

Identification of IgE-binding epitopes of the major peach allergen Pru p 3

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Background: Lipid transfer proteins (LTPs) are clinically relevant plant food panallergens and have been proposed as ideal tools to study true food allergy. Pru p 3, the major peach allergen in the Mediterranean area, is among the best-characterized allergenic members of the LTP family. Its diagnostic value for *Rosaceae* fruit allergy has been demonstrated both in vivo and in vitro.

Objective: We sought to locate major IgE-binding epitopes of Pru p 3.

Methods: A serum pool and individual sera from patients with peach allergy and positive skin prick test results to Pru p 3 were used. Three-dimensional modeling was achieved by using experimentally available structures of Pru p 3 homologues as templates. Theoretical prediction of potential IgE-binding regions was performed by selecting specific residues on the molecular surface displaying prominent electrostatic potential features. Point mutants of Pru p 3 were constructed by standard polymerase chain reaction procedures with the appropriate primers. Mutants were expressed in *P. pastoris* by means of the pPIC 9 vector and purified from the corresponding supernatants by gel-filtration chromatography followed by RP-HPLC. IgE binding by Pru p 3 mutants was tested by immunodetection and quantified by ELISA and ELISA inhibition assays. Synthetic peptides (10 mer; 5 amino acids overlapping) covering the full Pru p 3 sequence were used to detect IgE epitopes by ¹²⁵I-anti-IgE immunodetection.

Results: Pru p 3 showed a 3-dimensional structure comprising 4 α -helices and a nonstructured C-terminal coil (residues 73 to 91). Regions around amino acids in positions 23 to 36, 39 to 44, and 80 to 91, particularly residues R39, T40, and R44, K80 and K91, were predicted as potential antibody recognition sites according to their relevant surface and electrostatic properties.

Point mutants K80A and K91A were found to have an IgE-binding capacity similar to that of recombinant Pru p 3, but the triple mutant R39A/T40A/R44A showed a substantial decrease (approximately 5 times) of IgE binding. IgE immunodetection of synthetic peptides led to the identification of Pru p 3 sequence regions 11 to 25, 31 to 45, and 71 to 80 as major IgE epitopes.

Conclusions: Main IgE-binding regions of the Pru p 3 amino acid sequence were identified. The three major ones comprised the end of an α -helix and some residues of the following inter-helix loop. These data can help to search for Pru p 3 hypoallergenic forms. (J Allergy Clin Immunol 2003;112:599-605.)

Key words: Fruit allergy, lipid transfer protein, peach allergen, IgE-binding epitopes, site-directed mutagenesis, 3-dimensional modeling

Lipid transfer proteins (LTPs) have emerged as a highly relevant family of plant panallergens, particularly in the Mediterranean area.^{1,2} Their association with severe and systemic clinical symptoms in patients allergic to certain foods,¹ frequently nonsensitized to pollen, together with their resistance to gastric digestion³ and stability to heat treatments,⁴ have led to propose LTPs as ideal tools to investigate true food allergy.¹ The LTP family is widely distributed throughout the plant kingdom and is involved in defense mechanisms against pathogens and probably also in the transport of cuticular precursors to the outer layers of plant organs.^{5,6} According to its ubiquitous distribution, allergenic members of the LTP family have been identified mainly in *Rosaceae* fruits⁷ but also in vegetables,³ tree nuts,⁸ and cereals.⁹ Moreover, several main pollen allergens also belong to this protein family.^{8,10} Cross-reactions among allergenic LTPs have been demonstrated both in vitro and in vivo.^{3,8,11}

The LTP family comprises mainly basic 9-kD polypeptides (90 to 95 amino acids long) with 8 conserved cysteine residues and a wide range of sequence identity (30% to 95%) among different members.⁵ The experimentally determined 3-dimensional (3-D) structures of cereal LTPs¹²⁻¹⁴ have led to propose an all- α -type compact structure with 4 α -helices and a nonstructured C-terminal coil, firmly held by 4 disulfide bridges, as the standard 3-D folding of plant LTPs.⁵ Despite these structural data, no B-cell or T-cell epitopes of any allergenic member from plant foods have been mapped. Only a partial study focused on the N-terminal sequence (1 to

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Supported by Dirección General de Investigación, MCYT (project BMC 2002-00196).

