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Characterization of Profilin and Polcalcin Panallergens From Ash Pollen

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Abstract

Background: Ash (*Fraxinus excelsior*) is an important source of allergenic pollen in temperate areas of Europe. Profilin and polcalcin are 2 important panallergens involved in cross-reactivity between different sources.
Objective: To clone and produce Fra e 2 (profilin) and Fra e 3 (polcalcin) as recombinant proteins and evaluate their immunological properties using the natural forms obtained from ash pollen.
Methods: Total RNA from ash pollen was used as a template to obtain the specific complementary DNA (cDNA) sequences of the 2 panallergens. The cDNA-encoding sequences were cloned into the pET11b expression vector and used to transform BL21 (DE3) *Escherichia coli* cells. Proteins were expressed, purified by chromatography, and characterized structurally by circular dichroism, mass spectrometry, and immunologically by western blot and ELISA using profilin and polcalcin polyclonal antibodies and human sera from ash pollen–sensitized patients.
Results: Profilin and polcalcin amino acid sequences from ash pollen showed a high degree of identity with homologous allergens from different sources. The cDNA-encoding allergen sequences were expressed as nonfusion recombinant proteins and purified to homogeneity. Secondary structure values were similar to those obtained from other members of these families. Allergenic properties of the recombinant allergens were observed to be equivalent to those of the natural counterparts of *F excelsior* pollen.
Conclusions: Fra e 2 and Fra e 3 recombinant allergens might be used in clinical diagnosis to determine profilin- and polcalcin-specific IgE levels present in the sera of ash pollen–sensitized patients, thus facilitating the finding of the sensitizing source in areas with complex sensitization profiles.

Key words: Ash pollen. Panallergens. Polcalcin. Profilin. Recombinant allergen.

Resumen

Antecedentes: El polen de fresno (*Fraxinus excelsior*) es una importante fuente alergénica en zonas cálidas de Europa. La profilina y polcalcina son 2 panalérgenos implicados en reactividad cruzada.
Objetivos: Clonar y producir Fra e 2 (profilina) y Fra e 3 (polcalcina) como alérgenos recombinantes. Comparar sus propiedades inmunológicas con sus formas naturales del polen de fresno.
Métodos: El RNA total de polen de fresno se utilizó como molde para obtener los cDNAs específicos de ambos panalérgenos. Dichos cDNAs se clonaron en el vector de expresión pET11b y se transformaron células de *Escherichia coli* BL21(DE3). Las proteínas se caracterizaron mediante dicroísmo circular, espectrometría de masas, inmunodetección en membrana y ELISA utilizando anticuerpos policlonales frente a profilina y polcalcina y sueros de pacientes alérgicos al polen de fresno.
Resultados: Las secuencias de aminoácidos de la profilina y polcalcina de polen de fresno presentaban una identidad de secuencia elevada con alérgenos homólogos. Dichos alérgenos se expresaron como proteínas recombinantes independientes y se purificaron a homogeneidad. Los valores de estructura secundaria fueron similares a los de otros miembros de estas familias. Las propiedades alergénicas de los alérgenos recombinantes resultaron ser equivalentes a los de sus homólogos naturales del polen.
Conclusiones: Los alérgenos recombinantes Fra e 2 y Fra e 3 podrían usarse en diagnóstico clínico para determinar los niveles de IgE específicos para profilina y polcalcina en los sueros de los pacientes sensibilizados al polen de fresno, facilitando así la identificación de la fuente sensibilizante en áreas donde los pacientes presentan perfiles alergénicos complejos.

Palabras clave: Polen de fresno. Panalérgenos. Polcalcina. Profilina. Alérgeno recombinante.

Introduction

The Oleaceae family is composed of approximately 400 species. From an allergenic perspective, the most representative genus is *Olea*. *Olea europaea* (olive tree) is the second most common allergenic pollen in Mediterranean countries and the most common one in some large Spanish regions [1-4].

Ash (*Fraxinus excelsior*), another Oleaceae species, is mainly distributed throughout central Europe. Due to the widespread use of this tree as a source of wood, ash pollen has become an important source of allergens. Furthermore, given the high structural similarity between members of the Oleaceae family, these pollens are good candidates for studying cross-reactivity processes [5]. To date, the presence of the allergens profilin, polcalcin, and Fra e 1 (a member of the Ole e 1–like family) have been described in *F excelsior*, but only Fra e 1 has been characterized [5-7].

Profilin and polcalcin are known panallergens involved in cross-reactivity processes. They are small acidic proteins with molecular masses of 14 to 15 kDa and 9 kDa, respectively. Profilins are actin-binding proteins that regulate microfilament dynamics and mediate changes in polymerization in the microtubule cytoskeleton; they are also implicated in multiple signaling pathways, including those involving the Ena/VASP protein family [8]. Polcalcins, by contrast, belong to a 2 EF-hand Ca^{2+}-binding protein family and are involved in signaling processes, neuronal exocytosis, and pollen tube growth [9,10]. Both profilins and polcalcins have been described as panallergens in several pollen sources such as *Betula verrucosa, Chenopodium album*, and *O europaea* [7,9,11-13]. Profilin panallergens have also been identified in foods and latex [14]. Both allergens are highly conserved among species and their amino acid sequences share a high degree of sequence identity with their counterparts in other allergenic sources (60%-90% for polcalcins and >75% for profilins). This accounts for the high level of cross-reactivity observed between members of the same family.

