Mitigation of peanut allergic reactivity by combined processing: Pressured heating and enzymatic hydrolysis

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Abstract

Among food allergens, peanut is one of the most critical. This study evaluates peanut allergenic features after the combination of heat, pressure, and enzymatic digestion under sonication, by immunodetection using serum IgE of sensitized patients and mass-spectroscopy. In the studied population, there was a predominance of patients sensitized to Ara h 9 (a1LTP) followed by sensitization to seed storage proteins (Sprot, Ara h 1, 2, 3, and 6). The Sprot sensitized patients showed higher reactivity. The enzyme E5 was efficient for inducing protein fragmentation and allergenic reactivity reduction when it was used combined with pressured heating treatments such as autoclave and Controlled Instantaneous Depressurization (DIC). Only a few Ara h 1 and Ara h 3 peptides were identified after enzymatic digestion of DIC peanut samples. The combination of pressured heating treatments and enzymatic hydrolysis was the most efficient method to strongly mitigate or even eliminate the allergenic potential of peanut. Our findings set a possibility for a group of patients in which their allergy could be treated with a processed less-allergenic peanut and consequently less risky, more easy and quicker desensitization treatment.

Industrial relevance: The findings identify innovative thermal, pressure and enzymatic processing conditions highly effective to mitigate or even abolish the allergenic potency of peanut, which may be relevant for consumers, clinicians, regulatory agencies and the food industry. The applications of processed peanut with reduced IgE binding potency for tolerance induction might be a convenient strategy.

1. Introduction

Among food allergies, peanut allergy is one of the most severe, with high prevalence in Western countries (2% in children and 0.6% in adults). US and UK studies have suggested an increase in peanut allergy in the last decades (Sicherer & Sampson, 2018). In Spain, peanuts are the third food most frequently involved in allergic reactions after walnut and hazelnut. However, in some European countries peanut is the most prevalent (Haroun-Díaz et al., 2017; Lyons et al., 2020). Most legume and tree nut allergens belong to seed storage proteins, including legumins (11-13S globulins), vicilins (7S globulins), and 2S albumins. Other allergens, such as profilins, lipid transporter proteins (LTPs) and class 10 pathogenesis-related proteins (PR-10), have well-known biological functions and they are considered to be panallergens (Crespo, James, Fernandez-Rodriguez, & Rodriguez, 2006). In peanut, at least 11 allergens have been identified: Ara h 1 (7S vicilin), Ara h 3 and Ara h 4 (11S legumins), Ara h 5 (profilin), Ara h 2, Ara h 6 and Ara h 7 (2S albumins), Ara h 8 (PR-10), Ara h 9 (LTP), Ara h 10 and Ara h 11 (oleosins).

Abbreviations: DIC, Controlled Instantaneous Depressurization; RT, room temperature.
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Food is subjected to a wide variety of processes to guarantee food safety and quality, improve its organoleptic characteristics and prepare it for consumption. Food processing causes a series of biochemical transformations and interactions between different compounds and can dramatically influence their allergenic properties, by modifying both conformational and linear epitopes (Rahaman, Vasiljevic, & Ramchandran, 2016; Shriver & Yang, 2011). The elimination of natives epitopes or the generation of new ones (neoallergens) is a usual consequence of the conformational change of proteins derived from processing (Jiménez-Saiz, Benedé, Molina, & López-Exposito, 2015). The sensitivity to processing of each protein, its structure, the condition and type of the treatment applied, as well as the food matrix, will determine the extent of processing effect on its allergenic potential (Maleki, 2004; Sathe & Sharma, 2009). The modifications on legume and nut allergenicity produced by boiling, roasting, and pressure-cooking have been previously assessed finding that processing with heat treatments under pressure affect considerably the in vitro IgE binding potency (Alvarez-Alvarez et al., 2005; Cabanillas et al., 2012; Cuadrado et al., 2009; Cuadrado et al., 2011; Maleki, Chung, Champagne, & Raufman, 2000; Schmitt, Nesbit, Hurlburt, Cheng, & Maleki, 2010). Peanut processing can differentially affect its immunoreactivity. According to some reports, boiling or frying peanuts reduces its IgE-immunoreactivity (Beyer, Morrow, Xiú-Min, & Bardina, 2001), although other authors reported that soluble Ara h 1 and Ara h 2 major allergens were decreased throughout boiling and frying without generating hypoallergenic peanuts (Comstock, Maleki, & Teuber, 2016). As previously reported, roasted peanut has 100 times more IgE-binding capacity than untreated peanut (Maleki et al., 2000). These studies demonstrated that, in roasted peanuts, the structural alterations suffered by Ara h 1 and Ara h 2 allergens enhanced their allergenic potential. Cabanillas, Maleki, et al. (2012) demonstrated that IgE recognition of roasted peanuts decreased significantly with autochlate at harsh conditions (2.6 bar, 30 min) and that most of α-helical structure was disrupted after autoclaving. The immunoreactivity reduction can be explained because most of the IgE epitopes are placed on the α-helical regions of major peanut allergens (Ara h 1, 2 and 3) (Mueller, Maleki, & Pedersen, 2014).

When Depressurization Instant Controlled (DIC) treatments at 3 bars (1 and 3 min) and at 6 bars for 1 min, were evaluated on untreated raw and roasted peanut and lupin samples, their immunoreactive pattern were similar compared to control raw samples (Cuadrado et al., 2011; Guillamón et al., 2008). Nevertheless, DIC treatment at 6 bars during 3 min produced a considerable reduction of Ara h 1. Moreover, some bands are affected differently in untreated and roasted peanuts, such as the Ara h 3 acid subunit (37 kDa) (Cuadrado et al., 2011).

