
Pollen and plant food profilin allergens show equivalent IgE reactivity

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Background: Profilins are commonly involved in polysensitization of allergic patients; therefore, appropriate markers should be used in component-resolved diagnosis.

Objective: To evaluate the immunological equivalence between profilins from pollens and plant-derived foods, to be used in component-resolved diagnosis.

Methods: Specific immunoglobulin (Ig) G antibodies against pollen and fruit profilins, as well as sera from patients allergic to mustard, melon, or olive pollen, were used. Purified profilins from mustard seeds, fruit melon, and chenopod and birch pollen were assayed in immunoblotting, enzyme-linked immunosorbent assay (ELISA), and ELISA inhibition assays.

Results: Significant correlation was found in the response of purified profilins by ELISA and immunoblotting for both specific IgG and IgE. The highest levels of IgE binding were obtained for olive pollen–allergic patients, which could be related to the route of sensitization. The responses of individual patients to profilins were also similar and independent of the sensitizing source. The inhibition between pairs of allergens was generally higher than 70%, indicating that profilins share most of the IgE epitopes. Modeling of mimotopes in the conformational structure of the implicated profilins supports their strong cross-reactivity obtained experimentally.

Conclusions: No correlation exists between the level of IgE response of individual patients to specific profilins and the corresponding theoretical sensitizing source, suggesting that the sensitization could be attributable to any profilin present in the environment of the patients. This would bear out the use of most profilins as a common marker for polysensitization in component-resolved diagnosis and for therapeutic approaches.

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INTRODUCTION

Pollen and plant-derived foods are very allergenic sources that contain allergens from a small number of protein families.^{1,2} Profilins, which belong to one of these families,³ are 14 kDa (131–134 amino acid residues) and play a role in the reorganization of the cytoskeleton of eukaryotic cells by regulating actin's polymerization.⁴ Plant profilins, even those from phylogenetically unrelated sources, display highly conserved amino acid sequences (identities higher than 70%) and very similar 3-dimensional structures.³ Most profilins are considered minor allergens, because only 10 to 30% of patients allergic to pollens or plant foods have specific immunoglobulin (Ig) Es.³ However, almond profilin displays a

prevalence of 50% in patients with almond allergy,⁵ and more than 70% of patients hypersensitive to melon and orange have IgE to Cuc m 2 and Cit s 2, respectively.^{6,7}

The high structural similarity of profilins would account for a common immunologic behavior against specific antibodies, and therefore a large number of cross-reactions involving profilins have been reported.^{8–10} Consequently, their role in the polysensitization of patients is generally accepted. Nevertheless, evidence exists for a partial cross-reactivity of profilin from birch pollen with those from cherry, pear, celery, and peanut,¹¹ as well as between profilins from *Parietaria* and grass pollen¹²; a lack of cross-reactivity is seen between profilins from latex and *Mercurialis annua* pollen, two members of the *Euphorbiaceae* family.¹³ Studies performed combining 3-dimensional modeling with IgE immunoassays indicated the existence of species-specific IgE epitopes¹⁴ as well as remarkable differences in the IgE-binding capacity for different epitopes.¹⁵ Accurate diagnosis of allergy in patients suffering from polysensitization is crucial to design a successful specific immunotherapy. Conventional techniques that make use of complete allergenic extracts render results that are easily misunderstood. However, the promising strategy of component-resolved diagnosis that employs purified allergens would allow us to resolve the specific allergenic profile of the patient.¹⁶ It will be a valuable tool in clinical practice when panallergens are the

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sensitization agent, but this approach requires an appropriate panel of allergenic molecules.¹⁷

In this work, we compare the immunologic behavior of profilins from pollen- and vegetable-derived foods to facilitate the diagnosis by component-resolved techniques. For this purpose, we used specific IgG antibodies obtained against several profilins as well as sera from allergic patients recruited by their sensitivity to specific pollen- or plant-derived foods. Discussion of the results on the basis of the previously reported epitopes is also included.

