JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Evaluation of the Impact of Sequential Microwave/Ultrasound Processing on the IgE Binding Properties of Pru p 3 in Treated Peach Juice

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ABSTRACT: Peach lipid transfer protein (LTP) can cause severe allergic reactions to peach-allergic patients. It belongs to the nonspecific LTPs family, a class of proteins extremely resistant both to proteolytic digestion and to high temperatures. Food processing can either drop or increase the allergenicity, depending on the process and on the food. As far as peach-derived products (pulp, juice) are concerned, it has been previously shown how thermal treatment performed in an autoclave does not decrease LTP allergenicity. In this work, it was attempted to investigate whether sequential microwave and ultrasound processing could affect the allergenicity of peach juice. Incubation with specific anti-Pru p 3 serum showed how treating peach peel with microwave at 140 °C and with ultrasound does not eliminate Pru p 3 IgE binding properties. The application of MW/US protocol on peach pulp appeared to be insufficient for the reduction of IgE binding to Pru p 3.

KEYWORDS: *peach, Pru p 3, microwave, ultrasounds, IgE binding*

INTRODUCTION

Lipid-transfer proteins (LTP) are present in high amounts in higher plants. They form a protein family of basic polypeptides of ±9 kDa, the members of which are located extracellularly, usually associated with plant cell walls, and possess a broad lipid-binding specificity closely related to their three-dimensional structure. The nsLTP fold is characterized by a compact domain composed of 4 α-helices, firmly held by a network of 4 conserved disulfide bridges. This fold presents a large internal tunnel-like cavity, which can accommodate different types of lipids. Lipid transfer proteins are involved in several plant defense mechanisms, none of which has been yet extensively described.[1,2]

Several members of the nsLTP family have been identified as relevant allergens in plant foods and pollens.[3] Despite that they are widely distributed among plants, their clinical relevance is largely confined to the Mediterranean area. LTPs are normally considered true food allergens because they are able to sensitize via the gastrointestinal tract, and their IgE reactivity is often associated with severe systemic symptoms.[4] Pru p 3 is the major allergen in peach, and it is also the most recognized by allergic patients sera in Spain.[5] Most of the peach-allergic patients who are sensitized to Pru p 3 present systemic symptoms after peach ingestion, while patients with oral allergy syndrome (OAS) mostly present profilin-Bet v 1-related sensitization.[6] It has been recently reported how in patients with allergy to fruits belonging to the Rosaceae family, the detection of high levels of Pru p 3 specific IgE can be regarded as a warning for systemic reactions.[7]

In 2003, García-Casado and co-workers were able to identify the IgE-binding epitopes on the major peach allergen Pru p 3, positioning them between the end of an α-helix and some residues of the following interhelix loop.[8] Such epitopes present a high degree of conservation (>80%) among proteins of the Rosaceae family.[9] LTPs present a high resistance to both heat treatment and digestive proteolytic attack, as previously shown for many food plants.[10−14] However, it was recently reported how such proteins, in peach, appear to be resistant to pepsin and chymotrypsin, whereas the action of trypsin was able to generate low and high molecular weight peptides. The last ones consisted of the full Pru p 3 protein, with the disulfide bridges still intact, but they were lacking the smaller peptides. The intact protein and the high molecular weight peptides were found to be recognized by patients' sera, whereas the small peptides were found to be not reactive.[15]

Food processing technologies have a significant impact on the allergenicity and antigenicity of allergenic foods. Recent studies have attempted to determine the effect of processing on certain food allergens, to assess involved risks for the allergic consumer and to find methods to reduce or abolish allergenic activity of food allergens, as a prerequisite for the production of non- or hypoallergenic foods. However, not all attempts to reduce or eliminate food allergenicity through food processing have been successful. Physical processing typically affects the three-dimensional structure of proteins; it may either destroy existing epitopes on a protein or generate new ones (neoallergen formation), as a result of change in protein conformation.[16,17] Therefore, the processing may sometimes increase the allergenicity, as has been seen for roasted peanuts[18]

Received: May 10, 2012
Revised: July 25, 2012
Accepted: August 13, 2012
Published: August 13, 2012

or for irradiated gluten and wheat dough.[19] Any single process on its own is very unlikely to be sufficient to substantially reduce (or entirely eliminate) the allergenicity of an allergenic food, but combinations of various treatments (such as chemical lye peeling combined with ultrafiltration, or high-pressure treatment in the presence of proteolytic enzyme) have proven rather effective in producing hypoallergenic peach juice[10,20] and hypoallergenic rice.[21] Understanding the impact of food processing and food structure on allergenic potential is central to manage allergen risks in the food chain. Knowledge of how processing or food structure may alter threshold doses of allergens able to elicit an allergic reaction is highly relevant for managing allergens in a factory environment.[22] However, current knowledge of the impact of food processing on allergen structure indicates that there are no clear rules regarding how different allergens respond to food processing.

