

Influence of enzymatic hydrolysis on the allergenic reactivity of processed cashew and pistachio



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ABSTRACT

Cashew and pistachio allergies are considered a serious health problem. Previous studies have shown that thermal processing, pressurization and enzymatic hydrolysis may reduce the allergenic properties of food by changing the protein structure. This study assesses the allergenic properties of cashew and pistachio after thermal treatment (boiling and autoclaving), with or without pressure (autoclaving), and multiple enzymatic treatments under sonication, by SDS-PAGE, western blot and ELISA, with serum IgE of allergic individuals, and mass spectroscopy. Autoclaving and enzymatic hydrolysis under sonication separately induced a measurable reduction in the IgE binding properties of pastes made from treated cashew and pistachio nuts. These treatments were more effective with pistachio allergens. However, heat combined with enzymatic digestion was necessary to markedly lower IgE binding to cashew allergens. The findings identify highly effective simultaneous processing conditions to reduce or even abolish the allergenic potency of cashew and pistachio.

1. Introduction

Food allergy affects 6–8% of children and approximately 2% of the population. It has been estimated that food allergy causes approximately 30,000 anaphylactic reactions and 2000 hospitalizations annually in the U.S. (Jerschow, Lin, Scaperotti, & McGinn, 2014). This increase in the prevalence and severity of food allergy has led to growing concerns by consumers and the food industry (Taylor & Hefle, 2001). Peanuts and tree nuts are the leading cause of fatal anaphylactic reactions caused by foods (Bock, Munoz-Furlong, & Sampson, 2001) and have a significant impact on the quality of life (Primeau et al., 2000). The prevalence of allergy to nuts is estimated to be approximately 1% in the UK and in the U.S. (Sicherer, Munoz-Furlong, Godbold, & Sampson, 2012). In Spain nuts are ranked second among foods most frequently involved in anaphylactic reactions (Fernandez-Rivas, 2009). Most nut allergens are seed storage proteins such as vicilins (7S globulin subunits composed of approximately 50–60 kDa), legumins (11–13S globulins composed of acidic subunits of 30–40 kDa and basic subunits of 15–20 kDa) and 2S albumins (15–20 kDa)

(Crespo, James, Fernandez-Rodriguez, & Rodriguez, 2006). Other allergens of nuts with known biological function, such as lipid transfer proteins (LTP), profilins and pathogenesis-related, class 10 (PR-10) proteins are considered panallergens because they contribute to the allergenicity of a large group of pollen, nuts, seeds, fruits and other plants (Radauer & Breiteneder, 2007). A common feature of the nut allergenic proteins is their resistance to proteolysis and denaturation (Roux, Teuber, & Sathe, 2003).

Cashew (*Anacardium occidentale*) allergy is thought to be the second most allergic nut in the U.S. and may cause more severe reactions than peanuts (Clark, Anagnostou, & Ewan, 2007). Three allergenic proteins of cashew have been identified and characterized: Ana o 1 (7S vicilin, 50 kDa) (Wang et al., 2002), Ana o 2 (11S legumin, 55 kDa) (Wang, Robotham, Teuber, Sathe, & Roux, 2003) and Ana o 3 (2S albumin, 14 kDa) (Robotham et al., 2005). Pistachio (*Pistacia vera*) is another well characterized nut for its allergenic potential and cross-reactivity with cashew and mango (Noorbakhsh et al., 2011). Similar to other nuts, 4 of the five pistachio major allergens identified correspond to seed storage proteins: Pis v 1 (2S albumin, 7 kDa), Pis v 2 (11S legumin, 32 kDa), Pis

Abbreviations: HRP, Horseradish peroxidase; RT, room temperature

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Table 1

The description of the processing treatments and protein content, determined by LECO analysis, for cashew and pistachio samples of this study.

Code	Sample	Treatment	Protein content (g/100gr)
PDF 1	Defatted Flour Pistachio	Untreated (Control)	34.34
PDF 2	Defatted Flour Pistachio	Boiled 30 min	35.51
PDF 3	Defatted Flour Pistachio	Boiled 60 min	37.37
PDF 4	Defatted Flour Pistachio	Autoclaved 120 kPa, 15 min	41.08
PDF 5	Defatted Flour Pistachio	Autoclaved 120 kPa, 30 min	41.92
PDF 6	Defatted Flour Pistachio	Autoclaved 256 kPa, 15 min	43.20
PDF 7	Defatted Flour Pistachio	Autoclaved 256 kPa, 30 min	43.35
CDF 1	Defatted Flour Cashew	Untreated (Control)	33.06
CDF 2	Defatted Flour Cashew	Boiled 30 min	40.17
CDF 3	Defatted Flour Cashew	Boiled 60 min	37.71
CDF 4	Defatted Flour Cashew	Autoclaved 120 kPa, 15 min	36.63
CDF 5	Defatted Flour Cashew	Autoclaved 120 kPa, 30 min	35.41
CDF 6	Defatted Flour Cashew	Autoclaved 256 kPa, 15 min	42.90
CDF 7	Defatted Flour Cashew	Autoclaved 256 kPa, 30 min	37.81

v 3 (7S vicilin, 55 kDa) and Pis v 5 (11S legumin, 36 kDa) and a superoxide dismutase, Pis v 4 (25.7 kDa) (Ahn, Bardina, Grishina, Beyer, & Sampson, 2009; Ayuso, Grishina, & Ahn, 2007; Willison et al., 2008).

Food processing, particularly heat treatment, may alter the allergenicity of foods, and can therefore be useful in controlling allergenic risk (Besler, Steinhart, & Paschke, 2001; Maleki, 2004). Thermal treatment changes the structure, function, digestibility, solubility and immunogenicity of proteins, and therefore the overall allergenicity of the food (Cabanillas, Pedrosa et al., 2012; Liu et al., 2010; Maleki, 2004; Maleki & Hurlburt, 2004; Maleki, Schmitt, Galeano, & Hurlburt, 2014; Mueller, Maleki, & Pedersen, 2014; Nesbit, Hurlburt, Schein, Cheng, & Maleki, 2012; Noorbakhsh et al., 2010; Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010a). These effects depend on the temperature, type and duration of the treatment, the intrinsic characteristics of the protein and the physicochemical conditions of the micro-environment (Davis, Smales, & James, 2001; Maleki, Chung, Champagne, & Raufman, 2000; Nesbit et al., 2012; Schmitt et al., 2010; Wal, 2003). There are no general rules about the effect of processing on allergenicity. Processing can lead to the generation of new allergenic epitopes (neoallergens), as well as abolish the existing epitopes (Cuadrado et al., 2009; Maleki et al., 2000; Nesbit et al., 2012; Álvarez-Álvarez et al., 2005). Several studies have evaluated the changes induced by roasting, boiling, microwave heating, and pressure-cooking on legume and nut allergenicity, showing that processing based on pressure and certain heat treatments seems to have an important impact on in vitro IgE binding capacity (Cabanillas, Maleki et al., 2012; Cuadrado et al., 2009, 2011; Maleki et al., 2000; Schmitt et al., 2010; Álvarez-Álvarez et al., 2005). A study conducted with raw, dry roasted and steamed pistachio showed that immunoreactivity decreases only when roasting is performed with steam (Noorbakhsh et al., 2010). More recently, it has been shown that the multi-enzyme systems that combine the action of endo and exopeptidases cause significant destruction of IgE epitopes in peanut and lentil (Cabanillas, Pedrosa et al., 2012; Cabanillas et al., 2010). Cabanillas et al. (2014) also found that walnuts subjected to high pressure were more susceptible to gastric and duodenal digestion.

