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Examining the effect of High Pressure Processing on the allergenic potential of the major allergen in peach (Pru p 3)

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ABSTRACT

The aim of this work is to study the effect of High Pressure Processing (HPP) on the allergenicity of the main protein involved in peach allergy (Pru p 3). Results obtained showed that most pressure/time combinations slightly enhanced *in vitro* IgE-binding to Pru p 3 and peach extract. Moreover, additional tests were carried out by means of skin prick tests on peach allergic patients. Different from *in vitro* results, *in vivo* effects evidenced that HPP (600 MPa/5 min) can either reduce or increase Pru p 3 allergenicity, depending on the particular sensitisation of each patient. Notwithstanding this variability, it is highly remarkable that the skin response to pressurized peach extract was stronger in more than half of individuals. These results would suggest a higher risk of HPP-treated peach products to elicit an allergic reaction. However, it has been also proved that matrix plays an important role in peach allergenicity modification. Consequently, further investigations are needed before extrapolating results to more complex products.

Industrial relevance: Peach allergy is one of the most frequent allergies in Mediterranean countries among adult patients. However, up to date, there is no information about the effect of HPP on peach allergenicity. This study increases understanding about the impact of processing on an important fruit allergen in order to advance knowledge-based ways to managing allergens risks in industry, as well as it opens new opportunities of research in other technologies or strategies in the attempt to reduce peach allergenicity.

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1. Introduction

Peach (*Prunus persica*) is responsible for an important number of food allergies in Europe (Pastorello et al., 1999; Sánchez-Monge, Lombardero, García-Sellés, Barber, & Salcedo, 1999) and it is the fruit most frequently involved in allergic reactions in the adult patients of the Mediterranean area (Fernández-Rivas et al., 2003). In this region, sensitization to Pru p 3, identified as the major allergen in peach, has been detected in >60–90% of patients allergic to peach (Fernández-Rivas et al., 2003; Gamboa et al., 2007; Pastorello et al., 1999; Sánchez-Monge et al., 1999). Pru p 3 is a non-specific lipid-transfer protein (LTP) and, as many other food allergens, it has been characterised to be very resistant to heat treatments and digestion or enzymatic hydrolysis (Asero et al., 2000; Brenna et al., 2000; Gaier et al., 2008). This high resistance of Pru p 3 is probably the cause of the severity of this fruit allergy. Besides, non-specific LTPs have been identified as allergens in various *Rosaceae* fruits (e.g. apple, apricot,

plum and cherry), and results in an extensive cross-reactivity of Immunoglobulins E (IgE) among these fruits, even with LTPs from botanically distant plants (Asero et al., 2002; Garino, Coisson, & Arlorio, 2016), increasing the risk of peach allergic patients to suffer an allergic reaction when eating fruit products.

Among all abnormal immune response caused by food, clinical studies have confirmed that Pru p 3 allergy is an IgE-regulated allergy (Linhart et al., 2013; Rossi, Monasterolo, Canonica, & Passalacqua, 2009). The reaction mechanism of a food IgE-mediated allergy usually starts by the recognition of the epitopes of the antigen (allergen) by IgE antibodies found in an allergic patient (who has been previously sensitized to the allergen).

Accordingly, previous researchers have focused their work on processing methods and alternative processing technologies in an attempt to inhibit allergen immunoreactivity (IgE recognition) by altering/removing protein epitopes. For instance, thermal treatment, enzymatic hydrolysis, chemical peeling, ultrafiltration, reducing chemical agents and sequential microwave combined by ultrasound processing have been tested against peach allergens (Brenna et al., 2000; Cavatorta et al., 2010; Garino et al., 2012; Gómez-Casado et al., 2015). As said

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before, Pru p 3 is very resistant to thermal treatments and hydrolysis, and only chemical lye peeling and ultrafiltration, which mean protein removal, were effective to reduce peach allergenicity in peach products (Brenna et al., 2000). No other common food processing technologies have been tested to assess their impact on peach allergen Pru p 3, but it is known that proteins structure, and consequently, IgE epitopes, could be affected by other technologies (Besler, Steinhart, & Paschke, 2001; Mills, Sancho, Rigby, Jenkins, & Mackie, 2009; Verhoeckx et al., 2015).

High Pressure Processing (HPP) is widely used in food industry since this non-thermal technology inactivates pathogenic and spoilage micro-organisms, while maintains unaltered the nutritional and organoleptic characteristics of products (Barba, Esteve, & Frígola, 2012; Barba, Terefe, Buckow, Knorr, & Orlén, 2015). HPP is also known to affect the structure of proteins, and it has been probed to modify the allergenic potential and digestibility of several food proteins including legumes, grains, fruits and vegetables (Huang, Hsu, Yang, & Wang, 2014; Kato, Katayama, Matsubara, Omi, & Matsuda, 2000; Li, Zhu, Zhou, & Peng, 2012; Somkuti & Smeller, 2013). However, up to our knowledge, no studies have been performed about HPP effect in peach proteins allergenicity. Taking into account that HPP is frequently used to treat fruit products, such as purée, coulis, or juices that may content allergenic proteins, it is important to research into the effect of this technology on Pru p 3 allergenicity in order to provide knowledge-based ways to managing allergens risks during processing to food manufacturers.

