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Identification of a 36-kDa olive-pollen allergen by *in vitro* and *in vivo* studies

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Background: Ole e 1 has been considered the major allergen of olive (*Olea europaea*) pollen. Some other relevant allergens (Ole e 2, 3, 4, and 6) have been recently described. This work aimed to study the IgE-binding frequency of a 36-kDa protein from *O. europaea* pollen in a large population of olive-allergic patients, its allergenic reactivity *in vivo*, and its presence in olive pollens of different origin, as well as in other relevant allergenic pollens.

Methods: Identification of IgE-binding components from *O. europaea* pollen extracts was elucidated by inhibition of SDS-PAGE immunoblotting using recombinant profilin (Ole e 2) and Ole e 1 molecules. The IgE-binding frequency of the 36-kDa protein was estimated by Western blot in a sample of 120 sera from olive-allergic patients. The cutaneous test with the 36-kDa protein was performed by intradermoreaction in allergic patients and control subjects.

Results: Exactly 83% of the sera from *O. europaea*-allergic patients recognized a protein with an apparent molecular weight of 36 kDa, under reducing conditions. It was detected by sera from monosensitized and polysensitized patients, showing a higher IgE frequency than the major allergen Ole e 1 (59%) and the minor profilin (Ole e 2) allergen (27%). Similar reactivity rates (79%) was found by intradermal test. Extracts from olive pollens collected in California presented a much higher amount (around 16-fold on average) of the 36-kDa protein than those from pollens of Spanish origin. The presence of similar allergens was detected only in closely related species (*Syringa*, *Fraxinus*, *Ligustrum*), and not in other common allergenic pollens.

Conclusions: The 36-kDa protein constitutes a major allergen for olive-sensitized patients, but it is not equally represented in *O. europaea* pollens of different origins.

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Abbreviations: BSA: bovine serum albumin; PVDF: polyvinylidene difluoride; SDS–PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS: Tris borate saline buffer.

Allergic sensitization to olive (*Olea europaea*) pollen has a significant incidence in some Mediterranean European countries, such as Italy, Spain, and France, as well as in some regions of North Africa and North America (1). This pollen is a major cause of both rhinoconjunctivitis and asthma in areas where the intensive cultivation of the olive tree causes elevated airborne concentrations of it.

The allergens from *O. europaea* pollens were earlier characterized in the works of Blanca et al. (2) and Vela et al. (3), but, from the beginning, the research was focused on a native protein of 50–65 kDa which showed two bands of 17 and 19 kDa under SDS–PAGE conditions (3–8). This allergen was first named Ole e 1 by Villalba et al. (9); since then, with the advent of new technologies, analysis of its structure has been completed. Thus, its amino-acid sequence was established by microsequencing (10); later, its cDNA sequence was analyzed (11), and it was finally cloned and successfully expressed (12). The glycosylation of one of its constitutive monomers has also been recently investigated (13), and even its immunodominant T-cell epitopes have been established (14).

The presence of other allergens unrelated to Ole e 1 in olive pollen has more recently been studied by different groups. Thus, Batanero et al. (15) described the allergen Ole e 3 as a protein consisting of a single polypeptide chain of 9.2 kDa. Later, Ole e 6 was detected and determined by the same group to be another low-molecular-mass allergen of 5830 Da (calculated from the deduced amino-acid sequence) (16). The plant panallergen profilin has been also found in *O. europaea* pollen, and three full-length cDNAs encoding this allergen (Ole e 2) were isolated and sequenced, and the recombinant proteins were expressed in *E. coli*, as reported in a previous work by our group (17). Some molecular properties of the native profilin, such as its molecular mass of 15 kDa, have been studied by Ledesma et al. (18).

Finally, two new allergens, Ole e 4 and Ole e 5, have been purified and characterized, and their IgE-binding frequency evaluated with a sample of 20 sera from Spanish olive-allergic patients (19). Ole e 5, with an apparent molecular mass of 16 kDa, showed an IgE-binding frequency by immunoblot of 35%, while Ole e 4, of 32 kDa, showed 80%, by far higher than the Ole e 1 frequency. The authors used olive pollen collected in California (USA) and suggested

the existence of variability in allergen content with respect to the collection area of the raw material.

