

Ole e 3, an olive-tree allergen, belongs to a widespread family of pollen proteins

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An allergen has been isolated from a saline extract of olive tree (*Olea europaea*) pollen. The protein consists of a single polypeptide chain of 9.2-kDa, as determined by mass spectrometry. It contains neither tryptophan nor tyrosine residues, and displays an acidic isoelectric point. The secondary structure of the protein, estimated from the analysis of the circular-dichroism spectrum in the peptide-bond region, is composed of 52% α -helix, 10% β -strand, 29% β -turn and 9% non-regular conformation. The N-terminal end of the protein is blocked. Amino-acid-sequence data have been obtained from peptides produced by CNBr treatment of the native allergen. A partial sequence of 36 amino acids has thus been elucidated. The protein exhibits sequence similarity with pollen allergens from *Brassica* species and contains a Ca^{2+} -binding motif. The isolated protein displays IgE-binding activity against sera of patients allergic to olive-tree pollen. It has been named Ole e 3, according to the recommendations of the IUIS Nomenclature Committee. IgG ELISA inhibition assays with polyclonal antibodies specific for Ole e 3 reveal the presence of proteins similar to Ole e 3 in the pollen from non-related plant species, which may explain allergic cross-reactivity processes.

Keywords: allergen; olive pollen; *Olea europaea*; Ole e 3; protein characterization.

Type-I allergy is a clinical disorder that affects more than 15% of the human population in developed countries. Olive pollinosis is one of the most important causes of type-I allergy in Mediterranean countries, where the olive tree is widely cultivated [1]. Ole e 1 has been shown to be a major allergen from olive pollen, since the IgE of more than 70% of olive-allergic patients recognize this protein [2–4]. In addition to Ole e 1, other allergenic proteins have been detected in saline extracts of the olive pollen [5–7], some of which exhibit low molecular masses. Preliminary studies on these molecules were performed with pools of sera from patients hypersensitive to olive pollen. Lauzurica et al. [2] showed the existence of an IgE-reacting protein of 8 kDa upon SDS/PAGE of the pollen extract. Proteins of 7, 9, 14, 15 and 16 kDa, present in olive pollen, have been immunostained with individual sera from olive allergic patients [8]. However, isolation or molecular characterization was not performed for these allergens.

Allergic cross-reactivities have been described among *Oleaceae* members, such as olive, ash and privet [9–13] as well as between olive-pollen and grass-pollen proteins [8]. In addition to profilin, which is an important and well defined panallergen involved in cross-reactions [14], other proteins contained in pollen from different species that have conserved sequences could be responsible for allergic processes in individuals not previously sensitized to a given biological source.

In spite of the knowledge of a high number of allergenic structures, little is known about their B-cell and T-cell epitopes and the mechanism of induction of allergy. The protein allergens frequently exhibit molecular masses lower than 40 kDa. Among them, a few allergens with very low molecular mass (< 10 kDa)

have been characterized from different biological sources. The interest in these very small allergens has increased in the last few years since their lower structural complexity would facilitate the study of the structure/allergenicity relationships [15–17]. Therefore, these proteins can be used as simple models to understand the molecular mechanisms involved in allergy.

In this work, we describe the isolation and characterization of a low-molecular-mass allergen from olive pollen with a significant incidence among the allergic individuals, and present in the pollen of different species. A partial amino acid sequence of this allergen is reported.

MATERIALS AND METHODS

Saline-extract preparation. Pollens from different sources were obtained from Allergon AB. The pollen, (5% mass/vol.) was extracted three times for 1 h at room temperature with 50 mM ammonium bicarbonate, pH 8.0, 1 mM phenylmethylsulphonyl fluoride, then centrifuged at 12000×g for 20 min at 4°C. The lyophilized supernatant was stored at –20°C. Yellow-mustard seed extract was prepared as reported [18].

Purification of the allergen. The lyophilized saline extract was dissolved in 0.2 mM ammonium bicarbonate, pH 8.0, and subjected to gel filtration on a Sephadex G-75 column (2.8 cm×93.0 cm) equilibrated in the same buffer (48 ml/h flow rate). IgE-binding activity of the fractions was assessed by SDS/PAGE and immunoblotting analysis with a pool of sera of individuals allergic to olive pollen. The fractions selected were lyophilized and chromatographed on a FPLC Superdex 75 (Pharmacia) column (1 cm×30 cm) equilibrated in 0.1 M ammonium bicarbonate, pH 8.0. The protein was eluted at a flow rate of 1 ml/min, and 0.5-ml fractions were collected. IgE-binding activity of the fractions was determined. In both chromatographies, the absorbance was measured at 280 nm. Allergenic fractions

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Abbreviation. MALDI, Matrix-assisted laser desorption/ionization.

were lyophilized and subjected to reverse-phase HPLC in a nucleosil C₁₈ column. An acetonitrile gradient (from 15% to 55%) in 0.1% trifluoroacetic acid was employed for the elution of the samples. Absorbance was measured at 214 nm and fractions of 0.5 ml were collected. The fractions containing the allergen were lyophilized and stored at -20°C.

Amino acid analysis and protein-concentration determination. Protein samples (1–2 nmol) were hydrolyzed with 5.7 M HCl at 105°C for different lengths of time (24, 48 and 96 h) as described [4]. Hydrolyzed samples were analyzed on a Beckman system 6300 amino acid analyzer. The cysteine content was determined as cysteic acid after performic acid oxidation. Tryptophan was spectrophotometrically measured [19]. The protein concentration of purified samples was determined by amino acid analysis. Total protein determinations of saline extracts were achieved according to Lowry et al. [20].