Thus, the main objective of this work was to study the effect of HPP treatments on the *in vitro* (IgE-binding capacity) and *in vivo* (skin) immunoreactivity of isolated Pru p 3 and peach extract.

2. Materials and methods

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

Peach extract and isolated Pru p 3 were obtained from fresh peach peel. 500 g of peach (*Prunus persica*) peels were homogenized in 500 mL of extraction buffer (10 mM phosphate buffer, pH 7.0, 2% polyvinyl-polypyrrolidone, 2 mM EDTA, 10 mM sodium diethyldithiocarbamate, 3 mM sodium azide). After homogenizing, the mixture was centrifuged at $10,000 \times g$ at 4 °C for 30 min, and the supernatant was thoroughly dialysed against PBS (14 mM NaCl, 2 mM KCl, 1 mM KH_2PO_4 , 8 mM Na_2HPO_4 , pH 7.4) in a dialysis tubing with a MW cut-off of 3500 Da (Fisherbrand, Thermo Fisher Scientific, Waltham, MA, USA). Afterwards, the extract was concentrated in a 3K Macrosep centrifugal concentrator according to manufacturer instructions (MW cut-off of 3000 Da, Pall Life Sciences, Port Washington, NY, USA) to a protein content of 1 mg/mL. Peach extract was aliquoted and frozen at –20 °C until used.

From peach extract, Pru p 3 was isolated using the same chromatographic methods and conditions described by Díaz-Perales et al. (2003). Briefly, a first step cation-exchange chromatography was performed on a Waters Accell™ Plus CM SepPak[®] cartridge (Waters Corp., Milford, MA, USA). Washing was carried out with 20 mM formic acid, pH 4.0, and the retained material was then eluted with 0.75 M NaCl in the same buffer (1 mL/min). The resulting retained was repurified by RP-HPLC on a Nucleosil 300-C4 column (7 × 250 mm; particle size 5 µm; Teknokroma, Barcelona, Spain) with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (10% during 5 min and 10–100% in 150 min; 0.5 mL/min). Fraction containing Pru p 3 was concentrated by lyophilisation, and suspended for treatments in PBS at a concentration of 1 mg/mL (1000 ppm). The proper isolation of the Pru p 3 protein was checked by SDS-PAGE following the method described below.

Protein content in both extract and isolated protein was determined according to Bradford method (Bradford, 1976) with premixed reagents (Pierce, Waltham, MA, USA). Resulting colour was read on a spectrophotometer (Varioskan Flash, Thermo Scientific) at 595 nm. A standard

curve was created with known quantities of bovine serum albumin (BSA) protein.

2.2. High hydrostatic pressure and thermal treatments

A schematic diagram of sample preparation and experimental design can be seen in Fig. 1. Isolated Pru p 3 or peach extract were sealed in polyethylene bags after exclusion of air bubbles and treated by HPP (400, 500 or 600 MPa/22 °C/for 0, 5 or 10 min holding time) in a Wave 6000/55HT equipment (Hiperbaric, Burgos, Spain) using water as pressurisation fluid. Inlet water temperature was set between 4 °C (± 0.5 °C) for 600 MPa treatments and 10 °C (± 0.5 °C) for 400 MPa treatments. So, taking into account adiabatic heating, the temperature theoretically increased up to 22 °C in all treatments (final temperature). Temperature of the vessel and the plugs was also held at 22 °C. Moreover, the equipment used has two temperature probes that protrudes from each plug inside the vessel, and that allow to measure the temperature inside the vessel during the treatment, that never exceeded 25 °C. The rate of pressure increase was about 170 MPa/min and the pressure release was immediate at the end of the pressure holding time.

Thermal treatments were carried out separately in order to compare processing effects and confirm the stability of this protein. Samples of 300 µL were placed in 1.5 mL Eppendorf® Safe-Lock Microcentrifuge tubes in order to assure a fast heat transfer. Thermal treatments were performed in a Lauda Controller E300 Baths EcoLine 012 (Lauda-Königshofen, Germany) at 80, 90 and 100 °C for 5, 10 or 15 min.

2.3. SDS-PAGE and immunoblotting

Proteins from extract were separated by 10–15% SDS-PAGE (PhastGel System, Pharmacia, Uppsala, Sweden) according to manufacturer instructions: Samples were diluted in 10 mM Tris-HCl (pH 8.0), containing 1 mM EDTA, 2.5% SDS and 5% beta-mercaptoethanol, and boiled for 5 min. After electrophoresis, one gel was stained in Coomassie blue R. The second gel was used for immunoblotting analysis.

Immunochemical tests were performed with a pool of sera from 9 allergic peach patients from the Allergy Services of the Basurto Hospital (Bilbao, Spain). Patients were selected on the basis of documented allergic reactions to peach (see Section 2.5 for detailed description of symptoms and allergy status) and high levels of sensitisation: Pru p 3 specific IgE median value: 11.4 kU/L; Interquartile range: 5.8–146 kU/L; Median value of total IgE = 243 kU/L; interquartile range: 22–985 kU/L. Inclusion in this study was made with the approval of the Ethical committee of the Hospital and under individual informed consent.