Allergic proteins are less susceptible than other proteins to proteolytic action, but enzymatic digestion can modify their allergenicity. Enzymatic digestion is considered an efficient process to disrupt both conformational and sequential epitopes (Fritsche, 2009), and it can be an alternative to intact proteins for developing hypoallergenic formulations for allergic patients (Clemente, 2000). However, depending on the digestion conditions and the type of enzymes used, peptides of different sizes can be obtained with variable allergenicity (Fritsche, 2009; Walker Smith, 2000). The combination of endo- and exopeptidases action on peanuts and lentils provokes a significant reduction of IgE binding epitopes in both peanuts and lentils (Cabanillas et al., 2010; Cabanillas et al., 2012). According to Cabanillas et al. (2014), high pressure applied to walnuts causes more susceptibility to duodenal and gastric digestion. The combined effect of autoclaving and enzymatic digestion under sonication is highly effective to produce a remarkable decrease in the allergic capacity of pistachio and cashew pastes being pistachio more affected than cashew (Cuadrado et al., 2018). Although analyzing the clinical relevance of these findings require further studies, this combined enzymatic method could be a procedure of choice to produce hypoallergenic protein hydrolysates. However, up to now the combined effects of pressured heating and enzymatic hydrolysis on peanut allergenicity have not been analyzed.

The current work aims to evaluate the allergenic potential and IgE immunoreactivity of peanut proteins after heating combined with high pressure (autoclave and DIC), enzymatic hydrolysis and ultrasound treatment, by means of in vitro immunossays and skin prick tests (SPT).

2. Materials and methods

2.1. Patients and sera

Sera obtained from 27 patients sensitized to peanut (positive specific IgE to peanut and skin prick test) who were attended during 2020–2022 in the allergy department of any of the three Spanish hospitals (Hospital Universitario Cruces, Fundación Jiménez Díaz, and Hospital Universitario Princesa), included in this study were collected (see Table 1 suppl).

Skin prick test (SPT) was carried out according to standard method (Malling, 1993) with raw peanut extract (unprocessed sample or control) and five peanut processed samples: boiling 60 min (B60), autoclaving at 256 kPa, 30 min (AU), DIC treated peanut during 2 min (DIC), and 1 h enzymatically digested raw and DIC peanut samples, SPT was carried out in duplicate and a negative (PBS) and positive (histamine dihydrochloride) controls were used. SPT mean wheal diameters were calculated, and values at minimum 3 mm above than negative control were assumed positive.

Peanut specific IgE and total serum IgE levels were measured by ImmunoCAP® (ThermoFisher Scientific, Upsala, Sweden). Specific IgE to peanut allergens were also determined and collected (Ara h 1, Ara h 2, Ara h 3, Ara h 6, Ara h 8 and Ara h 9). Following the manufacturer’s recommendations, a slgE value at least above 0.35 kUA/L was assumed as a positive result.

The clinical importance of the positive sensitization was evaluated through a detailed anamnesis. Diagnosis of peanut allergy was made after a predictable clinical history of peanut allergy together with positive specific IgE to peanut and/or skin prick test. Symptoms of the reactions were grouped into local or systemic symptoms. Afterward, all studied patients were divided into peanut allergic (n = 22) and peanut sensitized (n = 5) with a positive skin prick test and slgE >0.35 but who tolerate the ingestion of peanuts.

This study was approved by the Ethics Committees of HU Cruces, Fundación Jiménez Díaz, and HU Princesa, in accordance with regulations of the review boards of their institutions (Permissions No. CBV1839/2 M, PIC164-18, 3798, respectively).

2.2. Material and processing experiments

In this study, peanuts (Arachis hypogea, variety Virginia) obtained from Productos Manzanares (Spain) were used. Seven food-grade enzymes from Amano (Amano Enzyme Europe Ltd., Agno, Switzerland) were used to digest peanut samples (E1-E7): E1 (endoprotease; Thermoase PC10F), E2 (exoprotease, ProteAX), E3 (proprietary, Protin SD–NY10), E4 (exopeptidase, Peptidase R), E5 (alkalase-like, Protin SD–AY10), E6 (proprietary, Protease M “Amano” SD) and E7 (proprietary, Protease P “Amano” 3SD). The seven maintain their stability at pH 7 and 50–55 °C.

The workflow of the treatments and evaluations are summarized in Fig. 1.

Whole peanut seeds dipped in double distilled water (at 1:5 w/v) ratio were subjected to:

- Boiling (100 °C, 60 min).
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- Autoclaving 138 °C, 256 kPa, for 30 min in a food-grade equipment (Compact 40 Benchtop Autoclave, Priorclave, London, UK).
- Controlled Instantaneous Depressurization (DIC) treatment was applied to whole peanut seeds at La Rochelle University (LaSiE). The DIC procedure was performed according to a factorial design already reported (Haddad, Louka, Gadouleau, Jubel, & Allaf, 2001). In that procedure, moistened whole peanuts are subjected to vapor pressure (7 bar) for a short time (120 s) under elevated temperature (170 °C), in a processing chamber and afterwards, subjected to an instant pressure decline to a vacuum at around 50 mbar. The instant pressure fall, at a rate ΔP/Δt above 5 bar/s, produces an instantaneous cooling and simultaneously, an auto-vaporization of water in the product, which stops degradation.
- Untreated, boiled, autoclaved and DIC treated peanut seeds were milled and defatted (34 mL of n-hexane /g of flour, 4 h). Defatted flour from untreated peanuts was the control sample for processed samples. The nitrogen content of the samples was measured by LECO analysis following standard methods and the total protein amount was estimated as N x 5.3 (AOAC, 2003). The results, at least in duplicate, are collected in Table 2 suppl. Most of processed samples showed greater protein content than the raw peanuts, probably explained by a dry matter reduction of the processed samples.
- Peanut soluble protein extract was obtained according Cabanillas, Pedroso, et al. (2012). Defatted flour was extracted twice in a solution of 0.05 M Tris-HCl, 0.05 M NaCl, pH 8.0, at a 1:10 w/v ratio for 1 h, by stirring at 4 °C. After centrifugation (27,000 g, for 20 min, at 4 °C) the supernatants were dialyzed against H2O (cut-off point, 3.5 kDa) for 48 h at 4 °C and freeze dried.
- Digestion of peanut protein extract treated with Tris buffer (2 mg/mL) was performed by incubation with each enzyme at 1 mg/mL of PBS (pH 7.4) during 19 h at 55 °C and taking aliquots at the following time points: 0, 1, 2, 3 and 19 h. ES enzyme was selected for further experiments in peanut after SDS-PAGE analysis.
- Experiments of the enzymatic hydrolysis of whole peanut paste (in contrast to soluble extract) incubated in an ultrasound bath. The whole peanut paste was made after blending defatted peanut flour with double distilled H2O (0.5 g/mL) and maintained at −20 °C. 0.5 g of raw whole peanut paste with 10 mL of double distilled H2O and 110 μL of Amano enzyme (1 mg/mL) were subjected to sonication (Ultrasons, Selecta, Barcelona, Spain) in plastic tubes (15 mL) at 55 °C over 1 h. At the beginning of the experiment, a 100 μL aliquot was taken before adding the enzyme (no E, untreated control), and it was also taken at several time points after incubation with enzymes in a ultrasound bath, and then heated in sample buffer for 15 min at 65 °C. These control samples were evaluated by SDS-PAGE and immunoblossays. The experiments were performed in duplicate.