Profilin has been reported as a major allergen in pollen and food sources such as *C album* and *Cucumis melo*, respectively [15,16], and in populations polysensitized to olive [13] or grass [17] pollens. Polcalcins are described as minor allergens in most of the sources analyzed but they still recruit a high percentage of specific IgE, ranging from 10% to 40% in allergic patients. Irrespective of their designation as major or minor allergens, both profilins and polcalcins are important markers of polysensitization, and at least in the case of profilins, previous sensitization to aeroallergens from pollens results in susceptibility to subsequent exposure to certain food counterparts, triggering oral allergy syndrome [18]. Indeed, sensitization to latex profilin has been reported to occur through primary sensitization to pollen or food allergens [19]. Polcalcins are involved in processes that occur during pollen germination. They have only been described in pollen grains, and accordingly have not been associated with pollen-food cross-reactivity [20].

In the present study we describe the recombinant expression and characterization of profilin and polcalcin panallergens from ash pollen. Our findings could facilitate the standardization of these allergens in extracts, improve component-resolved diagnosis of allergy to pollens from the Oleaceae family [21], and facilitate the design of effective treatments for polysensitized patients. The improvement of current diagnostic and treatment approaches is particularly important in areas in which individuals are exposed to multiple pollen types due to the overlap of different allergenic sources, which hampers patient diagnosis [17,22].

Methods

Plasmids and Cells

A TOPO-TA Cloning kit (Invitrogen) was used to obtain partial and full complementary (c) DNA-encoding sequences from Fra e 2 and Fra e 3 according to the manufacturer´s instructions. cDNA sequences were subcloned into the pET11b plasmid (Novagen) and used to transform *Escherichia coli* DH5α F´ cells (Invitrogen). The recombinant proteins were then expressed in BL21 (DE3) cells (Agilent Technologies).

Food and Pollen Protein Extracts

Pollens from the indicated species were purchased from Allergon (Ängelholm). Pollen protein extracts were obtained by saline extraction as described previously [23]. The indicated protein extracts from nuts and fruits were also obtained as previously described [24].

Sera and Antibodies

Twenty-five patients from Strasbourg in France who were sensitized to ash pollen, as demonstrated by prick test, with specific IgE to ash pollen extract between class 1 and 6 as determined by the CAP-RAST method (Amersham Pharmacia Biotech), with no migratory background to or from Mediterranean areas, and with ash pollen as a primary Oleaceae sensitizer, were recruited from the pulmonology department of Hôpital Lyautey (Hôpitaux Universitaires de Strasbourg). This randomly selected patient population displayed a similar level of Fra e 1 positivity to that described in previously published results [7,25], ie, they had demonstrated ash pollinosis and no bias to any specific allergens. All patients and nonatopic controls gave written informed consent. The protocols used for experimental work with mice were approved by the ethics committee of the Complutense University in Madrid, Spain, and the methodology relating to the use of human sera was approved by the ethics committee of Hôpital Lyautey in Strasbourg, France.

Rabbit polyclonal antisera against profilin Ole e 2 (Ole e 2 pAb) or polcalcin Ole e 3 (Ole e 3 pAb) from *O europaea* pollen were generated in response to weekly injections (100 µg) of the allergen in complete Freund´s adjuvant. The production of these antibodies was supervised by staff at the animal facility at the Fundación Jiménez Díaz in Madrid, Spain, and performed in accordance with Spanish legislation and the guidelines of the ethics committee of Fundación Jiménez Díaz and Complutense University. Horseradish peroxidase-labeled goat anti-rabbit polyclonal IgG was purchased from Bio-Rad and mouse monoclonal anti-human IgE was kindly donated by ALK-Abelló. Horseradish peroxidase-labeled goat anti-mouse pAb was purchased from

Pierce Chemical Co and horseradish peroxidase-labeled rabbit anti-mouse pAb was acquired from Dako.

Cloning and Recombinant Expression of Fra e 2 and Fra e 3

Total RNA from ash pollen was isolated as previously described [6] and used as a template to obtain cDNA by reverse transcription using the SMART RACE cDNA amplification kit in accordance with the manufacturer´s instructions (BD Biosciences/Clontech). Amplification of specific sequences from Fra e 2 and Fra e 3 was performed using degenerate oligonucleotides spanning the different sequences of previously described profilin and polcalcin allergens and a nonspecific primer (Universal Primer Mix, UPM) included in the kit.

The 2 proteins were cloned following a 2-step process. An initial polymerase chain reaction (PCR) round was performed with the UPM primer and the following degenerate oligonucleotides: 5´-atacatATGTCNTGGCARRCNTAYGT-3´, encoding MSW S/A YV for Fra e 2 (*NdeI* restriction site underlined); and 5´-atgaattcTTARAANATYTTNGCNACRTCYTT–3´, encoding KDVAK I/M F for Fra e 3 (*Eco*RI restriction site underlined). Next, the cDNA of Fra e 2 was cloned in a second round of PCR using the same degenerate sense primer and the antisense oligonucleotide 5´-CTCATTGANCAGGGCCTGTAGCTCGAGTCC-3´, encoding LIDQGL, whose sequence was obtained from the sequencing of the first PCR round. For Fra e 3, the second round of PCR was performed using the previously obtained cDNA and the following specific sense and antisense primers, respectively: 5´-atacatATGGCCGACGATCCACAGGAAGT-3´ (MADDPQE;NdeI restriction site is underlined) and 5´-aaagaattcctaGAAGATTTTTGCAACATCCTTGAC-3´ (VKDVAKIF; *Eco*RI restriction site is underlined), deduced from the previously obtained cDNA sequence. Both cDNA sequences were cloned into PCR2.1 (Invitrogen) and then digested with *Nde*I and *Eco*RI and subcloned into the pET11b plasmid (pET11b/Frae2.1 and pET11b/Frae3). *E coli* DH5α F´ cells were transformed to expand these plasmids. Next, the recombinant constructs were used to transform *E coli* BL21(DE3) cells to express the allergens as recombinant proteins.

