



Lipid transfer proteins from Rosaceae fruits share consensus epitopes responsible for their IgE-binding cross-reactivity

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Abstract

Four IgE-binding epitopes have been characterized that cover a large area ($\geq 40\%$) of the molecular surface of lipid transfer protein allergens of Rosaceae (apple, peach, apricot, and plum). They mainly correspond to electropositively charged regions protruding on the molecular surface of the modeled apple (Mal d 3), apricot (Pru ar 3), and plum (Pru d 3) allergens. Two of these epitopes consist of consensus epitopes structurally conserved among the lipid transfer protein allergens from the Rosaceae. Their occurrence in different lipid transfer protein allergens presumably accounts for the IgE-binding cross-reactivity often observed among different Rosaceae fruits. In this respect, LTP consist of phylogenetically- and structurally-related pan allergens. However, the IgE-binding cross-reactivity due to fruit lipid transfer protein has varying degrees of clinical relevance and this cross-reactivity is not necessarily accompanied by a cross-allergenicity to the corresponding fruits.

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Non-specific lipid transfer proteins (nsLTP) have been recently characterized as major allergens of fruits and, especially, of Rosaceae fruits (apple, apricot, peach, and plum) [1–5]. Plant LTP allergens belong to the LTP1 group of family 14 of pathogenesis-related (PR-14) proteins [6]. They consist of small-sized basic proteins of 9 kDa (91 amino acid residues, $pI \geq 9$) built up from a core of four α -helices stabilized by four conserved disulfide bridges and a loosely coiled C-terminal tail [7,8] that confers a

“saxophone-like” fold to the protein. This tightly packed three-dimensional structure most probably accounts for the high resistance to thermal denaturation and proteolysis previously reported for these plant allergens [9]. In Northern European countries, LTP from Rosaceae fruits trigger hypersensitive reactions, essentially contact dermatitis, in individuals previously cross-sensitized by pollen LTP [10]. With the exception of the plum fruits, the skin of other Rosaceae fruits usually contains the higher amounts of LTP [11,12], thus making the allergen directly available to trigger contact dermatitis in sensitized people. However, they are also capable to achieve the sensitization of susceptible individuals *via* a stimulation of the lymph nodes of the gastro-intestinal tract immune system, especially in people from Mediterranean countries [13,14]. The often reported IgE-binding cross-reactivity towards different Rosaceae fruits obviously depends on the conserved three-dimen-

Abbreviations: IgG, immunoglobulin G; IgE, immunoglobulin E; Mal d 3, *Malus domestica* (apple) LTP allergen; nsLTP, non-specific lipid transfer protein; OAS, oral allergy syndrome; Pru ar 3, *Prunus armeniaca* (apricot) LTP allergen; Pru d 3, *Prunus domestica* (plum) LTP allergen; Pru p 3, *Prunus persica* (peach) LTP allergen; TBS, tris buffered saline (pH 7.0); TBSTw, TBS containing 0.1% (v/v) Tween 20 (pH 7.0).

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sional structure of LTP allergens, that presumably share common IgE-binding epitopes [15]. Except for Pru p 3 [16], the peach LTP allergen, the IgE-binding epitopes of other fruit LTP have not been characterized to date. Here we present a structural analysis of the IgE-binding epitopes on the molecular surface of LTP allergens from apple (Mal d 3), apricot (Pru ar 3), peach (Pru p 3), and plum (Pru d 3) showing the occurrence of a consensual IgE-binding epitope that most probably accounts for the currently observed IgE-binding cross-reactivity among Rosaceae fruits.

Materials and methods

IgE and IgG antibodies. Polyclonal IgG antibodies against Mal d 3 and Pru p 3 were raised in rabbit as previously described [17]. Blood samples were drawn after informed consent of five patients allergic to Rosaceae fruits (as checked by skin prick testing using peach extract as allergen source and specific IgE measurement to peach ≥ 3.0 UI) and pooled sera were used for both Western blotting and SPOT assay experiments.