The main objective of this study is to assess the digestibility and human serum IgE binding capacity of both soluble and insoluble pistachio and cashew proteins after thermal treatment in combination with high pressure (autoclaving), enzymatic digestion and ultrasound treatments.

2. Materials and methods

2.1. Patients and sera

Sera from 7 patients (P1-P7) with pistachio and cashew allergy, confirmed on the basis of either a convincing history or recently documented reaction after nut ingestion, were used in this study (see Table 1 suppl). The study was approved by the Ethics Committee of Tulane Health Science Center (New Orleans, LA, USA) in accordance with the rules and regulations of the institutional review board.

2.2. Plant material and processing

Cashew (*Anacardium occidentale*, type 320) obtained from Productos Manzanares (Spain) and pistachio (*Pistacia vera*, variety Kerman) from the Germoplasm Bank of Institut de Recerca i Tecnologia Agroalimentàries (IRTA-Mas de Bover, Tarragona, Spain) were used in the study. Whole nuts seeds were immersed in distilled water (1:5 w/v) and boiled (100 °C, 30 and 60 min) or autoclaved using an autoclave Compact 40 Benchtop (Priorclave, London, UK) at 121 °C, 120 kPa, for 15 and 30 min and at 138 °C, 256 kPa, for 15 and 30 min. Untreated, boiled and autoclaved nut seeds were ground and defatted with n-hexane (34 ml/g of flour) for 4 h, shaken, and air-dried after filtration of the n-hexane. Defatted flour from untreated cashew and pistachio were the controls for boiled and autoclaved samples. The nitrogen contents of the samples were determined by LECO analysis according to standard procedures based on Dumas method (AOAC, 2003). The total protein content was calculated as $N \times 5.3$ (AOAC, 2003). The analyses were carried out in duplicate and the results summarized in Table 1. The protein content of processed samples was higher than in the raw ones. This could be related to the reduction of dry matter (i.e. carbohydrates) in these processed samples.

Seven food-grade proteases (Amano Enzyme Europe Ltd., Agno, Switzerland) were tested for digestion of cashew and pistachio samples and are referred to as E1-E7 in this study: E1 – Thermoase PC10F (endoprotease), E2 – ProteAX (exoprotease), E3 – Protin SD – NY10 (proprietary), E4 – Peptidase R (exopeptidase), E5 – Protin SD – AY10 (alkalase-like), E6 – Protease M “Amano” SD (proprietary), E7 – Protease P “Amano” 3SD (proprietary). All are stable at 50–55 °C and pH 7. The first three were recommended by Amano for the potential to digest allergenic proteins (E1-E3). Digestion of cashew and pistachio protein extracted with buffered saline borate (BSB, 0.1 M H₃BO₃, 0.025 M Na₂B₄O₇, 0.075 M NaCl, 1% w/v PVP, pH 8.45) (2 mg/ml) was carried out by incubation with 1 mg/ml PBS pH 7.4 of each enzyme at 55 °C over a period of 19 h while taking samples at various time points (0, 1, 2, 3 and 19 h). After SDS-PAGE analysis, E3 and E5 or E1, E3, E5 and E7 enzymes were selected for further experiments in pistachio and in

cashew, respectively. Such assays included the enzymatic digestion of whole nut paste (as opposed to soluble extract) under sonication (ultrasound disruption). The whole nut paste was prepared by mixing pistachio and cashew defatted flour with distilled water (0.5 g/ml) and kept at -20°C . Cashew and pistachio paste was subjected to ultrasound equipment consisting of a seven frequency multiSONIK-2 Ultrasonic Generator (Blackstone-Ney Ultrasonics, Jamestown, NY, USA) and controlled according the method described by Easson et al. (2015). A thermocouple system was installed in conjunction with the ultrasound instrument to maintain a steady temperature of 55°C . Fifteen ml plastic tubes, containing 0.5 g of untreated pistachio or cashew paste in 10 ml of distilled water followed by 110 μL of protease enzymes (1 mg/ml, Amano Enzyme, Switzerland), were sonicated for 1 h for pistachio or 2 h for cashew at 55°C and 40 kHz ultrasound frequency at 980 W of power. A 100 μL aliquot was removed at the beginning of the experiment prior to adding the protease enzyme (untreated, no E control), and at various time points, after sonication with enzymes, and boiled at 65°C for 15 min in sample buffer and analyzed by SDS-PAGE and western blot as control sample. All the experiments were performed at least in duplicate and the figures are representative of these replicate experiments.

2.3. Protein electrophoresis and IgE immunoblot experiments

Protein electrophoresis of the defatted flours was carried out as previously described (Cabanillas, Maleki et al., 2012). Defatted flours from untreated and treated cashew and pistachio were dissolved in standard electrophoresis sample buffer. The same amount of protein (20 μg) calculated from LECO analysis from each sample was electrophoresed in 4–20% and 16% Tris-glycine gels. Proteins were visualized with GelCode Blue Stain Reagent (Thermo Scientific, Waltham, MA, USA). For western blot, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA, USA). After blocking with 2% non-fat milk in PBS (1 h at 37°C), membranes were incubated overnight at 4°C with individual sera from 7 patients with pistachio and cashew allergy (P1-P7) (1:10, 1:5 or 1:3 dilution) washed and then treated with Horseradish peroxidase (HRP) conjugated mouse anti-human IgE (1:10,000 dilution for 30 min at RT) (Sigma, Saint Louis, MO, USA). Detection of IgE-binding proteins was achieved by means of enhanced chemiluminescence, according to the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA). The signal was measured using CCD camera system (Fuji Photo Film Co., Ltd., Duluth, GA, USA).