Qualitative detection of IgE binding proteins was investigated by Immunoblotting. Proteins separated in the gel were transferred to a nitrocellulose membrane (0.2 µm, Sigma-Aldrich, St. Louis, MO, USA). After blocking with 1% Bovine Serum Albumin (BSA) in PBS (w/v) for 1 h, the membrane was incubated with 1:10 diluted pool of sera in PBS. Bound IgEs were detected using 1:100 diluted Goat anti-human Kappa-HRP IgG (SouthernBiotech, Birmingham, Alabama, USA) and insoluble (4-chloro-1-naphthol) HRP-substrate.

2.4. Competitive inhibition enzyme linked immunosorbent assay (ciELISA)

ELISA inhibition assays were carried out as described in Díaz-Perales et al. (2003) with slight modifications. Briefly, microtiter plate wells were coated with 100 µL/well of 5 µg/mL Pru p 3 or 100 µL/well of 10 µg/mL peach extract diluted in $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer 0.05 M, pH 9.6. After incubation overnight at 4 °C, the wells were washed three times with PBS with 0.05% (v/v) Tween 20 (PBST). Then, wells were blocked with 300 µL of 1% (p/v) skimmed milk powder in PBS and incubated for 2 h at room temperature (RT). After blocking, plates were incubated with 100 µL of a mixture (1:1) of the corresponding dilution of untreated or treated peach samples and a 1:25 dilution (previously optimised) of the same pool of sera described before (pool of sera

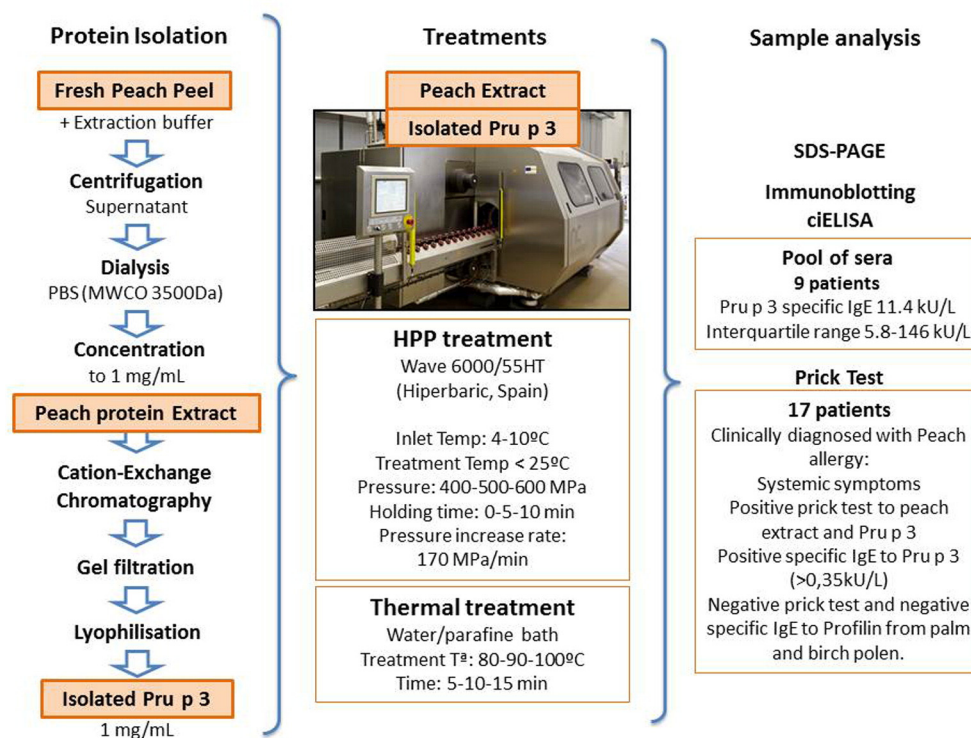


Fig. 1. Schematic diagram of sample preparation, Pru p 3 isolation and experimental design.

from 9 peach allergic patients). As negative control, a mixture (1:1) of PBS (0 ppm protein) and patient serum were also assayed. Each sample was analysed by triplicate in two independent experiments. The competitive reaction took place in the wells for 2 h at RT. Plates were then washed three times with PBST and incubated with 100 µL of goat anti-human Kappa-HRP (diluted 1/4000 in PBS) for 1 h at RT. Finally, plates were washed again with PBST, and 100 µL per well of TMB substrate were added. The enzyme reaction was stopped after 15 min by adding 50 µL of 2 M H₂SO₄ per well, and the absorbance of each well was determined at 450 nm using a microplate reader Thermo Scientific™ Varioskan Flash (Waltham, MA, USA). Modifications in IgE-binding to the protein (sample) due to the treatments with respect to untreated samples (native Pru p 3 or extract) and negative control (0% protein) was calculated for each sample as follows (adapted from Díaz-Perales et al., 2003):

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

With OD_{sample} defined as the value of absorbance of the treated sample, OD_{0%} defined as the value of control (untreated sample) and OD_{100%} defined as the absorbance of the negative control. Thus, a positive value ranging from 0 to 100% of IgE binding inhibition indicates a reduction of IgE binding to treated proteins (a hypothetical reduction of the allergenicity), while negative values indicate an increasing on protein IgE recognition (theoretical increase of allergenicity).