The aims of the present work were to determine the IgE-binding frequency of a detected 36-kDa protein in a large population of allergic patients polysensitized and mono-sensitized to *O. europaea* by immunoblot analysis after precise elucidation of the IgE-binding pattern obtained with *O. europaea* pollen extract; to evaluate the *in vivo* correlation of these findings by cutaneous tests with the isolated allergen; and to assay the presence of this protein in extracts obtained from olive pollens collected in different geographic areas, as well as in extracts from other common allergenic pollens.

Material and methods

Extract production

O. europaea pollen used for the IgE-binding frequency study by immunoblot was purchased from New England Antigenics (Portland, ME, USA), and the collection area was stated to be California. For comparison of the 36-kDa protein content in *O. europaea* extracts, pollens collected from different places were also used: California (Allergon AB, Engelholm, Sweden), Murcia (Spain) (Juan Antonio Jimenez Allergen Pollens, Madrid), Tarragona (Spain) (Biopol, Barcelona), Córdoba (Spain) (University of Córdoba, Department of Plant Biology and Ecology), and Seville (Spain) (kindly provided by Dra. Pilar Conde, Seville). In all cases, after checking adequately their purity (more than 98%), pollen grains were defatted with diethyl ether and afterward extracted by magnetic stirring (24 h at 4°C) in 0.1 mol/l of phosphate buffer, pH 8.0, at 15% w/v. Extracts were clarified by centrifugation at 5600g for 30 min, filtered through a 0.45-μm pore diameter membrane, and dialyzed by ultracentrifugation in a Pellicon System (Millipore Corp., Milford, MA, USA) with a 5-kDa cutoff. The dialyzed extracts were sterilized by filtration through a 0.22-μm pore diameter membrane and freeze-dried.

Patients

Fourteen patients showing clinical manifestation of rhinitis and/or asthma and being diagnosed with allergy to olive pollen by anamnesis, cutaneous provocation, specific IgE (CAP, Pharmacia Upjohn, Sweden), and histamine-release tests were chosen for cutaneous testing. Five of them were monosensitized to *O. europaea* pollen and nine polysensi-

tized to other pollen allergens. Eleven control nonatopic subjects were also tested to ascertain the threshold of specific responses.

Human sera

Human sera were collected from 120 patients selected by having a clinical history consistent with hypersensitivity to olive pollens, positive cutaneous test, and specific IgE equal to or higher than class 3 score (≥ 3.5 kU/l). Out of the 120 sera, six corresponded to monosensitized patients, 77 to patients having IgE also to grass pollen, and 37 to patients polysensitized to other nongrass pollens. Sera from nonatopic subjects were used to ascertain the specificity of *in vitro* experiments.

SDS-PAGE immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (20). Polyacrylamide concentrations of 12.5% and 3% were used for separating and stacking gels, respectively. Proteins dissolved in 0.125 M HCl-Tris buffer, pH 6.8, were dissociated with 0.1% SDS and 5% (v/v) β -mercaptoethanol by treatment at 100°C for 5 min. An amount of 20 μ g of protein, estimated by Bradford's method (21), was applied per well. After electrophoresis, gels were stained by diffusion in 0.1% Coomassie brilliant blue R-250 dissolved in methanol/acetic acid/distilled water (4:1:5). Destaining was also performed by diffusion in the same mixture without dye. Separated protein bands were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore), essentially by the method of Towbin et al. (22). Immunochemical staining was performed by first blocking the Immobilon membrane with 9% (w/v) defatted milk in 50 mM Tris-buffered saline TBS (1 h at 37°C). After three washes with TBS, the blots were incubated with 2.5 ml of undiluted human serum (16 h at 4°C). The blots were washed three times with TBS and incubated with 2.5 ml of 1:50 diluted rabbit immunoglobulins to human ϵ -chains (Dako, Glostrup, Denmark) conjugated to peroxidase. In the case of IgG Western blot with rabbit polyclonal antiserum anti-36-kDa olive protein (1:3000 dilution), goat immunoglobulins to rabbit IgG (Dako) conjugated to peroxidase were used at 1:1000 dilution. Bound peroxidase was detected by the addition of 3 ml of freshly prepared 0.06% w/v of 4-chloro-1-naphthol solution (Bio-Rad, Richmond, CA, USA) and 0.01% (v/v) H_2O_2 in TBS. After 30 min, the blots were rinsed in distilled