2.4. ELISA inhibition assays

Polystyrene microtiter plates (Immulon 4 HBX, Thermo Scientific, Waltham, MA, USA) were coated with 50 μL /well of raw cashew or pistachio protein extracts (10 $\mu\text{g}/\text{ml}$ or 0.5 $\mu\text{g}/\text{well}$ in 0.1 M NaHCO_3) and incubated for 1 h at 37°C . In parallel, a serum from a pistachio and cashew allergic patient (#5, 1:2 dilution) was preincubated with untreated raw (control) or enzymatically treated raw, boiled and autoclaved cashew or pistachio samples as inhibitors (final concentrations: 10, 5 and 2.5 $\mu\text{g}/\text{ml}$) 1 h at 4°C with rocking. Serum preincubated with PBS was also included (un-inhibited serum). Wells were washed with PBS containing 0.5% Tween-20 and blocked with 250 μL /well of PBS containing 3% non-fat milk (1 h at 37°C). The serum, preincubated with the different inhibitors, or un-inhibited serum was then added to the wells (50 μL /well) of the pre-coated ELISA plates for 1 h. After washing the wells, 50 μL /well of a Horseradish peroxidase (HRP) conjugated mouse anti-human IgE (1:1000 dilution) (Sigma, Saint Louis, MO, USA) were added and incubated for 1 h. After washing, the peroxidase reaction was developed with 100 μL of peroxidase substrate (SureBlue TM, KPL, Gaithersburg, MD, USA). After 30 min, the reaction was stopped with 100 μL of 4 N HCl, and the optical density (O.D.) was measured at 450 nm. The percentage of the decrease in IgE binding (or

inhibition) was calculated with the formula: $[(1 - (\text{AI}/\text{AN})) \times 100]$ where AI is the absorbance value obtained from raw cashew or pistachio samples preincubated with inhibited sera (raw or enzymatically hydrolyzed samples) and AN is the absorbance value of raw samples (cashew or pistachio) incubated with sera in the absence of inhibitor. All tests were performed in triplicate and the graphs are representative of these experiments. To test any significant differences between untreated (control) and treated samples for each inhibitor concentration, the inhibition data were subjected to one-way analysis of variance (ANOVA) and the means were compared by Duncan's multiple range test using the Statgraphic Centurion XVI.I software (Statpoint Tech. Inc., Warrenton, VA, USA). The table below each graph (Fig. 4) shows the comparison by Duncan's multiple range test between means of untreated and treated samples for each inhibitor concentration. Means in the same column followed with the same superscript are not significantly different ($P > 0.05$).

2.5. Liquid chromatography, mass-spectroscopy (LC/MS/MS)

Cashew and pistachio proteins from untreated, boiled for 60 min and autoclaved samples (120 kPa, 30 min and 256 kPa, 30 min) were digested by enzymatic treatment with enzyme 7 (cashew) and enzyme 5 (pistachio). The digestion resistant protein bands were excised from the gel following electrophoretic separation and identified by LC/MS/MS. Proteins were in-gel digested with trypsin (Sequencing Grade Modified, Promega, Madrid, Spain) according to Shevchenko, Tomas, Havlis, Olsen, and Mann (2006). Briefly, excised gel bands were washed with 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v) and incubated then placed in 10 mM dithiothreitol in 100 mM NH_4HCO_3 at 56°C for 45 min followed by alkylation of the reduced sulfhydryl groups with 55 mM iodoacetamide in 100 mM NH_4HCO_3 at 25°C for 30 min in the dark. Proteins were digested by adding 50 μL of trypsin (130 $\mu\text{g}/\text{ml}$) and incubating at 37°C overnight. Peptides were eluted in extraction buffer (1:2 (v/v) 5% formic acid/acetonitrile) incubating at 37°C for 15 min and 0.1% trifluoroacetic acid was added for further analysis. The tryptic digests were analyzed via LC/MS/MS, using an Agilent 1260 LC system, an Agilent Chip-cube interface and an Agilent 6520 Q-TOF tandem mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Data files were transferred to an Agilent workstation equipped with Spectrum Mill software (Agilent Technologies, Santa Clara, CA, USA) which extracted, sequenced, and searched databases such as TrEMBL and NCBI.

3. Results

3.1. Assessing the efficiency of enzymes to digest cashew and pistachio protein

Seven enzymes from Amano Enzymes Ltd. were used for digestion of pistachio and cashew proteins in order to reduce the IgE binding properties. These enzymes were chosen for their higher temperature stability and differences in enzymatic properties. These 7 enzymes (E1-E7 at 1 mg/ml each) were each incubated with raw pistachio and cashew extracts for various amounts of time in order to identify the enzymes with the highest digestion ability. Digestion of pistachio proteins is shown as an example of this selection in Fig. 1A. The left panel shows the SDS-PAGE analysis of pistachio BSB extracts subjected to the longest digestion time (19 h) for each enzyme. Enzyme 3 and 5 (E3 and E5) were determined to be the most efficient in digesting pistachio proteins and used in further experiments. In the middle panel, autoclaved pistachio BSB extract (120 kPa, 15 min) incubated with E3 or E5 (1 mg/ml each) for 19 h is shown. In order to enhance digestion and reduce the processing time, the digestion experiments with whole pistachio paste were carried out in the presence of ultrasound treatment for 1 h at a frequency of 40 kHz, temperature of 55°C and power of 980 watts at 100%. Due to the differences in digestion products for E3 and

