

A Major Allergen from Pollen Defines a Novel Family of Plant Proteins and Shows Intra- and Interspecific Cross-Reactivity¹

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Olive tree (*Olea europaea*) pollen is a main cause of allergy associated with extensive areas of Europe and North America. Ole e 10, a small (10.8 kDa) and acidic (pI 5.8) protein, has been identified as a major allergen from the olive pollen, isolated, and characterized. Circular dichroism analysis gave 17% α helix, 33% β sheet, and 21% β turn for its secondary structure. Based on amino acid sequences of tryptic peptides, the protein was cloned and sequenced. The allergen consists of a single polypeptide chain of 102 aa, with a signal peptide of 21 residues. Ole e 10 showed homology with the C-terminal domain of another olive allergen, Ole e 9 (1,3- β -glucanase, 53% identity), with deduced sequences from *Arabidopsis thaliana* genes (42–46% identity) and with polypeptide segments (Cys boxes) of proteins involved in yeast development (Epd1/Gas-1p/Phr2 families; 42–43% similarity). Ole e 10 showed 55% prevalence for olive-allergic patients and exhibited an IgE response dependent on its conformation. Remarkable IgE cross-reactivity was detected with Ole e 9, but no correlation was observed between the individual IgE responses to both allergens. Ole e 10 shares IgE B cell epitopes with proteins from *Oleaceae*, *Gramineae*, *Betulaceae*, *Chenopodiaceae*, *Cupressaceae*, *Ambrosia*, and *Parietaria* pollens, latex, and vegetable foods, such as tomato, kiwi, potato, and peach. These data indicate that Ole e 10 is a new pan-allergenic plant protein that shows notable intra- and interspecific IgE cross-reactivity and is a powerful candidate to be involved in pollen-latex-fruit syndrome. *The Journal of Immunology*, 2004, 172: 3644–3651.

Type-I allergy is an immunological disorder that appears as a response to basically innocuous Ags, so-called allergens, and is mediated by the production of IgE Abs against such allergenic molecules (1). Patients affected by IgE-mediated allergies suffer from rhinitis, conjunctivitis, hay fever, asthma, dermatitis, and even anaphylactic shock. These symptoms cause health damage and great economic loss by absenteeism from work. A detailed characterization of the molecular nature of allergens would allow improvement of the degree of allergen standardization and thus help to obtain more safe and effective protocols for diagnosis and therapy of the allergic diseases.

Pollen from grasses, weeds, and trees constitutes one of the main sources of inhalant allergens frequently associated with geographical and seasonal patterns of allergic diseases. Pollen from the olive tree (*Olea europaea*) is a major cause of inhalant type I allergy in countries of the Mediterranean coast (2, 3) and is an important allergen in the western U.S., South America, South Africa, and areas of Australia where this tree is also extensively cultivated (4). The olive tree belongs to the *Oleaceae* family, which comprises other members present in temperate areas of Europe and North America, such as ash, lilac, and privet, enlarging the territory of influence of *Oleaceae* pollinosis (5). Indeed, a high degree

of structural and immunological similarity has been observed among allergens from different *Oleaceae* pollens (2, 6–8).

Olive pollen extracts contain a high number of allergenic components, from which up to nine (Ole e 1 to Ole e 9) have been characterized (9, 10). Regarding their prevalence, Ole e 1 is the main allergen from olive pollen, affecting >70% of the hypersensitive population (11, 12). However, other proteins, such as Ole e 9, are also major allergens, because they exhibit an incidence of >50% among the olive-allergic population (10). The reported olive allergens display molecular masses between 5.5 and 46 kDa, which are within the range of 5–50 kDa proposed for proteins with allergenic capability. Three allergens of low molecular mass (<11 kDa) have been isolated and characterized from this pollen: Ole e 3 (9.2 kDa), Ole e 6 (5.5 kDa), and Ole e 7 (9.9–10.3 kDa). However, when olive pollen IgE-reactive proteins were resolved by two-dimensional electrophoretic techniques, isoelectrofocusing (IEF)³ followed by SDS-PAGE, a new and significant allergenic component appeared at ~10 kDa (13).

Allergens from unrelated sources that share structural similarities are frequently involved in cross-reactivity processes (14). Thus, several families of plant pollen proteins have been reported to show high IgE cross-reactivity: 1) profilins, a family of ubiquitous cytoskeletal proteins of eukaryote organisms with conserved three-dimensional structure (15, 16); 2) Bet v 1-like allergens, a putative pathogenesis-related family of proteins found in trees of the *Fagales* order, such as birch, alder, and hazel (17), and in plant derived-food as fruits, vegetables, and spices (18); and 3) calcium-binding proteins of two EF-hand motifs that are specifically expressed in pollen tissue (polcalcins) (19). Immunological cross-reactivity may explain why patients who were sensitized against a concrete allergenic source exhibit clinical symptoms after contact with materials of unrelated origin. Definition of structural

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³ Abbreviations used in this paper: IEF, isoelectrofocusing; CD, circular dichroism; MS, mass spectrometry; TFA, trifluoroacetic acid.

similarities related to the immunological cross-reactivity among allergens may substantially help to develop simplified protocols in which a limited number of highly pure molecules is required for component-based diagnostics and immunotherapy (14, 20).

In the present study we report the detection, purification, and molecular characterization of a novel allergen from olive pollen, Ole e 10, with high clinical prevalence. This allergen constitutes the first member of a new family of plant proteins. A cDNA encoding this protein was cloned and sequenced. IgE binding dependence on the native conformation was determined using individual sera from allergic patients. Studies on intra- and interspecies cross-reactivity were performed.