water and air-dried. Antigen-antibody binding was identified by purple-stain deposition (23). Band patterns underwent image analysis in the BIO-IMAGE System (Millipore, Bedford, MA, USA). The system allows high-performance digital scanning of gels and membranes at a resolution of 600 dots per inch, which can be processed by the Whole Band Analyzer program. The relative mobility of a set of protein standards (LMW Calibration Kit, Pharmacia Biotech, Uppsala, Sweden) was plotted logarithmically against their molecular masses, and the resulting curve was used to estimate the molecular mass of the resolved bands in the different samples analyzed. Quantification of bands was expressed in terms of integrated intensity, defined as the volume of a band calculated by summing the volume of each pixel within the boundaries of a band (area times height) and subtracting the background for each pixel.

SDS-PAGE immunoblotting inhibition

For immunoblot-inhibition studies, 1-ml volumes of different pool sera from olive-allergenic patients were incubated overnight at 4°C, with 10 μ g purified recombinant Ole e 1 or profilin. The preadsorbed sera were then used for immunoblot experiments. The cloning, expression, and purification of both recombinant olive allergens were achieved according to experimental techniques published previously (17).

Glycoprotein detection

The possible glycoprotein nature of the 36-kDa protein was assayed by immunodetection, using the DIG Glycan detection kit (Boehringer Mannheim Biochemica, Mannheim, Germany), based on the method of Haselbeck (24), according to the manufacturer's instructions. Briefly, 10 μ g protein was dissolved in 20 μ l of 0.1 M sodium acetate buffer, pH 5.5, and then 10 μ l of 15 mM sodium metaperiodate was added, the mixture being incubated for 20 min at room temperature, protected from light. Excess periodate was destroyed with 10 μ l of 20 mM sodium disulfide. After 5 min at room temperature, 5 μ l of digoxigeninsuccinyl- ϵ -amidocaproic acid hydrazide was added and incubated for 1 h. The resulting sample was subjected to SDS-PAGE and Western blot (as described above). The incorporated digoxigenin was then detected on the PVDF membrane by an enzyme immunoassay. The membrane was incubated with polyclonal sheep anti-digoxigenin-alkaline phosphate (10 μ l in 10 ml of TBS, 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5) and then washed three times with TBS and immersed in Boehringer substrate solution for 60 min.

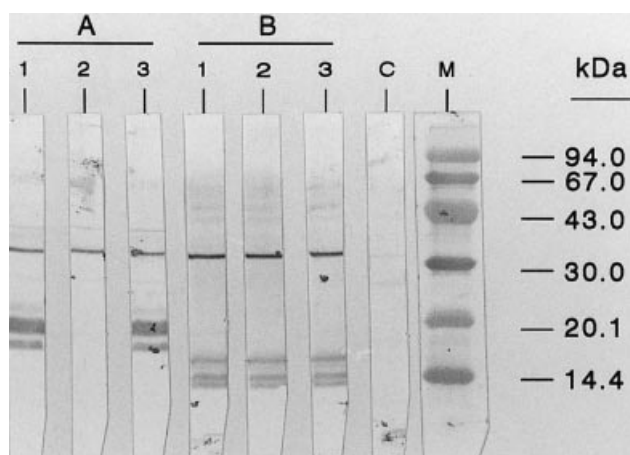


Figure 1. SDS-PAGE immunoblotting of *O. europaea* pollen extracts incubated with pool of sera from olive pollen-allergic patients recognizing Ole e 1 bands (A), and with pool of patient sera recognizing profilin bands (B). Sera pools were preincubated with buffer alone (lane 1), rOle e 1 (lane 2), and BSA (lane 3). C) *O. europaea* pollen extract incubated with control serum pool of nonatopic patients; M) molecular mass markers.

