

Molecular and structural analysis of the panallergen profilin B cell epitopes defined by monoclonal antibodies

Juan A. Asturias¹, Nuria Gómez-Bayón¹, M. Carmen Arilla¹, Luis Sánchez-Pulido², Alfonso Valencia² and Alberto Martínez¹

¹Research and Development Department, Bial-Arístegui, Alameda Urquijo 27, 48008 Bilbao, Spain

²Proteins Design Group, National Center of Biotechnology-CSIC, 28049 Madrid, Spain

Keywords: allergen, continuous epitopes, IgE-binding site, sunflower, three-dimensional structure

Abstract

Interactions of five mouse mAb (10A4, 5F2, 9A7, 9G4 and 3H8) and sunflower profilin were characterized using synthetic overlapping peptides. All the continuous B cell epitopes analyzed in this work were 6–10 amino acids in length, and clustered at the N- and C-terminal α -helices and a two-stranded segment composed of residues 40–50. Mutational analysis of the epitopes revealed that single amino acid changes within these peptides had dramatic effects on IgG-binding characteristics. A three-dimensional molecular model of sunflower profilin was generated by homology modeling based on the crystal structure of *Arabidopsis thaliana* profilin. All but one of the murine B cell epitopes defined in this work were located on the surface of the profilin molecule in the α -helices (10A4 and 3H8) or in the turns (5F2 and 9G4). In contrast, 9A7 epitope was located in the profilin core and partially buried by the C-terminal. Two mAb (5F2 and 10A4) inhibited the binding of anti-profilin human IgE up to 52%. In contrast, mAb 3H8 seemed to enhance the binding of anti-profilin IgE of sera from allergic patients.

Introduction

Identification of antigenic sites and characterization of the interaction between these sites and their antibodies can yield valuable information. Antigenic sites can be divided into two structural categories: continuous (segmental sites consisting of short sequence motifs) and discontinuous (assembled topographic sites consisting of residues far apart in the primary sequence, but brought together in the surface of the native protein) epitopes (1), albeit the physiological role of continuous epitopes has been questioned (2).

Profilins, ubiquitous cytosolic actin-binding proteins with molecular masses of 12–15 kDa, act at a critical control point in signaling pathways initiated by events at the plasma membrane, and play a crucial role in regulating the activity in the microfilament system and intracellular calcium levels (3–5). When pollen is hydrated, as when it contacts a human mucus membrane following inhalation, pollen tube formation is initiated and large amounts of profilin are released. Profilins have been identified as allergens in several plant species of trees, grasses and weed pollens, and in many fruits and vegetables (6–9). This widespread cross-reactivity has led to the designation of profilins as 'pan-allergens' (6,10) and

although profilin only elicits IgE antibody responses in 20% of the positive pollinic patients (6), their pan-allergenic characteristics make of them important allergens. Crystal structures of two plant profilins, for *Arabidopsis* and birch, have been recently described (11,12), and the overall folds of both proteins are similar to that of the mammalian and amoebae profilins (13–16). The profilin is composed of three α -helices, seven β -strands and 10 turns constituting two hydrophobic cores separated by a central six-stranded β -sheet (11,12).

We have used a structurally well-defined protein antigen such as pollen profilin and several anti-profilin mAb as a model for studies on the interaction of protein antigen with its antibodies.

Methods

Pollen extracts

Pollen extracts from *Helianthus annuus*, *Cynodon dactylon*, *Mercurialis annua*, *Betula verrucosa*, *Nicotiana tabacum* and

Olea europaea were produced as follows. Defatted pollen was extracted by magnetic stirring (24 h at 4°C) in 0.1 M phosphate buffer, pH 8 at 15% (w/v). The extract was clarified by centrifugation at 5600 *g* for 30 min, filtered through 0.45-mm pore diameter membranes and dialyzed by ultrafiltration in a Pellicon System (Millipore, Bedford, MA) with a 5000 Da cut-off.

Production of recombinant sunflower profilin

Escherichia coli BL21 (DE3) (17) cells containing the pKN172-derived recombinant plasmids (18) were grown in LB with 200 µg/ml ampicillin. Expression and purification of soluble recombinant sunflower pollen profilin (Hel a 2) were performed as previously described (19).

mAb and antisera

The production of profilin-specific mAb has been previously described (18). Briefly, 8- to 10-week-old BALB/c mice were immunized i.p. with 500 µl saline solution containing 50 µg of sunflower profilin emulsified in complete Freund's adjuvant. The same amount of antigen and incomplete Freund's adjuvant were given every 15 days over 2 months with a final 50 µg i.p. dose without adjuvant. Mice were sacrificed 3 days later, and the spleen cells were isolated and mixed with SP₂O myeloma cells using a modification of the method described by Köhler and Milstein (20). Supernatants were screened by ELISA on day 11 and positive hybridomas containing antibodies of the desired specificity cloned by limiting dilution. Isotype detection was done using a commercial kit (Boehringer Mannheim, Mannheim, Germany).

Synthetic peptides

Individual peptides were synthesized on a derivatized cellulose membrane (21) according to manufacturer's instructions (SPOTs; Genosys Biotechnologies, Pampisford, UK). Synthesis was performed using Fmoc chemistry on marked spots on a cellulose membrane in which coupling reactions are followed by a change of color as an indicator that the reaction has occurred. Briefly, 9 µl of the appropriate Fmoc-amino acid derivatives, dissolved in 1-methyl-2-pyrrolidone, were spotted to the membrane (twice) and allowed to react for 15 min. The membrane was washed 3 times in 20 ml *N,N*-dimethyl formamide (DMF) for 2 min, with 800 µl acetic anhydride being added to the last wash. The membrane was again washed 3 times with 20 ml DMF for 2 min and then placed in 20 ml of 20% piperidine in DMF for 5 min. The membrane was washed 5 times in 20 ml DMF for 2 min. Then, 200 µl of 1% bromophenol blue was added to DMF and the membrane was incubated until the spots turned blue. Cycles of coupling, blocking and deprotection were repeated until peptides of the desired length were synthesized. The membrane was washed and then placed in 20 ml DMF with 400 µl acetic anhydride to acetylate the peptide. The membrane was washed 3 times with 20 ml DMF and 3 times with 20 ml methanol, and air-dried. Finally, the membrane was incubated for 1 h in a solution of 5 ml dichloromethane, 5 ml trifluoroacetic acid and 250 µl triisobutylsilane, and washed 4 times with 20 ml dichloromethane, 3 times with 20 ml DMF and 4 times with 20 ml methanol. Membranes were either tested immediately or stored at -20°C until needed.

