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Review. Plant food allergens: peach non-specific lipid transfer protein Pru p 3 as a model

G. Salcedo[1]*, R. Sánchez-Monge[1], A. Díaz-Perales[1] and L. F. Pacios[2]

[1] *Unidad de Bioquímica. Departamento de Biotecnología. ETS Ingenieros Agrónomos. Universidad Politécnica. Madrid. Spain*

[2] *Unidad de Química y Bioquímica. Departamento de Biotecnología. ETS Ingenieros de Montes. Universidad Politécnica. Madrid. Spain*

Abstract

Plant non-specific lipid transfer proteins (LTPs) are plant defence proteins that constitute a relevant panallergen family present in both plant foods and pollens. Their high resistance to proteolytic digestion, thus being probably primary sensitizers by ingestion, and association with systemic and severe clinical symptoms, have led to propose LTPs as a model of true food allergens. Peach Pru p 3, the prototypic member of the family, has been extensively studied at the biochemical, immunological and clinical level. IgE-binding regions of Pru p 3 have been identified by an experimental and modelling based approach.

Additional key words: cross-reactivity, model allergen, plant defence protein, plant food allergy.

Resumen

Revisión. Alergenos de alimentos vegetales: la proteína de transferencia de lípidos de fruto de melocotón, Pru p 3, como modelo

Las proteínas de transferencia de lípidos (LTPs) de plantas son proteínas de defensa, y constituyen una familia de panalergenos, con relevancia tanto en alimentos vegetales como en pólenes. Su inusual resistencia a la digestión proteolítica, que posibilita que actúen como agentes de sensibilización primaria por ingestión, junto a su asociación con síntomas clínicos sistémicos y severos, justifica que se hayan propuesto como modelo de alergenos alimentarios. La LTP de melocotón, denominada Pru p 3, es el prototipo de esta familia de alergenos, y ha sido ampliamente estudiada a nivel bioquímico, inmunológico y clínico. Las regiones (epitopos) de esta proteína responsables de la unión a IgE han sido identificadas por distintas técnicas experimentales complementadas por modelado tridimensional.

Palabras clave adicionales: alergeno modelo, alergia a alimentos vegetales, proteínas de defensa, reactividad cruzada.

Introduction[1]

Allergic diseases, including rhinitis, conjunctivitis, dermatitis, asthma and anaphylaxis, are provoked by the inhalation, ingestion or contact of allergens, which induce in some subjects an adverse immunological response mediated by immunoglobin E (IgE) (Holgate *et al.*, 2006). The prevalence of these diseases has been greatly increased in developed countries, nowadays affecting up to 30% of their population. In the case of food allergy, the actual prevalence appears to be around 3% of the adult population and 6-8% in young childrens (Burks and Ballmer-Weber, 2006).

The identification, isolation and biochemical and immunological characterization of allergens have been essential for basic studies to understand how these molecules induced IgE antibody production, thus triggering allergic reactions. Besides, purified allergens (natural or recombinant) are currently introduced to standardize and enhance diagnostic tools, as well as to rationalize protocols of immunotherapy (Valenta *et al.*, 1999; Valenta, 2002). Furthermore, the availability of cDNA

* Corresponding author: gabriel.salcedo@upm.es
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[1] Abbreviations used: Ig (immunoglobulin), LTP (lipid transfer protein), PB (Poisson-Boltzmann), PR (pathogenesis -related), RMSD (root mean square deviation).

clones and recombinant forms of major allergens, together with the mapping of their T-cell and B (IgE) epitopes, have allowed the generation of hypoallergenic variants (i.e. by site-directed mutagenesis of critical amino acid residues responsible for IgE-binding, but not for T-cell activation). Such engineered vaccines would eventually present lower IgE reactivity, thus reducing the risk of severe IgE-mediated side-effects in specific immunotherapy (Ferreira *et al.,* 2002). Additionally, the identification of the major sensitizing allergens can help to predict cross-reactivity between plant foods and pollens, or different plant sources (i.e. fruits and latex) (Van Ree, 2004), and whether or not can be expected a reduction of allergenic potency by food processing (i.e. heat treatment) or digestion (Shate *et al.,* 2005). Finally, evaluations of the putative risk of transgenic foods include a decision protocol mainly based on potential homology of the transgenic protein and known allergens (Goodman *et al.,* 2005).

