

Mechanisms of allergy

Identification, isolation, and characterization of Ole e 7, a new allergen of olive tree pollen

M. Luisa Tejera, MSc, Mayte Villalba, PhD, Eva Batanero, PhD, and Rosalía Rodríguez, PhD *Madrid, Spain*

Background: Olive tree (*Olea europaea*) pollen is an important cause of pollinosis in countries of the Mediterranean area and California.

Objective: The aim of this study was to identify and purify a new allergen of olive tree pollen.

Methods: Detection of a pollen allergen was done with individual allergic sera by immunoblotting and ELISA tests. Two allergenic fractions were isolated from olive pollen extract by using gel filtration and reverse-phase HPLC. Molecular characterization was achieved by acid hydrolysis and amino acid analysis, as well as by mass spectrometry. Sequencing of the N-terminal end of the allergen was carried out by Edman degradation of the polypeptide chain. Allergenic characterization was performed with sera from subjects with olive allergy by means of ELISA and immunoblotting after SDS-PAGE.

Results: The new allergen Ole e 7 exhibits a high degree of polymorphism. Its molecular mass is in the range of 9875 d to 10,297 d. Twenty-one amino acid residues from the N-terminal end of 2 isoforms of the allergen have been sequenced revealing no homology with proteins contained in database banks.

Ole e 7 has an average frequency of about 47% in patients with olive allergy. The strategy of purification of Ole e 7 can be useful on the isolation of new allergens.

Conclusions: A new olive pollen allergen of clinical significance has been purified and characterized, contributing to the study of the complete allergogram of the olive tree pollen. (*J Allergy Clin Immunol* 1999;104:797-802.)

Key words: *Olea europaea*, allergen, olive pollen allergy, Ole e 7, amino acid sequence

Olive pollinosis is a major cause of respiratory allergy in Mediterranean countries and areas of North America.¹⁻³ More than 70% of the patients allergic to olive pollen are hypersensitive to Ole e 1,^{4,5} one of the main polypeptide

Abbreviation used

RP: Reverse phase

components of the pollen grain and a major allergen that significantly contributes to the overall allergenicity of the olive pollen extracts.⁶ Ole e 1 has been structurally and immunologically characterized,⁷ as well as sequenced and expressed in *Escherichia coli*⁸ and *Pichia pastoris*.⁹ In addition to Ole e 1, other allergens of olive pollen have been detected, isolated, and analyzed.¹⁰⁻¹⁴ On the basis of their amino acid sequences, a biochemical activity can be attributed to some of these allergens, such as profilin (Ole e 2), polcalcin (Ole e 3), and superoxide dismutase (Ole e 5). However, the primary structures of Ole e 4 and Ole e 6 exhibit no homology with any other known protein, and therefore a biologic role cannot be suggested for them.

Protein extracts obtained from allergenic sources usually contain a mixture of molecules responsible for hypersensitivity processes. However, only a few allergens are present at high levels, and most of the allergenic proteins are minor components the detection and characterization of which become frequently difficult. This problem is increased in vegetable extracts because they contain high amounts of pigments, carbohydrates, and low-solubility materials that make the analysis of the allergenic contents difficult. Although the minor protein components could be powerful allergens and of a high prevalence, they can be masked by the presence of major contributors. Therefore a correct diagnosis and an adequate design of the therapy protocol require a deep knowledge of the complete allergenic composition of the corresponding biologic extract. We have analyzed the low molecular weight protein fraction of the olive pollen extract, and a highly prevalent new allergen of 10 kd has been characterized.

METHODS

Saline extract preparation and isolation of Ole e 1, Ole e 3, and Ole e 6

Olive tree pollen (Allergon AB, Engelholm, Sweden) was extracted 3 times for 15 minutes at room temperature with 50 mmol/L ammonium bicarbonate (pH 8.0) containing 1 mmol/L phenylmethylsulfonyl fluoride, followed by centrifugation at

From the Departamento de Bioquímica y Biología Molecular, Facultad de Química, Universidad Complutense, Madrid.

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Reprint requests: Rosalía Rodríguez, PhD, Departamento de Bioquímica y Biología Molecular, Facultad de Química, Universidad Complutense, 28040 Madrid, Spain.