Antibody staining of membrane-bound peptides

The peptide-coupled membranes were washed 3 times with Tris-buffered saline (TBS) and then blocked overnight at 4°C either with TBS plus 0.05% Tween 20 (TBS-T) containing 1% BSA and 5% sucrose or with a proprietary blocking agent as recommended by the manufacturers (with similar results in each case). After three washes with TBS-T the membrane was then incubated with mAb appropriately diluted in TBS-T for 4 h at room temperature. The membrane was washed 3 times with TBS-T and reacted with peroxidase-conjugated anti-mouse IgG (diluted 1:20,000 in TBS-T) for 2 h at room temperature. After washing, the membrane was incubated for 1 min with ECL detection reagent, before being exposed to ECL film (both from Amersham Pharmacia Biotech, Little Chalfont, UK) for various times from 30 s to 5 min. After development of the film, membranes were stripped off the antibody by treatment 5 times with 8 M urea/1% SDS for 10 min and then another 5 times with ethanol:water:acetic acid (5:4:1) for 10 min. Finally, they were washed 2 times with methanol for 10 min and either probed immediately or stored at -20°C until needed. IgG binding was measured by densitometry using Diversity database software (Biorad, Richmond, CA).

Inhibition assays

The ability of anti-profilin mAb to inhibit binding between profilin-specific human IgE from patients' sera and profilin was determined as follows. Microtiter plates were coated with 200 ng/well of natural sunflower profilin in 0.1 M bicarbonate buffer (pH 9.6) and saturated with blocking buffer (PBS supplemented with 0.05% Tween 20 and 1% BSA). Then, 100 µl of each mAb (at different dilution to render them equipotent) was added and incubated for 90 min at 37°C. The plate was then washed 3 times with PBS plus 0.05% Tween 20, and 100-µl aliquots of profilin positive sera from sunflower-allergic patients were added and incubated overnight at 4°C. The amount of IgE bound to profilin was measured with peroxidase-conjugated rabbit anti-human IgE antibody as previously described (22).

The ability of mAb to competitively inhibit the binding of mAb 9A7 to sunflower profilin was tested as follows: microtiter plates coated with profilin were simultaneously incubated with peroxidase-labeled 9A7 mAb and competitive mAb at different concentrations (0.1–100 µg/ml).

Analytical methods

Proteins were analyzed by SDS-PAGE under reducing conditions (23) and visualized by Coomassie brilliant blue R-250 staining (24). For immunoblotting experiments, after SDS-PAGE proteins were electrophoretically transferred to PVDF membranes (Hybon-P; Amersham Pharmacia Biotech) (25). Membranes were incubated at 37°C for 60 min with mAb diluted 1:2 to 1:10. Immunodetection was performed as described above using anti-mouse IgG antibodies linked to horseradish peroxidase and ECL reagents (Amersham Pharmacia Biotech).

Homology modeling

A three-dimensional model of sunflower profilin was constructed based on its strong sequence homology (71% of

```

Hel a 2:      MSWQAYVDEHLMCDIEGTGQHLSAAAILGLDGTVWAQSAKFPQFKPEEMKGIIEKFDEAG 60
              MSWQ+YVD+HLMCD+EG   HLT+AAILG DG+VWAQSAKFPQ KP+E+ GI K+F+E G
PRO1_ARATH:  MSWQSYVDDHLMCDVEGN--HLTAAAILGQDGSVWAQSAKFPQLKPQEIDGIKKDFEEPG 58

Hel a 2:      TLAPTGMFIAGAKYMLVQGEFGAVIRGKKKGAGGICIKKTGQAMIMGIYDEPVPAPGQCNMV 120
              LAPTG+F+ G KYMV+QGE GAVIRGKKKG GG+ IKKT QA++ G YDEP+ GQCN+V
PRO1_ARATH:  FLAPTGLFLGGEKYMVIQGEQGAVIRGKKKGPGGVTIKKTNQALVFGFYDEPMTGGQC�NLV 118

Hel a 2:      VERLGDYLLE 130
              VERLGDYL+E
PRO1_ARATH:  VERLGDYLIE 128

```

Fig. 1. (A) Alignment of the sunflower (Hel a 2) and *Arabidopsis* (PRO1_ARATH) profilin sequences. Similar amino acids are marked by '+'.

identical amino acids and 14% of similar amino acids; Fig. 1) with the solved crystal structure of *A. thaliana* profilin (12) using the Swissmodel (26,27) and the WhatIf (28) programs. Structural comparisons and visualization were carried out with the Insight II version 98.0 Molecular Modeling System (Molecular Simulations, Burlington, MA).

Results

Multiple IgG-binding epitopes on the Hel a 2 allergen

Individual peptides spanning the entire sunflower profilin were synthesized as a series of 14-amino-acid peptides which overlapped by 7 amino acids. The peptides were then incubated with several mAb, rabbit experimental serum anti-profilin, pre-immune control serum and irrelevant mAb against bacterial proteins (Arilla, unpublished results). Incubation with pre-immune control serum or irrelevant mAb revealed no non-specific binding to the synthesized peptides (data not shown). Ten anti-profilin mAb were tested with the Hel a 2 peptides and five of them (10A4, 5F2, 9A7, 9G4 and 3H8) were chosen for further study. Figure 2 represents IgG-binding activity of these mAb and the rabbit anti-profilin serum (RAPS). RAPS recognized several peptides, but with different reactivity (Fig. 2B, right). Most of the reactivity peptides were localized at the N- and C-termini (residues 1–28 and 100–133 respectively) and a central region (residues 36–63). All mAb but one (9G4) recognized the same peptides as the RAPS did, suggesting that the studied mAb has been generated against a good representation of the murine IgG binding epitopes.

The exact amino acid sequence of the IgG-binding regions was determined by synthesizing small peptides (10 amino acids long offset by 2 amino acids) representing the C-terminal IgG-binding region. By these means, it was possible to identify individual binding epitopes corresponding to mAb 9A7, 9G4, 3H8 (Fig. 3) and 5F2 (data not shown). The size of the epitopes ranged from 6 to 10 amino acids in length. Two epitopes, corresponding to mAb 9A7 and 9G4, were found in the region of amino acid residues 100–115 and partially overlapped within each other.