Sequence analysis. N-terminal Edman degradations of native or alkylated protein (1 nmol) and peptides (400 pmol) were performed on an Applied Biosystems model 477A sequencer (Applied Biosystems). The phenylthiohydantoin-amino-acid derivatives were analyzed by means of a 120 A on-line phenylthiohydantoin-amino-acid analyzer and the standard Applied Biosystems program.

CNBr treatment of native protein (50 µg) was performed in 70% formic acid during 24 h at room temperature, with 500-fold excess of the reagent over methionine in the dark and under nitrogen. Reverse-phase HPLC of the sample was performed on nucleosil C₈ column developed with a gradient from 10% to 45% acetonitrile in 0.1% trifluoroacetic acid. The CNBR treatment, the separation of the peptides and their Edman degradations were performed twice, with different preparations of the protein.

PAGE and IEF. SDS/PAGE was performed according to Laemmli [21] in 15% polyacrylamide gels. Proteins were stained with 0.1% Coomassie blue in 40% methanol, 10% acetic acid. Molecular-mass determinations were done according to Weber and Osborn [22] by means of protein markers provided by Sigma.

IEF was performed on pH 3 to 9 gradient, with a 5% polyacrylamide gel containing Pharmalyte carrier ampholytes (PhastGel IEF 3–9, Pharmacia LKB Biotechnology) for 30 min at 2000 V and 3.5 W in a PhastSystem apparatus (Pharmacia LKB Biotechnology). The isoelectric point (pI)-calibration kit was PhastGel IEF 3–9. Staining was achieved with Coomassie blue reagent.

Mass-spectrometry analysis. Molecular-mass determination of the allergen was performed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Matrix surfaces were made by fast evaporation on the sample probe of 0.3 µl of a saturated solution of sinapinic acid in acetone [23]. Subsequently, 1 µl protein solution was mixed in a 1:2 ratio with aqueous matrix solution [sinapinic acid in 0.1% trifluoroacetic acid/acetonitrile (2:1, by vol.)] and deposited on top of the fast-evaporation matrix film. The protein/matrix solution was dried at ambient temperature and washed with pure water prior to mass analysis. Mass analysis was performed in the linear mode on a Bruker REFLEX time-of-flight mass spectrometer (Bruker-Franzen Analytik). The acceleration voltage was 30 kV. Spectra were acquired as the sum of ion signals generated by irradiation of the protein/matrix deposit by 100–150 pulses from a 337-nm N₂ laser. Mass calibration was accomplished by means of the known masses of matrix-ion signals. Errors in mass accuracy were less than 0.05%.

Circular-dichroism analysis. Circular-dichroism spectrum was obtained on a Jasco J-715 spectropolarimeter fitted with a 150-W xenon lamp and connected to a Weslab RTE-111 ther-

mostabilizer bath. The spectra in the far-ultraviolet region were recorded at 50 nm/min scanning speed and the accumulation of four spectra was achieved. The sample was analyzed in 0.1-cm optical-path cells and in 20 mM ammonium bicarbonate, pH 8.0, at 25°C. The protein concentration was 0.10–0.15 mg/ml. Mean residue mass ellipticities were calculated based on 110 as the average molecular mass/residue, obtained from the amino acid composition, and expressed in terms of $\theta(\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1})$. Secondary-structure estimations were performed by computer fit according to the method of Perczel et al. [24].

Human sera and polyclonal antibody production. Sera from untreated donors were used. All the patients had a well documented clinical history of allergy to olive-tree pollen, with positive skin and radioallergosorbent tests of class 3–6 to *Olea europaea* allergen extract. A pool of six of these sera with radioallergosorbent-test classes of 4–5, which reacted with protein bands of 8–13 kDa of the olive-pollen extract, was prepared. A pool of sera of four non-allergic individuals was used as control.

Polyclonal antiserum against the purified antigen was prepared by immunizing New Zealand rabbits over an 8-week period by multidermal injections of the protein (50 µg) [25].

Immunological assays. Protein bands on SDS/PAGE were transferred onto nitrocellulose membranes (BioRad) as described [13]. Blots were saturated in 140 mM NaCl/10 mM sodium phosphate, pH 7.2 (NaCl/P_i) containing 3% non-fat milk and 0.1% Tween 20 (buffer A), and incubated with the human sera diluted tenfold in buffer A, followed by reaction with mouse anti-human IgE (5000-fold diluted). The blots were incubated with horseradish-peroxidase-labelled goat anti-mouse IgG (diluted 5000-fold in 1% non-fat milk, 0.05% Tween 20). The peroxidase reaction was developed by means of the excited-chemiluminescence Western-blotting reagent (Amersham).

ELISA titration was performed as previously described [26]. Microtiter plates were coated with 100 µl antigen (1 µg/ml) in NaCl/P_i and incubated at 4°C overnight. The wells were washed with NaCl/P_i/0.5% Tween 20, and saturated with NaCl/P_i containing 3% non-fat milk, 0.1% Tween 20. Plates were incubated with human sera (diluted tenfold), then with 100 µl mouse anti-human IgE diluted 5000-fold in buffer A. The plates were incubated with horseradish-peroxidase-labelled goat anti-mouse IgG (diluted 5000-fold). The peroxidase reaction was developed by means of fresh enzyme substrate (0.03% H₂O₂ and 0.63 mg/ml *o*-phenylenediamine in 0.1 M sodium citrate, pH 5.0) and by measuring the absorbance at 492 nm. Alternatively, the wells were coated with the purified antigen and incubated with different dilutions of the rabbit polyclonal antibody obtained against the antigen, then with horseradish-peroxidase-labelled anti-rabbit IgG (BioRad) diluted 3000-fold.

