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ORIGINAL PAPER

Effect of thermal and ultrasound treatments on denaturation and allergenic potential of Pru p 3 protein from peach

Ana P. Tobajas[1,3] · Ana Agulló-García[2,3] · José L. Cubero[2,3] · Carlos Colás[2,3] · Alba Civera[1,3] · Santiago Condón[1] · Lourdes Sánchez[1] · María D. Pérez[1,3]

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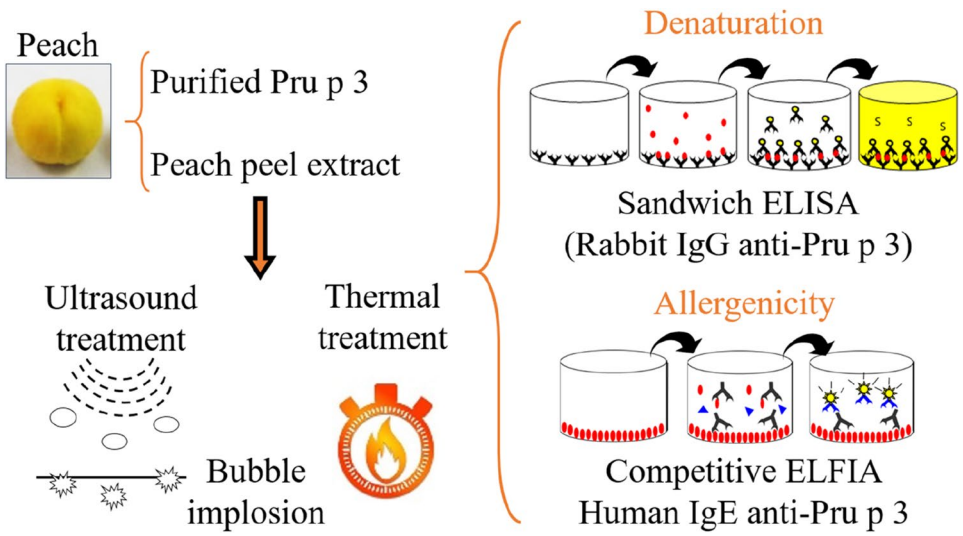
Abstract

The effect of thermal and ultrasound treatments on denaturation and allergenicity of Pru p 3, the major peach allergenic protein, was determined. The degree of denaturation of Pru p 3 was estimated by sandwich ELISA using specific rabbit IgG, that was previously developed. Validation of ELISA test showed high sensitivity and specificity, and acceptable results of precision and robustness. Allergenicity of Pru p 3 was determined by immunofluorescent assay using three pools of sera from peach allergic individuals. Denaturation of Pru p 3 was dependent on the intensity of the thermal treatment applied and the treatment medium. Thus, the degree of denaturation of Pru p 3 treated at 95 °C for 40 min was about 60% and 95%, for the protein heated in peach extract and in buffer, respectively. Ultrasound treatments denatured Pru p 3 up to 60%, being dependent on amplitude and pressure. However, both heat and ultrasound treatments at the most severe conditions applied inhibited less than 10% the IgE-binding of Pru p 3. These results indicate that although heat and ultrasound treatments induce a considerable denaturation of Pru p 3, they are not effective in reducing its allergenicity.

Graphical abstract



Keywords Pru p 3 · Peach · Thermal · Ultrasound · Denaturation · Allergenicity

✉ María D. Pérez
dperez@unizar.es

Extended author information available on the last page of the article

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Introduction

The prevalence of fruit allergy estimated by oral challenge test has been reported to range between 0.1 and 4.3%, being higher in adults than in children [1]. Studies performed in Southern Europe showed that fruits belonging to the Rosaceae family are the main elicitor of allergic reactions. Furthermore, peach (*Prunus persica*) is the most common fruit associated to IgE-mediated hypersensitivity in the Mediterranean area [2, 3] and the first triggering food that is subsequently associated to other Rosaceae fruit allergies, such as apple, due to their cross-reactivity [4].

In the Mediterranean area, sensitization to Pru p 3 has been observed in 60–90% of peach allergic patients; therefore, it has been recognized as the major peach allergen [5]. Pru p 3 belongs to the non-specific lipid transfer protein (LTP) family [6]. It is a small basic protein of about 9 kDa and its main structural motif is represented by an α-helical compact domain, where four helices are connected by short loops and firmly held by four disulphide bridges [7]. This compact structure makes the protein highly resistant to thermal treatments, pH changes and proteolysis by digestive enzymes [8]. Therefore, Pru p 3 is considered a true food allergen as it induces sensitization via gastrointestinal tract and its IgE reactivity is often associated to severe systemic symptoms, frequently producing an anaphylactic shock. In fact, it has been recently indicated that high levels of specific IgE to Pru p 3 in peach allergic patients can be regarded as a warning for severe systemic reactions [9].

Processing technologies could have a significant impact on the allergenic potential of foods. Processing may induce in proteins different effects like denaturation, aggregation, hydrolysis or cross-linking to other components, and these changes can alter the IgE binding to certain allergenic proteins. Because of these modifications, processing may destroy existing epitopes or generate new ones (neoallergen formation). This means that processing may influence allergenicity either positively, negatively or may not have any effect at all [10]. With the high prevalence of peach allergy in Southern Europe and Mediterranean population, researchers have actively sought processing technologies that can reduce the allergenicity of peach allergens.

Thermal treatments are commonly used in food industry for preservation and extension of the self-life of foods. It was shown that treatment of peach nectar at 121 °C for 30 min did not decrease the reactivity of Pru p 3 with IgE of peach allergic patients [11], while treatment of peach extract at 100 °C for 15 min induced about 25% inhibition of IgE-binding [12]. Using Circular Dichroism (CD) spectroscopy and high field Nuclear Magnetic Resonance (NMR) spectroscopy, Gaier et al. (2008) [13], observed that Pru p 3 protein was unable to refold after heating to 95 °C under neutral conditions but readily refolded after heating at pH 3, which are the conditions usually applied in peach products like juices, nectars, jams, etc.