Peptide synthesis and SPOT assays for the determination of overlapping IgG- and IgE-binding peptides. Overlapping 15-mer peptides, frameshifted by three residues, corresponding to the entire amino acid sequences of Mal d 3, Pru ar 3, Pru d 3, and Pru p 3, respectively, were prepared by using the SPOT technique [18]. The protocol previously described in detail [19] was followed, with the exception of the utilization of the MultiPep automatic SPOT synthesizer (Intavis). Briefly, peptides were assembled using the Fmoc chemistry on a cellulose membrane harboring an amino polyethylene glycol moiety. The C-terminal residue of each peptide was coupled to the activated membrane. After Fmoc deprotection, the following amino acids were sequentially added. At the end of the synthesis, side-chain protecting groups were removed by a trifluoroacetic acid treatment while the linkage of the peptides to the membrane was maintained.

The membrane was soaked overnight into 20 mL of Tris-buffered saline (TBS) containing 2 mL blocking buffer (Roche) and 1 g sucrose (pH 7.0), and then washed with TBS containing 0.1% (v/v) Tween 20 (TBSTw). A 1:10 (v/v) diluted pool of patient sera was added in the presence of an anti-protease cocktail (Roche) and the membrane was incubated in a moist chamber for 2 h. After three washes with TBSTw (pH 7.0) the membrane was stirred in a 1:4000 dilution of mouse monoclonal anti-human IgE labeled with alkaline phosphatase (Sigma) for 1 h. After two washes with TBSTw (pH 7.0) and two washes with 10 mM citric acid containing 137 mM NaCl and 3 mM KCl (pH 7.0) (CBS buffer), the interacting peptide spots were colored for 30 min by adding the bromochlorolindolylphosphate (BCIP) substrate for alkaline phosphatase (Promega) in CBS buffer containing 5 mM MgCl₂ and 0.7 mM thiazolyl blue tetrazolium bromide. The membrane was washed three times with deionized water and dried for scanning. The membrane can be used repeatedly after a regeneration step consisting in one wash in dimethyl-formamide for 1 min, three washes in deionized water, three washes in 8 M urea containing 1% (w/v) SDS and 1% (w/v) β -mercaptoethanol and three subsequent washes in a mixture of ethanol–CH₃COOH–H₂O (50:10:40, v/v).

Molecular modeling and mapping of IgG- and IgE-binding epitopes on the molecular surface. Molecular modeling of Mal d 3 and other LTP was carried out on a Silicon Graphics O2 R10000 workstation, using the programs InsightII, Homology and Discover3 (Accelrys, San Diego, CA, USA). The atomic coordinates of the maize LTP Zea m 14 [20] (RCSB Protein Data Bank code 1MZL) and Pru p 3 (PDB code 2ALG) [16] were used to build the three-dimensional model of the allergens. The percentages of both identity ($\sim 35\%$) and homology ($\sim 75\%$) Zea m 14 shares with other LTP allowed us to build accurate three-dimensional models using the X-ray coordinates of the maize and peach LTP as templates. Steric conflicts were corrected during the model building procedure using the rotamer library [21] and the search algorithm implemented in the

homology [22] to maintain proper side-chain orientation. An energy minimization of the final models was carried out by 50–100 cycles of steepest descent using Discover3. PROCHECK [23] was used to assess the geometric quality of the three-dimensional models. As an example, all the residues of Mal d 3 were correctly assigned on the best allowed regions of the Ramachandran plot with the exception of residues Ser19 and Pro25 which are located in the generously allowed regions of the plot (result not shown). Cartoons were drawn with PyMOL (W.L. DeLano, <http://pymol.sourceforge.net>). Electrostatic potentials were calculated and displayed with GRASP [24] as previously described [17]. A clip of 8–16 Å slab across the molecular surface of the modeled allergens was used to display the overall shape and size of the fatty acid harboring tunnel-like cavity of LTP.