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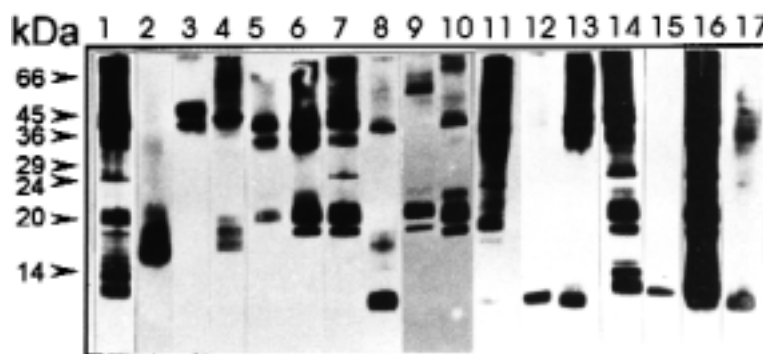


FIG 1. SDS-PAGE of olive pollen extract and immunostaining with sera of 17 patients allergic to olive pollen after transference to membranes of nitrocellulose, showing the variability of the allergograms.

12,000g for 20 minutes at 4°C. The lyophilized supernatant was stored at -20°C.

The olive allergens Ole e 1,¹⁵ Ole e 3,¹⁰ and Ole e 6¹¹ were isolated as previously described.

Human sera

Sera from 164 untreated donors (all of them displaying a well-documented clinical history of allergy to olive tree pollen) with positive skin test and RAST (classes 3 to 6) responses to *Olea europaea* allergen extract were used. Pooled sera from 4 nonallergic individuals was used as control.

Purification of the allergen

The lyophilized saline extract was dissolved in 0.2 mol/L ammonium bicarbonate (pH 8.0) and subjected to gel filtration on a Sephadex G-75 column equilibrated in the same buffer. Fractions eluting at approximately 10 kd were selected and assayed for IgE-binding activity by SDS-PAGE and immunoblotting with a pool of allergic sera. The lyophilized material was chromatographed on a reverse-phase (RP) nucleosil C-18 HPLC column. An acetonitrile gradient in 0.1% (vol/vol) trifluoroacetic acid was used for the elution. Absorbance was measured at 214 nm, and fractions containing IgE-binding activity were collected and lyophilized. A Superdex 75 (Pharmacia & Upjohn, Inc, Kalamazoo, Mich) molecular exclusion column equilibrated in 0.1 mol/L ammonium bicarbonate (pH 8.0) was used as the final chromatographic step. The column was eluted at a flow rate of 1 mL/min, and the absorbance was monitored at a wavelength of 280 nm. IgE-binding activity of the fractions was also determined.

Amino acid analysis and protein concentration

Protein samples (1 to 2 nmol) were hydrolyzed and quantitatively analyzed as described.⁷ The cysteine content was measured as cysteic acid after performic acid oxidation. Tryptophan was spectrophotometrically determined.¹⁶ Total protein concentration of saline extracts was estimated according to the method of Lowry et al.¹⁷ The protein content of purified samples was determined by amino acid analysis.

Sequence analysis

N-terminal Edman degradations of native and alkylated protein (0.2-0.5 nmol) were performed on an Applied Biosystems model 477A sequencer (Applied Biosystems, Foster City, Calif) equipped with a 120A on-line phenylthiohydantoin-amino acid derivative analyzer and the standard Applied Biosystems program. Homology

searches on Protein Sequence Data Banks was carried out by using the BLAST program.¹⁸

Polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli¹⁹ in 15% (wt/vol) polyacrylamide gels. Proteins were stained with 0.1% (wt/vol) Coomassie blue stain in 40% (vol/vol) methanol and 10% (vol/vol) acetic acid. Apparent molecular weight determinations were done according to the method of Weber and Osborn²⁰ by using protein markers provided by Sigma (MW-SDS-70L).

Mass spectrometry analysis

Molecular mass determination of the allergen was performed by means of matrix-assisted laser-desorption ionization mass spectrometry. The protein was dissolved in aqueous matrix solution (sinapinic acid in 0.1% [vol/vol] trifluoroacetic acid and acetonitrile) and left to dry at ambient temperature. Mass analysis was performed in the linear mode on a Bruker REFLEX time-of-flight mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany). Spectra were acquired as the sum of ion signals generated by 100 to 150 pulses from a 337-nm N₂ laser. Mass calibration was accomplished by using the known masses of matrix ion signals. Mass accuracy is better than 0.05%.