E coli BL21(DE3) transformed cells were induced with 0.4 mM isopropyl-β-thiogalactoside at 37ºC for 4 hours and then centrifuged at 6000 g and 4ºC for 20 minutes. The cell pellets were resuspended in 20 mM ammonium bicarbonate (AB) containing 1 mM phenylmethylsulfonyl fluoride and lysed by 3 cycles of freezing in liquid N_2 and thawing at 42ºC. Debris was removed after centrifugation at 12 000 g for 20 minutes at 4ºC and the supernatant containing the recombinant proteins was collected and lyophilized.

Purification of rFra e 2 and rFra e 3

The first step of the purification of both Fra e 2 and Fra e 3 consisted of size-exclusion chromatography in a Sephadex G-50 medium column (39 x 5.75 cm) equilibrated in 0.2 M AB [pH 8.0]. For the purification of Fra e 3, fractions containing the recombinant protein were then loaded onto a DEAE-cellulose column and eluted with an ionic strength gradient ranging from 0.1 to 0.5 M AB [pH 8.0]. Both recombinant proteins were then separately purified to homogeneity in a final step using reverse-phase high-performance liquid chromatography (HPLC) in a Nucleosil C18 column with an acetonitrile gradient in 0.1% trifluoroacetic acid. All the chromatographic stages of Fra e 2 and Fra e 3 protein purification were visualized by 17% SDS-PAGE in the absence of 2-mercapthoethanol according to standard procedures.

Structural Characterization and Sequence Analysis Predictions

Purified recombinant proteins were lyophilized and reconstituted in 50 mM sodium phosphate [pH 7.5] at a concentration of 200 µg/mL to record the far-UV CD spectra using a JASCO J-715 spectropolarimeter. CDNN CD spectra deconvolution software (Applied Photophysics) was used to determine the composition of the secondary structure. The experimental molecular mass was determined by mass spectrometry using a Bruker Reflex IV MALDI-TOF apparatus (Bruker-Franzer Analytic). The theoretical molecular masses corresponding to the sequences obtained by cloning were calculated using the ProtParam tool [26].

The homology of the polcalcin and profilin sequences obtained were analyzed using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information [27] and sequences were aligned using the GeneDoc software (http://www.nrbsc.org/). Modeling of the 3D structure of Fra e 2 and Fra e 3 was accomplished using the homology-modeling server of the Swiss Institute of Bioinformatics [28], using Bet v 2 (PDB: 1cqa) and Che a 3 (PDB: 2opo) as templates for profilin [29] and polcalcin [30], respectively.

Immunological Characterization

After SDS-PAGE, the proteins were transferred to nitrocellulose membranes (Amersham Biosciences) and immunostained with polyclonal antibodies against Ole e 2 (1:10 000) or Ole e 3 (1:5000), or with individual human sera from either ash pollen-sensitized patients or nonatopic controls (diluted 1:10), and then incubated with mouse monoclonal anti-human IgE. The membranes were then incubated with the corresponding peroxidase-labeled secondary antibodies. For the immunoblotting inhibition assays, Ole e 2 pAb or Ole e 3 pAb, or a pool of sera (n=5) were preincubated with phosphate buffer solution, and 1 or 5 µg of purified proteins were used as inhibitors. Finally, proteins were visualized using the chemiluminescent ECL Western blotting reagent (Amersham Biosciences).

Indirect ELISA was performed in triplicate in 96-well plates coated with 0.1 µg of purified recombinant protein. Assays were performed using individual human sera (1:10), a pool of 5 human sera (1:10), or polyclonal antibodies against Ole e 2 or Ole e 3 (1:10000 and 1:5000, respectively), previously adsorbed to various protein extracts as inhibitors (20 or 200 µg). Binding of human IgE was detected by incubation with mouse monoclonal anti-human IgE (1:5000) followed by horseradish peroxidase-labeled goat anti-mouse Ig (1:2500). Absorbance was measured at 492 nm in an iMark microplate absorbance reader (Bio-Rad) after color production with the peroxidase substrate o-phenylenediamine (Sigma-Aldrich).

Mean absorbance values of over 0.1 were considered positive, and the percentage of inhibition was calculated using the following formula: inhibition (%) = [1-(absorbance at 492 nm with inhibitor/absorbance at 492 nm without inhibitor)] x 100.

Results

Cloning, Recombinant Production, and Purification of Ash Profilin and Polcalcin

Natural profilin and polcalcin were detected by immunoblotting of ash pollen protein extract with specific polyclonal antisera directed against profilin or polcalcin (Figure 1). Profilin appeared as a group of multiple bands of approximately 14 kDa and polcalcin appeared as a 10-kDa band.

cDNA sequences of profilin and polcalcin form *F excelsior* were obtained by PCR as described in the Methods section. After cloning and sequencing, 2 different DNA sequences were obtained for Fra e 2 and 3 for Fra e 3 (Figure 2A and B). Fra e 2.1 and 2.2 were composed of 402 and 399 nucleotides (134 and 133 amino acids), respectively, while the 3 polcalcin isoforms were composed of 252 nucleotides (84 amino acids) each. Fra e 2.1 showed a high degree of sequence identity with plant-related profilins, while the DNA sequence of Fra e 2.2 was identical to that previously described for Amaranthaceae profilin [31]. Fra e 2.1 was thus selected for recombinant expression. The 3 DNA sequences obtained for Fra e 3 exhibited 6 silenced nucleotide changes with no modifications to their amino acid sequences. The only amino acid sequence deduced from the 3 isoforms is shown in Figure 2B. Comparison of Fra e 2.1 with Ole e 2 revealed high sequence identity (96%) and similarity (99%). Moreover,