In the past years, new sustainable and environmental friendly techniques for the preservation of foods, such as ultrasounds-assisted or microwave processing, have rapidly developed, because they offer a series of advantages in term of productivity, yield, and selectivity, improving the processing time and the quality, and reducing chemical and physical hazards.[23] The use of microwaves (MW) and ultrasounds (US) in pasteurization has proven effective in the inactivation both of the microbial population and of the enzymes responsible for the deterioration of fruit juice,[24,25] while, in combination with heat, US can accelerate the rate of sterilization of foods. Combined microwave (MW) and ultrasound (US) irradiation, whether simultaneous or sequential, has proven to be an effective technique in process chemistry, organic synthesis,[26] pollutant degradation,[27] and food processing: for instance, the simultaneous application of heat and USs on raw milk before fermentation allowed one to significantly improve the rheological and qualitative properties of yogurt, demonstrating an effective impact on proteins structure.[28] Recently, the combination of thermosonication with pulsed electric fields has been employed for the pasteurization of orange juice, improving its storage life without affecting the sensory acceptability by consumers.[29] The combined use of MW and US aimed at the reduction of allergenicity of foods has not yet been reported in the literature.

The focus of the present work was to evaluate the impact of MW/US sequential processing on Pru p 3 IgE binding capacity, treating pulp meant for the production of peach juice.

MATERIALS AND METHODS

Sample Materials. Nectarines (actually a peach variety) from the "Sweet Red" cultivar (yellow pulp) were kindly provided by the "Consortium for research, experimenting and popularization for the market gardening of Piedmont" (CRESO, Manta di Saluzzo, Italy), and they belonged to the 2009 harvest. Peel samples were manually removed from peach fruits with a knife, while pulp was homogenized using a common blender and stored at −20 °C immediately after arrival, before all of the technological processing with MW and US took place.

Physical Processing. Microwave irradiation was carried out in a multimodal professional reactor synthWAVE (Milestone, Bergamo, Italy). Samples were irradiated under mechanical stirring. Software "easy wave" enables the operator to monitor the power/temperature protocol in real time. Ultrasounds were then applied using a high-intensity US device, a probe system with a titanium horn (frequency is 21 kHz), developed by Danacamerini sas (Torino, Italy). In this device, the electronic generator acting on the oscillating circuit continuously adjusts the US frequency (±0.5 kHz maximum variation) to the actual resonance value of the reaction system (which is a function of the viscosity, dissolved gases, acoustic impedance, and so on). This value corresponds to the frequency that maximizes the US output for a given power setting (lock frequency system).[30]

Peel material was ground in a mortar, and distilled water (1:1) was added prior to the MW treatment. The mixture was heated in a MW oven at 140 °C for 30 min under constant stirring. Afterward, a sonication protocol was applied for 15 min at 150 W, developing a final temperature of 88 °C. All pulp samples (treated and untreated) were initially diluted 1:1 with distilled water, prior to MW irradiation. Treated samples were heated in a MW oven at 100 °C for 30 min with constant stirring. Afterward, they underwent a sonication protocol for 30 min at 150 W, developing a final temperature of 88 °C.

Protein Extraction Protocol. Protein extraction protocol was carried out both on treated and on untreated peach material. Protein extraction from peel samples was performed as follows: 50 mL of extraction buffer (Tris Glycine 0.01 M and PBS buffer 50 mM, pH 7.5) was added to 5 g of ground lyophilized peel powder and stirred for 1 h at room temperature. Samples were then centrifuged at 4600*g* for 20 min at 4 °C, and supernatants were collected and dialyzed overnight versus distilled water using 1 kDa cutoff membranes (Spectra/Por Regenerated Cellulose Dialysis Tubing 1K MWCO), to eliminate the excess of salts. After the dialysis, a 50 mM solution of three bivalent cations ($CaCl_2$, $MgCl_2$, $MnCl_2$) was added to samples, to obtain a final concentration of 5 mM, following Jona and Fronda protocol.[31] To allow pectin precipitation, the pH was set to 7.0 using NaOH, and samples were left to stir for 2 h at room temperature. Samples were then centrifuged at 13 000*g* for 20 min at 4 °C, and the supernatants were once again dialyzed overnight versus distilled water using 1 kDa cutoff membranes. Dialyzed samples were then filtered by syringe using 0.22 μm cutoff filters. Finally, to reduce the volumes obtained, peel samples were concentrated using 3 kDa cutoff membranes (Microcon, Amicon, U.S.).