Materials and Methods

Biological materials and protein extracts

Pollen from olive (*Olea europaea*), ash (*Fraxinus excelsior*), privet (*Ligustrum vulgaris*), lilac (*Syringa vulgaris*), mugwort (*Artemisia vulgaris*), chenopod (*Chenopodium album*), birch (*Betula verrucosa*), ryegrass (*Lolium perenne*), Bermuda grass (*Cynodon dactylon*), Timothy grass (*Phleum pratense*), pellitory (*Parietaria judaica*), cypress (*Cupressus sempervirens*), and ragweed (*Ambrosia trifida*) were purchased from Allergon-Pharmacia (Ängelholm, Sweden). Pollens (2%, w/v), except olive pollen, were extracted as previously described (21).

Protein extracts from kiwi (*Actinidia chinensis*), tomato (*Lycopersicon esculentum*), and potato (*Solanum tuberosum* L.) were obtained as previously described (22). Latex (*Hevea brasiliensis*) and peach (*Prunus persica*), peel and pulp, extracts were provided by Drs. G. Salcedo (Universidad Politécnica, Madrid, Spain) and J. F. Crespo (Hospital 12 de Octubre, Madrid, Spain), respectively. Extracts were tested by SDS-PAGE and Coomassie blue staining, and their total protein content was determined (23). Ole e 9 allergen was isolated as previously described (10).

Isolation of olive pollen allergen

Olive pollen was homogenized (1.2%, w/v) and extracted for 15 min in 50 mM ammonium bicarbonate, pH 8.0, containing 1 mM PMSF. After centrifugation at $12,000 \times g$ for 20 min at 4°C, the supernatant was discarded, and the pellet was extracted with the same buffer (2%, w/v) three times for 1 h at room temperature. Supernatants were collected, lyophilized, dissolved in 0.2 M ammonium bicarbonate (pH 8.0), and fractionated on a size exclusion Sephadex G-75 (Pharmacia Biotech, Uppsala, Sweden) column. Fractions containing proteins in the range of 5–12 kDa were pooled, lyophilized, and chromatographed on a Sephadex G-50 superfine (Pharmacia Biotech) column equilibrated in 0.2 M ammonium bicarbonate, pH 8.0. A reverse phase HPLC (Nucleosil C₁₈ column) was used for the final purification. The elution was performed with an acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid (TFA).

Electrophoresis and carbohydrate detection

SDS-PAGE was performed in 15% (w/v) polyacrylamide gels (24) and alternatively using 2-ME reducing treatment of the sample. Proteins were stained with 0.1% (w/v) Coomassie brilliant blue R-250 or were blotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Arlington Heights, IL). Carbohydrate detection of blotted proteins was performed using biotinylated Con A lectin (Pierce, Rockford, IL) and staining by HRP reaction (12). Apparent molecular mass determinations were made using protein markers (MW-SDS-70L; Sigma-Aldrich, St. Louis, MO).

Amino acid analysis, protein concentration, and pI determination

Protein samples were hydrolyzed with 5.7 M HCl and analyzed on a System 6300 amino acid analyzer (Beckman Coulter, Fullerton, CA). Cysteine content was determined as cysteic acid after performic acid oxidation (12). The protein concentration of purified samples was determined by amino acid analysis. The pI of the protein was determined using the Protean IEF system from Bio-Rad (Richmond, CA), with strip gels (4% T and 3% C; Bio-Rad) containing immobilized pH gradients from 3–10 and 3–6, according to the manufacturer's protocol. Standard proteins (IEF standards; pI range, 4.54–9.6) were used to calibrate the strips.

Mass spectrometry (MS) analysis and circular dichroism measurements

Samples (1 µg) were analyzed in a Reflex II MALDI-TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) as previously described (10). The equipment was externally calibrated employing singly, doubly, and triply charged signals from cytochrome c (12,360 Da).

Circular dichroism (CD) spectra were obtained on a Jasco J-715 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) fitted with a 150-W xenon lamp and connected to a Weslab RTE-111 thermostabilizer bath. Far UV spectra were registered in the range of 190–250 nm, and near UV spectra were registered at 250–330 nm. Samples were analyzed in PBS, pH 7.2, at 25°C, and the protein concentration was 0.55 mg/ml. Mean residue mass ellipticities were calculated based on 106 as the average molecular mass/residue, obtained from the amino acid composition, and expressed in terms of θ (degrees per square centimeter per decimoles). Secondary structure estimations were performed according to the method of Perczel et al. (25).

Proteolytic treatment and amino acid sequence analysis

Protein samples were reduced with DTT and alkylated with iodoacetamide (12). The carboxyamidomethylated protein (0.2–0.5 nmol) was then digested with sequence grade trypsin or chymotrypsin (12). An enzyme: substrate ratio of 1:100 (w/w) was used at 37°C for 2 h. Soluble peptides were fractionated by RP-HPLC on a Nucleosil C₁₈ column with an acetonitrile gradient in 0.1% TFA. N-terminal Edman degradation of peptides were performed on a 494 sequencer (PE Applied Biosystems, Foster City, CA).

PCR-based cloning strategy of Ole e 10 cDNA

The T2 peptide sequence obtained by Edman degradation after tryptic digestion was used to design an antisense degenerate oligonucleotide, OL10B (5'-YTGRTACCANGARTTCATNGC-3'), which was included as primer in the cloning procedure. The SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA) allowed the synthesis of a double-stranded cDNA from total RNA from olive pollen using the 5'-RACE cDNA synthesis primer included in the kit. This cDNA was used as template in a PCR amplification with OL10B and a nonspecific primer UPM (5'-CTAATAC-GACTCACTATAGGCAAG CAGTGGTAACAACGACAGAGT-3'), and a fragment of 330 bp was obtained, purified, and cloned using the TOPO TA cloning kit (Invitrogen, Groningen, The Netherlands). Based on the sequence of this fragment, a nondegenerate sense oligonucleotide, OL10F (5'-ATGCGAGGAACCGCAGGTGTG-3'), corresponding to the first seven amino acid residues of the open reading frame of the protein, was synthesized and used to determine, together with the UPM primer, the full-length of the nucleotide sequence of Ole e 10. Nucleotide sequencing was conducted according to the method described by Sanger et al. (26) adapted for PCR. Homology searches were performed using the BLAST program, and multiple amino acid sequence alignments were performed using the CLUSTAL W program.

