

# Purification, characterization, and partial sequencing of two new allergens of *Olea europaea*

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**Background:** The inhalation of olive (*Olea europaea*) pollen is an important cause of allergic respiratory diseases in southern Europe and California.

**Objective:** The aims of this study were to characterize the allergenic composition of *O. europaea* pollen collected in California and to purify two important allergens.

**Methods:** One hundred grams of *O. europaea* pollen was extracted dialyzed in 10 kd cut-off membranes and lyophilized. Allergen characterization was done by sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotting. Two allergens were isolated by gel filtration, ion exchange, and hydrophobic interaction chromatography and sequenced.

**Results:** Ole e 4 has an apparent molecular weight, under reducing conditions, of 32 kd and pIs between 4.65 and 5.1. The N-terminal was blocked and the analysis of the amino acid sequence of two internal regions revealed no homology with other known proteins. Ole e 5 has a molecular weight of 16 kd and pIs between 5.1 and 6.5. The amino acid sequence of the N-terminal showed a high degree of homology with superoxide dismutase of several plant species. Ole e 4 and Ole e 5 had an IgE binding frequency by immunoblot of 80% and 35%, respectively.

**Conclusions:** Olive pollen extracts have a heterogeneous composition, with several important allergens. One of these allergens showed a high degree of homology with a superoxide dismutase. (J Allergy Clin Immunol 1998;101:210-6.)

**Key words:** *Olea europaea*, allergen, Ole e 1, Ole e 4, Ole e 5, superoxide dismutase, allergic rhinitis, asthma

*Olea europaea* pollen is one of the most important aeroallergens in some regions of North America as well as in Mediterranean countries.<sup>1-4</sup>

Early studies suggested the presence of a major allergen (Ole e 1) in *O. europaea* extracts.<sup>5-11</sup> Ole e 1 is well characterized and consists of two components of 15-17 and 17-21 kd,<sup>5, 7, 12</sup> with an IgE binding frequency greater than 80%.<sup>7, 13</sup> Its amino acid sequence was established by Villalba et al.,<sup>10</sup> who sequenced a single

## Abbreviations used

|           |                                                          |
|-----------|----------------------------------------------------------|
| PBS:      | Phosphate-buffered saline                                |
| SDS-PAGE: | sodium dodecylsulfate-polyacrylamide gel electrophoresis |

polypeptidic chain of 145 amino acid residues with a calculated molecular mass of 16.3 kd. Ole e 1 shows sequence similarities with proteins that are involved in pollen maturation, germination, and/or pollen tube growth of tomato and corn. It has been suggested that Ole e 1 could play a role in the elongation of the pollen tube.<sup>10</sup> It has been suggested that Ole e 1 contributes significantly to the total allergenicity of olive pollen extracts<sup>14</sup> and that its concentration is closely related to the allergenic activity.<sup>14, 15</sup> Ole e 1-like allergens are present in pollen from other species of the Oleaceae family.<sup>16, 17</sup>

In addition to Ole e 1, other allergens have been detected in olive pollen.<sup>18, 19</sup> An allergen of 9.5 kd by mass spectrometry (Ole e 3) was recently isolated, characterized, and partially sequenced. This allergen has an IgE recognition rate of 20% and is also found in pollen from nonrelated species.<sup>20</sup> It bears two Ca<sup>+2</sup> binding sites that could be involved in reproductive pollen functions. It has been suggested that Ole e 3 may correspond to an allergen previously reported by Lauzurica et al.<sup>12, 20</sup>

The objectives of this study were to investigate the allergenic composition of *O. europaea* pollen collected in California and to characterize two important allergens. Two new allergens of 32 and 16 kd were purified and named Ole e 4 and Ole e 5, respectively, according to the recommendations of the International Union of Immunological Societies Allergen Nomenclature Subcommittee.<sup>21, 22</sup>

## METHODS

### Allergenic extracts

One hundred grams of *O. europaea* pollen was purchased from Biopol (Spokane, Wash.). The acetone-defatted pollen was extracted 1:20 (wt/vol) in 125 mmol/L ammonium bicarbonate solution for 4 hours at 4° C. The resulting suspension was centrifuged at 13,000 rpm for 30 minutes and sterile filtered. The extract was extensively dialyzed in membranes with a nominal cut-off of 10 kd (Visking, U.K.) and lyophilized. The freeze-dried preparation contained 15 ng of Ole e 1 per microgram of protein.