Plant food allergens

A large number of plant food allergens has been identified so far (Breiteneder and Radauer, 2004). However, all of them can be assigned to only 31 different protein families, being most allergens included in a few types of seed storage or plant defence proteins (Chapman *et al.,* 2007; see Table 1). Prolamins from cereal flours and vicilins, legumins and 2S albumins from legumes and nuts are the main allergenic storage proteins. Several groups of pathogenesis-related proteins, such as chitinases, thaumatins, PR-10 proteins and lipid transfer proteins (LTPs), have been described as allergens, mainly in fruits and vegetables. Besides, a panallergen family constituted by profilins, which are proteins involved in the regulation of the actin cytoskeleton, has been identified in many plant foods as responsible for cross-reactivity with pollens.

The LTP allergen family

The non-specific lipid transfer protein family of allergens includes basic polypeptides of 9 kDa (90-95 amino acid residues), with 8 conserved cysteines forming 4 disulphide bridges, which are crucial to explain the high stability and allergenic potency of these proteins (see for reviews Kader, 1996; Van Ree, 2002; Pastorello and Robino, 2004; Salcedo *et al.,* 2004, 2007). The LTP family is ubiquitously present throughout the plant kingdom, encoded by a multigene family showing complex, specific tissue and developmental expression patterns in most plant species investigated. However, the most abundant members of the family are extracellular proteins, associated with cell walls, and mainly accumulated in epidermal tissues surrounding aerial organs (leaves, fruits, etc.) (García-Olmedo *et al.,* 1995; Marzban *et al.,* 2005; Ahrazem *et al.,* 2007). This localization has relevant clinical implications, such as the higher allergenic potency of peels than pulps of Rosaceae fruits in the Mediterranean population (Fernández-Rivas and Cuevas, 1999).

The original designation as LTPs was based on their potential to bind and transfer lipids between membranes in *in vitro* assays, but their extracellular localization

Table 1. Major types of plant food allergens

Type/family	Representative foods
Seed Storage proteins	
Prolamins	Wheat, rye
Vicilins	Soybean, peanut, lentil
Legumins	Soybean, peanut, hazelnut
2S albumins	Mustard, sunflower, walnut
Defence proteins	
Chitinases and hevein-like proteins (PR[1] 3,4,8)	Avocado, chestnut, banana
Thaumatins (PR 5)	Kiwi, apple, cherry
Bet v 1 family (PR 10)	Celery, carrot, apple
Lipid transfer proteins (PR 14)	Peach, apple, wheat
α-Amylase inhibitors	Wheat, rye, rice
Proteases	Kiwi, melon

[1] PR: pathogenesis-related proteins.

Table 2. Plant sources containing allergenic members of the LTP family included in the List of Allergens (IUIS, Allergen Nomenclature Sub-Committee)

Plant foods
— Fruits: apple, apricot, cherry, grape, lemon, orange, peach, plum, tomato, strawberry
— Vegetables: asparagus, cabbage, lettuce
— Cereals: maize, wheat
— Nuts: chestnut, hazelnut, walnut
Pollens
— Mugwort, olive, parietaria, plane, ragweed
Other plant sources
— Latex

has led to consider very unlikely a similar *in vivo* role (in intracellular trafficking of membrane lipids). In fact, different lines of evidence now available support the involvement of LTPs in plant defence mechanisms against phytophatogenic bacteria and fungi (García-Olmedo *et al.,* 1995, 1998; Salcedo *et al.,* 2007). Such evidence includes their *in vitro* activity against phytopathogens and permeabilization of fungal spores, induction by bacterial and fungal infection, enhancement of tolerance to bacterial pathogens in transgenic plants expressing LTPs, binding to fungal elicitin receptors, and promotion of long-distance signalling (probably interacting with oxylipins) linked to systemic-acquired resistance. LTPs are now forming the PR-14 family of pathogenesis-related proteins. Other possible function proposed for the LTP family, such as the transport of monomers (i.e. of cutin) during the assembly of hydrophobic polymers in surface protective layers (Douliez *et al.,* 2000), is fully compatible with its implication in plant defence.