ELISA inhibition assays were performed in triplicate over the antigen-coated and blocked wells prepared as above described. Aliquots of 100 µl antigen-specific rabbit polyclonal antiserum (diluted 200 000-fold in buffer A), previously incubated with extracts of different pollens (500 µg or 50 µg total protein) as inhibitors, were added to the wells. Binding of IgG was detected by horseradish-peroxidase-labelled anti-rabbit IgG antibody diluted 3000-fold in buffer A. The peroxidase reaction was performed as described above.

RESULTS

The allergenic composition of the olive-pollen extract was analyzed by immunoblotting with 270 sera from patients suffering from allergy to olive-tree pollen. 52 of these sera (19.3%) showed IgE-binding to protein bands of 8–13 kDa. Six of these positive sera, of radioallergosorbent-test classes 4–5, were used

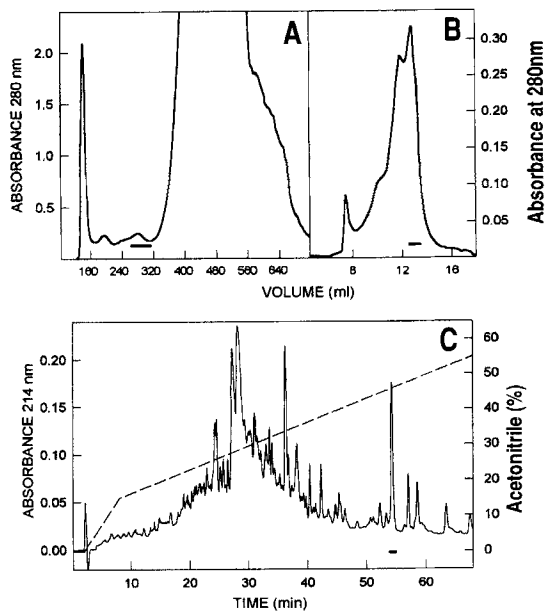


Fig. 1. Isolation of Ole e 3. Elution profiles of the chromatographic steps during the purification procedure. (A) Gel-filtration on Sephadex G-75. (B) FPLC on a Superdex 75 column. (C) HPLC on a nucleosil C₁₈ reverse-phase column. The pooled fractions that contained the allergen are indicated by bars. The dotted line represents the acetonitrile gradient.

to prepare a pool to isolate the most significant allergen of low molecular mass from the olive pollen and to be used for further immunological assays.

Isolation of a low-molecular-mass allergen. An allergen from olive-tree pollen has been isolated by means of three purification steps. The saline extract was chromatographed on a gel-filtration Sephadex G-75 column (Fig. 1a). High-molecular-mass proteins and most of the pigmentary material were separated from the allergenic polypeptides of low molecular mass. A good resolution rendered the complete separation of Ole e 1 from allergens smaller than 18 kDa. The fractions containing allergens of 8–13 kDa were chromatographed on a Superdex 75 FPLC column (Fig. 1b), which reduced the number of polypeptide contaminants of similar molecular mass. An additional reverse-phase HPLC step was carried out to isolate the allergen (Fig. 1c). At each purification step, the allergenic components were detected by SDS/PAGE and Western blot analysis with the pool of allergic sera mentioned above. The SDS/PAGE pattern and immunoblotting of the samples at different steps of the isolation procedure are shown in Fig. 2. The pool of allergic sera recognized Ole e 1 and other protein bands around 8–13 kDa in the olive-pollen extract (Fig. 2b, lane 1), which were removed during the three chromatographic steps.

The isolated protein was judged homogeneous by HPLC with an extended acetonitrile gradient from 30% to 50% in 0.1% trifluoroacetic acid and by mass spectrometry. The purified protein was named Ole e 3, according to the IUIS Allergen Nomenclature Committee [27].

Molecular characterization of Ole e 3. The molecular mass of the allergen, determined by MALDI mass spectrometry was 9.203 kDa (Fig. 3). SDS/PAGE gave a similar result (9.5 kDa). After addition of 20 mM dithiothreitol and incubation at 56°C for 15 min, the mass of the protein obtained by MALDI mass spectrometry did not change, indicating that the protein consists of a single polypeptide chain, which was also deduced by SDS/

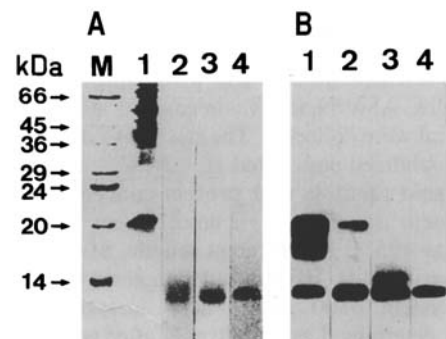


Fig. 2. SDS/PAGE analysis of the purification steps. (A) Coomassie blue staining of the 15% acrylamide gels. (B) IgE-binding analysis with a pool of sera from patients allergic to olive pollen. Lanes 1, crude extract of olive pollen; lanes 2, fractions containing Ole e 3 eluted from the Sephadex G-75 column; lanes 3, Ole e 3 eluted from the Superdex 75 column; lanes 4, Ole e 3 eluted from the HPLC column. M, molecular-mass markers.