2.3. Electrophoresis of proteins and immunoblot assays

Samples (20 μg protein per lane) in Laemmli sample buffer (Bio-Rad, CA, USA) with β-mercaptoethanol (Bio-Rad, CA, USA) were boiled at 60 °C for 10 min, and loaded in 4–20% Tris-HCl linear gradient gels (Bio-Rad, CA, USA) according to Cuadrado et al. (2018). Separated proteins were stained with Coomassie Brilliant Blue R250. The western blot was carried out by transference to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA, USA). Mouse IgG anti-Ara h 9 (LTP) (kindly provided by Dra. A. Diaz-Perales) diluted in blocking solution (PBS with 2% non-fat milk, 1:500) was incubated with membranes of PVDF for 1 h. After washing, the membranes were incubated with goat anti-mouse antibody alkaline phosphatase (AP) conjugated (1:5000) (Sigma, Saint Louis, MO, USA). Enhanced chemiluminescence using ECL substrate was used for visualization of IgE-binding proteins, following the recommendations of manufacturer (Thermo Scientific, Waltham, MA, USA). The signal was determined using a ChemiDoc (Bio-Rad, CA, USA).

Fig. 1. Workflow of thermal/pressure processing, preparation of peanut pastes, enzymatic treatments and in vitro and in vivo evaluations done.

![Workflow Diagram]

- Hospitals
- Patients
- IgE sera
- SPTs
- Western Blot
- ELISA Inhibition
2.4. Ara h 1, Ara h 2, Ara h 3, Ara h 6 and Ara h 8 detection

Ara h 1, Ara h 2, Ara h 3, Ara h 6 and Ara h 8 were detected in Western blot and ELISA using specific antibodies. For Western blotting, samples (20 μg protein per lane) were loaded in 12% SDS-PAGE gels and transferred into PVDF membranes (Bio-Rad, Hercules, CA, USA) using a semi-dry transfer equipment (Trans-Blot® TurboTM Transfer System, Bio-Rad, Hercules, CA, USA) at 0.5 A at RT for 30 min. Afterwards, blocking was carried out in 5% non-fat dry milk in PBS with 0.5% Tween 20 (PBS-T) for 1 h at RT with stirring. Then, membranes were incubated individually with the following mouse monoclonal antibodies: anti-Ara h 1 (clone 2C12, stock: 2.7 mg/mL), anti-Ara h 2 (clone 1C4, stock: 2 mg/mL), anti-Ara h 3 (clone 1E8, stock: 1.1 mg/mL), anti-Ara h 6 (clone 3BB: stock: 2 mg/mL), and with the polyclonal antibody rabbit anti-Ara h 8 (PA-AH8) (Inbio, Cardiff, UK) (all used at 1:5000 dilution, incubated overnight at 4 °C with shaking). After washing in PBS-T, the detection of the monoclonal antibodies mouse anti-Ara h 1, Ara h 2, Ara h 3, and Ara h 6 was carried out using the secondary antibody Horseradish Peroxidase (HRP)-conjugated rabbit anti-mouse IgG (stock: 1.3 mg/mL, diluted 1:2500) (Dako, Glostrup, Denmark) at RT for 1 h with stirring. The detection of the polyclonal antibody rabbit anti-Ara h 8 was carried out using the secondary antibody HRP-conjugated goat anti-rabbit IgG (stock: 0.4 mg/mL, diluted 1:2500) (Enzo Life Sciences, Inc., Farmingdale, New York, USA) at RT for 1 h with stirring. Detection by enhanced chemiluminescence (ECL) was applied following the recommendations of manufacturer (SupersignalTM West Pico Plus Chemiluminescent Substrate, Thermo Fisher Scientific, Madrid, Spain). To measure the signal an ImageQuantTM LAS 4000 (GE Healthcare, Little Chalfont, UK) was applied.

ELISA were carried out by coating wells (100 μL/well) with samples (8 μg/mL) in 96-well high-bind plates (Costar, Cambridge, MA, USA) and incubating the microplates overnight at 4 °C. Plates were then washed with PBS-T and incubated at RT for 1 h with blocking solution (PBS-T containing 3% non-fat dry milk) (Bio-Rad, Hercules, CA, USA). Afterwards, plates were incubated separately with the same monoclonal and polyclonal antibodies detailed above (2 h at RT). All primary antibodies were used at 1:5000 dilution. The plates were then washed. The same secondary antibodies described above for western blot were used for the detection of these monoclonal or polyclonal antibodies. Afterwards, the plates were washed and then the peroxidase reaction was developed using the secondary antibody HRP-conjugated goat anti-rabbit IgG (stock: 20 μg/mL) in 96-well high-bind plates (Costar, Cambridge, MA, USA) (1 h at 37 °C). After washing, the plates were incubated with 100 μL/well of a mouse anti-human IgE - HRP conjugated (1:1000 dilution) (Sigma, Saint Louis, MO, USA) (1 h at 37 °C). Then the plates were washed and the reaction of peroxidase was developed with peroxidase substrate (100 μL) (SureBlue TM, KPL, Gaithersburg, MD, USA). 100 μL of 1% HCl were added to stop the reaction after 30 min, and the optical density (O.D.) at 450 nm was determined. The IgE binding inhibition percentage was determined according to the formula: [(1 – (AI / AN)] x 100, where AI means the O.D. value of raw peanut samples preincubated with inhibited sera (raw or thermally or enzymatically digested samples), and AN means the O.D. value of raw peanut samples incubated with un-inhibited sera. All tests were carried out at least in triplicate. The inhibition data were analyzed by analysis of variance (ANOVA) and Duncan’s test utilizing the Statgraphic Centurion XVI-I programme (Statpoint Tech. Inc., Warrenton, VA, USA).