Besides their concern in the context of plant desease, the LTPs have gained a new area of interest in relation to human health since 1999, as a relevant panallergen family involved in IgE-mediated reactions to both plant foods and pollens (Pastorello and Robino, 2004; Salcedo *et al.,* 2004, 2007; Zuidmeer and Van Ree, 2007). Allergenic members of the family have been identified in an increasing number of plant foods, including fruits, vegetables, nuts and cereals, as well as in latex (Table 2). All these allergenic LTPs show amino acid sequence identities from 92% to 45% to the fruit peach (*Prunus persica*) allergen Pru p 3, which is selected as model member of the family (Fig. 1; see below). This structural similarity provides the molecular basis for the wide cross-reactivity found among most LTP allergens from plant foods (Díaz-Perales *et al.,* 2000; Salcedo *et al.,* 2007). However, such cross-reactivity seems to be highly restricted between members whose IgE epitopes have substantially diverged, such as peach Pru p 3 and wheat Tri a 14 (Fig. 1; Palacin *et al.,* unpublished). Interestingly, reactive LTPs have been also detected in some pollens (Table 2), thus suggesting an additional role as inhalant allergens (Salcedo *et al.,* 2007; Zuidmeer and Van Ree, 2007). Those from mugwort (*Artemisia vulgaris*) and plane tree (*Platanus acerifolia*) show over 40% of sequence identity with Pru p 3, and cross-react with homologous members from some foods, thus being potentially responsible for plant food-pollen cross-sensitization (Palacin *et al.,* 2006; Lauer *et al.,* 2007). In contrast, allergenic LTPs from olive (*Olea europea*), ragweed (*Ambrosia artemisiifolia*) and *Parietaria judaica* pollen display lower sequence identities with Pru p 3 and have not been implicated in pollen-food cross-sensitization.

The LTP family has been proposed as a model of true food allergens based on three properties that are tightly linked: uncommon resistance to proteolytic digestion, ability to sensitize by the oral route and association with systemic and severe clinical symptoms (Van Ree, 2002; Salcedo *et al.,* 2007). The LTP allergens assayed so far in *in vitro* models of gastrointestinal digestion show a high proteolytic stability, thus retaining their immunological reactivity and eliciting responses similar to those displayed by the untreated (native) allergens (Asero *et al.,* 2000; Scheurer *et al.,* 2004; Vassilopoulou *et al.,* 2006). This *in vitro* behaviour

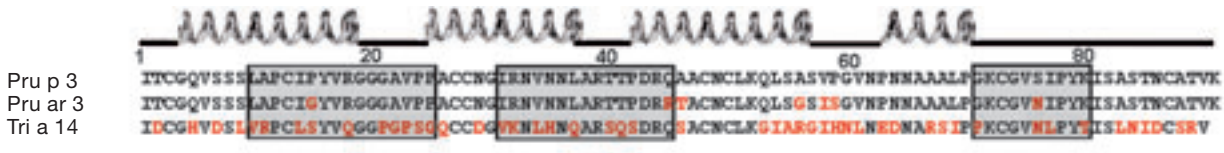

Figure 1. Alignment of amino acid sequences of LTP allergens from peach (Pru p 3), apricot (Pru ar 3; 92% identity to Pru p 3) and wheat (Tri a 14; 45% identity to Pru p 3). Regions corresponding to the IgE epitopes defined in Pru p 3 (Garcia-Casado *et al.,* 2003) are boxed, and the 4 α-helices (AAAA) and loops (—) located in this allergen (Pasquato *et al.,* 2006) are indicated.

suggests that the allergenic motifs of LTPs remain active in the gastrointestinal tract and can interact with the associated immune system to trigger sensitization and systemic symptoms. In fact, a link between LTP sensitization and severe allergic reactions has been described in patients (mainly from the Mediterranean area; see below) with allergy to several plant foods, such as peach, apple or cherry (Fernández-Rivas *et al.,* 2003, 2006; Reuter *et al.,* 2006).