Patients and immunological assays

This study included 150 patients, all of them living in Jaén (southern Spain), who fulfilled the following criteria (27): 1) seasonal rhinitis and/or bronchial asthma from late April to June, 2) a positive skin prick test to *O. europaea* pollen extract (ALK-Abelló, Madrid, Spain) and specific IgE values >0.7 kU/l measured by Pharmacia Biotech, and 3) no previous *O. europaea* immunotherapy. The patients were also tested against other aeroallergens, including allergenic pollens such as those from ryegrass, cypress, mugwort, chenopod, and *P. judaica*. Thirteen patients (8.7%) showed a positive response only to olive pollen.

IgE-reactive proteins transferred to nitrocellulose membranes were immunostained as previously described (28). Briefly, membranes were incubated with allergic sera (diluted 1/10), followed by reaction with mouse anti-human IgE (diluted 1/5000; donated by Dr. M. Lombardero, ALK-Abelló, Madrid, Spain) and then with HRP-labeled goat anti-mouse IgG (Pierce; diluted 1/5000). The peroxidase reaction was developed using the ECL Western blotting reagent (Amersham Pharmacia Biotech) as previously described (28).

IgE quantification was performed by indirect ELISA (29) in microtiter 96-well plates (Costar, Cambridge, MA) coated with 100 µl of purified protein (1 µg/ml in PBS). Plates were incubated with individual allergic sera (diluted 1/10) for 2 h. Binding of IgE was detected by mouse anti-human IgE Abs (diluted 1/5000), followed by HRP-labeled goat anti-mouse IgG (diluted 1/5000) as described above. Peroxidase reaction was developed with *o*-phenylenediamine reagent (29) and the OD was read at 492 nm. All determinations were conducted in duplicate. An OD under 0.1

was considered a negative response. Negative controls were performed using a pool of sera from nonallergic patients or coating wells with mustard allergen Sin a 1.

For ELISA inhibition experiments, a pool of allergic sera (diluted 1/10), previously incubated with either saline extracts (500 μ g of total protein) or different concentrations of pure proteins as inhibitors, was added to the allergen-coated wells. Bound IgE was detected as described above.

Results

Isolation, structural characterization, and partial amino acid sequencing of Ole e 10

A new protein has been purified from olive tree pollen after three chromatographic steps. The saline extract obtained from the pollen after discarding the protein released at 15 min was applied onto a gel filtration Sephadex G-75 column (Fig. 1A). Fractions containing IgE-binding proteins of low molecular mass (5–12 kDa) were pooled, lyophilized, and chromatographed in a Sephadex G-50 superfine column (Fig. 1B). An additional reverse phase HPLC step (Fig. 1C) rendered the allergen with a homogeneity degree >95% and a yield of ~ 80 μ g/g of dried pollen. Aliquots taken from different steps of the purification procedure were analyzed by SDS-PAGE (Fig. 1D). The purified protein exhibited an electrophoretic pattern with two bands of 10.4 and 11.6 kDa, which represent 90 and 10%, respectively, of the total protein, determined by densitometric analysis of the stained gel. Immunoblotting assays were performed with a pool of sera from patients allergic to olive tree pollen, showing that both protein bands, 10.4 and 11.6 kDa, were recognized by the IgE Abs (Fig. 1D). They gave relative IgE responses of 52 and 48%, respectively, as determined by densitometric scanning of the ECL film.

Mass spectrometry analysis of the purified protein rendered two peaks of 5,395 and 10,789 Da, corresponding, respectively, to MH_2^{+2} and MH^+ species of the allergen (Fig. 2A). The protein was subjected to reductive treatment with 2-ME to determine how its electrophoretic mobility and IgE recognition were affected by this disulphide-disrupting reagent. SDS-PAGE of the reduced protein showed a single band of 14.0 kDa molecular mass, which was

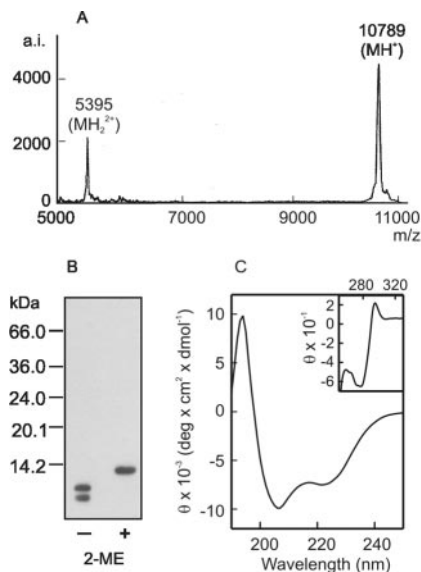


FIGURE 2. Molecular characterization of Ole e 10. A, MS analysis of purified Ole e 10. The mass/charge ratio (m/z) is shown. The intensity of the signals is shown as arbitrary intensity (a.i.). B, Analysis of the electrophoretic behavior and IgE-binding capability of Ole e 10 subjected to reductive treatment. Purified protein samples in the absence (lane $-$) and the presence (lane $+$) of 2-ME were subjected to SDS-PAGE, transferred to membranes, and immunostained with sera from olive-allergic patients. C, CD studies of Ole e 10 were performed in PBS at 25°C using 0.55 mg/ml protein for both far and near UV spectra.

recognized in immunoblotting by the pool of allergic sera (Fig. 2B). The change in electrophoretic mobility of both protein bands to a higher and single molecular mass value in response to 2-ME indicates a defolding of the tertiary structure as a consequence of the rupture of potential cystine bridges. IEF analysis of the allergen also gave a single band with a pI of 5.8 (data not shown). Binding assay of Con A lectin to the purified protein after SDS-PAGE separation and transference to membranes gave a negative result (data not shown), indicating the absence of mannose-containing glycans in the allergen. Edman degradation of the protein rendered no N-terminal sequence, indicating blockage of the polypeptide chain. Based on MS, IEF, reductive treatment, and sequencing experiments, it can be hypothesized that the two bands of the purified protein separated in SDS-PAGE correspond to two different conformations of the protein. According to the standard nomenclature of allergens, this protein was called Ole e 10.