2.6. Mass-spectrometry (MALDI-TOF/TOF) analysis of proteins bands

Digestion of peanut proteins from raw, boiling for 60 min, autoclaving (256 kPa for 30 min), and DIC samples, was performed by enzymatic treatment with enzyme 5. After electrophoretic separation, the protein bands resistant to digestion were cut from the gel and subjected to MALDI-TOF/TOF MS analysis for protein identification. Firstly, excised bands were incubated sequentially with 50 mM N,N,N′,N′-tetramethylethylene diamine (TMEDA) and acetonitrile. Then the samples were reduced with 10 mM/L of dithiothreitol for 1 h at 37 °C. Afterwards, for alkylation of sulfhydryl groups, 55 mM/L of iodoacetamide were added for 30 min at 25 °C in the dark. Finally for protein trypsin digestion, incubation with 12.5 ng/μL sequencing grade trypsin (Roche Molecular Biochemicals) in 25 mM ammonium bicarbonate (pH 8.5) was carried out overnight at 37 °C. After digestion, the peptide in the supernatant was collected and 1 μL was spotted onto a MALDI target plate. MS analyses were carried out in a 4800 Analyzer MALDI-TOF/TOF (Applied Biosystems, MDS Sciex, Toronto, Canada) at the Proteomics Unit of Complutense University of Madrid. The MALDI-TOF/TOF operated in positive reflector mode with an accelerating voltage of 20,000 V. All mass spectra were calibrated internally using peptides from the auto digestion of trypsin. For peptide mass fingerprint protein identification a homemade Data Base with the Arahis hypogaea allergens (64 sequences; 18,385 residues) downloaded from allergome platform (https://www.allergome.org) was searched using Mascot 2.8 (www.matrixscience.com) through the Protein Pilot v4.5 Software (ABSciex). Search parameters were: Peptide mass tolerance, 80 ppm; Carbamidomethyl Cystein as fixed modification and oxidized methionine as variable modification, up to 1 missed trypsin cleavage site.

In all protein identification, the probability scores were greater than the score fixed by mascot as significant with a p-value minor than 0.05. In parallel, peptides from digestion of protein extract of all peanut samples have been analyzed by liquid nano-chromatography coupled to a high-resolution mass spectrometer (Q-Exactive HF). This proteomic analysis was carried out in the Proteomics Unit of Complutense University of Madrid.

2.7. Liquid chromatography mass-spectroscopy (LC/MS/MS) experiments

The peptides from in solution digest of samples were analyzed by RP-LC-ESI-MS/MS in a Vanquish Neo System coupled to the Q-Exactive HF mass spectrometer through the Nano-Easy spray source (all from Thermofisher Scientific). Peptides were loaded first onto a Acclaim PepMap 100 Trapping column (Thermo Scientific, 20 mm × 75 μm ID, 3 μm C18 resin with 100 Å poro size) using buffer A (mobile phase A: 2% acetonitrile, 0.1% formic acid) and then were separated and eluted on a C18 reverse phase analytical column (Thermo Scientific Easy Spray Column, PepMap RSLC C18 de 500 mm × 75 μm ID, 2 μm, 100 Å poro size) with
an integrated spray tip. A gradient of 2% to 35% Buffer B (100% acetonitrile, 0.1% formic) was selected for analysis. The samples were analyzed in Q-exactive HF in a full-MS Data Dependent Acquisition (DDA) method in a mass range of 350–1800 Da and in positive mode. So, from each full MS scan, the MS2 scans were selected for the top 15 most abundant precursors with charges of 2–4 for higher energy collisional dissociation (HCD) fragmentation, with dynamic exclusion of 20 s and an isolation mass window of 2 m/z. The threshold to trigger MS2 scans was 1.3E4; the normalized collision energy (NCE) was 27%; the resolved fragments were scanned at mass resolution of 30,000, and AGC target value of 2E5 in a ITmax of 120 ms. The MS/MS data acquired were analyzed using Proteome Discoverer software v.2.4 (Thermo Scientific) with search engine MASCOT 2.8. The searches were performed assuming trypsin digestion with up to 2 missed cleavage sites, using a fragment ion mass tolerance of 0.02 Da and an ion precursor tolerance of 10 ppm. Carbamidomethylation was specified as fixed modifications, and Oxidation of methionine as variable modifications. The acceptance criteria for proteins identification were a FDR < 1% and at least one unique peptide identified with high confidence (CI > 99%, p < 0.01).

3. Results

3.1. Proteomic investigation by electrophoresis

With the aim to decrease the IgE binding potency of peanut, seven proteases from Amano Enzymes Ltd. were selected and tested for enzymatic hydrolysis of peanut proteins. These enzymes were chosen taking into account their differences in enzymatic properties and stability at high temperature. Each enzyme (E1-E7 at 1 mg/mL each) was incubated with untreated peanut extracts for several time points, and so to select the enzymes with the greatest hydrolysis capacity. The hydrolysis assays with whole peanut paste were performed in an ultrasound bath at 55°C for 1 h, to try to reduce the processing time and enhance digestion. The results of these experiments are shown in Fig. 1 supplementary. The electrophoretic results of peanut Tris-HCl extracts applied during the maximum digestion time (19 h) for each enzyme are shown in panel A. The most efficient enzyme for peanut proteins digestion resulted in Enzyme 5 (Amano Protease SD – AY10) and was used in additional experiments. Because of the similarity in digestion products for E5 at different times (1 h, 2 h, 3 h and 19 h, Fig. 1B suppl), 1 h treatment was selected for use in additional assays.