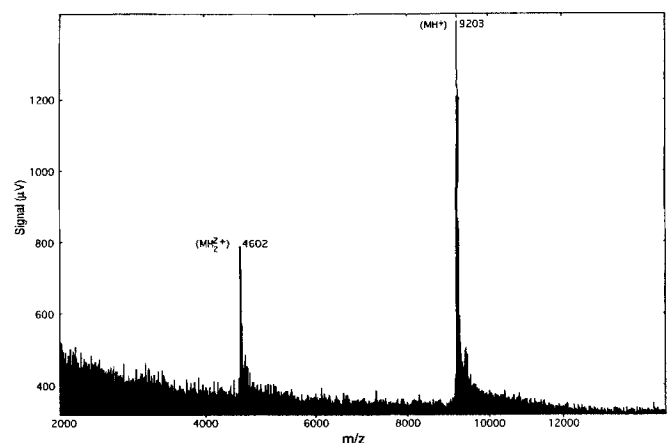


Fig. 3. MALDI-MS analysis of Ole e 3. The mass spectrum exhibits the single charged (MH⁺) and doubly charged (MH₂⁺) protein molecular ions. The minor signals on the high-mass side of the protein signals correspond to protein-matrix adducts.

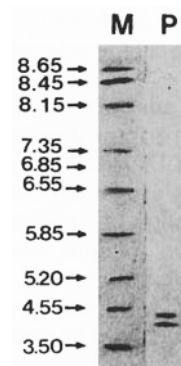


Fig. 4. Analytical IEF of Ole e 3. Coomassie blue staining of a 5% polyacrylamide gel. IEF was performed in a pH gradient from 3 to 9. M, calibration-kit markers; P, Ole e 3 allergen.

PAGE of the allergen after treatment with 5% 2-mercaptoethanol for 20 min at 80°C.

The pI of Ole e 3 was determined by IEF (pI 3–9). Two bands of similar intensity were distinguished at pI 4.2 and pI 4.3 (Fig. 4). Both bands were positive for the binding of IgE from the allergic sera (data not shown). The absence of a hetero-

Table 1. Amino acid composition of Ole e 3. Compositions of Bra r II [28] and BETV4 (accession number S54819) were deduced from the nucleotide sequence of their cDNAs.

Amino acid	Amount in Ole e 3	Number of residues in		
		Ole e 3	Bra r II	BETV4
	%			
Cys	0.2 ^a	0	0	0
Asx	15.7	13	15	13
Thr	4.6	4	3	4
Ser	5.3	5	6	3
Glx	12.0	10	6	9
Pro	2.6	2	0	2
Gly	9.8	8	7	8
Ala	10.1	8	10	9
Val	5.9	5	2	2
Met	3.4	3	4	3
Ile	5.6	5	6	6
Leu	5.3	5	4	5
Tyr	0	0	2	0
Phe	6.9	6	5	7
His	1.5	1	2	2
Lys	6.3	5	8	7
Arg	4.9	4	3	5
Trp	0 ^b	0	0	0
Total	—	84	83	85

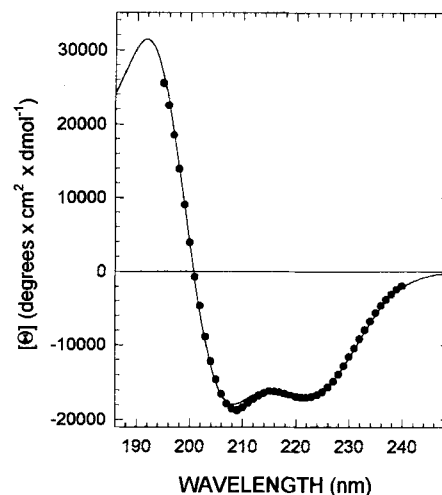
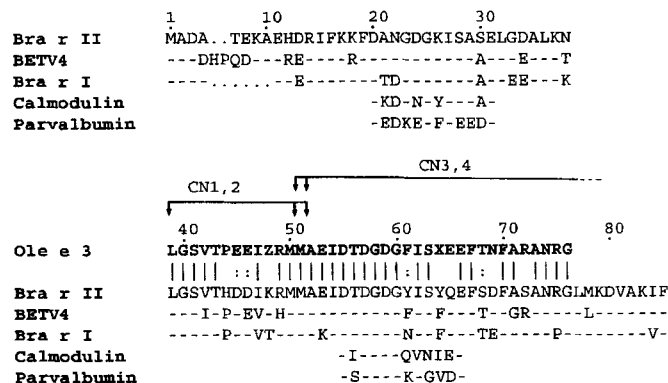
^a Determined by oxidation with performic acid.^b Determined spectrophotometrically.

geneous signal on mass spectrometry suggests that these two bands are charge isoforms of the protein resulting from either partial deamidation or amide/acid residue substitution.

The quantitative amino acid composition of the allergen was determined after acid hydrolysis. The number of residues given in Table 1 was calculated on the basis of the molecular mass obtained by mass spectrometry (9.2 kDa). The protein has a high content of acidic residues, as expected from the pI value, and contains no tyrosine residues. The protein lacks tryptophan residues, which results in an unusual near-ultraviolet absorbance spectrum, in which the typical band around 280 nm is absent.