Protein extraction from pulp samples was performed as follows: samples were left to stir in extraction buffer as described above at room temperature for 30 min, they were centrifuged at 4600*g* for 20 min at 4 °C, and the supernatants were collected. Extraction buffer was added to the pellets, just enough to enable the stirring, and samples were left at room temperature for at least 2 h. Once again, samples were centrifuged at 4600*g* for 20 min at 4 °C, and the supernatants were joined to those previously collected and dialyzed overnight versus distilled water using 1 kDa cutoff membranes. After the dialysis, bivalent cations solution was added as previously described for peels. Samples were then centrifuged at 13 000*g* for 20 min at 4 °C, and the supernatants were once again dialyzed overnight versus distilled water using 1 kDa cutoff membranes. Dialyzed samples were then filtrated by syringe using 0.22 μm cutoff filters. Finally, to reduce the volumes obtained, samples were lyophilized.

Total Protein Quantification. At first, protein extracts were quantified as mg/albumin equivalent according to Bradford,[32] using the reagent from Sigma-Aldrich Srl (Milan, Italy). As far as pulp extracts are concerned, quantification of protein using the Bradford method was unreliable when compared to the sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS PAGE) protein profile. For this reason, elemental analysis on lyophilized extracts from both treated and untreated pulp samples was performed (Redox snc, Monza, Italy).

Serum Samples. To detect the presence of peach LTP in the analyzed extracts, polyclonal rabbit anti-Pru p 3 sera were used.[33] Immunoblots assays were performed using sera from allergic patients, provided by the Regional Allergology Network and selected mainly on the basis of their availability. Among them, 5 sera were expected to be positive on our peach extracts, while 3 were expected not to show any binding. Serum number 1 displayed positivity to LTP both by SPT and by PBPT; moreover, RAST highlighted the presence of specific anti-rPru p 3 immunoglobulins. Sera number 2 and 3 were also positive to LTP on SPT, although LTP was not tested neither by PBPT nor by RAST; moreover, PBPT performed using peach showed negative results in serum number 3. Serum number 4 was not tested for LTP, but it displayed positivity on PBPT for peach. Sera number 5 and 6 were never tested for LTP or peach, although the patient number 5

Table 1. Clinical Characteristics of the Sera Used[a]

patient no.	sex	year of birth	clinical history	positive on SPT	positive on PBPT	RAST (kUA/L)
1	M	1971	food allergic, U	LTP, mu, pn, pc NT, so	ap, ar, ch, co, gb, LTP, pc, pl	pn (0,43), rPru p 3 (1,12), wa (0,40)
2	M	1971	food allergic, U	LTP, pc NT	ch, LTP NT, pc, pl	NT
3	F	1993	food allergic, U	be, bp, cm, co, gr, ha, LTP, mu, pc NT, ol	al, ap, ba, cn, ki, LTP NT, me, pc NEG, pn, to, wa	NT
4	M	1964	asthmatic, food allergic, OAS	gr, ha, LTP NT, pc NT, tr	LTP NT, pc	NT
5	M	1974	asthmatic, food allergic, OAS, U; symptoms after peach consumption	ch, co, gr, ha, LTP NT, ol, pc NT, pe, pn, so, to, tr	al, ap, ki, LTP NT, pc NT, pl	al (3,69), ap (43,00), co (1,34), ki (0,55), wa (3,14)
6	F	1963	pollen and drug allergic, OAS	LTP NT, mu 3+, pc NT, so	NT	NT
7	M	1983	grass and food allergic, U	be, ce, co, LTP NT, pc NEG, sh, so 2+, to	ap, ki, LTP NT, pc NT	ap (0,41), co (21,6), gr (50,80), ha (0,54), rPhl p 5 (17,20), rPru p 1 NEG, rPru p 3 NEG
8	F	1964	pollen and food allergic, OAS	LTP NEG, pc NT, rHev b 8, tr	ap, ca, ki, LTP NT, pc NT	NT