Immunologic assays

Protein bands in SDS-PAGE were transferred onto nitrocellulose membranes (Bio-Rad, Richmond, Calif) as described.⁷ Briefly, blots were saturated in PBS containing 3% (wt/vol) nonfat milk and 0.1% Tween 20 (blocking buffer) and incubated with the human sera diluted 10-fold in blocking buffer, followed by reaction with mouse anti-human IgE (diluted 5000-fold) kindly donated by Dr M. Lombardero (ALK-Abelló, Madrid). Afterward, blots were incubated with horseradish peroxidase-labeled goat anti-mouse IgG (Pierce, Rockford, Ill) diluted 5000-fold in PBS containing 1% (wt/vol) nonfat milk and 0.05% (vol/vol) Tween 20. The peroxidase reaction was developed by using the ECL Western-blotting reagent (Amersham Corp, Buckinghamshire, England).

ELISA titration was performed as described.¹⁰ Briefly, microtiter plates were coated with 100 µL of antigen (1 µg/mL) in PBS (pH 7.2) and incubated at 4°C overnight. Wells were washed with PBS and 0.5% (vol/vol) Tween 20 and saturated with the blocking buffer used in immunoblotting assays. Plates were incubated with the human sera at different dilutions and then with 100 µL of mouse anti-human IgE diluted 5000-fold. Afterward, plates were incubated with horseradish peroxidase-labeled goat anti-mouse IgG (diluted 5000-fold). Finally, the peroxidase reaction was developed by using fresh enzyme sub-

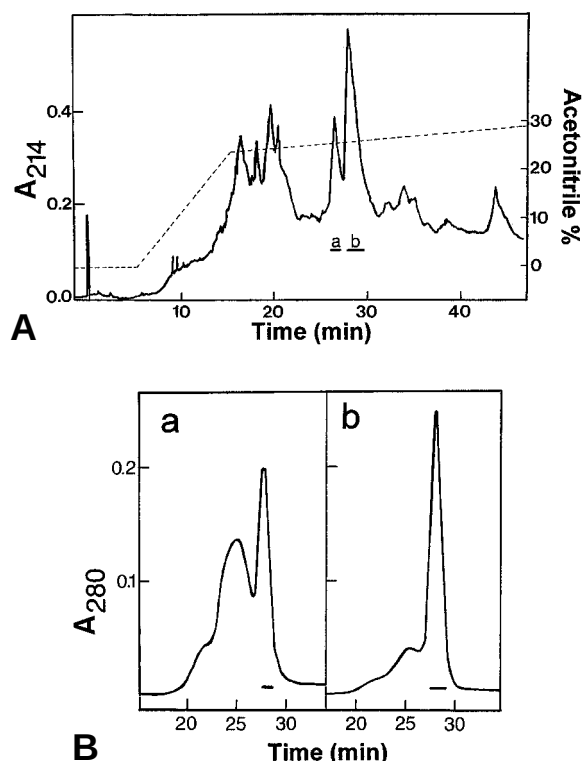


FIG 2. Isolation of Ole e 7. **A**, Elution profile of the RP-HPLC on a nucleosil C-18 column. **B**, Superdex 75 gel permeation chromatography of samples a and b from the HPLC column. Fractions containing IgE-binding activity are denoted by a bar.

strate (0.03% [vol/vol] H₂O₂ and 0.63 mg/mL of *o*-phenylenediamine in 0.1 mol/L sodium citrate, pH 5.0) and measuring the optical density at 492 nm.

RESULTS

Detection of a new olive pollen allergen

The sera of 164 patients, allergic to olive tree pollen as determined by skin prick tests, were assayed for IgE binding to olive pollen extracts in immunoblotting. A random selection of these blots is shown in Fig 1, in which, besides the diversity of allergenic patterns, small but significant differences on the bands around 10 kd can be observed. Seventy-two (44%) sera showed recognition of bands lower than 13 kd and were assayed by ELISA for specific IgE binding to Ole e 3- and Ole e 6-coated wells. Among them, 12 sera did not recognize Ole e 3 (9.2 kd) or Ole e 6 (10.0 kd). These sera were pooled and used for the purification of new olive allergens of low molecular mass.