In order to determine whether the proximity of epitopes of mAb 9G4, 9A7 and 3H8 could interfere the binding, these three mAb and two other mAb, whose epitopes were more distant, were studied. The ability of mAb 10A4, 5F2, 9G4 and 3H8 to competitively inhibit the binding of peroxidase-labeled

9A7 is shown in Fig. 4. Even at high concentration, mAb 5F2, 3H8 and 10A4 had little effect on the binding of 9A7. In contrast, mAb 9G4 showed some cross-reactivity with 9A7 because an inhibition of 54% was found at 100 µg/ml. Both mAb had similar dissociation coefficients: 9A7, 0.45 nM and 9G4, 0.40 nM.

Epitope mapping of mAb 9G4

The amino acids essential to IgG binding in epitope 9G4 were determined by synthesizing peptides corresponding to sequenced plant profilins or mutated peptides with Ala substitutions at each position (Fig. 5). Positions 108, 112 and 114 seem to be important for the binding. mAb 9G4 did not recognize peptides with substitution of Ala for Tyr at position 108 and substitution of Met or Ala for Val112 reduced drastically the 9G4 binding. In contrast, the substitution of Glu or Ala for Asp at position 109 and an Ala for Pro 111 resulted in increasing IgG binding. Western blot experiments of pollen extracts with mAb 9G4 gave similar results (data not shown). No profilin band was detected at 14–15 kDa on grass pollen extracts which contained the sequence identical to peptide 2 (Val112 → Met, Ala113 → Thr). Low-intensity profilin bands were detected in *Olea* and *Mercurialis* extracts which contained the sequence of peptide 5 (Asp109 → Glu, Ala113 → Thr).

mAb specificity relative to human IgE: epitope mapping of mAb 10A4 and 5F2

mAb were examined for their ability to block IgE binding to sunflower profilin of five serum samples from sunflower allergic patients with high levels of IgE anti-profilin. Results shown in Fig. 6 demonstrated that mAb 5F2 and 10A4 inhibited the binding of anti-profilin human IgE up to 52%. In contrast, mAb 3H8 seemed to enhance the binding of anti-profilin IgE of sera from allergic patients. An effect of synergy (67% inhibition) was observed when mAb (5F2 and 10A4) were used together for inhibition of IgE binding (data not shown).

Overlapping dodecapeptides of the N-terminal region of Hel a 2 were probed for binding to mAb 10A4. Only the two first spots were positive, although a low positive signal (53% of the signal of spot 1) could be detected in spot 2, showing that the two first residues are also important for binding (Fig. 7A). mAb 10A4 reacted with peptides synthesized as 10mers, 9mers, 8mers and 7mers, but less efficiently with a 6mer peptide

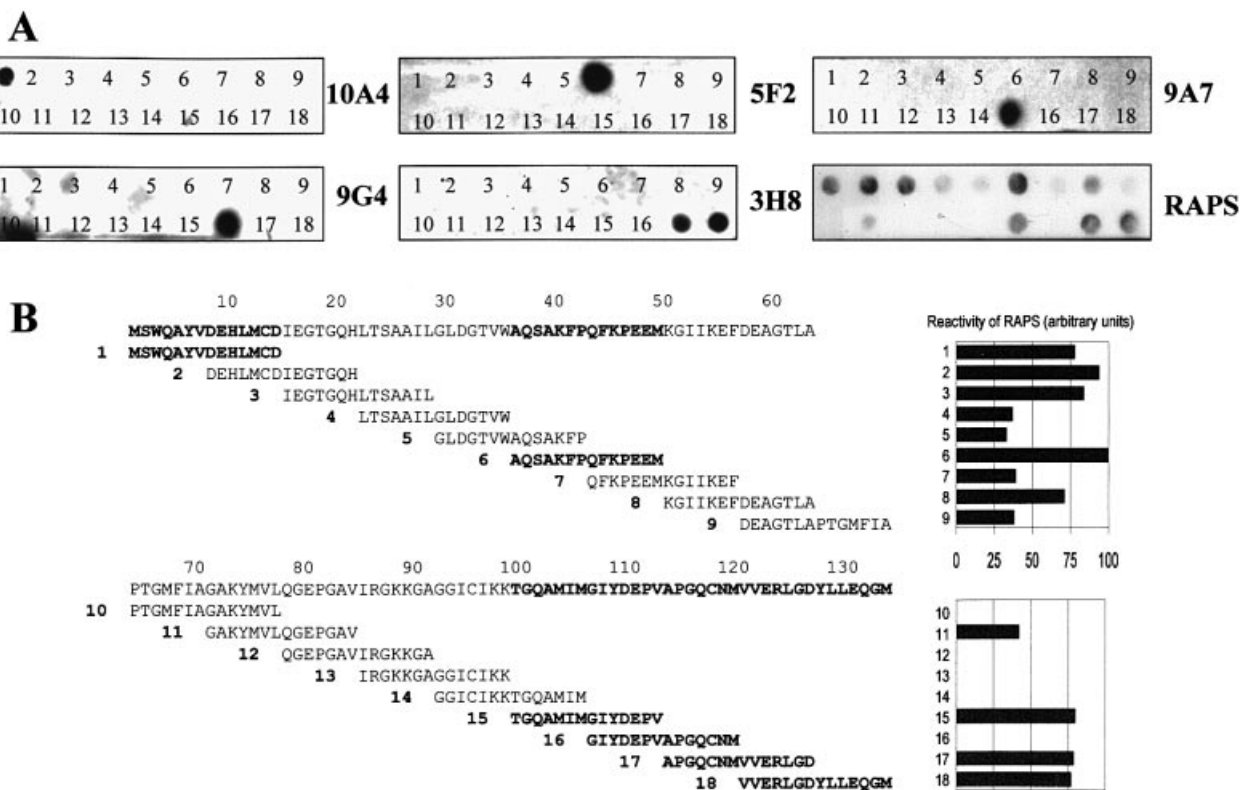


Fig. 2. (A) Reactivity of mAb and RAPS with synthetic Hel a 2 peptides. Each spot represents a separate 14mer peptide as described in (B). (B) Overlapping 14-amino-acid peptides of Hel a 2 synthesized on the SPOTs membrane. Peptides in bold represent spots that were positive with the individual mAb. Reactivity of the antiserum to the peptides, quantified by densitometry, is represented on the right-hand side of the panel.

