

Olive-pollen profilin. Molecular and immunologic properties

Ledesma A, Rodríguez R, Villalba M. Olive-pollen profilin. Molecular and immunologic properties.

Allergy 1998; 53: 520–526. © Munksgaard 1998.

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Olive-pollen profilin has been isolated and characterized as a significant allergen. Its molecular properties, such as a molecular mass of 15 kDa; amino-acid composition; and secondary repetitive structure percentages of 15% α -helix, 33% β -strand, 20% β -turn, and 32% random coil, have been determined. Its allergenic capability, a recognition frequency estimated at 24% of olive-hypersensitive patients, and high cross-reactivity with all the pollen used have been found. The presence of conformational epitopes in the olive profilin, as well as a high structural and immunologic similarity to other pollen sources such as birch and ash, can be established from these studies.

Key words: allergen; allergenic cross-reactivity; olive-tree pollen; profilin.

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Accepted for publication 20 January 1998

Profilin is a ubiquitous protein that has been detected in various animal and plant tissues. Profilins interact with skeleton proteins of the cell, being involved in polymerization of actin filaments (1, 2) and signal transduction events through phosphoinositide pathways (3). Profilin has been identified as a panallergen because of its allergenicity; it can induce type-I hypersensitivity as a constituent of both pollens (4) and fruits (5). Sera from hypersensitive patients have been shown to contain IgE antibodies which recognize profilin from birch, wheat, celery, or *Phleum* (6).

Although profilins are not major allergens, they cause significant cross-reactivity among related and unrelated sources of allergens (7). About 20% of pollen-allergic patients are sensitized to profilins (8), but these proteins seem to have greater relevance in pollen–food cross-sensitizations (9). The cross-reactivity could be due to the similarity between their amino-acid sequences, which reaches up to 80% identity among pollen profilins (10).

Allergy to olive (*Olea europaea*) pollen is a clinical disorder of great incidence in Mediterranean countries (11, 12). Most of the allergic patients (about 70% in the studied populations) are sensitized to Ole e 1, the major allergen described in olive pollen (13, 14). However, many other IgE-reactive bands have been detected in SDS–PAGE of the saline extract of this pollen (15, 16), some of them exhibiting a molecular mass under 16 kDa that could be attributed to profilin. In fact, profilin

has been already detected in peach and apple (17) and its cDNA cloned and sequenced (18). In this work, we report the isolation of olive-pollen profilin. The analysis of its physicochemical and immunologic properties allowed us to assess its similarity to other known profilins and, thus, to establish the cross-sensitivity of olive pollen with other pollen sources.

Material and methods

Pollen extracts

Pollens from *O. europaea*, *Betula verrucosa*, *Zea mays*, *Cynodon dactylon*, and *Fraxinus excelsior* were obtained from Allergon AB. Pollen (5% w/v) was extracted with 50 mM ammonium bicarbonate, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride. The pellet was twice re-extracted in the same solution. Total protein determinations of saline extracts were carried out according to Lowry et al. (19).

Human sera and rabbit antiprofilin antibodies

Sera from donors allergic to olive pollen and not undergoing immunotherapy were used. The sera were selected by their recognition of 14–16-kDa protein bands of the olive-pollen extract. A serum from a patient allergic to Der p 1 was used as control.

A polyclonal rabbit antiserum obtained against recombinant birch profilin, kindly donated by Dr R. Valenta, was used in the purification steps of the antigen. On the other hand, a specific rabbit polyclonal antiserum was raised against olive-pollen profilin by the intra-lymph-node technique (20). An amount of 50 µg of the purified olive profilin was used for immunization. One month later, the rabbit was boosted with the same dose of antigen.