The circular-dichroism spectrum of the allergen, in the far-ultraviolet region (185–250 nm) showed a high content of periodically ordered structure, mainly α -helix (Fig. 5). Application of the convex-constraint-analysis method of Perczel et al. [24] to this spectrum gave the following secondary structure composition: 52% α -helix, 10% β -strand, 29% β -bend and 9% non-regular conformation.

The analysis of the N-terminal amino acid sequence of the native allergen by automatic Edman degradation was unsuccessful for three preparations of the protein. 1 nmol protein was applied at each sequence run, 50 pmol being the minimum level of detection. The same result was obtained for a sample of the reduced and iodoacetamide-treated protein. This finding could be due to blockage of the N-terminal amino group of the polypeptide chain of the allergen. The nature of this blockage was not investigated. Native Ole e 3 was treated with CNBr and the reaction products chromatographed on a reverse-phase HPLC C₈ column. Six peptides were purified. Attempts to obtain the N-terminal sequence of two of these peptides, which display nearly identical amino acid composition, were unsuccessful. They probably correspond to the N-terminal end of the protein and could contain the amino acid residue involved in the charge heterogeneity. The sequence obtained by automatic Edman degradation of peptide CN-1 was LGSVTPPEIZR (Fig. 6), although its amino acid composition revealed the presence of one

**Fig. 5. Circular-dichroism spectrum of Ole e 3 in the far-ultraviolet region (190–250 nm).** ● indicates the theoretical values for the secondary structure, determined according to the method of Perczel et al. [24].**Fig. 6. Sequence data of Ole e 3 and comparison with those of pollen proteins.** Four CNBr peptides were sequenced: CN1, CN2, CN3, and CN4. Their sequences were aligned with those deduced from cDNAs of the pollen allergens Bra r I and Bra r II from *Brassicaceae* [30] and with that of a putative allergen (BETV4) from birch pollen. Dots represent gaps, and dashes identities with Bra r II. Vertical lines indicate identities between Ole e 3 and Bra r II, and : indicates conservative substitutions. Ca²⁺-binding sites of calmodulin (residues 90–107 and 129–140) and parvalbumin (residues 51–62 and 90–101) were also aligned.

homoserine residue. The amino acid sequence of CN-2 was identical to that of CN-1 but with an extra Met residue (Fig. 6). The sequences obtained for peptides CN-3 and CN-4 were identical except that CN-4 contained an extra Met at the N-terminal position. Since the whole protein contains three Met residues, this partial sequence data allows the prediction of the overlapping of the CN peptides given in Fig. 6.

Antigenic capacity of Ole e 3. In addition to the immunoblotting analysis (Fig. 1), the IgE-binding activity of the purified protein was examined by ELISA titration, with Ole e 3 fixed to wells, by means of the pool of olive-allergic sera (Fig. 7a). Non-allergic sera were used as negative control. ELISA with 52 olive hypersensitive sera, which were positive for bands of 8–13 kDa on Western blot analysis, gave 50 positive responses against the purified allergen (data not shown).

A polyclonal antibody with specificity for Ole e 3 was obtained in rabbit by immunization with the purified protein. An ELISA titration with fixed Ole e 3 of this antiserum is shown in

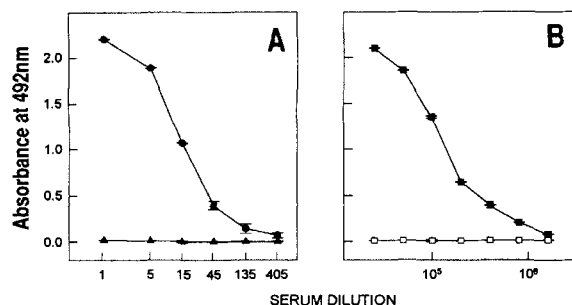


Fig. 7. IgE and IgG titration of Ole e 3. (A) Different dilutions of a pool of six sera from patients allergic to olive pollen (●) were used to assay the IgE-binding to wells coated with Ole e 3. A non-allergic sera pool was used as control (▲). (B) ELISA titration of the binding to wells coated with Ole e 3 by means of a specific rabbit polyclonal antibody raised against Ole e 3 (■). The response of Ole e 1 is also indicated (□).

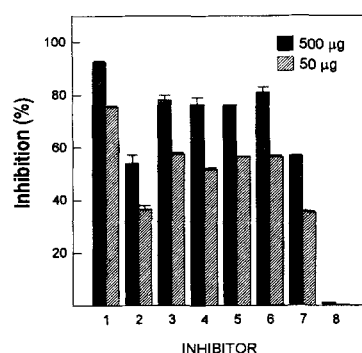


Fig. 8. Occurrence of proteins similar to Ole e 3 in pollen from different species. ELISA inhibition of the binding to wells coated with Ole e 3 was measured by means of a rabbit antiserum specific for Ole e 3 previously incubated with pollen extracts (500 µg or 50 µg total protein, calculated by the method of Lowry et al. [20]) from (1) olive tree, (2) mugwort, (3) birch, (4) ryegrass, (5) rose, (6) pine, (7) cypress. Mustard-seed saline extract (8) was used as control.

Fig. 7b, in which the response against the major allergen of olive pollen, Ole e 1, is also given.

Widespread occurrence of proteins similar to Ole e 3 in pollens. Allergenic pollen from species botanically non-related to the olive tree were tested for the presence of proteins similar to Ole e 3 [*Gramineae*, ryegrass (*Lolium perenne*); *Betulaceae*, birch (*Betula verrucosa*); *Compositae*, mugwort (*Artemisia vulgaris*); *Pinaceae*, pine (*Pinus sylvestris*); and *Cupressaceae*, cypress (*Cupressus arizonica*)]. Rose (*Rosa rugosa*) pollen, from the *Rosaceae* family, was selected as a pollen for which allergic reactions have not been described. In addition, yellow-mustard-seed extract was assayed as a non-pollen plant tissue containing allergenic components [18].