The content of the secondary structure of Ole e 10 was determined by obtaining its far UV CD spectrum (Fig. 2C). Application of the convex-constraint analysis method (25) to this spectrum gave a 17% α helix structure, 33% β sheet, 21% β turn, and 29% coil. The near UV spectrum displayed a negative band centered at 280 nm and a positive peak with a maximum at 295 nm, indicating the existence of an asymmetric environment for the aromatic residues of the allergen and, therefore, the existence of a defined three-dimensional folding.

To obtain peptide fragments of Ole e 10 that would allow us to design oligonucleotide primers for its cloning, the allergen was subjected to proteolytic treatments. The alkylated protein was digested alternatively with trypsin and chymotrypsin. The soluble resulting peptides were separated in a C_{18} reverse phase HPLC column (Fig. 3, A and B). Major peptides obtained from each treatment (T1 to T3 and C2 to C4) were sequenced by Edman degradation (Fig. 3C). Attempts to obtain the N-terminal sequences of T4 and C1 peptides were unsuccessful.

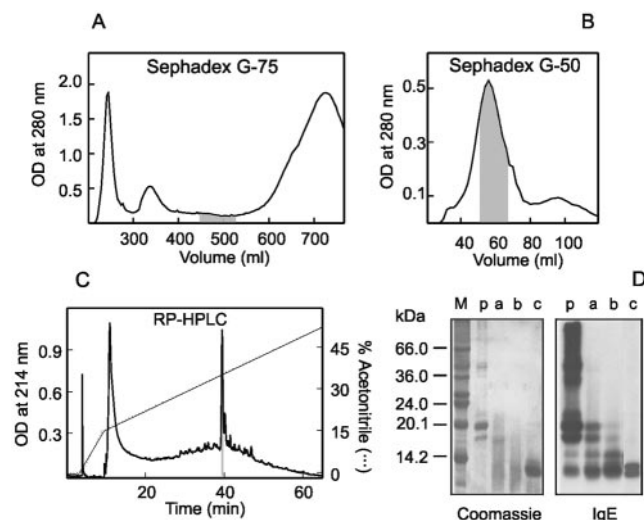


FIGURE 1. Purification of Ole e 10. Elution profiles of gel filtration chromatographies (A and B) and reverse phase HPLC C_{18} column (C); fractions containing the protein are shaded. D, SDS-PAGE analysis of the purification steps: lane p, pollen extract; lanes a–c, protein obtained from the shaded fractions of elution profiles A, B, and C, respectively; lane M, molecular mass markers. The samples were stained with Coomassie Blue or transferred to nitrocellulose membranes and reacted with a pool of sera from patients allergic to olive pollen.

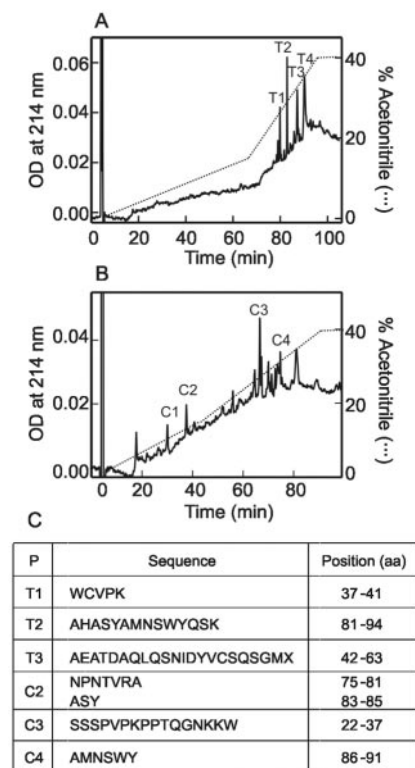


FIGURE 3. Determination of internal sequences of Ole e 10. Reverse phase HPLC separation of peptides obtained from Ole e 10 by treatment with trypsin (A) or chymotrypsin (B), with acetonitrile gradients in 0.1% TFA. C, Amino acid sequences of peptides obtained by trypsin (T1–T3) and chymotrypsin (C2–C4) proteolysis; the position in the complete sequence of Ole e 10 is indicated.

Cloning and sequencing of cDNA encoding Ole e 10

Ole e 10-specific cDNA was obtained after cDNA synthesis and PCR amplification from total RNA isolated from olive pollen. An antisense primer, designed on the basis of the amino acid sequence of T2 peptide, and a nonspecific primer, UPM, were used in the first step of PCR cloning. This experiment resulted in a cDNA fragment with an estimated size of 330 bp that would correspond to the N-terminal portion of the allergen. Based on this partial sequence of Ole e 10, a nondegenerate primer was designed and used together with the UPM primer to obtain the full-length of the cDNA encoding Ole e 10 (Fig. 4). Five cDNA clones encoding the whole protein were sequenced, and no differences were observed

in the deduced amino acid sequences (123 residues). The molecular mass of the amino acid sequence deduced from the clone (12,878 Da) was notably higher than that obtained by MS for the protein purified from the pollen (10,789 Da). This difference suggested the existence of a putative signal peptide at the N-terminal region of the unprocessed polypeptide chain. The difference in molecular mass between mature and immature forms of the allergen suggested the location of the cleavage position between Thr²¹ and Ser²² (Fig. 4). MS analysis of the tryptic-treated Ole e 10 confirmed that Ser²² was the N-terminal amino acid residue of the mature protein. Therefore, the first 63-nt segment from the ATG start codon codes for a signal peptide, and the mature protein consists of 102 aa residues with a molecular mass deduced from the sequence of 10,791 Da, which is consistent with the MS result. All amino acid sequences obtained by Edman degradation of tryptic and chymotryptic peptides (Fig. 3) were contained in the polypeptide inferred by the nucleotide sequence. The polypeptide sequence of the allergen contained a putative *N*-glycosylation consensus site, but the presence of mannose-containing oligosaccharides in the mature allergen should be disregarded because of its negative response to Con A lectin.