Besides their resistance to proteolytic digestion, LTPs also resist heat treatments (up to 100-120ºC) without a significant lost of their IgE-binding capacity (Brenna *et al.,* 2000; Scheurer *et al.,* 2004). Both characteristics lead to find active LTP allergens in processed plant-derived products, such as beer, wine, juices and jams (García-Casado *et al.,* 2001; Duffort *et al.,* 2002; Salcedo *et al.,* 2007).

The unexpected geographical profiles of LTP sensitization found across Europe is other characteristic of this panallergen family with a relevant significance in the clinical practice (Fernández-Rivas *et al.,* 2003, 2006; Reuter *et al.,* 2006). The population from South European countries (i.e. Spain, Italy) with allergy to several plant foods (and even mugwort or *Parietaria* pollen), usually present a high rate (above 50%) of sensitization to the corresponding LTP allergen, whereas very low prevalences (under 15%) are detected in similar patients from Central and Northern Europe. Differences in dietary habits (i.e. time and amount of Rosaceae fruit, mainly peach, introduction in the diet) and exposure to pollens (i.e. mugwort or olive *versus* birch) have been claimed to explain the different geographical profiles of LTP sensitization. However, some exceptions to the role of LTPs as dominant allergens in the Mediterranean countries have been recently reported. Thus, the members present in orange and tomato seem to be minor allergens (sensitization rates below 50% in groups of patients with allergy to the corresponding fruit) (Ahrazem *et al.,* 2005; Le *et al.,* 2006), and the LTP from chestnut shows high reactivity in chestnut allergic patients without associated latex allergy, but not in those suffering a chestnut-latex syndrome (Sanchez-Monge *et al.,* 2006).

Peach Pru p 3 as model of plant food LTP allergens

Peach represents the major cause of allergic reactions induced by plant food in the Spanish adult population (Red Vegetalia-FIS, unpublished), and seems to be the starting point for LTP sensitization in many patients. Accordingly, peach LTP Pru p 3 has been identified as the most prevalent plant food allergen in Spanish subjects with allergy to these foods (around 40% of sera showing specific IgE to Pru p 3; Red Vegetalia-FIS, unpublished). Consequently, Pru p 3 has been a rising focus of studies at the biochemical, immunological and clinical level, being probably the best characterized allergenic LTP from plant foods at present (Salcedo *et al.,* 2007). Since the isolation of its natural form 8 years ago (Pastorello *et al.,* 1999; Sánchez-Monge *et al.,* 1999), its clinical relevance has been demonstrated (Fernández-Rivas *et al.,* 2003), its encoding cDNA isolated and expressed in *Pichia pastoris* (Díaz-Perales *et al.,* 2002), and quantitation methods based on specific mono- and polyclonal antibodies to the purified allergen developed (Duffort *et al.,* 2002). Pru p 3 levels have been determined in several peach and nectarine cultivars, varying from 5.5 to 41.1 $\mu g\ g^{-1}$ of fresh weight in whole fruits and exhibiting approximately 250-fold higher concentration in peels than in pulps (Ahrazem *et al.,* 2007).

To dispose of the full amino acid sequence, as well as recombinant form of Pru p 3 have opened several research opportunities. Firstly, a direct comparison of the natural and recombinant versions of the allergen has supported their equivalent folding, and IgE-binding and biological capacity, thus validating rPru p 3 as a tool for the diagnosis of *Rosaceae* fruit allergy and LTP sensitization (Díaz-Perales *et al.,* 2003). Secondly, it constitutes the background to start a structural search for Pru p 3, including its 3-D modelling, epitope mapping and engineering by site-directed mutagenesis.