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### Total protein determination

The protein concentration of the *O. europaea* extract and their fractions was estimated by the Biuret-Lowry method (Sigma Diagnostics; St. Louis, Mo.), following the manufacturer's instructions. An extract containing 1 mg of lyophilized material per milliliter had a corresponding total protein concentration of 1 mg/ml.

### Human sera

Serum samples were collected from Spanish individuals with allergic rhinitis and/or asthma and skin tests positive to a standardized olive pollen extract (C.B.F. LETI, S.A.). The presence of specific IgE (classes 3 to 4) was confirmed by CAPFEIA system (Pharmacia Biotech; Uppsala, Sweden). All samples were stored in 1 ml aliquots at  $-20^{\circ}\text{C}$  and pooled just before use.

### Gel filtration chromatography

The *O. europaea* extract was fractionated by gel filtration chromatography with a  $90 \times 3$  cm Sephadex G-75 column equilibrated in phosphate-buffered saline (PBS) 0.01 mol/L (pH 7.4). The column was previously calibrated with dextran blue and a mixture of proteins with known molecular weights: peroxidase (44 kd), soybean trypsin inhibitor (29 kd), and lysozyme (14.3 kd). In each run, 300 mg of lyophilized extract was applied at a flow rate of 50 ml/hr; 8.9 ml fractions were collected. The elution profile was followed by continuous monitoring at 280 nm and divided into four pools of fractions in descending order of molecular weights. These pools were dialyzed overnight against distilled water and lyophilized. After measurement of their protein concentrations, these fractions were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### Ion exchange chromatography

Three hundred milligrams of lyophilized material from fraction 2 were resuspended in Tris/HCl 0.01 mol/L (pH 6.08) and applied to a  $20 \times 1.6$  cm diethylaminoethyl cellulose column containing 15 ml of gel, previously equilibrated in the same buffer at a flow rate of 50 ml/hr. The unbound proteins were washed obtaining a first peak according to the monitoring at 280 nm. Three new peaks were obtained by increasing the ionic strength to 0.05, 0.3, and 1 mol/L NaCl. The fractions from each peak were pooled and extensively dialyzed against distilled water, lyophilized, resuspended, and analyzed by SDS-PAGE.

### Hydrophobic interaction chromatography

Twenty milligrams of lyophilized material was resuspended in Tris/HCl 0.05 mol/L buffer (pH 7.4) containing 5 mol/L NaCl and applied to a  $20 \times 1.6$  cm column with 5 ml of phenylsepharose CL4B gel (Pharmacia Biotech). This column was previously equilibrated in the same solution at a flow rate of 50 ml/hr. Fractions of 10 ml were collected and monitored at 280 nm. Stepwise elution of the column took place using the former buffer with decreasing concentrations of NaCl (5, 2, and 0.5 mol/L and distilled water). A peak for each step was obtained. The fractions were pooled, dialyzed against distilled water for 3 days at  $4^{\circ}\text{C}$ , lyophilized, redissolved, and their protein content was determined and analyzed by SDS-PAGE.

### SDS-PAGE and immunoblotting

SDS-PAGE was performed essentially according to the method of Laemmli<sup>23</sup>; 14% wt/vol polyacrylamide separating gel and a 4% wt/vol stacking gel were used. The chromato-

