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Effect of high pressure and pulsed electric field on denaturation and allergenicity of Pru p 3 protein from peach

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ABSTRACT

The effect of high pressure (HP) and pulsed electric field (PEF) treatments combined or not with heat on denaturation and allergenicity of Pru p 3, the major allergenic protein of peach, was studied. Denaturation of Pru p 3, determined by ELISA using rabbit IgG, occurred when the protein was treated at 500 and 600 MPa at 20 °C and at 400 MPa at 50 °C. PEF treatment at 25 kV/cm at 50 °C denatures Pru p 3. Allergenicity of Pru p 3 was estimated *in vitro* by a competitive fluorescent immunoassay using three pools of sera from peach allergic patients. Any treatment applied did not show to influence the binding of Pru p 3 to IgE. When HP and PEF treated samples were tested by the prick test, the skin response was dependent on the particular sensitization of each patient, obtaining an increased reaction in more than 50% of individuals.

1. Introduction

Fruits are considered to be among the primary elicitors of food allergy in humans. In a meta-analysis study based on challenge tests, it was estimated that about 0.1–4.3% of the population are hypersensitive to fruits (Zuidmeer et al., 2008). Studies about allergy prevalence conducted in France, Germany, Denmark, Italy and Spain have shown that fruits are the most common elicitors of food allergies in adults and children (Vanga, Jain, & Raghavan, 2018). The report published by the Spanish Society of Clinical Alergology and Immunology (SEAIC) has shown that fruits belonging to the *Rosaceae* family induced 59.4% of reactions produced by fruits and 25.7% of all reactions to foods (Ojeda, Sastre, Olaguibel, & Chivato, 2018).

Allergy to *Rosaceae* fruits presents geographical differences in the clinical presentation that are related to the different pattern of allergens involved (Fernández-Rivas et al., 2006). In Central and Northern Europe, peach allergy is often related with birch pollinosis, and sensitization is due to cross reactivity between Pru p 1 and Bet v 1. Pru p 1 is a protein very sensitive to processing and proteolysis by digestive proteases and induces generally mild symptoms that affect mouth and throat (oral allergy syndrome, OAS), where the labile protein is still intact (Wang, Vanga, & Raghavan, 2019). In the Mediterranean countries (Spain, Italy, Israel, etc) in patients not sensitized to birch pollen, peach allergy is mainly caused by the allergenic protein Pru p 3. Symptoms related to Pru p 3 are more severe with frequent involvement of urticaria and/or anaphylaxis.

Pru p 3 belongs to the family of Lipid Transfer Proteins (LTP). The proteins of this family are the most important allergens of the *Rosaceae* fruits including also apple (Mal d 3), plum (Pru d 3), cherry (Pru av 3) and apricot (Pru ar 3). Systemic manifestations caused by these proteins are based on the cross reactivity with Pru p 3, which is considered as the primary sensitizer (Hassan & Venkatesh, 2015).

Pru p 3 has a molecular weight of 9 kDa and a basic isoelectric point. The protein contains eight cysteine residues, which form four disulfide bridges that maintain protein conformation and makes it highly resistant to thermal treatment, pH changes and proteolysis (Sinha et al., 2014).

Food processing technologies could have a significant effect on immunoreactive epitopes of allergenic proteins. Processing could destroy epitopes present on the protein or generate new epitopes because of structural modifications. Many studies have been carried out in the last decade to determine the effect that technological treatments have on food allergenicity, with the aim of designing methods that reduce or eliminate allergenic activity, as a prerequisite for the production of hypoallergenic foods (Ekezie, Cheng, & Sun, 2018).

Therefore, the effect of thermal treatments of peach products or pure Pru p 3 has been shown to decrease (Brenna et al., 2000) or increase (Lavilla, Orcajo, Díaz-Perales, & Gamboa, 2016) the reactivity of Pru p 3 with the IgE from peach allergic patients.

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In the last few years, several studies have been focused on the study of the effect of non-thermal technologies on allergenic proteins. These alternative technologies are widely used in food industry as they inactivate pathogenic and spoilage bacteria producing minimal changes in organoleptic and nutritive properties of food. These treatments could induce a series of structural modifications (reversible or irreversible) in allergenic proteins that lead to denaturation, aggregation or gelation, which alter the conformational epitopes that are recognized by IgE, thus decreasing protein allergenicity (Ekezie et al., 2018; Huang, Hsu, Yang, & Wang, 2014).

Garino et al. (2012) reported that the sequential microwave heating and ultrasound processing does not seem to have a decreasing effect on the IgE reactivity of Pru p 3. Recently, the effect of high pressure (HP) treatment on the allergenicity of Pru p 3 has been studied. Results showed that treatment at pressure up to 600 MPa for 10 min at 25 °C slightly enhanced *in vitro* IgE-binding to Pru p 3 and peach extract. Furthermore, when using the prick test, these authors found that HP treated samples showed in many patients an increase of the wheal diameter suggesting an increase of allergenicity (Lavilla et al., 2016).

The most effective treatment reported up to now that produced a decrease of Pru p 3 allergenicity is the combination of chemical lye peeling of fruits and ultrafiltration of a limpid peach juice through membranes of 10 kDa cut-off (Brenna et al., 2000).

To our knowledge, no studies have been carried out to determine the effect of pulsed electric field treatments (PEF) on denaturation and allergenicity of Pru p 3. The effect of HP treatments at 22 °C on Pru has been examined but no studies have been reported on the effect of combined heat and HP treatments on this protein.

In this study, we determined the effect of PEF and HP treatments combined or not with heat on denaturation and allergenicity of Pru p 3. The degree of denaturation was estimated as the loss of reactivity of Pru p 3 with rabbit IgG using a sandwich ELISA that we have previously developed. The allergenicity was determined *in vitro* by a competitive immunofluorescent technique using pools of sera from allergic patients to peach, and *in vivo* by the prick test.