[a]OAS, oral allergy syndrome; U, urticaria; SPT, skin prick test (2−3+: 2−3 three times the histamine control); PBPT, prick by prick test; RAST, radio allergo-sorbent test (sIgE cut-off value >0.35 kUA/L); al, almond; ap, apple; ar, apricot; ba, banana; be, beans; bp, birch pollen; ca, carrots; ce, celery; ch, cherry; cm, cereals mix; cn, coconut; co, corn; gb, green beans; gr, grass; ha, hazelnut; ki, kiwi; me, melon; mu, mugwort; ol, olive; pc, peach; pe, pea; pl, plum; pn, peanut; sh, shrimp; so, soybean; to, tomato; tr, tree pollen (birch, alder, hazel); wa, walnut; NT, not tested; NEG, negative.

reported symptoms after peach consumption. Sera number 7 and 8 displayed negative results, either by RAST toward rPru p 3 (serum number 7) or by SPT toward LTP (serum number 8). Immunological features of the sera used in the immunoblot assay are reported in Table 1.

SDS-PAGE and Blotting. Gel electrophoresis and blotting experiments were performed independently in the laboratory of Allergy of the Academic Medical Center of Amsterdam (The Netherlands), and at the Food chemistry, biotechnology and nutrition unit of DISCAFF in Novara (Italy), following different complementary approaches.

In the first approach, total proteins from peel and pulp were resuspended in LDS loading buffer (Invitrogen, Carlsbad, CA) and separated by SDS-PAGE (NuPAGEs 4−12% Bis-Tris gel, Invitrogen, U.S.) using reducing running conditions. Proteins were then visualized via a silver staining procedure.[34] Western blotting was performed by transferring the proteins semidry to nitrocellulose (0.2 μm) on a Novablot electrophoretic transfer apparatus, according to the manufacturers' protocol (Invitrogen, U.S.). After being blocked with PBS/5% skimmed milk powder/0.02% Tween-20 for a minimum of 10 min, the blots were washed three times (PBS/0.02% Tween-20) and incubated overnight with 1 μL of polyclonal rabbit anti-Pru p 3 serum in 30 mL of PBS/0.02% Tween-20/0.5% skimmed milk powder. After being washed five times (PBS/0.02% Tween-20), blots were incubated for 4 h with goat antirabbit IRDye800 (Li-Cor, Providence, RI) and washed as before. The IRDye800-labeled proteins were detected using the Odyssey V3.0 (Li-Cor).

In the second approach, lyophilized protein extracts from pulp were resuspended in SDS buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) to a final concentration of 10 mg/mL, according to the quantification data obtained through elemental analysis, using the formula:

$$\text{total protein concentration} = \text{nitrogen concentration} \times 6.25$$

Resuspended protein samples were dissolved (2:1) in Laemmli sample buffer[35] with 2-mercaptoethanol (20%), and separated by SDS-PAGE with 4−15% Mini-PROTEAN TGX Precast Gels (SDS-PAGE; Mini-PROTEAN III Cell, Bio-Rad, Richmond, CA). Blotting was performed by transferring the proteins to nitrocellulose (0.45 μm Protran Nitrocellulose Transfer Membranes, Perkin-Elmer Inc., U.S.) on a Bio-Rad electrophoretic transfer apparatus, according to the manufacturers' protocol (Mini-PROTEAN II Cell, Bio-Rad, Richmond, CA.). After being blocked with PBS/5% skimmed milk powder/0.02% Tween-20 for 1 h, the blots were washed three times with PBS/0.02% Tween-20 and incubated overnight with antiserum. The presence of Pru p 3 on blot was monitored by adding polyclonal rabbit anti-Pru p 3 serum 1:30 000 (v/v) in 30 mL (or 3 mL when strips were used) of PBS/0.02% Tween-20/0.5% skimmed milk powder. To monitor human IgE binding to peach immunoblot, 3 mm wide blot strips were incubated overnight with 100 μL of human serum in 3 mL of PBS/0.02% Tween-20/0.5% skimmed milk. After being washed five times with PBS/0.02% Tween-20, blots were incubated for 4 h with horseradish peroxidase (HRP)-labeled goat antirabbit IgG (Bio-Rad Laboratories, Richmond, CA) or HRP-labeled antihuman IgE (KPL, Gaithersburg, MD). Detection of HRP-label was performed with enzymatic chemiluminescence. Blots were exposed to X-ray film (Hyperfilm ECL; GE Healthcare, U.S.).

