

Characterization of peach thaumatin-like proteins and their identification as major peach allergens

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Clinical & Experimental Allergy

Summary

Background Peach is the most important fruit related to food allergy in the Mediterranean area. Pru p 3, its lipid transfer protein, has been described as the principal allergen responsible for cross-reactivities with other foods and pollen and the severity of clinical symptoms.

However, the involvement of other allergenic families cannot be ruled out. Thaumatin-like proteins (TLPs) have been described as food allergen in several fruits, such as apple, cherry, kiwi and banana, and pollen.

Objective To identify members of the TLP family in peach fruit and to characterize putative allergens.

Methods Through two-dimensional (2D) electrophoresis of peach extract and immunodetections with a pool of peach-allergic patients, IgE-binding spots were identified and the corresponding proteins purified and characterized as allergens by *in vitro* and *in vivo* assays.

Three isoforms, belonging to the TLP family, were purified by different chromatographic systems and characterized by *N*-terminal amino acid sequences, molecular weight determination (MALDI) and enzymatic activity analysis (β -1,3-gluconase test and inhibition growth of fungi). In the same way, their IgE-binding capacity and allergenic activity were tested by ELISA assays, basophil activation tests and skin prick tests (SPT).

Results Two peach-TLPs, Pru p 2.0101 and Pru p 2.0201, were identified as IgE-binding spots by 2D electrophoresis. Another peach-TLP, Pru p 2.0301, was cloned and produced as recombinant protein in a yeast system. The three isoforms were purified and characterized as TLPs by immunoblotting with anti-chestnut TLP antibodies and anti-plant *N*-asparagine complex glycan (anti-cross-reactive carbohydrate determinant). All of them showed β -1,3-gluconase activity and inhibition of fungal growth. The three TLPs were recognized by around 50% of the sera from 31 patients analysed in ELISA experiments. All three gave a positive response to an SPT and/or in basophil activation experiments.

Conclusion Three isoforms, belonging to the TLP family, were identified in peach as principal allergens. Their prevalence, observed in *in vitro*, *ex vivo* and *in vivo* analyses, suggests that they are important allergens and should therefore be included in the routine diagnosis of peach allergy, at least in the Mediterranean area.

Keywords cross-reactivity, food allergy, peach allergy, PR5, Pru p 2.0101, Pru p 2.0201, Pru p 2.0301, thaumatin-like proteins

Submitted 22 March 2010; revised 18 May 2010; accepted 4 June 2010

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Cite this as: A. Palacín, L. Tordesillas, P. Gamboa, R. Sanchez-Monge, J. Cuesta-Herranz, M. L. Sanz, D. Barber, G. Salcedo and A. Díaz-Perales, *Clinical & Experimental Allergy*, 2010 (40) 1422–1430.

Introduction

Peach is the most prevalent plant food allergy in the Mediterranean area, affecting over 40% of patients with this type of sensitization [1, 2]. The majority of them (80%) recognize Pru p 3 [3, 4], a lipid transfer protein (LTP)

that is present mainly in the peel of the fruit. Recently, Pru p 3 was identified as the most important plant food allergen in Italian patients, being recognized by 9.8% of a broad allergic population [5]. LTPs are considered to be true food allergens, being markers of severe symptoms [6, 7]. However, other allergens have been described in

peach, such as Pru p 1 [8], a Bet v 1-like protein and Pru p 4 [9], a profilin.

Thaumatococcus-like proteins (TLPs) are panallergens and may be responsible for cross-reactivity among foods and pollen [10]. These allergens have been identified in several food allergies such as those of apple [11], cherry [12], pepper [13], olive [14], grape [15] and kiwi [16]. In pollen, TLPs have proved to be important sensitizers in cedar [17], cypress [18] and some species of juniper [19].

TLPs are proteins with a molecular weight of 20–30 kDa, and a very compact three-dimensional (3D) structure maintained by eight disulphide bridges [10, 20]. This highly conserved 3D folding helps their resistance to the process of digestion in the gut and heat treatments at acidic pHs [21, 22]. This protein family is comprised of plant defence proteins (pathogenesis-related protein; PR5) (especially against fungal attacks). There are two types of TLPs, differing with respect to their cellular location: acidic, which are directly driven to the apoplastic space, and basic, which are transported to the vacuole and ejected from the cell under some stress conditions [20].