In the last decades, with the development of non-thermal processing technologies, such as high pressure and pulse electric field treatments, researchers have explored their impact on allergenic proteins and the possibility of producing hypoallergenic foods using them [14, 15].

Ultrasonication is a versatile technology with broad food applications like pasteurization, enzyme inactivation, extraction and homogenization among others [15]. The use of ultrasound treatment, alone or in combination with mild heating, has proven to be effective to inactivate bacteria and enzymes responsible for the deterioration of fruit juice [16]. These effects arise from acoustic cavitation generated during sonication due to the formation, growth and implosion of bubbles during the propagation of sound waves in a liquid media [17]. Ultrasound effects may induce changes in tertiary and secondary structure and/or formation of intra or intermolecular interactions in allergenic proteins, which might influence its potential allergenicity. However, only few studies have been conducted on the effect of ultrasound treatment on food allergens from animal or plant origin, and those performed have reported dissimilar results [18–21].

To our knowledge, there is only one study to determine the effect of sequential microwave heating (140 °C, 30 min) and ultrasound treatment (26 kHz, 150 W, 30 min) on denaturation and allergenicity of peach peel and pulp extracts using Western-blotting [22]. This study showed that the combination of both treatments did not decrease the binding of Pru p 3 to rabbit specific IgG or to human IgE from individual sera of peach allergic patients.

The aim of this study was to evaluate exclusively the effect of ultrasound treatment of peach extracts performed at different pressures (manosonication), amplitudes and holding times on denaturation and allergenicity of Pru p 3. Denaturation was estimated by an ELISA technique using rabbit specific antibodies to Pru p 3 and allergenicity by an immunofluorescent assay using three pools of sera from peach allergic individuals. For comparison, thermal treatments at different temperatures and holding times were also performed.

Materials and methods

Preparation of peach extract and isolation of Pru p 3

Extracts were prepared from fresh peel of peach belonging to the Spanish indigenous variety "amarillo tardío" clone Calante, as described by Tobajas et al. (2020) [23] with some modifications. Briefly, peel samples were homogeneized in

10 mM sodium phosphate buffer (pH 5.6) containing 2 mM EDTA, 10 mM sodium diethyldithiocarbamate (DIECA), 2% solid polyvinylpolypyrrolidone (PVPP) and 3 mM sodium azide at a ratio 1:2 (w:v), stirred for 2 h at 4 °C and centrifuged at 12,000 × *g* for 30 min. The supernatant was dialyzed against the same phosphate buffer without additives for 48 h at 4 °C and applied to a SP-Sepharose column (5 × 2 cm). Retained proteins were eluted using the same phosphate buffer with 1 M NaCl and fractions obtained pooled and subjected to ultrafiltration using a membrane of 30 kDa molecular cut-off. The permeate was collected and concentrated using a membrane of 3 kDa. Protein profiles of peach fractions were analyzed by SDS-PAGE and the purity of Pru p 3 was determined by densitometry of stained gels. The electrophoretic band with a molecular weight of about 9 kDa was manually excised from gel and was analyzed by mass spectrometry MALDI-TOF/TOF as previously described by Tobajas et al. (2020) [23].

SDS-PAGE and western-blotting

SDS−PAGE in 4–20% precast polyacrylamide gels, under reducing conditions with mercaptoethanol, was carried out in a Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories) according to Laemmli, (1970) [24]. Proteins were stained with Coomassie blue. Western-blotting using anti-Pru p 3 antiserum was performed according to the procedure described by Benfeldt et al. (1995) [25].

Obtention and conjugation of rabbit anti-Pru p 3 antibodies

Rabbits were immunized with purified Pru p 3 to obtain antisera as indicated by Wehbi et al. (2005) [26].

The titer of the antisera was determined using an indirect non-competitive ELISA and characterized using Western-blotting. Specific antibodies against Pru p 3 were isolated by affinity chromatography using a HiTrap NHS activated HP column of 1 mL (GE Healthcare, Farfield, Connecticut, USA) coupled with Pru p 3, as previously described by Segura-Gil et al. (2019) [27]. Then, purified antibodies were labeled with horseradish peroxidase (HRP) using the Lightning-Link HRP antibody labeling kit (Innova Biosciences, Cambridge, UK).

Patient sera

Peach allergic individuals ($n = 22$) were voluntary recruited at the Allergy Department of the University Hospital Lozano Blesa of Zaragoza (Spain). Patients with a positive prick test (ALK-Abelló S.A., Madrid, Spain) and with specific IgE against Pru p 3 higher than 0.35 kU/L determined by the ImmunoCAP FEIA system (ThermoFisher Scientific/Phadia, Uppsala, Sweden) were selected. Allergic individuals to Pru p 3 had different symptomatology and, therefore, three different pools of sera were prepared: pool of patients that presented an anaphylactic shock (ANS) ($n = 7$), an oral allergy syndrome (OAS) ($n = 5$) and at least one of these four symptoms: urticaria, angioedema, asthma, abdominal pain, in an acute outbreak (ALOS) ($n = 10$).

Thermal treatment

A volume of 100 µl of purified Pru p 3 protein (1 mg/mL) or peach peel extract (protein content of 0.3 mg/mL), both in phosphate buffer pH 5.6 was added in glass tubes of 1.2 ml capacity (diameter of 8.2 mm and height of 40 mm), and they were placed in a thermostatic bath (± 0.1 °C). Samples were treated at different temperatures (75, 85 and 95 °C), taking samples in duplicate at different treatment times (15 s and 5, 10, 20 and 40 min). The tubes were immediately cooled by immersion in an ice water bath. At least two independent experiments were performed for each treatment and samples analyzed by triplicate.

