

cDNA cloning and heterologous expression of the major allergens from peach and apple belonging to the lipid-transfer protein family

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Summary

Background Lipid-transfer proteins (LTPs) have been identified as major allergens of *Rosaceae* fruits in populations living in areas virtually free of *Fagales* trees, such as several Mediterranean communities. Pru p 3 and Mal d 3, the allergens from peach and apple, respectively, have a main clinical relevance in these areas.

Objective To isolate and characterize cDNAs for Pru p 3 and Mal d 3, and to produce recombinant Pru p 3 in the yeast *Pichia pastoris*.

Methods cDNAs for both allergens were isolated by polymerase chain reaction using non-degenerated primers. Expression of Pru p 3 was performed in *P. pastoris* using the pPIC 9 vector. The recombinant product was purified by gel-filtration chromatography followed by RP-HPLC. Immunodetection and immunoblot inhibition assays were carried out with sera from peach-allergic patients.

Results The cDNAs for both Pru p 3 and Mal d 3 showed a 273 open reading frame coding for the 91 amino acid mature polypeptides. The deduced amino acid sequences exhibited N-terminal regions fully identical to those previously determined for the natural peach and apple allergens. Pru p 3 was expressed in *P. pastoris* at 20 mg/L of culture medium. The recombinant allergen showed the same N-terminal sequence (plus a glutamic acid added for proper extracellular expression) and apparent molecular size as natural Pru p 3. Both the recombinant and natural forms of Pru p 3 displayed similar immunoglobulin (Ig)E-binding capacity in immunodetection and immunoblot inhibition assays.

Conclusions Comparison of the complete primary structures of mature Pru p 3 and Mal d 3 deduced from their corresponding cDNA clones supports the close relationship between both allergens. Recombinant Pru p 3 binds IgE *in vitro* like its natural counterpart. Therefore, it can be a useful tool for specific diagnosis and structural studies.

Keywords lipid-transfer proteins, plant allergen, cloning, recombinant allergen, peach, apple

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Introduction

A wide array of limitations has been pointed out with regard to the current forms of allergy diagnosis and immunotherapy using crude food or pollen extracts. Several of these problems can seemingly be overcome by replacing crude extracts by either individual or cocktails of recombinant allergens [1,2]. This approach has already been carried out in some cases, such as the use of recombinant (r)Bet v 1 and Bet v 2 for birch pollen diagnosis [3]. Furthermore, both allergens are also useful tools to study the association between birch pollinosis and allergy to some fruits (e.g. apple, peach) and vegetables (e.g. carrot, celery), frequently found in central and northern Europe and in North America [3,4]. The presence of Bet v 1 and Bet v 2

homologues in the associated plant foods is the main factor responsible for these allergenic cross-reactivities [5].

In the Mediterranean area *Rosaceae* fruits, particularly peach, are among the most frequent causes of food-induced allergic reactions [6,7]. Most of this area is virtually free of birch trees, and the majority of the *Rosaceae* fruit-allergic patients living in it do not show specific immunoglobulin (Ig)E to Bet v 1. Lipid-transfer proteins (LTPs) have been identified as major peach (Pru p 3), apple (Mal d 3) and apricot allergens in this Mediterranean population [8,9]. Allergenic LTPs have also been located and characterized in other plant foods, such as chestnut, maize and carrot, and in *Artemisia* pollen [10–12]. Cross-reactions between Pru p 3 and other reactive LTPs have been demonstrated *in vitro* [10–12]. The strong resistance of LTPs to heat treatment and to pepsin digestion [12,13] point to them as potentially severe food panallergens.

The allergenic LTPs of plant foods belong to a family of 9 kDa (90–95 amino acids) polypeptides involved in plant defence mechanisms against pathogens [14,15]. Most members of

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the family contain eight conserved cysteins forming four disulphide bridges which stabilize a postulated 3-dimensional structure with four α -helices and a C-terminal tail [14]. The LTP family is widely distributed throughout the plant kingdom, but knowledge of these polypeptides varies greatly among different botanical groups. Thus, whereas clones and recombinant products of cereal LTPs are available, neither cloning nor heterologous expression of the relevant allergens (LTPs) from *Rosaceae* fruits have been published. We report here the isolation and sequencing of cDNA clones for Pru p 3 and Mal d 3, and the purification of rPru p 3 expressed in the yeast *Pichia pastoris*.