Cutaneous tests

The 36-kDa protein from *O. europaea* pollen extract was isolated by electroelution. After SDS-PAGE, the gel was slightly stained; then, the visualized 36-kDa band was cut with a razor blade, minced, placed in an elution tube, and eluted with a BioRad model 422 electroeluter at 10 mA/tube over 4 h. Afterward, the collected protein was dialyzed and lyophilized. Protein was reconstituted in phenolated saline solution (0.5% phenol) at 10 and 1 µg/ml and sterilized by filtration through a 0.22-µm diameter pore membrane. Cutaneous tests were performed, in duplicate, by intradermoreaction. Nonatopic subjects were also tested as controls. A biologically standardized complete extract of *O. europaea* pollen (10 µg/ml) was applied in parallel to the purified allergen. Wheal areas were recorded after 20 min, transferred to a translucent tape, and later measured by digitalization by means of computer-aided design software. The skin test was considered positive when the wheal diameter induced by the allergen was 3 mm greater than the negative control, thus proving that the wheal area was at least twofold larger than the average value obtained for control subjects at the same concentration.

Polyclonal antiserum

Immunization was performed according to Gallart et al. (25) in white male adult New Zealand rabbits. Weekly injections of 1 mg of electroeluted 36-kDa protein, with Freund's complete

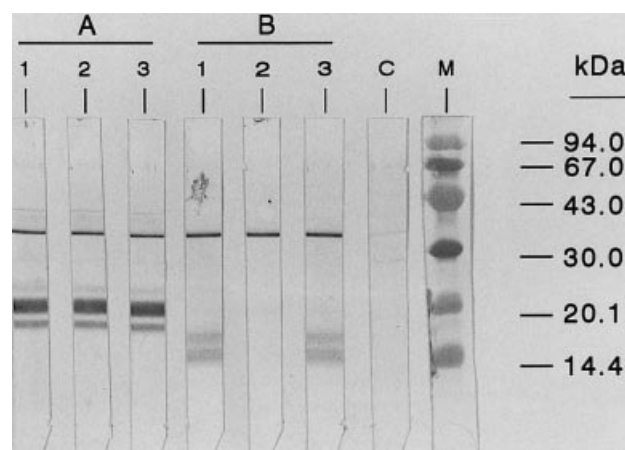


Figure 2. SDS-PAGE immunoblotting of *O. europaea* pollen extracts incubated with pool of sera from olive pollen-allergic patients recognizing Ole e 1 bands (A) and with pool of patient sera recognizing profilin (Ole e 2) bands (B). Sera pools were preincubated with buffer alone (lane 1), rOle e 2 (lane 2), and BSA (lane 3). C) *O. europaea* pollen extract incubated with control serum pool of nonatopic patients; M) molecular mass markers.

adjuvant, were given subcutaneously. After eight injections, the animals were bled, and the serum was collected.

Results

To study the IgE-binding frequency of the 36-kDa protein from *O. europaea* pollen extracts in raw materials of well-established content of this allergen, we did two experiments of SDS-PAGE immunoblotting inhibition, using an extract of pollens collected from the California area and the recombinant(r) olive allergens Ole e 1 and Ole e 2 (profilin). The aim of these assays was to identify precisely the main IgE-binding bands detected in the Western blots of olive extracts. Two pool sera obtained from aliquots of 120 sera from Spanish olive-allergic patients were used for the experiments. One serum pool corresponded to sera recognizing the presumably Ole e 1 bands at 18 and 20 kDa (pool A) and another one to sera recognizing the presumably Ole e 2 bands, at 15.2, 16, 17, and 17.8 kDa (pool B). Fig. 1 shows the IgE-binding band pattern observed before and after preadsorbing both pool sera with rOle e 1. It could be noted that IgE-binding bands at 18 and 20 kDa were completely inhibited, while the four bands at 15.2–17.8 kDa maintained their intensity. The intense IgE-binding band at 36 kDa, as well as other bands showing minor intensities, also remained unaltered.

The results found in the inhibition with rOle e 2 can be seen in Fig. 2. In this case, the only IgE-binding bands to be

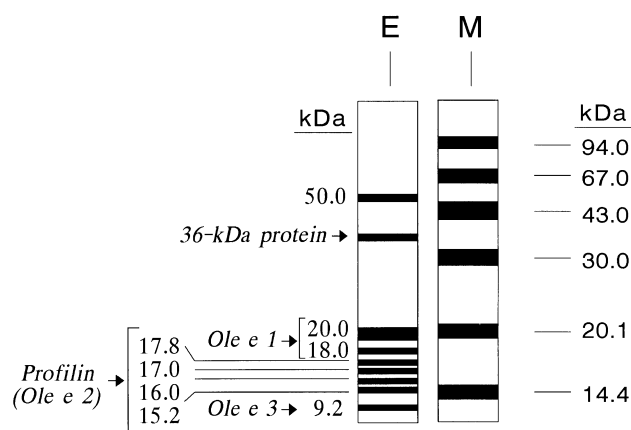


Figure 3. Schematic representation of main IgE-binding bands of *O. europaea* pollen extract (E). M) Molecular mass markers.