2. Materials and methods

2.1. Preparation of peach extract and purification of Pru p 3

Peach (*Prunus persica*) extract from the Spanish indigenous "amarillo tardío" variety, clone Calante, was prepared from fresh peel. A given amount of peach peels was homogeneized in 10 mM sodium phosphate buffer (pH 6) containing 2 mM EDTA, 10 mM sodium diethyldithiocarbamate, 2% solid polyvinylpolypyrrolidone and 3 mM sodium azide at a ratio 1:2 (w:v) using an ultraturrax. The mixture was stirred for 2 h at 4 °C and centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was dialyzed against 50 mM sodium acetate buffer, pH 6 for 48 h at 4 °C using dialysis tubing with a MW cut-off of 3.5 kDa.

Pru p 3 was isolated by cation exchange chromatography on a SP-Sepharose column (5 × 2 cm). A volume of 80 mL of dialyzed peach extract was applied onto the column and the column washed with the same buffer until absorbance at 280 nm was < 0.02. Retained proteins were eluted using the same buffer containing 1 M NaCl. The eluted fractions were mixed, concentrated by ultrafiltration and applied onto a Sephadex G-50 column (77 × 1 cm) and afterwards, the column was washed with acetate buffer.

Peach extract and chromatographic fractions were analysed by SDS-PAGE under reducing conditions according to Laemmli (1970) using 4–20% precast polyacrylamide gels on a Mini PROTEAN Tetra Cell (Bio-Rad, Milan, Italy). Coomassie blue was used to stain the gels.

2.2. Mass spectrometry analysis of protein spots

The electrophoretic band with a molecular weight of about 9 kDa was manually excised from gel. Protein for analysis was in-gel reduced, alkylated and digested with trypsin according to Sechi and Chait (1998). Briefly, the sample was reduced with 10 mM dithioerytritol in 25 mM ammonium bicarbonate for 30 min at 56 °C and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 15 min in the dark. Finally, sample was digested with 12.5 ng/μl sequencing grade trypsin (Roche Molecular Biochemicals, Barcelona, Spain) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37 °C. After digestion, the supernatant was collected and 1 μl was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.6 μl of a 3 mg/mL of α-cyano-4-hydroxy-cinnamic acid matrix (Sigma, Misuri, USA) in 50% acetonitrile were added to the dried peptide digest spots and allowed again to air-dry at room temperature.

MALDI-TOF MS analyses were performed in a 4800 Plus Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, MDS Sciex, Toronto, Canada) at the Genomics and Proteomics Center of Complutense University of Madrid.

For protein identification NCBInr without taxonomy restriction was searched using MASCOT 2.3 through the software Global Protein Server v 3.6 (AB Sciex, Madrid, España). Search parameters were: Carbamidomethyl Cystein as fixed modification and oxidized methionine as variable modification.

2.3. Obtaining and conjugation of anti-Pru p 3 antibodies

Antisera were obtained in rabbits by immunization with purified Pru p 3 as previously described by Wehbi et al. (2005). All procedures performed with animals were approved by the Ethic Committee for Animal Experiments from the University of Zaragoza (Project Licence PI 30/19). The care of animals was performed in agreement with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Specific antibodies to Pru p 3 were purified by immunoadsorption using a HiTrap NHS activated HP column of 1 mL (GE Healthcare, Farfield, Connecticut, USA) previously coupled with Pru p 3. A volume of 15 mL of antisera was applied onto the column and the column washed with 10 column volumes of 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.14 mM KCl and 0.14 M NaCl, pH 7.4 (PBS). Bound antibodies were eluted with 0.1 M HCl glycine buffer, pH 2.8, which contained 0.5 M NaCl and immediately neutralized with 0.5 M Tris buffer, pH 8.0, to reach a pH of 7.4. Conjugation of purified antibodies to Pru p 3 with horseradish peroxidase (HRP) were performed using the Lighting-link HRP conjugation Kit (Innova Biosciences, Cambridge, UK).

2.4. HP and PEF treatments

Samples of Pru p 3 (1 mg/mL in 50 mM sodium acetate buffer, pH 6.0) and peach extract were introduced into Eppendorf tubes without headspace and subjected to pressure treatment at 400 MPa, 500 MPa or 600 MPa for 5 and 10 min at 20 °C, 50 °C and 80 °C. Treatments were applied using a discontinuous isostatic equipment from Stansted Fluid Power FPG 11500 B (Stansted, Essex, United Kingdom). The pressure transmitting fluid, composed by a mixture of ethanol and castor oil (70/30, v/v), was previously equilibrated at the temperature to be used in the corresponding treatments. Temperature increase due to adiabatic heating was approximately 3 °C per 100 MPa. The rate of pressure increase was approximately 240 MPa/min and pressure release time was lower than 30 s. An automatic device was employed to set and record the pressure, time and temperature during each pressurization cycle.

Pru p 3 samples (1 mg/mL in 50 mM sodium acetate buffer, pH 6.0) were subjected to 50 square waveform pulses of 3 μs at 25 kV/cm for treatment at 20 °C and 50 °C. Treatments were applied in a PEF equipment and treatment chamber supplied by ScandiNova (Modulator PG, ScandiNova, Uppsala, Sweden), as previously described by Saldaña et al. (2010). Samples were treated in a tempered batch parallel-electrode treatment chamber. The distance between electrodes was 0.25 cm and the electrode area was 1.76 cm^2. Frequency of pulse delivery

was 1 Hz. The temperature of the treatment solution was measured with a thermocouple before and after treatment and differences found were less than 2 °C. The energy per pulse (W) and the total specific energy (kJ/kg) was calculated considering the electrical conductivity and the mass of each treatment medium (Luengo, Condón-Abanto, Álvarez, & Raso, 2014).