Materials and methods

Sera from allergic patients

A pool of seven sera from patients allergic to peach were used in the immunodetection and immunoblot inhibition assays. All patients showed a positive case history to this fruit, positive skin prick test (SPT) to peach extract and purified natural Pru p 3, and specific IgE levels to peach from 30.3–2.7 kU/L.

Pooled sera from three subjects not sensitized to *Rosaceae* fruits (negative SPT and undetectable specific IgE to peach, apple and plum) were tested as negative control.

Specific IgE levels were determined by the Pharmacia CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden). SPTs were carried out with a commercial preparation (ALK-Abello, Madrid, Spain) and purified Pru p 3, using standard procedures. Weal areas 7 mm² greater than that produced by the saline control were considered positive.

Isolation of cDNAs encoding Pru p 3 and Mal d 3

Total RNA of peel from mature peaches or apples was prepared by the method of Lagrimini et al. [16]. The reverse transcription was carried out with 5 µg of total RNA using the oligonucleotide primer TCGACTCTAGAGGATCCGAATTCAAGC (T)₁₅, and the First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, Piscataway, NY, USA).

The cDNA obtained was amplified by PCR using the following oligonucleotide 5'-primers: 5'-TAACATGTGGCC AAGTGTCCAGCGCACTTGCACCATGC-3' for Pru p 3, and 5'-TAACATGTGGCCAAGTGACCAGCAGCCTTGC ACCATGC-3' for Mal d 3. Both primers were designed from the N-terminal amino acid sequence of each allergen and the nucleotide sequence of a cDNA for almond LTP (accession number X96714). For the reverse direction the 3'-primer 5'-CTGCAGGTCGACTCTAGAGGATCC (complementary to that used for reverse transcription) was applied. The amplification was performed with Taq-Gold DNA polymerase (Perkin-Elmer, Roche Molecular System, Branchburg, NY, USA) as recommended by the manufacturer. The PCR program was 7 min denaturation at 96°C, followed by 30 cycles each of 1 min denaturation at 96°C, 1 min annealing at 55°C and 1 min extension at 72°C, and final extension for 12 min at 72°C. Re-amplification was carried out using the same program, except annealing at 65°C.

The PCR products were cloned into pGEM TEasy vector (Promega, Madison, WI, USA). These constructs were used to transform DH5 α F' *Escherichia coli* cells, and the DNAs from several clones were sequenced using the ABI PRISM Dye

Terminator Kit and a ABI 377 sequencer (Perkin-Elmer Biosystem, Warrington, UK).

Expression of rPru p 3 in *Pichia pastoris*

The coding region of the Pru p 3 cDNA was amplified by PCR with Taq-Gold DNA polymerase (Perkin-Elmer), a sense primer 5'-CCGTCTCGAGAAAAGAGAGAAATAACATG TGGCC-3' (*Xho*I restriction site underlined) and an anti-sense primer 5'-CCTCGAATTCTCACTTCACGG-3' (*Eco*RI restriction site underlined). The sense primer allows fusion of the Pru p 3 encoding region in-frame with the sequence coding for the pre-prosequence of the *Saccharomyces cerevisiae* α -factor present in plasmid pPIC 9. The signal peptidase cleavage site lies between Arg and Glu, and it was recreated by the sequence AGA GAA located before the first codon (ATA) for mature Pru p 3.

The PCR product was purified, digested with *Xho*I/*Eco*RI restriction enzymes and inserted into the same sites of the pPIC 9 vector (pPIC 9/Pru p 3) to transform DH5 α F' *E. coli*, and the DNA from several clones was sequenced to confirm the in-frame arrangement described above.