The surface occupied by the previously characterized or predicted sequential IgG- and IgE-binding epitopes of LTP allergens was calculated with GRASP and displayed on the molecular surface of the proteins with PyMOL. Their overall conformation on the molecular surface was displayed with PyMOL.

Results

All the modeled LTP allergens share a very conserved three-dimensional structure built up from four α -helical segments ($\alpha 1$ – $\alpha 4$) that adopt a saxophone-like fold with an extended C-terminal tail (Fig. 1A). Eight conserved Cys residues participate in four disulfide bridges which contribute to stabilize the overall fold. According to their basic character (net charge of 7–8), they all exhibit a predominantly electropositively charged surface (Fig. 1B). They contain a cranked tunnel-like cavity that has been shown to unspecifically accommodate different fatty acid chains and fatty acid derivatives (Fig. 1D). Mal d 3, Pru p 3, and Pru ar 3 nicely superimpose with a rms value of 0.48 Å for 91 superimposed α -carbons (Fig. 1C). Obviously, the best superimposition was obtained for α -helices except for helix $\alpha 1$ which displays some discrepancy among the three allergens.

Epitope mapping using rabbit polyclonal anti-Mal d 3 IgG revealed four IgG-binding amino acid sequence stretches distributed along the polypeptide chain of Mal d 3 (Fig. 2A). They correspond to stretches 22AVP24 (epitope #1), 35INGLARTTADRQ46 (epitope #2), 62VNPNNA67 (epitope #3), and 77NVPYKISTSTNCATVK92 (epitope #4), respectively. Except for the IgG-binding epitope 3, they contain electropositively charged (Arg and Lys) residues. Both IgG-binding epitopes cover a large part of the accessible surface (2550 Å²) that represents $\sim 50\%$ of the total accessible surface (5108 Å²) of Mal d 3. Other nsLTP allergens yielded an identical distribution for epitopes 2 and 3 whereas epitope 1 constantly corresponds to a shorter but still charged stretch of amino acid residues (Fig. 3). However, the accessible surface occupied by the three IgG-binding epitopes still amounts for almost 50% of the total accessible surface in Pru p 3, Pru ar 3, and Pru d 3.

Epitope mapping performed with different IgE-containing sera from patients allergic to Rosaceae fruits instead of using rabbit polyclonal IgG, revealed a closely similar distribution of four IgE-binding epitopes along the amino