METHODS

Human Sera, Antibodies, and Allergens

Sera from 6 patients sensitized to mustard (*Sinapis alba*) seeds and with specific (s)IgE to mustard profilin rSin a 4 (group B), sera from 7 patients sensitized to melon and sIgE to melon profilin rCuc m 2 (group C), and sera from 7 patients sensitized to olive pollen and with sIgE to olive and chenopod profilins (Ole e 2 and Che a 2) (group D) were used in this study. These sera were individually used or combined in pools. A pool of sera ($n = 6$) from nonatopic subjects was used as a negative control (pool A). All patients suffered symptoms against each allergenic source. Written informed consent was obtained from patients and control subjects, and the study was approved by the Ethics Committee of the Hospital Fundación Jiménez Díaz (Madrid).

Polyclonal serum (IgG-pAb) against nOle e 2 was prepared by immunizing a New Zealand white rabbit by weekly injection of the protein in complete Freund's adjuvant. Mice IgG-pAb specific to Sin a 4 was prepared by weekly intraperitoneal injection of mice BALBc with 4 μ g of Sin a 4 preincubated for 1 hour with Al(OH)₃. After 21 days of treatment, serum was obtained by centrifugation of the blood. Rabbit IgG-pAb against pear profilin Pyr c 4 was provided by Dr Scheurer (Paul-Ehrlich Institute, Langen, Germany).

The rCuc m 2, rChe a 2, and rSin a 4 were obtained as previously described,^{6,18,19} and rBet v 2 (birch profilin) was purchased from Biomay (Wien, Austria). Birch profilin was selected as a model member of this allergen family. Chenopod profilin represents a member very close to Ole e 2 (93% amino acid sequence similarity), which is a significant allergen in Spain. Sera from patients allergic to olive pollen were positive to Ole e 2 and Che a 2. Cuc m 2 and sera from patients allergic to melon were included because they represent the prototype of allergy to vegetable-derived food mediated by profilin in the Spanish population. Sin a 4 from mustard and patients allergic to mustard seeds were included because no allergy to mustard has been associated with allergy to profilin. The polyclonal antisera employed were specific to Ole e 2, mustard profilin Sin a 4, or pear profilin Pyr c 4, which are three profilins with different interests: a pollen very common in the Mediterranean area, a significant allergenic spice, included in the study, and a representative fruit of the Rosacea family, respectively.

Electrophoresis and Immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed in 17% polyacrylamide gels in the presence of 2-mercaptoethanol. Proteins were visualized by Coomassie blue staining or transferred to nitrocellulose membranes (Amersham, Buckinghamshire, United Kingdom). The protein concentration was determined by using bicinchoninic acid (Pierce, Rockford, Illinois).

Immunodetection of proteins in membranes was achieved as described,¹⁹ by using pools of sera from patients allergic to mustard, or melon, or olive pollen (diluted 1:5), or IgG-pAb to Ole e 2, to Sin a 4, or to Pyr c 4 (diluted 1:5,000). Bound IgE antibodies were detected with mouse anti-human IgE antibodies (diluted 1:5,000) followed by horseradish peroxidase-labeled goat anti-mouse IgG antiserum (diluted 1:5,000; Pierce). Reaction to pAb against Ole e 2 or Pyr c 4 (diluted 1:5,000) was detected by horseradish peroxidase-labeled goat anti-rabbit IgG (diluted 1:3,000; BioRad, Richmond, California) or in the case of pAb against Sin a 4 (diluted 1:2,500) by horseradish peroxidase-labeled goat anti-mouse IgG (diluted 1:5,000; Pierce). The peroxidase reaction was developed using the enhanced chemiluminescence Western blotting reagent (Amersham).

Enzyme-Linked Immunosorbent Assay Experiments

Enzyme-linked immunosorbent assay (ELISA) plates were coated with 100 μ L/well of protein (2 μ g/mL) and incubated with patients' sera (diluted 1:5) or with the different pools of sera (diluted 1:5).¹⁹ Binding of IgE was detected as described for immunoblotting. Immunoglobulin G titrations were performed with increasing dilutions of each pAb. Peroxidase reaction was developed using fresh enzyme substrate and measuring the optical density (OD) at 492 nm. Each value was calculated as the mean of 2 determinations after blank subtraction.

For ELISA inhibition assays, after being coated with 100 μ L of each profilin (2 μ g/mL), the plates were incubated with the 3 pools of sera (diluted 1:5) previously adsorbed with 1 μ g/mL of each profilin.²⁰ The plates were incubated with mouse anti-human IgE and horseradish peroxidase-labeled goat anti-mouse IgG as described. The percentage of inhibition was calculated as: $(1 - OD_{492nm} \text{ with inhibitor} / OD_{492nm} \text{ without inhibitor}) \times 100$. All the determinations were carried out as duplicates.