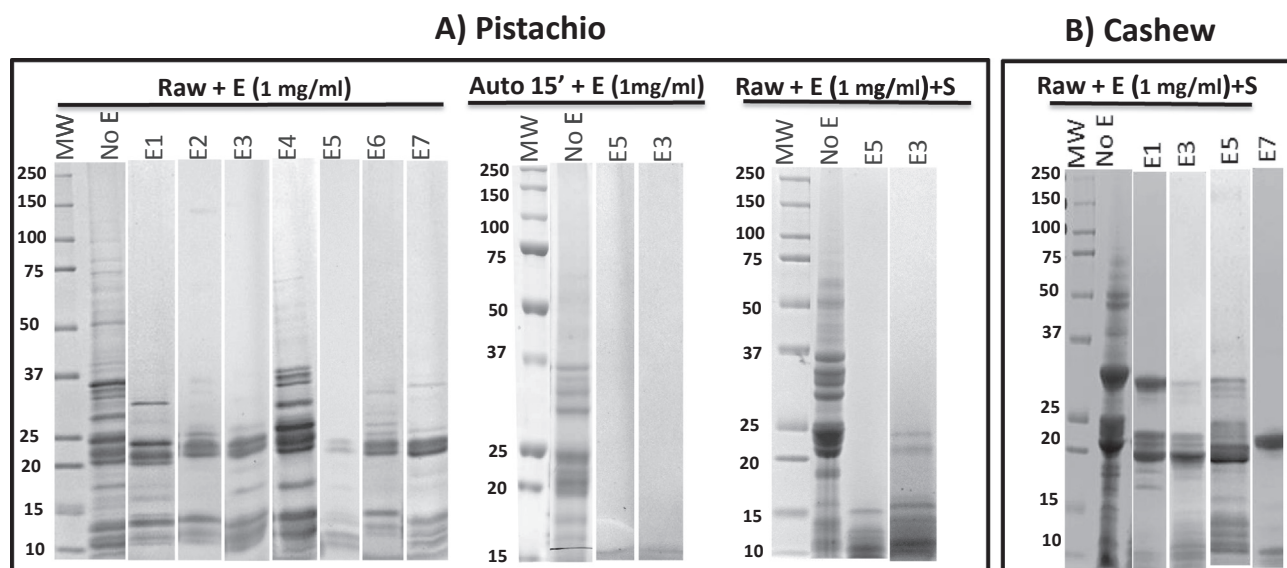


Fig. 1. A) SDS-PAGE analysis of the ability of various enzymes to digest pistachio proteins. Seven enzymes (E1–E7) were used at 1 mg/ml concentration to digest pistachio BSB extracts untreated (Raw, left panel) during 19 h without ultrasound treatment. Middle panel: Autoclaved pistachio BSB extract (120 kPa, 15 min) digested with enzymes E3 and E5 during 19 h without ultrasound treatment. Right panel: Digestion experiments of whole pistachio paste with enzymes E3 and E5 subjected to 1 h of ultrasound treatment (S). B) SDS-PAGE of digestion experiments of whole cashew paste with enzymes E1, E3, E5 and E7 subjected to 2 h of ultrasound treatment (S). Molecular weight marker (MW) and undigested pistachio or cashew (No E) are also shown. Each lane was loaded with 20 µg of protein. 4–20% Tris-glycine gels were used.

E5, E5 was selected for use in further experiments (Fig. 1A, right panel). Similar experiments were carried out to identify the most effective enzymes for the digestion of whole cashew paste with E1, E3, E5 and E7 (2 h, 40 kHz) and enzyme 7 (E7) was selected for further experiments (Fig. 1B). Multiple two-enzyme combinations of the seven enzymes were also used in digestion experiments for both cashew and pistachio extracts (data not shown). It was determined that the digestions were no more efficient with any two-enzyme combinations than the single enzymes selected for further experiments.

3.2. Electrophoretic characterization of processed cashew samples

The SDS-PAGE patterns of the proteins in paste made from untreated (control), boiled (boil), and autoclaved (AU) cashew before and after 2 h of simultaneous ultrasound treatment and digestion with the Amano Protease P 3DS (E7), are presented in Fig. 2A (lanes 1 to 7). Similar complex protein banding patterns were observed in raw, boiled and autoclaved at 120 kPa samples with bands ranging from 7 to 100 kDa. Cashew autoclaved at 256 kPa for 15 min and 30 min showed less defined stained bands and more smearing, most likely due to a combination of heat and pressure-induced aggregation and degradation of proteins. The protein migration patterns of untreated cashew (control) and thermally treated (boiling and autoclaving) after enzymatic digestion with E7 are also shown in Fig. 2A (lanes 8–14). The cashew protein patterns before and after enzymatic hydrolysis were very different with an important decrease in proteins higher than 25 kDa. Bands around 20 kDa and lower molecular weights were still present after enzymatic treatment of raw and boiled samples. However, many of the bands appeared to be digested with E7 in most of the autoclaved samples, as seen in lanes 12–14.

The samples were also analyzed for differences in IgE binding by immunoblot using individual sera from 6 patients (P1–P6) with clinical allergy to cashew (Fig. 2B). Previous studies have shown that certain physical processing can alter protein solubility affecting the extraction of soluble proteins by traditional protein extraction methods (Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010), therefore defatted flours were directly solubilized in sample buffer and used for the SDS-PAGE and immunoblot analysis carried out in this study. Since the samples that had been autoclaved for 15 min (lanes 4 and 6) seemed to alter the

protein profile less than the samples autoclaved for 30 min, they were not assessed for IgE binding by all patient sera. However, all blots contain raw, boiled 60 min and autoclaved (at 120 kPa, 30 min and 256 kPa, 30 min) samples before and after enzymatic treatment (lanes 1, 3, 5, 7, 8, 10, 12 and 14) for comparison purposes. Similar allergenic proteins were recognized by IgE in raw, boiled and autoclaved at 120 kPa cashew before enzyme treatment when sera from patients P1 to P5 were used in immunoblots (Fig. 2B). However, serum IgE from patient P6 recognized a different pattern of bands from 12 to 75 kDa in untreated cashew (lane 1) than in thermally processed cashew. Autoclaved cashew at 256 kPa (lanes 6 and 7) showed a similar IgE blot pattern to SDS-PAGE (Fig. 2A) with an overall reduction in the distinct IgE-reactive bands with increased time of treatment.

The results showed that numerous proteins from untreated and heat treated cashew samples were digested after 2 h of incubation with Amano Protease P 3DS (E7) under sonication; however, a few digestion resistant, IgE binding proteins could be visualized with sera from patients P1 (at and below 20 kDa, lanes 8, 10 and 12), P4 (10 kDa) and P5 (14 and 50 kDa). Autoclaved cashew proteins at 256 kPa showed higher susceptibility to digestion since no IgE-binding bands could be detected in 5 out of 6 sera used (lane 14).

3.3. Electrophoretic characterization of processed pistachio samples

The SDS-PAGE patterns of the proteins in paste made from untreated (control), boiled (boil), and autoclaved (AU) pistachio, before and after 1 h of simultaneous ultrasound treatment and digestion with the Amano Protease Protin (E5), are presented in Fig. 3A. Multiple bands can be seen in control sample with molecular weights between 6 and 100 kDa. Less defined bands were observed after autoclaving with an increase in protein aggregation and therefore higher molecular weight smears, particularly visible in samples autoclaved at 256 kPa (lanes 6 and 7). Digestion of treated samples with E5 enzyme (lanes 8–14) resulted in hydrolysates with an important decrease of higher MW proteins, above 15 kDa, and an increase of low molecular weight bands in the treated samples (lanes 9–14).