Most data about the allergenicity of food TLPs come from *in vitro* assays. In fact, the published prevalence of these proteins is highly variable, depending on the method used. Mal d 2 was revealed to be a major allergen with a 75% positive response in apple-allergic patients when its allergenicity was analysed by immunodetection blot under reducing conditions [23]. However, when non-reducing methods were used, its response was reduced, showing 66% and 15% recognition by ELISA [11] and RAST, respectively. Act d 2, the kiwi thaumatocin, was recognized by 64% of Spanish patients [24] and 53% of Central European kiwi-allergic people by ELISA [25]. When its allergenicity was tested by skin prick test (SPT), more than 50% of patients showed a positive response with Act d 2 [24]. Recently, two new TLPs have been identified as allergens: Mus a 4, in banana (Palacin A, Quirce S, Sánchez-Monge R, Bobolea I, Díaz-Perales A, Martín-Muñoz F, Pascual C, Salcedo G, unpublished data) and Tri a-TLP in wheat [26].

In the case of pollen, TLPs behave as minor allergens such as Jun a 3 and Cry j 3, recognized by 36% of patients [19] and 27% of pollinic patients [17], respectively. The cypress TLP appears to be an exception, with a positive response in 63% of patients in Spain [18].

The allergenicity of TLPs has been related to their type of glycosylation [10], although their importance has not been clarified. Oberhuber et al. [11] reported that 25% of patients sensitized to Mal d 2 appeared to recognize complex sugar antigens. Asparagine-linked complex glycans from plants have been identified as cross-reactive carbohydrate determinants (CCDs). These broadly cross-reacting glycan epitopes are enhancers of false-positive responses in *in vitro* assays [27] that are not reflected in *in vivo* activity or clinical symptoms.

In our search for new peach allergens reported here, three TLPs were identified and characterized as important allergens. Two of them were purified from fruit, and the third was produced as a recombinant protein in *Pichia pastoris* yeast. They were characterized as new allergens by analysing their allergenic activity *in vitro* and *in vivo*. The results obtained highlight their importance in peach allergy in the Mediterranean area.

Methods

Patients and sera

Sera from 31 patients with allergy to peach selected at the Allergy Service of the Fundación Jiménez Díaz (Madrid) and from Hospital de Basurto (Bilbao) were used (age 16–46 years; sex 55% female, 45% male). All patients had a convincing clinical history of immediate allergic reactions after peach ingestion (urticaria/angioedema or anaphylactic symptoms); a positive response in the SPT using a commercial peach peel extract (ALK-Abello, Madrid, Spain); and a positive response to peach by an open oral challenge, except those patients who had suffered from anaphylaxis [28]. Most selected patients (64%) also had pollinosis, being mainly sensitized to grass pollen.

SPTs with the purified proteins were performed by dissolving proteins in glycerol:PBS (v:v) at 50 µg/mL for TLPs or 20 µg/mL for Pru p 3 [29].

Written informed consent was obtained from all patients and the ethics committees of the corresponding hospitals approved the study (Fundación Jiménez Díaz, Madrid; Hospital de Basurto, Bilbao, Spain).

Peach extract and purification of thaumatocin-like proteins

Whole fruits (peel and pulp) of peach (*Prunus persica*) were cut into pieces and were extracted with phosphate-buffered saline (0.1 M sodium phosphate, pH 7.0 and 0.5 mol/L NaCl; 1×1:5 (w/v), 1 h, 4 °C). After centrifugation (12 000 g, 30 min, 4 °C), the supernatant was dialyzed (cut-off point 3.5 kDa) and freeze-dried. Protein concentration was quantified according to the method of Bradford [30].

The basic TLP (Pru p 2.0101) was purified from the peach extract by cation-exchange chromatography on a Bio-Scale™ Mini Macro-Prep® High S column (BioRad, Hercules, CA, USA) equilibrated with 20 mM formic acid, pH 4 and eluted with 75 mM NaCl in the same buffer. The acidic protein (Pru p 2.0201) was obtained by anion-exchange chromatography on a Bio-Scale™ Mini Macro-Prep® High Q column (BioRad) equilibrated 20 mM ethalonamine, pH 9 and eluted with 1 M NaCl in the same buffer.

Act d 2, kiwi TLP and Pru p 3, peach LTP, were purified as previously described [24, 31].

The purified proteins were quantified by a commercial bicinchoninic acid test (Pierce, Cheshire, UK). The purity of the proteins was tested by SDS-PAGE, N-terminal

amino acid sequencing with an Applied Biosystems 477A gas-phase sequencer (Applied Biosystems, Foster City, CA, USA), and mass spectrophotometric analysis with a Biflex III Spectrometer (Bruker-Franzen Analytik, Bremen, Germany), using standard methods.