Sequence Alignment and Three-Dimensional Modeling

Multiple sequence alignments were performed with the ClustalW program.²¹ The 3-dimensional structures of Sin a 4, Che a 2, and Cuc m 2 (accession numbers EF626939, Q84V37, and Q7X9Q0, respectively) were modeled by using the Swiss-Model Protein Modelling Server²² and the structure of Bet v 2 (P25816, PDB 1cqaA) as a template. Graphical processing of the 3-dimensional structures was accomplished with PDB viewer PyMOL program.²³

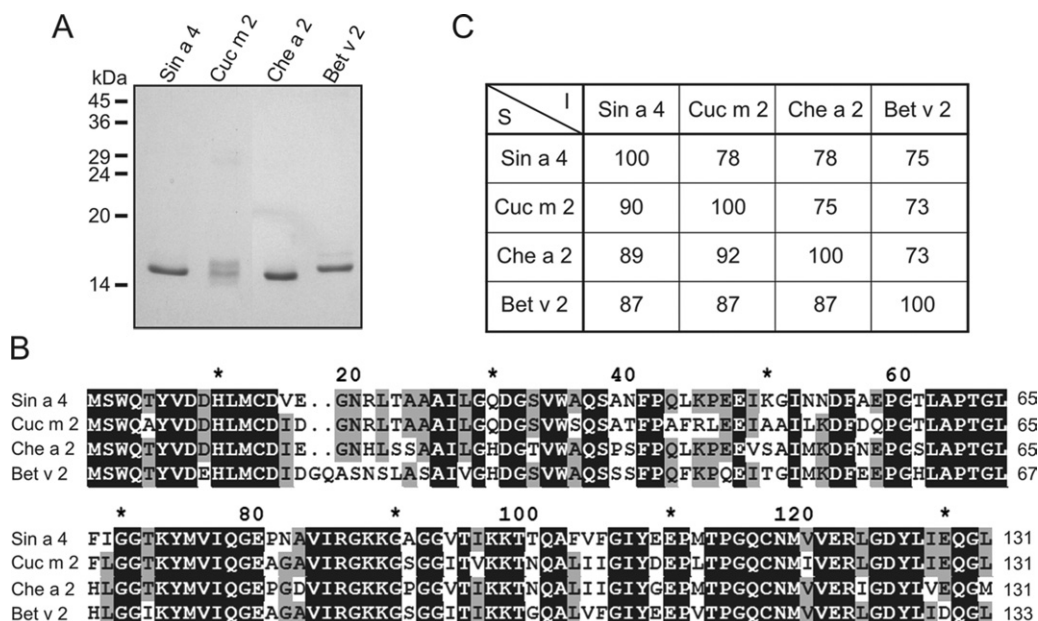


Figure 1. A, Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Coomassie blue staining of recombinant Sin a 4, Cuc m 2, Che a 2, and Bet v 2. B, Alignment of the amino acid sequences of the 4 profilins: Sin a 4, Cuc m 2, Che a 2, and Bet v 2 (accession numbers EF626939, Q7X9Q0, Q84V37, and P25816, respectively). Residues conserved in all proteins are shown in *black*, and those conserved in 3 sequences are in *gray*. C, Percentages of amino acid sequence identities (I) and similarities (S).

RESULTS

Structural Relationships among Mustard, Melon, Birch, and Chenopod Profilins

Two profilins from pollens (Che a 2 from chenopod and Bet v 2 from birch) and 2 from plant-derived foods (Cuc m 2 from melon and Sin a 4 from mustard) have been analyzed in this study. These proteins were obtained by recombinant technology and purified to homogeneity (Fig 1A). The presence of more than 1 band in their electrophoretic analysis, such as those obtained for Cuc m 2 and Bet v 2, is frequently reported for purified profilins.²⁴ Alignment of the amino acid sequences and the percentage of identity and similarity between pairs of the included profilins are shown in Figure 1B, C. Values higher than 70% identity and 85% similarity were found in all comparisons.