All samples were analyzed with IgE-immunoblot using a serum from 6 patients with clinical allergy to pistachio (P1, P2, P3, P4, P5 and P7). Fig. 3B shows the IgE-binding patterns to raw and processed pistachio

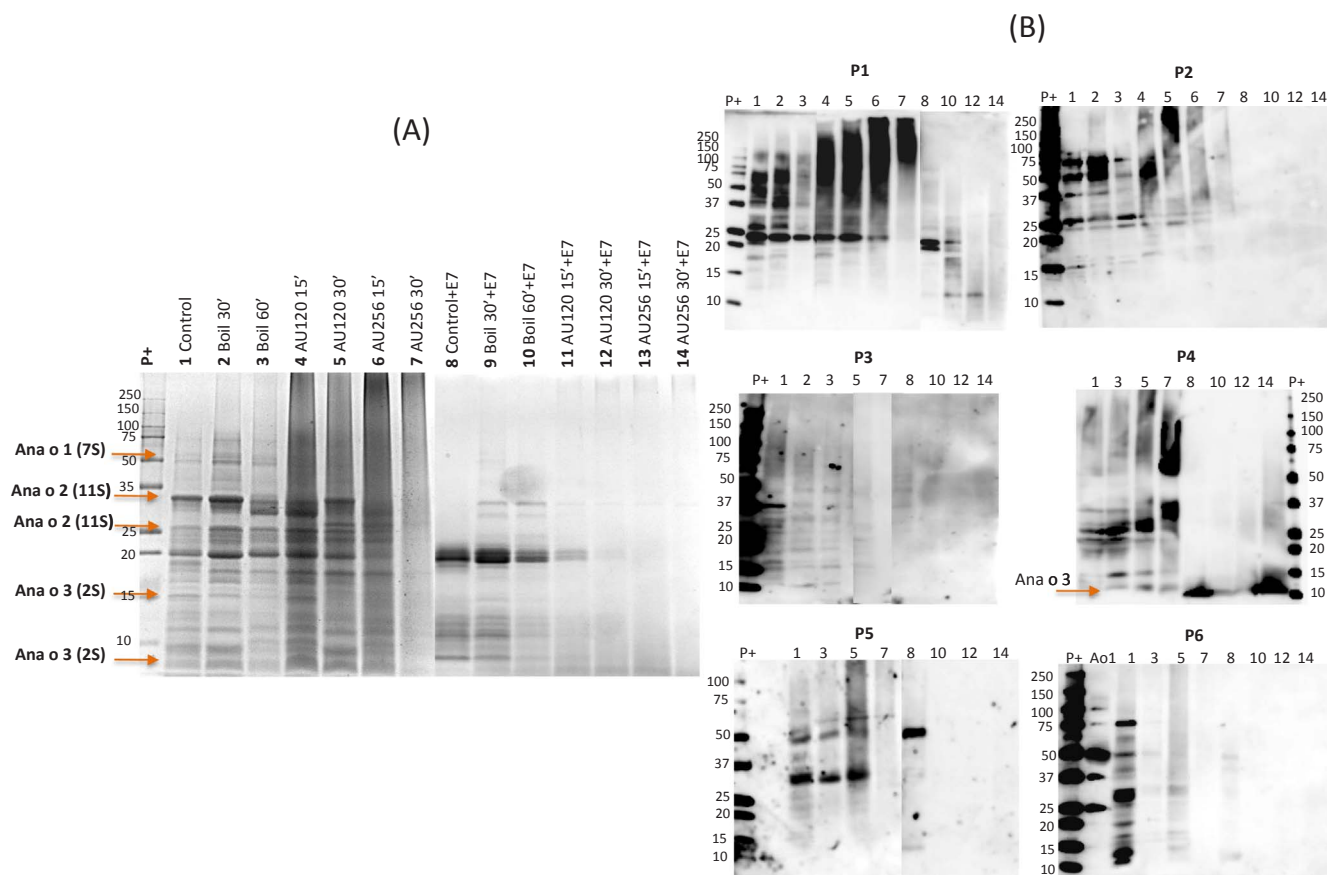


Fig. 2. SDS-PAGE (A) and IgE immunoblots (B) of untreated cashew paste (control; lanes 1 and 8) and processed cashew paste: boiled for 30 min (lanes 2 and 9) or 60 min (lanes 3 and 10); autoclaved at 120 kPa for 15 min (lanes 4 and 11) or 30 min (lanes 5 and 12); autoclaved at 256 kPa for 15 min (lanes 6 and 13) or 30 min (lanes 7 and 14) before and after incubation with enzyme 7 (Protease P, Amano 3DS), during sonication (2 h, 40 kHz). IgE immunoblots were carried out using individual sera from 6 patients allergic to cashew (P1–P6). Each lane was loaded with 20 μ g of protein. 16% Tris-glycine gels were used.

samples before and after enzymatic treatment with E5. As mentioned above for cashew, only representative samples were subjected to immunoblots. Raw pistachio showed a pattern of IgE-binding to proteins in the range of 12–100 kDa. Pistachio IgE immunoreactivity appeared reduced with autoclaving at 256 kPa for several patient sera, but not after boiling or autoclaving at 120 kPa. Most proteins from untreated and processed pistachio samples were digested after 1 h of sonication in the presence of E5. Serum IgE from 2 out of 6 sera (P1 and P4) were still able to detect and bind to several proteins of varying molecular weights and smears as high as 75 kDa in the case of P4. No IgE reactive protein bands were detected after digestion of autoclaved pistachio at 256 kPa for all patient sera, except P4.

3.4. ELISA

In order to assess the IgE reactivity of cashew and pistachio hydrolysates, a competitive inhibition ELISA assay was carried out using serum from patient P5 with clinical allergy to pistachio and cashew. In this ELISA experiment, raw cashew or pistachio was used as a solid phase and raw and enzymatically treated raw, boiled and autoclaved cashew or pistachio samples were used as inhibitors. The percentage of the decrease in IgE binding was calculated as described in methods section and shown in Fig. 4A and B. The table below each graph shows the comparison by Duncan's test between means of untreated and treated samples for each inhibitor concentration. Raw cashew competed for IgE before and after hydrolysis with enzyme E7 (92.35% and 73.41%, respectively). However, the processed and digested cashew samples bound significantly lower IgE levels than the raw sample. Boiled (60 min) and autoclaved (256 kPa, 30 min) cashew hydrolysates

samples inhibited only 24.16% of the IgE binding to raw cashew (Fig. 4A). Meanwhile, the effect was more pronounced with pistachio samples and all of the enzymatically treated samples. Raw as well as boiled and autoclaved pistachio were significantly weaker competitors for IgE binding compared to untreated raw pistachio (Fig. 4B). These findings are consistent with the reduction in IgE binding seen in the western blot analysis (Figs. 2B and 3B).