RESULTS

As previously declared, the main aim of the project was to evaluate the impact of a strong combined physical treatment on the stability of peach main allergen Pru p 3 obtained from pulp extract that was subjected to sequential MW and US.

In a preliminary stage, to evaluate the impact of the proposed treatment on the antibody binding activity of Pru p 3, we decided to start from peach peel, where the LTP content is much higher than in pulp. Total proteins fraction from untreated control and treated peel samples were extracted and loaded on 4−12% SDS-PAGE to compare the protein profiles (Figure 1, left). High molecular weight (HMW) proteins present in the untreated sample disappeared after treatment, and only a very intensive signal was still present around 10 kDa. When specific anti-rPru p 3 antibody was used to detect the presence of peach LTP, both samples displayed a clear signal in the Western blot experiment. The signal detected in the untreated sample, however, seemed to be originated both from the band around 12 kDa and from the band at 8 kDa, while in the treated sample only the lowest band was detected by the antibody (Figure 1, right).

Because the final aim of the project was to evaluate a new processing method aimed at physically treating peach pulp meant for the juice production, the focus of the research was moved to the pulp. Because of the occurrence of Maillard reaction's products, which cause the browning of the material (probably increased by the occurrence of the carbohydrates caramelization) and the resulting spoiling of the look and taste

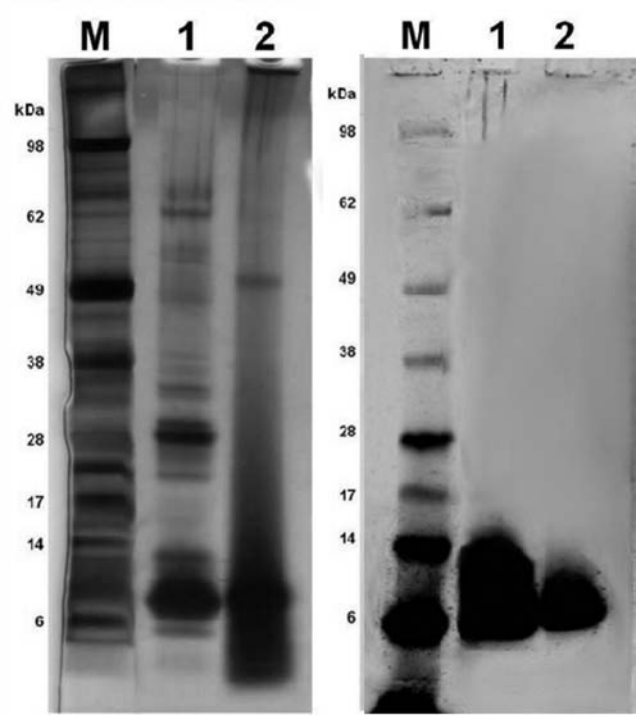

Figure 1. 4−12% SDS-PAGE (left panel) and Western blots (right panel) of peach peel total protein extract. M: marker. Lane 1: untreated control. Lane 2: peach peel heated for 30 min at 140 °C in MW and sonicated for 15 min at 150 W and 26 kHz. Protein staining was performed with silver staining. Blots were probed with rabbit anti-Pru p 3, and detected using the Odyssey V3.0 system.

of the drink,[36] it was not possible to apply the same thermal protocol used for the peach peel to the pulp. A temperature of 100 °C was chosen as the upper limit for the MW heating, while the sonication was prolonged to 30 min. These conditions were selected after setting up a study (data not shown), performed to limit the browning of the peach material (particularly peach pulps), correlated to the development of the Maillard reactions. We tried to select, among different protein extraction methods,[37,38] a protocol that yielded a clear protein fingerprint in the range of 6−17 kDa where presumably the LTP protein is present (data not shown). The chosen protocol has been described in the Material and Methods. The setting up of the total protein extraction method from the pulp was laborious, and several trials were carried out before its final optimization. The main problem was the low quality of the SDS/PAGE protein profiles, due to the high presence of carbohydrates, especially pectins, largely represented in this fruit matrix, and hence the removal of this interfering matter was addressed in detail. Pectins are a class of polysaccharides defined by the presence of galacturonic acid, in which the variable degree of methylation can influence their gelling properties.[39] Moreover, the gelling activity is enhanced by cold temperatures,[40] and the fruits used in our analyses were immediately stored at −20 °C after arrival, to preserve them. Several protocols attempting to remove pectins were employed, including a treatment with a pectinase from *A. niger*, which did not result in satisfying results. The only effective way to clear the pectins fraction away was to apply the method developed by Jona and Fronda.[31] This is based on the use of a bivalent cations solution, which is able to cause pectins precipitation. To precipitate them efficiently, we needed to raise pH up to neutrality, probably decreasing in this way the yield of LTP extraction.[41] However, we succeeded in obtaining around 12 mg of total proteins from 100 g of pulp (0,012%), a lower amount as compared to the 0.03% previously reported from peach.[42]