Received for publication March 31, 2003; revised April 29, 2003; accepted for publication May 1, 2003.

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0091-6749/2003 \$30.00 + 0

doi:10.1067/mai.2003.1638

Abbreviations used

LTP: Lipid transfer protein
PCR: Polymerase chain reaction
SPT: Skin prick test

30 residues) of the major *Parietaria judaica* allergen Par j 1.0101 has been published.¹⁰ The disulfide bonds between cysteines 14 and 29 and residues between positions 20 and 30 were identified as being essential for IgE binding in this study.

Pru p 3, the major allergen and LTP from peach,^{7,15} appears to be a suitable model to undertake the identification of IgE epitopes of allergenic LTPs of plant foods. Peach is among the most frequent causes of food allergy in the Mediterranean population, and Pru p 3 induces positive in vivo reactions (SPT) in >90% of patients allergic to this fruit,¹¹ thus indicating that it could be a useful tool for the diagnosis of *Rosaceae* fruit allergy in such a population. Furthermore, the recombinant allergen (rPru p 3) has been produced, and its immunologic reactivity has been shown to be equivalent to that of natural Pru p 3.^{15,16} This background warrants the search for hypoallergenic forms of the allergen and for the molecular basis of its cross-reactivity with allergenic LTPs from other plant sources. Both objectives require previous knowledge of Pru p 3 T-cell and B-cell epitopes for a rational approach. We report the identification of potential IgE-binding regions of Pru p 3, following a dual and complementary approach, based on 3-D modeling and on the analysis of selected site-directed mutants and synthetic peptides.

METHODS**Patients and sera**

Seven patients allergic to peach were selected during a prospective study performed at Hospital Virgen de la Arrixaca, Murcia (Spain). All patients showed immediate hypersensitivity to peach according to their clinical history, positive SPT to peach extract and purified Pru p 3, and specific IgE levels to peach >3.5 kU/L. Clinical data of the selected patients were previously reported.¹⁶ Three healthy subjects with negative SPT responses and no specific IgE to peach, apple, and plum were tested as negative control patients. Written informed consent was obtained from all subjects included in this study.

Total and specific IgE levels were determined by the Pharmacia CAP system FEIA (Pharmacia Diagnostics, Uppsala, Sweden). SPTs were carried out with a commercial preparation (ALK-Abeló, Madrid, Spain) or with purified natural Pru p 3 (20 µg/mL), following conventional procedures. Wheal responses 7 mm² greater than those produced by the saline control were considered positive.

A serum pool or individual sera from the selected patients or control patients were used in the IgE immunodetection and ELISA assays.

Three-dimensional modeling and theoretical prediction of potential IgE-binding regions of Pru p 3

The 3-D structure of Pru p 3 was determined by using the services of the Swiss-Model Protein Modeling Server,¹⁷⁻¹⁹ choosing as templates 5 experimentally available structures of Pru p 3 homologues with amino acid sequence identity between 53% and 62%. These known structures belong to nonspecific LTPs from maize (x-

ray diffraction data with PDB codes 1MZL and 1MzM¹³ and a set of NMR data, code 1AFH²⁰) and rice (X-crystal geometry with code 1RZL¹⁴ and a set of NMR structures, code 1BV2²¹). The graphical processing of the 3-D-modeled structure, as well as the calculation of the electrostatic potential mapped onto the molecular surface of the protein, were accomplished with Swiss-Pdb Viewer software.¹⁹ Values of total solvent-accessible surface areas for the whole exposed surface and individual area contributions from the 91 residues were calculated with the Arvomol program.²² The surface profiles to determine protuberant residues were calculated and selected as explained elsewhere.²³