Production of Pru p 2.0301 as a recombinant protein in Pichia pastoris

Total RNA from peach was obtained by Chang and Cairney [32]. The reverse transcription was carried out with 1 µg of total RNA using the oligonucleotide primer 5'-TCGACTCTAGAGATCCGAATTCAAGCT-3' and the high capacity cDNA reverse transcription kit (Applied Biosystems). The cDNA obtained was amplified by polymerase chain reaction (PCR) using the forward primer: 5'-ATGATGAAGACCCTAGGAGCAGTCCTCAGC-3' and the reverse primer: 5'-TCATGGGCAGAAAGTAATGAGCTAGT-3', designed from previously reported cDNA (accession no. EU424255). The amplification was carried out with Taq-Gold DNA polymerase (Perkin-Elmer, Branchburg, NY, USA) as recommended by the manufacturer. PCR products were cloned into pGEM T-Easy vector (Promega, Madison, WI, USA), and several clones were sequenced using the ABI PRISM Dye Terminator kit and an ABI 3000 sequencer (Perkin-Elmer Biosystem, Warrington, UK). The production of recombinant protein was performed in *P. pastoris* yeast following the manufacturer's recommendations (Invitrogen Corporation, Paisley, UK).

The recombinant protein, Pru p 2.0301, was purified from yeast supernatants. The dialysed supernatants were fractionated by gel filtration on a Superdex 75 Hiload 16/60 column (Pharmacia, GE Healthcare, Barcelona, Spain) in 0.1 M acetate ammonium (1 mL/min), followed by RP-HPLC on Europa protein C4 column (25×0.7 mm; particle size 5 µm; Teknokroma, Barcelona, Spain). Elution was performed with a gradient of acetonitrile in 0.1% trifluoroacetic acid (0% for 10 min, and 0–100% for 90 min; 0.5 mL/min).

Two-dimensional gel electrophoresis

Two-dimensional electrophoresis (2DE) of peach extract (150 µg) was performed as described in Görg et al. [33] on an Immobiline DryStrip gradient (pH 3–10; 7 cm; Amersham Biosciences, Uppsala, Sweden). Isoelectric focusing was performed in IPGPhor-I (Amersham Biosciences, Uppsala, Sweden), to a final value of 8000 Vh. SDS-PAGE in the second dimension was performed in 15% polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie Blue staining or electrotransferred to polyvinylidene difluoride membranes to perform immunodetection assays, as described below.

Immunodetection assays

Samples (20 µg of protein from peach extract and 5 µg of purified allergens) were separated on SDS-PAGE (Invitro-

gen, Carlsbad, CA, USA). Proteins were electrotransferred to polyvinylidene difluoride membranes. After blocking, membranes were incubated with a pool of 31 sera from patients with peach allergy (1 : 5 dilution; overnight), and with goat anti-human IgE-peroxidase conjugate (Biosource, Camarillo, CA, USA; 1 : 3000 dilution). IgE-binding components were detected by means of chemiluminescence (Amersham Biosciences, Little Chalfont, UK). A pool of sera ($n = 5$) from patients with dust mite, but not plant food or pollen allergy, was assayed as a negative control (data not shown).

Alternatively, blocked membranes were immunodetected with rabbit polyclonal antibodies to chestnut TLP (1 : 30 000 dilution; 1 h; kindly provided by Dr L. Gomez, ETSIM, UPM, Madrid, Spain) or with anti-Pru p 3 antibodies (dilution 1 : 2000; 1 h), or with anti-complex asparagine-linked glycan serum (1 : 30 000 dilution; 1 h; [34]), and then treated with goat anti-rabbit IgG-peroxidase conjugate (Dako, Carpinteria, CA, USA; 1 : 10 000 dilution) and revealed by means of chemiluminescence (Amersham Biosciences, Little Chalfont, UK).

The inhibition immunoblots were performed as described above with only a slight modification: the pool of sera was pre-incubated with purified protein (10 µg/mL) before incubation with the blot.

Endo-β-1,3-glucanase activity

Endo-β-1,3-glucanase activity was assayed by the method described in Menu-Bouaouiche et al. [35], using carboxymethylated Pachyman (CM-Pachyman, Megazyme, Wicklow, Ireland) as a substrate. Proteins (20 µg) were incubated for 2 h in 0.3 mL of 0.5% (w/v) CM-Pachyman in 50 mM sodium acetate (pH 4 and 5), 100 mM NaCl containing 0.05% (w/v) sodium azide (pH 4.0 and pH 5.0) or 20 mM potassium phosphate buffer (pH 6.0), 100 mM NaCl containing 0.05% (w/v) sodium azide. Four millilitre of 0.1% (w/v) tetrazolium blue, 50 mM NaOH and 0.5 M sodium potassium tartrate was added to 50 µL of samples and heated in boiling water. The quantity of liberated D-glucose equivalents was estimated by absorbance at 660 nm. Assays were performed in duplicate. Results are expressed as nkat/mg of protein (1 kat corresponds to the formation of 1 mol of D-glucose equivalent/s). Pru p 3 (peach LTP) and Act d 2 (kiwi TLP) were used as controls.