2.5. Sandwich ELISA

Microtiter plates (Nunc, Roskilde, Denmark) were coated with 125 μl of a solution of rabbit anti-Pru p 3 antibodies (1 μg/mL) in 50 mM sodium carbonate buffer, pH 9.6, and incubated overnight at 4 °C. Then, wells were blocked with 300 μl of ovalbumin at 3% (w/p) in PBS at room temperature (RT) for 2 h. Before using, wells were washed three times with PBS containing 0.05% (v/v) Tween-20 (PBST) and incubated with 100 μl per well of standards or samples diluted in PBS containing 5% sucrose and 0.1% bovine serum albumin (BSA) for 1 h at RT. After washing five times with PBST, wells were incubated during 1 h at RT with 100 μl of anti-Pru p 3 antibodies labelled with peroxidase diluted 1/70000 in the same buffer. After washing five times, 100 μl/well of a commercial substrate containing tetramethylbenzidine (TMB) were added. After incubation for 30 min at RT, the enzymatic reaction was stopped by adding 50 μl of 2 M H_2SO_4 and the absorbance was measured at 450 nm using a microplate reader ELISA Multiskan MS (Labsystem, Helsinki, Finland).

2.6. Patients and skin prick test

A number of 22 adult patients allergic to fruit LTP were voluntary recruited at the Allergy Department of the University Hospital Lozano Blesa of Zaragoza (Spain). Selected patients had a clinical diagnosis of LTP allergy: symptoms after ingestion of peach, a positive prick test with peach LTP (ALK-Abelló S.A., Madrid, Spain) and specific IgE to Pru p 3 higher than 0.35 kU/L (ImmunoCAP FEIA system, ThermoFisher Scientific/Phadia, Uppsala, Sweden). Among selected patients, 5 patients presented an oral allergy syndrome (OAS), 10 presented at least one of these four symptoms (urticaria, angioedema, asthma, abdominal pain) in an acute outbreak (ALOS) and 7 presented an anaphylactic shock (ANS). Prior to the prick test, all subjects were given a questionnaire and signed an informed consent for using the results of this study. The prick tests were performed at the Hospital Lozano Blesa after the study protocol was approved by the clinical research ethic committee of Aragón (CEICA) (Projects PI15/0323 and PI17/0351). All samples were tested in a unique session for each patient.

The skin prick tests were performed on the volar surface of the forearm by using a lancet of 1 mm-tip, according to the EAACI recommendations (Dreborg, 1989). The prick test was performed with samples of pure Pru p 3 untreated and treated by high pressure (600 MPa for 10 min at 20, 50 and 80 °C) and pulsed electric field (25 kV/cm at 20 °C and 50 °C). A positive control of histamine hydrochloride (10 mg/mL) and a negative control of saline solution were also tested in all patients. The major and minor diameters of the wheal were measured in millimeters and the product of both diameters was calculated for each patient and treatment. The test was regarded as positive when one of the diameters of the wheal was higher than 3 mm or greater than that produced by the negative control. For each patient, the percentage of the increase or decrease in the product of the diameters of treated samples respect to the untreated sample was calculated. After the prick test, a blood sample was taken from each patient and after clotting and centrifuging, serum was stored at − 20 °C until analysis.

2.7. Competitive and non-competitive inhibition enzyme linked fluorescent immunoassay (ELFIA)

Pru p 3 ImmunoCAP (Reference f420, Thermo Fisher Scientific) was used for sIgE measurements in a Phadia 100 system with individual patient sera or with three pool of sera, in a non-competitive format, according to the manufacturer protocol for sIgE determination. The three pool of sera corresponded to patients that showed one of these three types of symptomatology: OAS, ALOS and ANS.

In order to compare the binding of sIgE to untreated Pru p 3 and HP and PEF treated samples, a competitive assay was designed using Pru p 3 ImmunoCAP. In this assay, untreated or treated Pru p 3 samples diluted 1/40 in PBS were mixed with the pool of sera (1/1, v/v) and sIgE was determined. As negative control, a mixture of PBS with the patient pool sera (1/1, v/v) was also assayed. The changes in IgE-binding to Pru p 3 induced by treatments (sIgE sample) with respect to untreated sample (sIgE 0%) and negative control (buffer, sIgE 100%) was estimated for each sample as follows:

$$\text{IgE binding}\,(\%) = \frac{\text{sIgE sample} - \text{sIgE0\%}}{\text{sIgE100\%} - \text{sIgE0\%}} x100$$

Therefore, a value lower than 100% suggest a lower IgE recognition (decreased allergenicity) and a value higher than 100% suggests a higher IgE recognition (increased allergenicity).

2.8. Statistical analysis

For prick test data, statistical analyses were performed with SPSS version 15.0 for Windows (Statistical Package for the Social Sciences (SPSS) Inc., Chicago, IL, USA). Descriptive statistics, frequencies, percentages, graphs and charts were used to summarize the data. After determining that data was not normally distributed by the Kolmogorov-Smirnov and Shapiro-Wilk W tests, non-parametric tests were used for the analysis. We used the Wilcoxon signed-rank test to determine differences between control and each treatment. A significance level of $p < 0.05$ was predefined in all cases.

For Pru p 3 concentration obtained by ELISA, data were analyzed for statistical significance with GraphPad Prism 5 software, by using a Kolmogorov-Smirnov normality test and a one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison Test.