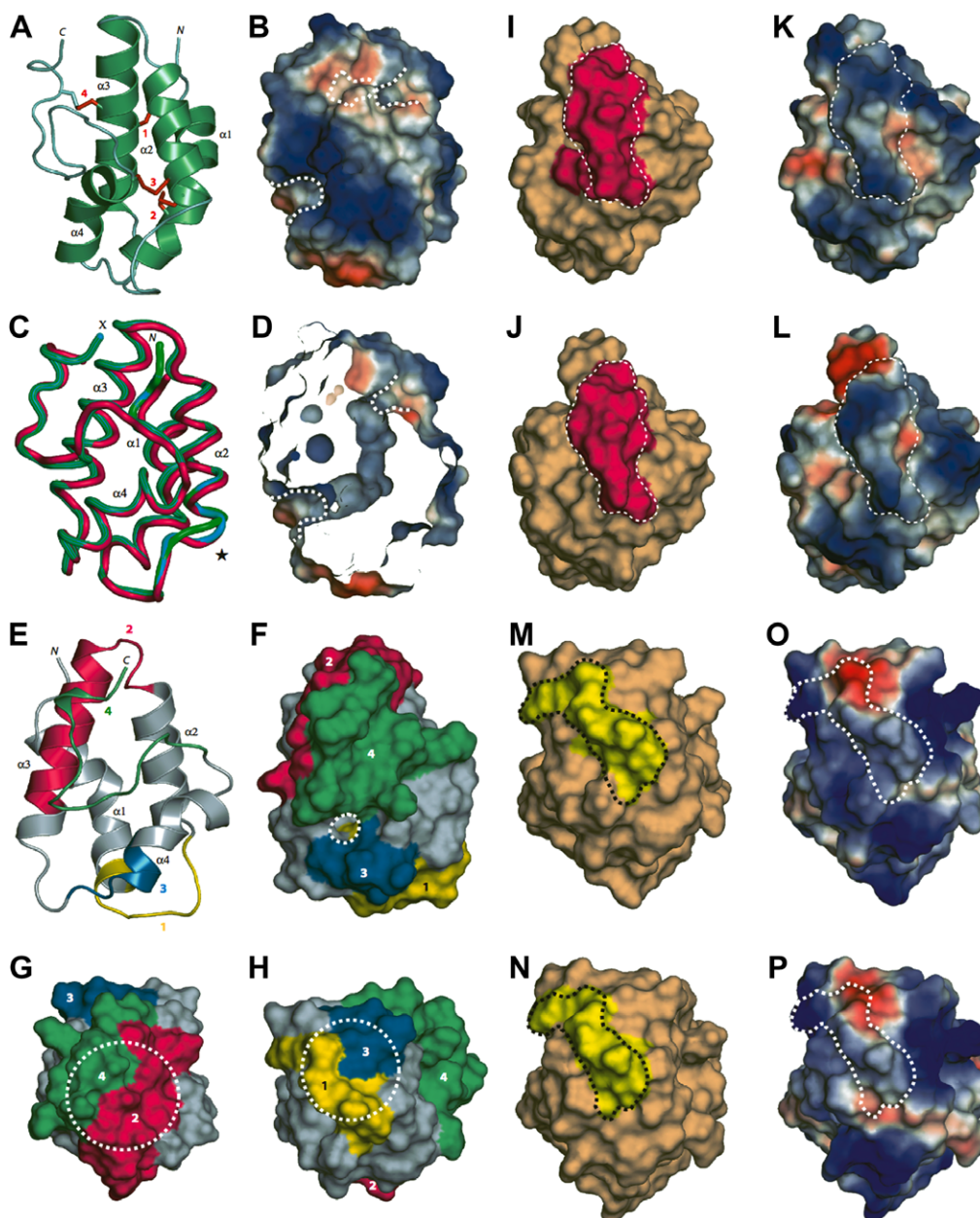


Fig. 1. (A) Ribbon diagram showing the overall three-dimensional fold of Mal d 3. The four α -helices ($\alpha 1$ – $\alpha 4$) and the four conserved disulphide bridges are labeled 1, 2, 3, and 4, respectively. N and C correspond to the N- and C-termini of the polypeptide chain. (B) Mapping of the electrostatic potentials on the molecular surface of Mal d 3 shown in a different orientation to reveal both extremities (dashed white lines) of the tunnel-like cavity. Electropositively- and electronegatively-charged regions are colored blue and red, respectively. Neutral regions are colored white. (C) Superimposition of Mal d 3 (green), Pru p 3 (pink), and Pru ar 3 (cyan). The major fold discrepancy occurring at the beginning of helix $\alpha 1$ is indicated by a star (*). (D) Clip view showing the overall cranked shape of the tunnel-like cavity. Both extremities of the tunnel are indicated by dashed white lines. (E and F) Localization of the IgE-binding epitopes #1 (1, yellow); #2 (2, pink); #3 (3, blue); and #4 (4, green) on the ribbon diagram (E) and the molecular surface (F) of Mal d 3. One aperture of the tunnel-like cavity is indicated by a dashed white circle on the molecular surface (B and F). (G and H) Coalescence (open dashed white circle) of IgE-binding epitopes #2–#4 (G) and #1–#3 (H) susceptible to create more extended epitopic areas on the molecular surface of Mal d 3. (I–L) Conformation of the consensual IgE-binding epitope #2 (pink surface) characterized on the molecular surfaces of Mal d 3 (I) and Pru ar 3 (J). This epitope corresponds to a preferentially electropositively-charged area as shown upon mapping of electrostatic potentials on the molecular surfaces of Mal d 3 (K) and Pru ar 3 (L). All nsLTP allergens are similarly oriented and the common epitope core is delineated by a dashed white line. (M–P) Conformation of the consensual IgE-binding epitope #1 (yellow surface) characterized on the molecular surfaces of Mal d 3 (M) and Pru p 3 (N). This epitope corresponds to a rather charged area as shown upon mapping of electrostatic potentials on the molecular surfaces of Mal d 3 (O) and Pru p 3 (P). All allergens are similarly oriented and the common epitope core is delineated by a dashed black or white line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acid sequences (Fig. 2B) and on the molecular surface of the assayed LTP allergens (Fig. 1E and F). However, some discrepancies were observed that mainly concern both the