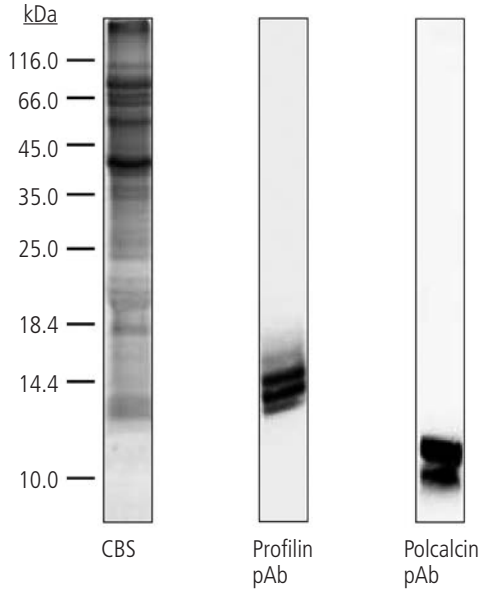

Figure 1. Identification of natural profilin and polcalcin proteins in the *Fraxinus excelsior* pollen protein extract using polyclonal antisera (pAb) raised against olive pollen profilin or polcalcin allergens. In total, 50 µg of total protein extract were separated by SDS-PAGE and stained with Coomassie Blue (CBS) or alternatively transferred to nitrocellulose membranes. Molecular weight markers are shown.

identity and similarity scores of 71% to 85% and 85% to 92% were obtained when Fra e 2.1 was compared with allergenic profilins from other sources, including those from unrelated families such as *C album* and *B verrucosa*, and from foods such as *Arachis hypogea, Capsicum annuum*, and *Lycopersicum esculentum* (Figure 2A). Similar findings were observed when Fra e 3 was compared with polcalcins from both related species such as *O europaea* (91%/97%) and *Syringa vulgaris* (85%/94%) and unrelated families such as *B verrucosa, C album, Brassica napus*, and *Nicotina tabacum*, with sequence identity and similarity values ranging from 61% to 81% and 82% to 92%, respectively (Figure 2B).

The selected cDNA-encoding sequences were cloned into the pET11b plasmid and used to transform BL21(DE3) *E coli* cells for recombinant expression of the proteins. The production of the recombinant allergen was visualized by SDS-PAGE. Fra e 2 and Fra e 3 were detected in the soluble fraction after bacterial lysis, with the highest yield obtained after 4 hours of induction at 37ºC (Figure 3A). Two chromatographic steps were required for the purification of rFra e 2 and 3 for rFra e 3. Although the proteins appeared to be homogeneous by SDS-PAGE, small amounts of contaminants were removed in a final chromatographic step, in both cases consisting of reverse-phase HPLC with an acetonitrile gradient (Figure 3B and C). For both proteins, this procedure rendered about 10 mg of recombinant protein per liter of cell culture.

Molecular Characterization of Fra e 2 and Fra e 3

Experimental molecular masses of 14261.8 and 9242.1 Da were determined by mass spectrometry for rFra e 2 and rFra e 3, respectively (Figure 4A). These values differed from the respective theoretical molecular masses of 117.4 and 87.3 Da, probably due to the processing of the N-terminal methionine by the bacterial machinery in both recombinant proteins.

Analysis of the CD spectra revealed correct folding of the 2 proteins. β-sheet content in rFra e 2 and α-helix content in rFra e 3 were higher than that described in previous reports of the secondary structures of the corresponding resolved crystal structures (Figure 4B) [29,30].

Immunological Characterization of Ash Pollen Panallergens

Antigenic validation of the recombinant proteins was achieved by IgG immunoblotting inhibition experiments using natural profilin and polcalcin contained in the pollen protein extract (Figure 4C). rFra e 3 completely inhibited IgG binding to natural polcalcin, while rFra e 2 induced 85% of IgG binding to natural profilin; in both cases 5 µg of each recombinant protein was used as an inhibitor. These results indicate the equivalence of both recombinant proteins to the natural forms present in pollen.

IgE binding to both recombinant proteins was tested by ELISA using sera from 25 randomly obtained ash pollen–sensitized patients from Strasbourg, France (Figure 5A) [7,25]. According to the selection criteria, this cohort should be representative of patients with ash pollinosis, with no bias towards any specific allergen. This experiment revealed that 52% and 16% of the sensitized patients possessed specific IgE