Construction, heterologous expression, and purification of Pru p 3 point mutants

Site-directed mutagenesis to generate Pru p 3 point mutants was performed by using appropriate primers for PCR amplification and the cDNA encoding Pru p 3 previously obtained¹⁵ as template. The primers used were K80A mutant 5'-GAATTCTCACTTCACGGTG-GCCAGTTGGTGGAGGCGCTAATCGCGTAAGG-3'; K91A mutant 5'-GAATTCTCACGCCACGGTGGCGC-3' (*Eco* R I restriction site underlined). In both mutants, the previously described sense primer for Pru p 3 cDNA amplification¹⁵ (5'-CCGTCTCGA-GAAAAGAGAAATAACATGTGGCC-3'; *Xho* I restriction site underlined) was also used for amplification. The R39A/T40A/R44A triple mutant was generated with primers 5'-CTG-GGCGTCAGGGGTGGCCGCTGCC-3' and 5'-GGCAGC-GGC-CACCCCTGACGCCAG-3', using the sense (see above) and anti-sense primers (5'-CCTCGAATTCTCACTTC ACGG-3') previously described¹⁵ for reamplification. The PCR program consisted of 5 minutes' denaturation at 95°C followed by 30 cycles each of 30 seconds' denaturation at 94°C, 30 seconds' annealing at 55°C, and 30 seconds' extension at 72°C and final extension for 5 minutes at 72°C. Reamplification was carried out with the use of the same program.

The PCR products were purified from agarose gels, digested with *Eco* RI and *Xho* I restriction enzymes, and inserted into the same sites of the pPIC 9 vector to transform DH5αF' *Escherichia coli* cells. The DNAs from several clones corresponding to each mutant were sequenced with the ABI PRISM Dye Terminator Kit and an ABI 377 sequencer (Perkin Elmer Biosystem, Warrington, United Kingdom) to confirm the introduced mutation.

Expression of Pru p 3 point mutants in GS115 *Pichia pastoris* cells was performed as previously described for recombinant Pru p 3.¹⁵ Isolation of the mutants as well as of rPru p 3 from the corresponding culture medium was achieved by gel filtration followed by reverse-phase HPLC, using the same columns and conditions as described for rPru p 3.¹⁵ Purified proteins were quantified by using the bicinchoninic acid method.²⁴

Immunodetection assays

Samples were separated by SDS-PAGE on 10% to 20% Tricine precast polyacrylamide gels (Invitrogen, DeSchelp, The Netherlands) under reducing conditions and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes, as previously reported.²⁵ After washing and blocking, membranes were incubated with a serum pool from patients allergic to peach (1:3 dilutions) and then with alkaline phosphatase-conjugated monoclonal anti-human IgE (clone GE-1, Sigma, St Louis, Mo; 1:500 dilution) and developed by adding 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium solution.

Alternatively, replica-blocked membranes were immunodetected with rabbit polyclonal antibodies to natural Pru p 3¹⁵ (1:2000 dilution), then treated with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma; 1:1000 dilution) and finally with a 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium solution.