abolished were the four at 15.2, 16, 17, and 17.8 kDa, corresponding to profilin, whereas the other detected bands remained unaltered.

From these results and previous bibliographic data, a schematic pattern of the IgE-binding bands encountered in the Western blot analysis of the 120 sera from olive-allergic patients was built (Fig. 3) to evaluate the frequencies of reactivity of each allergen in the studied population. The representative SDS-PAGE immunoblotting shown in Fig. 4 revealed the individual IgE-binding pattern found after analysis of 120 sera from olive pollen-allergic patients. A sharp and often intense IgE-binding band could be detected at 36 kDa (indicated by an arrow), with a frequency found by this *in vitro* technique of 83% (100/120). Within the same population, IgE reactivity to the Ole e 1 doublet band (20 and 18 kDa) was shown to be 59%, while the recognition frequencies of any of the bands at 15.2–17.8 kDa, corresponding to profilin, was 27%. An unidentified band at 50 kDa and another one at 9.2 (probably Ole e 3) were detected in a lower but significant number of patients. Analysis of allergograms in relation to the three groups of sensitized patients revealed that two out of the six sera from monosensitized patients recognized the 36-kDa band. In the group of patients polysensitized to grass pollens, 70 out of 77 sera recognized the 36-kDa band (91%), and in the group of patients polysensitized to nongrass pollen allergens, 23 out of 37 sera detected such a band (62%).

The results of cutaneous tests performed by intradermoreaction with two concentrations of the electroeluted 36-kDa protein and *O. europaea* extract are presented in Table 1, which shows the wheal areas obtained for the 25 tested individuals and the average values calculated for each group of patients (monosensitized and polysensitized patients, as well as control subjects). According to the expressed criteria of positivity, 11 out of the 14 olive-allergic

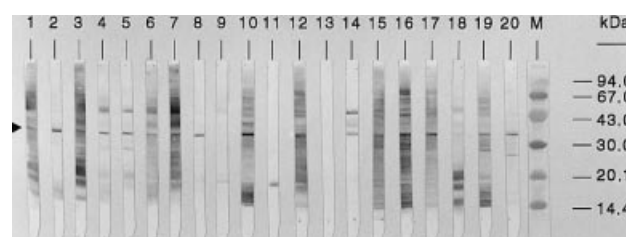


Figure 4. SDS-PAGE immunoblotting of *O. europaea* pollen extracts incubated with representative of 20 sera out of 120 olive-allergic patients involved in study. M) Molecular mass markers. Arrowhead indicates IgE-binding band corresponding to 36-kDa protein.

patients were test positive (79%). The 36-kDa allergen at 10 µg/ml concentration produced a certain degree of false positive response, as evidenced in control subjects. The isolated 36-kDa protein showed glycoprotein nature, as it yielded a positive band when assayed by means of the DIG Glycan kit, evaluated in comparison with negative and positive control proteins.

To study the relative content of the 36-kDa allergen in *O. europaea* pollens of different geographic origin, allergenic extracts produced from 11 different olive-pollen batches were evaluated by Western blot with a serum pool from sera of those allergic patients which recognized mainly the 36-kDa IgE-binding band. As shown in Fig. 5A, extracts in lanes 1, 7, 8, and 9 exhibited the most intense IgE-binding bands at 36 kDa, all of them corresponding to different batches of olive pollen from California. The mentioned band was only slightly detected on extracts blotted in lanes 3, 5, and 6, the first two corresponding to Spanish pollens and the last one to a batch of Californian origin. And finally, the 36-kDa band was negligible in extracts blotted in lanes 2, 4, 10, and 11, all obtained from pollens collected in Spain. The integrated intensity of the 36-kDa band in the PVDF membranes corresponding to the 11 different *O. europaea* extracts was evaluated by image analysis. On average, the intensity of the band from Californian pollens was 16-fold higher than that of the Spanish pollens.