The SDS-PAGE profiles of the paste proteins obtained from raw (control), boiling for 60 min (B60), autoclaving at 256 kPa for 30 min (AU) and DIC treated peanut during 2 min (DIC), before and after 1 h of hydrolysis with the Enzyme 5 (Amano Protease Protin) under sonication treatment, are presented in lanes 1 to 8 of Fig. 2A. Band protein profiles were complex in raw and boiled, with similar bands (7 to 65 kDa). Peanut subject to autoclaving at 256 kPa for 30 min had few sharp stained bands and more smearing, probably because of aggregation and degradation of proteins induced by heat and pressure combination. The DIC treatment for 2 min showed only band proteins of around 37, 25, and 15 kDa. After incubation for 1 h with E5 Amano Protease in an ultrasonic bath, the findings indicated that many proteins of untreated (raw) and heat treated peanut samples were hydrolyzed, although some digestion resistant bands were detected (Fig. 2A).

3.2. Protein band identification

In order to determine the identity of peanut proteins from raw (control), boiling for 60 min, autoclaving and DIC treated samples before and after digestion with E5 enzyme, these samples were electrophoresed in a 4–20% Tris-glycine gel. Mass spectrometry analysis (MALDI-TOF/TOF) was performed on 26 protein bands manually excised and tryptic digested (Fig. 2 supplementary). The peptide identification data of these peanut samples are summarized in Table 3 supern. The seven bands of untreated peanut (bands #1 to 7) were identified by peptide mass fingerprint search as from Ara h 1, Ara h 2, Ara h 3, Ara h 6 and Ara h 7, being the Ara h 1 peptides the most abundant. When the peptides from raw peanut sample (control) have been analyzed by liquid nano-chromatography coupled to a high-resolution mass spectrometer Q-Exactive HF, 12 peptides from Ara h 8, 3 peptides from Ara h 9 and 2

![Fig. 2. Protein electrophoresis and detection of peanut ns LTP in untreated and treated peanut samples. A: SDS-PAGE of control (untreated) (lane 1), Boil (boiled 100 °C, 60 min) (lane 2), AU (138 °C, 256 kPa, 30 min) (lane 3), DIC (170 °C, 700 kPa, 120 s) (lane 4) and enzymatically treated (ES) peanut samples (lanes 5–8). B: IgG immunoblots were carried out using anti-Ara h 9 (LTP). Each lane was loaded with 20 µg of protein. 4–20% Tris-glycine gels were used and Precision Plus was used a MW marker (P+ lanes).](image-url)
from Ara h 11 were identified. Four bands out of 5 excised from DIC treated peanut sample were identified as Ara h 3 and one band as Ara h 1. Seven out of 9 bands excised from raw peanut digested with enzyme were identified as Ara h 1, Ara h 3 was identified in 4 bands and Ara h 2 in one band. The bands # 24, 25, and 26 from DIC enzymatically digested were identified as Ara h 3 and the band # 25 was also identified as Ara h 1. Ara h 1 was identified in band # 25, but was not detected in bands # 22 and 23, which suggest its degradation into smaller peptides. These data indicate that degradation of the proteins is taking place over heating pressured processing and enzymatic digestion, and that when DIC treated are digested only a minimum of resistant fragments could be identified indicating that only some peptides from Ara h 1 and Ara h 3 peanut allergens persist even thermal and enzymatic digestion.

3.3. Clinical experiments (SPT and immunoblotting results)

3.3.1. Patient SPT

Of the total studied population, five patients (18.5%) were asymptomatic upon peanut exposure and 22 (81.5%) patients were allergic (Table 1 supp). Specifically, seven (31.8%) reported oral allergy syndrome, 15 (68.2%) systemic symptoms (3 with anaphylaxis). It should be noted that since there was no significant differences between the allergic and sensitized groups neither in the SPT wheel size with peanut nor in the specific IgE to peanut values, they were included in a unique group of patients sensitized to peanut. Our study population's average age is 29.6 ± 2.9 years, with a higher prevalence of females (16 namely 59.2%).

Mean total IgE was 990.1 kU/L (±317.7, range from 6.8 to 7600 kU/L) and peanut specific IgE (sIgE) was 9.44 kU/L (±3.3, range from 0.33 to 100kU/L). In terms of the allergens profile, there was a predominance of patients with sensitization to Ara h 9, nStP (77.7%) followed by sensitization to seed storage protein, Ara h 1, Ara h 2, Ara h 3 or Ara h 6 (18.5%, 40.7%, 11.1% and 25.9% respectively) and lastly Ara h 8, PRI0 (7.4%). Details are provided in Table 1 supp. All 27 studied patients had positive SPT to raw peanut (control) with a mean wheal of 9.2 mm² (±0.89) (Fig. 3). Moreover, in these 27 patients, boiling for 60 min (B60), autoclaving at 256 for 30 min (AU) and DIC treated for 2 min (DIC) peanut processed samples were applied for SPT determination. Additionally, in 19 out of these 27 patients, 1 h enzymatically treated raw and DIC peanut samples (Ct Enz and DIC + Enz) were tested. The SPT with the five processed samples showed significantly lower wheal’s size than untreated peanut (P < 0.05) (Fig. 3).

A wheal size reduction of >50% was demonstrated after Ct + Enz to 4.0 mm² (±0.98) and after B60 to 3.5 mm² (±0.98). Even lower values were obtained after high-pressure procedures: AU to 2.5 mm² (±0.96), and DIC to 2.3 mm² (±0.92). The lowest wheal size mean was obtained after DIC + Enz to 1.4 mm² (±0.67).