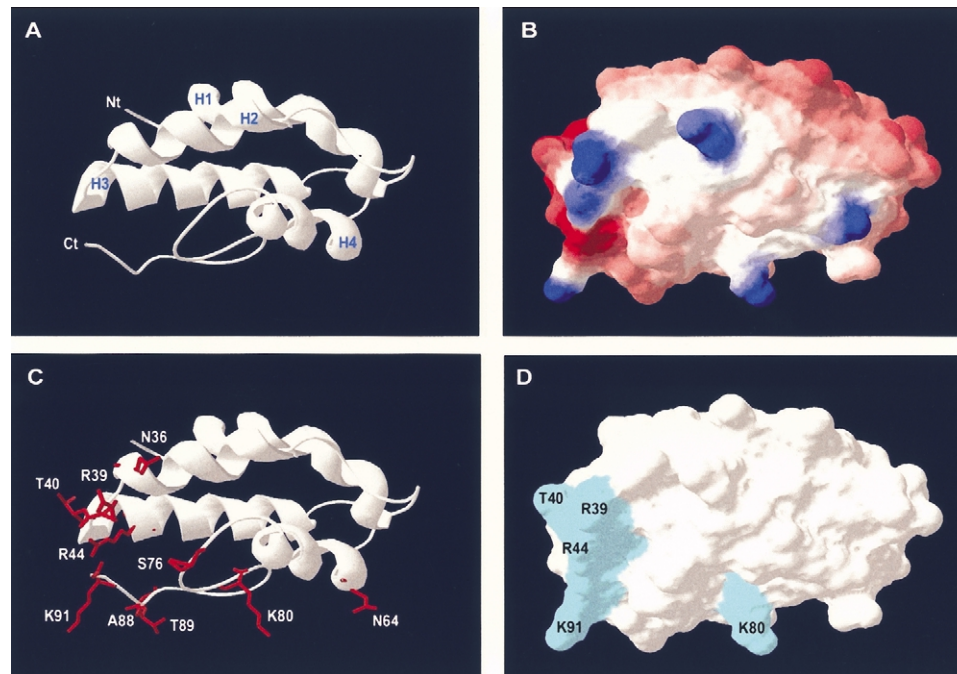


FIG 1. Three-dimensional modeled structure of Pru p 3. **A**, Backbone ribbon-type representation with four helices marked H1 to H4. **B**, Molecular surface with electrostatic potential mapped onto surface coded by using a range from 0.0 (deep red), 2.0 (white), to 4.0 (deep blue) in units of kT/e. **C**, Model A, including side chains of residues identified on theoretical grounds as potential IgE-binding regions (see text). **D**, Residues in loop 2 (R39, T40, R44) and lysines (K80 and K91) highlighted over the molecular surface displayed in **C**.

Specific IgE binding and ELISA inhibition assays

Specific IgE binding to rPru p 3 and its mutants was carried out as previously described,¹⁶ using 1:3 serial dilutions (range, 1:1 to 1:3⁵) of a serum pool from 7 patients allergic to peach and 2 µg/mL of purified protein. PBS buffer with 1% BSA was tested as the negative control as well as a serum pool from healthy subjects (OD below 0.05 units when tested toward rPru p 3 or its mutants). All tests were performed in triplicate.

ELISA inhibition assays were carried out by the same method, with the use of rPru p 3 (2 µg/mL) as the solid phase and 25 µL of the serum pool (1:9 dilution), previously preincubated with 25 µL of serial dilutions of rPru p 3 or of each mutant (range, 2 to 0.002 µg/mL) for 3 hours at 25°C. All tests were performed in triplicate.

Dot-blot analysis of solid phase-bound synthetic peptides

Synthetic peptides (17 decapeptides overlapping by 5 amino acids) spanning the entire Pru p 3 sequence and covalently bound to a cellulose membrane (SPOTs) were commercially obtained from Jerini AG (Berlin, Germany). The SPOT membranes were wetted in ethanol, washed with PBS-Tween buffer (0.1 mol/L sodium phosphate, pH 7.0, 0.15 mol/L NaCl, 0.05% Tween-20), and blocked with the same buffer containing 1% blocking agent (Amersham Biosciences, Little Chalfont, United Kingdom; code RPN2125) for 16 hours at 25°C. The membrane was then incubated with serum pool from patients allergic to peach diluted (1:3) in blocking buffer (16 hours), afterward with ¹²⁵I-labeled anti-human IgE (ALK-Abel-ló) for 4 hours at 25°C, and finally revealed by autoradiography (48 hours' exposure at -80°).

SPOTs comprising selected reactive peptides as well as synthetic alanine substitution mutants were also analyzed by the same method using individual sera or the serum pool from allergic patients.

RESULTS

The secondary structure of the modeled Pru p 3 protein comprised four α-helices conserved in the experimentally available structures of other LTPs and a large C-terminal coil between residues 73 and 91 (Figs 1, A, and 5, A). The 8 cysteine residues present, also conserved in most homologues, probably formed 4 disulfide bridges in locations 3-50, 13-27, 28-73, and 48 to 87 (not shown). Basic residues (4 arginines and 4 lysines) prevailed over acid residues (only one aspartate), which determined the electrostatic features of the protein. In fact, the electrostatic potential calculated on the molecular surface (Fig 1, B) is positive everywhere, with a half-side dominated by apolar residues, which correspond to the apolar end of the hydrophobic cavity existing in the homologues chosen as templates (see Methods section). The half-side displayed showed an overall intermediate electrostatic potential (white regions) with several prominent polar residues exhibiting large positive values (blue regions).