Response of the Profilins to Specific IgG Antisera

Specific polyclonal antisera obtained against profilins from olive tree pollen or pear and mustard seed were able to distinguish these profilins, in both immunoblotting and ELISA (Fig 2A, B). The high reactivity of several profilins against some specific pAb allowed us to detect the presence in immunoblotting of minor bands of approximately 14 kDa as well as a dimmer form of 30 kDa (Fig 2A). In immunoblotting, Sin a 4-specific antiserum is the most reactive against the 4 proteins (Fig 2A). By contrast, IgG anti-olive profilin recognized Bet v 2, whereas the binding is remarkably lower to melon and mustard profilins. Similarly, the binding of Pyr c 4-specific antiserum to mustard, melon, and

chenopod profilins is low, but the binding is high to Bet v 2. This profilin shows the best response both against pollen-specific antiserum (anti-Ole e 2) and against fruit specific antiserum (anti-Pyr v 4). Chenopod profilin shows very different behavior against the two specific antisera of plant-derived foods, in both immunoblotting and ELISA. Interestingly, Sin a 4-specific antiserum recognized better the bands of 15 to 16 kDa from Cuc m 2 and Bet v 2 than that of 14 kDa (Fig 2A), suggesting that the antiserum better binds a specific conformation of the allergen.

IgE Response of Allergic Sera to Purified Profilins

The sera from four groups of patients were used to analyze the individual response and the response in pool to the profilins included in the study. The responses obtained in ELISA for the pools of sera were consistent with those obtained in immunoblotting (Fig 3A, B). The highest responses, either with pools or with individual sera, were obtained with patients allergic to olive pollen (pool D). The responses of individual patients against each profilin were very variable and did not seem to be associated with the corresponding sensitizing source. For instance, serum 5 from group D (patients allergic to olive pollen) recognized preferentially Che a 2 and Sin a 4, whereas serum 7 recognized better Sin a 4, Cuc m 2, and Bet v 2 (Fig 3E); serum 4 from group B showed a higher response to Che a 2 than to Sin a 4 (Fig 3C). Apart from some exceptions (such as serum 4 from group B, or sera 2 and 4 from group C, or sera 5 and 7 from group D), the response of each individual serum to specific profilins was

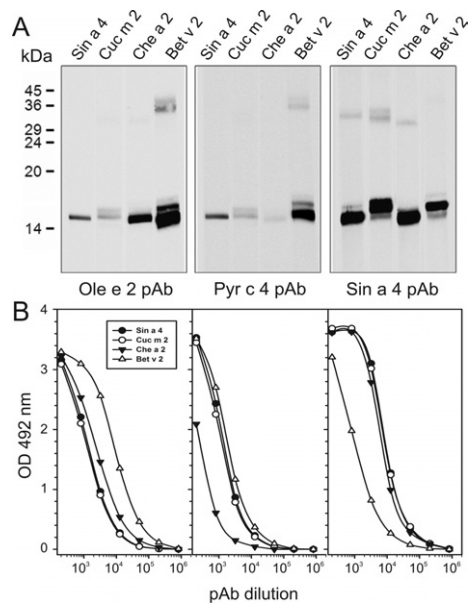


Figure 2. Analysis of the IgG reactivity. A, Immunoblotting of purified profilins using Ole e 2-specific pAb, Pyr c 4-specific pAb, and Sin a 4-specific pAb. B, ELISA titration of Sin a 4, Cuc m 2, Che a 2, and Bet v 2, using Ole e 2-specific pAb, Pyr c 4-specific pAb, and Sin a 4-specific pAb.

similar. The best responses of some individual sera do not correlate with the theoretical source of hypersensitization of the patients. Thus, sera 2 and 4 from group B gave higher response to Che a 2 than to Sin a 4 (Fig 3C), and serum #2 from group C gave higher response to Sin a 4 than to Cuc m 2 (Fig 3D).