3.5. Proteins identified after enzymatic treatment of processed pistachio and cashew samples

Cashew and pistachio proteins from untreated, boiled for 60 min and autoclaved samples (120 kPa, 30 min and 256 kPa, 30 min) after digestion by treatment with E7 enzyme (cashew) and E5 enzyme (pistachio) were loaded in a 16% Tris-glycine gel in order to determine the extent of enzyme. The major bands were tryptic digested in order to carry out the analysis by mass spectroscopy (LC/MS/MS). Finally, twelve digestion resistant bands of pistachio and twelve digestion resistant bands in cashew were manually excised for LC-MS/MS identification (Fig. 5). Table 2 [supp](#) summarizes the polypeptide identification data of these pistachio and cashew samples. A peptide mass fingerprint search allowed us to identify all the resistant bands of pistachio (1–12) as Pis v 1, Pis v 2.01 or Pis v 5. The reason why the Pis v 1 peptides are so few is most likely due to the fact that the digested fragments, smaller than 8–10 kDa, ran off of the bottom of the gel. The bands # 4 and 7 had very few peptides identified and there were no peptides for Pis v 2 or Pis v 5 identified in band #10. Similar pattern is found with bands # 2, 5, 8, and 11 as well as for bands # 3, 6, 9 and 12. The bands # 13, 16, 19 and 22 (20 kDa) were identified as Ana o 2. and

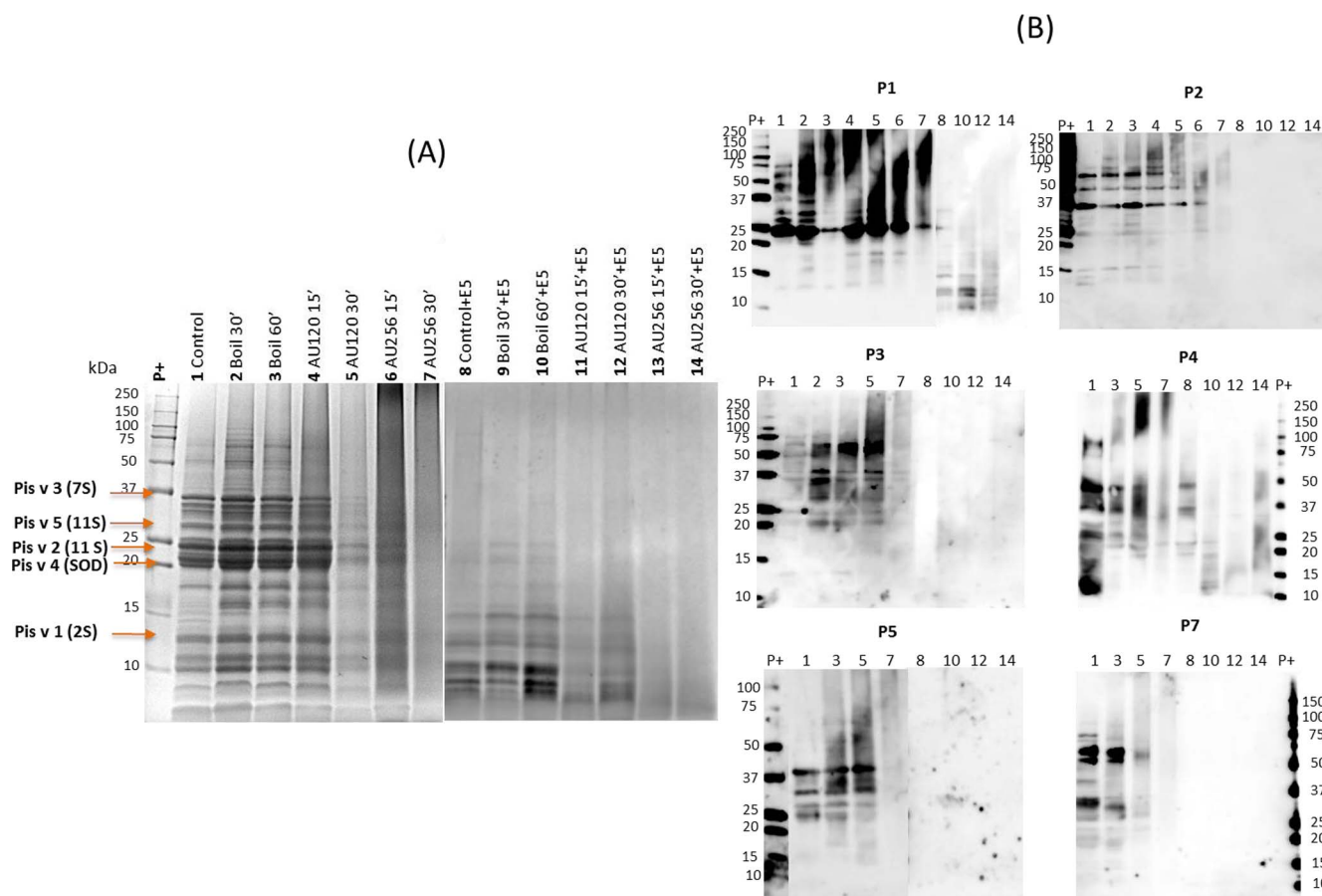


Fig. 3. SDS-PAGE (A) and IgE immunoblots (B) of untreated pistachio paste (control; lanes 1 and 8) and 6) and processed pistachio paste: boiled for 30 min (lanes 2 and 9) or 60 min (lanes 3 and 10); autoclaved at 120 kPa for 15 min (lanes 4 and 11) or 30 min (lanes 5 and 12); autoclaved at 256 kPa for 15 min (lanes 6 and 13) or 30 min (lanes 7 and 14) before and after sonication (1 h, 40 kHz) with enzyme 5 (Protin, Amano). IgE immunoblots were carried out using individual sera from 6 patients allergic to pistachio (P1–P7). Each lane was loaded with 20 µg of protein. 16% Tris-glycine gels were used.

the bands # 17, 20 and 23 were also identified as Ana o 2, except one peptide which was identified as Ana o 1 (this could be an artifact, but the scores are around 83–84). Ana o 3 is detected in bands # 14 and 17, but not in bands # 20 and 23, suggesting that is degraded into smaller fragments. This is confirmed because Ana o 3 fragments are found in bands # 15, 18, 21 and 24. These findings show that the proteins are getting degraded over thermal treatment and enzymatic digestion and when autoclaved samples at 256 kPa are digested (bands # 10, 11, 12, 22, 23 and 24) only very few resistant peptides could be identified indicating that some fragments of pistachio and cashew allergens survive even thermal and enzymatic treatment.

4. Discussion

In the present study, we have shown for the first time that IgE binding capacity of cashew proteins substantially decreased after pressure treatment at 256 kPa, 138 °C. We also found that some IgE-binding proteins from cashew were stable following boiling and autoclaving at 120 kPa. In accordance with these results, Venkatachalam et al. (2008) found that simple food processing treatments (autoclaving at 121 °C, blanching, microwave heating, roasting, gamma irradiation and pH) may not be sufficient to eliminate or substantially reduce cashew nut allergenicity as Ana o 1, Ana o 2 and Ana o 3 survive the processing conditions tested. Our findings indicate that enzymatic digestion with Amano Protease P 3DS (E7) during 2 h of sonication reduced the number of bands recognized by IgE of cashew allergic patients sera. Ana o 2 (36 kDa and 20 kDa bands) recognition was almost abolished but still some digestion resistant proteins were recognized by

50% of sera tested (Fig. 2B) and Ana o 3 (~8 kDa band) is still detected by IgE in one of the immunoblots (Fig. 2B). However, a combination of both, enzymatic hydrolysis under sonication and thermal treatment, was a more effective method to reduce the allergenic reactivity of cashew. The results of competitive inhibition ELISA (Fig. 4A) confirmed the greater reduction of IgE binding capacity of boiled and autoclaved cashew allergenic proteins compared to raw cashew.