3. Results and discussion

3.1. Pru p 3 purification

Purification of Pru p 3 was performed by cationic exchange chromatography on a SP-Sepharose column and gel filtration on a Sephadex G-50 column. Fig. 1a shows the electrophoretic profile of pooled protein fractions eluted with 1 M NaCl from the SP-Sepharose column. This pooled fraction contains a major band of about 9 kDa, which is the expected molecular weight of Pru p 3 (Sinha et al., 2014) and some minor bands of higher molecular weight. The band of 9 kDa were cut from the gel and analyzed by MALDI-TOF mass spectrometry. Results obtained gave 8 matching proteolytic peptides with sequence coverages of 92% indicating that fragments correspond to Pru p 3. Pru p 3 of the pooled fraction obtained from SP-Sepharose had a purity degree of about 80% as determined by densitometry (Fig. 1a, Lane 1). This Pru p 3 enriched fraction was used to determine the effect of technological treatments on Pru p 3. The gel filtration of this enriched fraction rendered Pru p 3 with a degree of purity higher than 96% (Fig. 1a, Lane 2). This purity degree was considered good enough to immunize rabbits to obtain antibodies for ELISA.

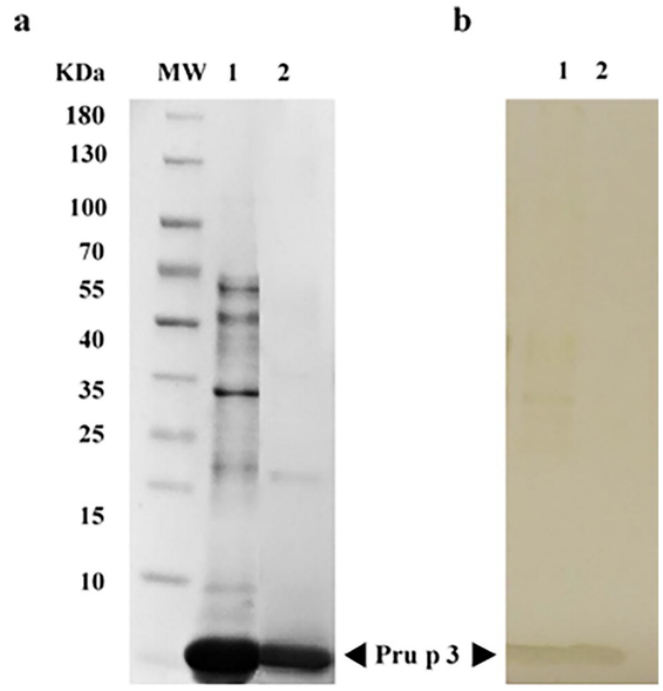

Fig. 1. SDS-PAGE in 4–20% polyacrylamide gel under reducing conditions (a) and Western-blotting using rabbit antiserum against Pru p 3 (b) of Pru p 3 enriched fraction and purified Pru p 3. MW, molecular weight marker. Lane 1, Pru p 3 enriched fraction corresponds to the pooled fractions eluted from SP-Sepharose with 1 M NaCl. Lane 2, purified Pru p 3 was obtained after gel filtration on Sephadex G-50 of Pru p 3 enriched fraction.

3.2. Development of the sandwich ELISA assay

The specificity of antiserum to Pru p 3 was determined by Western-blotting. Results showed that antibodies mainly recognized the 9 kDa protein of enriched Pru p 3 fraction and pure Pru p 3 (Fig. 1B).

Calibration curves were obtained by plotting the absorbance values versus the concentration of Pru p 3, which were adjusted to a polynomial curve that was linear within the range between 2.5 and 100 ng/mL ($r^2 \geq 0.980$) (Supplementary Fig. 1). The Limit of Detection (LOD) and Limit of Quantification (LOQ) of the ELISA assay were calculated as the protein concentration corresponding to the mean absorbance of ten replicates of the blank plus 3 and 10 times its standard deviation, obtaining values of 3.7 ng/mL and 12.4 ng/mL, respectively. The LOD of the developed sandwich ELISA is higher than that reported by Duffort et al. (2002) for an indirect sandwich format, which was of 0.1 ng/mL. However, the sensitivity of our ELISA is higher than that indicated by Carnés, Fernández-Caldas, Gallego, Ferrer, and Cuesta-Herranz (2002) for a competitive ELISA, as they obtained a calibration curve linear from 125 to 1000 ng/mL.

3.3. Effect of HP on denaturation and allergenicity of Pru p 3

Several pressures and holding times at different temperatures were applied to peach extract or to pure Pru p 3 and the degree of denaturation of Pru p 3 was determined by measuring the loss of reactivity with its specific rabbit antibodies using the sandwich ELISA. This technique allows determining the denaturation of a protein in a complex food extract, avoiding the use of pure protein in which the influence of other components is not considered (Tremblay, Laporte, Leonil, Dupont, & Paquin, 2003).

It should be considered that Pru p 3 antibodies employed in this study were purified by affinity chromatography using a gel matrix coupled with the native protein. Therefore, it is expected that the isolated antibodies recognize preferentially conformational epitopes that are displayed on the surface areas of the native form of the protein (Wehbi et al., 2005). For this reason, antibody binding reactions could be perturbed by changes in the conformational structure of a protein, such as those produced by technological treatments. Therefore, immunological techniques offer a way to determine the effect of HP and PEF on protein denaturation, because the loss of immunoreactivity is related to the loss of structure.

Immunochemical techniques have been previously used to determine the effect of processing treatments on allergenic proteins from different foods. Thus, the degree of pressure-induced denaturation has been estimated for Mal d 1 and Mal d 3 from apple (Johnson et al., 2010) and the kinetic parameters of pressure-treated β-lactoglobulin from bovine milk has been obtained (Mazri, Sánchez, Ramos, Calvo, & Pérez, 2012).