Inhibition Experiments

Enzyme-linked immunosorbent assay inhibition assays of the IgE binding to Sin a 4, Cuc m 2, Che a 2, and Bet v 2 were performed with the three pools of sera and using the four profilins as inhibitors (Fig 4). Interestingly, inhibition values higher than 70% were obtained in most cases. Self-inhibitions rendered the highest inhibition level for all profilins, although, in some experiments, alternative inhibitors reached similar values: pool B against Sin a 4, with Che a 2 and Bet v 2 as inhibitors (Fig 4A); or pools B, C, and D against Che a 2, with Sin a 4 and Bet v 2 as inhibitors (Fig 4C). Che a 2 was the poorest inhibitor when sera from patients allergic to melon were used. Bet v 2 was a good inhibitor (>80% of inhibition) for all pool of sera, although the allergen displays the lowest amino acid sequence similarity with the remaining profilins.

A Common Mimotope in All Analyzed Profilins

Tordesillas et al²⁵ have recently located an IgE mimotope (conformational IgE-binding region) in the melon profilin Cuc m 2 (Fig 5). Thirteen of the 14 residues that constitute the mimotope of Cuc m 2 were located in the equivalent positions of Sin a 4, Che a 2, and Bet v 2 (Fig 5). Variability

was only found in positions 5 (T/A), 9 (D/E), and 122 (L/I), 2 of them (positions 9 and 122) being conservative changes in the amino acid sequence. These data support the strong cross-reactivity displayed experimentally by the profilins herein studied (Figs 2–4).

DISCUSSION

Ubiquitous allergens with conserved structures are responsible for incomplete and erroneous diagnoses when total extracts from allergenic sources are used by standard procedures.^{10,26–28} Novel systems using a component-resolved strategy could refine the identification of the sensitizing agent, which is of great importance for polysensitized patients.^{16,17,29–34} The decision about how many or which members from a family of panallergens have to be included in the tests seems to be of crucial relevance to obtain the most accurate results without unnecessary enlarged costs. In addition, the diagnosis should determine the specific immunotherapy and therefore the efficacy of the vaccine.³⁵ Profilins are among the most ubiquitous and cross-reactive allergens,^{3,8,9} and at least 39 allergenic profilins have been identified to date.²⁸ The similarity between their amino acid sequences is approximately 90%, which can explain the high degree of cross-sensitization that profilins induce among inhalant and plant-derived food allergenic sources.^{8,9} Thus, profilin sensitization has been proposed as a risk factor for allergic reactions to multiple pollen and food allergen sources,²⁹ as well as a marker of a more severe grass pollen sensitization.³⁰ In this work, we explore the possibility of classifying profilins in different categories according to their vegetable original sources—pollen or food allergens—or, in other words, according to the vehicle of sensitization: inhalation or ingestion. This would simplify the use of purified profilins in component-resolved diagnosis without loss of efficiency.

The analysis of the IgG reactivity of specific polyclonal antisera to profilins showed a variety of responses, and the differences were associated neither with the origin—pollen or food—of the specific antigens employed to obtain each antiserum nor to their amino acid sequence similarities. Thus, Pyr c 4-specific and Sin a 4-specific polyclonal antisera rendered completely different binding behavior to the tested profilins. This indicates that IgG antisera can discriminate among antigens even if they display small differences in amino acid sequence and conformation.

Regarding the IgE binding capacity of pools and individual sera, the responses to different profilins were similar, in general. No preferential binding of IgE to Che a 2 or to Bet v 2 was found for sera from patients allergic to pollen; and no preferential IgE reactivity to Sin a 4 or Cuc m 2 was obtained for sera from patients allergic to mustard or melon, respectively. This again supports the idea that the IgE epitopes are highly conserved in the members of this panallergen family, as previously reported.^{3,25} However, our results have also shown that some patients may display a very different degree of IgE reactivity to distinct profilins (Fig 3C, D, E). These patients do not belong to the same group, and the discrimi-