Ultrasound treatment

Treatments were performed in a MTS resistometer provided with a treatment chamber of 100 mL as described by Raso et al. (1998) [28]. The bottom of the chamber is reached by the tip of a sonication horn of an ultrasound generator Digital Sonifier® 450 (Branson Ultrasonic, Danbury, CT, EE. UU). Samples of peach peel extract diluted 1/10 in phosphate buffer pH 5.6 were treated with ultrasonic waves at a frequency of 20 kHz and different amplitudes (43, 72 and 100 µm). Treatments were carried out at 25 ± 1 °C and at different relative pressures (0, 50 and 100 kPa) for different times (0, 0.5, 1, 1.5, 2, 3, 4, 6 and 8 min). The power of treatments applied ranged between 22 and 128 W. Temperature control during the experiments was achieved by dissipating excess heat evolved during sonication by circulating cool water through the cooling coil. The temperature of treatment medium was continuously monitored by a thermocouple (ALMEMO, Ahlborn, Germany). At least two independent experiments were performed for each treatment and samples analyzed by triplicate.

Sandwich enzyme-linked immunosorbent assay (ELISA)

The concentrations of Pru p 3 in untreated and treated samples of fruit extracts and purified Pru p 3 were determined using a sandwich ELISA assay that was previously developed Tobajas et al. (2020) [23]. Briefly, wells of microtiter plates were coated with 120 µl of anti-Pru p 3 antibodies (1 µg/ml). After incubation overnight at 4 °C, wells were

blocked with 300 µl of ovalbumin at 3% (w/v) for 2 h and washed with distilled water. Before using, wells were washed with 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.14 mM KCl and 0.14 M NaCl, pH 7.4 (PBS) containing 0.05% 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. After washing with PBST, wells were incubated with 100 µl of anti-Pru p 3 antibodies labeled with peroxidase diluted 1/65,000 in the same buffer and after washing again, wells were added with 100 µl/well of substrate containing tetramethylbenzidine (TMB). After 30 min, the enzymatic reaction was stopped with 2 M H_2SO_4 (50 µl/well) and the absorbance was read at 450 nm.

Validation of sandwich ELISA to Pru p 3

The sandwich ELISA developed was validated following standardized procedures established by the Association of Official Analytical Chemists (AOAC) [29, 30] and the validation guidance of EURACHEM [31].

The Limit of Detection (LOD) and the Limit of Quantification (LOQ) were calculated as the mean concentration of Pru p 3 from ten replicates of the zero standard plus 3 and 10 times the standard deviation (SD), respectively. Cross-reactivity was tested analyzing extracts of several fruits (nectarine, paraguayan, apricot, apple, cherry, pear, white plum, strawberry, grape, pineapple, kiwi, tomato, orange). The precision parameters and robustness were determined using a commercial pineapple juice spiked with 0.01 and 0.02% of commercial peach juice. Repeatability was estimated analyzing ten replicates of the same extract in one run. Intra-assay reproducibility was estimated analyzing ten extracts of the same sample in one experiment. Inter-assay reproducibility was determined assaying three extracts of the same sample in three different days. Robustness was determined applying small deliberate changes to the normal conditions in a single experiment (Supplementary Table 1). A Youden matrix was designed, which makes use of a fractional factorial design. The standard deviation of the differences (SDi) was calculated as previously described [32].

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

The presence of specific IgE (sIgE) against Pru p 3 was determined in the three pools of sera from allergic patients using Pru p 3 ImmunoCAP (Reference f420, Thermo Fisher Scientific) in a Phadia 100 system. Assays were performed using a non-competitive format following manufacturer instructions for sIgE determination.

A competitive assay using Pru p 3 ImmunoCAP was used to compare the binding of sIgE to untreated and ultrasound or thermal treated peach peel extracts. For thermal treatments, purified Pru p 3 protein was also analyzed. Samples were mixed with each of the three pools of sera (1/1, v/v) and sIgE was determined. A mixture containing PBS and the patient pool sera was also assayed, as negative control. 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 as follows:

$$\text{IgE binding (\%)} = \frac{\text{sIgE sample} - \text{sIgE 0\%}}{\text{sIgE 100\%} - \text{sIgE 0\%}} \times 100$$

Statistical analysis

Data were analyzed for statistical significance with GraphPad Prism 8 software, using the Kolmogorov–Smirnov normality test and one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison Test.

Results and discussion

Characterization of Pru p 3 and antisera

The electrophoretic profile of peach extract and purified Pru p 3 is shown in Fig. 1a. Pure Pru p 3 showed the presence of a well-defined band of molecular weight of about 9 kDa whose degree of purity was higher than 95% as determined by densitometry. Results obtained by mass spectrometry gave 8 matching proteolytic peptides with sequence coverages of 92% indicating that fragments correspond to Pru p 3. The titer of antisera obtained, determined by a non-competitive indirect ELISA using wells coated with Pru p 3, ranged between 1/2000 and 1/8000 depending on the animal and bleeding. The immunoreactivity of antisera to Pru p 3, determined by Western-blotting mainly recognized Pru p 3 in samples of the pure protein and peel extract (Fig. 1b).

Development and validation of the sandwich ELISA to determine Pru p 3

The concentration of coating and labeled antibodies as well as incubation temperature and time of the different steps of the assay were optimized to obtain the best sensitivity and a good relationship between concentration and optical density of the standards.