The binding of IgG specific for Ole e 3 to the olive-antigen-coated wells was assayed in the presence of two concentrations of the extracts. The ELISA inhibition data are shown in Fig. 8, in comparison with the activity of olive-pollen extract. All the pollens contain polypeptides that share antigenic determinants with the purified allergen, as they were able to inhibit, by at least 50%, the capability of the antiserum to bind Ole e 3. Rose and cypress extracts were less potent inhibitors than the other pollens. Mustard-seed extract gave a negative reaction with the antiserum.

DISCUSSION

Most of the natural sources of clinically relevant allergens contain several components able to induce type-I allergy. This feature is remarkable for pollen from trees, weeds and grasses. Only one allergenic protein, Ole e 1, has been purified from olive-tree pollen, an important cause of pollinosis in the Mediterranean area. This allergen was characterized, cloned and expressed in *Escherichia coli* [4, 26, 28]. Although several allergenic protein bands have been detected in olive pollen by immunoblotting assays [2, 5–8], the proteins have not been isolated. The availability of a large number of sera from patients allergic to this pollen, and the selection of those with the highest reaction against low-molecular-mass allergens, allowed us to isolate Ole e 3, an olive allergen, against which nearly 20% of patients allergic to olive pollen had specific IgE.

By means of gel-permeation and reverse-phase HPLC, a 9.2-kDa allergen has been purified. Ole e 3 exhibits a high solubility in diluted saline medium, since more than 50% of the protein is released from the pollen during the first 15 min of saline extraction. This solubility is in accordance with the high content of amino acid residues with polar side chains (Table 1). High solubility is a property that has been generally linked to the allergenic condition of pollen proteins [29]. Ole e 3 may correspond to the olive allergen detected by Lauzurica et al. [2], because of the similarity of its reported molecular mass (8 kDa) and pI (4.5). However, the iodination of tyrosines described for that protein [2] is not possible for Ole e 3 since it lacks this type of residue. The possibility of other allergens of 8–13 kDa cannot be discarded, since a small percentage of sera, which are sensitive on immunoblotting to proteins of this molecular mass, did not react with Ole e 3 in ELISA.

The partial sequence of Ole e 3 can be aligned with those deduced from the cDNAs of the *Brassicaceae* pollen allergens Bra r I and Bra r II as well as with that of a putative allergen from birch pollen (BETV4, accession number S54819) (Fig. 6). The compared segments of these proteins exhibit high similarity (about 80% identity). Moreover, the experimental data for the secondary structure of Ole e 3 (65% α -helix) is in agreement with the theoretical values obtained on the basis of the deduced amino acid sequences of Bra r II and BETV4. Therefore, Ole e 3 and the putative proteins deduced from the three cDNA considered could be members of a family of pollen proteins with highly conserved structures. Analysis of proteins similar to Ole e 3 in the pollen of six allergenic and one non-allergenic species, by means of polyclonal antibody specific for Ole e 3, revealed its presence in all assayed extracts. This finding suggested widespread occurrence of this family of proteins in the pollen tissue, which would be responsible for the IgG-binding inhibition by these extracts (Fig. 8). The high level of inhibition by the saline extracts could be due to structural similarity of the members of this family of proteins.

Two Ca^{2+} -binding sites, which were similar to those of Ca^{2+} -binding proteins such as calmodulin, were proposed in the sequence of Bra r I and Bra r II [30]. One of these EF-hand Ca^{2+} -binding motifs is located in the region of Ole e 3 that was sequenced. Moreover, the secondary-structure data of the olive-pollen allergen, which is enriched in α -helix conformation, are in agreement with the helix-loop-helix structures obtained by X-ray and NMR analysis of EF-hand Ca^{2+} -binding proteins [31]. Thus, it can be postulated that the above potential family of pollen proteins may have a function in this tissue that is related to its Ca^{2+} -binding ability. The birch allergen Bet v III, which also contains Ca^{2+} -binding sites [32], is unlikely to belong to this family of pollen proteins because of its molecular mass (26 kDa), the presence of four Ca^{2+} -binding sequences, and the

absence of sequence similarity with Bra r I and Bra r II except for the EF-hand motifs [30].

Ca²⁺ metabolism has a critical role in pollen germination and pollen-tube growth [33]. Thus, intracellular Ca²⁺ levels and their signalling effects must be tightly controlled. Many Ca²⁺-binding proteins are involved in this control [34]. Proteins, such as Ole e 3, that bear two Ca²⁺-binding sites could play an important role in cellular processes that affect the reproductive pollen functions.