Anti-fungal activity

Anti-fungal activity was estimated essentially as described by Vitali et al. [36]. One hundred microlitre of spore suspension (approximately 10^6 spores/mL) of *Fusarium oxysporum* or *Plectosphaerella cucumerina* were ground in potato dextrose broth (Sigma; St. Louis, MO, USA) in 96-well microplates and incubated for 48 h at 23 °C. TLPs were added to culture at 20 µg/150 µL. Act d 2 was used as TLP control. The medium with fungal suspension was used as a

negative control. The anti-fungal effect was monitored as OD at 660 nm.

Specific immunoglobulin E determination and enzyme linked immunosorbent inhibition assays

Specific IgE of the 31 individual sera (1:5 dilution) to natural Pru p 2.0101 and Pru p 2.0201 and recombinant Pru p 2.0301 (5 µg/mL as solid phase) was measured by ELISA following a previously described method [31]. Pru p 3, the major peach allergen, and Act d 2, the kiwi TLP, were used as control model allergens. Pineapple bromelain (Sigma-Aldrich) was used as a marker of plant *N*-asparagine complex glycan antigens (complex carbohydrate determinants; CCDs). Blocking solution (Sigma) was used as a negative control and specific IgE levels > 0.14 OD units [mean (OD) + 3 SD of the negative control value] were considered positive. All tests were performed in triplicate.

ELISA inhibition assays were carried out by the same method, except that the pool of sera was incubated beforehand with 10, 5, 2 and 1 µg/mL of the natural or recombinant TLPs as inhibitors (3 h at 25 °C). All tests were performed in triplicate.

Basophil activation test

The basophil activation test (BAT) was performed as described previously [37, 38]. After blood cell separation, 50 µL of the patient's cell suspension was incubated with 50 µL of four final concentrations of the tested samples: 25, 10, 1 and 0.1 µg/mL for Pru p 2.0101, Pru p 2.0201, Pru p 2.0301 and Pru p 3. In order to evaluate background basal values without stimulation (negative control), 50 µL of stimulation buffer (*N*-2 hydroxyethylpiperazine-*N*-2-ethansulphonic acid 20 mM, NaCl 133 mM, KCl 5 mM, CaCl₂ 7 mM, MgCl₂ 3.5 mM, bovine serum albumin 1 mg/mL, pH 7.4) containing

IL3 (2 ng/mL) and heparin (10 µL) (5000 UI/mL; ROVI, Madrid, Spain) as added to the cell suspension in another well. As a positive control, a monoclonal anti-IgE receptor antibody (Bühlmann, Allschwil, Switzerland) was used at a final concentration of 1 µg/mL.

A positive response was recorded when the antigen-specific response was ≥ 20% of the basal level, and the stimulation index (antigen-specific response/basal level) was ≥ 2.

Results

Identification of thaumatin-like proteins in peach fruit

The presence of TLPs was established by 2DE on pH gradient strips from pH 3–10, following the manufacturer's recommendations. SDS-PAGE separation and Coomassie staining yielded a map of spot proteins (Fig. 1a). Several spots, which were recognized by anti-chestnut TLP, were cut out and analysed by fingerprinting in a mass spectrometer (MS/MS). Two peach TLPs, one acidic and the other basic (gi 190613905; gi 190613911 [39]), were identified by means of the fragmentation of trypsin peptides (Fig. 1b). Two of the spots, II and III, were matched with the same protein, corresponding to a basic peach TLP. By contrast, the two spots had different molecular weights, possibly due to a differential post-translation processing, as has been described for other basic TLPs [40].

The incubation with a serum pool from allergic patients showed IgE-binding spots overlapping with the TLP region.

Purification of the allergenic TLPs of peach

The native peach TLPs identified by IgE immunodetection were purified using several ionic chromatographic methods, resolving as unique bands in SDS-PAGE (Fig. 2a).