Presuming that an IgE-binding region should exhibit both a protuberant local surface and an electrostatically active local molecular domain, we searched for residues showing large changes of the electrostatic potential with

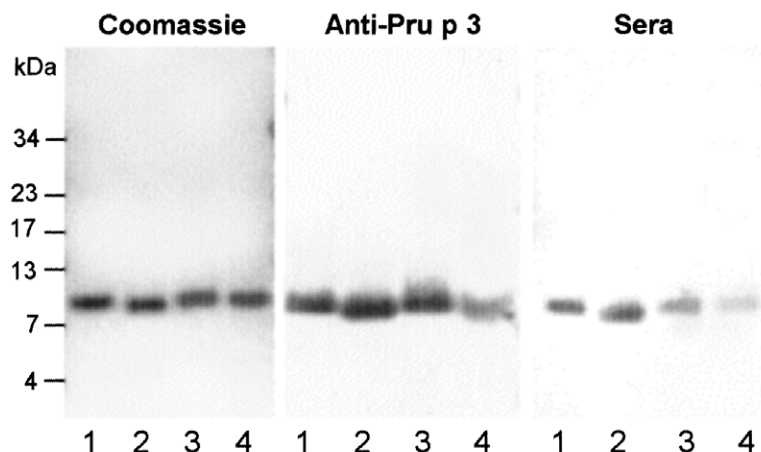


FIG 2. Protein staining (Coomassie) and immunodetection with anti-Pru p 3 rabbit polyclonal antibodies and IgE antibodies of a pool of sera from patients allergic to peach of purified recombinant Pru p 3 (1) and its mutants K80A (2), K91A (3), and R39A/T40A/R44A (4).

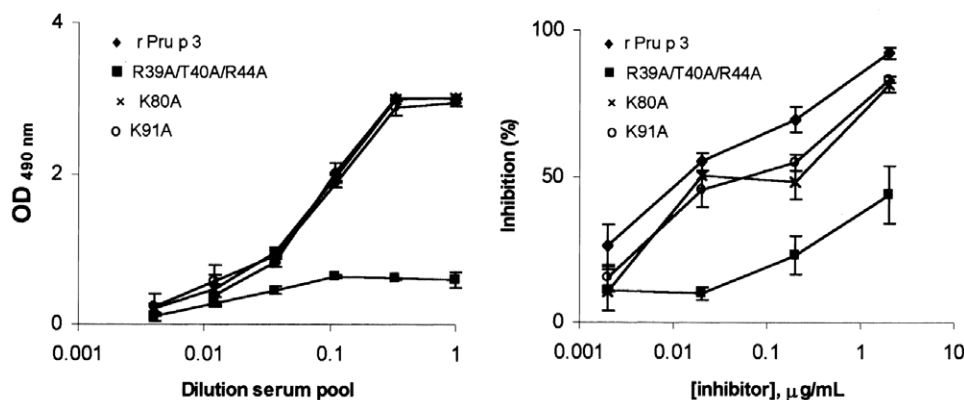


FIG 3. IgE binding to rPru p 3 and its mutants K80A, K91A, and R39A/T40A/R44A (left). ELISA inhibition with rPru p 3 in the solid phase and rPru p 3 and its mutants K80A, K91A, and R39A/T40A/R44A as inhibitors (right). A pool of sera from patients allergic to peach was used in both assays. Means ($n = 3$) and SDs (bars) are represented.

respect to nearby intermediate values with simultaneous percentages on surface area exposure >60%.²³ The residues whose side-chains are displayed in Fig 1, C, all of them located on the polar half-side surface, happen to fulfil these conditions. Among these, the 5 residues with the largest quantitative values according to the criteria considered are the two arginines (R) 39 and 44, threonine (T) 40, and the two lysines (K) 80 and 91. All of these residues are located near the region of loop 2 except for K80. On the basis of this theoretical approach, we considered the residues highlighted in Fig 1, D, over the molecular surface as the best candidates to potentially harbor antibodies.