As it can be seen in Fig. 2, in the case of high pressure treatments of peach extract performed at 20 °C, treatment at 400 MPa did not induce denaturation of Pru p 3 whereas a significant decrease in the amount of immunoreactive protein was observed at higher pressures. The higher decrease of immunoreactive protein at 20 °C was obtained after treatment at 500 MPa for 10 min, which was about 40%, and a small increase was detected at 600 MPa.

The effect of pressure treatment combined with thermal treatment of peach extract was also evaluated (Fig. 2). Pru p 3 was very susceptible to denaturation when it was pressure treated at 50 °C, obtaining a degree of denaturation at 400 MPa higher than 50% respect to untreated protein. Furthermore, a similar denaturation was observed when treatments were performed at higher pressures (500 and 600 MPa) at that temperature. At all pressures and temperatures assayed, it was observed that the decrease in immunoreactive Pru p 3 concentration took place mainly in the first 5 min of treatment and at 10 min further reduction was very low.

These results are in agreement with those reported by Johnson et al. (2010) for Mal d 3, the homologous protein belonging to the LTP family in apple. These authors determined reactivity of Mal d 3 by ELISA using rabbit specific antibodies and found that the effect of pressure at 20 °C on Mal d 3 was minimal, whereas the protein showed a marked loss of of immunoreactivity (about 60–70%) when treated at pressures between 400 and 800 MPa at 80 °C. A good correlation was observed in that study between loss of immunoreactivity and changes in the secondary structure of Mal d 3 determined by circular dichro-

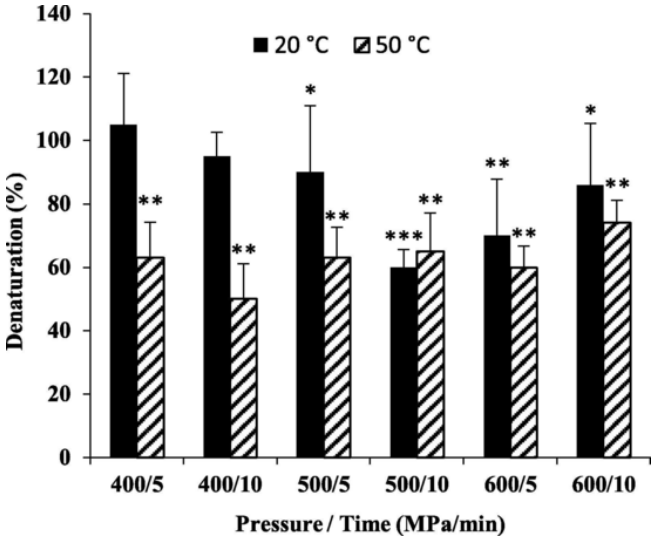

Fig. 2. Effect of HP treatment of peach peel extract on the denaturation of Pru p 3 protein determined by sandwich ELISA using rabbit specific IgG (a). Results are the mean ± standard deviation of data from two experiments analysed by triplicate and are expressed as percentage with respect to untreated sample (100%). Asterisks indicate significant differences with respect to untreated sample. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

ism. These authors did not found changes in secondary structure of Mal d 3 after pressure treatment at 20 °C, whereas a significant unfolding of α-helix to random coil were observed at 80 °C, especially above 400 MPa.

The effect of pressure treatment of apple peel on Mal d 3 was studied by Husband et al. (2011) and denaturation was determined by Western blotting using a pool of apple allergic patients. These authors showed that pressure processing at 700 MPa for 10 min at 20 °C caused a decrease of about 70%, determined by densitometry, in the amount of immunoreactive Mal d 3 and this protein was still visualized after the same treatment performed at 115 °C.

When pure Pru p 3 was treated at 600 MPa for 10 min, the denaturation of the protein was about 25% at 20 °C and about 50% at 50 °C and 80 °C (Fig. 3a). The reactivity of Pru p 3, even at the more extreme conditions, indicates that the protein maintains a significant residual immunoreactivity with the antibodies. This fact could be due to that pressure-induced denaturation does not affect some of the discontinuous conformational epitopes of the protein, and/or that some antibodies recognize continuous linear epitopes that are not affected by pressure.