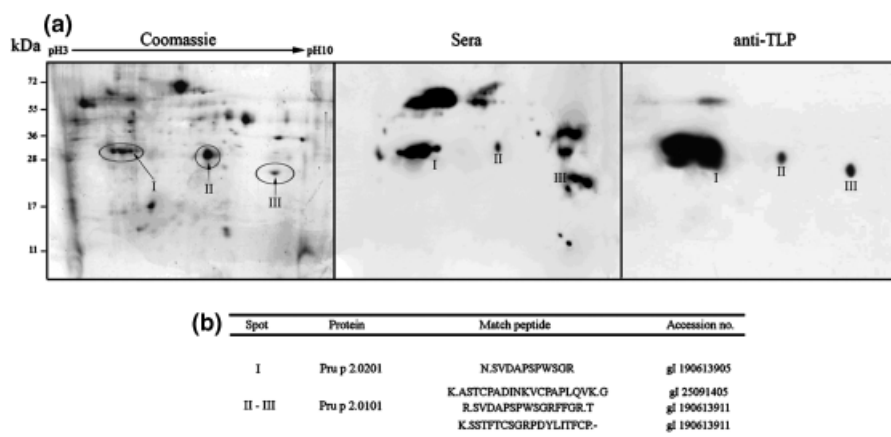


Fig. 1. Two-dimensional gel electrophoresis of peach-fruit extract. Proteins were separated on pH gradient-dried strips (pH 3–10) following SDS-PAGE. (a) Coomassie staining (Coomassie). Replica gels were electrotransferred to Immobilon-P membranes and incubated with a pool of sera (Sera); or with antibodies produced against chestnut thaumatin-like proteins (TLP) (anti-TLP). The identified TLP-spots are indicated by Roman numerals. (b) Identification of the peach TLPs: accession number and amino acid sequences.

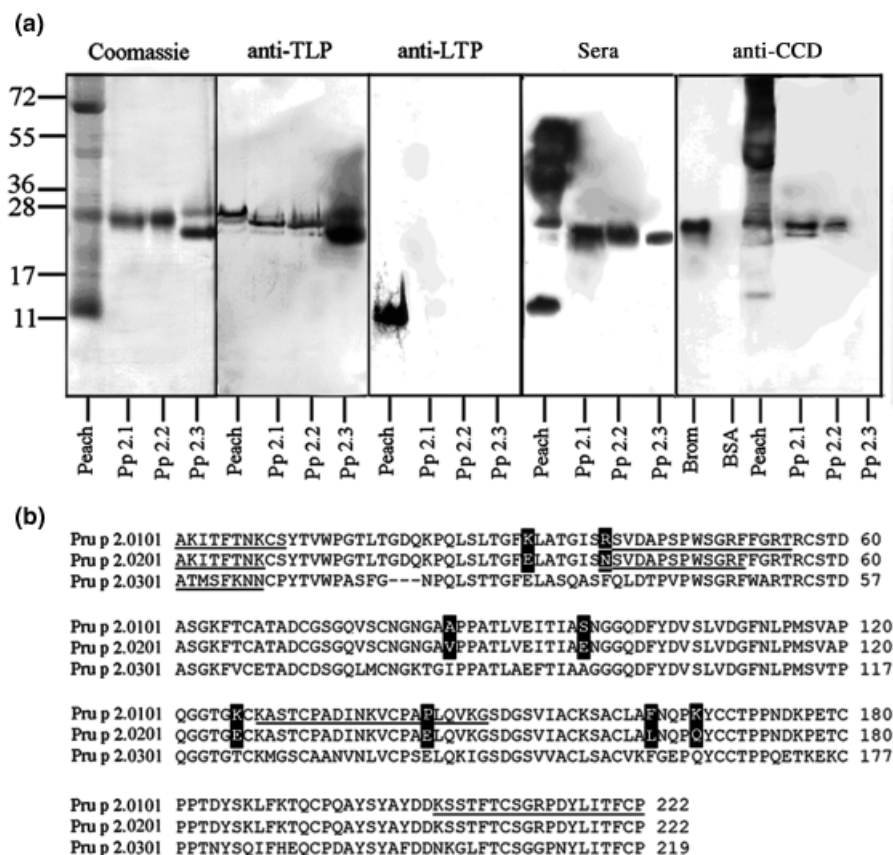


Fig. 2. Purification of peach TLPs. (a) Peach extract (20 µg) or purified proteins (5 µg), Pru p 2.0101 (Pp2.1), Pru p 2.0201 (Pp2.2) and Pru p 2.0301 (Pp2.3) were separated on SDS-PAGE and stained with Coomassie Blue R125 (Coomassie). Replica gels were electrotransferred to Immobilon-P membranes and incubated with anti-chestnut Thaumatin-like proteins (TLP) antibodies (anti-TLP), or with anti-Pru p 3 antibodies (anti-LTP), or with a pool of sera from peach patients (Sera), or anti-plant *N*-complex-glycans (anti-CCD). (b) Amino acid sequences of the three characterized TLPs. The *N*-terminal sequence and the peptides identified by fingerprinting are underlined. The amino acids differing between Pru p 2.0101 and Pru p 2.0201 are highlighted in bold.

Purity of the proteins was confirmed by serum immuno-detection, MS and *N*-terminal amino acid sequences.

The native isoforms corresponded to the basic and acidic TLPs that shared the same *N*-terminal and 96% amino acid sequence identity. Their differences were confined to eight charged amino acids, which were responsible for the great disparity in pI (basic pI 8.3; acidic pI 4.8; Fig. 2b). Their molecular weight and identification by fingerprinting enabled us to confirm their nature. These peach TLPs showed homology with other food allergens from the same protein family. Mal d 2, apple TLP, were the closest allergens, with 85% identity; Pru av 2 shared 71% of residues in common with peach TLPs; and Act d 2 had 40% identity with them.