Ten micrograms of pPIC9/Pru p 3 were digested with *Bgl*II restriction enzyme, and the larger purified fragment was used to transform GS115 *P. pastoris* cells by electroporation following the Invitrogen manual recommendations (Invitrogen Corporation, De Schelp, the Netherlands). Transformed cells were grown on minimal glucose plates at 30°C during 3–4 days, and screening for positive clones was performed by patching the colonies in replica-plating on minimal glucose vs. minimal methanol plates.

The selected transformants were cultured in buffered glycerol complex medium for 48 h at 30°C. Cells were then collected by centrifugation, and expression of the recombinant protein was induced by resuspension in buffered methanol complex medium. Maximum expression was achieved after 4 days.

Isolation of recombinant and natural Pru p 3

The culture medium obtained after induction was dialysed against 0.1 M ammonium acetate, and freeze-dried. This preparation (6 mg of protein) was fractionated by gel-filtration on a Superdex HR 75 16/26 column (Pharmacia Biotech), eluting with 0.1 M ammonium acetate (1 mL/min). Fractions enriched in rPru p 3 were pooled, freeze-dried, and then separated by RP-HPLC on a Nucleosil 300-C4 column (8 × 250 mm; particle size 5 µm; Sugerlabor, Madrid, Spain). Elution was performed with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (10% during 5 min and 10%–100% in 150 min; 1 mL/min).

Peaks containing rPru p 3 in each chromatographic separation were identified by SDS-PAGE and immunodetection. Protein concentration was determined according to Bradford [17].

N-terminal amino acid sequencing of rPru p 3, transferred onto polyvinylidene difluoride membrane after SDS-PAGE, was carried out by standard methods with an Applied Biosystems 477 A gas-phase sequencer.

Natural Pru p 3 was purified from defatted skins of peach [10]. After extraction with Tris-HCl buffer (0.1 M Tris, pH 7.5, 10 mM ethylene diamine tetraacetic acid (EDTA); 1:5 (w/v); 1 h; 4°C), the remaining material was washed with H₂O, and then re-extracted with 1.5 M LiCl (1:5 (w/v); 1 h; 4°C). The LiCl extract was dialysed against H₂O, freeze-dried, and fractionated by cation-exchange chromatography on an Acell Plus CM

Waters™ SepPak^R cartridge (Waters Corp, Milford, MA, USA). Elution was 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).

Immunodetection and immunoblot inhibition assays

Samples were separated by SDS-PAGE on 4%–20% Tris-HCl precast polyacrylamide gels (Bio-Rad, Hercules, CA, USA) by using reducing conditions, and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes, as previously reported [18]. After washing and blocking, membranes were incubated with a serum pool from peach-allergic patients or with control sera (1 : 3 dilutions), and then with alkaline phosphatase-conjugated monoclonal anti-human IgE (clon GE-1, Sigma (St Louis, MO, USA); 1 : 500 dilution) and developed by adding 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium solution.

Alternatively, blocked membranes were immunodetected with rabbit polyclonal antibodies to natural Pru p 3 (1 : 2000 dilution), then treated with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma; 1 : 500 dilution) and finally with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution.

Immunoblot inhibition assays were performed as described above, except that the pool of sera from peach-allergic patients was pre-incubated for 3 h at room temperature (25 °C) with each of the inhibitors (5 µg of purified natural and rPru p 3, and bovine serum albumin as negative control).

Results

Non-degenerated oligonucleotide primers were designed for PCR amplification of peach and apple cDNAs. The primers were based on the known N-terminal amino acid sequences of Pru p 3 and Mal d 3 [8], as well as on the full nucleotide encoding sequence of almond LTP (accession number X96714), the deduced amino acid sequence of which shared > 80% identity with the reported partial sequences of the peach and apple allergens. The PCR yielded a predominant fragment of approximately 350 bp in each fruit. Isolation and sequencing of the cDNAs revealed a 273-bp open reading frame coding for a 91 amino acid polypeptide (Fig. 1) in both peach and apple. The N-terminal amino acid regions deduced from the Pru p 3 and Mal d 3 cDNAs were fully identical to those previously determined for the natural peach and apple allergens, respectively [8, Fig. 1]. Additionally, the complete sequence deduced for Pru p 3 presented only a single change at position 76 (S→H), when compared with the full amino acid sequence of this allergen previously obtained by direct protein sequencing [19]. Moreover, the calculated molecular weights for Pru p 3 (9177 D) and Mal d 3 (9084 D) were in agreement with those experimentally determined by matrix-assisted laser desorption/ionization (MALDI) analysis for the natural peach (9138 D) and apple (9078 D) allergens [8].