Purification of the antigen

The lyophilized saline extract was dissolved in 0.2 M ammonium bicarbonate, pH 8.0, and subjected to gel filtration on a Sephadex G-75 column. The absorbance at 280 nm was measured. The fractions were assessed with the anti-birch-profilin polyclonal antibody in immunoblotting analysis. Fractions containing positive bands in the range of 14–16 kDa were pooled, loaded in a poly-(L-proline) column (21), and eluted with TBS-ATP containing 6 M urea. The eluted material was dialyzed against distilled water at 4°C overnight. This material was lyophilized and dissolved in 20 mM sodium phosphate, containing 20 mM NaCl, pH 7.6, and loaded onto a Superdex-75, HR 10/30 gel filtration column. The elution was done on an FPLC system (Pharmacia). Absorbance was measured at 280 nm.

The amino-acid composition of the isolated protein (1–2 nmol) was determined by acid hydrolysis and amino-acid analysis as described (13). The protein concentration of the purified antigen was determined by amino-acid analysis. NH₂-terminal Edman degradations of native protein were performed on an Applied Biosystems model 477A sequencer (Applied Biosystems), as previously described (13).

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed according to Laemmli (22) in 15% polyacrylamide gels. Profilin samples were reduced by using β-mercaptoethanol 5% (v/v) and heating at 80°C for 20 min. Protein was stained with silver, as previously described (23). Molecular weight determination was done according to Weber & Osborn (24), by using protein markers provided by Sigma. In immunoblotting assays, we used prestained molecular weight markers provided by Bio-Rad, and their molecular masses are indicated in Fig. 1.

Circular-dichroism (CD) analysis

The CD spectrum was obtained on a Jasco J-715 dichrograph fitted with a 150-W xenon lamp. The

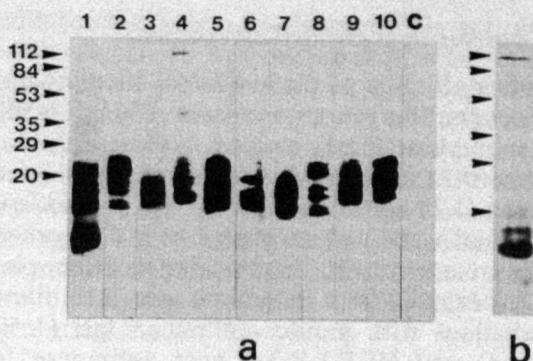


Fig. 1. a) IgE recognition of olive-pollen extract (50 µg) by 10 individual sera from patients allergic to olive pollen (lanes 1–10). Serum of Der p 1-allergic patient (c) was used as control. b) Detection of profilin in crude extract with polyclonal antibody obtained against recombinant birch profilin. Molecular mass markers are indicated by arrowheads.

sample was analyzed in 0.1-cm optical-path cells, in 20 mM sodium phosphate and 20 mM sodium chloride, pH 7.6. The spectra in the far-ultraviolet region were recorded at 50 nm/min scanning speed, and the accumulation of four spectra was achieved. The protein concentration was in the 0.05–0.1 mg/ml range. Mean residue mass ellipticities were calculated on the basis of 111 as the average molecular mass/residue and expressed in terms of Θ (degree $\times$ cm² $\times$ dmol⁻¹). Secondary structure estimations were performed by computer fit by the method of Perczel et al. (25).

Immunoblotting

Protein bands in SDS-PAGE were transferred to nitrocellulose membranes (Bio-Rad) with an LKB NovaBlot 2117 apparatus (Pharmacia LKB), as described by Batanero et al. (26). After transference, the nitrocellulose sheet was saturated in 140 mM sodium chloride and 10 mM sodium phosphate (PBS), pH 7.2, containing 3% nonfat milk and 0.1% Tween 20 (blocking buffer), for 1 h. The membrane was incubated with patient sera 1:10 diluted, for 2 h at 25°C. After washing, the strips were incubated with mouse antihuman IgE (1:5000 diluted) in blocking buffer for 1 h. Blots were washed, incubated with horseradish-peroxidase (HRP)-labeled goat antimouse IgG, washed again, and developed with the ECLTM Western-blotting reagent (Amersham, UK), over Hyperfilm sheets (Amersham).

The immunostainings with polyclonal antibodies raised against recombinant birch profilin (1:20 000 diluted) and against olive-pollen profilin (1:50 000 diluted) were followed by incubation with HRP-labeled goat antirabbit IgG.