3.3.2. Immunoblotting

Western blots were carried out using specific IgG anti-ns LTP (Ara h 9, 10 kDa). The results showed that some immunoreactive proteins were recognized when IgG anti-LTP was incubated with untreated peanut (control) and boiled samples (Fig. 2B). The presumed Ara h 9 protein (10 kDa) was not recognized after autoclaving at 256 kPa, 30 min nor after DIC processed. After treating peanut samples with protease E5, the detection of one band around 10 kDa was possible in control and boiled but not for peanut samples that were subjected to heat and pressure treatments before the enzymatic treatment (lanes 5 to 8 of Fig. 2B).

IgE binding pattern was also analyzed by Western blot using individual sera from 27 patients sensitized to peanut (Fig. 3 supp). Defatted flours were solubilized in sample load buffer and were directly applied for the SDS-PAGE and western blot analyses performed in this study taking into account former studies reporting that food processing can modify the solubility of proteins reducing the amount of soluble proteins extracted by traditional protein extraction methods. Samples from untreated (control), boiling 60 min, autoclaving at 256 kPa, 30 min and DIC treated for 2 min before and after 1 h of enzymatic digestion were included in all blots (lanes 1 to 8) for comparison purposes (Fig. 3 supp).

In Fig. 4 are collected immunoblots of the 27 individual sera patients grouped by the processing type applied to the peanut samples. The figure showed results of untreated (control peanut), and the five processed peanut samples also applied to the patients for SPT analysis (boiling for 60 min, autoclaving at 256 kPa for 30 min, DIC treated for 2 min and the 1 h enzymatically untreated control raw and DIC peanut samples). Recognition of allergenic proteins by IgE was similar in untreated (control) and boiled peanut samples before enzyme digestion when individual sera from the 27 patients were applied in the western blots (Fig. 4). IgE immunoblot pattern was similar to SDS-PAGE pattern (Fig. 2A) in autoclaved and DIC peanut samples with a remarkable mitigation of IgE-reactive bands.

After incubation for 1 h with E5 Amano Protease in ultrasound bath, the findings indicated that many proteins of untreated (raw) and heat treated peanut samples were hydrolyzed, although some digestion resistant, IgE immunoreactive proteins were detected with sera from patients n. #2, 3, 5, 9, 10, 15, 18, 21, 27, 31 and 32 (mainly at and below 25 kDa). The highest susceptibility to digestion corresponded to DIC treated peanut proteins since no IgE-immunoreactive bands could be visualized with most of 27 sera applied (Fig. 4).

3.3.3. Ara h 1, Ara h 2, Ara h 3, Ara h 6 and Ara h 8 detection in untreated and treated peanut

Protein profiles of untreated (control), and treated peanut samples before and after enzymatic hydrolysis with the Enzyme 5 under sonication are shown in Fig. 5A. The protein patterns of untreated and boiled peanut samples (lanes 1 and 2) were similar. Specifically, these samples presented multiple defined bands with a wide range of molecular weights. Peanut samples subjected to heat and pressure treatments (lanes 3 and 4: AU and DIC) showed a decrease in defined stained bands and a widespread smear along the lanes that could potentially be linked to protein fragmentation. Protein profiles of untreated and boiled peanut after enzymatic digestion with protease E5 (lanes 5 and 6) showed relevant protein fragmentation with a remarkable reduction in stained bands above 30 kDa approximately. However, the fragmentation by enzymatic treatment with E5 was stronger for samples subjected to heat and pressure treatments (lanes 7 and 8: AU and DIC).

Western blots were carried out using specific antibodies in order to analyze alterations in the recognition of major peanut allergens in untreated (raw) and treated peanut samples. The results showed that monoclonal anti-Ara h 1, anti-Ara h 2, anti-Ara h 3 and anti-Ara h 6 antibodies, and a polyclonal anti-Ara h 8 antibody recognized the
respective allergens Ara h 1 (7S globulin) (64 kDa, and a fragment of 30 kDa), Ara h 2 (2S albumin) (19 kDa), Ara h 3 (11S globulin) (37 kDa), Ara h 6 (2S albumin) (15 kDa), and Ara h 8 (PR-10) (17 kDa) in untreated peanut (control) (Fig. 5 B1 – 5 F1). The sole application of heat and pressure treatments (lanes 3 and 4: AU and DIC treatments) to peanut samples led to a decrease in the detection of Ara h 1, accompanied by an increase in the smearing observed in the high molecular weight region. Interestingly, in samples additionally treated with the enzyme E5, Ara h 1 detection decreased, especially in the case of peanut samples subjected to boiling, AU, and DIC treatment (Fig. 5 B1). In the
showed a reduction in the detection of these allergens in western blot, which was more marked for AU than for DIC treatment (Fig. 5 C1).

Injection to heat and pressure treatments alone (AU and DIC treatments), case of western blots performed with antibodies against Ara h 2, 3, 6, and 8, the results showed that these allergens were similarly detected in untreated and boiled peanut samples. However, peanut samples subjected to heat and pressure treatments alone (AU and DIC treatments), showed a reduction in the detection of these allergens in western blot, which was more marked for AU than for DIC treatment (Fig. 5 C1—F1).

After treating peanut samples with protease E5, the recognition of these allergens in western blot seemed to decrease especially for Ara h 3 and Ara h 6 for all the peanut samples tested (Fig. 5 D1 and SE1). In the case of Ara h 2 and Ara h 8, the enzymatic treatment also induced a decrease in the detection of these allergens by western blot, but less marked and only relevant for peanut samples pressured heating before the enzymatic treatment (Fig 5 C1 and F1).