Proteins were extracted both from untreated and from MW/US treated fruit pulp and loaded on SDS-PAGE as described. The profile was similar to the one obtained by loading fruit peel extracts, even though some of the HMW proteins were still visible after silver staining (Figure 2). Western blot confirmed

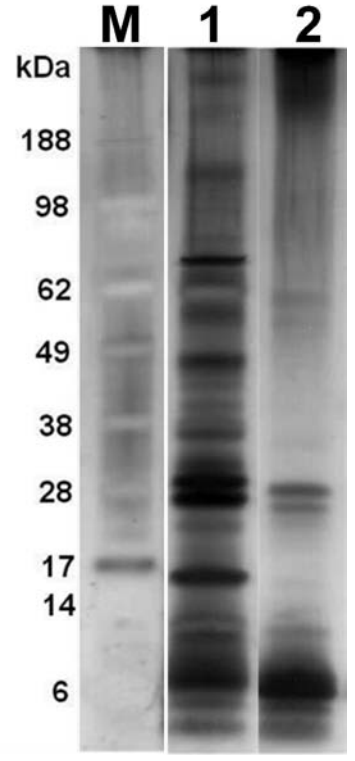

Figure 2. 4−12% SDS-PAGE of peach pulp total protein extract. Lane 1: untreated control. Lane 2: peach pulp heated for 30 min at 100 °C in MW and sonicated for 30 min at 150 W and 26 kHz. Protein staining was performed with silver staining.

the presence of Pru p 3 both in the untreated and in the treated sample. According to the results displayed in Figure 3, MW/US treated sample seemed to have an even greater affinity to the rabbit antisera than did the untreated one.

It was evaluated by 4−15% SDS-PAGE followed by immunoblot whether the combined physical treatment of peach pulp had an impact on the IgE binding characteristics to bind Pru p 3. Eight sera were employed in the further analysis, of which the clinical characteristics are shown in Table 1. Four of them were obtained from either peach or LTP-sensitized patients (sera nos. 1−4), while two were tested negative either on skin prick test or on radio sorbent allergo test (RAST, sera nos. 7−8). The last two sera were not tested for LTP or peach, although one of the two was obtained from a patient displaying symptoms (itching, urticaria, dyspnea, dysphonia) after ingesting peaches (serum no. 5). Results are displayed in Figure 4: in none of the employed sera was highlighted a clear observed loss (or even a sign of it) in the intensity of the 10 kDa band, relative to the presence of LTP; rather, in some sera the intensity seemed to rise.

dx.doi.org/10.1021/jf302027e | J. Agric. Food Chem. 2012, 60, 8755−8762

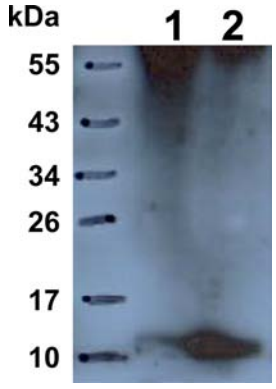

Figure 3. Western blots of peach pulp protein extracts probed with rabbit anti-Pru p 3. Lane 1: untreated pulp control. Lane 2: peach pulp heated for 30 min at 100 °C in MW and sonicated for 30 min at 150 W and 26 kHz. Detection was performed with enzymatic chemiluminescence.