IgE binding by individual allergic sera

Relative affinities of the individual allergic sera to the isolated allergen were estimated by IgE binding of solid-phase-fixed antigen, as described by Batanero et al. (26). A volume of 100 μ l of the antigen (1.25 μ g/ml) in PBS, pH 7.2, was added to individual wells and incubated at 4°C overnight. After treatment with the blocking solution, plates were incubated with individual sera 1:10 diluted. Incubations with mouse antihuman IgE (1:5000 diluted) and HRP-labeled goat antimouse IgG were carried out. The peroxidase reaction was developed with fresh enzyme substrate (0.03% H₂O₂, and 0.63 mg/ml of *o*-phenylenediamine in 0.1 M sodium citrate, pH 5.0), and the absorbance was measured at 492 nm.

Results*Evidence of profilin presence in the olive-pollen extract*

Immunoblotting analyses of olive-pollen crude extract were carried out with 191 IgE-specific sera from patients allergic to olive pollen, in order to detect a profilin-like protein (Fig. 1a). Twenty-four percent of the sera (46/191 tested) recognized protein bands of 14–16 kDa, which is the molecular mass of profilin (4, 27). A specific rabbit polyclonal antibody raised against the birch recombinant profilin was also used to check blots with transferred olive-pollen extract. Fig. 1b shows the reaction of the antibody to the 14–16-kDa protein band. These results support the presence of profilin in the olive-pollen extract.

Isolation and purification of the olive profilin

The isolation and purification of the olive profilin was performed in three chromatographic steps. Gel filtration chromatography on a Sephadex G-75 column was carried out to allow the enrichment of the profilin and remove polypeptides of higher molecular mass and pigment components. The pooled fractions that contained the allergen were loaded into a poly-(L-proline) Sepharose 4B affinity column, where profilin was specifically retained. The eluted fraction has an abnormally high content of proline, indicating a coelution besides profilin of poly-L-proline peptides from the matrix. These contaminants were eliminated through additional gel filtration FPLC chromatography. After each purification step, the presence of profilin in the eluted fractions was tested by SDS-PAGE and Western blot analysis, with both a pool of allergic sera and the polyclonal antibody specific for birch

recombinant profilin. The final yield of the purified protein was around 100 μ g/g of dried pollen.

Molecular characterization

Two main close bands of around 15 kDa molecular mass were observed by silver staining of the purified protein after SDS-PAGE. Both bands could bind the antiprolin polyclonal antibody, as well as the IgE from a pool of sera from five subjects allergic to olive pollen (Fig. 2). Olive-pollen profilin was blocked at the NH₂-terminal end, as the attempt to obtain the amino-acid sequence of the allergen by automatic Edman degradation failed to release any phenylthiodantoin amino-acid derivative after five cycles of degradation.

The quantitative amino-acid composition of this allergen was determined after standard acid hydrolysis. The total number of residues given in Table 1 was calculated according to the total content of glutamic acid plus glutamine residues present in the other profilins. In Table 1, the amino-acid composition of the olive profilin is compared with those of the other pollen profilins.

The CD spectrum of the purified protein was determined as a three-dimensional property of its structure (Fig. 3). The results obtained for the regular secondary structure, 15% α -helix, 33% β -strand, 20% β -turn, and 32% random conformation, agree with the molecular data of many members of the profilin family, such as *Acanthamoeba* (28) and human profilins (29).

IgE binding of purified profilin with individual sera

The allergenic reactivity of the purified protein was assayed by analyzing the IgE-binding capacity of the protein by immunoblotting (Fig. 4A) and

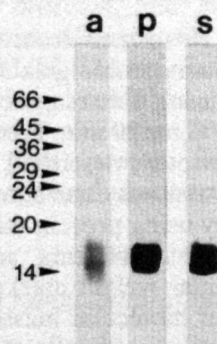


Fig. 2. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified profilin. a) Silver staining; p) immunostaining of protein transferred to nitrocellulose membranes with rabbit polyclonal antiserum raised against birch profilin; s) immunostaining with pool of sera from patients allergic to olive pollen.