The deduced primary structures for Pru p 3 and Mal d 3 showed 82% identity, thus confirming the close relationship between both allergens. Comparison with other LTPs, IgE-binding capacity of which had been demonstrated [8–12], revealed very different degrees of similarity. Allergenic LTPs from plant foods, such as apricot, maize, chestnut and carrot,

Pru p 3

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1   ATA ACA TGT GGC CAA GTG TCC AGC AGC CTT GCA CCA TGC ATA CCC TAC GTG   51
   I T C G Q V S S S L A P C I P Y V
52  AGA GGC GGT GGA GCT GTG CCT CCA GCT TGC TGC AAC GGC ATT AGG AAC GTC   102
   R G G G A V P P A C C N G I R N V
103 AAC AAC TTG GCA AGG ACC ACC CCT GAC CGC CAG GCC GCT TGC AAC TGC CTG   153
   N N L A R T T P D R Q A A C N C L
154 AAA CAG CTT TCC GCC AGC GTC CCC GGA GTC AAC CCT AAC AAT GCC GCA GCG   204
   K Q L S A S V P G V N P N N A A A
205 CTT CCC GGC AAG TGT GGA GTT AGT ATT CCT TAC AAG ATT AGC GCC TCC ACC   255
   L P G K C G V S I P Y K I S A S T
256 AAC TGC GCC ACC GTG AAG TGA   276
   N C A T V K *

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Mal d 3

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1   ATA ACA TGT GGC CAA GTG ACC AGC AGC CTT GCA CCA TGC ATT GGC TAC GTG   51
   I T C G Q V T S S L A P C I G Y V
52  AGG AGT GGC GGA GCT GTC CCT CCA GCT TGC TGC AAT GGA ATC AGA ACC ATT   102
   R S G G A V P P A C C N G I R T I
103 AAC GGC TTG GCC AGG ACC ACC GCT GAC CGC CAG ACT GCT TGC AAC TGC CTG   153
   N G L A R T T A D R Q T A C N C L
154 AAG AAT CTT GCC GGC AGC ATC AGT GGT GTT AAC CCT AAC AAT GCA GCA GGG   204
   K N L A G S I S G V N P N N A A G
205 CTT CCT GGA AAG TGT GGA GTC AAC GTC CCC TAC AAG ATC AGC ACC TCC ACC   255
   L P G K C G V N V P Y K I S T S T
256 AAC TGC GCC ACC GTG AAG TAA   276
   N C A T V K *

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Fig. 1. Nucleotide and deduced amino acid sequences of coding regions for mature allergens Pru p 3 and Mal d 3. The N-terminal amino acid sequences previously reported by Sanchez-Monge et al. [11] are underlined. Numbers indicate nucleotide positions (bp). The nucleotide sequences have been submitted to EMBL Database under accession numbers AJ 277163 (Pru p 3) and AJ 277164 (Mal d 3).

presented from 93%–52% sequence identity with Pru p 3, and those from *Artemisia* and *Parietaria* pollens, 41% and 29%, respectively (Fig. 2).

The high similarity among Pru p 3, Mal d 3 and Pru ar 3 led us to choose the former as a model for heterologous expression of allergenic lipid-transfer proteins from *Rosaceae* fruits. Thus, the pPIC 9 expression plasmid carrying the Pru p 3 cDNA was used to transform *P. pastoris* GS115 cells. Maximum expression of extracellular rPru p 3 was achieved four days after induction with methanol (not shown). The yield was around 20 mg of recombinant allergen per litre of culture supernatant.

