, their susceptibility to digestion were characterized.

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5 References

- [1] Sicherer, S. H., Muñoz-Furlong, A., Godbold, J. H., Sampson, H. A., US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J. Allergy Clin. Immunol.* 2010, 125, 1322–1326.
- [2] Straus, S. H., Doerksen, A. H., Restriction fragment analysis of pine phylogeny. *Evolution.* 1990, 44, 1081–1096.

- [3] Jin, T., Albillos, S. M., Chen, Y. W., Kothary, M. H. et al., Purification and characterization of the 7S vicilin from Korean pine (*Pinus koraiensis*). *J. Agric. Food Chem.* 2008, **56**, 8159–8165.
- [4] Nergiz, C., Dönmez, I., Chemical composition and nutritive value of *Pinus pinea* L. seeds. *Food Chem.* 2004, **86**, 365–368.
- [5] García-Menaya, J. M., Gonzalo-Garijo, M. A., Moneo, I., Fernández, B. et al., A 17-kDa allergen detected in pine nuts. *Allergy* 2000, **55**, 291–293.
- [6] Fine, A. J., Hypersensitivity reaction to pine nuts (pinon nuts–pignolia). *Ann. Allergy* 1987, **59**, 183–184.
- [7] Nielsen, N. H., Systemic allergic reaction to pine nuts. *Ann. Allergy* 1990, **64**, 132–133.
- [8] Armentia, A., Quintero, A., Fernández-García, A., Salvador, J., Martín-Santos, J. M., Allergy to pine pollen and pinon nuts: a review of three cases. *Ann. Allergy* 1990, **64**, 49–53.
- [9] Jansen, A., Vermeulen, A., Dieges, P. H., van Toorenbergen, A. W., Allergy to pine nuts in a bird fancier. *Allergy* 1996, **51**, 741–744.
- [10] Beyer, A. V., Gall, H., Peter, R. U., Anaphylaxis to pine nuts. *Allergy* 1998, **53**, 1227–1228.
- [11] Roux, N., Hogendijk, S., Hauser, C., Severe anaphylaxis to pine nuts. *Allergy* 1998, **53**, 213–214.
- [12] de las Marinas, D., Vila, L., Sanz, M. L., Allergy to pine nuts. *Allergy* 1998, **53**, 220–222.
- [13] Ibáñez, M. D., Lombardero, M., San Ireneo, M. M., Muñoz, M. C., Anaphylaxis induced by pine nuts in two young girls. *Pediatr. Allergy Immunol.* 2003, **14**, 317–319.
- [14] van de Scheur, M. R., Bruynzeel, D. P., Acute anaphylaxis after pine nut skin testing. *Ann. Allergy Asthma Immunol.* 2004, **92**, 93.
- [15] Bock, S. A., Sampson, H. A., Atkins, F. M., Zeiger, R. S. et al., Double-blind, placebo-controlled food challenge (DBPCFC) as an office procedure: a manual. *J. Allergy Clin. Immunol.* 1988, **82**, 986–997.
- [16] Noe, D., Bartemucci, L., Mariani, N., Cantari, D., Practical aspects of preparation of foods for double-blind, placebo-controlled food challenge. *Allergy* 1998, **53**, 75–77.
- [17] Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., Mann, M., In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* 2006, **1**, 2856–2860.
- [18] Murzin, A. G., Brenner, S. E., Hubbard, T., Chothia, C., SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J. Mol. Biol.* 1995, **247**, 536–540.
- [19] Breiteneder, H., Mills, E. N., Molecular properties of food allergens. *J. Allergy Clin. Immunol.* 2005, **115**, 14–23.
- [20] Moreno, F. J., Clemente, A., 2S Albumin storage proteins: what makes them food allergens? *Open Biochem. J.* 2008, **2**, 16–28.
- [21] Mills, E. N., Jenkins, J., Marigheto, N., Belton, P. S. et al., Allergens of the cupin superfamily. *Biochem. Soc. Trans.* 2002, **30**, 925–929.
- [22] Koppelman, S. J., Hefle, S. L., Taylor, S. L., de Jong, G. A., Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: a comparative in vitro study and partial characterization of digestion-resistant peptides. *Mol. Nutr. Food Res.* 2010, **54**, 1711–1721.
- [23] Pantoja-Uceda, D., Palomares, O., Bruix, M., Villalba, M. et al., Solution structure and stability against digestion of rproBnlb, a recombinant 2S albumin from rapeseed: relationship to its allergenic properties. *Biochemistry* 2004, **43**, 16036–16045.
- [24] Suhr, M., Wicklein, D., Lepp, U., Becker, W. M., Isolation and characterization of natural Ara h 6: evidence for a further peanut allergen with putative clinical relevance based on resistance to pepsin digestion and heat. *Mol. Nutr. Food Res.* 2004, **48**, 390–399.
- [25] Moreno, F. J., Maldonado, B. M., Wellner, N., Mills, E. N., Thermostability and in vitro digestibility of a purified major allergen 2S albumin (Ses i 1) from white sesame seeds (*Sesamum indicum* L.). *Biochim. Biophys. Acta* 2005, **1752**, 142–153.
- [26] Moreno, F. J., Rubio, L. A., Olano, A., Clemente, A., Uptake of 2S albumin allergens, Ber e 1, and Ses i 1, across human intestinal epithelial Caco-2 cell monolayers. *J. Agric. Food Chem.* 2006, **54**, 8631–8639.