Calibration curve obtained for the determination of Pru p 3 is shown in Fig. 2. The best fit was obtained when plotting the absorbance values versus the concentration of

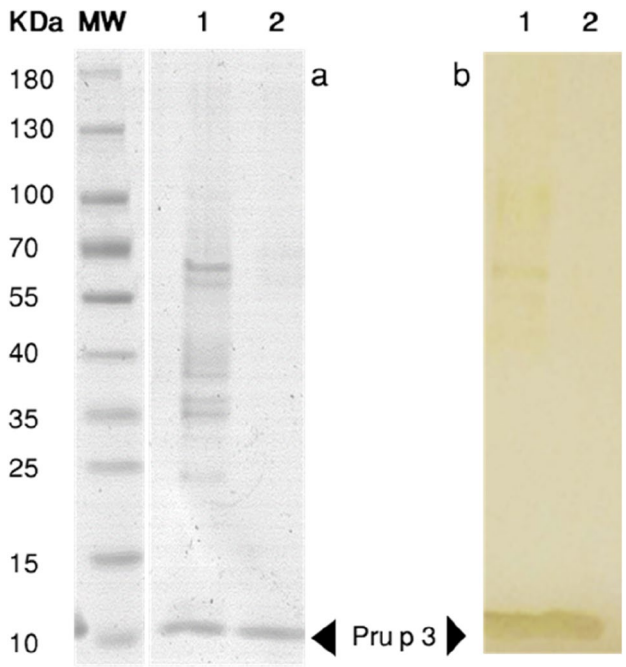

Fig. 1 SDS-PAGE in 4–20% polyacrylamide gel under reducing conditions (**a**) and Western-blotting using rabbit antiserum to Pru p 3 (**b**). MW, molecular weight marker. Lane 1, peach peel extract. Lane 2, purified Pru p 3

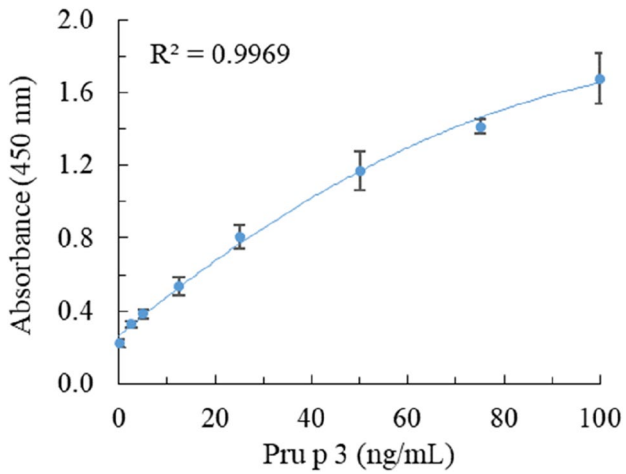

Fig. 2 Calibration curve obtained for the determination of Pru p 3 by sandwich ELISA using rabbit specific IgG. Standards were prepared with purified Pru p 3. Each data point represents the mean of 10 measurements of the absorbance at 450 nm

the standards within a range of concentrations from 2.5 to 100 ng/mL, which was adjusted to a polynomial curve. All assays gave coefficients of regression (R^2) higher than 0.986. The concentration of Pru p 3 in samples was calculated using the calibration curve of each assay.

The validation of the sandwich ELISA was carried out following the procedures established by the Association of Official Analytical Chemists (AOAC) [29]. The LOD and LOQ of the ELISA assay were found to be 2.6 ng/mL and 8.6 ng/mL, respectively. The LOD is greater than that obtained by Duffort et al. (2002) [33] using an indirect sandwich ELISA to Pru p 3, which reported a value of 0.1 ng/mL. However, the sensitivity of our ELISA is better than the indirect competitive ELISA to Pru p 3 developed by Carnes et al. (2002) [34] as they reported a working range from 0.125 to 1 µg/ml.

Specificity of developed ELISA, shown in Fig. 3, showed a considerable high cross-reactivity with nectarine and paraguayan (23% and 38%, respectively) probably because they are varieties of peach (*Prunus persica*, variety *nucipersica* and *platycarpa*, respectively). The rest of the fruits gave cross-reactivity lower than 5%. These results indicate that the developed technique is capable of detecting LTP from different peach varieties, which would allow a better detection of these proteins in mixed fruit products, being able to ensure their presence and thus avoiding allergic reactions.

The precision parameters of the sandwich ELISA were determined using a commercial pineapple juice spiked with 0.01 and 0.02% of commercial peach juice. Results obtained gave coefficients of variation that ranged from 10.4 to 12.1% for repeatability, from 8.2 to 13.9% for intra-assay reproducibility and from 9.6 to 13.0% for inter-assay reproducibility (Table 1). These values are within the acceptance limits established by the AOAC for food allergens [30]. For the determination of the robustness, the value of the standard deviation of the differences (SDi) was 1.482 and 0.913, for samples spiked with 0.01 and 0.02% of peach

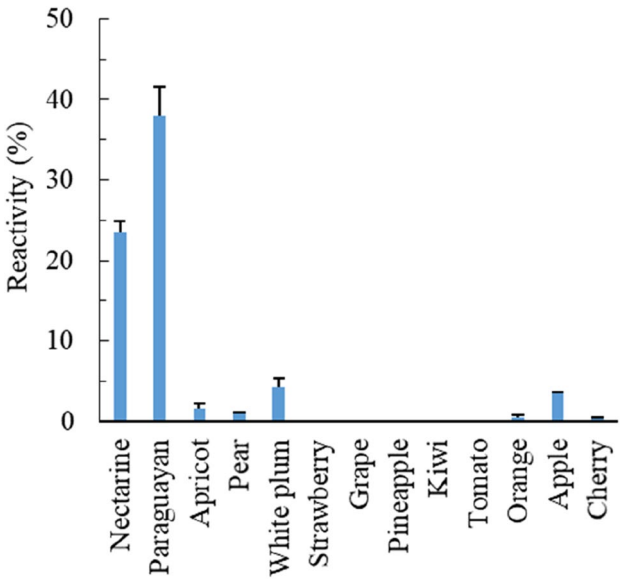

Fig. 3 Cross-reactivity of different fruit extracts analyzed by sandwich ELISA to determine Pru p 3. Results correspond to the percentage of reactivity respect to peach extract (100%)