Isolation of new IgE-binding antigens from olive pollen

The olive pollen extract was chromatographed on a gel filtration column, and fractions of around 10 kd were pooled, lyophilized, and further fractionated by HPLC (Fig 2, A). Two main and closely positioned protein peaks showed high IgE-binding capability. The 2 allergenic

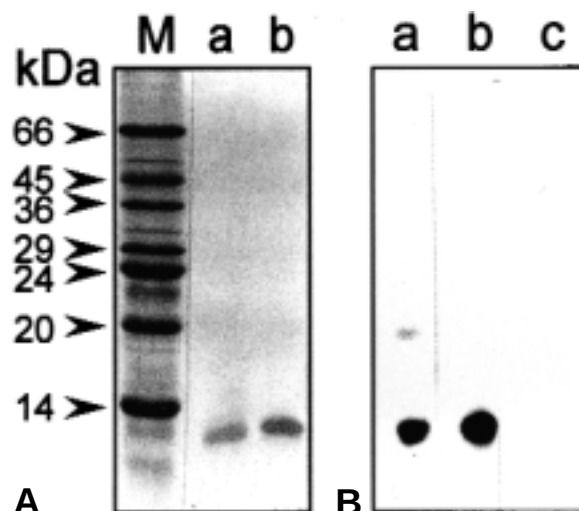


FIG 3. SDS-PAGE analysis of fractions a and b from Fig 2. **A**, Coomassie blue staining. **B**, IgE-binding analysis by Western blot staining with sera from patients allergic to olive pollen. In lane c a mixture of samples a and b were stained with a pool of nonallergic sera. M, Molecular mass protein markers.

TABLE I. Amino acid composition of the allergenic fractions a and b calculated by acid hydrolysis and automatic analysis

Amino acid	Fraction a	Fraction b
Cys*	6	6
Asx	10	10
Thr	5-6	5-6
Ser	8-9	8-9
Glx	9	5-6
Pro	3-4	4
Gly	6	6
Ala	10	10
Val	5	5
Met	2-3	2-3
Ile	4	5
Leu	4	4-5
Tyr	1-2	2
Phe	0	0
His	1	1
Lys	8	8
Arg	2	2-3
Trp†	1	1
Total	85-90	84-90

*Determined after oxidation with performic acid.

†Determined spectrophotometrically.

peaks were then separated in a new gel filtration column (Fig 2, B). The 2 obtained fractions (a and b) displayed an apparent molecular mass of 10 kd, as determined by SDS-PAGE (Fig 3, A). The proteins were transferred to membranes of nitrocellulose after SDS-PAGE and immunostained with the pool of olive hypersensitive sera (Fig 3, B).

Molecular characterization

The amino acid compositions of the 2 protein peaks are shown in Table I. A strong similarity can be deduced from these data.