Alignment of the Ole e 10 amino acid sequence with those of proteins contained in the GenBank/EMBL database (Fig. 5) revealed homology (from 42–46% identity) with amino acid sequences deduced from several reported genes of *Arabidopsis thaliana* that would codify proteins of different lengths (e.g., 110, 119, 121, 129, 132, and 256 aa residues; two examples are shown in Fig. 5). Protein products derived from the expression of these genes have not been reported to date, and their putative biological function is unknown. Ole e 10 also showed identity with the C-terminal segment of long 1,3- β -glucanases, a family of pathogenesis-related proteins from vegetable sources to which the olive allergen Ole e 9 belongs. Thus, Ole e 10 showed 53% identity with the corresponding region of Ole e 9 and 27% identity with that from *A. thaliana* (A6 protein). Finally, Ole e 10 showed limited, but significant, similarity with polypeptide segments from proteins involved in yeast development, such as Epd1 of *Candida maltosa*, Gas-1p of *Saccharomyces cerevisiae*, and Phr2 of *Candida albicans* (43, 42, and 40% similarity, respectively; Fig. 5).

Immunological characterization of Ole e 10

The frequency of sensitization to Ole e 10 was analyzed with 150 sera from olive-allergic patients (mean age, 23.2 years). We determined the capability of IgE binding to purified Ole e 10-coated wells by ELISA. Eighty-three sera (55% of the total) gave OD values at 492 nm >0.1 , and thus they were considered able to bind

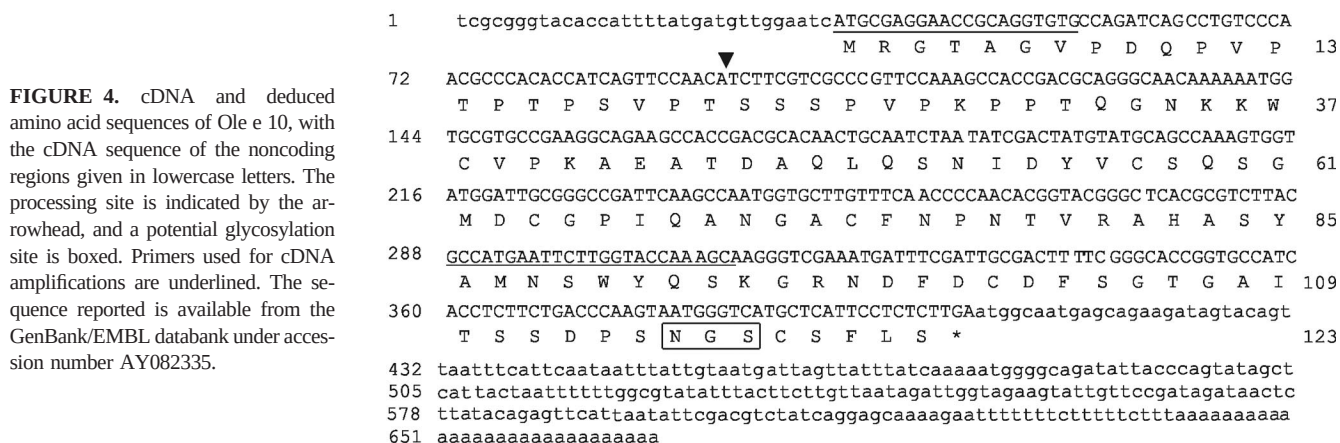


FIGURE 4. cDNA and deduced amino acid sequences of Ole e 10, with the cDNA sequence of the noncoding regions given in lowercase letters. The processing site is indicated by the arrowhead, and a potential glycosylation site is boxed. Primers used for cDNA amplifications are underlined. The sequence reported is available from the GenBank/EMBL databank under accession number AY082335.