**TABLE I.** IgE binding prevalence of 20 Spanish sera to allergenic components in an *O. europaea* extract

| Allergen (kd) | IgE prevalence (%) |
|---------------|--------------------|
| 70            | 75                 |
| 45            | 75                 |
| 42            | 50                 |
| 32            | 80 (Ole e 4)       |
| 28            | 60                 |
| 20            | 40 (Ole e 1)       |
| 16            | 35 (Ole e 5)       |
| 10            | 100                |

graphic pools of fractions and the starting material were run in a solution of Tris-HCl 62.5 mmol/L buffer containing 0.1% SDS. Samples under reduced conditions were dissolved in a solution of Tris-HCl 62.5 mmol/L buffer (pH 6.8), 10% glycerol, 2% SDS, and 5% of 2- $\beta$ -mercaptoethanol. Before application to the gel, samples were heated at  $95^{\circ}\text{C}$  for 10 minutes. In some cases, samples were run in native conditions in a solution of Tris-HCl 62.5 mmol/L buffer (pH 6.8), 10% glycerol, and 2% SDS without heating. Bio-Rad low range standards (Bio-Rad; Richmond, Calif.) were used as molecular weight markers. The run was carried out in a refrigerated system and the gel was stained with 0.1% Coomassie brilliant blue (R250) in water: methanol:acetic acid (50:40:10) and destained in the same solution.

The immunoblotting was performed in a Tank Blot (Bio-Rad) apparatus. Proteins of the gel were electroblotted to Immobilon-P membranes (Millipore, Bedford). After transferring, the blots were allowed to dry completely and the sheets incubated with a pool of sera diluted 1:6 vol/vol in a blocking buffer consisting of 1% human serum albumin in PBS 0.1% Tween 20 for 2 hours at room temperature. For the assay of individual sera, the sheets were cut into strips and incubated with an individual serum sample diluted at one-third vol/vol in the former blocking solution. After five washing steps with PBS 0.1% Tween 20, the sheets were incubated with horseradish peroxidase-labeled mouse monoclonal anti-human IgE at 2  $\mu\text{g/ml}$  (anti-IgE-HRP; Ingenasa; Madrid, Spain) for 2 hours at room temperature. After five washing steps, the enzymatic reaction of the peroxidase was developed by adding a solution of 0.1 mol/L citrate buffer (pH 5.0) containing 10 mmol/L ethylenediamine tetraacetic acid and 1% dextran sulfate (1 ml), tetramethylbenzidine (TMB; Boehringer Mannheim, Germany) in dimethyl sulfoxide (DMSO; Merck, Germany) (20  $\mu\text{l}$ ) and 1 mol/L  $\text{H}_2\text{O}_2$  (2  $\mu\text{l}$ ).

### Isoelectric focusing

Whole *O. europaea* extract and its two purified allergens were subjected to analytical isoelectric focusing in a Bio-Rad apparatus (Biophoresis) according to the manufacturer's instructions. Gels were prepared with pH 3-5 (Serva; Heidelberg, Germany) pH 5-8 ampholytes (Biolyte, BioRad) at a dilution (1:40) previously found to be suitable for focusing pollen allergens. Gels were previously fixed with a solution containing 4% 5-sulfosalicylic acid dihydrate, 12.5% trichloroacetic acid, and 30% methanol for 30 minutes and stained with Coomassie brilliant blue (R-250) in 27% ethanol, 10% acetic acid and