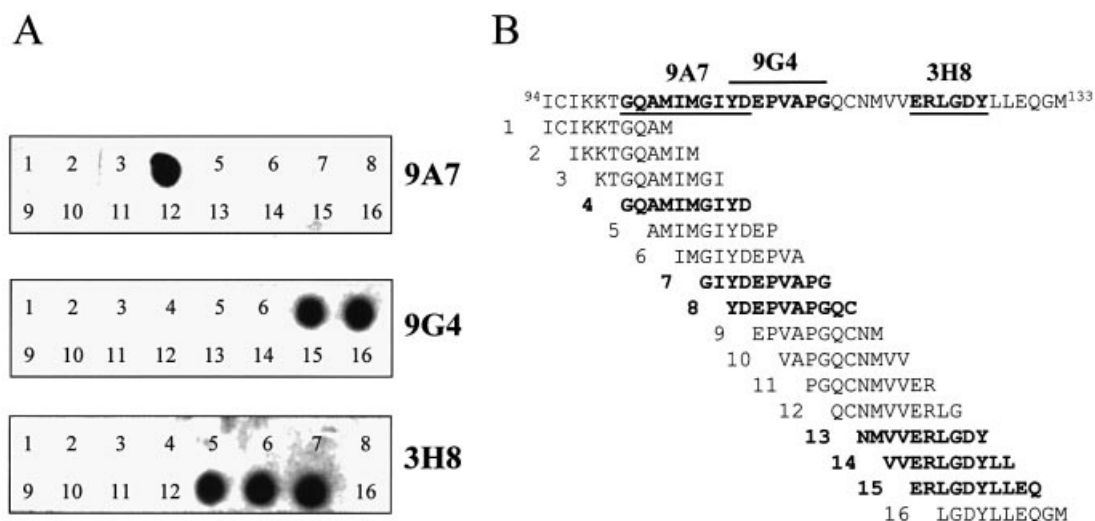


Fig. 3. Identification of IgG epitopes recognized by 9A7, 9G4 and 3H8 on the Hel a 2 allergen. (A) Detailed epitope mapping of mAb was performed on the IgG-binding region which had been previously identified in Fig. 1 by synthesizing 10-amino-acid long peptides offset from each other by 2 amino acids. (B) Amino acid sequence (residues from 94 to 133) of Hel a 2 tested in (A). Amino acids in bold correspond to common IgG-binding amino acids of the spot shown in (A).

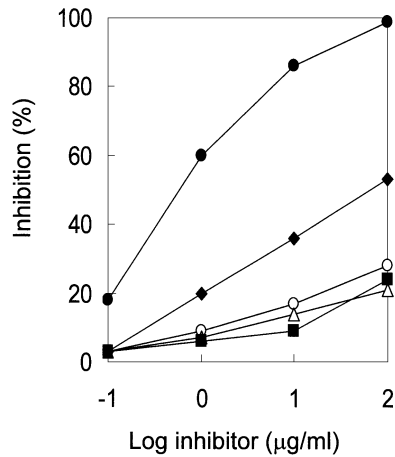


Fig. 4. Ability of mAb 9A7 (solid circles), 9G4 (diamonds), 5F2 (open circles), 10A4 (triangles) and 3H8 (squares) to competitively inhibit the binding of peroxidase-labeled 9A7. Inhibition with homologous mAb 9A7 at 100 $\mu\text{g/ml}$ was considered 100%.

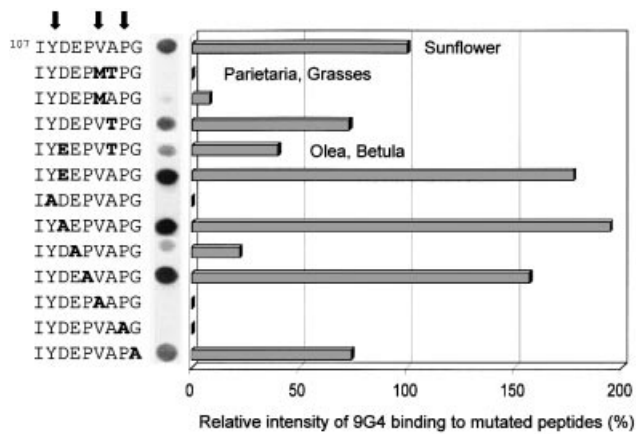


Fig. 5. Effect of single amino acid changes to the 9G4 epitope. IgG binding to mutated peptides was calculated by densitometry and represented as relative intensity (wild-type peptide was 100%). Amino acids in bold represent residues changed from the Hel a 2 sequence. Vertical arrows indicated important residues involved in IgG recognition.

(Fig. 7B). Both experiments demonstrated that the epitope recognized by mAb 10A4 is MSWQAYV, although some binding could be detected with shorter peptides.

mAb 5F2 recognized profilin from different pollen extracts. A single IgG-binding protein of 14–16 kDa was detected by 5F2 in sunflower extracts, but in other extracts, in addition of the monomeric profilin, some bands of higher molecular mass (30, 46 and 59 kDa) were detected (Fig. 8A). Binding differences between mAb 5F2 and plant profilins were further investigated at the epitope level using peptides corresponding to sunflower (peptide 1), pellitory (peptide 2), birch (peptide 3), wheat (peptide 4), maize and Bermuda grass (peptide 8) pollen profilin (Fig. 8B). All the sequences, with the exception of that corresponding to birch profilin (peptide 3, 62% binding), had

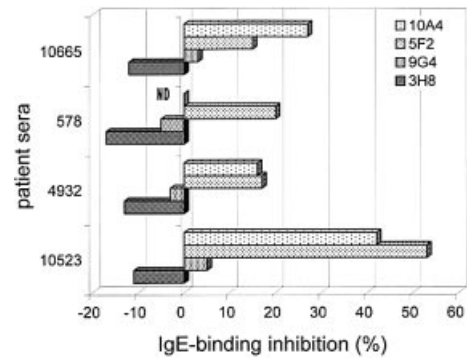
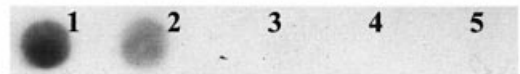


Fig. 6. Ability of mAb 10A4, 5F2, 9G4 and 3H8 to competitively inhibit the binding of human IgE in sera from sunflower-allergic patients. IgE anti-profilin-containing serum samples were diluted conveniently to render them equipotent (i.e. giving optical density values of 0.5–0.6).