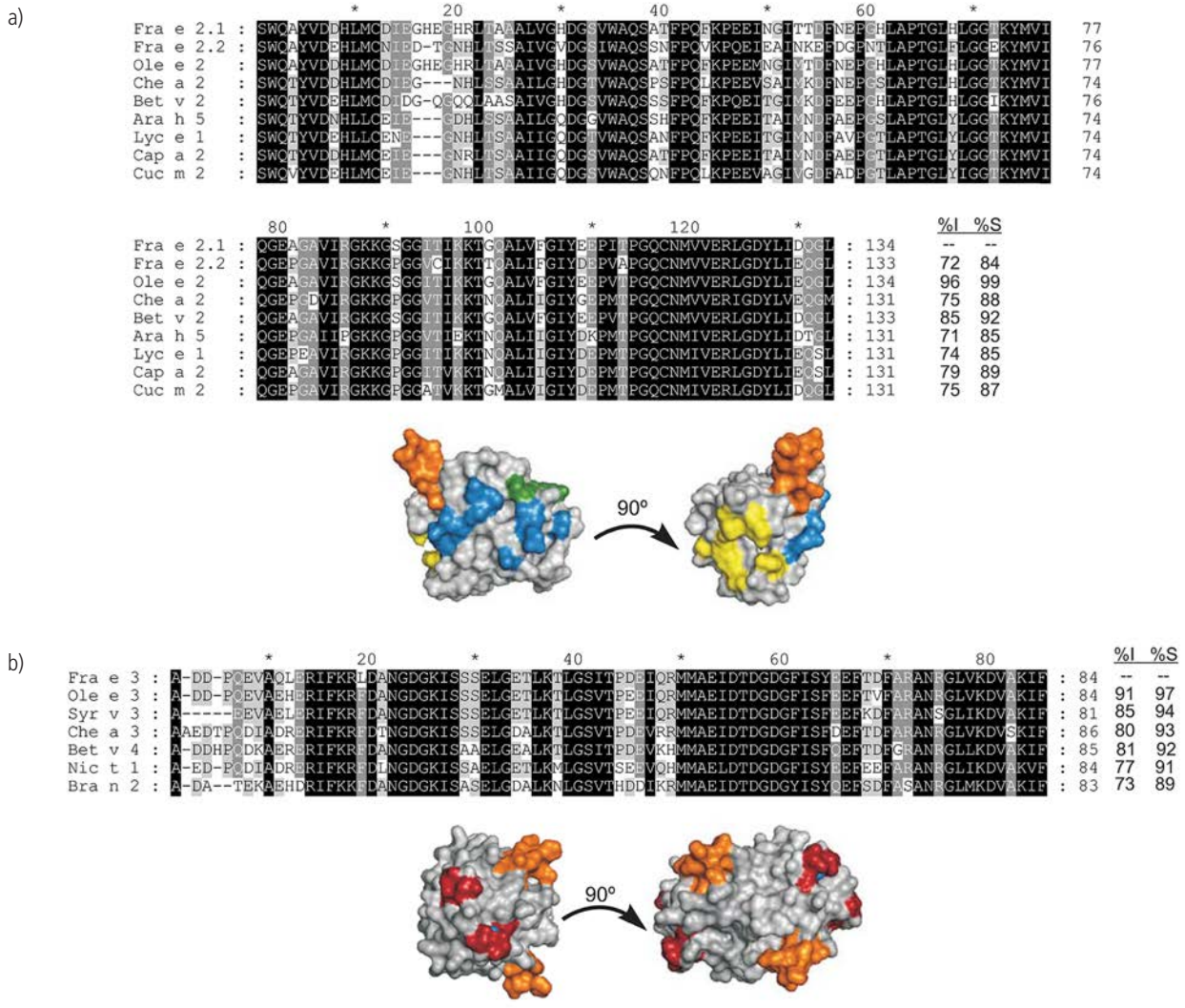

Figure 2. Comparison of the amino acid sequences of profilin and polcalcin from related and unrelated allergenic sources. A, Alignment of amino acid sequences of Fra e 2 isoforms with pollen and food profilins (top); 3D-modeling of Fra e 2 (bottom). In orange, highly variable region; blue, actin-binding site; green, IP3-binding site and yellow, poly-L-proline-binding site. B, Alignment of Fra e 3 amino acid sequence with pollen polcalcins (top); 3D-modeling of Fra e 3 (dimer) (bottom). In orange, highly variable region; red, calcium-binding site; and blue, calcium ion. Percentages of identity and similarity are shown in each case in comparison with Fra e 2.1 (Accession number KC920922) and Fra e 3 (KC920923), respectively.

to rFra e 2 and rFra e 3, respectively. Median values of 0.175 and 0.619 were obtained for the 15 sera reactive to rFra e 2 and the 4 sera reactive to rFra e 3, respectively.

Three individual sera and a nonatopic control were tested by immunoblotting using rFra e 2 and rFra e 3 (Figure 5B), revealing specific IgE binding to the recombinant proteins. The validation of rFra e 2 and rFra e 3 against their natural counterparts was confirmed by immunoblotting inhibition assays of ash pollen extract using a pool of sera from patients allergic to each allergen and the recombinant proteins as inhibitors (Figure 5C). The recombinant forms resulted in complete inhibition of IgE binding to the natural profilin and polcalcin present in the *F excelsior* pollen protein extract.

Finally, IgE cross-reactivity of ash pollen profilin and polcalcin was analyzed in ELISA inhibition experiments using a pool of sera from ash pollen–sensitized patients and pollen and plant-derived food extracts (200 μg) as inhibitors (Table). The protein integrity of polcalcin in pollen and profilin in pollen and plant-derived food extracts was tested before being used in subsequent experiments (data not shown). For ash pollen profilin, IgE binding inhibition of 50% to 70% was observed, except in the cases of tomato (*L esculentum*), peanut (*A hypogea*), and *Artemisia vulgaris* extracts, for which values of over 20% were observed. Small differences in IgE binding to pollen profilin were observed when pollen (mean [SD] inhibition, 69% [15%]) and plant-derived foods (58% [17%]) extracts were used as inhibitors. For ash pollen polcalcin, IgE-binding inhibition values of 50% to 85% were observed, with 38% inhibition detected for *A vulgaris*.