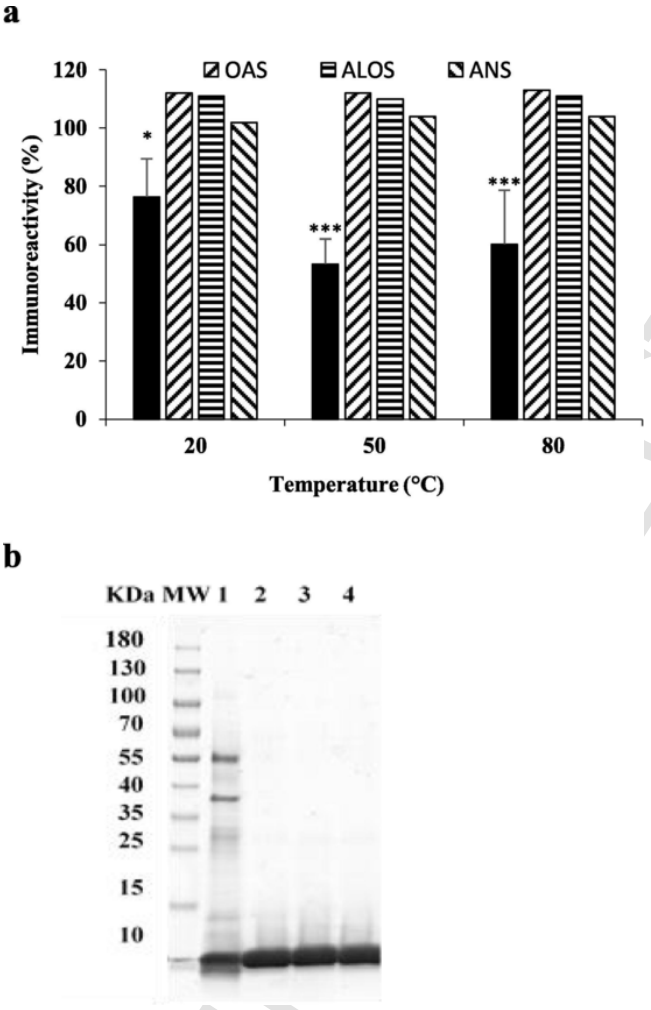

Fig. 3. (a) Effect of HP treatment of purified Pru p 3 at 600 MPa for 10 min at different temperatures on the denaturation of the protein determined by sandwich ELISA using rabbit specific IgG-antibodies (black bars) and on the allergenicity of Pru p 3 determined by ELFIA using human sera from peach allergic patients (hatched bars). The three pools of sera assayed corresponded to patients that showed one of these three types of symptomatology: OAS, Oral allergy syndrome; ALOS, at least one of these four symptoms (urticaria, angioedema, asthma, abdominal pain) in an acute outbreak; ANS, anaphylactic shock. Results are expressed as percentage with respect to untreated sample (100%). (b) SDS-PAGE (4–20%) under reducing conditions of Pru p 3 samples untreated and treated at 600 MPa for 10 min at different temperatures. MW, molecular weight marker. Lane 1, untreated sample. Lane 2, treatment at 20 °C. Lane 3, treatment at 50 °C. Lane 4, treatment at 80 °C.

When comparing denaturation of Pru p 3 treated at 600 MPa for 10 min in the extract (Fig. 2) and in the purified protein (Fig. 3a), it was observed that the protein shows a higher barosensitivity when treated in the purified form, mainly at 50 °C. These results suggest that there is some components present in the food matrix that exerts a protective effect on denaturation of Pru p 3 during processing. These results are in accordance with those previously reported for Mal d 3 (Husband et al., 2011) and Pru p 3 (Lavilla et al., 2016). The plausible reason for the higher stability of these proteins treated in a complex medium has been attributed to the presence of other components, as it has been demonstrated for pectin, which prevents the pressure-induced modification of Mal d 3 (Husband et al., 2011).

When samples of untreated or pressure treated Pru p 3 were analyzed by SDS-PAGE, no apparent changes were observed in the intensity of the Pru p 3 band respect to the untreated sample (Fig. 3b), as it was previously indicated by Lavilla et al. (2016). In contrast, we observed that high molecular weight proteins present in untreated samples disappear after treatment indicating a severe denaturation and/or aggregation of those proteins.

The effect of HP treatment on allergenicity of Pru p 3 was also studied using *in vitro* and *in vivo* techniques. For the *in vitro* technique, the ImmunoCAP system was modified to perform a competitive assay. All sera were previously analyzed using a non-competitive format. When performing allergenicity studies, it is usual to work with a pool of patient sera. However, we considered more relevant to prepare three pools of sera by selecting patients, which presented different severity of allergic symptomatology (oral allergy syndrome, some systemic symptom or anaphylactic shock). Therefore, sera were selected to prepare pools with those presenting a IgE level against Pru p 3 higher than 1.30 kU/L. The level of specific IgE in these three pools were 5.43, 6.84 and 9.86 kU/L, respectively.

As it can be observed in Fig. 3a, HP treatment of Pru p 3 at 600 MPa for 10 min seems no to affect the IgE binding of the pooled sera from patients to the protein or even induce a small increase of IgE reactivity, although statistical significance was not determined. These results suggest that HP treatment maintains or even may slightly increase the allergenic potential of Pru p 3. These results agree with those obtained by Lavilla et al. (2016) as these authors also found that most pressure/time combinations applied at 20 °C to purified Pru p 3 or peach extract enhanced *in vitro* IgE-binding to the protein.

On the other hand, in this study skin prick tests were performed with peach allergic patients in order to know the effect of technological treatments on *in vivo* allergenicity of Pru p 3. The number of samples to be tested by the prick test was limited in order to avoid patient discomfort. These samples included purified Pru p 3 subjected to the most severe pressure treatment (600 MPa for 10 min) combined or not with heat (20, 50 and 80 °C).

Table 1 shows the specific IgE value to Pru p 3 determined in patient sera, the symptoms reported by patients in the questionnaire and the product of the wheal diameters obtained for untreated and treated HP samples for each patient. The expression of the results as the product of the major and minor diameters is given as several patients gave an irregular area or developed a pseudopod during the skin prick test (Vohlonen et al., 1989). Differences in allergenicity with respect to the corresponding untreated sample (100%) were also included.

As it can be observed in Table 1, there is not a relationship between values of specific IgE and the product of the diameters of the wheal (r^2 = 0.007). Likewise, the severity of the symptoms reported by patients seems to be related neither with the value of specific IgE nor with the diameter product of the wheal. These results suggest that the allergenic potential of Pru p 3 depends on the sensitization of each patient and it can be assessed only on an individual basis.

When comparing the mean ranks of the wheal diameter product obtained for samples of untreated and pressure treated Pru p 3 sam-

Table 1
Effect of HP treatment at 600 MPa for 10 min at 20 °C (HP20), 50 °C (HP50) and 80 °C (HP80) on the allergenicity (product of diameters) of purified Pru p 3 determined by the skin prick test in peach allergic patients. Changes in allergenicity are also expressed as the percentage of the wheal diameter product respect to untreated sample (100%).