Another isoform of peach TLP-family was predicted by Chen et al. [39], but neither of them was found in the fruit protein extract. However, a third TLP member (EU424255) was produced as a recombinant in the yeast *P. pastoris*. This protein could be of great interest due to the high amino acid sequence identity with the cherry allergen, Pru av 2 (82%) [12]. The purified protein was resolved as two

bands in SDS-PAGE (Fig. 2a), although both were recognized by anti-chestnut-TLP antibodies and corresponded to the same *N*-terminal amino acid sequences. This double band could correspond with different levels of yeast glycosylation of the recombinant protein.

The third TLP isoform shared around 66% and 67% identity with the basic and acidic forms of the other purified peach TLPs, respectively. By contrast, it was more distant from Mal d 2 (67% identity), but shared the same degree of identity with Act d 2 as their peach homologues (33%).

The three TLP-isoforms were recognized by IgE from a pool of sera from peach-allergic patients (Fig. 2a; sera). These results led the IUIS allergen nomenclature committee to name these isoforms as Pru p 2.0101 (basic TLP), Pru p 2.0201 (acidic TLP) and Pru p 2.0301 (recombinant).

The native isoforms were recognized by anti-plant *N*-asparagine-linked glycan antibodies (Fig. 2a, anti-CCD), suggesting that they could be glycosylated, as described in other food TLP allergens, such as Mal d 2 or Act d 2 [11, 22, 24]. However, the recombinant TLP was not detected with the same antibodies.

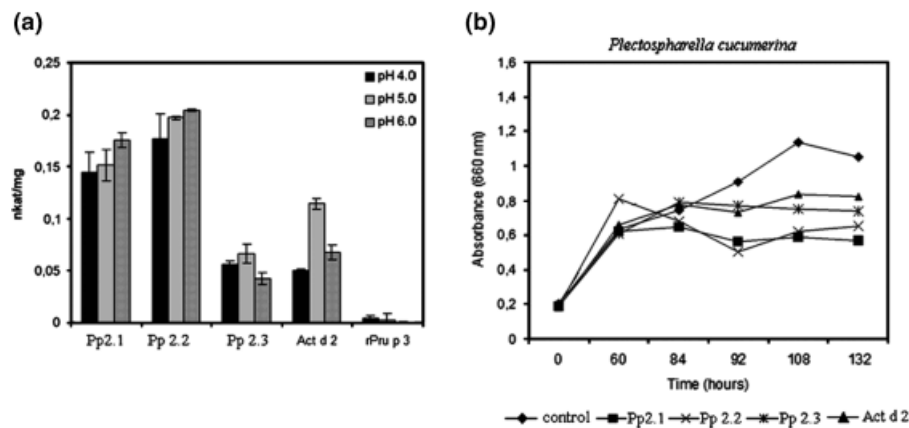


Fig. 3. Analysis of enzymatic activity of the peach TLPs. (a) Endo- β 1,3-glucanase test using carboxy-methylated Pachyman as a substrate. Purified proteins (20 μ g) were incubated with the synthetic substrate at pH 4, 5 and 6. The amounts of generated D-glucose equivalents (as nkat/mg of protein) were estimated by measuring the absorbance at 660 nm. Pru p 3 and Act d 2 were used as controls, respectively. Results are from duplicate measures. (b) Spores from *Plectosphaera cucumerina* were grown under standard conditions. After 48 h, each TLP (20 μ g) was added and the culture was continued for 0–132 h. The absorbance values obtained at 600 nm are shown.

The enzymatic properties of the three TLPs were checked for endo- β 1,3-glucanase activity [35] (Fig. 3a). All of them were able to degrade the used substrate, CM-Pachyman. In the three cases, the level of activity was comparable with other allergens of the same family, described previously using similar assays [35, 41]. Both native proteins, Pru p 2.0101 and Pru p 2.0201, were more active at pH 6 than at other pHs. By contrast, Pru p 2.0301 preferred pH 5, although the activity was much lower (between 60% and 70%) than the others.

On the other hand, the capacity of peach TLPs to inhibit the growth of two species of fungi, *Fusarium oxysporum* and *P. cucumerina*, was examined. Peach TLPs inhibited the growth of *P. cucumerina* (20–50%), when they were present in the media at a concentration of 3.5 mM (Fig. 3b). The native proteins achieved a 55% inhibition of the growth of this fungus, while the recombinant inhibited the growth of *P. cucumerina* by 36%, the same level as Act d 2, the kiwi TLP that inhibited growth by a 32%. However, *F. oxysporum* was able to grow even in the presence of the proteins, suggesting pathogenic specificity (data not shown).