2.5. Patients and skin prick test

For skin prick test (SPTs), 17 peach allergic patients, seven of them with LTP syndrome (multiple plant-food sensitizations), were voluntarily recruited from local patients with a clinical diagnosis of IgE mediated food allergy: systemic symptoms (urticaria/angyoedema and digestive or respiratory symptoms after ingestion of peach), positive prick test with peach extract and isolated Pru p 3 (Laboratorios Bial, Bilbao, Spain), positive (>0.35 kU/L) specific IgE to Pru p 3 (ImmunoCAP FEIA

system, Thermo Fisher Scientific) and negative prick tests and negative specific IgE against Profilin from palm and birch pollen (Laboratorios Bial, Bilbao, Spain). More specifically, the 17 peach allergic patients had a median value of 8.61 kU/L of Pru p 3 specific IgE (interquartile range: 5 kU/L–8.6 kU/L). Each patient signed the corresponding informed consent for using results, and tests were performed with the Basurto Hospital Ethical Committee approval. SPTs were performed by on the volar surface of the forearm by using a standard 1-mm-tip lancet, following the recommendations of the EAACI (Dreborg, 1989). Histamine hydrochloride (10 mg/mL) and physiological serum were used as positive and negative controls, respectively. Patients were skin prick tested with untreated, pressurized (600 MPa/10 min) and thermally treated (100 °C/10 min, as control) samples (both Pru p 3 and extract). In order to avoid patients discomfort only the strongest treatments were tested. The wheal areas were measured in millimetres squared. The SPT was considered positive when the mean wheal diameter was 3 mm (area, 7 mm²) or greater than that produced by the negative control, and the differences between untreated and treated samples were calculated for each patient as the % of increase or decrease in wheals area.

2.6. Statistical analysis

The statistical analysis was carried out in SPSS (version 16 for Windows). Means were compared with Wilcoxon test for paired data. In all cases the comparisons were bilateral, with a p value of <0.05 considered to be statistically significant.

3. Results and discussion

As previously stated, the main objective of this project was to evaluate the impact of High Pressure Processing (HPP) on the allergenicity of peach main allergen (Pru p 3) obtained from peel extract.

In a preliminary stage to evaluate the impact of HPP, SDS-Electrophoresis and immunoblotting were performed (Fig. 2). SDS-PAGE of isolated Pru p 3 showed a proper protein isolation (Fig. 2a).

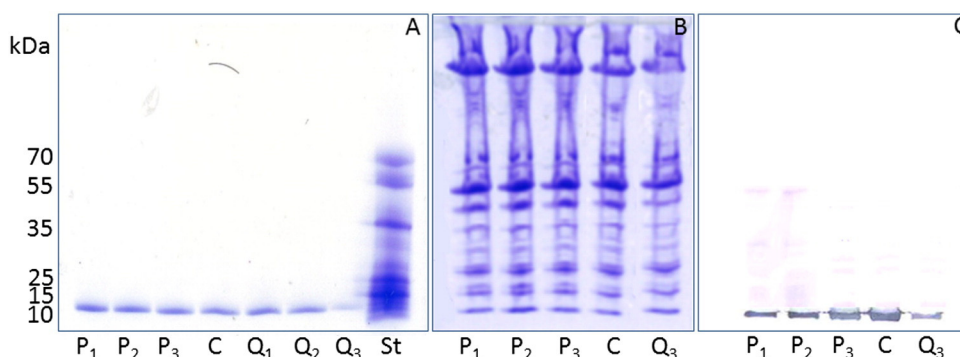


Fig. 2. A) SDS-PAGE of isolated Pru p 3, B) SDS-PAGE of peach peel protein extracts and C) Immunoblotting of peach peel protein extracts subjected to by different treatments: C: untreated control; Q1: thermal processing (80 °C/5 min); Q2: thermal processing (90 °C/10 min); Q3: thermal processing (100 °C/5 min); P1: 400 MPa/10 min; P2: 500 MPa/10 min; P3: 600 MPa/10 min.

However, no apparent changes in protein structure due to the treatment were observed except for the protein treated at 100 °C/15 min, which exhibited a clearer protein band. Contrarily, SDS-PAGE results from protein extract (Fig. 2b) showed that both thermal processing and HPP slightly modified the protein profile of the peach extract. The band corresponding to Pru p 3 ($\approx$ 9–13 kDa measured by SDS-PAGE according to Pastorello et al., 1999 and Sánchez-Monge et al., 1999) can be clearly seen in electrophoretic profile of peach extract. Contrarily to isolated protein, Pru p 3 bands seemed to remain unaltered in all cases, probably due to a protective effect of the matrix.

When peach extracts were analysed by immunoblotting, the band matching to Pru p 3 molecular weight appeared as the most relevant immunoreactive protein (Fig. 2c). Although this method is only qualitative, high pressure treatments (especially at 400 and 500 MPa) seemed to cause a slight increase of the specific Pru p 3 recognition by IgEs from patient sera (samples P1 and P2). Contrarily, heat treatment at 100 °C (Q) had a slight apparent effect in reducing peach IgE-binding, showing more blurred band intensity than untreated control (C).