Patient	kU/L	Symptoms	Wheal diameter product				Allergenicity (%)		
			Control	HP20	HP50	HP80	HP20	HP50	HP80
LTP006	9,98	OAS	128	96	120	140	75	93	109
LTP009	2,59	OAS	72	96	70	80	133	97	111
LTP014	6,83	OAS	558	315	800	900	56	143	161
LTP019	4,22	OAS	64	180	264	176	281	412	275
LTP021	3,51	OAS	440	400	350	400	90	79	90
LTP001	1,09	ALOS	280	220	144	70	78	51	25
LTP002	0,03	ALOS	48	56	84	56	116	175	116
LTP003	15,1	ALOS	35	112	60	105	320	171	300
LTP004	2,56	ALOS	108	96	70	117	88	64	108
LTP005	0,35	ALOS	375	140	252	375	37	37	100
LTP016	0,12	ALOS	54	84	35	60	155	64	111
LTP018	0,6	ALOS	42	128	64	90	304	152	214
LTP022	6,21	ALOS	100	200	240	250	200	240	250
LTP007	9,01	ALOS	20	24	30	24	120	150	120
LTP012	1,34	ALOS	90	168	112	48	186	124	53
LTP008	1,57	ANS	180	160	81	200	88	45	111
LTP010	0,92	ANS	112	84	60	48	75	53,6	42
LTP011	2,03	ANS	112	90	144	240	80	128,6	214
LTP015	0,75	ANS	200	264	392	448	132	196	224
LTP013	4,99	ANS	108	132	210	90	122	194,4	83
LTP017	13,8	ANS	120	260	144	120	232	240	114
LTP020	18,6	ANS	70	320	360	300	457	514,3	428

Symptoms correspond to oral allergy syndrome (OAS), at least one of these four symptoms (urticaria, angioedema, asthma, abdominal pain) in an acute outbreak (ALOS) or anaphylactic shock (ANS).

ples, no significant differences were found with any of the temperatures of treatment assayed ($Z = -1.055$, $p = 0.291$; $Z = -1.185$, $p = 0.236$; $Z = -1.867$, $p = 0.062$ for pressure treatments at 20, 50 and 80 °C, respectively).

Furthermore, when HP treated Pru p 3 samples were compared with untreated protein, an increase of the reaction (product of the diameters) was found in 50% of patients for samples pressure-treated at 20 °C and 50 °C, and in 64% of patients for sample treated at 80 °C. A reduction of the reaction also occurred in 45%, 50% and 27% for the same groups of patients (Supplementary Table 1).

Our results are in good agreement to those previously reported by Lavilla et al. (2016). These authors reported that the prick test performed with purified Pru p 3 treated at 600 MPa at 20 °C showed an increase of the wheal area in 35% of peach allergic individuals and a decrease in 41% of them. Likewise, they did not found significant differences in the wheal area of pressurized samples compared to the untreated control sample in 24% of patients.

3.4. Effect of PEF treatments on denaturation and allergenicity of Pru 3

Results obtained on the effect of PEF treatments on the purified Pru p 3 have shown that treatment at 25 kV/cm at 20 °C did not appear to affect the reactivity of the protein with specific rabbit polyclonal IgG as determined by sandwich ELISA. These results are in good agreement to those reported in the analysis of Mal d 3 by circular dichroism, as no changes in the secondary structure was indicated for the protein treated at 35 kV/cm at 20 °C. However, when PEF treatment was combined with heating at 50 °C, we observed a decrease of immunoreactive protein more than 50% (Fig. 4a).

Regarding the effect of PEF treatment on *in vitro* allergenecity of Pru p 3, we found that treatment performed at both temperatures (20 °C or 50 °C) did not induce changes in the IgE binding to the protein when using the three pool of sera from allergic patients (Fig. 4a). These results are in good agreement to those previously reported for other allergenic proteins, which showed no effect of PEF treatment on the properties of them. Thus, it has been shown that PEF treatment does not change the secondary structure of Mal d 3 from apple and Ara h 2,6 from peanut at field strengths applied of 30 and 35 kV at 20 °C (Johnson et al., 2010). Likewise, no changes in immunoreactivity of β-lactoglobulin were found when milk or whey were subjected to PEF treatments of different intensity at 20 °C (De Luis et al., 2009).

The analysis by SDS-PAGE of samples subjected to PEF treatments showed that the band of Pru p 3 as well as bands of about 25 and 38 kDa remained unaltered whereas the intensity of the bands of about 45–60 kDa decreased markedly (Fig. 4b).

Results obtained in the prick test are shown in Table 2. When comparing the mean of the wheal product of the diameters estimated for untreated and PEF treated samples, no significant differences were found at both temperatures assayed ($Z = -0.348$, $p = 0.728$; $Z = -1.060$, $p = 0.289$; for treatments at 20 and 50 °C, respectively).

It was also observed an increase of the reaction in 41% and 55% of patients and a decrease of the reaction in 50% and 40% when PEF-treated Pru p 3 was compared with untreated protein at 20 °C and 50 °C, respectively (Supplementary Table 2). As in the case of pressure treatment, we did not observed a relationship between values of specific IgE, given in Table 1 and the diameter's product of the wheal obtained for PEF treatments ($r^2 = 0.057$).

4. Conclusions

Results derived from this study indicate that HP and PEF treatments combined with heat induces a considerable denaturation of Pru p 3, as determined by ELISA using rabbit specific IgG obtained against the native form of the protein. However, both technologies combined or not with heat did not change the reactivity of Pru p 3 with the IgE of peach allergic patients. These results indicate that IgG and IgE probably recognize different epitopes of the protein. Therefore, when using immunoassays to detect allergenic proteins in food, it should be considered that the reduced IgG immunoreactivity could not reflect a reduction in hazard to the allergic patient.