Similarly to cashew, IgE immunoreactivity of pistachio was strongly reduced with thermal treatment with high pressure (autoclaving at 256 kPa), but not after boiling or autoclaving at 120 kPa. These findings are also in agreement with our previous results with other tree nuts and legumes (Cabanillas, Maleki et al., 2012; Cabanillas et al., 2014; Cuadrado et al., 2009; Álvarez-Álvarez et al., 2005). In contrast, the influence of enzymatic digestion on pistachio allergens was more pronounced. Most of immunoreactive proteins from untreated and processed pistachio samples were digested with Protin (E5) in conjunction with 1 h of sonication (Fig. 3B). In this case, the antigenicity of pistachio was almost abolished after digestion of raw and thermally treated samples (Fig. 4B). After the enzymatic treatment, the results of protein identification by MS analysis (Fig. 5 and Table 2 supp) indicate that the allergens are being degraded over thermal treatment and enzymatic digestion. Enzymatic digestion of processed samples produced few resistant peptides indicating that some fragments of pistachio and cashew allergens survive even following thermal and enzymatic treatments.

Cabanillas et al. (2010, 2012) concluded that sequential hydrolysis of lentil and peanut by endo- and exoproteases results in an important proteolytic destruction of IgE-binding proteins. Cabanillas et al. (2014) demonstrated higher susceptibility to digestion of pressure treated

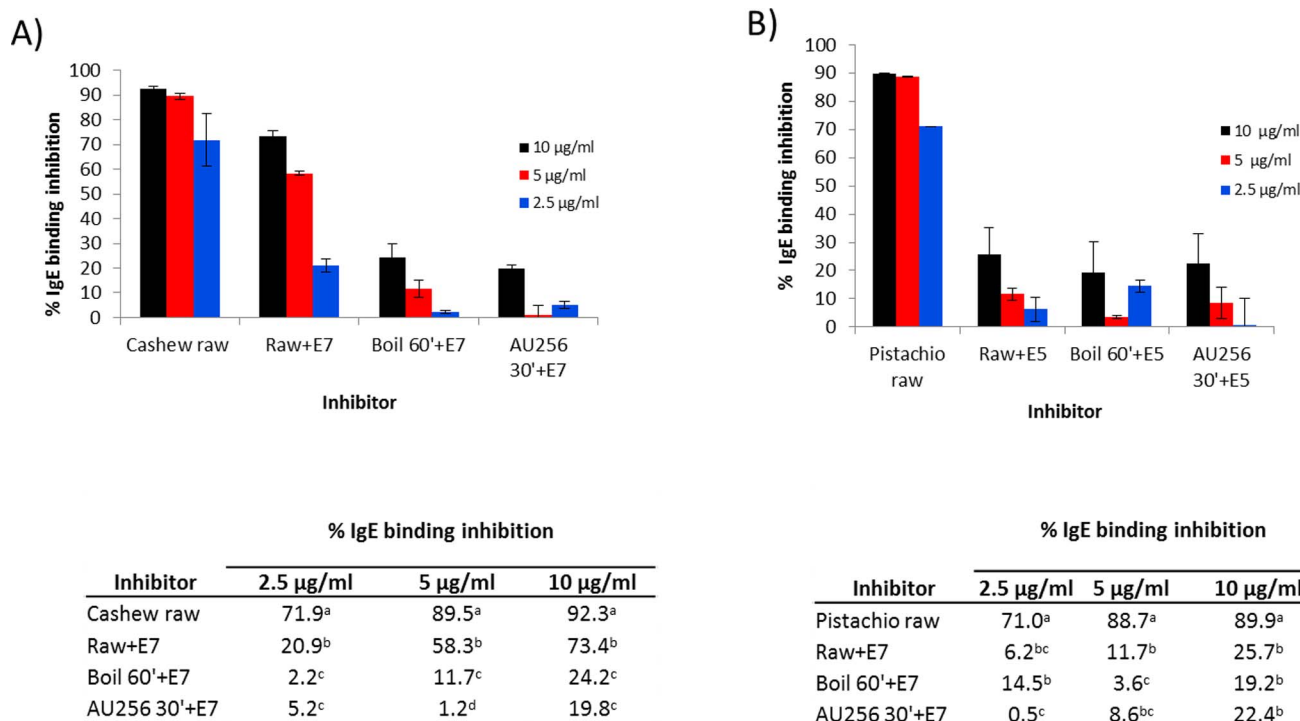


Fig. 4. Competitive inhibition ELISA of IgE binding to immobilized raw cashew (A) and raw pistachio (B) by three different concentrations (10 µg/ml, 5 µg/ml; 2.5 µg/ml) of untreated raw (control) or enzymatically treated raw, boiled and autoclaved cashew or pistachio samples as inhibitors. Panels A and B show the percentage of inhibition of IgE binding (mean ± SD) by the indicated concentrations of cashew and pistachio samples. The table below each graph shows the comparison by Duncan's multiple range test between means of untreated and treated samples for each inhibitor concentration. Means in the same column followed with the same superscript are not significantly different. ($P < 0.05$).

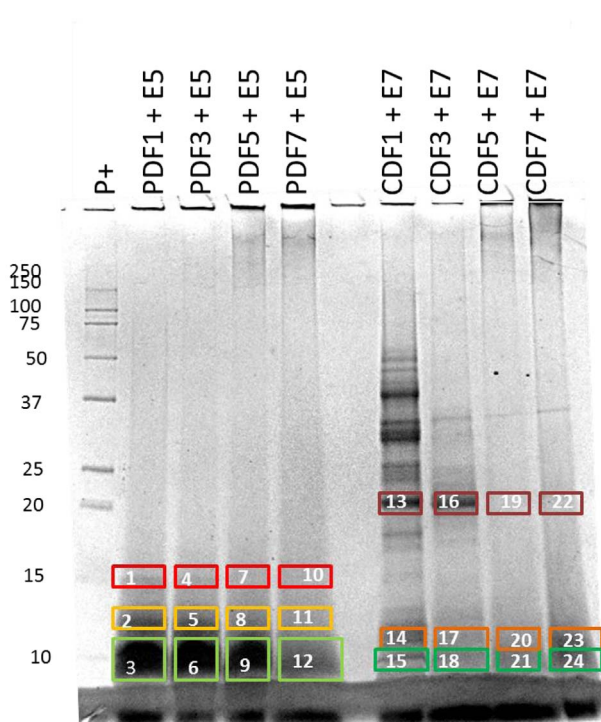


Fig. 5. SDS-PAGE (16% Tris-glycine) of untreated, boiled (60 min) and autoclaved (120 kPa, 30 min and 256 kPa, 30 min) pistachio paste (PDF1, PDF3, PDF5, PDF7) and cashew paste (CDF1, CDF3, CDF5, CDF7) after incubation with enzyme 5 (1 h, 40 kHz) and enzyme 7 (2 h, 40 kHz), respectively. Twelve digestion resistant bands of pistachio and twelve digestion resistant bands in cashew (squared) were manually excised for LC-MS/MS identification. Each lane was loaded with 20 µg of protein.

walnut proteins. That is in agreement with the present results with cashew and pistachio showing that enzymatic hydrolysis is more efficient on autoclaved cashew and pistachio samples.