Table 1 Results of the precision study performed with the sandwich ELISA using rabbit specific IgG for the determination of Pru p 3 in pineapple juice spiked with 0.02 and 0.01% of peach juice

	0.02%		0.01%	
	Mean	CV (%)	Mean	CV (%)
Repeatability	24.0	10.4	11.9	12.1
Intra-assay reproducibility	24.9	8.3	11.6	13.9
Inter-assay reproducibility	24.4	12.9	12.1	9.6
Intra-assay				
Day 1	28.0		13.2	
Day 2	22.0		12.4	
Day 3	23.1		10.9	

Values are expressed in ng of Pru p 3/ml of juice

juice, respectively. These values are lower than the standard deviation of the values of inter-assay reproducibility (3.17 and 1.17, respectively), which suggests that the sandwich ELISA is robust according to the criteria established by Karageorgou and Samanidou (2014) [32].

Effect of thermal treatment on denaturation and allergenicity of Pru p 3

The effect of thermal treatment at several temperatures and holding times on denaturation of Pru p 3 naturally present in peach peel extracts or as pure protein in phosphate buffer, both at pH 5.6, was determined. The degree of denaturation of Pru p 3 was estimated by measuring the loss of reactivity with its specific rabbit antibodies using the sandwich ELISA. This technique relies on the modifications that take place in protein structure induced by thermal treatments, which decreases the immunoreactivity, mainly due to the damage of conformational epitopes of the protein that interact with IgG. These ELISA techniques have been widely used to determine the effect of technological treatments on denaturation of allergenic LTP like Pru p 3 [23] or Mal d 3 [35]. The use of immunochemical techniques present the advantage of determining the extent of denaturation of a specific protein not only in the purified form, but also in a complex food such as fruit juice in which the effect of other food components is also considered [36].

As it is shown in Fig. 4, denaturation of Pru p 3 depends on the intensity of the thermal treatment applied. Treatment at 75 ºC and 85 °C up to 40 min of peel extract did not show significant differences in the concentration of immunoreactive protein respect to the untreated sample. However, at 95 °C, the degree of denaturation increased with the time of heating, obtaining values of immunoreactive Pru p 3 of about 77% and 40% after 20 and 40 min of treatment, respectively, respect to untreated sample (100%) (Fig. 4a). Regarding the results obtained, heating produced

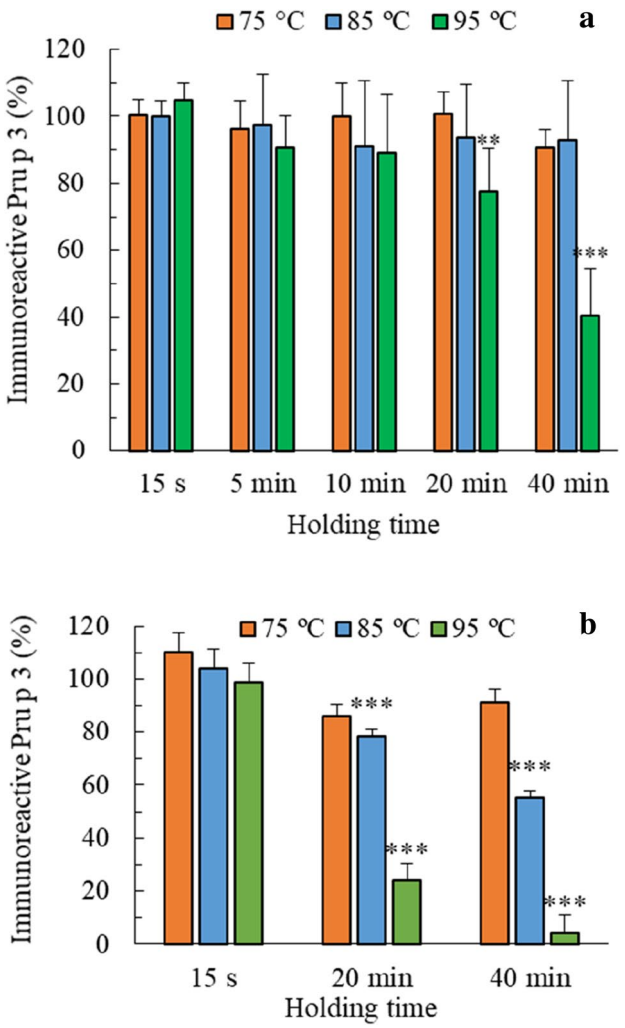

Fig. 4 Effect of thermal treatment of peach peel extract (**a**) or purified protein (**b**) on the denaturation of Pru p 3 determined by sandwich ELISA using rabbit specific IgG. Values are the mean ± SD of data from two experiments analyzed by triplicate and are expressed as percentage with respect to untreated sample (100%). Asterisks indicate significant differences with respect to corresponding untreated samples (*$p < 0.05$), **$p < 0.01$, ***$p < 0.001$)

a more marked denaturation on the pure protein than when it was treated in the extract. Thus, treatment of the pure protein for 40 min at 85 °C decreased the amount of immunoreactive protein to 55% and at 95 °C to 5% (Fig. 4b). Results obtained for the thermal treatment of the pure protein are in good agreement to those reported for Mal d 3 recognition by rabbit IgG using an ELISA technique, as a decrease of immunoreactivity to 40% and 10% was found after heating the pure protein at 90 and 100 °C for 10 min at pH 7.0, respectively [35]. Differences in the degree of denaturation of Pru p 3 treated in buffer or in peach extract are probably due to the influence of other components of the fruit. Those components can exert a protective effect

on its denaturation during processing as it has been shown for pectin, which prevents the pressure-induced modification of Mal d 3 [37].