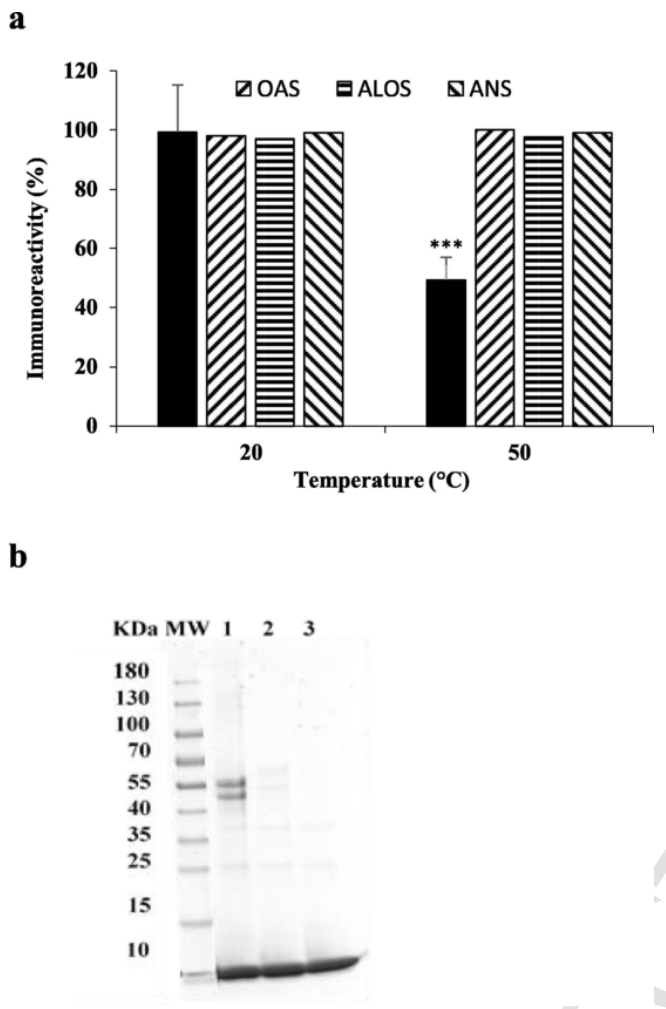

Fig. 4. (a) Effect of PEF treatment of Pru p 3 at 25 kV/cm at 20 °C and 50 °C on the denaturation of the protein determined by sandwich ELISA using rabbit specific IgG-antibodies (black bars) and on the allergenicity of Pru p 3 determined by ELFIA using human sera from peach allergic patients (hatched bars). The three pools of sera corresponded to patients that showed one of these three types of symptomatology: OAS, Oral allergy syndrome; ALOS, at least one of these four symptoms (urticaria, angioedema, asthma, abdominal pain) in an acute outbreak; ANS, anaphylactic shock. Results are expressed as percentage with respect to untreated sample (100%). (b) SDS-PAGE (4–20%) under reducing conditions of Pru p 3 samples untreated and treated at 25 kV/cm at 20 °C and 50 °C.MW, molecular weight marker. Lane 1, untreated sample. Lane 2, treatment at 20 °C. Lane 3, treatment at 50 °C.

The skin prick test revealed that both processing could alter the allergenicity of peach proteins, by either reducing or increasing its allergenic potential, depending on the individual. This different behavior is probably related with the release and/or masking of the epitopes that are specifically recognized by each individual, as the result of the unfolding caused by each processing technology.

The demand of natural and healthy products has triggered the need for the development of a number of non thermal approaches to food processing of which HP and PEF technologies has proven to be very valuable. In fact, several commercial products like fruit juices treated by these technologies are currently available on the market. Therefore, the possibility of using allergenic proteins, as Pru p 3, subjected to different technological treatments for the diagnosis of food allergies either *in vivo* or *in vitro* should be considered, as the modification of the protein induced by those treatments could alter its allergenic potential for some patients.

The risk for peach allergic patients is not always reduced by the use of the assayed technologies, provided the conditions used in our study. Therefore, it is necessary to perform more research exploring other technologies, such as enzymatic treatment combined or not with heat pressure, to know their effectiveness in reducing the allergenic potential of Pru p 3.

Table 2
Effect of PEF treatment at 25 kV/cm at 20 °C and 50 °C on the allergenicity (product of diameters) of purified Pru p 3 determined by the skin prick test in peach allergic patients. Changes in allergenicity are also expressed as the percentage of the wheal diameter product of untreated sample (100%).

Patient	Wheal diameter product			Allergenicity (%)	
	Control	PEF20	PEF50	PEF20	PEF50
LTP006	180	160	154	88	85
LTP009	56	84	128	150	228
LTP014	630	960	750	152	119
LTP019	160	80	144	50	90
LTP021	252	176	168	69	66
LTP001	63	63	88	100	139
LTP002	63	40	128	63	203
LTP003	72	36	41	50	58
LTP004	98	112	144	114	146
LTP005	200	126	200	63	100
LTP016	54	30	70	55	129
LTP018	200	63	72	31	36
LTP022	176	162	120	92	68
LTP007	25	20	15	80	60
LTP012	110	0	80	0	72
LTP008	63	160	160	254	254
LTP010	35	70	50	200	142
LTP011	48	96	176	200	366
LTP015	208	390	351	187	168
LTP013	54	224	120	414	222
LTP017	224	192	105	266	145
LTP020	175	120	300	68	171

Symptoms correspond to oral allergy syndrome (OAS), at least one of these four symptoms (urticaria, angioedema, asthma, abdominal pain) in an acute outbreak (ALOS) or anaphylactic shock (ANS).

CRediT authorship contribution statement

Ana P. Tobajas: Investigation, Writing - original draft. **Ana Agulló-García:** Investigation, Data curation. **José L. Cubero:** Formal analysis. **Carlos Colás:** Conceptualization, Resources. **Isabel Segura:** Visualization. **Lourdes Sánchez:** Methodology. **Miguel Calvo:** Writing - review & editing. **María D. Pérez:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126745.

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