Therefore, alanine (A) substitution mutants K80A and K90A, as well as the triple mutant R39A/T40A/R44A (designed because of the proximity of the three residues), were produced by standard PCR procedures with the appropriate oligonucleotide primers. The actual mutations introduced were confirmed by DNA sequencing of

at least 3 different clones for each mutant. The corresponding constructs inserted in the pPIC9 vector were used to transform GS115 *P. pastoris* cells, and after induction, the mutants were purified from the culture medium of each transformed yeast clone. All three mutants showed a single protein band of the expected molecular size in SDS-PAGE analysis and were clearly recognized by rabbit anti-Pru p 3 and human IgE antibodies (Fig 2), thus indicating their correct heterologous expression.

The IgE-binding capacity of each site-directed mutant was determined by direct ELISA and ELISA inhibition assays (Fig 3). Both alanine substitution mutants of K80 and K91 showed an immunologic behavior very similar to that of rPru p 3. By contrast, a drastic reduction of IgE-binding capacity and inhibition potency (at least approximately 5 times) was found for the triple mutant R39A/T40A/R44A, thus indicating the role of this amino acid region as a relevant IgE epitope of Pru p 3.

TABLE I. IgE binding of selected synthetic peptides of Pru p 3 with seven individual sera from patients allergic to peach

Peptide no.	Serum number						
	1	2	3	4	5	6	7
1 ITCGQVSSSL	–	–	–	–	–	–	–
2 VSSSLAPCIP	–	–	+	–	–	–	–
3 APCIPYVRGG	++	++	++	++	++	++	++
4 YVRGGGAVPP	–	–	+	–	–	+	–
6 ACCNGIRNVN	–	+	–	–	–	+	–
7 IRNVNNLART	++	++	++	+	++	+	++
8 NLARTTPDRQ	+	–	++	++	–	–	–
14 AAALPGKCGV	+	–	+	–	–	–	–
15 GKCGVSIPYK	++	++	++	+	+	++	++
16 SIPYKISAST	–	+	++	–	–	–	–
17 ISASTNCATVK	–	–	+	–	–	–	–

The intensity of each peptide in each SPOT was quantified by densitometry, using peptide 12 (nonreactive) as negative control (background). Responses were classified with respect to the peptide showing maximum intensity (100%) in each SPOT, according to the following criteria: <40% = –; 40% to 70% = +; 70% to 100% = ++.

A complementary approach to identify sequential IgE-binding regions of the peach allergen was explored by analyzing SPOTs with 10 mers synthetic peptides (overlapping 5 amino acids), which spanned the entire Pru p 3 sequence (Fig 4, A). The serum pool from patients allergic to peach recognized 14 of the 17 peptides, but main responses were observed for the overlapping peptide pairs 3-4 (positions 11 to 25), 7-8 (positions 31 to 45) and, to a lesser extent, 15-16 (positions 71 to 85). Selected synthetic peptides reacting with the serum pool were then tested with 7 individual sera from allergic patients (Fig 4, B, and Table I). Taking into account the 7 reaction patterns obtained, peptide 3 showed the most prominent recognition profile. Therefore, alanine substitutions of the 3 polar residues (C13, Y16, and R18) included in this peptide were tested with the serum pool from patients allergic to peach (Fig 4, C). No drastic reduction (responses approximately 50% of the original peptide 3) of IgE binding was found for any of these single substitutions. By contrast, the triple-change C13A/Y16A/R18A fully abolished the IgE recognition.

Two additional IgE-binding regions were also detected (Table I). Together, peptides 7 and 8 highly reacted with the individual sera. However, a differential response was recorded, as peptide 7 was strongly recognized by 5 sera, whereas peptide 8 showed only relevant responses with 2 of them. The third region was represented by peptide 15, which reacted with all 7 individual sera, with prominent intensity in 5 cases. The location of these three IgE-binding regions (peptides 3, 7, and 15) in the amino acid sequence and the 3-D structure of Pru p 3 is illustrated in Fig 5. Interestingly, the end of an α -helix together with the beginning of its neighbor loop were comprised in the three peptides.