The presence of proteins similar to the 36-kDa allergen in other common allergenic pollens was assayed also by Western blotting, but in this case with a polyclonal antiserum raised against electroeluted protein (Fig. 6). Besides *O. europaea* pollen, the extracts obtained for 10 pollen from different species were evaluated: four from grasses (*Phleum pratense*, *Lolium perenne*, *Cynodon dactylon*, and *Dactylis glomerata*), three from weeds (*Parietaria judaica*, *Artemisia vulgaris*, and *Mercurialis annua*), and three from different species of the Oleaceae family (*Syringa vulgaris*, *Fraxinus excelsior*, and *Ligustrum vulgare*). The

Table 1. Results of intradermal tests performed with *O. europaea* pollen extract and isolated 36-kDa protein

	Wheal area (mm ²)				
Patient no.	Olive extract	36-kDa protein (1 µg/ml)	36-kDa protein (10 µg/ml)	Histamine	36-kDa protein positivity
Polysensitized					
1	36	3	51	53	No
2	276	162	247	275	Yes
3	24	–	31	62	No
4	44	–	18	87	No
5	363	–	366	70	Yes
6	143	257	121	223	Yes
7	128	–	122	91	Yes
8	100	13	182	121	Yes
9	102	25	95	170	Yes
Mean ± SEM	135 ± 38	51 ± 31	137 ± 38	128 ± 26	–
Monosensitized					
10	208	95	47	90	Yes
11	83	40	29	92	Yes
12	44	65	63	155	Yes
13	75	11	81	109	Yes
14	277	7	64	187	Yes
Mean ± SEM	137 ± 45	44 ± 17	57 ± 7	127 ± 19	–
Negative control group					
15	–	–	–	93	No
16	–	–	–	112	No
17	–	–	–	165	No
18	–	–	62	103	No
19	–	–	–	96	No
20	–	–	–	173	No
21	–	–	64	312	No
22	–	–	59	140	No
23	–	–	80	182	No
24	–	–	84	168	No
25	7	–	6	157	No
Mean ± SEM	–	–	32 ± 11	154 ± 19	–

polyclonal antiserum allowed the detection of the 36-kDa IgG-binding band only in extracts from *O. europaea*, *S. vulgaris*, and *F. excelsior*, and at a lower intensity in *L. vulgare*. Some additional bands were detected in the olive-pollen extracts, especially at high molecular mass.

Discussion

The aim of improving the quality of olive-pollen allergenic extracts, intended for use in the diagnosis and treatment of

hypersensitivity to this important aeroallergen in Mediterranean and North American countries, has stimulated the search for new olive allergens, other than Ole e 1, in order to comprehend the array of components that should be contained and controlled in *O. europaea* preparations.

Recently, two new allergens, Ole e 4 (32 kDa) and Ole e 5 (16 kDa), have been purified and characterized from olive pollen collected in California (19). Surprisingly, the IgE-binding prevalence of Ole e 4, assayed by Western blot within the sample of 20 sera from olive-allergic patients, reached 80%, a prevalence by far higher than the 40% of the

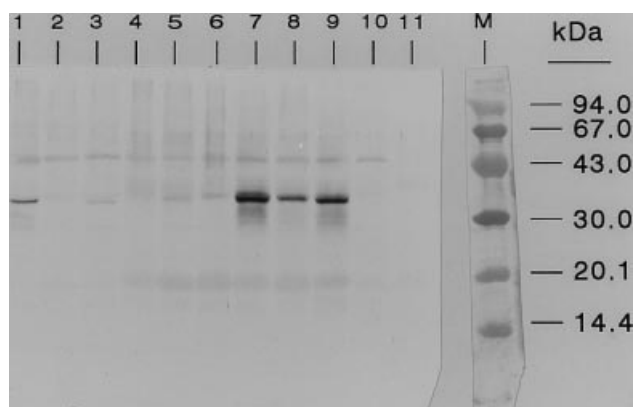


Figure 5. SDS-PAGE immunoblotting of *O. europaea* extracts produced from different batches of olive pollens incubated with serum pool made up of sera mainly reacting to 36-kDa allergen. Lane 1) batch a, origin California (Allergon); 2) Tarragona, Spain (Biopol); 3) Córdoba, Spain (Córdoba University); 4) batch a, Murcia, Spain (J.A. Jimenez); 5) batch b, Murcia, Spain (J.A. Jimenez); 6) batch a California (New England Antigenics); 7) batch b, California (Allergon); 8) batch c, California (Allergon); 9) batch b, California (New England Antigenics); 10) batch c, Murcia, Spain (J.A. Jimenez); 11) Seville, Spain (Dra. P. Conde); M) molecular mass markers.