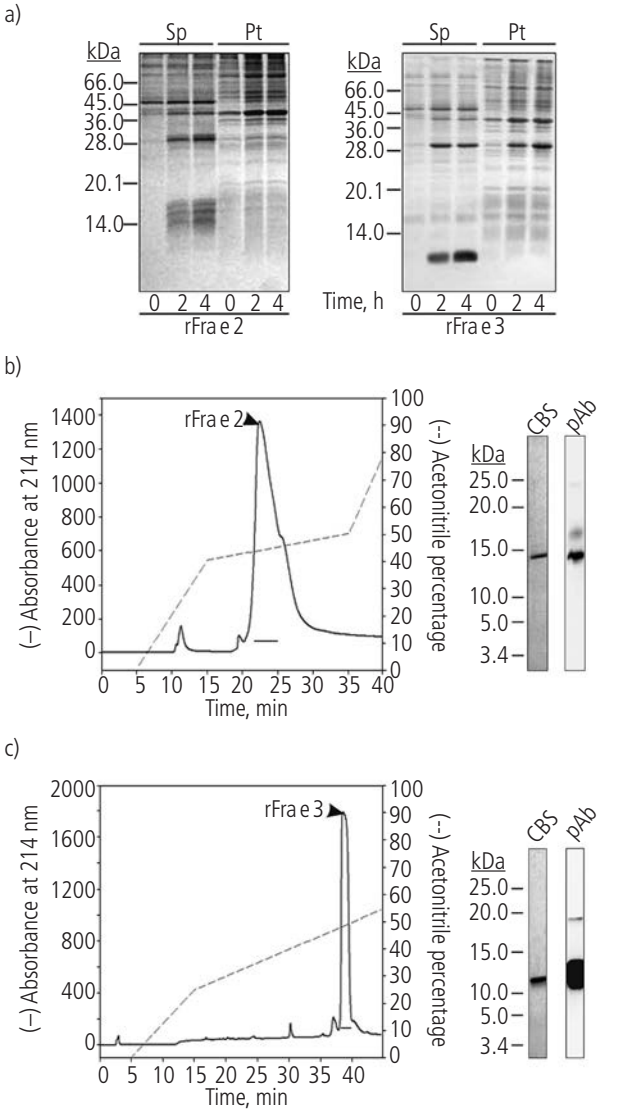

Figure 3. Recombinant production and purification of Fra e 2 and Fra e 3; 17% SDS-PAGE with Coomassie Blue staining (CBS) of supernatant (Sp) and pellet (Pt) obtained after cell lysis at different induction times (A). Last step of purification consisted of reversed-phase high-performance liquid chromatography with an acetonitrile gradient for rFra e 2 (B) and rFra e 3 (c). The line in each chromatogram highlights the fractions pooled for each protein. Pooled fractions containing the recombinant proteins purified at homogeneity (0.5 µg) were run in 17% SDS-PAGE with CBS or alternatively transferred to nitrocellulose membranes and immunostained with the corresponding polyclonal antibody (pAb) (right panel). Molecular weight markers are shown.

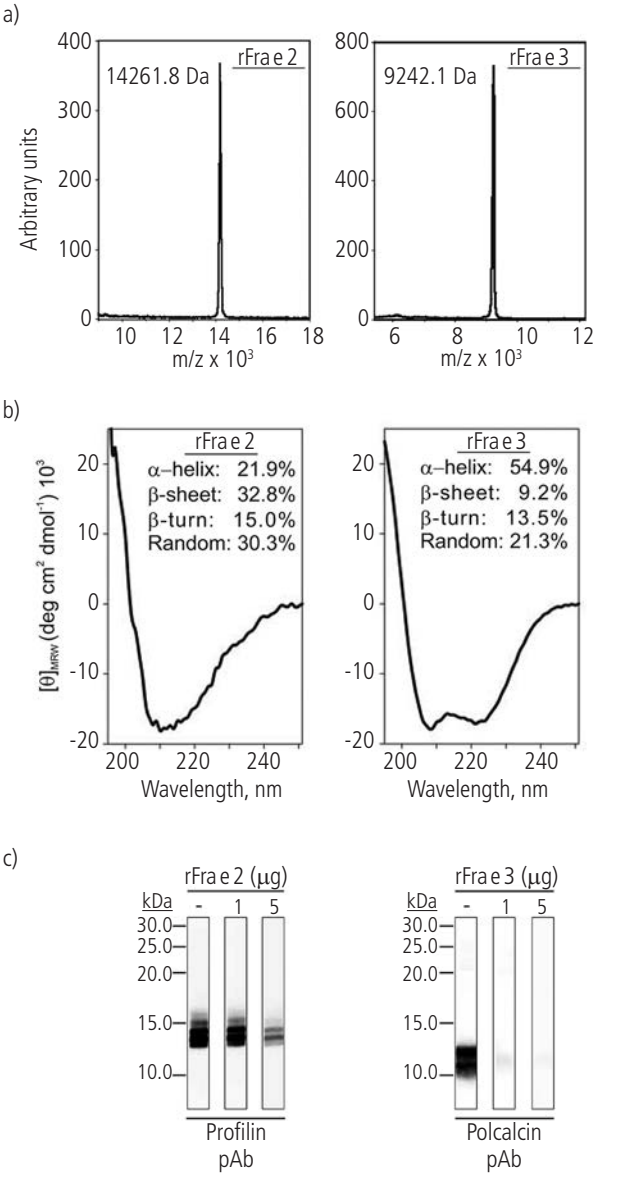

Figure 4. Molecular and immunological characterization of rFra e 2 and rFra e 3. A, Mass spectrometry in arbitrary units. B, CD spectra and percentages of secondary structure obtained by deconvolution. C, IgG-binding inhibition assays by immunoblotting with the corresponding polyclonal antisera (pAb) raised against olive pollen profilin or polcalcin to 50 µg of total ash pollen protein extract using 1 or 5 µg of the recombinant purified proteins as inhibitors. (-), without inhibitor. Molecular weight markers are shown.

Discussion

Ash tree is the main source of allergenic pollen in temperate areas of Europe. In these regions, allergies to ash pollen have gone largely unnoticed because of the overlapping flowering periods of ash tree and *B verrucosa* [4], with ash pollination naturally preceding birch pollination [32].Thus, the diagnosis of sensitization to both birch and ash and of polysensitization in general is quite complex and very often not established. Panallergens are families of ubiquitous proteins with close structural similarities involved in cross-reactivity processes. These allergenic molecules are responsible for the majority of diagnostic errors in allergic patients. Although further studies are required to determine the relevance of some of these molecules (eg, profilins) as inducers of allergic symptoms, these proteins constitute important tools for allergy diagnosis given their involvement in cross-reactivity reactions. IgE reactivity to profilin is associated with multiple pollen sensitizations and pollen-food syndromes [33]. Moreover, as polcalcin is a pollen-specific protein, its allergenic relevance