DISCUSSION

According to reported estimations, the total content of LTP in a peach can vary between 4 and 24 mg/kg,[41] while other sources set the upper limit to 48 mg/kg. Internal variability of the LTP quantity is also quite broad, but no significant differences were observed among nectarines and peaches.[43] We decided to start with Sweet Red nectarine peel, because, on average, the total protein content of the peel is 2 times higher than that in the pulp, while the LTP contribution to the total protein fraction is 7 times bigger in peel than in pulp.[42] In a recent report, the exclusive localization of the LTP protein in the outer part of the fruit, such as peel and the thin layer of pulp immediately under it, has been assessed by matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI MSI).[44] Therefore, the use of peel as starting material, in a preliminary step of our investigation, was thought to ease the recovery of Pru p 3 after the application of the treatment.

Because of their three-dimensional structure, all nsLTPs are described to be extremely resistant both to proteolysis and to thermal treatment.[2] The putative IgE binding sites are highly conserved among different plant species, allowing this particular class of proteins to be considered as panallergens.[9] The LTP protein has shown to keep its ability to bind IgE in peach even after a heat treatment at 121 °C for 30 min,[10] although no tests to quantitatively measure the amount of bound IgE were performed. More recently, LTPs from both maize and cherry displayed similar reactivity to IgE[12,13] and ability to induce histamine release[13] after the thermal treatment, even though circular dichroism demonstrated an altered secondary structure of the maize LTP after cooking it at 100 °C.[12] However, in all previously mentioned cases, the heating process was performed by normal boiling in water bath or autoclave, and no additional physical treatments were considered. Ultrasound is known to have a destabilizing effect on protein structure, and it has already been successfully used on shrimp to reduce allergenicity caused by tropomyosin sensitization.[45] To the best of our knowledge, no data regarding the use of MW and US on LTP's allergenic stability are reported. Previous studies reported how auxiliary energies, such as microwave or ultrasounds, can accelerate oil oxidation, thus reducing the shelf life;[46,47] therefore, apart from the observations on allergens inactivation, an accurate evaluation of the juice integrity, performed through sensorial and chemical analyses, should be taken into account before the scaling up.

Strong thermal treatment (140 °C for 30 min) carried out on peels, followed by US treatment, seemed to affect the binding activity of the rabbit anti-Pru p 3. While in the untreated sample two bands were observed at 6 and 14 kDa, there was only one band detected in the treated sample at ±8 kDa. However, we cannot exclude that the upper band around 14 kDa could be due to the presence of an artifact in the untreated sample, later eliminated by the treatment. The existence of a truncated form of grape-LTP (6 kDa) after in vitro digestion[48] also pointed toward a similar very stable peptide backbone present in the LTP molecule. Severe heat treatment (100 °C for 2 h) induced minor changes in LTP-protein structure even in apple peel, but a significant decrease in IgE-binding (30-fold) and in its ability to induce the release of histamine in basophils (100−1000-fold) was observed.[14] To compare the rabbit antiserum with the human IgE response, we tried to assess whether IgE binding activity toward Pru p 3 was also affected in peach pulp by the MW/US treatment.

Impact on allergens due to food processing must be carefully taken into account. During the international workshop organized in 2006 by the ILSI (International Life Sciences Institute) Health and Environmental Sciences Institute Protein Allergenicity, participants agreed that investigating food allergy mechanisms, validating appropriate methods for identifying allergenic proteins, and refining strategies to assess and manage the risks from food allergy were important issues before processing considerations are integrated into public-health decision-making for novel proteins.[49] Thermally or biochemically processed foods, or even genetically engineered novel

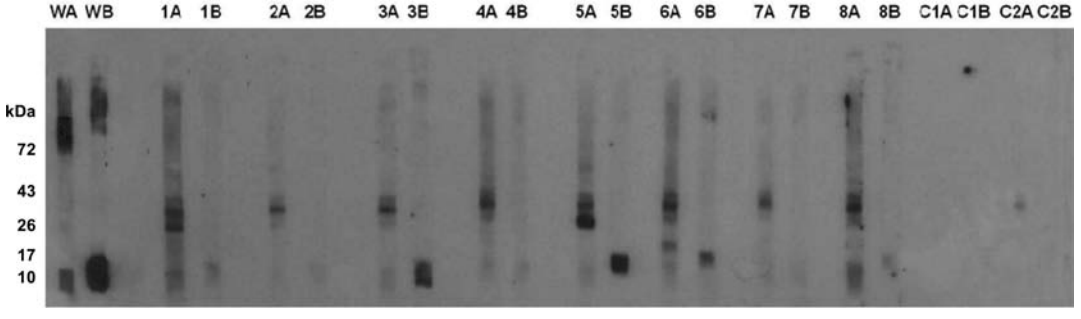

Figure 4. Immunoblots of peach pulp total protein extracts untreated (A) and heated for 30 min at 100 °C in MW and sonicated for 30 min at 150 W and 26 kHz (B). W = blots probed with rabbit anti-Pru p 3. 1−8 = blots probed using sera from 1 to 8. C1 = blots probed without rabbit anti-Pru p 3. C2 = blots probed without serum. Detection was performed with enzymatic chemiluminescence.