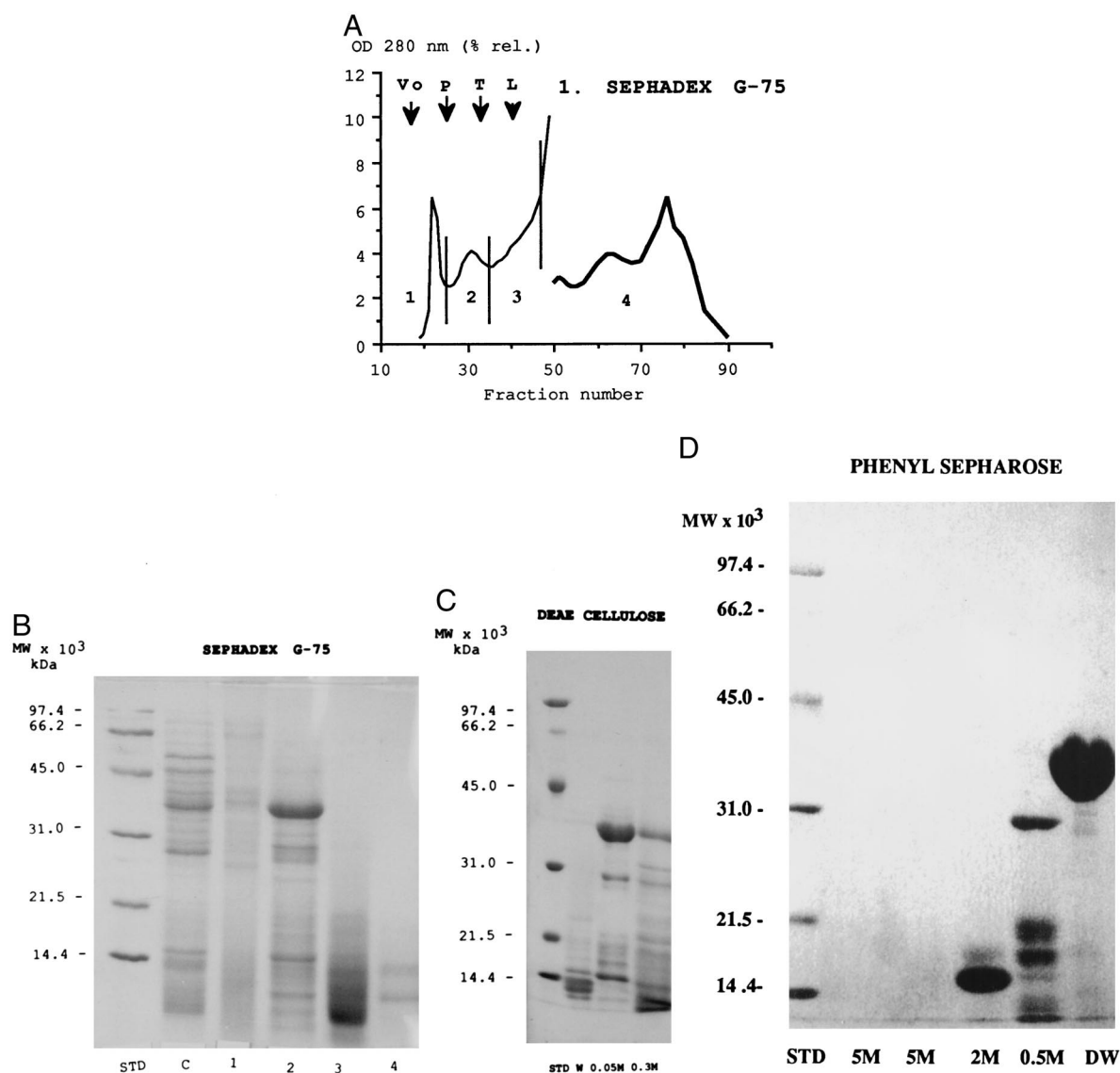


**FIG. 1.** Scheme of purification of Ole e 4 and Ole e 5 from *O. europaea* extract. **A**, Elution profile of gel filtration chromatography on Sephadex G-75 of extract. Molecular weight markers are *Vo*, dextran blue; *P*, peroxidase (44 kd); *T*, trypsin inhibitor (29 kd); and *L*, lysozyme (14.3 kd). **B**, SDS-PAGE analysis of extract (*C*) and four pool fractions obtained from gel filtration (1, 2, 3, and 4). **C**, SDS-PAGE analysis of protein fractions eluted at different concentrations of NaCl from DEAE-cellulose (*W*, washed column; 0.05 and 0.3 mol/L NaCl) arisen from pool fraction 2. **D**, SDS-PAGE analysis of protein fractions eluted from phenylsepharose with decreasing concentrations of NaCl (5, 2, and 0.5 mol/L NaCl and *DW*, distilled water) arisen from 0.05 mol/L fraction of the DEAE-cellulose. The Ole e 5 is located in 2 mol/L fraction and the Ole e 4 in *DW* fraction.

0.5% copper (II) sulfate for 2 hours in constant shaking; 12% ethanol, 7% acetic acid, 0.5% copper (II) sulfate was used for destaining gels. In some gels, silver staining was used.