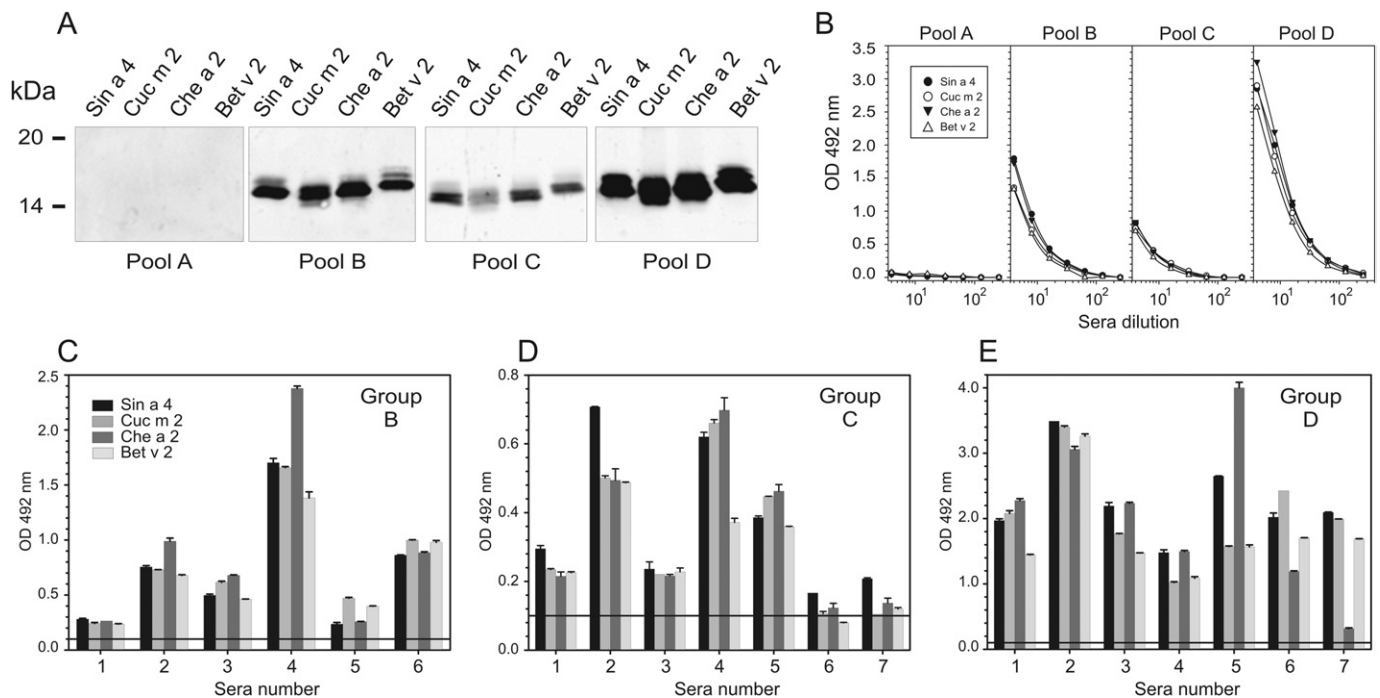


Figure 3. Analysis of the IgE reactivity. A, Immunoblotting of Sin a 4, Cuc m 2, Che a 2, and Bet v 2 with pools of sera: pool A, nonatopic patients; pool B, patients allergic to yellow mustard; pool C, patients allergic to melon; and pool D, patients allergic to olive pollen. The ELISA IgE-binding titration to the four profilins with pooled sera (B) or quantitation to individual sera (C–E) from the 3 groups of patients allergic to mustard (group B), to melon (group C), and to olive pollen (group D).

nated profilin is not always the same, thus supporting the existence of species-specific epitopes that can be recognized by some allergic patients.¹⁴ This observation would not modify our proposal for component-resolved diagnosis, because these patients would be correctly classified as profilin-sensitized individuals.

The IgE responses of pools of sera were similar in ELISA and immunoblotting. This is in agreement with previous results showing no major differences in the IgE-binding activity of profilins after denaturing treatments.²⁴ However, the observed values for a pool of sera are the average of the individual values. In this context, partial or total loss of IgE-binding activity to Ole e 2 (olive profilin) was observed in immunoblotting for individual sera from patients allergic

to olive pollen with strong ELISA responses.³⁶ Interestingly, the highest levels of specific IgE to profilin were obtained in sera from pollen-sensitized patients. This could be related to the route of sensitization: inhalation (no protein degradation) versus ingestion (potential allergen degradation). Sensitization to profilin from vegetable foods has been repeatedly associated with earlier sensitization to pollens, and thus many plant food-derived profilins have been shown to be involved in pollen-food syndromes.¹

Patients sensitized to a particular allergenic source did not show higher levels of specific IgE to the corresponding profilin than to the others. In fact, the antigenic and allergenic behavior of Che a 2 was more similar to those of Sin a 3 and Cuc m 2 than to that of Bet v 2. Differences cannot be