As for other known structures of LTPs, the main motif of Pru p 3 is the helical compact domain made up of four α-helices (H1-H4 in Fig. 2A) that involve 57 out of the 91 residues in the protein. Eight cysteines form four disulphide bridges that contribute to the structural rigidity of the protein by connecting helices H1 to H3 (Cys 3-Cys 50), H1 to H2 (Cys 13-Cys 27), H2 to H4 (Cys 28-Cys 73), and helix H3 to the C-terminal coil (Cys 48-Cys 87). These four packed helices leave a tunnel-like hydrophobic cavity that runs through the whole protein and is able to accommodate lipidic ligands (Pasquato *et al.,* 2006; Salcedo *et al.,* 2007). The non-polar end of this lipophilic cavity located near the loop H3H4 (Fig. 2B) consists of residues L10, A11, I14, L54, and V58. The polar end of the cavity is at the opposite side of the protein and involves residues N35 and R44 that presumably would anchor the carboxylic

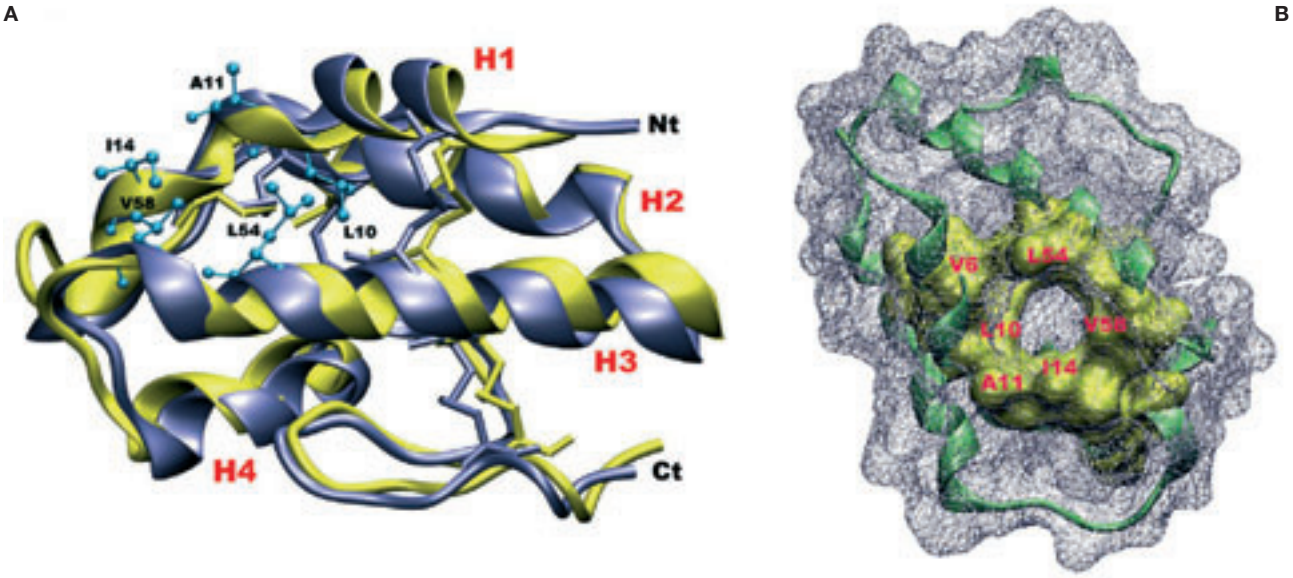

Figure 2. A: ribbon diagram of crystal (blue) and homology modelled (yellow) structures of Pru p 3 superposed. The four helices characteristic of LTPs are labelled H1-H4, Nt and Ct denote N- and C-termini, respectively, and the bonds represent four disulphide bridges. Balls-and-sticks (cyan) represent residues at the hydrophobic end of the lipid binding cavity for the crystal structure (their orientations are nearly coincident for the model structure). B: accessible surface of the whole protein (wireframe) and residues around the lipid binding cavity (yellow solid surface) for the crystal structure. Nonpolar residues labelled form the hydrophobic end of the cavity.

heads of fatty acids which are left exposed to the solvent (Pasquato *et al.,* 2006). These two residues happen to be included in the IgE-binding epitopes so far characterized in Pru p 3 (see below).