similar binding (90–110%). Mutated peptides with an Ala

A

1 MSWQAYVDEH¹⁰
2 WQAYVDEHLM
3 AYVDEHLMCD¹⁴
4 VDEHLMCDIE
5 EHLCDIEGT¹⁸



B

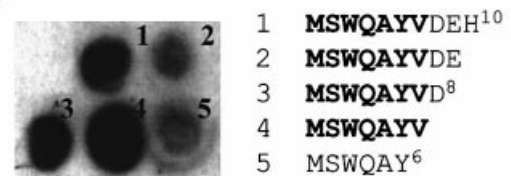


Fig. 7. Exact mapping of the 10A4 epitope. (A) Based on the IgG-binding region previously identified in Fig. 1, a detailed epitope mapping was performed by synthesized 10-amino-acid peptides offset from each other by 2 amino acids. (B) Binding activity of mAb 10A4 with decreasing length peptides. Amino acids in bold represent residues involved in IgG recognition.

substitution in each residue were probed for binding to mAb 5F2. Four residues were found to be important for IgG recognition: Phe41, Pro42, Lys45 and Glu48. All of them are voluminous or charged amino acids in which an Ala substitution completely avoided binding of mAb 5F2 (Fig. 8B). A clear correlation was found between residue conservation on this epitope among sequenced plant profilins and percentage of binding to the mutated peptides. The four important residues mentioned above are conserved in all profilins, while mutations of other residues less conserved had no effect on IgG binding.

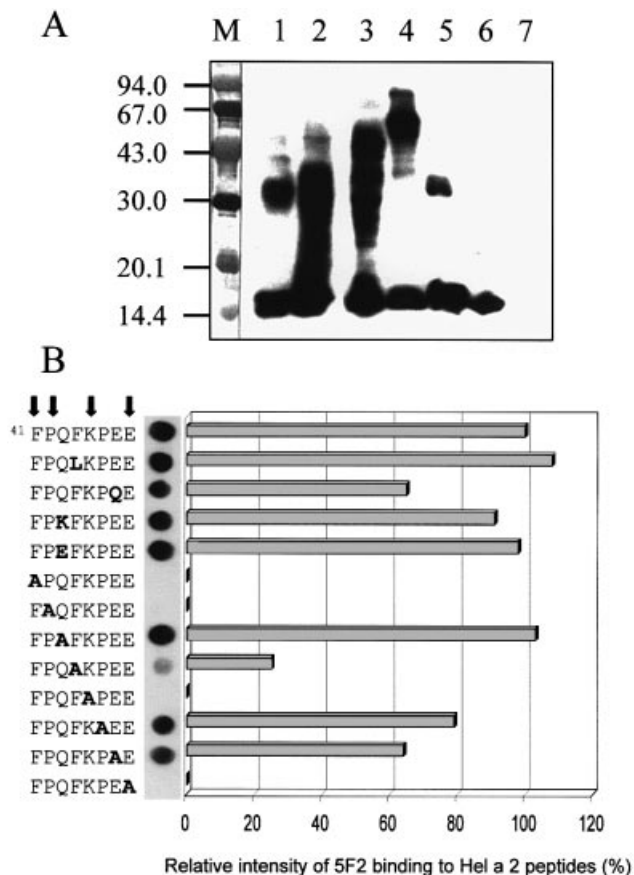


Fig. 8. Single amino acid changes to 5F2 mAb epitope and reactivity with other plant profilins. (A) Western blot of different pollen extracts incubated with 5F2: lane 1, *B. verrucosa* (birch); lane 2, *M. annua* (mercurial); lane 3, *C. dactylon* (Bermuda grass); lane 4, *P. pratense* (timothy grass); lane 5, *O. europaea* (olive tree); lane 6, *H. annuus* (sunflower); and lane 7, *Dermatophagoides pteronyssinus* (house dust mite). Lane M, molecular mass marker. (B) Effect of single amino acid changes to the 5F2 epitope peptides corresponding to sunflower, pellitory, birch, wheat, maize and Bermuda grass pollen profilins and mutated peptides synthesized with an Ala substituted for one of the amino acids in the peptide (mutated peptides from 6 to 13). IgG binding to mutated peptides was calculated by densitometry and represented as relative intensity (wild-type peptide was 100%). Vertical arrows indicate important residues involved in IgG recognition. Amino acids in bold represent residues changed from the Hel a 2 sequence.

Epitope mapping of mAb 3H8

The amino acids essential to IgG binding in epitope 3H8 were determined by synthesizing mutated peptides with Ala substitutions at each position or with changes derived from the variability of sequenced plant profilins. The sequence of the epitope 3H8 is highly conserved among sequenced plant profilins with the exception of those from *C. dactylon* and *N. tabacum*. The differences of mAb 3H8 binding to different plant profilins were further investigated at the epitope level using peptides corresponding to tree, grass and weed profilins. The microheterogeneity in this region was correlated with the binding properties of 3H8. An immunoblot strip containing the wild-type, mutated peptides and peptides

derived from plant profilin sequences is shown in Fig. 9(B). The mAb 3H8 did not either recognize the peptide or binding was drastically reduced when Ala was substituted for each of the amino acids at position 123–126, showing the importance of these residues in the binding of IgG. Changes of Leu124 for Ile did not affect binding, but substitution of Leu for similar but smaller amino acids, such as Ala, drastically reduced binding. Position 123 is very sensitive to amino acid changes—substitution of Ala for a positively charged amino acid, such as Arg, blocked IgG binding to the peptide, but surprisingly changes of Arg123 for Lys, presented in *C. dactylon* and *N. tabacum* profilins, also affected drastically the binding both as synthesized peptide as well as completed protein (Fig. 9A). The substitution of the apolar Ala for the polar Gly at position 126 seemed to be responsible for the lack of IgG binding to the mutated peptide.

Discussion

The interaction of sunflower profilin and specific mouse mAb was investigated. Profilin was chosen as a model for antigen–antibody interactions because profilins are potent allergens that elicit IgE antibody responses in 20% of the positive pollinic patients (7), which has led to their designation as pan-allergens (6), and they are structurally well-conserved eukaryotic proteins.

All the B epitopes of sunflower profilin found in this work are continuous or sequential epitopes, a term coined for peptide epitopes consisting of short sequence motifs (1). Although continuous epitopes have been reported for a number of allergens (29–33), the physiological role of continuous epitopes has been questioned (2). Murine IgG-binding sites analyzed in this work clustered at the N- and C-terminal α -helices and a two-stranded segment composed of residues 40–50. Similar results have been found for IgE-binding sites using a birch profilin epitope library probed with profilin-allergic patients' sera (34). In contrast, recently data suggested that IgE binding of soybean profilin (which respectively share a 75 and 71% identity with birch and sunflower profilins) is mediated by conformational epitopes (35). Experiments are now in progress to localize sunflower profilin IgE epitopes from allergic individuals sera using synthetic peptides bound to a membrane as reported herewith. Alternative methods, such as phage displayed peptide libraries, have been previously used, but only a mimotope whose sequence showed no homology with profilin sequences was found (36). The results found in the present work may be highly valuable since the murine model has been proved both to correlate and be predictive of human response (32,37).