Table 1. Amino-acid composition of olive-pollen profilin compared with birch (*Betula*) and maize isoform 1 profilins

Amino acid	Amount in olive profilin (%)	Number of residues in profilin		
		Olive	<i>Betula</i>	Maize I
Cys	1.4 ^a	2	2	2
Asx	9.9	13	9	7
Thr	5.9	8	6	9
Ser	5.6	7	9	5
Glx	12.7	16	16	17
Pro	6.3	9	6	7
Gly	13.2	17	20	18
Ala	8.6	11	8	9
Val	4.8	7	9	7
Met	3.3	5	5	7
Ile	5.5	8	11	10
Leu	6.0	8	9	10
Tyr	3.1	4	4	4
Phe	2.6	3	4	3
His	2.5	3	4	5
Lys	6.2	8	7	7
Arg	2.3	3	2	2
Trp	Not done	Not done	2	2
Total		132	133	131

^a Determined by oxidation with performic acid.

ELISA (Fig. 4B) with 14 individual sera from patients allergic to olive pollen which recognized protein bands of around 15 kDa with different intensities in the saline extract of the olive pollen. As shown in Fig. 4, several sera were positive to a band around 32 kDa, which could correspond to the dimer form of the protein. This band is very often observed in immunoblotting assays with the polyclonal antibody. These data indicated the strong tendency to aggregation of this protein.

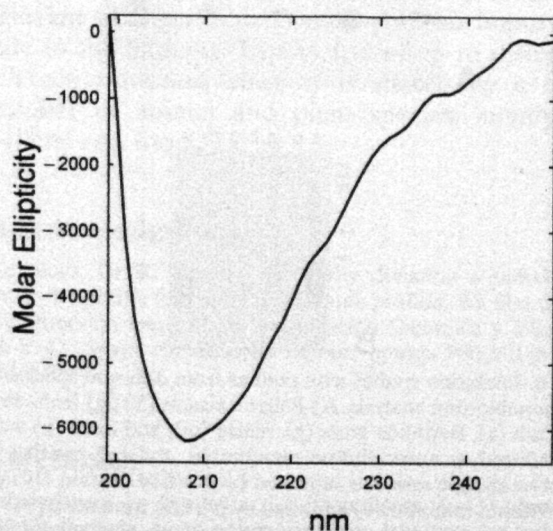


Fig. 3. Circular-dichroism spectrum of olive-pollen profilin in far-ultraviolet region (200–250 nm). Molar ellipticity is expressed as Θ (degrees $\times$ cm² $\times$ dmol⁻¹).

Serum from a patient sensitized to Der p 1, and a nonallergic serum, were included as negative controls. Each of the sera showed IgE reactivity in ELISA analysis, whereas only 11 of them reacted to nitrocellulose-blotted protein. Moreover, several sera exhibited intense response in ELISA, while a very weak or no signal was observed in immunoblotting. These results indicate the existence of conformational IgE epitopes in the olive profilin, since denaturing conditions during SDS-PAGE might affect the protein folding.

Cross-reactivity studies

The presence of this allergen was checked in blots of pollen extracts of olive, ash, birch, Bermuda grass, and maize, by using a specific antibody against olive-pollen profilin. In all cases, a positive band of around 15 kDa was bound by the antiserum (data not shown). Preincubation of the antiserum with the same amounts (600 μ g) of crude extracts inhibited binding to purified profilin to a varying degree (Fig. 5).