O. europaea major allergen Ole e 1 (19). In the present study, the presence of a 36-kDa allergen, which could be coincident with the described Ole e 4, was detected in *O. europaea* extracts. The allergenic reactivity of this allergen was evaluated by *in vivo* and *in vitro* techniques, in the latter case with a large collection of sera from olive-allergic patients showing high IgE-binding titers (RAST score of $\geq$ class 3), in order to provide the most clear immunoblotting patterns. For precise identification of the IgE-binding bands obtained in the Western blots of *O. europaea* extracts, two preliminary experiments of SDS-PAGE immunoblotting inhibition were carried out with rOle e 1 and rOle e 2; thus, identification of the main olive allergens giving rise to the most significant IgE-binding bands of olive blots was made possible (Figs. 1–3).

Under this scheme, the IgE-binding frequency of the main olive allergens was estimated from the analysis of the 120 blot patterns produced by incubation with the panel of olive-allergic patients. A frequency of 83% was found for the 36-kDa protein, in excellent agreement with the result of 80% reported by Boluda et al. (19), for Ole e 4, with a smaller group of patients. It seems that the frequency is even higher when evaluating the results of the group of sera from polysensitized patients (92%) and much lower in the group of sera from monosensitized patients (two out of six), albeit this was a rather small group with which to reach a substantial conclusion. No distinction was made between polyspecific and monospecific sera in the Ole e 4 study (19).

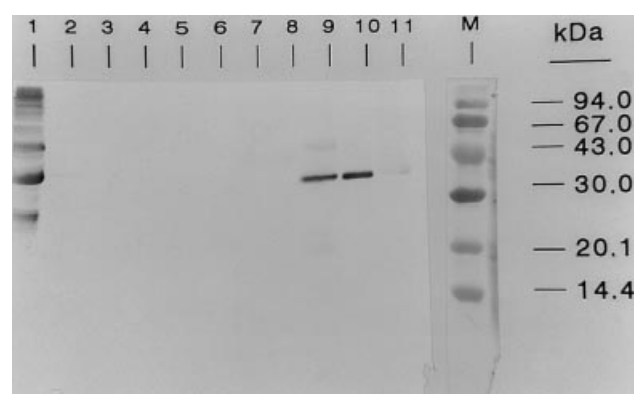


Figure 6. SDS-PAGE immunoblotting of different pollen extracts incubated with rabbit polyclonal antiserum specific to 36-kDa protein. Lane 1) *O. europaea*; 2) *P. judaica*; 3) *P. pratense*; 4) *L. perenne*; 5) *C. dactylon*; 6) *D. glomerata*; 7) *A. vulgaris*; 8) *M. annua*; 9) *S. vulgaris*; 10) *F. excelsior*; 11) *L. vulgare*; M) molecular mass markers.

The assay of allergenic reactivity of the 36-kDa protein *in vivo* by intradermal test provided a good correlation with the *in vitro* results, at least from the overall consideration of all the olive-allergic patients. Thus, the isolated allergen gave rise to positive cutaneous tests in 79% of the patients tested (Table 1). In the two groups of olive pollen-allergic patients, it can be observed that the allergen was positive in the five monosensitized patients tested and in only six out of the nine polysensitized ones, a result which does not match the *in vitro* results and should be investigated further with a larger group of patients. However, these preliminary results suggest that the 36-kDa allergen is specific for olive (or Oleaceae) pollen, as further corroborated by Western blot assays (Fig. 6); therefore, the cutaneous positivities found in the polysensitized group of patients should be due to reactivity to this pollen allergen, and not to cross-reactions among homologous components of the other sensitizing grass and weed pollens. It is also interesting to note that the positivity found in the *in vivo* test with the electroeluted 36-kDa protein indicates that the conformational epitopes of this monomeric protein are maintained after the electrophoretic procedure isolation. A certain degree of skin prick test reactivity was observed in the negative control group at the 10- μ g/ml concentration. It is already known that high concentrations lead to false positive results in intradermal testing, which are usually related to nonspecific irritant factors (26); in this case, it may be attributed to some traces of electrophoretic reagents not completely removed in the dialysis procedures.