length of the identified epitopic stretches and their reactivity towards the corresponding antibodies. As exemplified in Fig. 2, the strongly reactive IgE-binding epitope #1

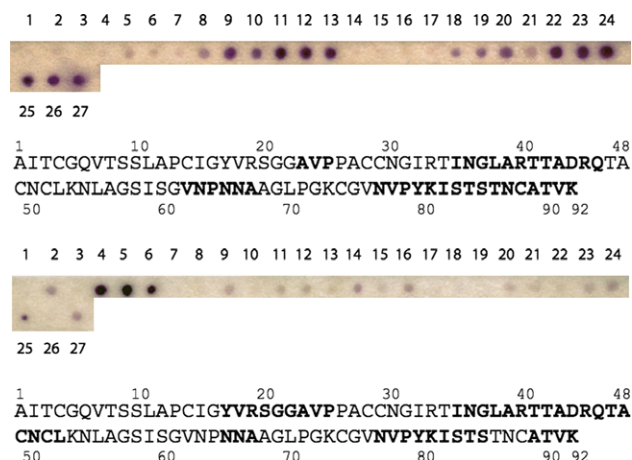


Fig. 2. (A) upper: IgG-binding peptides (dark spots) revealed on the SPOT membrane and, lower: mapping of the corresponding continuous IgG-binding epitopes (bold letters) along the amino acid sequence of Mal d 3. (B) upper: IgE-binding peptides (dark spots) revealed on the SPOT membrane and, lower: mapping of the corresponding continuous IgE-binding epitopes (bold letters) along the amino acid sequence of Mal d 3.

16YVRS¹⁶GGAVP²⁴ corresponds to the weakly reactive shorter IgG-binding epitope #1 22AVP²⁴ in Mal d 3. Conversely, epitope #2 35INGLARTTADRQ⁴⁶ strongly interacts with polyclonal rabbit IgG whereas its longer IgE-binding counterpart 35INGLARTTADRQTACNCL⁵² is much more less immunoreactive. Despite these discrepancies, a fairly good overlap occurs between IgG- and IgE-binding epitopic stretches characterized along the amino acid sequences of LTP allergens (see Fig. 3). The IgE-binding epitopes similarly cover a large surface (2700 Å²) that amounts about 53% of the molecular surface in Mal d 3. With a few exceptions, they still correspond to predominantly electropositively charged areas. This overlapping suggests a closely-related similarity and specificity of the human IgE- and rabbit IgG-epitopic regions in spite of a very different source for these immunoglobulins.

Discussion

Both polyclonal rabbit IgG and IgE-containing sera from allergic patients allowed us to identify four IgG/IgE-binding epitopes along the polypeptide chain of fruit LTP allergens using the SPOT technique. Most of these

epitopes contain electropositive (Arg, Lys) and, to a lesser extent, electronegative (Asp, Glu) residues due to the highly basic character ($pI \geq 9$) of LTP of plant origin. These charged residues are classically exposed on the molecular surface of allergens. However, the characterized IgE-binding epitopes moderately overlap on those previously characterized [16] or predicted [17] along the amino acid sequence of Pru p 3 except for the strongly charged exposed core of epitopes #1, #2, and #3. Such a discrepancy most probably depends on both the size (pentadecapeptides instead of decapeptides) of the synthetic peptides used in the SPOT technique to characterize the IgE-binding epitopes and the slightly different immunoreactivity of the IgE-containing sera of allergic patients used as probes.