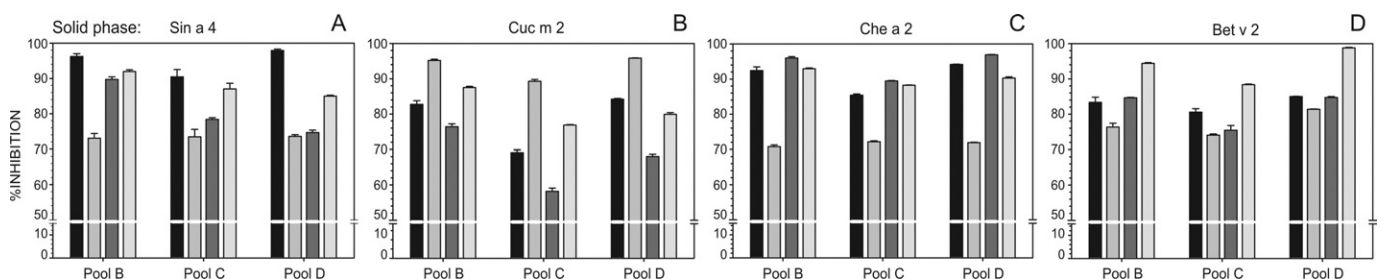


Figure 4. ELISA-inhibition experiments using the 3 pools of sera from allergic patients (see legend to Fig 3) and the four purified profilins as solid-phase (A–D) and as inhibitors (Sin a 4 ■, Cuc m 2 ■, Che a 2 ■, and Bet v 2 ■).

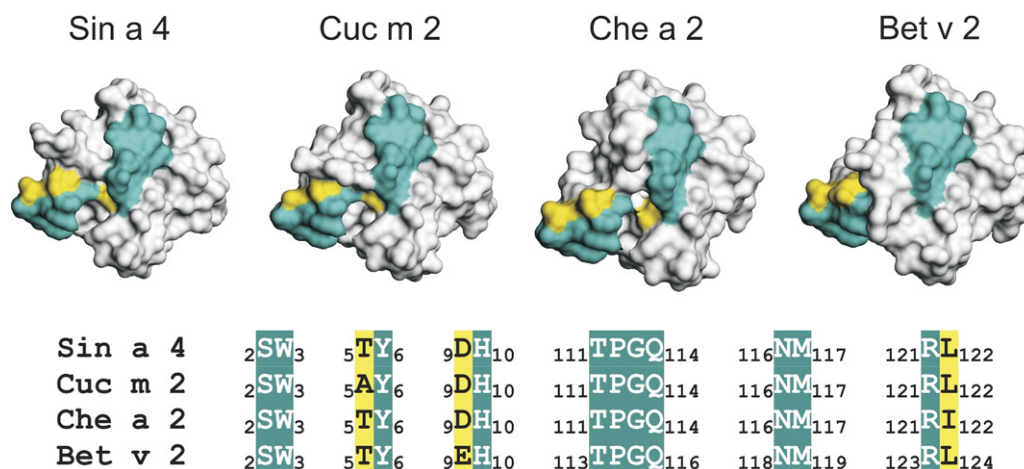


Figure 5. Location of mimotopes on the molecular surfaces of the 3-dimensional structures of Sin a 4, Cuc m 2, Che a 2, and Bet v 2 at the same orientation (upper row). Comparison of the amino acid sequences at the mimotope positions in the 4 profilins (lower row). Conserved residues are shown in blue; changed residues in yellow.

explained by divergence of the amino acid sequences: 87% for Bet v 2 versus 89% to 92% for the remaining proteins. Cuc m 2 showed the most different behavior when used as an inhibitor, and Bet v 2 showed a high inhibitory ability. This result is remarkable because it is not expected that the patients recruited for the study were in contact with birch pollen allergens in their geographic environments; no birch trees grow in the area of Madrid.

In summary, we cannot find any difference for profilins based on their biological origin. No correlation was seen between the IgE binding of individual patients to specific profilins and the corresponding theoretical sensitizing source, suggesting that sensitization could be attributable to 1 or several profilins from the environment of the patients. This would support the idea that many components of this family of proteins—avoiding non-cross-reactive members—can be used as diagnostic marker for polysensitization in component-resolved diagnosis and for therapeutic approaches.

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