The major allergen of *O. europaea* pollen, Ole e 1, was detected with 59% of the tested sera with an extract from olive pollen collected in California, a result which is higher

than the 40% frequency previously reported also using Californian pollen (19), and does not agree either with the 80% frequency reported in the works of Wheeler et al. (4) and Lauzirica et al. (27). These discrepancies reveal not only the great allergenic variability found in *O. europaea* extracts obtained from different sources, as already demonstrated (6, 28), but also the importance of patient selection to carry out immunoprint studies, especially taking into account the monosensitized or polysensitized character of the patients from which sera are collected.

However, the 27% IgE-binding frequency found for the plant panallergen profilin (Ole e 2) in *O. europaea* pollen extract is in agreement with the overall value of 20% quoted by Valenta et al. (29) within pollen-allergic individuals.

The variability in the content of the 36-kDa allergen in pollens of Californian and Spanish origin has been demonstrated by SDS-PAGE immunoblotting (Fig. 5). Six batches from four Spanish pollen suppliers and five batches from two suppliers of pollen of American origin were evaluated by using a pool of sera from patients mainly reacting to the 36-kDa IgE-binding band. Quantification of its presence by image analysis indicated that, although the allergen may be also detected in some of the batches of pollens collected in Spain, the content of the 36-kDa allergen is much higher in pollens of Californian origin, with the exception of one batch (Fig. 5, lane 6). The possibility that these differences could be attributed to the collection procedures or storage conditions of pollens should be ruled out, as these were exactly the same in all cases (vacuum collection, drying at 35°C in a hot-air oven and storage at 2–8°C). On the contrary, the content of Ole e 1 was similar in all the batches studied (data not shown).

The results obtained in the present study strongly suggest that the 36-kDa allergen detected in olive pollen is identical with the reported Ole e 4 (19). This is based on their coincidence in IgE-binding frequency, restricted occurrence in Californian pollens, and similar molecular mass. No data on the possible glycoprotein nature of Ole e 4 were provided in the work of Boluda et al. (19). Whether Ole e 4 and the 36-kDa allergen are the same protein or not will be determined by the cloning and sequencing of this allergen,

a task which is currently underway in our laboratory. Preliminary experiments showed that the electroeluted 36-kDa protein is blocked at its N-terminal, as reported for Ole e 4 (19). The cloning of the 36-kDa allergen and subsequent elucidation of its complete amino-acid sequence will also provide an idea of the physiologic role of this molecule in the pollen and thus shed light on the question of why this protein is differently expressed in plants subjected to different environmental conditions.

From the results shown in Fig. 6, it seems that molecules homologous to the 36-kDa allergen are not present in the grass, weed, and tree pollens evaluated by Western blot using a polyclonal antiserum specific to this protein, in spite of the fact that polysensitized patients also recognized this allergen with similar frequencies. On the contrary, the presence of proteins immunologically similar to the 36-kDa allergen can be detected in species closely related to *O. europaea*, such as those of the Oleaceae family: *S. vulgaris*, *F. excelsior*, and, to a lesser extent, *L. vulgare*. This result is consistent with the already detected IgE-binding band of 36.7 kDa involved in the cross-reactivity between pollen extracts of Oleaceae species (30). Additional IgG-binding bands detected in *O. europaea* extract at molecular masses other than 36 kDa could be due to the presence of protein aggregates, derivatives, or common epitopes present in different proteins.

The main conclusion of the present study is that the 36-kDa protein constitutes a major allergen for olive-sensitized patients, but is not equally represented in *O. europaea* pollens of different origin. The observed intraspecific variability in the allergenicity of *O. europaea* pollens suggests that we should either diversify the production of extracts using pollens from the same geographic areas of the patients to be diagnosed and treated, as suggested by Waisel (31), or use combined plant extracts of different sources.

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