Recombinant Pru p 3 was purified by a two-step chromatographic procedure (Fig. 3). The dialysed extracellular medium of the culture was first fractionated by gel-filtration chromatography (Fig. 3a). Fractions containing putative rPru p 3 (together with additional components) were freeze-dried and then separated by RP-HPLC (Fig. 3b). The peak including rPru p 3, as well as the homogeneity of the isolated recombinant polypeptide, were checked by SDS-PAGE. Purified rPru p 3 showed a single electrophoretic band, which migrated exactly the same as that of natural Pru p 3 (Fig. 4a). N-terminal sequencing of the purified rPru p 3 band rendered the expected sequence EITCG, corresponding to the four N-terminal residues of the mature allergen (Fig. 1) preceded by a glutamic acid that was added for proper extracellular expression of the protein (see Materials and methods).

Recombinant Pru p 3 was strongly recognized by polyclonal antibodies against natural Pru p 3 (Fig. 4b). Furthermore, both

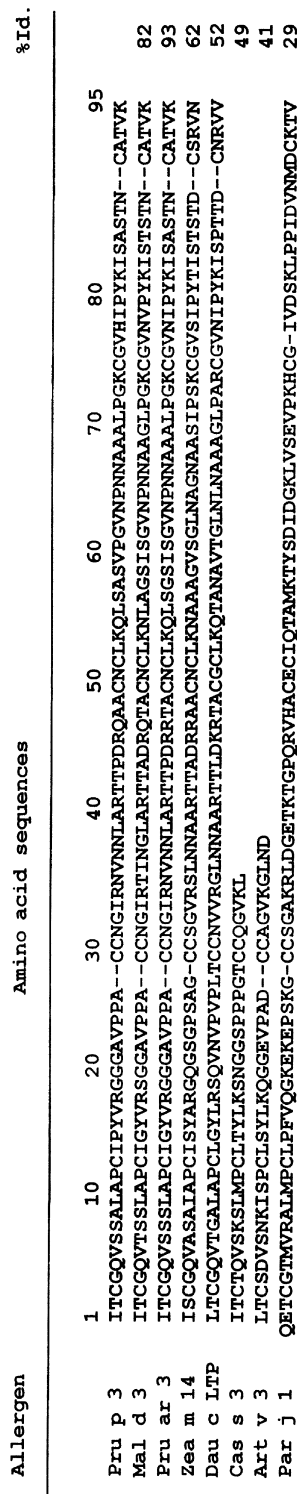


Fig. 2. Amino acid sequences of Pru p 3 and Mal d 3, aligned with those of allergenic LTPs from apricot (Pru ar 3; accession number P81651), maize (Zea m 14; accession number P19656), carrot (Dau c LTP; accession number P27631); chestnut (Cas s 3; only N-terminal sequence available; see [10]), *Artemisia vulgaris* pollen (Art v 3; only N-terminal sequence available; see [10]) and *Parietaria judaica* pollen (Par j 1; accession number P 43217). Gaps (–) to obtain the proper sequence alignment are indicated. Percentages of sequence identity (%Id) with respect to Pru p 3 are indicated at the right side of figure.