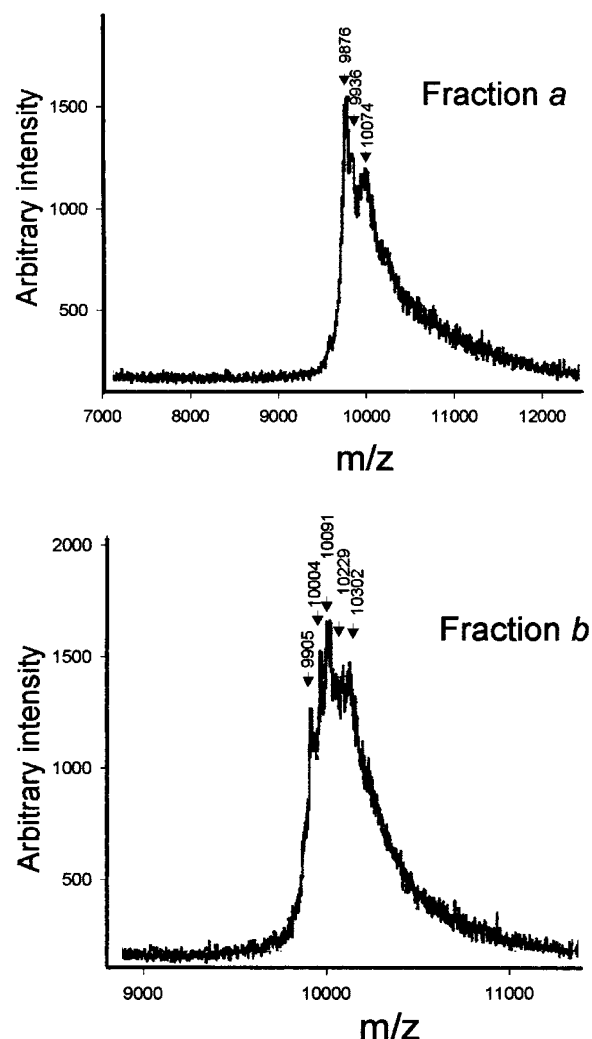


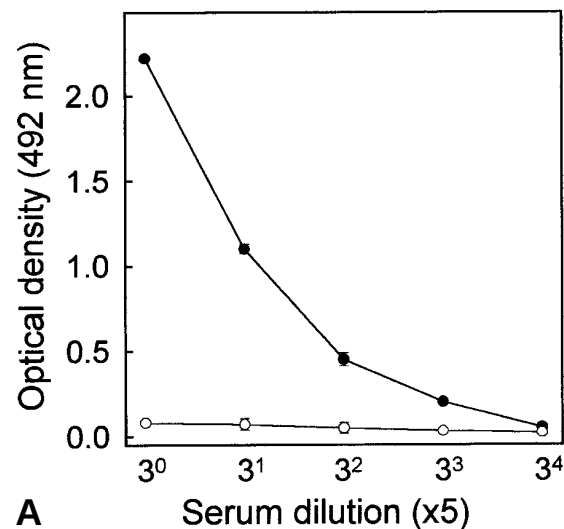
FIG 4. Matrix-assisted laser-desorption/ionization mass spectrometry analysis of the allergenic fractions a and b. The mass spectra exhibit the charged (MH^+) protein molecular ions.

The molecular mass of these 2 protein fractions is around 10 kd, as determined by mass spectrometry (Fig 4). This analysis reveals a significant degree of polymorphism for both samples. Three main polypeptides of 9876 d, 9936 d, and 10,074 d were detected in fraction a, and at least 5 polypeptides of 9905 d, 10,004 d, 10,091 d, 10,229 d, and 10,302 d were detected in fraction b. Considering these values and the amino acid composition, a length of about 88 amino acids can be estimated for these polypeptide chains.

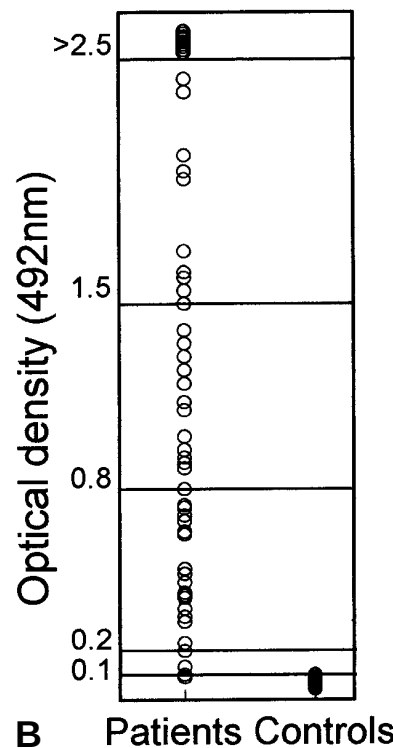
The NH_2 -terminal amino acid sequencing of these 2 samples was done by Edman degradation of the reduced and carboxyamidomethylated polypeptide chains. Fig 5 shows the strong similarity of both sequences. In addition, a degree of microheterogeneity was detected at positions 4 and 11 in fraction a and at positions 4, 5, 10, and 11 in the sequence of fraction b, which could explain the polymorphism observed by mass spectrometry. No

Fraction a APSQSTVTALLTSCVSYIDDQ
Fraction b APSQGTVTAKLTSCVSYKDXQ

FIG 5. NH_2 -terminal amino acid sequences of the allergenic fractions a and b. Positions containing different residues are boxed.



A



B

FIG 6. IgE-binding assays to Ole e 7. **A**, ELISA titration by using different dilutions of a pool of 6 sera from patients allergic to olive pollen (filled circles); a nonallergic sera pool was used as control (open circles). The values are means of 3 determinations, with the SD value being less than 0.04. **B**, IgE binding to Ole e 7 of the 77 positive sera. ODs under 0.1 were considered as negative responses (87 sera). Six nonallergic sera were used as controls. Each value is the mean of 2 determinations.

homology has been found with other known proteins from Protein Sequence Data Banks (SWISS-PROT, PIR, and GenPep).

From all these data, it can be inferred that fractions a and b compile a pool of isoforms of the same allergen. Those present in fraction a would be more hydrophilic than those in fraction b, as deduced from their relative retention on the RP-HPLC column (Fig 2, A). This polymorphic allergen has been named Ole e 7, according to the recommendations of the Allergy Nomenclature Subcommittee of the International Union of the Immunological Societies.^{21*}

Immunologic characterization of Ole e 7

A pool of 6 sera, which had been shown to recognize purified Ole e 7 in immunoblotting, was used for ELISA titration of the allergen. In Fig 6, A, the result is given in comparison with the binding assay of the same sera to Ole e 1.