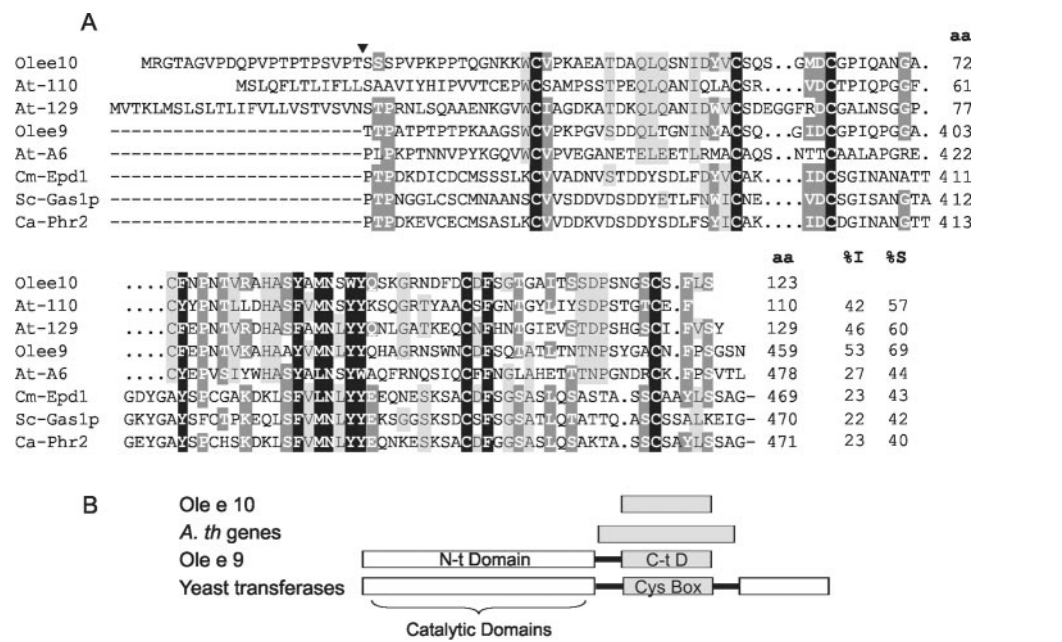


FIGURE 5. Alignment of Ole e 10 (Olee10) with proteins present in the databanks (A). Points indicate gaps; dashes indicate polypeptide regions not related to the Ole e 10 sequence. The arrowhead indicates the processing site in Ole e 10. Letters over black shading are conserved residues in all sequences; dark gray indicates residues conserved in at least six sequences; light gray indicates residues conserved in five sequences. At-110 and At-129 are deduced from genes determined in the genome of *A. thaliana* (accession no. AB015476 and AB008265, respectively). C-terminal segments of 1,3- β -glucanases from olive pollen (Olee9) and *A. thaliana* anthers (At-A6) are also compared (accession no. AF249675 and Q06915, respectively). Cm-Epd1, Sc-Gas1p, and Ca-Phr2 are polypeptide segments from glucanosyltransferases involved in yeast development from *C. maltosa*, *S. cerevisiae*, and *C. albicans*, respectively (accession no. AB005130, NC001445, and AF011386). I and S, Identity and similarity percentages of these sequences with that of Ole e 10. B, Topological representation of the alignment of molecules indicated in A; homologous regions are indicated in gray. N- and C-terminal domains of Ole e 9 (and other 46-kDa 1,3- β -glucanases) are indicated as well as the Cys box domain of the yeast enzymes.

to the purified allergen (Fig. 6A). Ole e 10 was able to bind IgE from nine of 13 sera sensitive only to *O. europaea* (69.2% prevalence in this population). These values define Ole e 10 as a major

allergen. The response of the same sera was also analyzed against whole olive pollen extract (Fig. 6A). No linear correlation between the responses was observed. This can be explained because the olive pollen contains at least nine more allergens, and some of them are major allergens (9). As Ole e 10 showed sequence similarity with the C-terminal domain of Ole e 9, IgE binding to this allergen was also analyzed in ELISA with the same 150 allergic sera (Fig. 6B). Sixty-three sera (42%) had IgE Abs reactive to Ole e 9, and 56 of them were reactive to both allergens, indicating a high degree of cosensitization. From the data in Fig. 6B, the existence of two populations of patients can be suggested: one of

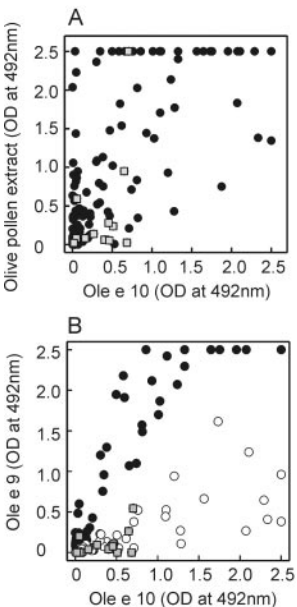


FIGURE 6. Analysis by ELISA (OD at 492 nm) of the IgE-binding capacity of Ole e 10 to 150 olive-allergic sera. Comparison with the IgE binding of total pollen extract (A) and Ole e 9 (B) to the same sera is shown. B: ●, IgE titer to Ole e 9 > IgE titer to Ole e 10; ○, IgE titer to Ole e 9 < IgE titer to Ole e 10. □, Values for the 13 sera sensitized exclusively to *Olea*.

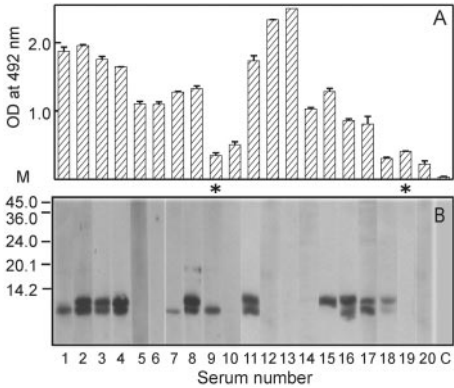


FIGURE 7. Influence of SDS-denaturing agent in the IgE-binding capability of Ole e 10. Responses of IgE from 20 individual allergic sera to purified Ole e 10 were obtained by ELISA (A) and compared with those obtained for the same sera by immunoblotting after SDS-PAGE (B). *, Monosensitized sera. Molecular masses (M) are indicated in kilodaltons.

them would exhibit a slanted sensitivity to Ole e 9, and the other would have a pronounced reactivity to Ole e 10.

The IgE binding responses obtained in ELISA for 20 sera against Ole e 10 were compared with those obtained after SDS-PAGE of the allergen and transference to membranes (Fig. 7). Eight sera (no. 5, 6, 10, 12, 13, 14, 19, and 20) completely lost their IgE-binding capability to Ole e 10 in denaturing conditions; several others, such as 1 and 7, were less reactive in the presence of SDS. These results suggest that a substantial part of the IgE epitopes of native Ole e 10 are of a conformational nature and thus are disturbed by SDS. Interestingly, sera 1, 7, 9, and 15 only recognized in immunoblotting one of the two bands of the allergen, suggesting that the IgE Abs from a population of patients can discriminate between epitopes present in one or another putative conformation of the allergen. The loss of reactivity in SDS did not seem to be associated with a specific conformation.