As seen in previous works, LTP allergens retain their allergenic properties in thermally processed foods (Brenna et al., 2000). However, the increase in IgE-binding caused by HPP that can be discerned from immunoblotting, suggest an increase in the ability of HPP-treated peach foods to elicit allergic reactions. Thus, these preliminary results evidence that the impact of food processing technologies must be considered and measured regarding the allergenic potential of foods in order to provide useful data for management of allergens risks in food industry (Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2014; Mills et al., 2009; Verhoeckx et al., 2015).

In any case, this immunochemical method is only qualitative and consequently, in order to obtain quantitative data and confirm the apparent intensification of IgE recognition caused by HPP, isolated Pru p 3 and peach extract samples were also tested by competitive ELISA.

As results show, in general, HPP has slight effect on IgE binding measured by ELISA in both isolated Pru p 3 and peach extract (Fig. 3). However, it can be seen that the allergenic potential of peach proteins

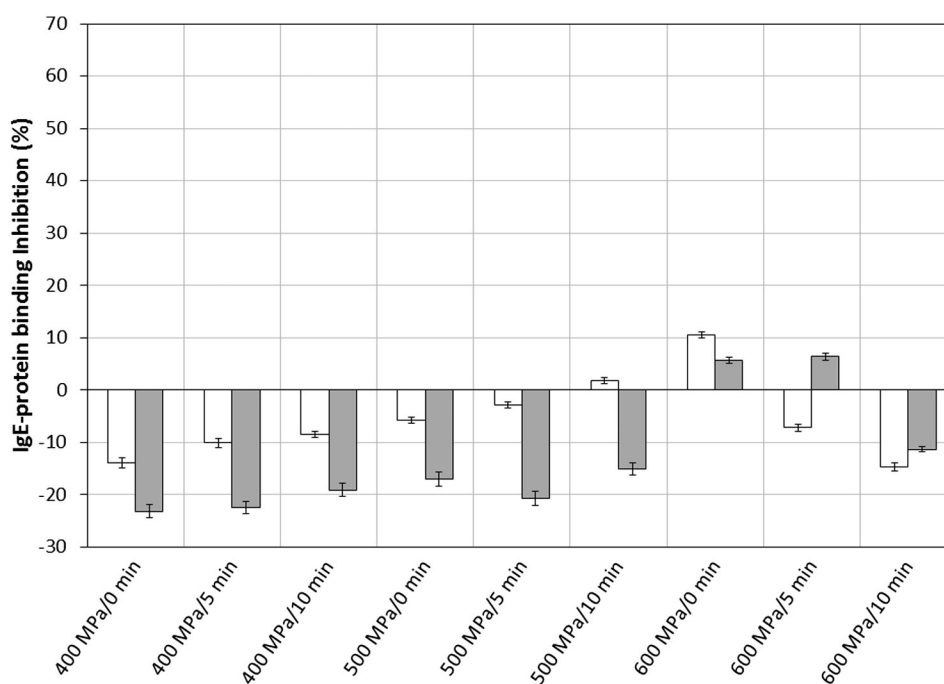


Fig. 3. Effect of different pressure treatments (MPa/min) on the recognition of isolated Pru p 3 (white columns) and peach peel protein extracts (grey columns) by a pool of peach allergic patients sera, measured by ciELISA. Results are expressed as the % of modification of IgE protein recognition caused by treatments, taken as reference untreated sample (0% inhibition). Results are mean values of results obtained from two independent experiments, analysed by triplicate (n = 6).

depends on the intensity of the treatments (pressure/time combination) and the food matrix in which the allergen is processed.

When Pru p 3 was treated isolated, the higher increase in IgE binding to the protein was observed at 400 MPa, independently of the time of processing, and 600 MPa/10 min treatments. Only one treatment showed to provoke a slight IgE inhibition (600 MPa for 0 min: which means pressure increase up to 600 MPa and immediate pressure release). When we observe peach peel extracts results, treatments at 400 and 500 MPa, independently of the time, increase IgE binding to Pru p 3 in all cases. These results confirm that high pressure may increase to some extent the allergenic potential of Pru p 3. Only in a narrow range of pressure/time combinations (600 MPa for very short times) results indicate that pressure would permit to reduce recognition by IgE. However, the impaired IgE-binding obtained with this treatment is insignificant from a practical point of view: <10% of inhibition is not relevant enough to be applied in food development for the allergic population.