All but one of the murine B cell epitopes defined in this work are located on the surface of the profilin molecule in the α -helices (10A4 and 3H8) or in the turns (5F2 and 9G4) (Fig. 10A). The hydrophobic core of epitope 9G4 is constituted by Tyr108, Val112 and Pro114. Any change in these positions prevents the peptide mimicking the folding which would occur in the natural profilin (Fig. 10B). In contrast to these surface localized epitopes, epitope 9A7 is located in the profilin core and partially buried by the C-terminal. Only the flanking residues of the epitope 9A7 (Gly100, Gln101 and Asp109) have limited accessibility. Whether this epitope is either fully

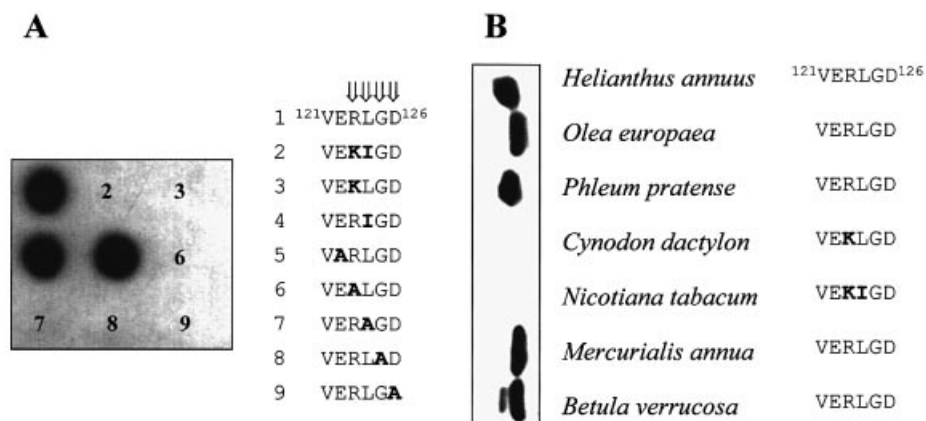


Fig. 9. Single amino acid changes to 3H8 mAb epitope and reactivity with other plant profilins. (A) Reactivity of mAb 3H8 to peptides corresponding to tobacco and Bermuda grass pollen profilin sequences, and mutated peptides synthesized with an Ala substituted for one of the amino acids in the peptide (mutated peptides 5–9). (B) Western blot of different pollen extracts incubated with 3H8. Amino acids in bold represent residues changed from the Hel a 2 sequence. Vertical arrows indicate important residues involved in IgG recognition.

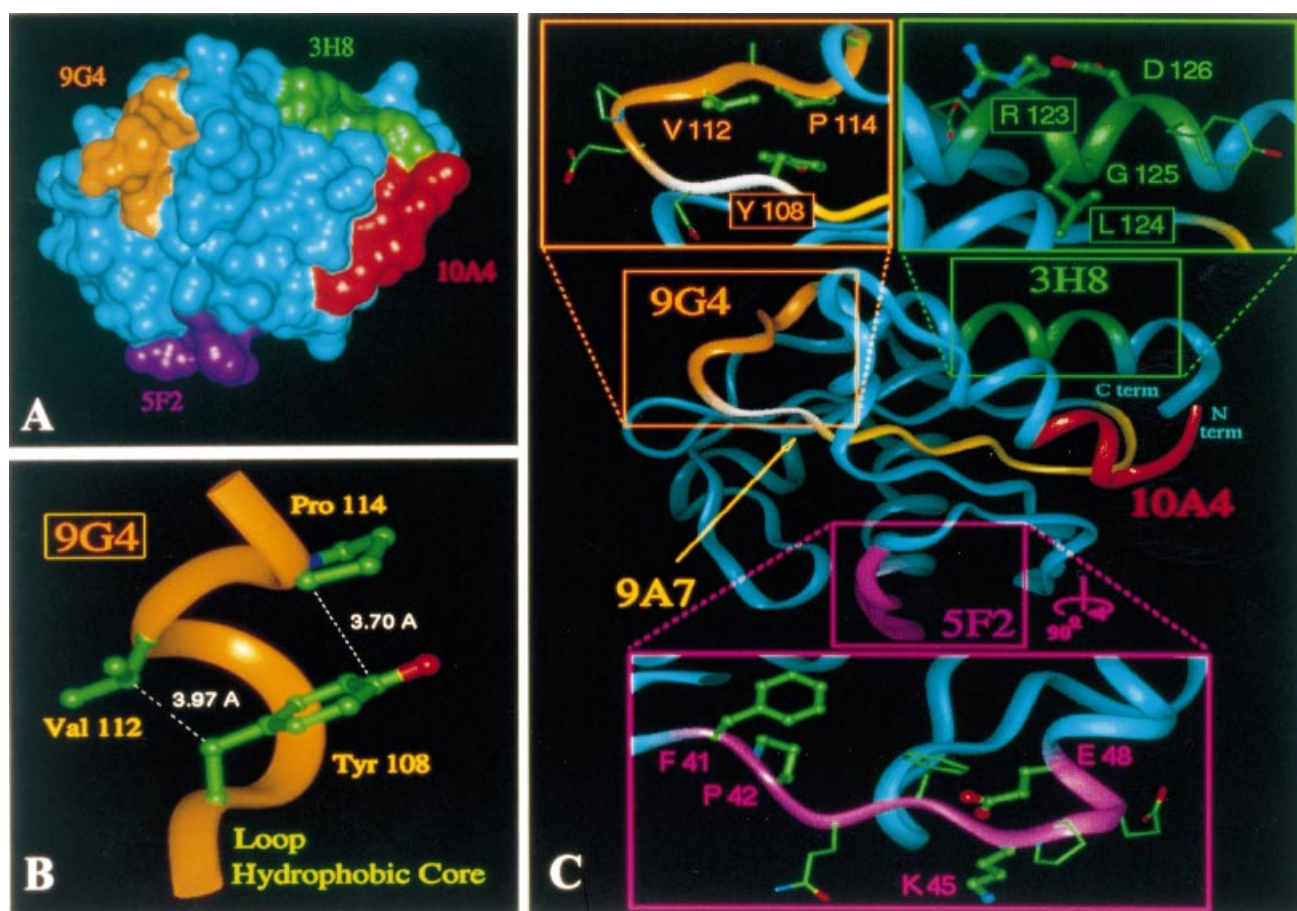


Fig. 10. (A) Summary of mAb epitopes on the Hel a 2 surface. (B) Detail of the hydrophobic core of epitope 9G4. (C) Ribbon diagram of Hel a 2 showing the sequential IgG-reactive epitopes defined by mAb 10A4, 5F2, 9G4, 9A7 and 3H8. The ribbon regions corresponding to the linear peptides are marked with different colors, and the overlapping region between 9A7 (yellow) and 9G4 (orange) is colored in white. The residues involved in the different epitopes are shown as sticks. The residues directly involved in IgG recognition, highlighted in the different insets, are displayed as thick sticks..