IgE cross-reactivity of Ole e 10 with Ole e 9 olive allergen, latex, and vegetable fruits

An equivolumetric mixture of individual sera, each exhibiting a positive response to Ole e 9 and Ole e 10, was used to analyze the capability of these Ags to inhibit IgE binding to Ole e 10. Ole e 10-coated wells were incubated with the serum pool preadsorbed with different amounts of the inhibitors (Fig. 8A). Ole e 10 was able to inhibit 87% of the binding, whereas Ole e 9 inhibited up to 52%.

Protein extracts from allergenic pollens, vegetables, fruits, and latex were also tested for the existence of cross-reactivity with Ole e 10. A pool of sera from allergic patients, which showed a positive IgE response to Ole e 10, were used in ELISA inhibition experiments with Ole e 10 fixed to wells and the protein extracts

(500 μ g of total protein) from different sources as inhibitors. Most extracts were able to inhibit a significant percentage of IgE binding to the purified allergen (Fig. 8B), indicating that they contain proteins sharing antigenic determinants with Ole e 10. Ash, privet, lilac (*Oleaceae* species) and cypress extracts, showed the highest inhibition values among pollens. This analysis was extended to nonpollen vegetable materials. Potato, latex, and kiwi extracts showed >30% of inhibition; pulp and peel of peach as well as tomato produced lower, but significant, inhibition.

Discussion

Olive pollen is the most important cause of respiratory allergy in southern Spain, with epidemics of pollen-induced exacerbation between late April and early June when patients are exposed to extremely high level pollen counts (>5000 grains/m³ in Jaén) (30). Thus, the existence of seasonal rhinitis and/or bronchial asthma as well as a positive skin prick test to olive pollen allergen were the primary inclusion criteria for the study. The majority of olive allergic patients (~90%) present associated taxonomically unrelated pollen allergies (31). However, inhibition assays of the IgE binding of olive-allergic sera to different pollen extracts suggest that *O. europaea* allergy should be a primary sensitization in those patients who are intensely exposed to its pollen (32).

The IgE binding pattern of the olive pollen is complex and diverse. Patients exhibiting high radioallergosorbent test values (4–6) for olive pollen extract are sensitive to a considerable number of its allergens (9). Thus, analysis of the allergogram of olive pollen, at least of their major allergenic components, is essential to design standardized mixtures for effective diagnosis and specific immunotherapy. Despite the high number of allergens characterized from olive pollen to date, new proteins are emerging as relevant Ags causing pollinosis. From two-dimensional electrophoretic analyses, a new allergen of acidic pI and low apparent molecular mass was detected (13). Taking into account the similarity of its molecular properties to those of Ole e 3 and Ole e 6, which are also acidic and small proteins, a procedure was designed to isolate the novel allergen. Purification of Ole e 10 was only possible by discarding very soluble proteins, which are released from the pollen after very short times of extraction.

As the N-terminal end of the purified Ole e 10 was not accessible to the Edman reagent, several peptides were obtained from two different proteolytic digestions and used to determine internal amino acid sequences. These sequences were then employed to clone the whole protein. Ole e 10 consists of a single polypeptide chain of 102 aa residues (10,791 Da), preceded by a putative signal peptide of 21 aa that should be processed during the post-translational maturation. Comparison of the primary structure of Ole e 10 with proteins submitted to the sequence databanks showed homology with a family of genes from *A. thaliana*, which would encode proteins of different polypeptide lengths, between 110–256 aa. The analysis of any protein product from these genes has not been reported to date, and their functional role is unknown. Hence, Ole e 10 would represent the first described and characterized member of a novel family of plant proteins. Fig. 5B shows a topological representation of the alignment of Ole e 10 and homologous polypeptides. Ole e 10 also displayed sequence similarity with the C-terminal portion of Ole e 9. Ole e 9 is a 1,3- β -glucanase (46 kDa) that consists of two defined domains (10, 33): the N-terminal domain (36 kDa), which contains the catalytic site, and the C-terminal domain (10 kDa), which has an unknown biochemical role. The latter corresponds to a Cys-enriched extension that is absent in the most abundant and widely spread 1,3- β -glucanases (M_r , ~36 kDa) (34, 35). Ole e 10 exhibits the highest sequence

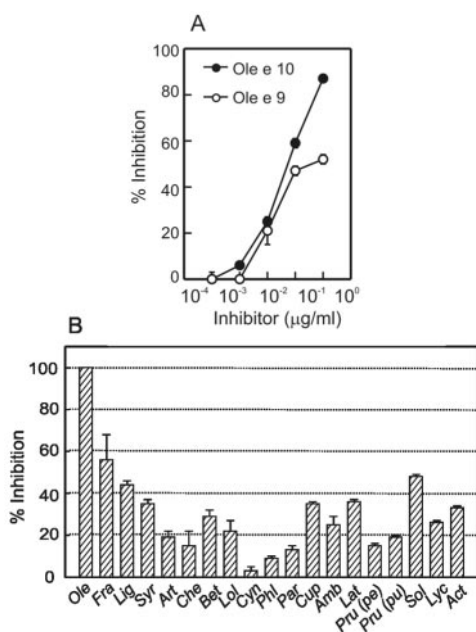


FIGURE 8. Cross-reactivity of Ole e 10. *A*, ELISA inhibition analysis of IgE binding to Ole e 10-coated wells using Ole e 9 (○) and Ole e 10 (●) as inhibitors. *B*, Inhibition ELISAs of IgE binding to Ole e 10-coated wells using extracts from different allergenic sources as inhibitors (500 μ g of total protein): pollen extracts from olive (Ole), ash (Fra), privet (Lig), lilac (Syr), mugwort (Art), chenopod (Che), birch (Bet), ryegrass (Lol), Bermuda grass (Cyn), timothy (Phl), *P. judaica* (Par), cypress (Cup), ragweed (Amb); saline protein extracts from peel and pulp of peach (Pru (pe) and Pru (pu)), potato (Sol), tomato (Lyc), and kiwi (Act); and natural latex extract (Lat).

identity or similarity (53 and 69%, respectively) with this C-terminal module of Ole e 9 among those homologous polypeptides found in the databanks. For other 1,3- β -glucanases, such as that from *A. thaliana* (A6-protein), the identity/similarity values fall dramatically to 27/44%. Finally, Ole e 10 showed homology to the so-called Cys Box domains from three families of 1,3- β -glucanotransferases involved in yeast development: Epd (essential for pseudohyphal development), Gas (glycophospholipid-anchored surface), and Phr (pH-regulated) proteins (36–38). Epd/Gas/Phr protein families belong to a novel family of glucanotransferases, family 72, whose members share common structural and functional characteristics (39–41).