#### Enzyme immunoabsorbent assay for specific IgE

Whole olive pollen extract and the two purified allergens were coated to the wells of flat-bottomed microtiter plates (Immulon 4, Chantilly). Allergen coating was done by dissolving allergenic preparations at 5 µg/ml in 0.2 mol/L sodium bicarbonate buffer (pH 9.2) and incubating 100 µl aliquots per well overnight in a humid chamber. The coated wells were blocked with 1% human serum albumin in PBS at room temperature for

30 minutes. After washing with PBS 0.1% Tween 20, 100 µl aliquots of a serum pool containing high titers of specific IgE to olive pollen allergen were incubated overnight at room temperature. After three washing steps, 100 µl of anti-IgE-HRP at 1 µg/ml diluted in a blocking solution containing PBS 0.1% Tween 20, 25% fetal calf serum, and 1% heparin were added and left for 2 hours at room temperature. After washing, the peroxidase was evaluated by incubation with a mixture of 0.16 mol/L acetate buffer (pH 6) (1 ml), TMB in DMSO (20 µl) and 1 mol/L H<sub>2</sub>O<sub>2</sub> (2 µl). The enzymatic reaction was stopped by the addition of 100 µl 2N sulfuric acid. Optical densities were read at 450 nm in a microplate reader

**TABLE II.** Amino acid sequence of two internal regions of Ole e 4

|                    |                                     |
|--------------------|-------------------------------------|
| Ole e 4 fragment 1 | ... A F A N T G V E I V ...         |
| Ole e 4 fragment 2 | ... S I D T Y L F S L Y D E D K ... |

(Titertek Multiskan, Finland) and plotted against serum dilution.

### IgE inhibition experiments

The olive pollen extract and the two purified allergens were tested for allergic potency by an enzyme immunosorbent assay inhibition. Briefly, 125  $\mu$ l aliquots of serial dilutions of the allergenic extract and purified allergens were mixed with 125  $\mu$ l of a serum pool from allergic patients at dilution of 1:32. These mixtures were gently shaken for 2 hours at room temperature. After the inhibition period, 100  $\mu$ l was incubated overnight in duplicate in *O. europaea* coated plates. Specific IgE bound to the wells was determined as before. Allergenic activities were evaluated by establishing the 50% inhibition points from double-log plots of percent inhibition versus protein content.

### Amino acid sequencing

Two hundred fifty micrograms of purified protein in 1 ml PBS were applied onto a PVDF membrane with the use of a ProSpin cartridge (Applied Biosystems) to determine the N-terminal protein sequence of Ole e 5. After thorough washing, the membrane was directly sequenced with an ABI 473A pulsed liquid phase protein sequencer (Applied Biosystems) with the use of modified cycles according to manufacturer's instructions.

The N-terminal of Ole e 4 was blocked. Ole e 4 was fragmented by trypsin digestion to obtain internal protein sequences. Gel slices containing the purified protein were washed in 200 mmol/L ammonium carbonate (pH 8.9) at 30° C for 20 minutes and hydrated with 200 mmol/L ammonium carbonate (pH 8.9) 0.02% Tween 20. Trypsin (sequencing grade, Sigma) was added at 1:4 (wt:wt) and incubated overnight at 30° C. Digestion was stopped with 1.5  $\mu$ l of trifluoroacetic acid (TFA). Two peptide extractions were done with the addition of 100  $\mu$ l 60% acetonitrile, 0.1% TFA at 30° C for 20 minutes, and concentrated in a speed/vac to a final volume of 20  $\mu$ l. Peptides were injected into a  $\mu$ RPC C2C18 SC (2.1  $\times$  10 mm) reverse-phase column connected to a SMART micro-HPLC system (Pharmacia). A trypsin digestion control was run in parallel. Peptides were separated at 100  $\mu$ l/min with the chromatographic conditions described elsewhere,<sup>24</sup> and fractions were collected by automatic integration of the peaks. Two peaks eluting at 71 and 74 minutes were sequenced. The selected fractions were then applied onto polibrene-coated glass fiber filters and subjected to sequencing.

Both sequences were registered in the SWISS-PROT sequence database with accession numbers P80741 and P80740 for the Ole e 4 and Ole e 5, respectively.

### Statistical analysis

The regression analysis of the IgE inhibition curves was assessed by linear regression ( $r_1$ ). The calculation was performed with the Cricket Graph program in an Apple Macintosh computer.