The 164 sera from patients allergic to olive were once more assayed by ELISA for direct IgE binding but now against the purified allergen Ole e 7 (Fig 6, B). Among them, 77 sera showed IgE binding to Ole e 7-coated wells, representing 47% of the sera that were used in this work. The recognition rate of the 164 initial sera obtained by direct ELISA to the allergens purified from the olive pollen Ole e 1, Ole e 3, and Ole e 6 were, respectively, 71%, 21%, and 32%. Therefore Ole e 7 is shown to be an allergen of significant relevance.

DISCUSSION

From the results shown in this work, as well as those from previous studies, it can be inferred that the olive pollen saline extract contains a complex mixture of allergenic proteins. To date, 6 allergens (Ole e 1 to Ole e 6) have been isolated and characterized, and some of them have been obtained as recombinant forms in *E coli* or *P pastoris*.^{7-14,22} Most of these allergens are important hypersensitizing agents, although their specific incidences seem to be dependent on different factors, such as the geographic area of the patients^{10,11} or the origin of the pollen samples used for the immunologic assays.^{14,22}

Lengthwise, through the purification and study of Ole e 1,^{7,8} olive profilin,¹³ Ole e 3,^{10,22} and Ole e 6,¹¹ we have found that the existence of major allergens can hide other ones with a strong allergenicity, although present in lower amounts. Boluda et al¹⁴ have described an IgE prevalence of 100% for allergenic components of 10 kd in the *O europaea* extract. Although our own data did not reach such high prevalence, they were higher than those obtained for Ole e 3 (10.0 kd) plus Ole e 6 (9.5 kd). Thus we studied the presence of more allergenic proteins of around 10 kd in the olive pollen extract.

For this purpose, we have fractionated the pollen extract by molecular size exclusion techniques; thus

allergens present in low amounts can be enriched in fractions that display a narrow range of molecular mass. To distinguish different allergens in spite of their similar molecular masses, we have used immunoblotting assays and individual allergic serum instead of pools to diminish the complexity of the allergenic patterns. These approaches have allowed the detection and isolation of a new olive pollen allergen, Ole e 7. It displays an apparent molecular mass in SDS-PAGE nearly identical to that of Ole e 6 (10.0 kd) and very similar to that of Ole e 3 (9.5 kd). A mixture of these 3 allergens totally inhibits the IgE binding of sera from patients allergic to olive pollen to protein bands of molecular mass from 8 kd to 13 kd in immunoblotting (data not shown). Moreover, the sum of the individual prevalence of Ole e 3, Ole e 6, and this new allergen, Ole e 7, accounts for the high value obtained for the overall protein bands of around 10 kd in the allergogram of the olive pollen. Ole e 7 has a significant clinical importance because 47% of the patients used in our study were sensitive to this allergen. The prevalence of Ole e 7 appears to be related to the geographic area, as has been also described for Ole e 6.¹¹ In fact, a value of more than 60% is obtained for patients from Jaen (South of Spain), whereas less than 20% is obtained for those in the Madrid area (Central Spain).

Ole e 7 is a very soluble protein because it is released from the olive pollen at the very early steps of the saline extraction. Eighty percent of the allergen is removed from the pollen in the first 15 minutes, and this short extraction time is crucial to isolate the allergen. Larger periods allow the extraction of other proteins that are difficult to eliminate in the chromatographic steps. For comparison, Ole e 6 requires 3 hours of saline extraction.

Ole e 7 consists of one polymorphic polypeptide chain of around 10 kd, which accounts for 88 amino acid residues. Polymorphism is a frequent feature of allergens that is remarkable in some major allergens, such as Bet v 1 from birch and Ole e 1 from olive. The number of isoforms of Ole e 7 is high, as deduced from the elution profile on HPLC, mass spectrometry data, and microheterogeneity of sequence. This fact compels the production of the recombinant allergen for both an accurate structural analysis and a precise definition of its specific B- and T-cell responses.

After a search into the protein Sequence Database Banks, it was concluded that the amino acid sequence of the NH₂-terminal end of Ole e 7 does not show significant similarity with any other known protein. The epitope analysis of this new and small antigen could provide valuable data about the minimal requirements for the allergic capability of polypeptides.

In summary, this work describes the detection, isolation, and characterization of a new allergen from olive tree pollen, which displays clinical significance and which had been concealed up to date because of its coincidence on molecular weight with Ole e 3 and Ole e 6. Thus this study is a contribution to the elucidation of the whole allergogram of the olive pollen extract.

*The sequence of the major component of fraction b was submitted to the SWISS-PROT Data Bank and appears with the accession No. P81430.

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