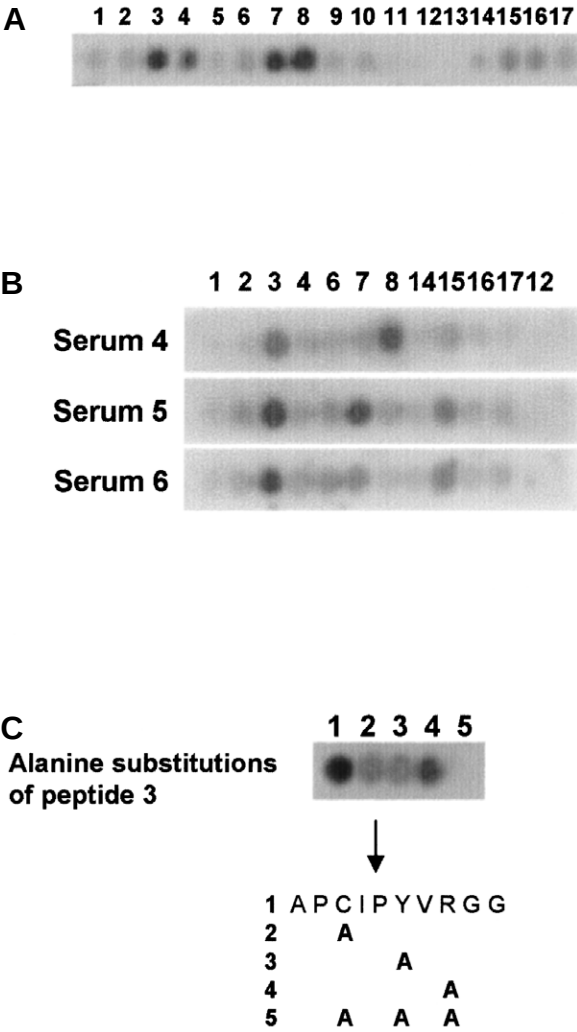


FIG 4. A, IgE immunodetection with a serum pool from patients allergic to peach of a SPOT with synthetic peptides (1 to 17) spanning the entire Pru p 3 amino acid sequence. B, Representative IgE-binding patterns with individual sera from allergic patients of SPOTs with selected synthetic peptides of Pru p 3. C, IgE binding of selected alanine substitutions of peptide 3 residues indicated below the Figure.

DISCUSSION

The 3-D modeling of Pru p 3 revealed an all- α -type compact structure with 4 α -helices covering 56% of its amino acid sequence, 3 interhelix loops, and a long non-structured C-terminal coil (Figs 1 and 5). This predicted 3-D structure fits the typical model proposed for plant LTPs⁵ and thus can serve as a tool to explore the structure/function (IgE-binding properties) relation of allergenic members from plant foods belonging to this protein family.¹ The quantification of surface area exposure together with the determination of electrostatically active local molecular domains allowed us to identify N36 (helix 1), R39 and T40 (loop 2), R44 (helix 3), N64 (helix 4), and S76, K80, A88, T89, and K91 (C-terminal