The CD analysis of Ole e 10 showed very similar contents of secondary structure to those obtained for the free recombinant C-terminal domain of Ole e 9 (16% α helix, 30% β sheet, 17% β turn) (33), thus supporting the homology between these molecules and suggesting closely related conformations for them. This could indicate a similar functional role for both polypeptides, with Ole e 10-like proteins operating as free molecules and C-terminal domains of Ole e 9-like glucanases (and even Cys boxes from 1,3- β -glucanotransferases) in working as covalent-bound domains, this without detriment of specific biochemical meanings for each individual protein family.

Interestingly, Ole e 10 displays a dual behavior in SDS-PAGE, exhibiting two forms of different mobilities. This could be explained by the existence of two spatial conformations for its three-dimensional structure because 1) the purified allergen showed a unique molecular mass peak by MS analysis; and 2) the reduced form rendered a single band in SDS-PAGE. The appearance of two conformations could be due to a cysteine-pairing isomerism. In fact, this effect takes place in small proteins containing several disulphide bridges in the core of the three-dimensional structure. Proteins such as the antifungal protein from *Aspergillus giganteus* and the basic pancreatic trypsin inhibitor have been shown to possess two patterns of disulphide bridge arrangements, which led to two different spatial conformations (42, 43). Finally and despite the existence of one N-glycosyl binding consensus site in the sequence of Ole e 10 (Asn⁹⁵ of the mature protein), the presence of a N-glycan moiety can be ruled out because the protein did not bind Con A lectin.

Ole e 10 is a relevant allergen because it displays 55% prevalence among olive pollen-allergic patients and 69.2% among those sensitized exclusively to *Olea*. Ole e 10 possesses conformational IgE epitopes that are perturbed in denaturing conditions, as a significant percentage of sera (seven sera, 35%, $n = 20$) completely lost their capability to bind the Ag in the presence of SDS. The allergen also displays IgE epitopes insensitive to this chemical agent, as the remaining 65% of the sera gave a positive response in the immunoblotting. For these sera, the IgE response does not seem to be significantly dependent on the disulphide bridge disruption, as 2-ME treatment of the Ag did not substantially modify the overall binding to the Abs (Fig. 2B). These results could indicate that these sera recognize linear IgE epitopes in Ole e 10. In terms of the IgE reactivity of the two bands of Ole e 10 resolved by SDS-PAGE, the individual sera were able to discriminate between the two forms of the allergen, indicating that they should exist in the pollen and are able to elicit IgE synthesis in hypersensitive patients.

Ole e 10 seems to exhibit a low intrinsic antigenicity, because no specific high titer polyclonal Abs were induced after different attempts to immunize rabbits with the pure Ag. Other olive allergens of similar and lower molecular mass, e.g., Ole e 3 (9.8 kDa) or Ole e 6 (5.5 kDa), were able to induce high titer antisera in identical conditions. However, many patients are sensitized to Ole e 10 in

natural conditions, and although it is released slowly from the pollen, it is highly soluble per se. We can speculate about an immunization pathway that would involve Ole e 10 associated with other proteins from the pollen cell, subcellular structures, or starch particles. Grote et al. (44) have demonstrated the expulsion of allergen-containing submicronic materials from grass pollens after hydration as a mechanism of allergen release. Ole e 10 could be expelled from the pollen linked to a protein or particle that behaves as a carrier, significantly increasing its potential antigenicity.

Ole e 10 shows IgE cross-reactivity with Ole e 9, another major allergen from olive pollen. It could be explained by the sequence similarity between Ole e 10 and the C-terminal domain of Ole e 9 (Fig. 5). However, ELISA inhibition results (Fig. 8) showed differences detectable by specific IgE, indicating that these proteins are immunologically distinguishable. In addition, a number of sera exhibited a higher IgE response to Ole e 10 than to Ole e 9 (Fig. 6B). These data indicate that Ole e 10 is an allergen per se. A notably allergenic capability has been reported for hevein, a small protein from latex and fruits, that constitutes the N-terminal module of both prohevein and class I chitinases (45–47). By contrast, Ole e 10 is not a part of Ole e 9, and a number of sensitizations were performed preferentially against one of these allergens indicating that both allergens may act as a primary sensitizer. These olive allergens thus illustrate a new form of intraspecific cross-reactivity.

Ole e 10 also exhibits IgE cross-reactivity with plant proteins from other pollens, latex, and vegetable foods, as their protein extracts are able to inhibit IgE binding to Ole e 10. The potency of the inhibition reached by each protein extract is highly variable and, unexpectedly, nonpollen tissues exhibited more capability than those of some pollens. However, it could be explained not only by the sequence diversity of the allergenic counterparts, but by their different content in the corresponding extract. The molecular analysis of putative Ole e 10-like proteins in other pollens, latex, peach, potato, kiwi, and other allergenic plant sources will clarify the importance of the allergenic interspecific cross-reactivity due to this protein family. It would also help to highlight its role on pollen-pollen, pollen-latex, pollen-fruit, and pollen-latex-fruit syndromes, which would be relevant for diagnosis and perhaps for specific immunotherapy of patients suffering from allergies to these materials.

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