Results obtained on the effect of heating on pure Pru p 3 at pH 5.6 are in accordance to those observed by Gaier et al. (2008) [13] using CD spectroscopy. These authors observed that when Pru p 3 was heated at neutral pH (pH 7.5), it underwent denaturation at 95 °C that started at about 85 °C and that the protein was not able to refold when the temperature returned to 25 °C, whereas the protein could refold upon heating and cooling at acid pH (pH 3). Likewise, this is also in agreement with results obtained by CD with the homologous Mal d 3 protein from apple, which showed a marked loss of structure, from α-helix to random coil, when heated to 90 °C or above [35]. These findings have been attributed to the cleavage of disulphide bonds of Pru p 3 under neutral or mild acidic conditions, as we observed in our study, and to the higher stability of disulphide structure at very acidic pH [13].

When samples of pure Pru p 3 subjected to thermal treatment were analyzed by SDS-PAGE, no apparent changes were observed in the intensity of the Pru p 3 band respect to untreated sample, except for the treatment at 95 °C for 40 min, which showed a considerable decrease in the intensity of the band (Fig. 5). These results are in good agreement to those reported by Lavilla et al. (2016) [12], that showed no apparent changes in Pru p 3 after treatment at 90 °C for 10 min, whereas after treatment at 100 °C for 15 min, the protein exhibited a protein band of lower intensity.

The effect of thermal treatment on allergenicity of Pru p 3 in peach extract and as pure protein in buffer was also determined using a competitive ELFIA technique and the three pools of sera from allergic patients. The level of specific IgE in these three pools of sera determined previously by a non-competitive assay was 5.43, 6.84 and 9.86 kU/L for OAS, ALOS and ANS, respectively. Results obtained indicated that all thermal treatments applied did not affect the binding of Pru p 3 to IgE, and only a slight inhibition, less than 10%, was observed after treatment at 95 °C for 40 min when assayed with the pool of sera from patients with ALOS, although the differences were not statistically significant (Supplementary Fig. 1a). These results suggest that Pru p 3 maintains its allergenic potential after the thermal treatments applied.

Our results are in accordance to those reported by Brenna et al. (2000) [11] as they found that thermal treatment applied to peach extract under harsh conditions (121 °C for 10–30 min) did not change its allergenicity when analyzed by immunoblotting using two pool of sera from individuals allergic to peach. They attributed this high thermostability to the presence of epitopes in linear amino acid sequences of the protein. In contrast, Lavilla et al. (2016) [12] using a competitive ELISA technique and a pool of sera from peach allergic patients showed that mild thermal treatment (80–90 °C) of Pru p 3 had only a slight effect on IgE recognition whereas increasing the temperature to 100 °C for 15 min caused 60% inhibition of binding to IgE. However, they found that only a 25% of inhibition was achieved when assaying peach extract subjected to that treatment, which suggest a protective effect of components present in peach extract on the allergenic potential of Pru p 3. Similar results have been also observed by Sancho et al. (2005) [38] on Mal d 3, showing that it is highly stable to treatments below 90 °C for 20 min, but exposure to much more severe conditions (100 °C for 2 h) resulted in alteration of the secondary structure with a considerable reduction in its IgE-binding capacity. Those authors also observed that heating purified Mal d 3 in the presence of glucose resulted in a lower reduction of its IgE-binding activity, suggesting that the presence of sugars in the fruits may contribute to maintain the allergenic activity of Mal d 3 in heat-processed foods [38].

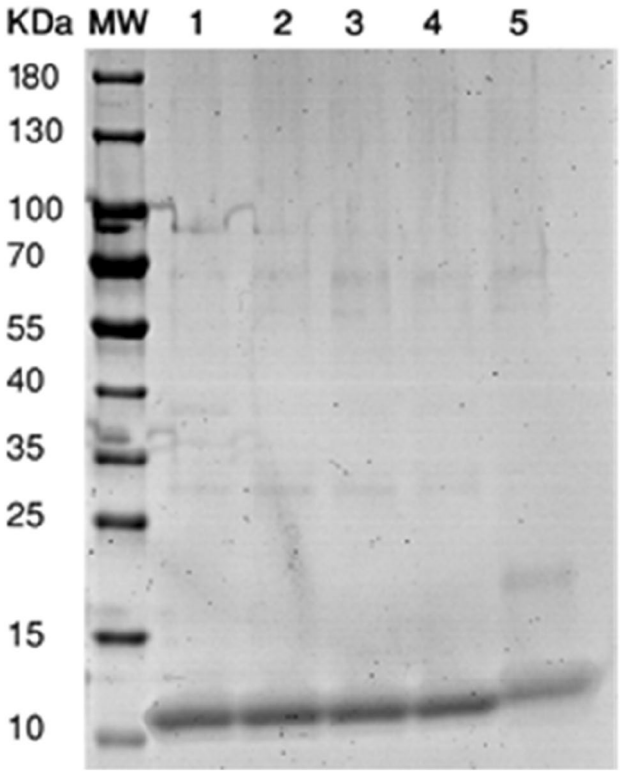

Fig. 5 SDS-PAGE in polyacrylamide gel (4–20%) under reducing conditions of thermal treated Pru p 3 samples. MW, molecular weight marker. Lane 1, untreated Pru p 3. Lane 2, 75 °C for 15 s. Lane 3, 95 °C for 15 s. Lane 4, 75 °C for 40 min. Lane 5, 95 °C for 40 min

Effect of ultrasound treatment on denaturation and allergenicity of Pru p 3

In this study, the effect of ultrasound treatment at different amplitudes, pressures and holding times on denaturation of Pru p 3 was determined using the sandwich ELISA. Ultrasound treatment was performed only on peach peel extract due to the large volume required to carry out the experiments.