As previously shown from ELISA measurements performed on Mal d 3 and Pru p 3 [17], our results confirm that IgE-binding epitope #2 corresponds to a consensual epitope conserved among the Rosaceae LTP allergens. Despite some discrepancy concerning the extent of the epitope on the molecular surface, its core structure exhibits a very similar conformation in other Rosaceae LTP (Fig. 1I–L). According to this structural conservation among the Rosaceae LTP, this epitope should be responsible for some IgE-binding cross-reactivity in the presence of IgE-containing sera from patients allergic to Rosaceae fruits. IgE-binding epitope #1 (16YVRS¹⁶GGAVP²⁴ in Mal d 3), which is strictly conserved in other Rosaceae LTP allergens, similarly exhibits a very similar conformation in Pru av 3, Pru d 3, and Pru p 3 (Fig. 1M–P) that suggests it should also correspond to another consensual epitope presumably responsible for some additional IgE-binding cross-reactivity among the fruit LTP. Moreover, the synthetic peptides corresponding to this (major) IgE-binding epitope #1 gave the strongest staining signal in our SPOT experiments (see Fig. 2B). In spite of some amino acid conservation, the two IgE-binding epitopes characterized on the surface of fruit LTP allergens exhibit rather different conformations from one allergen to another which most probably depends on changes in the rotamers of the amino acid residues in relation with their local environment.

As structurally- and phylogenetically-related proteins, plant LTP consist of pan allergens like profilins or Bet v 1 homologs, susceptible to cross-react in IgE-binding experiments [25]. The occurrence of closely-related LTP



Fig. 3. Overlap of the IgG-binding epitopes (upper line) and the IgE-binding epitopes (lower line) characterized with the SPOT technique along the amino acid sequences of Mal d 3 (A and B), Pru p 3 (C and D), Pru d 3 (E and F) and Pru ar 3 (G and H). Epitopic stretches are in black boxed white letters.

in vegetable foods and pollens might account for the rather high frequency of individuals sensitized to Rosaceae fruits, especially in the Mediterranean countries [25]. However, the discrepancy often observed between cross-reactivity and cross-allergenicity that separates asymptomatic subjects from allergic patients exhibiting systemic symptoms, urticaria or anaphylaxis, most likely depends on multiple factors including e.g. the level and polyclonality of the synthesized IgE, the polymorphism of the FcεRI receptors, the balance of T regulatory cells and Th1/Th2 cells, etc. [26].

One of the most striking results dealing with the topography of the IgE-binding epitopes consists in the large surface area they cover on the accessible surface of LTP allergens. In Mal d 3, the four identified IgE-binding epitopes cover about 50% of the total accessible surface. In addition, the close vicinity of epitopes #1 and #3 on the molecular surface, creates an extremely extended epitopic area that amounts for 38% of the total accessible surface in e.g. Mal d 3. In this respect, LTP readily differ from other plant allergens, e.g. the legumin and vicilin allergens from peanut or tree nuts, which exhibit less extended and more scattered IgE-binding epitopes over their molecular surface [27]. The coalescence of the two distantly arrayed epitopes #1 and #3 or #2 and #4 along the amino acid sequence is of paramount importance since it can contribute to build up new extended conformational epitopes that share amino acid residues from both linear epitopes. These structurally-related IgE-binding epitopes might possibly account for the strong allergenic propensity of the Rosaceae LTP, especially in Mediterranean countries [25].

Finally, the characterization of IgE-binding epitopes on the molecular surface of LTP open the way to the tailoring of recombinant hypoallergenic proteins by site directed mutagenesis with the goal of producing allergens that retain the T-cell propensity but are devoid of any consistent IgE-binding activity. Eventually, these hypoallergens should consist of useful tools for the immunotherapy of the LTP-mediated allergies to Rosaceae fruits.

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