accessible in some conditions to generate an immune response in mice or this mAb was produced against a partially denatured form of profilin remains to be investigated. Although mAb were generated from purified native profilin, the purification procedure included an elution step in 8 M urea (38) followed by renaturation during dialysis. Nevertheless, mAb 9A7 recognized sunflower profilin both as purified protein and as in raw extract. Partially hidden epitopes which could become immunologically available after denaturation are named cryptotopes or unfoldons (2).

Epitopes of mAb 10A4 and 3H8 overlap with some of the binding sites for proline-rich peptides (Trp3, Tyr6, Tyr127 and Leu128) and actin (Gly115) previously described (39), and one of them (mAb 10A4) inhibited allergic patients IgE binding (Fig. 10C). These observations are consistent with the idea that B cell epitopes overlap ligand sites due to the fact that native profilin, dissociated from the profilin-actin complex, is the molecule responsible for sensitization and profilin (ligand free) is easily extracted from pollen by aqueous solution, a situation which mimics contact with mucosal surfaces (34). None of the mAb epitopes described overlap with the plant-specific binding pocket located between Phe54 and Ile83 of *Arabidopsis* profilin structure, which was suggested as a major immunogen region of plant profilins (12). The epitope of mAb 5F2 partially overlaps with the epitope of mAb 4A6 described as birch profilin specific (40). In contrast to the later mAb, mAb 5F2 has a broad profile of profilin recognition and the three essential residues detected in this work are highly conserved among plant profilins. The high reactivity of mAb 5F2 with plant profilin has allowed us to use this mAb in a profilin-specific ELISA quantification assay (41).

In conclusion, we present a simple method for exact mapping of B cell epitopes and investigating essential residues involved in antibody binding, using peptides synthesized on a cellulose membrane, and its application to the analysis of profilin epitopes defined by mAb. Two mAb found in this work blocked IgE binding to profilin to different extents depending of the patient studied. The epitope of one of them, 5F2, is highly conserved among profilins, and could be involved in the clinical and serological cross-reactivity between pollen and plant foods.

Acknowledgements

This work was supported in part by Bial-Aristegui, and by grants FIT-090000-2001-83 from the Plan Nacional de I + D (Programa PROFIT, Ministerio de Industria y Energia, Spain) and TEI-0017-2001 from the Programa INTEK (Departamento de Industria, Agricultura y Pesca, Gobierno Vasco).

Abbreviations

Hel a 2	sunflower pollen profilin
DMF	<i>N,N</i> -dimethyl formamide
TBS	Tris-buffered saline
TBS-T	TBS plus 0.05% Tween 20
RAPS	rabbit anti-profilin serum

References

- Berzofsky, J. A. 1985. Intrinsic and extrinsic factors in protein antigenic structure. *Science* 229:932.
- Graeme Laver, W., Air, G. M., Webster, R. G. and Smith-Gill, S. J. 1990. Epitopes on protein antigens: misconceptions and realities. *Cell* 61:553.
- Baatout, S. 1996. Profilin: an update. *Eur. J. Clin. Chem. Clin. Biochem.* 34:575.
- Carlsson, I., Nyström, L. E., Sundkvist, F., Markey, F. and Lindberg, U. 1977. Actin polymerization is influenced by profilin, a low molecular weight protein in non-muscle cells. *J. Mol. Biol.* 115:465.
- Goldschmidt Clermont, P. J., Kim, J., Machesky, L. M., Rhee, S. and Pollard, T. D. 1991. Regulation of phospholipase C-1 by profilin and tyrosine phosphorylation. *Science* 251:1231.
- Valenta, R., Duchêne, M., Ebner, C., Valent, P., Sillaber, C., Deviller, P., Ferreira, F., Tejkl, M., Edelmann, H., Kraft, D. and Scheiner, O. 1992. Profilins constitute a novel family of functional plant pan-allergens. *J. Exp. Med.* 175:377.
- Valenta, R., Duchêne, M., Pottenburger, K., Sillaber, C., Valent, P., Bettelheim, P., Breitenbach, M., Rumpold, H., Kraft, D. and Scheiner, O. 1991. Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 253:557.
- Hirschwehr, R., Valenta, R., Ebner, C., Ferreira, F., Sperr, W. R., Valent, P., Rohac, M., Rumpold, H., Scheiner, O. and Kraft, D. 1992. Identification of common allergenic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J. Allergy Clin. Immunol.* 90:927.
- van Ree, R., Voitenko, V., Van Leeuwen, W. A. and Aalberse, R. C. 1992. Profilin is a cross-reactive allergen in pollen and vegetable foods. *Int. Arch. Allergy Immunol.* 98:97.
- Valenta, R., Duchêne, M., Vrtala, S., Valent, P., Sillaber, C., Ferreira, F., Tejkl, M., Hirschwehr, R., Ebner, C., Kraft, D. and Scheiner, O. 1992. Profilin, a novel plant pan-allergen. *Int. Arch. Allergy Immunol.* 99:271.
- Fedorov, A. A., Ball, T., Mahoney, N. M., Valenta, R. and Almo, S. C. 1997. The molecular basis for allergen cross-reactivity: crystal structure and IgE-epitope mapping of birch pollen profilin. *Structure* 5:33.
- Thorn, K. S., Christensen, H. E. M., Shigeta, R., Huddler, D., Shalaby, L., Lindberg, U., Chua, N. H. and Schutt, C. E. 1997. The crystal structure of a major allergen from plants. *Structure* 5:19.
- Metzler, W. J., Constantine, K. L., Friedrichs, M. S., Bell, A. J., Ernst, E. G., Lavoie, T. B. and Mueller, L. 1993. Characterization of the three-dimensional solution structure of human profilin: ¹H, ¹³C, and ¹⁵N NMR assignments and global folding pattern. *Biochemistry* 32:13818.
- Fedorov, A. A., Magnus, K. A., Graupe, M. H., Lattman, E. E., Pollard, T. D. and Almo, S. C. 1994. X-Ray structures of isoforms of the actin-binding protein profilin that differ in their affinity for phosphatidylinositol phosphates. *Proc. Natl Acad. Sci. USA* 91:8636.
- Vinson, V. K., Archer, S. J., Lattman, E. E., Pollard, T. D. and Torchia, D. A. 1993. Three-dimensional solution structure of *Acanthamoeba* profilin-I. *J. Cell Biol.* 122:1277.
- Cedergren-Zeppezauer, E. S., Goonesekere, N. C., Rozycki, M. D., Myslik, J. C., Dauter, Z., Lindberg, U. and Schutt, C. E. 1994. Crystallization and structure determination of bovine profilin at 2.0 Å resolution. *J. Mol. Biol.* 240:459.
- Studier, F. M. and Moffatt, B. A. 1986. Use of bacteriophage T7 polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 189:113.
- Arilla, M. C., Asturias, J. A., Gómez Bayón, N., Martínez, A., Martínez, J. and Palacios, R. 1997. Production and characterization of profilin monoclonal antibodies. *Allergol. Immunopathol.* 25:145.
- Asturias, J. A., Arilla, M. C., Gómez Bayón, N., Aguirre, M., Martínez, A., Palacios, R. and Martínez, J. 1998. Cloning and immunological characterization of the allergen Hel a 2 (profilin) from sunflower pollen. *Mol. Immunol.* 35:469.
- Köhler, G. and Milstein, C. 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur. J. Immunol.* 6:511.
- Frank, R. and Doring, R. 1988. Simultaneous multiple peptide