In conclusion, the results indicate that autoclave treatment and enzymatic hydrolysis under sonication separately induced a measurable reduction of the IgE binding properties IgE binding of pastes (as opposed to soluble extracts) made from treated cashew and pistachio nuts, being more effective with pistachio allergens. However, thermal treatment (boiling and autoclaving) combined with enzymatic digestion is necessary to substantially reduce IgE binding to cashew allergens. Such studies are important as a preliminary step prior to clinical oral challenge trials to confirm a possible decrease in allergenicity of these processed foods. The findings of this study identify treatment conditions to reduce or even abolish the allergenic reactivity of cashew and pistachio in order to produce hypoallergenic foods, which may be relevant for consumers, clinicians, regulatory agencies and food industry.

The use of processed cashew and pistachio with low IgE binding capacity might be a convenient strategy for tolerance induction. Evaluation of sensorial properties and clinical oral challenge trials are necessary to analyze organoleptic properties and to confirm the decrease of allergenicity of these processed nut pastes.

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Note

The authors declare no conflict of interest.

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.2017.08.120>.

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Table 1 supplementary. Immunological and clinical information of the 7 patients allergic included in this study.

Patient no.	Age/ Sex	IgE total (kU/L)	Ig E cashew (kU/L)	Ig E pistachio (kU/L)	Ig E peanut (kU/L)	Symptoms
# 1	58/F	13665	4.5	ND	26.1	C, EB, eye ulcers, itchy nose, CT, HF.
# 2	65/M	71000	ND	ND	>100	Severe eczema, hearing loss, pruritus, EB.
# 3	22/M	1200	17	8	>100	EB, TS, FS, LS, G, IM, H.
# 4	36/M	27.5	5.97	ND	8.45	Severe eczema.
# 5	26/M	362.9	10.1	5	>100	Anaphylaxis.
# 6	30/F	191	1.46	2.09	52.7	SOB, wheeze, severe asthma.
# 7	41/F	120	8.5	5.9	1.6	TS, CT, IT, IM, N, GI, V.

C, conjunctivitis; CT, chest tightness; EB, eye burn/itchy; FS, face swell; G, general itching; GI, gastrointestinal pain; H, hives; HF, hay fever; IM, itchy mouth; IT, itchy throat; LS, lip swell; N, nausea; SOB, shortness of breath; TS, throat swell; V, vomiting.
ND, not determined.

Table 2 supplementary. Cashew and pistachio proteins from untreated, boiled for 60 min and autoclaved samples (120 kPa, 30 min and 256 kPa, 30 min) treated by enzymatic digestion with enzyme 7 (cashew) and enzyme 5 (pistachio) and identified by LC/MS/MS.

Band No.	Protein identification	Mass (Da)	Matched peptides
1	Pis v 2.01/Pis v 5 (11S <i>P.vera</i>)	54,465	10
1	Pis v 1 (2S <i>P.vera</i>)	17,290	1
2	Pis v 2.01/Pis v 5 (11S <i>P.vera</i>)	54,465	22
2	Pis v 1 (2S <i>P.vera</i>)	17,290	1
3	Pis v 2.01/Pis v 5 (11S <i>P.vera</i>)	54,465	19
3	Pis v 1 (2S <i>P.vera</i>)	17,290	1
4	Pis v 2.01/Pis v 5 (11S <i>P.vera</i>)	54,465	3
5	Pis v 2.01/Pis v 5 (11S <i>P.vera</i>)	54,465	28
5	Pis v 1 (2S <i>P.vera</i>)	17,290	1
6	Pis v 2.01/Pis v 5 (11S <i>P.vera</i>)	54,465	21
6	Pis v 1 (2S <i>P.vera</i>)	17,290	1
7	Pis v 2.01/Pis v 5 (11S <i>P.vera</i>)	54,465	1
8	Pis v 2.01/Pis v 5 (11S <i>P.vera</i>)	54,465	6
9	Pis v 2.01/Pis v 5 (11S <i>P.vera</i>)	54,465	11
9	Pis v 1 (2S <i>P.vera</i>)	17,290	1
11	Pis v 2.01/Pis v 5 (11S <i>P.vera</i>)	54,465	2
12	Pis v 2.01/Pis v 5 (11S <i>P.vera</i>)	54,465	8
12	Pis v 1 (2S <i>P.vera</i>)	17,290	1
13	Ana o 2 (11S <i>A. occidentale</i>)	51,996	13
14	Ana o 2 (11S <i>A. occidentale</i>)	51,996	7
14	Ana o 3 (2S <i>A. occidentale</i>)	16,335	2
15	Ana o 2 (11S <i>A. occidentale</i>)	51,996	6
15	Ana o 3 (2S <i>A. occidentale</i>)	16,335	3
16	Ana o 2 (11S <i>A. occidentale</i>)	51,996	15
17	Ana o 1 (7S <i>A. occidentale</i>)	61,841	1
17	Ana o 2 (11S <i>A. occidentale</i>)	51,996	6
17	Ana o 3 (2S <i>A. occidentale</i>)	16,335	2
18	Ana o 2 (11S <i>A. occidentale</i>)	51,996	9
18	Ana o 3 (2S <i>A. occidentale</i>)	16,335	2
19	Ana o 2 (11S <i>A. occidentale</i>)	51,996	8
20	Ana o 1 (7S <i>A. occidentale</i>)	61,841	1
20	Ana o 2 (11S <i>A. occidentale</i>)	51,996	6
21	Ana o 1 (7S <i>A. occidentale</i>)	61,841	2
21	Ana o 2 (11S <i>A. occidentale</i>)	51,996	5
21	Ana o 3 (2S <i>A. occidentale</i>)	16,335	1
22	Ana o 2 (11S <i>A. occidentale</i>)	51,996	5
23	Ana o 1 (7S <i>A. occidentale</i>)	61,841	1
23	Ana o 2 (11S <i>A. occidentale</i>)	51,996	6
24	Ana o 1 (7S <i>A. occidentale</i>)	61,841	2
24	Ana o 2 (11S <i>A. occidentale</i>)	51,996	6
24	Ana o 3 (2S <i>A. occidentale</i>)	16,335	2