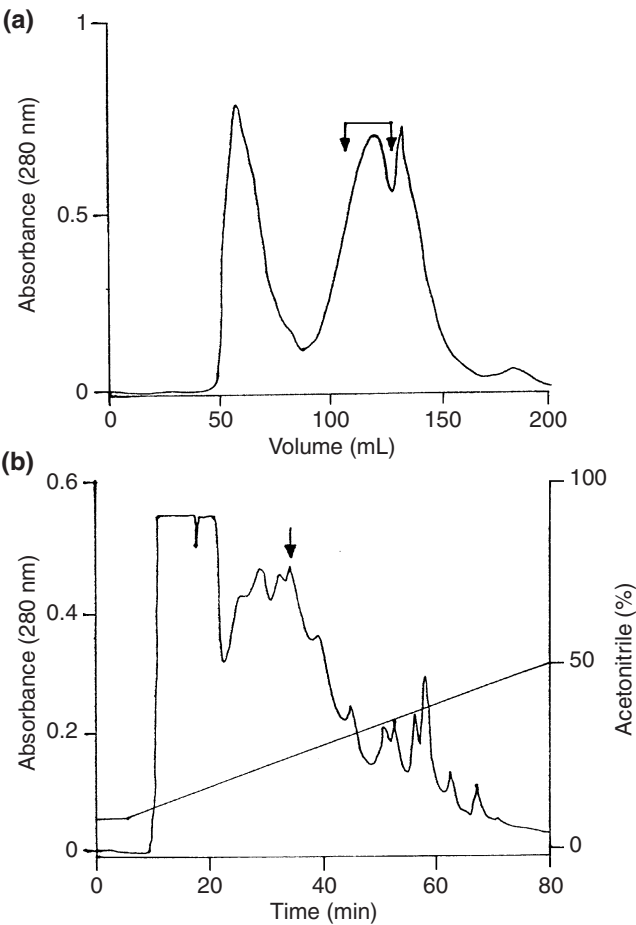


Fig. 3. Purification of recombinant Pru p 3. (a) Gel-filtration on a Superdex HR 75 16/26 column of the dialysed supernatant from *P. pastoris* culture expressing Pru p 3. Fractions enriched in rPru p 3 are indicated. (b) RP-HPLC fractionation on a Nucleosil 300-C4 column of the gel-filtration fractions indicated in panel (a). Arrow points to the position of the isolated rPru p 3.

forms displayed similar IgE-binding capacity when a pool of sera from peach-allergic patients was used for specific IgE immunodetection (Fig. 4c). This fact was strongly supported by immunoblot inhibition assays (Fig. 5). A full IgE-binding inhibition was obtained by using rPru p 3 as inhibitor against natural Pru p 3, both as purified protein or as major component of a protein preparation from peach peel. The same was true when natural Pru p 3 was tested as inhibitor against its recombinant counterpart.

Discussion

A relevant role of *Rosaceae* fruits, mainly peach, as plant foods causing allergic reactions, has been demonstrated in the Mediterranean area [7,20]. Lipid-transfer proteins have been identified as major allergens in several of these fruit, including peach, apple, apricot and cherry [8,9,21]. Besides their specific IgE-binding capacity *in vitro*, the *in vivo* reactivity of those from peach and apple (Pru p 3 and Mal 3, respectively) has also been proved [22]. The clinical relevance of both allergens in a population of Spanish patients with allergy to *Rosaceae* fruits has recently been highlighted by skin prick testing purified Pru p 3

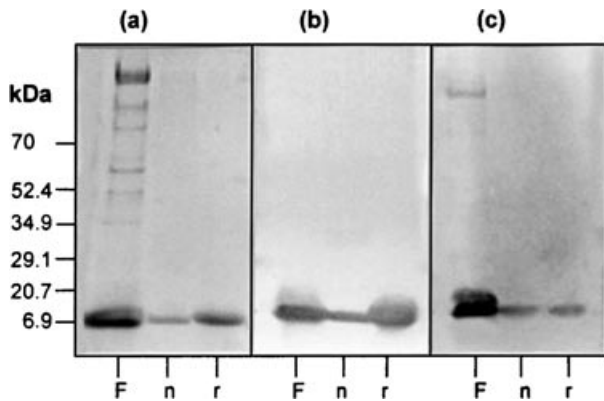


Fig. 4. SDS-PAGE fractionation of a protein preparation (5 µg of protein) enriched in Pru p 3 (F) from peach peel and of purified natural (n) and recombinant (r) Pru p 3 (1 µg). Replica gels were stained with Coomassie Blue (a) or electrotransferred to PVDF membranes and immunoblotted with polyclonal antibodies to nPru p 3 (b) or a pool of sera from peach-allergic patients (c).