Cross-reactivity analysis was carried out in immunoblotting by using a pool of five sera from patients whose IgE recognized this protein. Complete inhibition was observed when amounts of 50 μ g of the above pollen extracts were electrophoresed and transferred to nitrocellulose membranes, and the IgE-binding assay was performed with a previous

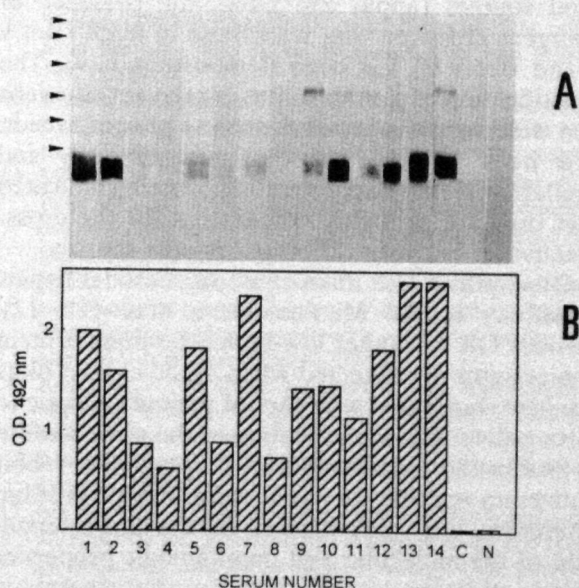


Fig. 4. IgE recognition of purified profilin by individual sera from patients hypersensitive to olive pollen. A) Immunoblotting assay responses for sera of 14 patients (lanes 1–14). B) ELISA responses with same sera are presented. Serum of patient allergic to Der p 1 (C) and serum of nonallergic subject (N), instead of allergic serum, were used as controls. Molecular mass markers are indicated by arrowheads, as in Fig. 1.

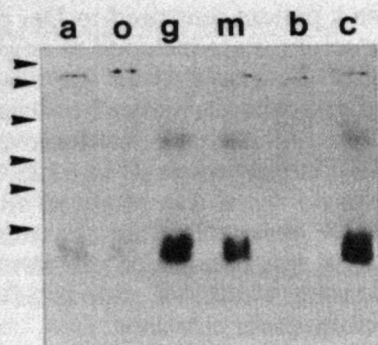


Fig. 5. Effect of preincubation with different extracts on rabbit antiprofilin antiserum immunodetection of purified profilin in immunoblotting analysis. Polyclonal antibody was preincubated with 600 μ g of following extracts: a) ash, o) olive, g) Bermuda grass, m) maize, b) birch, and c) PBS as control.

incubation with 10 μ g of the purified profilin (Fig. 6A). The same results were observed when the profilin (1 μ g) was transferred to the membranes, and 400- μ g amounts of the selected pollens were used as inhibitors (Fig. 6B).

Discussion

Two of the most significant allergic inducers are the inhalant and food allergens. These allergenic sources appear to be unrelated, but, as shown by Ebner et al. (30), they can exhibit cross-reactivities since they share common allergens. For particular food sources (apple and nuts), the presence of allergens cross-reacting with those in birch (Bet v 1 and Bet v 2) has been demonstrated (9). The identification of plant profilins as relevant allergens in a wide variety of sources such as grasses, weeds, and trees (4, 25), and the great ubiquity and similarity of these actin-binding proteins indicated that they are probably responsible for the cross-reactivities between different biologic sources.

Olive pollen is a main cause of seasonal hypersensitivity in the Mediterranean area (11, 12). Besides Ole e 1, other low-molecular-mass protein components are detected when crude extract blots are immunostained with sera of patients allergic to olive pollen. The identification of the olive profilin in the crude extract could be done with rabbit antiserum specific for birch recombinant profilin. Therefore, our study focused on the characterization of the molecular and immunologic properties of this allergen.

Profilin can be purified from a wide variety of animal and plant sources by taking advantage of its affinity for poly-L-proline (31). Like profilins from other sources, olive-pollen profilin binds specifically to this matrix. The yield of the purification (100 μ g/g of dried olive pollen) is higher

than that obtained for celery profilin (325 μ g/kg of crude extract).