plant variants, are highly likely to have altered protein patterns; therefore, these methods have a great potential to reduce food allergenicity. However, in some cases, these methods may fail to reduce allergenic potential, may even increase it, or may reveal neo-epitopes that were masked in the native protein, but which become accessible and/or reactive after denaturation/renaturation of the protein.[50] In peach, chemical peeling and ultrafiltration have previously been demonstrated to be effective means for the preparation of hypoallergenic purées,[20] even though the allergenic power is not completely quenched.

A new technological approach prospecting a further decrease in the ability of eliciting an allergic reaction in sensitive consumers would open chances for the commercialization of new hypoallergenic lines. Unfortunately, no evidence of such abatement, referring to human IgE binding ability, was observed when the MW/US protocol was applied to peach pulp. On the contrary, for some of the employed sera (numbers 3 and 5), the signal given by the IgE interaction with the protein appeared more intense in the treated samples than in the untreated ones. This fact could be due to a change in the three-dimensional conformation of the allergen, which brings a different presentation of the epitope. Another explanation could be that the aspecific binding of IgEs to peach high molecular proteins does not occur in the treated samples, thus increasing the chance of interaction with the LTP. One more reason could be that Pru p 3 might have been concentrated in the extract by the MW/US treatment: as shown in Figure 1, thermal treatment degraded several thermolabile proteins, which can be lost during the several steps of the extraction protocol, thus increasing the concentration of the thermostable ones. As reported in the Materials and Methods, samples were quantified through elemental analysis before loading them on gel; therefore, LTPs might be more represented in the total protein fraction extracted from the treated pulp.

Similar results were observed when innovative procedures, such as MW or γ-irradiation treatments, were employed with the aim of decreasing the allergenicity of tree nuts.[51,52] Even in those cases, allergenic proteins displayed their ability to resist to the treatment and to keep their immunological active structure and function. With respect to Pru p 3, recent findings demonstrated that the protein is sensitive to the thermal treatment, but that low pH conditions, characteristic of both the whole fruit and the juice, enable its complete refolding once the temperature falls. On the contrary, at neutral pH, Pru p 3 was unable to refold after heating, proving how the heating process can indeed affect its tertiary structure.[53]

Even though more precise investigations on the integrity of the LTP protein after these treatments are required, particularly concerning its folding and mass characterization, the results of our study suggest that the combination of MW and US mediated thermal processing does not seem to have a decreasing effect on the IgE reactivity of peach LTP. As was previously observed for almonds, cashew nuts, and walnuts major proteins, the data suggest that also Pru p 3 should be considered as an excellent marker protein for detecting nectarines in a multicomponent processed foods,[52] besides being also a sensitive and resistant marker of allergic danger for sensitized patients.

Finally, the risk for peach-allergic individuals (as well as for patients who clinically cross-react with the LTP's protein family) is not reduced by this combined approach, provided the conditions used in this study.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are indebted to Regione Piemonte (Ricerca Sanitaria Finalizzata 2008) for supporting this research. The International partnership was sponsored by Cariplo Foundation (Milan, Italy), within the NutriAl Network project (cod: 2009-2961). Special thanks go to Dr. Maurizio Galimberti from the Allergology and Clinical Immunology division of the "Ospedale Maggiore della Carità" of Novara for providing sera samples, and to Jaap Akkerdaas and Ronald van Ree from the Experimental Immunology Department of the Academic Medical Center of Amsterdam, for their help and support in the realization of the work.

ABBREVIATIONS USED

nsLTP, nonspecific lipid transfer protein; IgE, immunoglobulin E; OAS, oral allergy syndrome; SDS PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; HRP, horseradish peroxidase; kUA/L, kilounits of antibody per liter; HMW, high molecular weight; MW, microwave; US, ultrasounds; RAST, radio allergo sorbent test; LMW, low molecular weight

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