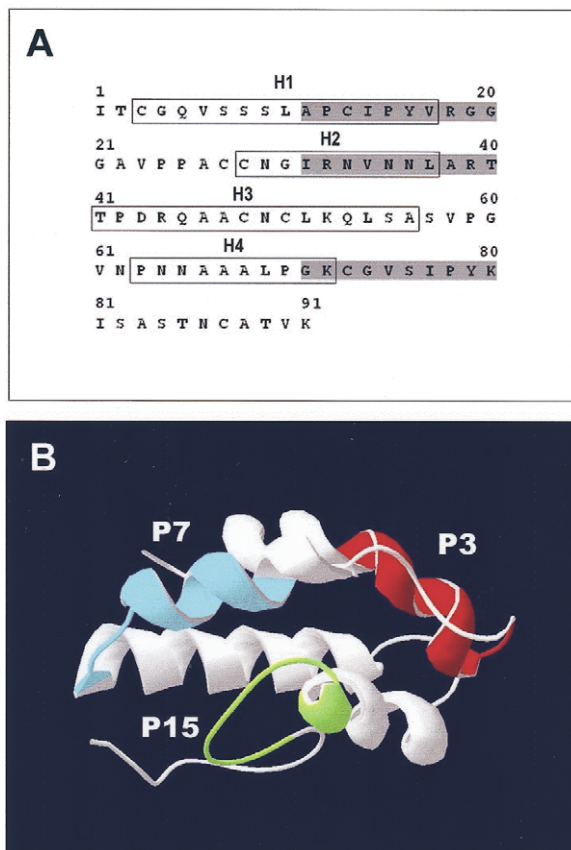


FIG 5. Location of major reactive synthetic peptides P3 (residues 11 to 20), P7 (residues 31 to 40), and P15 (residues 71 to 80) in the amino acid sequence (**A**) and the 3-D structure of Pru p 3 (**B**). The 4 predicted α -helices (H1 to H4) are indicated in **A**.

tail) as potential IgE-binding residues. Among them, the triplet R39/T40/R44 and lysines K80 and K91 were located as the most prominent candidates. To check this prediction, 3 site-directed mutants of Pru p 3 were generated, substituting the 5 residues by alanine, expressed in *P. pastoris* and purified from the corresponding culture medium. The recognition of each mutant by rabbit anti-Pru p 3 antibodies and by human IgE from patients allergic to peach (Fig 2 and 3) supports its proper expression and folding. The substitutions K80A and K91A did not affect the IgE binding to Pru p 3, but the triple substitution R39A/T40A/R44A strongly decreased (approximately 5 times) this binding. R39 and T40 residues are located in loop 2 and R44 at the beginning of helix 3. The three residues are included within the pair of synthetic reactive peptides 7-8 (positions 31 to 45), thus indicating that this Pru p 3 region represents an IgE epitope. The combination of these results suggests that the triple mutant can be a starting point to produce hypoallergenic forms of Pru p 3 for future immunotherapy. Obviously, complementary data, such as its capacity to induce T-cell proliferation and circular dichroism determinations to further confirm its proper folding, are needed to evaluate the actual value of this modified Pru p 3 form.

Besides amino acid sequence positions 31 to 45, the analysis of synthetic peptides (Fig 4 and Table 1) has allowed us to identify two additional Pru p 3 regions involved in IgE binding. The first of these regions consists of residues 11 to 20, represented by peptide 3, which showed the strongest response in SPOT analysis with individual sera. The three polar residues C13, Y16, and R18 appear to be relevant within this sequential IgE epitope. The region defined by peptide 3 could be extended until residue 25 if the high reactivity showed by peptide 4 with another detection method (chemiluminescence instead of ^{125}I -labeled anti IgE detection; results not shown) is considered. The relevance of this region as an IgE epitope is supported by previous data on the determination of an immunodominant IgE epitope of the major *Parietaria* allergen Par j 1.¹⁰ Despite the limited amino acid sequence identity between Pru p 3 and this pollen allergen (29%), the epitope located in Par j 1 overlapped the 11 to 25 amino acid region of Pru p 3 and includes a cysteine residue equivalent to C13, with an important role in IgE recognition. Moreover, this immunodominant region covers in both allergens the last half of helix 1 together with loop 1, thus suggesting that a common 3-D structure can be more determining from an immunologic point of view than sharing a highly identical amino acid sequence.

The third IgE-binding region of Pru p 3, identified by the strong responses of peptide 15, extends from the end of helix 4 (residue 71) to the 8th amino acid (residue 80) of the C-terminal coil. It comprises K80, one of the potential residues predicted to be involved in IgE recognition by the theoretical analysis of the Pru p 3 3-D structure (see above). Interestingly, no synthetic peptide covering part of helix 3 or loop 3 was found among the most reactive peptides in the SPOT analysis, probably because most of this region is mainly buried in the compact 3-D structure of Pru p 3.

In summary, residues 11 to 25, 31 to 45, and 71 to 80 of the Pru p 3 amino acid sequence have been detected as three main IgE-binding regions. Previous data on an IgE epitope of the pollen allergen Par j 1¹⁰ and a brief communication focused on the sequential epitopes of the latex LTP and allergen Hev b 12²⁶ suggest that the same regions identified in Pru p 3 can also be major IgE epitopes in most allergenic LTPs from pollens and plant foods.

The authors thank Linda Puebla (ALK-Abelló) for language revision.

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