Besides the specific binding to the poly-(L-proline) Sepharose, it is possible to find other features characteristic of every member of this family. The absence of the NH₂-terminal sequence has been reported for profilins such as those from *Acanthamoeba* (32) and calf spleen (33). In the silver-staining of the purified protein after SDS-PAGE, two bands appeared, the main one having a molecular weight of 15.6, which is in the range of birch (14.0), Bermuda grass (15.0), and maize (14.0 and 14.8) profilins. The presence of two bands indicates the existence of isoforms, as determined in celery (27) and maize (34), in which three profilin isoforms have been detected, two of them of different length. Like other members of this plant-protein family, olive-pollen profilin is an acidic protein, while in other genera, such as *Acanthamoeba*, profilins exist in both acidic (pI 5.5) and basic (pI 9.2) forms (35) that differ in their ability to bind the polyphosphoinositides.

The reaction of IgE-containing sera to olive-pollen allergens with the purified profilin confirms that this protein is an allergen, for which we propose the designation Ole e 2, according to the rules of the IUIS Allergen Nomenclature Committee (36). Of sera arbitrarily selected from patients allergic to olive pollen, 24% contained specific IgE

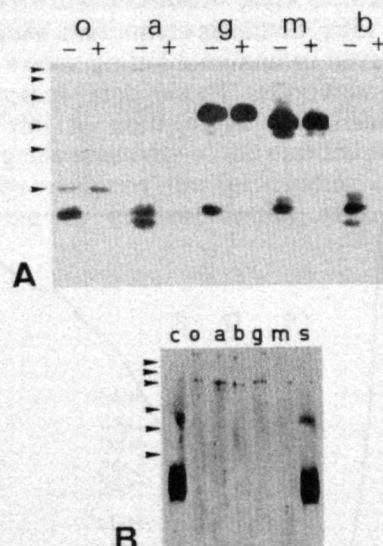


Fig. 6. Inhibition studies with pollens from different species by immunoblotting analysis. A) Pollen extracts (50 μ g) from olive (o), ash (a), Bermuda grass (g), maize (m), and birch (b) were transferred to nitrocellulose membranes, and IgE binding by pool of allergic sera was inhibited by purified profilin (10 μ g). -) Without inhibitor; +) inhibited by profilin from olive pollen. B) IgE binding with same pool of sera to purified profilin transferred to nitrocellulose, as inhibited by 400 μ g of same extracts of pollens used in A. (c) Incubation with PBS and (s) with *Sinapis alba* seed extract as controls.

for olive profilin, as detected after SDS-PAGE of the purified protein and immunoblotting. This frequency was increased when the binding assay was carried out under nondenaturing conditions. Therefore, the IgE of a population of allergic sera should be directed against conformational epitopes of the profilin.

One of the purposes of this study was to determine the cross-reactivities between profilins from diverse plant origins. In fact, there is a significant immunologic similarity among homologous profilins. The specific recognition of the profilin bands in different pollen extracts by a rabbit polyclonal antibody raised against purified olive profilin confirms a narrow structural relationship among these proteins. The inhibition assays using this polyclonal antibody lead to the conclusion that the relative affinity is higher in birch and ash (unrelated and related species, respectively), and lower in Bermuda grass, a fact which may be due to a lower immunologic relationship or to less abundance in the pollen. Allergic cross-reactivity can be observed in inhibition studies using a pool of sera allergic to olive pollen and the purified profilin as inhibitor. The presence of proteins cross-reacting with the purified profilin has been demonstrated for all the pollen sources tested, indicating the existence of shared IgE epitopes. The fact that less cross-reactivity was observed with the rabbit antiserum than with serum IgE could be due to a higher IgG titer against IgE or even a different route of sensitization to profilins from other pollens. These analyses confirm the similar immunologic behavior of pollen profilins, and this should be taken into consideration in the diagnosis of olive allergy, given the high prevalence of hypersensitivity to olive profilin.

Our study confirms that olive-pollen profilin is a relevant allergen which could play an important role in the patients' hypersensitivities to different allergenic sources, since it is shared by a high number of animal and plant species, mainly in pollens and foods.

Acknowledgments

We thank Dr R. Valenta for kindly donating a polyclonal antibody specific for recombinant birch profilin. We also thank the Dirección General de Investigación Científica y Técnica, M. E. C., Spain, for financial support (grants PB92/0195 and PM95/0074).

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