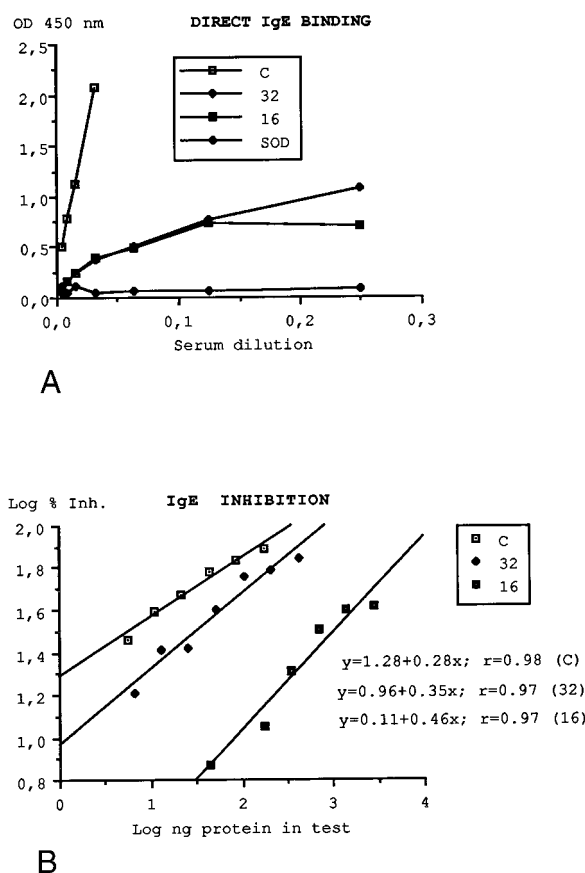


**FIG. 2.** A, Direct IgE binding to a whole *O. europaea* extract (C), Ole e 4 (32), Ole e 5 (16), and bovine SOD (SOD, *Bos taurus*). B, IgE inhibition experiments. The 50% inhibition points for the whole extract and Ole e 4 were 31.4 and 129 ng, respectively. The 40% inhibition point for Ole e 5 was 1.75  $\mu$ g.

## RESULTS

### Identification of olive pollen allergens by IgE immunoblotting

The sera of 20 individuals with allergies were used to evaluate IgE reactivity. The IgE binding pattern is shown in Table I. Various components in the range of 10 to 100 kd bound specific IgE in a variable frequency. Allergens with molecular weights of 10, 32, 70, 45, and 28 kd showed a high frequency of recognition, whereas the recognition rate for allergens with molecular weights of 42, 20 (Ole e 1) and 16 kd was lower.

### Purification of the Ole e 4 and Ole e 5

The *O. europaea* extract was fractionated by gel filtration chromatography and divided into four fractions (Fig. 1, A and B). Ole e 4 was concentrated in fraction 2. This fraction was further purified by ion-exchange chromatography (DEAE cellulose). A high amount of Ole e 4 as well as lower amounts of the 28 and 16 kd allergens were located in fractions eluted with 0.05 mol/L NaCl (Fig. 1, C). These fractions were