The characterization of putative IgE epitopes on the surface of Pru p 3 was accomplished using a homology modelled structure (García-Casado *et al.,* 2003) three years before the X-ray solved structure was published (Pasquato *et al.,* 2006). The reliability of our model is illustrated in Fig. 2A by superposing crystal and model structures: not only the architecture of the helical motif is accurately reproduced but the location of disulphide bridges as well as the geometry of the extended C-terminus coil and the interhelical loops are satisfactorily represented. A quantitative assessment of the similarity between both structures is provided by the low RMSD yielded by the superposition of the backbone atoms, 1.08 Å. Further comparison between solvent accessibility profiles for the whole sequence and surface features (areas and volumes of cavities) computed for both X-ray and model structures of Pru p 3 yields virtually identical results. Hence, the identification of putative epitopes performed on the model can be safely translated to the experimental structure.

The rationale behind our approach to search for epitopes in Pru p 3 was that IgE-binding regions should be local protuberant surfaces suggesting electrostatical activity. We therefore explored the contribution of every residue to the solvent accessible surface as well as the occurrence of local changes of electrostatical potential onto the surface. Five mostly positive residues were proposed as likely candidates involved in epitope activity: R39, T40, R44, K80, and K91. Site-directed alanine substitution single mutants K80A and K91A, as well as the triple mutant R39A/T40A/R44A were produced to evaluate their immunoreactivity. While both single mutants exhibited an IgE-binding behavior rather similar to that of the wild-type allergen, a drastic reduction of IgE-binding capacity and inhibition potency (at least five times) were observed for the triple mutant (García-Casado *et al.,* 2003). This result suggested that the region encompassing R39, T40 and R44 residues (highlighted in Fig. 3A) represents a relevant epitope of Pru p 3 (Fig. 3B). Despite the overall structural similarity of LTPs, significant differences among their electrostatical patterns are known (Pasquato *et al.,* 2006). Regarding Pru p 3, the dominance of basic residues (four arginines and four lysines) over acidic residues (just one aspartate) yields an overall positive character to the electrostatic potential, as Fig. 3C clearly illustrates (notice the scale of PB potential). There are, however, two regions that exhibit great changes between the extreme values of the PB range considered in small contiguous surface areas that are apparent in the bottom