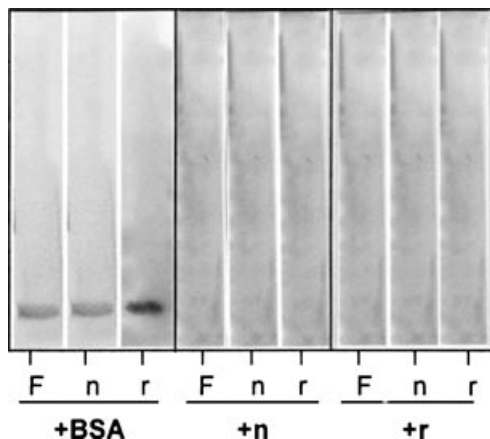


Fig. 5. Immunoblot inhibition assays with bovine serum albumin (+BSA; negative control) and purified natural (+n) and recombinant (+r) Pru p 3 as inhibitors (5 µg of each inhibitor). A protein preparation (3 µg of protein) enriched in Pru p 3 (F) from peach peel and purified natural (n) and recombinant (r) Pru p 3 (1 µg) separated by SDS-PAGE and electrotransferred to PVDF membranes, were immunodetected with a pool of sera from peach-allergic patients pre-incubated (3 h at room temperature) with each inhibitor.

and Mal d 3 in a group of 47 subjects suffering allergy to peach (Garcia-Selles et al. unpublished). The rates of positive skin prick tests for Pru p 3 and Mal d 3 were 91% and 77%, respectively. Furthermore, the reactivity to Mal d 3 increased to 92% when the subgroup of patients who reported adverse reaction to peach and apple were considered. Strong correlations were found between the skin responses to peach peel and Pru p 3 and peach extract and Pru p 3. The same was true between apple extract and Mal d 3, and between both purified allergens. Taking this fact into account, the lack of standardization of commercial fruit extracts and the non-availability of fresh fruit to perform prick-prick tests during long periods each year, to consider the use of recombinant allergenic LTPs as potential tools for diagnosis and immunotherapy seems to be warranted.

No clones have been reported for Pru p 3 and Mal d 3, the major peach and apple allergens. Therefore, cDNA clones coding for both mature allergens were isolated (Fig. 1) as a step prior to obtaining the recombinant products. The isolated

clones encode typical 91 residues mature LTP proteins, the correspondence with Pru p 3 and Mal d 3 of which is fully supported by the identity of their N-terminal regions and the close concordance of molecular sizes with those determined for the natural peach and apple allergens [8].

The complete amino acid sequences of mature Pru p 3 and Mal d 3 deduced from their corresponding cDNA clones have allowed to ascertain the close similarity among allergens of *Rosaceae* fruits belonging to the lipid-transfer protein family (Fig. 2). Moreover, the reported cross-reactions of Pru p 3 with other allergenic LTPs, such as those from chestnut, maize and carrot [10–12], illustrated by IgE-binding inhibition assays, can be explained by the substantial sequence identity of the peach allergen and its homologues present in the plant foods mentioned.

Expression of Pru p 3, chosen as a model *Rosaceae* LTP allergen, has been carried out in the yeast *P. pastoris*, as an extracellular non-fusion protein. This eukaryotic expression system allows the avoidance of the solubility problems provoked by the location of LTPs in inclusion bodies when expressed in *E. coli* [23, Diaz-Perales et al. unpublished]. rPru p 3 has been purified from the supernatants of transformed *P. pastoris* cultures (Figs 3 and 4). The recombinant allergen showed the expected N-terminal amino acid sequence, the same apparent molecular size in SDS-PAGE as its natural counterpart, and was strongly recognized by polyclonal antibodies against natural Pru p 3. All these data indicate that the recombinant protein is properly expressed. Interestingly, both natural and recombinant Pru p 3 have a similar IgE-binding capacity, as shown by immunodetection and immunoblot inhibition assays (Figs 4 and 5). Therefore, rPru p 3 can be a useful tool for future diagnostic and structural studies, including the epitope mapping and the production of hypoallergenic variants through site-directed mutagenesis.

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Erratum

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There was an error on p. 88 of this article. The sense primer sequence given on the third line of the section entitled 'Expression of rPru p 3 in *Pichia pastoris*' should read:

5'-CCGTCTCGAGAAAAGAGAAATAACATGTGGCC-3'