On the other hand, mild thermal treatment (80–90 °C) had only slight effect on IgE recognition (Fig. 4). Surprisingly, by increasing the temperature and the time of treatment, up to a 60% of inhibition of IgE-binding was obtained (100 °C/15 min) when the protein was treated isolated. Nevertheless, concerning the results obtained with peach extract, only a 25% of inhibition was achieved with the same treatment. Thus, there is a high effect of food matrix on the allergenic potential of Pru p 3 protein, clearly increasing its resistance, when it is subjected to a technological treatment. Taking into account that food proteins are usually treated in complex matrices, our results in peach extract show that this protein maintains or even increases its immuno-reactivity in peach products treated by both high pressure or thermal processing, even after strong processing conditions, confirming previous results (Brenna et al., 2000; Garino et al., 2012). Pru p 3 is characterised by four highly conserved intramolecular disulphide bonds. This structure is the responsible of its high resistance to heat and digestion. In fact, even reducing agents are not always able to impair the IgE-binding properties of this protein (Gómez-Casado et al., 2015). Consequently, this protein conformation would be also

responsible of Pru p 3 resistances to high pressure. In this work, no ostensible modification of Pru p 3 structure, measured by SDS-PAGE, was observed due to HPP processing. However, the slight increase in IgE-binding after a pressure treatment observed by *in vitro* methods (immunoblotting and ELISA) suggests that some protein unfolding is caused by pressure. This modification does not destroy protein or epitopes structure, but may make more accessible epitopes usually buried in the compact 3D structure.

Beyond *in vitro* results, in the last part of this study, skin prick tests (*in vivo* test) were performed with diagnosed peach allergic patients. In order to avoid patients discomfort during the procedure, only the treatments which had exhibited the highest impact on IgE-binding to isolated Pru p 3 (100 °C/15 min and 600 MPa/10 min) were studied compared to untreated samples.

Results of wheal diameters are related in Table 1 for each patient, and differences in allergenicity (%) are shown in Fig. 5 and Fig. 6 for isolated Pru p 3 and peach peel extracts, respectively.

As it can be seen, the allergenic potential of a food highly depends on personal sensitization and individual results were, as expected, very variable depending on the patient.

By one hand, when isolated protein was HPP treated, in comparison with raw proteins, a reduction of the reaction (wheal diameter) was observed in 41% of patients, no changes occurred in 24% and an increase of allergenicity was detected in 35% of individuals (Fig. 5). Contrarily, regarding the results obtained with peach protein extract (Fig. 6), immune skin reaction was increased by HPP in 53% of patients, while only in 33% of them the response was reduced. An important IgE-binding region has been suggested to be hidden in the structure of Pru p 3 (García-Casado et al., 2003). This fact would explain the high variability observed individually, since the suggested release of this epitope by unfolding caused by pressure could be the reason of the rise of allergenicity observed in some cases (probably those whose immune response is mainly directed against this specific epitope).

However, taken altogether skin prick tests data, the comparison of the media of the wheal sizes against Pru p 3 showed that between the control (untreated sample) and HPP treated protein there were no

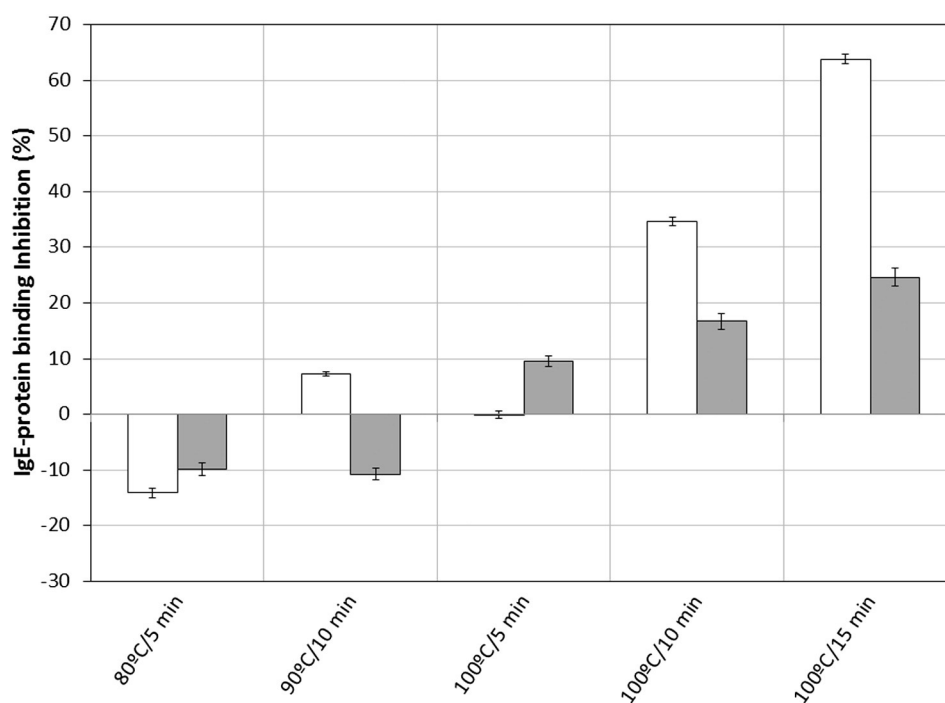


Fig. 4. Effect of different thermal treatments (°C/min) on the recognition of isolated Pru p 3 (white columns) and peach extracts (grey columns) by a pool of peach allergic patients sera, measured by ciELISA. Results are expressed as the % of modification of IgE protein recognition caused by treatments, taken as reference untreated sample (0% inhibition). Results are mean values of results obtained from two independent experiments, analysed by triplicate (n = 6).