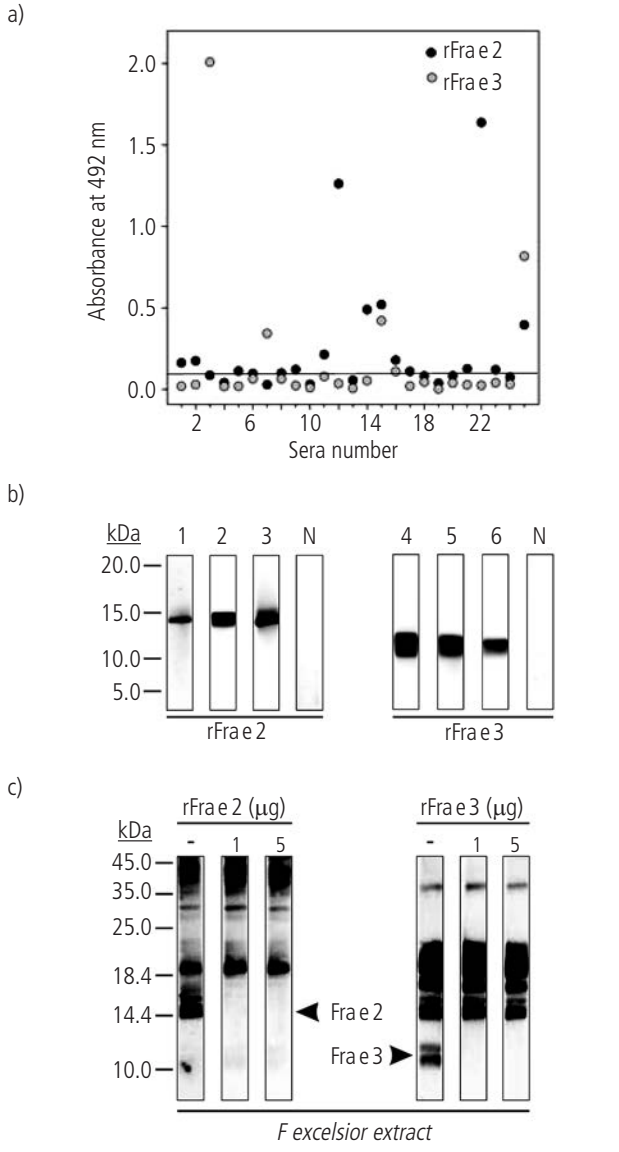

Figure 5. IgE-binding ability of rFra e 2 and rFra e 3. A, Determination of IgE binding from individual sera by ELISA. The horizontal line indicates the 0.1 cutoff value where sera values were considered positive. B, Immunoblotting of 0.5 µg of the recombinant proteins using individual sera from patients allergic to *Fraxinus excelsior* and a nonatopic serum (N). C, Inhibition of IgE binding to natural Fra e 2 and Fra e 3 from the pollen protein extract of *F excelsior* using 1 or 5 µg of rFra e 2 and rFra e 3 as inhibitors. (-), no inhibitor. Arrows indicate the electrophoretic mobility of natural Fra e 2 and Fra e 3. Molecular weight markers are shown.

has been exclusively associated with multiple pollen sensitizations, and not with food allergies [34].

It has been previously reported that panallergens from different sources do not exhibit complete immunological equivalence [35]. Thus, although sensitization can be diagnosed using any member of a family, specific immunotherapy could be improved by using the specific allergen against which a patient is actually sensitized [35,36]. Moreover, in addition to antibodies, immunotherapy involves T lymphocytes, which mediate distinct T-cell and antibody isotype responses to minimal amino acid differences in allergen isoforms. Therefore, the identification, cloning, and production of allergen isoforms from different allergenic sources are essential for their use in personalized desensitization protocols.

The panallergens analyzed in this study—profilins and polcalcins—are highly conserved proteins, and are thus involved in cross-reactivity processes between different sources. Panallergens enhance the allergenic character of different sources and increase the likelihood of the unexpected challenge (burst) of allergic symptoms in individuals in certain geographical locations with high concentrations of other pollens. For example, sensitization to Fra e 2 and Fra e 3 through *F excelsior* pollen in individuals living in northern and central Europe elicits allergic reactions on exposure to *O europaea* pollen, which is mainly distributed in southern Mediterranean Europe [37-39], where ash is rare. Importantly, the presence of specific IgE against these molecules in the serum of allergic patients leads to multiple sensitization processes to different sources, including food allergens and contact allergens (eg, latex) in the case of profilins and other pollens in the case of polcalcins.

The prevalence of 2 recombinant panallergens was 52% and 16% for profilin and polcalcin, respectively. The IgE-binding frequency for profilin indicated a role as a major allergen in ash-sensitized patients, which is consistent with previous findings in selected populations of allergic patients [15,16,40]. However, the IgE-binding frequency of polcalcin is not affected by other homologous allergens or by environmental pollen levels; it is considered a minor allergen in all populations studied, including the *F excelsior*–allergic population from Strasbourg analyzed in the present study. Remarkably, median ELISA values revealed that despite its low prevalence, Fra e 3 can recruit more IgE than Fra e 2 in ash pollen–sensitized patients.

The allergens presented here were immunologically validated against their natural counterparts using i) polyclonal antisera raised against olive pollen profilin and polcalcin and ii) a pool of sera from allergic patients. Inhibition experiments revealed that the recombinant profilin was unable to completely inhibit IgG binding to the natural profilin present in the extract, probably due to the presence of other isoforms recognized by the pAb. rFra e 3, by contrast, completely eliminated IgG binding to its natural counterpart.