Additionally, untreated and processed peanut samples were further analyzed by ELISA utilizing anti-Ara h 1, anti-Ara h 2, anti-Ara h 3 and anti-Ara h 6 monoclonal antibodies, and an anti-Ara h 8 polyclonal antibody. The results showed that, similar to the Western blot findings, the application of heat and pressure treatments alone, without hydrolysis with protease E5, led to a decline in the recognition of peanut allergens in the majority of cases, while the signal remained positive in a few cases, exceeding the positivity cut-off point. Similar to western blot, the combination of pressured heating treatments and enzymatic hydrolysis with E5 was the most efficient method to strongly decrease the recognition of Ara h 1, 2, 3, 6, and 8 up to levels below or near the cut-off point of positivity (Fig 5 B2—F2).

3.3.4. Reactivity to peanut seed storage proteins vs ns LTP

As described above, in our study population, there was a predominance of patients with sensitization to Ara h 9, (nsLTP) in terms of the type of peanut allergen sensitization profile (17 out of 27), followed by seed storage protein sensitization (Sprot), Ara h 1 (7S), Ara h 2 (S), Ara h 3 (11S) and/or Ara h 6 (2S) (10 out of 27) (Table 1 supplementary).

The SPT values of patients with Sprot profile were significantly different from LTP sensitized patients (Fig. 6 A). The wheel size means were significantly higher when peanut untreated (control) was applied to Sprot (11.75 ± 2.38) than to LTP sensitized patients (7.32 ± 0.70); as well as when SPT values of both patient groups were compared after application of boiled, autoclaved, DIC treated or enzymatically treated control and DIC peanut samples. Fig. 6 B, the IgE immunoblots of 27 individual sera of patients sensitized to peanut are shown, sorted according to the processing applied to peanut and to the type of peanut protein sensitization profile (Sprot or LTP). Although the immunoreactivity band pattern of both groups is different, the amount of IgE-immunoreactive bands is similar for untreated and boiled peanut samples. In both Sprot and LTP profiles, there is almost an elimination of the immunoreactive bands when samples autoclaved at 256 kPa for 30 min were analyzed whereas there were fewer reactive bands in the LTP than in the Sprot group after DIC treatment. The most relevant differences are in the response after 1 h of enzymatic treatment to untreated peanut, since in the LTP group there is an important reduction of immunoreactive bands in the 17 patients whereas in 5 out of 10 Sprot profile patients many reactive bands are still detected. DIC + Enz peanut samples show similar results for both patient groups with almost no reactive bands. In accordance with these results, the IgE immunoblots of pool sera (n = 10) of patient sensitized to Sprot (Fig. 6C) to LTP (n = 17) (Fig. 6D) showed different band patterns. There is an important reduction of immunoreactive bands when thermal pressured treatments (autoclave and DIC) and enzymatically treated peanuts were analyzed in LTP group. In the Sprot profile group the DIC peanut samples and control+enz showed higher reactivity than in the LTP profile group.

3.3.5. ELISA inhibition

Sera pool from 10 patients sensitized to Sprot peanut allergens was used to perform a competitive inhibition ELISA assay for evaluation of
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Fig. 6. A) SPT reactivity of patients sensitized to peanut nLTP (Ara h 9) or peanut storage proteins (Ara h 2 and Ara h 6 (2S), Ara h 1 (7S), and Ara h 3 (11S)). B) IgE immunoblots of 27 individual sera of patients sensitized to peanut ordered according to the processing applied to peanut and the type of peanut protein sensitization profile (Sprot or LTP). C) IgE immunoblots of pooled sera (n = 10) of patient sensitized to Sprot (patients no. #3, #4, #5, #8, #9, #15, #17, #21, #27, #31). D) IgE immunoblots of pooled sera (n = 17) of patients sensitized to LTP (#2, #6, #10, #11, #12, #13, #14, #16, #18, #19, #20, #25, #26, #28, #30, #32, #33).

Fig. 7. Competitive ELISA inhibition of the IgE binding to immobilized raw peanut by increasing concentration of untreated raw (control), boiled, autoclaved, DIC treated peanut, or 1 h enzymatically treated raw or DIC treated peanut samples, as inhibitors. Table summarizes the comparison by Dunncan’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).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>0.1 mg/ml</th>
<th>0.01 mg/ml</th>
<th>0.001 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanut raw</td>
<td>63.4a</td>
<td>66.9b</td>
<td>55.6a</td>
</tr>
<tr>
<td>Boil 60’</td>
<td>75.4a</td>
<td>49.9b</td>
<td>2.1d</td>
</tr>
<tr>
<td>AU138 30’</td>
<td>2.1c</td>
<td>3.2d</td>
<td>3.2d</td>
</tr>
<tr>
<td>DIC7b, 2’</td>
<td>25.0b</td>
<td>18.5c</td>
<td>26.6b</td>
</tr>
<tr>
<td>Peanut raw+E5</td>
<td>76.4a</td>
<td>71.7a</td>
<td>28.6b</td>
</tr>
<tr>
<td>DIC7b, 2’+E5</td>
<td>13.2b,c</td>
<td>15.2c</td>
<td>11.0c</td>
</tr>
</tbody>
</table>
the IgE binding capacity of peanut hydrolysates. Untreated peanut was used as a solid phase in this ELISA assay and it was preincubated with raw untreated (control), boiled, autoclaved, DIC treated peanut, or 1 h enzymatically treated raw or DIC treated peanut samples, as peanut inhibitors. The IgE binding inhibition whose percentage was determined as described in methods section is shown in Fig. 7. The means of untreated and processed peanut samples were compared by Duncan’s test for each inhibitor concentration and is summarized in the table below the graph (Fig. 7). Raw peanut before and after hydrolysis with enzyme E5 competed for IgE (63.4% and 76.4%, respectively). Nevertheless, the treated peanut samples compared to control raw peanut were significantly powerless competitors for IgE immunoreactivity. The minimum percentage of IgE binding inhibition to control untreated peanut forms corresponded to proteins from peanut subjected to autoclave (256 kPa, 30 min) and DIC treatment before and after 1 h of digestion with enzyme suggesting a mitigation in IgE binding potential for these treated peanut protein samples. These results are in concordance with the reduction in IgE binding detected for the same processed peanut samples (Fig. 4).