**TABLE III.** N-terminal sequence alignments of Ole e 5 with SOD sequences of different species

|                                         | 10              | 20                | 30                 | % homology<br>in 29 aa |
|-----------------------------------------|-----------------|-------------------|--------------------|------------------------|
| Ole e 5 ( <i>Olea europaea</i> )        | VKAVTVLNSSE     | PHGIVYFAQ         | EGDGPTTV . . . . . |                        |
| <i>Spinacia oleracea</i> (spinach)      | M-KAV-VL-SSEG-- | G-VYFAQEGD        | GPTTV . . . . .    | 79.3                   |
| <i>Ipomoea batatas</i> (sweet potato)   | VKAV-VL-SSEG--  | G---F-QEGD        | GPTTV . . . . .    | 72.4                   |
| <i>Zea mays</i> (maize)                 | VKAV-VL-SSEG--  | G---F-QEGD        | GPTTV . . . . .    | 72.4                   |
| <i>Oryza sativa</i> (rice)              | VKAV-VL-SSEG--  | G---F-QEGD        | GPTTV . . . . .    | 68.9                   |
| <i>Lycopersicum esculentum</i> (tomato) | VKAV--LNSSEG--  | G---F-Q-G--       | PTTV . . . . .     | 65.5                   |
| <i>S. cerevisiae</i> (baker's yeast)    | VKAV-VL-----    | -----TV . . . . . |                    | 27.5                   |
|                                         | 200             | 210               | 220                |                        |
| <i>Escherichia coli</i>                 | VKAV-VL-----    | G-----            | . . . . .          | 24.1                   |
|                                         |                 | 320               | 330                | 340                    |
| <i>Mus musculus</i> (mouse)             | TVK--TVL-----   | P---V-----        | . . . . .          | 24.1                   |
|                                         |                 | 280               | 290                | 300                    |
| <i>Homo sapiens</i>                     | FVK-VTVL-----   | G-----            | . . . . .          | 24.1                   |
|                                         | 14              | 20                | 30                 | 40                     |
| <i>Bos taurus</i> (bovine)              | V-A--VL-S-----  | G-----            | . . . . .          | 20.7                   |

submitted to hydrophobic interaction chromatography (phenylsepharose). Ole e 4 was purified in fractions eluted with distilled water according to SDS-PAGE patterns; Ole e 5 was purified in fractions eluted with 2 mol/L NaCl (Fig. 1, D).

#### Biochemical characterization of Ole e 4 and Ole e 5

The two purified allergens were run in SDS-PAGE under native and reduced conditions to verify whether or not they consisted of a single or several polypeptidic chains. Ole e 4 and Ole e 5 exhibited the same molecular weights under both conditions. Ole e 4 has at least two isoforms with pIs in the range of 4.6 to 5.1 detected by Coomassie brilliant blue and silver staining. The Ole e 5 has at least five isoforms within the pIs range of 5.1 to 6.5.

#### Immunochemical characterization of Ole e 4 and Ole e 5

The two purified allergens and the whole *O. europaea* extract were tested for specific IgE binding by immunoblotting; Ole e 4 revealed a stronger IgE binding capacity than did Ole e 5. Direct IgE binding experiments, comparing the whole extract with its corresponding isolated allergens, were done by coating the same protein amount per well (0.5 µg) (Fig. 2, A). The experiments revealed that IgE binding to the whole *O. europaea* extract was greater than to the two purified allergens and that the amount of IgE bound to Ole e 4 was greater than to Ole e 5 at a serum dilution of 1:4. However, at higher serum dilutions (1:8 to 1:256), IgE binding by both allergens was similar. IgE inhibition assays are shown in Fig. 2, B. Ole e 4 required four times more protein to produce 50% inhibition than the crude extract (129 and 31 ng, respectively). The whole extract reached a maximum of 92% inhibition; Ole e 4 reached 75% (Fig. 2, B). Ole e 5 required 1.75 µg to achieve 40% inhibition.

#### Partial amino acid sequencing of Ole e 4 and Ole e 5

The amino acid sequence of two internal regions of Ole e 4 and the N-terminal of Ole e 5 are shown in Tables II and III. The sequences of the two fragments of Ole e 4 did not show any homology with other known proteins on the basis of the Protein Sequence Data Bank explored (SWISS-PROT). However, the N-terminal sequence of Ole e 5 showed a high degree of homology with SODs of several plant species and less homology with SODs from distant phylogenetic species (Table III).

Bovine erythrocytes SOD (Sigma) was analyzed by SDS-PAGE in parallel with the two purified allergens, confirming that Ole e 5 and bovine erythrocyte SOD have a similar molecular weight. Bovine erythrocyte SOD was tested for specific IgE binding; no binding was detected (Fig. 2, A). Both SODs show a homology of 20.7% in 29 amino acids of the N-terminal.