Table 1

Effect of pressure (HPP: 600 MPa/10 min) and thermal treatment (Q: 100 °C/15 min) on the allergenicity (wheal diameter, mm²) of isolated Pru p 3 or Peach extract, measured by skin prick test in peach allergic patients. Allergenicity modification is expressed as the % of the wheal area of untreated samples (100% allergenicity = untreated sample).

Patient Id.	Isolated Pru p 3					Peach extract				
	C (mm ²)	HPP (mm ²)	% allergenicity (HPP/C)	Q (mm ²)	% allergenicity (Q/C)	C (mm ²)	HPP (mm ²)	% allergenicity (HPP/C)	Q (mm ²)	% allergenicity (Q/C)
B.I.F.	64	72	113	–	0	56	80	143	40	71
A.M.R.	20	28	140	–	0	24	50	208	35	146
S.M.C.	30	36	120	–	0	180	56	31	40	22
L.V.Z.	70	40	57	–	0	45	28	62	16	36
N.V.R.	32	30	94	–	0	80	88	110	12	15
A.R.P.	98	49	50	9	9	N.A.	N.A.	N.A.	N.A.	N.A.
M.M.A.	9	9	100	–	0	45	16	36	6	13
J.L.B.	30	30	100	–	0	N.A.	N.A.	N.A.	N.A.	N.A.
N.M.G.	36	36	100	–	0	63	36	57	20	32
D.P.G.	78	42	54	–	0	144	25	17	66	46
B.G.P.	56	30	54	–	0	96	96	100	16	17
B.S.E.	20	25	125	–	0	108	153	142	150	139
J.F.D.	42	80	190	–	0	112	220	196	56	50
A.R.S.J.	20	16	80	–	0	56	72	129	42	75
S.B.M.	36	25	69	–	0	36	66	183	30	83
E.C.B.	48	30	63	–	0	96	91	95	36	38
P.G.G.	60	80	133	–	0	60	143	238	72	120

N.A.: data not available.

significant differences ($Z = -0.79$; $p = 0.43$). Concerning the peach peel extract, results were very similar. By comparing the media of sizes of wheals obtained in prick tests, there were no significant differences between control and HPP samples ($Z = -0.35$; $p = 0.73$). Thus, these general *in vivo* results do not confirm the slight increase of allergenic potential of Pru p 3 by HPP, as it had been previously evidenced by immunoblotting and ELISA with a pool of sera. *In vitro* procedures showed an increase if IgE-binding to protein about 15–20%. However, this slight change in the ability of a food protein to bind IgE does not have to necessarily translate into altered *in vivo* allergenic functionality (Verhoeckx et al., 2015). No previous data have been found about the effect of high pressure in peach allergens, but similar related allergens appear to be also resistant to denaturation due to HP treatment, maintaining unaltered its allergenicity (Fernández, Butz, & Tauscher, 2009; Husband et al., 2011; Johnson et al., 2010).

On the other hand, heat treatment unexpectedly inhibited the skin allergenic reaction against the isolated protein in almost all cases (Table 1, Fig. 5). Thermal processing was also more effective than HPP in reducing allergenicity of peach peel extracts, as elucidated by

in vitro test, since 60% of the patients showed a reduced skin reaction against heated extracts compared to raw extract (Fig. 6). Indeed, there was a significant difference between the media of the wheal sizes caused by untreated isolated Pru p 3 and the media of sizes of wheals provoked by thermally treated samples ($Z = -3.62$; $p < 0.001$). Also, there were important differences between the reactivity of untreated and heated extracts ($Z = -2.67$; $p < 0.01$).

Thus, these results confirm previous *in vitro* results, showing that thermal processing can reduce Pru p 3 allergenicity. These results are also in agreement with previous researchers studying structural stability of isolated Pru p 3, who found that this protein was unable to refold after heating (95 °C) at neutral pH conditions (Gaier et al., 2008), as those studied in this work. This loss of structure due to thermal processing would explain the more effective loss of allergenicity (Toda et al., 2011), contrary to the effect obtained with high pressure. However, as it is seen, when Pru p 3 is treated in extracts and complex matrices, the protein is more resistant to denaturation and consequently, skin reaction reduction is less important than with isolated protein. Indeed, when the impact of heat processing on Pru p 3 allergenicity was studied