If the structural similarity between Ole e 3 and its counterparts in *Brassicaceae* and *Betulaceae* pollens is considered together with the presence of proteins similar to Ole e 3 in other allergenic pollens, this family of proteins could be involved in IgE cross-reactivities. Profilin and Bet v I allergens have been demonstrated to be responsible for cross-reactions among pollens, vegetables and fruits [35, 36]. Oligosaccharides contained in glycoallergens have been reported to have common structures that could contribute to cross-reactivity processes [35, 37]. However, certain cross-reactions have not been explained. The presence of proteins similar to Ole e 3 in pollens could justify the cross-reactivity that has been detected between cod parvalbumin (Gad d 1) and birch-pollen extracts, since peptides corresponding to positions 49–64 and 88–103 of parvalbumin inhibited the IgE-binding of allergic sera to birch-pollen extracts [38, 39]. These peptides, which seem to be involved in the IgE recognition of sera from patients allergic to cod [39], correspond to the two Ca²⁺-binding sites of parvalbumin, and exhibit high identity with the putative Ca²⁺-binding sites of Bra r I, Bra r II and BETV4 (Fig. 6). Therefore, the similarity, in at least several segments of their polypeptide chains, and the widespread distribution of this type of Ca²⁺-binding pollen protein could explain allergic cross-reactions, not only among pollens, but also between non-related biological sources.

A remarkable feature of the isolated allergen Ole e 3 is the absence of significant microheterogeneity. Polymorphism is a common property exhibited by many of the pollen allergens, such as Lol p 2 and Lol p 3, Amb a 1, Cor a 1, Ole e 1 and Bet v 1, and it renders structural and immunological analyses of these proteins extremely difficult. The absence of polymorphism and the small size of Ole e 3 make this protein a good model to analyze its structure/allergenicity relationships and its B-cell and T-cell epitopes.

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REFERENCES

- Bousquet, J., Cour, P., Guerin, B. & Michel, F.-B. (1985) Allergy in the Mediterranean area. I. Pollen counts and pollinosis of Montpellier. *Clin. Allergy* 14, 249–258.
- Lauzurica, P., Maruri, N., Galocha, B., González, J., Díaz, R., Palomino, P., Hernández, D., García, R. & Lahoz, C. (1988) Olive (*Olea europaea*) pollen allergens-II. Isolation and characterization of two major antigens. *Mol. Immunol.* 25, 337–344.
- Wheeler, A. W. (1992) Hypersensitivity to the allergens of the pollen from the olive tree (*Olea europaea*). *Clin. Exp. Allergy* 22, 1052–1057.
- Villalba, M., Batanero, E., López-Otín, C., Sánchez, L. M., Monsalve, R. I., González de la Peña, M. A., Lahoz, C. & Rodríguez, R. (1993) The amino acid sequence of Ole e 1, the major allergen from olive tree (*Olea europaea*) pollen. *Eur. J. Biochem.* 216, 863–869.
- Vela, G., Platas, C., Gurbindo, C., Tricas, L., Subiza, E., García, R. & Lahoz, C. (1982) Fractionation and biological characterization of *Olea europaea* pollen extract. *Int. Arch. Allergy Appl. Immunol.* 68, 289–294.
- Blanca, M., Boulton, P., Brostoff, J. & González-Reguera, I. (1983) Studies of the allergens of *Olea europaea* pollen. *Clin. Allergy* 13, 473–478.
- Rodríguez, R., Villalba, M., Batanero, E., Monsalve, R. I., López-Otín, C., Lahoz, C. & González de la Peña, M. A. (1995) Allergens from olive tree and related species, in *ECAC'95* (Basomba, A. & Sastre, J., eds) pp. 671–677, Monduzzi Editore, Bologna.
- Baldo, B. A., Panzani, R. C., Bass, D. & Zerboni, R. (1992) Olive (*Olea europaea*) and privet (*Ligustrum vulgare*) pollen allergens. Identification and cross-reactivity with grass pollen proteins. *Mol. Immunol.* 29, 1209–1218.
- Bousquet, J., Guerin, B., Hewitt, B., Lim, S. & Michel, F.-B. (1985) Allergy in the Mediterranean area. III. Cross-reactivity among *Oleaceae* pollens. *Clin. Allergy* 15, 439–448.
- Keneman, S. M., McCullough, J., Green, J. & Ownby, D. R. (1992) Evidence of cross-reactivity between olive, ash, privet and Russian olive tree pollen allergens. *Ann. Allergy* 69, 493–496.
- Obispo, T. M., Melero, J. A., Carpizo, J. A., Carreira, J. & Lombardero, M. (1993) The main allergen of *Olea europaea* (Ole e 1) is also present in other species of the oleaceae family. *Clin. Exp. Allergy* 23, 311–316.
- Martín-Orozco, E., Cárdbaba, B., del Pozo, V., de Andrés, B., Villalba, M., Gallardo, S., Rodríguez-García, M. I., Fernández, M. C., Alche, J. D., Rodríguez, R., Palomino, P. & Lahoz, C. (1994) Ole e 1: epitope mapping, cross-reactivity with other *Oleaceae* pollens and ultrastructural localization. *Int. Arch. Allergy Immunol.* 104, 160–170.
- Batanero, E., Villalba, M., López-Otín, C. & Rodríguez, R. (1994) Isolation and characterization of an olive allergen-like protein from lilac pollen. *Eur. J. Biochem.* 221, 187–193.
- Valenta, R., Duchêne, M., Pettenburger, K., Sillaber, C., Valent, P., Bettelheim, P., Breitenbach, M., Rumpold, H., Kraft, D. & Scheiner, O. (1991) Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 253, 557–560.
- Fehlner, P. F., Kochoumian, L. & King, T. P. (1991) Murine IgE and IgG responses to melittin and its analogs. *J. Immunol.* 146, 2664–2670.
- Metzler, W. J., Valentine, K., Roebber, M., Friedrichs, M., Marsh, D. G. & Mueller, L. (1992) Determination of the three dimensional solution structure of ragweed allergen Amb t V by NMR spectroscopy. *Biochemistry* 31, 5117–5127.
- Ghosh, B., Perry, M. P., Rafnar, T. & Marsh, D. G. (1993) Cloning and expression of immunologically active recombinant Amb a V allergen of short ragweed (*Ambrosia artemisiifolia*) pollen. *J. Immunol.* 150, 5391–5399.
- Menéndez-Arias, L., Moneo, I., Domínguez, J. & Rodríguez, R. (1988) Primary structure of the major allergen from yellow mustard (*Sinapis alba* L.) seed, Sin a I. *Eur. J. Biochem.* 177, 159–166.
- Bencze, W. L. & Schmid, K. (1957) Determination of tyrosine and tryptophan in proteins. *Anal. Chem.* 29, 1193–1196.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Weber, K. & Osborn, M. J. (1969) Reliability of molecular weight determinations by dodecylsulphate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244, 4406–4412.
- Vorm, O., Roepstorff, P. & Mann, M. (1994) Improved resolution and very high sensitivity in MALDI TOF of matrix surfaces made by fast evaporation. *Anal. Chem.* 66, 3281–3287.
- Perczel, A., Hollosi, M., Tusnady, G. & Fasman, G. D. (1991) Convex constraint analysis: a natural deconvolution of circular dichroism curves of proteins. *Protein Eng.* 4, 669–679.
- Vaitukaitis, J. L. (1981) Production of antisera with small doses of immunogen: multiple intradermal injections. *Methods Enzymol.* 73, 46–52.
- Batanero, E., Villalba, M. & Rodríguez, R. (1994) Glycosylation site of the major allergen from olive tree pollen. Allergenic implications of the carbohydrate moiety. *Mol. Immunol.* 31, 31–37.