#### DISCUSSION

This study describes the allergenic composition of olive pollen collected in the United States with Spanish sera and the characteristics of two purified allergens. The IgE binding pattern of this pollen differs from previous reports, especially when Spanish pollen was used.<sup>7, 13</sup> A great variability in the allergenic composition of *O. europaea* extracts has been described,<sup>8, 25</sup> and it has been demonstrated that olive trees from different cultivators produce pollen with different allergenic characteristics. In one of these studies,<sup>25</sup> Ole e 1 was only detected in 47% of the pollen extracts analyzed by immunoblotting. This report challenged the idea of Ole e 1 being the main allergen. These discrepancies are not clear but may be due to different soil nutrients, genetic differences, and strains and/or climatic conditions.

The IgE reactivity pattern described herein is more uniformly distributed than in other published reports, in which most allergens are mainly located in the low-

molecular-weight range. The SDS-PAGE profile of this extract is also different.<sup>5,9,10,16</sup> Surprisingly, the frequency of IgE binding to Ole e 1 was low (40%) in contrast with previous studies that reported an IgE binding frequency higher than 80%.<sup>7,13</sup> Such differences in allergenic composition can be attributed to the source of the pollen used and to the content of specific allergens. The Ole e 1 content of this extract was 15 µg of Ole e 1/mg of protein. This quantity can be considered as low because extract from other sources may contain up to three times more allergen. Extracts prepared with pollen collected in Spain regularly contain higher concentrations of Ole e 1. This fact is supported by current studies carried out in our laboratory that show differences in the allergenic composition of various lots of pollen collected in Spain and in the United States. The clinical implications of these findings remain to be established.

According to immunoblotting results, several allergens with molecular weights of 10, 32, 70, 45, 28, 42, 20, and 16 kd have been identified. Along with Ole e 1, some of these allergens (42, 28, and 10 kd) have been previously reported.<sup>4,12,13,18</sup> We concentrated our efforts toward the purification of Ole e 4 because this allergen fixed IgE at high serum dilutions (1:5 to 1:10). Both allergens are not attached by disulfide bridges or by noncovalent linkages, since they show the same molecular weight under native and reduced conditions. Ole e 4 has at least two isoforms in the pI range of 4.6 to 5.1. Ole e 5 has at least five isoforms with isoelectric points ranging from 5.1 to 6.5, similar to Ole e 1.<sup>12</sup> Thus Ole e 5 shows a higher degree of polymorphism than does Ole e 4.

IgE binding to Ole e 4 and Ole e 5 was studied by immunoblotting, direct IgE binding, and IgE inhibition. The allergenicity of Ole e 4 and Ole e 5 was not altered during the chromatographic steps because they retained their ability to bind specific IgE. Ole e 4 fixed higher amounts of IgE than did Ole e 5 by immunoblotting. However, these allergens captured similar amounts of specific IgE in solid-phase assays. In IgE inhibition experiments, Ole e 4 exhibited a relatively high inhibition capacity; the inhibition capacity of Ole e 5 was lower.

The N-terminal sequence of Ole e 5 shows a high degree of homology with SODs. Recently, an allergen isolated from *Aspergillus fumigatus* by recombinant technology has been identified as a manganese SOD that was able to elicit a specific immediate type allergic skin reaction as well as to induce proliferation of peripheral blood mononuclear cells.<sup>26</sup> It has been reported that in rare cases, the treatment with orgotein (a SOD from bovine liver) is able to produce an anaphylactic, IgE-mediated reaction.<sup>27-29</sup> According to our data, bovine SOD failed to bind IgE from a serum pool. This fact allowed us to conclude that the IgE binding sites of Ole e 5 are located on nonhomologous regions.

In summary, we describe the identification, purification, and characterization of two new allergens of *O.*

*europaea* pollen. Ole e 4 is an important allergen with an elevated IgE binding capacity and unknown function, according to sequencing data. Ole e 5 is a minor allergen that has a great homology with SOD. These results, together with previous publications, demonstrate that olive pollen contains a complex mixture of allergens that can vary largely, depending on the allergen source.<sup>25,30</sup> Further studies are needed to characterize the complex composition of olive pollen extracts.

Protein sequences were performed at the protein chemistry facilities of the Centro of Molecular Biology, Madrid, Spain. Ole e 4 and Ole e 5 have been submitted to WHO/IUIS Allergen nomenclature Subcommittee for their approval.

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