Characterization of the allergenic capacity of peach thaumatin-like proteins

The allergenic activity of the peach TLPs was evaluated by specific IgE determination and ELISA inhibition assays. The three isoforms were recognized by individual sera from peach-allergic patients (Fig. 4a). Pru p 2.0101 showed a positive response in 71% of the patients analysed, 77% of sera recognized the acidic isoform, Pru p 2.0201 and the recombinant Pru p 2.0301-bound IgE in 58% of tested sera. Pru p 3, the major allergen of peach, was recognized by 81% of patients, while Act d 2 showed a

positive response in 58% of the same sera. By contrast, only 29% of patients recognized pineapple bromelain, suggesting that plant CCD antigens are of little importance for the sensitization to peach TLPs.

The specificity of IgE-recognition was confirmed by ELISA inhibition assays (Fig. 4b). Every isoform could inhibit the IgE-binding capacity of the others by at least 50%. The highest inhibition (90–100%) was observed between the two native allergens, Pru p 2.0101 and Pru p 3 2.0201, suggesting strong cross-reactivity between them. By contrast, both native isoforms exerted level inhibition values when tested against the recombinant Pru p 2.0301, accordingly with the percentages of amino acid identities mentioned above.

Finally, the peach TLPs were tested by BAT and/or SPT (Table 1). Basophils from peach-allergic patients were activated in the presence of peach TLPs in at least 50% of cases. Pru p 2.0201 was the most active of the three isoforms, being recognized by 80% of patients, inducing Pru p 2.0101 and Pru p 2.0301 positive responses in 60% and 50% of cases, respectively.

In the same way, the native proteins Pru p 2.0101 and Pru p 2.0201 were used to study the allergenic activity of peach TLPs by SPTs (Table 1). In this case, 6/7 (86%) patients gave a positive response when Pru p 2.0201 was applied, compared with the low frequency of recognition obtained with Pru p 2.0101 (3/7; 43%).

Discussion

This study identified and characterized three isoforms of the TLP family as peach allergens. The three isoforms were purified and their identity assessed by N-terminal amino acid sequence and molecular weight and fingerprint determination by MS–MS spectrometry. Two of them were