Results obtained are shown in Fig. 6a–c. As it can be observed, for treatments performed at amplitudes of 43 and 72 µm, the decrease of reactivity of Pru p 3 with rabbit IgG was similar at the three pressures assayed, whereas in the sample treated at the highest amplitude (100 µm), the pressure of 100 kPa induced a higher decrease of immunoreactivity of Pru p 3. Furthermore, the decrease in immunoreactive Pru p 3 took place mainly in the first minute of treatment and at longer times, reduction was very low, being the difference between 1.5 and 8 min less than 27% of total immunoreactivity loss. When considering the factor of pressure, the degree of denaturation obtained after 8 min at 100 kPa was of 23%, 37% and 59% for the amplitudes of 43, 72 and 100 µm, respectively. These results confirm that the combined effect of pressure with ultrasound treatment (manosonication) increases the denaturation of Pru p 3 protein in peach extracts.

In the study of Garino et al. (2012) [22], authors determined the effect of sequential treatments of nectarine pulp extracts with microwave heating at 140 °C for 30 min and ultrasound for 30 min at 150 W on denaturation of Pru p 3 by Western-blotting using a specific rabbit antiserum. Our results using a quantitative ELISA indicate that the concentration of immunoreactive Pru p 3 decreases when peach peel extract is subjected to ultrasound treatment, suggesting that the structure of Pru p 3 must have been modified. This modification seems to have altered some conformational epitopes that are recognized by specific rabbit IgG, while maintaining other epitopes, lineal and probably some conformational, which are not affected by the processing.

When peach extracts subjected to ultrasound treatments were analyzed by SDS-PAGE, no visible changes in the protein profile and in the intensity of the bands were observed respect to the untreated extract (Fig. 7). This finding agrees with that found by Garino et al. (2012) [22], as they did not observe any changes in the intensity of Pru p 3 after processing with sequential microwave heating and ultrasound processing.

Results obtained on the effect of ultrasound treatment on allergenicity of Pru p 3 showed that only the more severe treatment applied (100 kPa and 100 µm of amplitude) induced a slight decrease, between 5 and 10%, of reactivity of the protein with the IgE of the pool sera from patients with SAO and ALOS (Supplementary Fig. 1b). These results

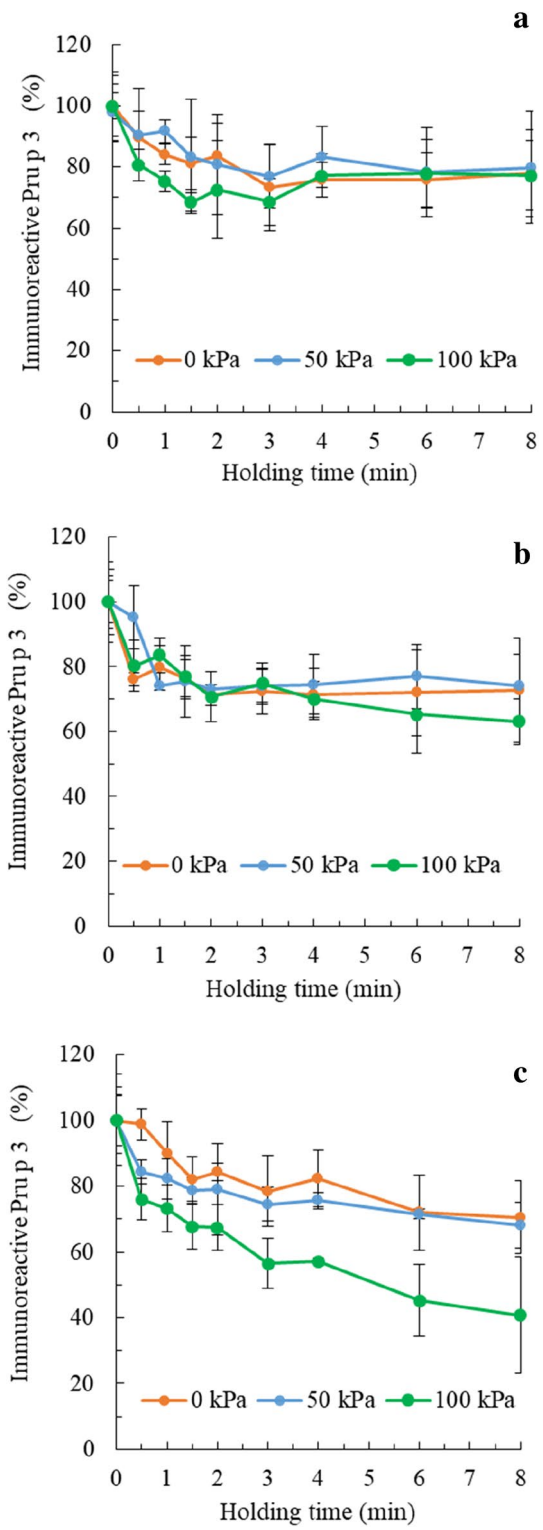

Fig. 6 Effect of ultrasound treatment of peach peel extract at amplitude of 43 (**a**), 72 (**b**) and 100 µm (**c**) and at different pressures on denaturation of Pru p 3 determined by sandwich ELISA using rabbit specific IgG. Results are the mean ± SD of data from two experiments analyzed by triplicate and are expressed as percentage with respect to untreated sample (100%)