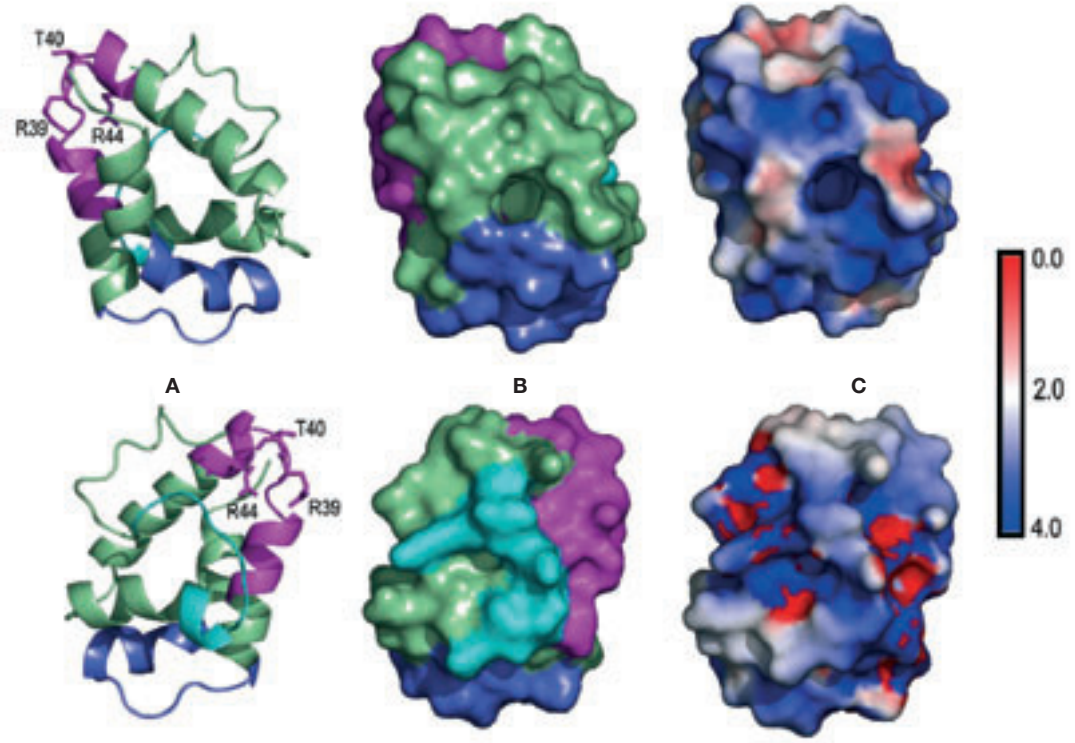

Figure 3. A: ribbon diagram of the crystal structure of Pru p 3 showing three linear epitope sequences identified in the modelled structure: 11-25 (blue), 31-45 (magenta), and 71-80 (cyan). Sidechains for residues 39, 40 and 44 in the second epitope are also depicted. B: solvent-accessible surfaces at the orientation in A. C: PB electrostatic potential mapped onto the surfaces in B. Bottom views are derived from upper views by a 180º rotation about a vertical axis. Scale bar in electrostatic units of kTe^{-1} (k: Boltzmann's constant, T: absolute temperature, e: electron charge).

view of Fig. 3C. One of these regions encompasses R39, T40, R44 including too the effect of the negative D43 which is however relatively buried (it has 54% exposure to the solvent) as its primary role seems to establish an ionic attraction with the near positive N terminal amino group lending thus further stability to the fold. It must be stressed that both D43 and R44 are strictly conserved in LTPs whereas R39 and T40 are well conserved in *Rosaceae* LTPs and only partially conserved in cereal LTPs (Pasquato *et al.,* 2006).

The IgE-binding regions of Pru p 3 were also investigated by analyzing a library of 10-mer synthetic peptides (overlapping five amino acids) which screened the entire sequence of the protein (García-Casado *et al.,* 2003). The main responses found by using a serum pool from patients allergic to peach were observed for overlapping peptides corresponding to sequence segments 11-25, 31-45, and 71-80 indicated in Fig. 3A and their surface showed in Fig. 3B. The first segment spans half the helix H1 and the coil between helices H1 and H2 and its surface shows a relatively homogeneous electropositive character. The second segment spans the end of helix H2, the loop H2H3 and the beginning of helix H3 and includes residues R39, T40 and R44 identified individually before along with D3, the unique acidic amino acid. The third segment spans the end of helix H4 and part of the large C-term coil and includes K80, also predicted as one of the five individual reactive residues. The surfaces of both segments 31-45 and 71-80 happen to exhibit relatively large electrostatic changes in nearby regions, one feature which was initially conjectured as indicative of IgE-binding behavior. It must be also remarked that these three sequences nicely match three of four linear epitopes recently predicted for Pru p 3 in a study conducted to identify a consensual IgE-binding epitope occurring in several plant LTPs (Borges *et al.,* 2006).

In a further attempt to sharpen the IgE-binding behavior of Pru p 3 we have tried to identify a consensus epitope (Diaz-Perales *et al.,* unpublished). To this end, specific IgEs purified from two different serum pools of patients with peach allergy were used to select phage clones from a random display peptide phage library. Seventeen distinct peptides with Pru p 3-specific IgE

binding capacity were identified. Peptide alignments revealed two overlapping consensual sequences: L37 R39 T40 P42 D43 R44 A46 P70 S76 P78 Y79 and N35 N36 L37 R39 T40 D43 A46 S76 I77 P78. Note that R39, T40, and R44 are three of the residues proposed to be involved in epitope activity on theoretical grounds. Furthermore, all the residues in the consensual sequences except A46 and P70, are contained in segments 31-45 and 71-80 identified on the basis of the reactive peptides discussed above. Those consensual sequences are thus mapped onto the same regions of Pru p 3 surface spanning the loop H2H3 and the C-terminal coil. These findings agree rather well with the IgE epitopes described before by our group.

Joint consideration of the structural data summarized above, together with the mapping of the T-cell epitopes of Pru p 3, can help to produce hypoallergenic variants of this allergen for a safer immunotherapy in a near future.

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