4. Discussion

The effects that individual treatments such as boiling, pressured heating in food-grade autoclaves (AU treatment) or DIC® technology, applied each of them alone or in combination with enzymatic hydrolysis, have on the allergenic content of peanut were analyzed in this study. Pressured heating treatments, chosen in this study, were selected based on previous studies that demonstrated that AU treatment at 138 °C, 256 kPa for 30 min seemed to reduce IgE binding in certain foods (Cabanillas et al., 2015; Cabanillas, Maleki, et al., 2012; Cuadrado et al., 2018; Cuadrado, Sanchiz, Vicente, Ballesteros, & Linacero, 2020; Lopez et al., 2012; Sanchiz et al., 2018). Regarding DIC treatment, the best conditions used previously to reduce IgE reactivity were steam pressure of 700 kPa for 2 min (Vicente et al., 2020).

Peanut processing can differentially affect its final allergenicity. According the outcomes already present in the literature about the impact of different technologies, boiling peanuts reduces its IgE-immunoreactivity (Beyer et al., 2001). Cabanillas, Maleki, et al. (2012) concluded that IgE recognition of peanuts decreased significantly with autoclave at harsh conditions (138 °C, 30 min). DIC treatment at 6 bars during 3 min produced a considerable reduction of Ara h 1 (Cuadrado et al., 2011). The combination of endo- and exopeptidases action on peanuts provokes a significant reduction of IgE immunoreactivity (Cabanillas, Pedrosa, et al., 2012). The findings of this study showed that heat and pressure treatments (AU and DIC treatments) alone induced protein fragmentation in peanut samples, evidenced by an intense smear in SDS-PAGE. Boiled peanut, however, did not show major differences compared with the untreated peanut sample. When the enzymatic treatment with the protease E5 was included after the above-mentioned thermal treatments, the protein fragmentation observed was more marked. The analyses of the allergen content of peanut by immunoaasays, applying monoclonal and polyclonal antibodies, showed a strong reduction of the content of peanut allergens Ara h 1, Ara h 2, Ara h 3, Ara h 6, and Ara h 8, in samples that were processed under the combination of heat, pressure (AU and DIC treatments) and hydrolysis with protease E5.

Our results are according to reported data demonstrating that a combination of processing methods, such as autoclave treatments and enzymatic hydrolysis, was required to obtain a substantial decrease in IgE reactivity on pistachio and cashew pastes. In this study, different proteases were used and the most efficient enzymes for reducing the IgE reactivity of pistachio and cashew were the Amano Proteases E5 and E7, respectively (Cuadrado et al., 2018). The present study went one step further and analyzed for the first time the combination of heat, pressure, and enzymatic hydrolysis on peanut allergenicity.

Processing by enzymatic hydrolysis has different applications in the food industry. For instance, there are partially or extensively hydrolyzed infant formulas based on milk or soy that are considered hypoallergenic. These formulas seem to diminish the risk of atopic diseases, in comparison to non-hydrolyzed formulas, due to decreasing allergenic epitopes (Alexander & Cabana, 2010; Cabana, 2017; Halken et al., 2000; Salvatore & Vandenplas, 2016). In this way, enzymatic hydrolysis seems to be an encouraging processing method for the modulation of allergenicity. In the case of peanut, previous studies using peanut protein extracts demonstrated that hydrolysis with the endopeptidase alcalase (but not using an exopeptidase flavourzyme individually) induced a relevant reduction in IgE immunoreactivity of peanut proteins (Cabanillas, Pedrosa, et al., 2012). In this present work, the enzyme E5 was efficient in inducing protein fragmentation and allergenic content reduction, especially when it was used combined with treatments of heat and pressure (AU and DIC treatments). Only hardly any resistant peptides identified as Ara h 1 and Ara h 3 were found after Enzyme 5 digestion of DIC samples.

In conclusion, our present findings indicate that the combination of pressured heating treatments (autoclave and DIC) and enzymatic digestion was the most efficient method to strongly decrease or even abolish the allergenic potency of peanut. Heat/pressure treatments (autoclave and DIC) and enzymatic digestion under sonication independently produced a remarkable mitigation of the IgE immunoreactivity potential of pastes obtained from processed peanut, being more effective with ns LTP (Ara h 9) than with Sprot (Ara h 1, 2, 3 and 6) peanut allergens. However, DIC treatment at 7b during 2 min in combination with enzymatic hydrolysis is required for significant mitigation of IgE immunoreactivity against Sprot peanut allergens. Therefore, the combination of DIC (heat/pressure) and enzymatic digestion strongly reduce the immunoreactivity of Ara h 1, 2, 3, 6, 8 and 9 allergens. Moreover, the present study is mainly focused on the results obtained from immunoblotting against allergenic people sera and Skin Prick Test, which being markers of clinical reactivity demonstrated the real allergenicity of the final treated peanut products. Consequently, these findings are essential as a precursor stage before oral challenge test assays in order to confirm the mitigation of allergenic capacity of these processed peanuts. The production of hypoallergenic foods is relevant for food industry, regulatory agencies, clinicians and consumers. The results of the present study identify the processing condition and procedure to mitigate or even abolish the allergenicity of peanut and therefore, are essential for producing hypoallergenic peanuts. With the combination of treatments including extensive enzyme hydrolysis and denaturation techniques, it seems likely that some allergic subjects could consume a processed peanut food with much reduced risk. The clinical involvement of these findings is still pending to be discovered. Other kind of studies is needed for those in vivo discoveries (protocolized, approved by the ethical committee, placebo-controlled clinical oral challenge). However, our study’s finding set a possibility for a group of patients in which their allergy could be treated with a processed less-allergenic peanut (or even may other nuts) and consequently less risky, more easy and quicker desensitization treatment. In the future, with those in vivo studies we could ascertain the patient’s profile for each treatment.

The applications of processed peanut with reduced IgE binding potency for tolerance induction might be a convenient strategy. Clinical oral challenge and evaluation of sensorial properties trials are required to evaluate the organoleptic properties of these processed peanut pastes and to confirm the mitigation of their allergenic reactivity.

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