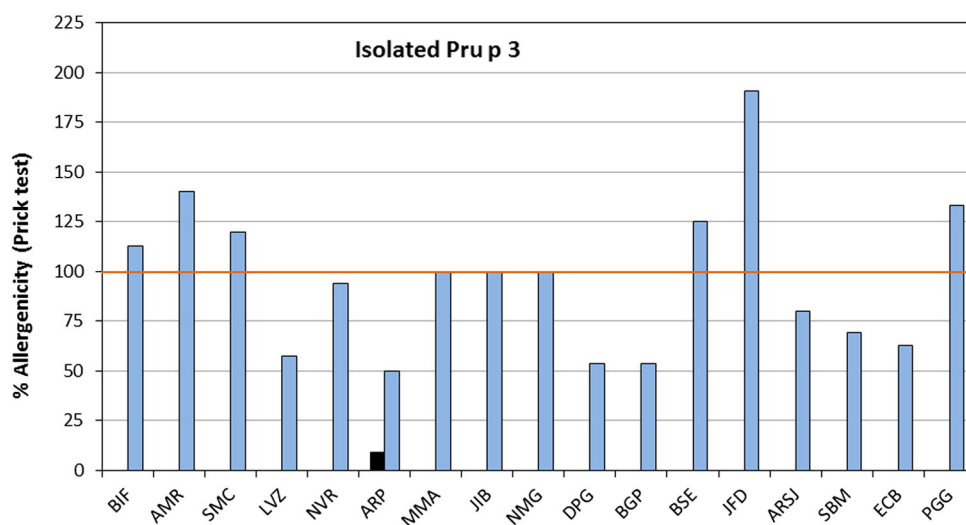


Fig. 5. Effect of pressure (HPP: 600 MPa/10 min, grey bars) and thermal treatment (Q: 100 °C/15 min, black bars) on the allergenicity of isolated Pru p 3, measured by skin prick test. Allergenicity modification is expressed as the % of the wheal area of untreated samples (100% allergenicity = untreated sample).

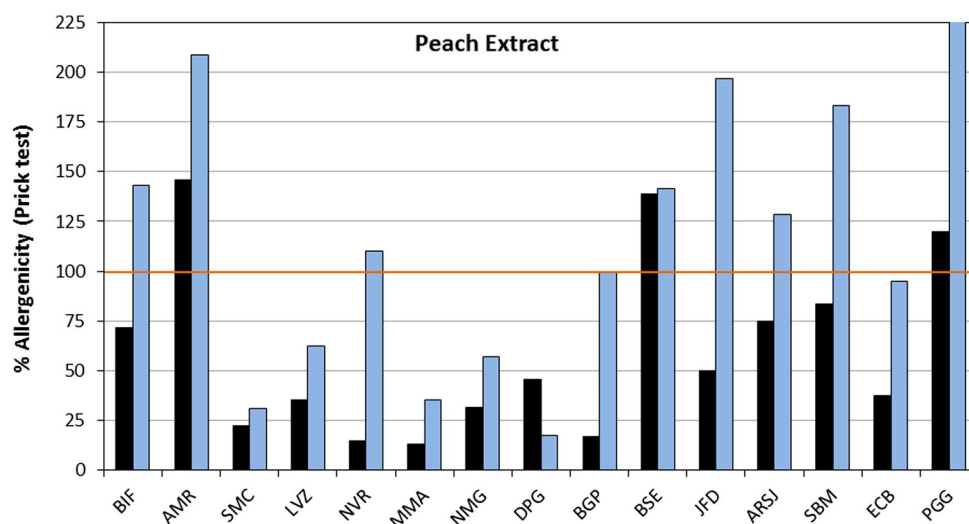


Fig. 6. Effect of pressure (HPP: 600 MPa/10 min, grey bars) and thermal treatment (Q: 100 °C/15 min, black bars) on the allergenicity (wheal diameter, mm²) of peach peel extract, measured by skin prick test. Allergenicity modification is expressed as the % of the wheal area of untreated samples (100% allergenicity = untreated sample).

in juices or nectar (Brenna et al., 2000; Garino et al., 2012), no changes were observed. These differences, likewise observed in our work, are clearly due to the influence of food matrix in allergen modification during processing. The higher resistance of allergenic proteins to processing and digestion due to matrix has been also previously described for other proteins (Maleki, 2004; Nowak-Węgrzyn & Fiocchi, 2009; Sancho et al., 2005; Schulten, Lauer, Sheurer, Thälhammer, & Bohle, 2011), and confirms the fact that the ability of a technology to reduce the allergenicity of a protein particularly depends on the environmental conditions. Other technologies with the ability of completely affecting the structure of this protein could achieve an important reduction of peach allergenicity. For instance, combination of high pressure with other technologies or strategies (heat, enzymes...) has been already shown to induce a reduction of immunoreactivity in similar cross-reactive proteins (Chung, Houska, & Reed, 2013; Husband et al., 2011; Johnson et al., 2010; Somkuti & Smeller, 2013).

Our results open an interesting research scope, with a need of deeper studies in order to better understand the modifications that lead to a loss of Pru p 3 allergenic properties. Further studies in isolated protein should be performed in order to investigate the structural changes during the pressure treatment, the irreversibility of the changes, not counting to understand potential interactions with other component that may increase peach allergenicity. Moreover, further research has to be accomplished with other technologies and other peach-containing food matrices with the objective of reducing its allergenicity.

4. Conclusion

In this work, results obtained by ELISA and skin prick test showed that HPP can alter the allergenic potential of peach proteins, by either reducing or increasing its allergenicity, depending on the patient. Nevertheless, in most cases this alteration reaches to an increase of the allergenicity, instead of the desired reactivity reduction, probably due to the fact that pressure processing releases a major IgE epitope, usually hidden in Pru p 3 structure. This fact reinforces the idea that processing is an important factor to be taking into account in allergen risk management in food industry. The high variability in results observed with pressurized samples in peach allergic population, together with the effect of food matrix and the higher effectiveness of intense thermal treatment in reducing allergenic reaction to isolated Pru p 3, points to further investigations to explore other treatments (e.g. high pressure/high temperature combination) or strategies to reduce peach allergenicity more effectively.

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