27. King, T. P., Hoffman, D., Löwenstein, H., Marsh, D. G., Platts-Mills, T. A. E. & Thomas, W. (1994) Allergen nomenclature, *Bull. W. H. O.* **72**, 797–803.
28. Villalba, M., Batanero, E., Monsalve, R. I., González de la Peña, M. A., Lahoz, C. & Rodríguez, R. (1994) Cloning and expression of *Ole e 1*, the major allergen from olive tree pollen. Polymorphism analysis and tissue specificity, *J. Biol. Chem.* **269**, 15217–15222.
29. Vrtala, S., Grote, M., Duchêne, M., van Ree, R., Kraft, D., Scheiner, O. & Valenta, R. (1993) Properties of tree and grass pollen allergens: reinvestigation of the linkage between solubility and allergenicity, *Int. Arch. Allergy Immunol.* **102**, 160–169.
30. Toriyama, K., Okada, T., Watanabe, M., Ide, T., Ashida, T., Xu, H. & Singh, M. B. (1995) A cDNA clone encoding an IgE-binding protein from *Brassica* anther has significant sequence similarity to Ca^{2+} -binding proteins, *Plant. Mol. Biol.* **29**, 1157–1165.
31. Strynadaka, N. C. J. & James, M. N. G. (1989) Crystal structures of the helix-loop-helix calcium-binding proteins, *Annu. Rev. Biochem.* **58**, 951–998.
32. Seiberler, S., Scheiner, O., Kraft, D., Lonsdale, D. & Valenta, R. (1994) Characterization of a birch pollen allergen, *Bet v III*, representing a novel class of Ca^{2+} -binding proteins; specific expression in mature pollen and dependence of patients' IgE binding on protein-bound Ca^{2+} , *EMBO J.* **13**, 3481–3486.
33. Hepler, P. K., Millar, D. D., Pierson, E. S. & Challaham, D. A. (1994) Calcium and pollen tube growth, in *Pollen-pistil interactions and pollen tube growth* (Stephenson, A. G. & Kao, T. H., eds) pp. 111–123, American Society of Plant Physiologists Rockville TN.
34. Roberts, D. M. & Harmon, A. C. (1992) Calcium-modulated proteins: targets of intracellular calcium signals in higher plants, *Annu. Rev. Plant. Physiol. Plant Mol. Biol.* **43**, 375–414.
35. Calkhoven, P. G., Aalbers, M., Koshte, V. L., Pos, O., Oei, H. D. & Aalberse, R. C. (1987) Cross-reactivity among birch pollen, vegetables and fruits as detected by IgE antibodies is due to at least three different cross-reactive structures, *Allergy (Cph.)* **42**, 382–390.
36. Ebner, C., Birkner, T., Valenta, R., Rumpold, H., Breitenbach, M., Scheiner, O. & Kraft, D. (1991) Common epitopes of birch pollen and apples. Studies by western and northern blot, *J. Allergy Clin. Immunol.* **88**, 588–594.
37. Batanero, E., Villalba, M., Monsalve, R. I. & Rodríguez, R. (1996) Cross-reactivity between the major allergen from olive pollen and unrelated glycoproteins: evidence of an epitope in the glycan moiety of the allergen, *J. Allergy Clin. Immunol.* **97**, 1264–1271.
38. Elsayed, S., Titlestad, K., Apold, J. & Aas, K. (1980) A synthetic hexadecapeptide derived from allergen M imposing allergenic and antigenic reactivity, *Scan. J. Immunol.* **12**, 171–175.
39. Elsayed, S., Ragwarsson, O., Apold, J., Floorvaag, E. & Vik, H. (1981) Allergenic synthetic peptide corresponding to the second calcium-binding loop of cod allergen M, *Scan. J. Immunol.* **14**, 207–211.