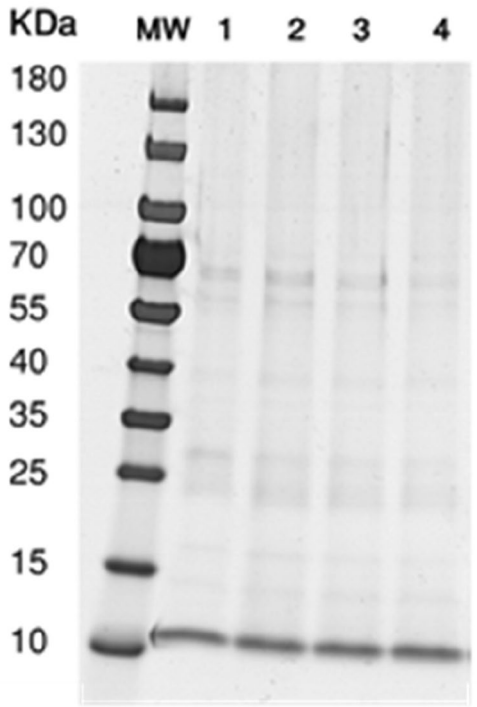

Fig. 7 SDS-PAGE (4–20%) under reducing conditions of peach peel extracts treated with ultrasounds at different pressures and amplitudes for 8 min. MW, molecular weight marker. Lane 1, untreated peach peel extract. Lane 2, 100 kPa and 43 µm. Lane 3, 100 kPa and 72 µm. Lane 4, 100 kPa and 100 µm

suggest that, as in the case of thermal processing, ultrasound treatments applied to Pru p 3 maintain its allergenic properties. Our results agree with those obtained by Garino et al. (2012) [22] as they did not observe loss in the intensity of the Pru p 3 band or even an increase with some sera when analyzing nectarine extracts subjected to microwave heating and ultrasound treatment, by Western-blotting using sera from peach allergic patients. This fact was attributed to the conformational change of Pru p 3, which produces a different exposure of epitopes or to the concentration of thermostable proteins like Pru p 3 and the loss of thermolabile ones during processing.

The absence of effect of ultrasound treatment on other allergenic proteins has been reported for milk proteins like β-casein [39] and for octopus tropomyiosin [40]. Those studies showed that ultrasound treatments were not effective in reducing the content and the "in vitro" allergenicity since no significant differences were found in IgG/IgE-binding values between the untreated and treated samples by ELISA using rabbit antisera or a pool of sera from allergic patients to the corresponding proteins.

In contrast, other studies have indicated that ultrasound treatment has the capacity to largely reduce the "in vitro" allergenicity. Thus, treatment of a shrimp allergen (tropomyosin) for 30–180 min with a ultrasonicator (30 kHz and 800 W) decreased its allergenicity approximately 81–88%, as determined by ELISA and immunoblotting inhibition analysis [18]. Likewise, the ultrasound treatment of kiwifruit treated with a ultrasonicator (20 kHz and 400 W) for 16 min induced changes in the secondary structure of the Act d 2 allergen, and also resulted in 50% reduction of immunoreactive allergen content determined by IgG binding using an ELISA and of the binding to IgE using Western-blotting [41].

Conclusions

The reason underlying the use of food processing to reduce the allergenicity of proteins is mainly due to the ability of certain technological treatments to produce considerable changes in the structure of food allergens, in particular by destroying conformational IgE epitopes [17].

Results obtained in this study indicate that thermal and ultrasound treatments have the ability to alter the structure of Pru p 3, as they induce a loss of reactivity with specific rabbit IgG obtained against the native form of the protein. In contrast, the impaired IgE-binding of Pru p 3 obtained with both treatments is insignificant and then, they seems not to be relevant enough to be applied in the development of hypoallergenic foods for peach allergic consumers. However, as changes in IgE reactivity of treated Pru p 3 respect to untreated protein is an indirect measurement of allergenicity, more direct techniques should be assayed like basophil histamine release, activation of markers on basophils, a cell line model and/or skin prick test to better know the implications of processing treatments on allergy risks.

On the other hand, our findings suggest that Pru p 3 could be considered a sensitive and resistant marker protein to detect peach varieties in processed foods that could help to prevent its consumption by sensitized patients. The ELISA test developed may be also used by Public Health Laboratories to verify the presence Pru p 3 in a suspected food that has caused an allergic reaction in a sensitized individual. Further research has to be performed with other processing technologies with the aim of reducing Pru p 3 allergenicity.

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conceptualization, resources. AC: visualization. SC: Writing—review and editing. LS: methodology. MDP: conceptualization, supervision.

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Declarations

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All procedures carried out 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 scientific purposes.

Compliance with Ethics requirements Peach allergic individuals filled a questionnaire and signed an informed consent before the blood draw, which began after the approval of the protocol by the Clinical Research Ethic Committee of Aragón (CEICA) (Projects PI15/0323 and PI17/0351).

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Authors and Affiliations

Ana P. Tobajas[1,3] · Ana Agulló-García[2,3] · José L. Cubero[2,3] · Carlos Colás[2,3] · Alba Civera[1,3] · Santiago Condón[1] · Lourdes Sánchez[1] · María D. Pérez[1,3]

[1] Departamento de Producción Animal y Ciencia de Los Alimentos, Facultad de Veterinaria, Instituto Agroalimentario de Aragón (IA2), (Universidad de Zaragoza-CITA), C/Miguel Servet, 177, 50013 Zaragoza, Spain

[2] Servicio de Alergia, Hospital Clínico Universitario Lozano Blesa, Zaragoza, Spain

[3] Instituto de Investigación Sanitaria Aragón (IIS Aragón), Zaragoza, Spain