- synthesis under continuous flow conditions on cellulose paper discs as segmental solid synthesis. *Tetrahedron* 44:6031.
- 22 Shen, H. D., Wang, S. R., Tang, R. B., Chang, F. Z. N., Su, S. N. and Han, S. H. 1988. Identification of allergens and antigens of Bermuda-grass pollen by immunoblot analysis. *Clin. Allergy* 18:401.
 - 23 Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* 227:680.
 - 24 Weber, K. and Osborn, M. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244:4406.
 - 25 Towbin, H., Staehelin, I. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. USA* 76:4350.
 - 26 Guex, N. and Peitsch, M. C. 1997. Swiss-Model and the swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 18:2714.
 - 27 Peitsch, M. C. 1996. ProMod and Swiss-Model: Internet-based tools for automated comparative protein modeling. *Biochem. Soc. Trans* 24:274.
 - 28 Vriend, G. 1990. WhatIf: a molecular modeling and drug design program. *J. Mol. Graph.* 8:52.
 - 29 Banerjee, B., Wang, X. D., Kelly, K. J., Fink, J. N., Sussman, G. L. and Kurup, V. P. 1997. IgE from latex-allergic patients binds to cloned and expressed B cell epitopes of prohevein. *J. Immunol.* 159:5724.
 - 30 Beezhold, D. H., Kostyal, D. A. and Sussman, G. L. 1997. IgE epitope analysis of the hevein preprotein; a major latex allergen. *Clin. Exp. Immunol.* 108:114.
 - 31 Stanley, J. S., King, N., Burks, A. W., Huang, S. K., Sampson, H., Cockrell, G., Helm, R. M., West, C. M. and Bannon, G. A. Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2. 1997. *Arch. Biochem. Biophys.* 342:244.
 - 32 Slater, J. E., Paupore, E. J. and O'Hehir, R. E. Murine B-cell and T-cell epitopes of the allergen Hev b 5 from natural rubber latex. 1999. *Mol. Immunol.* 36:135.
 - 33 Burks, A. W., Shin, D., Cockrell, G., Stanley, J. S., Helm, R. M. and Bannon, G. A. 1997. Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in pea nut hypersensitivity. *Eur. J. Biochem.* 245:334.
 - 34 Fedorov, A. A., Ball, T., Valenta, R. and Almo, S. C. 1997. X-ray crystal structures of birch pollen profilin and Phl p 2. *Int. Arch. Allergy Immunol.* 113:109.
 - 35 Rihs, H. P., Chen, Z., Rueff, F., Petersen, A., Rozynek, P., Heimann, H. and Baur, X. 1999. IgE binding of the recombinant allergen soybean profilin (rGly m 3) is mediated by conformational epitopes. *J. Allergy Clin. Immunol.* 104:1293.
 - 36 Leitner, A., Vogel, M., Radauer, C., Breiteneder, H., Stadler, B. M., Scheiner, O. and Kraft, D. and Jensen-Jarolim, E. 1998. A mimotope defined by phage display inhibits IgE binding to the plat panallergen profilin. *Eur. J. Immunol.* 28:2921.
 - 37 Beezhold, D. H., Hickey, V. L., Slater, J. E. and Sussman, G. L. 1999. Human IgE-binding epitopes of the latex allergen Hev b 5. *J. Allergy Clin. Immunol.* 103:1166.
 - 38 Lindberg, U., Schutt, C., Hellsten, E., Tjäder, A. and Hult, T. 1988. The use of poly(L-proline)-Sepharose in the isolation of profilin and profilactin complexes. *Biochim. Biophys. Acta* 967:391.
 - 39 Perelroizen, I., Marchand, J. B., Blanchoin, L., Didry, D. and Carlier, M. F. 1994. Interaction of profilin with G-actin and poly(L-proline). *Biochemistry* 33:8472.
 - 40 Wiedemann, P., Giehl, K., Almo, S. C., Fedorov, A. A., Girvin, M., Steinberger, P., Rudiger, M., Ortner, M., Sippl, M., Dolecek, C., Kraft, D., Jockusch, B. and Valenta, R. 1996. Molecular and structural analysis of a continuous birch profilin epitope defined by a monoclonal antibody. *J. Biol. Chem.* 271:29915.
 - 41 Asturias, J. A., Arilla, M. C., Aguirre, M., Gómez Bayón, N., Martínez, A., Palacios, R., Sánchez Gascón, F. and Martínez, J. 1999. Quantification of profilins by a monoclonal antibody-based sandwich ELISA. *J. Immunol. Methods* 229:61.