Polymorphism is a common feature of profilin and polcalcin allergens and different allergenic capacities have been described for distinct isoforms [41]. Remarkably, experiments using the pool of sera revealed complete inhibition of IgE binding to the natural counterpart allergens in ash pollen extract, indicating that rFra e 2 and rFra e 3 contain all the major epitopes recognized by the IgEs present in the serum of ash pollen–sensitized patients. Analyses of the amino acid sequences of profilin in the present study and previously reported sequences for profilins from other sources have shown a highly variable region involving amino acids 17 to 22. This region of the molecule appears to be critical for the cell functionality of the protein and for the binding of IgE epitopes described to date [42]. Furthermore, it is located at the surface of the protein, and thus may constitute a potential interaction region for the IgE antibodies of particular allergic patients. The

Table. IgE Cross-Reactivity With a Pool of Sera From Patients Allergic to Fra e 2 and Fra e 3

	Percentage of Inhibition[a]				Accession Number (Identity and Similarity, %)	
	rFra e 2–coated wells		rFra e 3–coated wells			
Extract name	20 µg	200 µg	20 µg	200 µg	Profilin	Polcalcin
Sinapis alba (mustard)	58	59	-	-	ABU95412.1 (80/89)	-
Prunus persica (peach)	9	56	-	-	Q8GT40 (80/91)	-
Solanum tuberosum (potato)	66	66	-	-	-	-
Persea americana (avocado)	66	67	-	-	-	-
Lycopersicon esculentum (tomato)	2	5	-	-	Q93YG7 (74/85)	-
Capsicum annuum (pepper)	58	61	-	-	Q93YI9 (79/89)	-
Brassica oleracea (cauliflower)	54	59	-	-	-	-
Cucumis melo (melon)	63	63	-	-	Q5FX67 (75/87)	-
Pistacia vera (pistachio)	32	63	-	-	-	-
Juglans regia (walnut)	45	64	-	-	-	-
Prunus dulcis (almond)	48	63	-	-	Q8GSL5 (79/90)	-
Arachis hypogea (peanut)	1	12	-	-	Q9SQI9 (71/85)	-
Robinia pseudoacacia (black locust)	61	67	54	69	-	-
***Fraxinus excelsior* (ash)**	**58**	**72**	**83**	**85**	-	-
Ligustrum vulgare (common privet)	41	48	74	85	-	-
Olea europaea (olive)	66	67	71	81	ABC47412.1 (96/99)	O81092 (91/97)
Betula verrucosa (birch)	69	69	37	52	P25816 (85/92)	Q39419 (81/92)
Lolium perenne (perennial ryegrass)	64	66	61	61	-	-
Platanus acerifolia (planetree)	61	62	52	85	-	-
Salsola kali (Russian thistle)	41	52	45	57	C6JWH0 (76/88)	-
Chenopodium album (lamb´s quarter)	40	52	50	56	Q84V37 (75/88)	Q84V36 (80/93)
Artemisia vulgaris (mugwort)	1	19	18	38	Q8H2C9 (76/88)	A0PJ17 (61/82)
Parietaria judaica (pellitory)	36	55	56	62	Q9XG85 (76/88)	B5QST3 (73/85)
Brassica napus (rapeseed)	57	61	52	60	-	-

[a]IgE-binding inhibition (%) to rFra e 2– or rFra e 3–coated wells with 20 or 200 µg (total protein) of indicated extracts as inhibitors. Inhibition results with ash pollen extract are highlighted in bold. Accession numbers and identity (%) and similarity (%) of the sequence profilin and polcalcin are indicated.

C-terminal of the protein is the area with the greatest capacity to recruit IgEs [43], although this has not been described for polcalcin IgE epitopes. The alignment of sequences from different sources reveals a region spanning amino acids 4 to 13 of the N-terminal as the most variable part of the protein. These amino acids are not part of the Ca2+-binding site of the protein and constitute a very exposed region of the molecule. The observed variability in these regions between allergens from different sources and even isoforms from the same source could account for distinct IgE-binding patterns [41].

Comparison of profilin and polcalcin with the corresponding members of the Oleaceae family, with which they share the greatest phylogenetic similarities, revealed a very high degree of sequence identity (2 and 5 amino acid changes with respect to *O europaea* profilin and polcalcin, respectively). ELISA inhibition experiments revealed equivalent allergenic properties in the Oleaceae profilins analyzed, except for that of *Ligustrum vulgare*. Although the profilin sequence of *L vulgare* is unknown, a high level of sequence identity and similarity with respect to other Oleaceae counterparts is expected. The low level of inhibition observed in the presence of rFra e 2 is probably due to the small amounts of natural profilin present in the *L vulgare* pollen extract. On the other hand, food profilins showed similar inhibition values, except for *L esculentum* and

A hypogea, for which low levels of inhibition were observed, possibly due to differences in the highly variable region of the profilins or amino acid changes in the full sequence. The inhibitory effect of ash pollen polcalcin on Ig binding was comparable in all allergenic sources tested except for *A vulgaris* polcalcin, probably due to the phylogenetic distance between the 2 sources, which share less sequence identity (61%) and similarity (82%) than that found between ash pollen polcalcins and those from other sources.

In summary, the present study describes the cloning of the main panallergens from *F excelsior* pollen, their expression as recombinant proteins, their purification, and their structural and immunological characterization. Both allergens exhibited a high degree of sequence identity with homologous proteins from related and unrelated sources, as well as similar immunological characteristics. Contrary to some previous reports [44], recent analyses of profilin have demonstrated differences in the IgE-binding ability of profilin isoforms from the same *S kali* pollen source [41]. These differences may be even more significant between species and families. The availability of these recombinant panallergens could facilitate *i*) the study of the IgE-binding capabilities of critical and essential amino acids, *ii*) the identification of primary sensitizers, *iii*) the clinical diagnosis of polysensitization to multiple sources, *iv*) the standardization of allergen extracts used in immunotherapy, and *v*) personalized desensitization of sensitized patients.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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