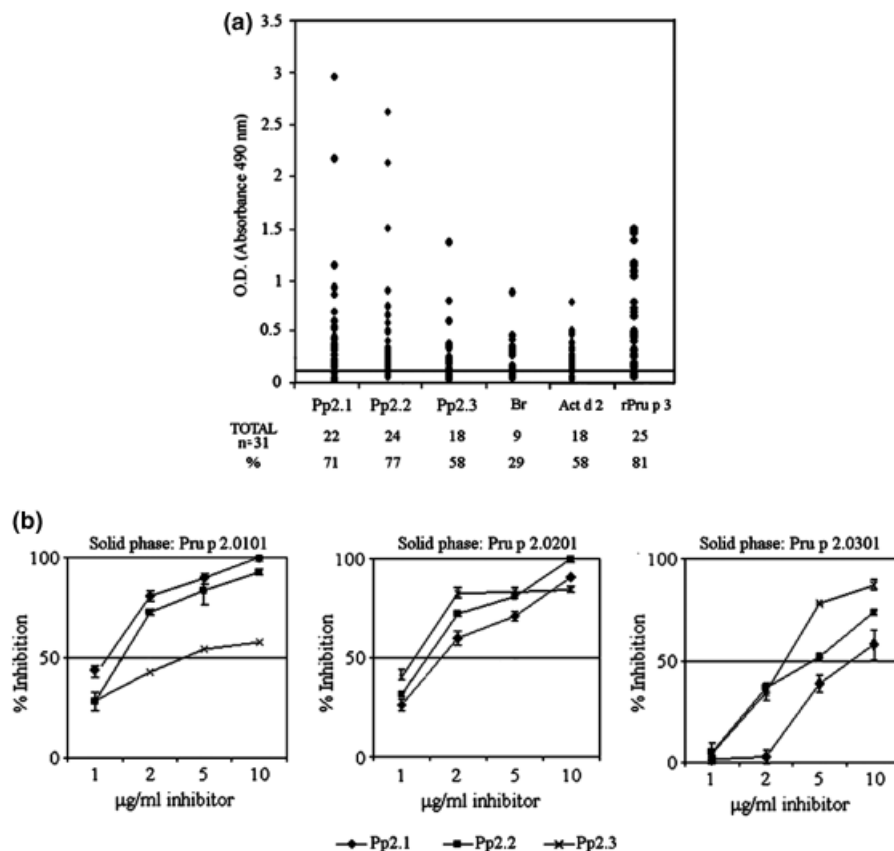


Fig. 4. Allergenic activity of the peach TLPs. (a) Specific IgE binding (as absorbance at 490 nm) of purified TLPs (5 µg/mL), Pru p 2.0101 (Pp2.1), Pru p 2.0201 (Pp2.2), Pru p 2.0301 (Pp2.3) and control proteins (Bromelain, Br; Act d 2, and Pru p 3; 5 µg/mL) to individual sera from peach allergic patients (dilution 1 : 5). Positive results were considered for values greater than 0.14 OD units [mean (OD) + 3 SD of negative control; horizontal line]. (b) Inhibition capacity of purified TLPs (as reduction in IgE-binding capacity; %). The pool of sera (dilution 1 : 5) was added after its pre-incubation with 10, 5, 2, and 1 µg/mL of TLPs as inhibitors (3 h at 25 °C). All tests were performed in triplicate.

Table 1. Results of skin prick tests (SPT) and basophil activation tests (BAT) in peach-allergic patients

Patients	SPT			BAT			
	Pp2.1*	Pp2.2	Pp3	Pp2.1	Pp2.2	Pp2.3	Pp3
1				+	+	+	+
2				—	+	+	+
3				—	—	—	+
4	—	+	+	—	+	+	+
5	+	+	+	—	—	—	+
6	+	+	+	+	+	—	+
7	—	+	+	+	+	—	+
8	—	+	+	+	+	+	+
9	—	—	+	+	+	—	—
10	+	+	+	+	+	+	+
Total (%)	43	86	100	60	80	50	90

*Pru p 2.0101, Pp2.1; Pru p 2.0201, Pp2.2; Pru p 2.0301, Pp2.3; Pru p 3, Pp3.

purified from the peach fruit, while the third was produced as a recombinant protein.

The three purified isoforms had different allergenic capacities. Pru p 2.0201 was the most active, giving positive

responses as the same level as Pru p 3, the main LTP allergen. It was recognized by 77% of the sera analysed by ELISA. More than 80% of patients reacted with this allergen when BAT or SPTs were performed. Pru p 2.0101 exhibited positive recognition in 71% of cases by ELISA and around 50% by BAT or SPTs. The differences in the level of recognition between Pru p 2.0101 and Pru p 3 2.0201 could not be explained by the differences in their sequences. The two natural isoforms share 96% of their residues, with only eight changes in charged amino acids. By contrast, the recombinant protein, Pru p 2.0301, was further to the others (66% of identity), being recognized by around 50% of peach patients in ELISA and BAT assays. For this reason, TLPs could be considered important allergen in peach.

The native isoforms were glycosylated based on their recognition by anti-CCD antibodies, and by MS-MS spectrometry, which may explain why they resolved as several bands after incubations with antibodies. This has also been described in other allergenic TLPs such as Act d 2 [24] and Mal d 2 [22, 41]. However, Pru p 2.0301, the only recombinant protein, migrated as a double band in

Coomassie-stained, although it was not recognized by anti-CCD antibodies.

The presence of *N*-complex sugar antigens appears not to be very relevant to the allergenic capacity of the purified proteins. Only 29% of the peach-allergic patients showed a positive response to pineapple bromelain, which was used as a CCD marker in this work. Thus, the recognition of peach TLPs should principally be due to protein epitopes, although the role of antigen sugars cannot be ruled out.

Act d 2, the kiwi TLP used as a model of this family, displayed 58% positive recognition of peach-allergic patients with ELISA experiments, although it only shares around 30% identity with the peach TLPs. This result could explain the cross-reactivity between peach and kiwi observed by SPT without any parallelism with the clinical symptoms [1, 2].

The close identity with other food TLPs described as allergens, such as Mal d 2 or Pru av 2, suggests cross-reactivity among these, although more detailed studies are required. Likewise, further work is required to evaluate the role of this family as panallergens and their importance as mediators of cross-reactions. The great stability of the members of this family to protease digestion and heat treatment at acid pHs could favour their permanence in foodstuff (after cooked or industrial processing), and thereby their allergenic potential, although these proteins have not been found in processed foods such as jam or juice [21]. On the other hand, the presence of members of this family described as pollen allergens could explain the high prevalence of pollinosis (50%) in the patients studied.

In summary, different variants of a new peach allergen from the TLP family has been described. Our results indicate how far we are from completing the allergenic map of common food allergies. On the basis of the reactivity and prevalence of the three TLP-isoforms, Pru p 2.0101, Pru p 2.0201 and Pru p 2.0301 should be included in routine tests for allergy diagnosis.

Acknowledgements

Financial support from the Ministerio de Ciencia e Innovación (grant BIO2009-07050) and the Instituto de Salud Carlos III, RETICS 2007 (RD07/0064/03), Spain, is acknowledged.

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