

Cloning and expression of the panallergen profilin and the major allergen (Ole e 1) from olive tree pollen

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Background: Olive tree (*Olea europaea*) pollen allergy is one of the main causes of allergy in Mediterranean countries and some areas of North America.

Objective: To clone olive allergens and to characterize immunologically the purified recombinant allergens.

Methods: Full-length complementary deoxyribonucleic acid (cDNA) strands encoding olive allergens (Ole e 1) were cloned by polymerase chain reaction amplification and sequenced. Recombinant proteins were produced in *Escherichia coli* by the use of two different expression systems. Immunoreactivity of the recombinant proteins was tested by ELISA and Western blot with serum from patients with allergy to olive.

Results: Significant sequence polymorphism was found in both allergens. The panallergen profilin was expressed as a nonfusion protein and was purified to homogeneity after a single step of affinity chromatography with a poly-L-proline Sepharose column. One cDNA encoding an Ole e 1 isoform was expressed as a fusion protein consisting of the glutathione S-transferase of *Schistosoma japonicum* and Ole e 1. The fusion protein was purified to homogeneity by gel filtration chromatography and affinity chromatography with a glutathione-Sepharose column, and digested with thrombin. Both recombinant allergens shared B cell epitopes with the corresponding natural allergens.

Conclusion: IgE-reactive Ole e 1 and olive profilin expressed in bacteria were purified after simple chromatographic procedures and may be useful for diagnostic purposes.

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Key words: Ole e 1, profilin, recombinant allergens, olive tree pollen, cDNA cloning, expression, IgE binding

Atopic (IgE-mediated) allergy is a clinical disorder that affects up to 20% of the population in developed countries. Allergens for diagnostic and therapeutic pur-

Abbreviations used

BSA:	Bovine serum albumin
cDNA:	Complementary deoxyribonucleic acid
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetic acid
GST:	Glutathione-S-transferase
IPTG:	Isopropyl-thio- β -galactoside
mRNA:	Messenger ribonucleic acid
PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
PLP-Sepharose:	poly-(L-proline)-CNBr activated Sepharose
PVDF:	Polyvinylidene difluoride
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

poses are extracted from natural sources, such as pollens. The allergenic content of these extracts may vary depending on environmental conditions. Furthermore, the allergenic content may be unstable due to proteases, and it is difficult to characterize. These problems can now be overcome by cloning and expression of the genes coding for the more common allergens, as the recombinant products are expected to represent pure, well-defined, and reproducible compounds.¹

Pollen allergies depend on environmental aspects such as geographical and seasonal conditions. Among seasonal allergies, olive tree (*Olea europaea*) pollen allergy is an important environmental health problem in Mediterranean countries and some areas of North America.² The major allergen from olive tree pollen (Ole e 1), which produces IgE reactivity in >70% of patients allergic to olive, exhibits glycosylated (20 kDa) and nonglycosylated (18.5 kDa) variants.³ A complementary deoxyribonucleic acid (cDNA) encoding Ole e 1 has been recently cloned.⁴ Profilins, a ubiquitous family of proteins that control actin polymerization in eukaryotic cells,⁵ are prominent allergens in pollen of trees, grasses, and weeds and are recognized by 20% of all patients allergic to pollen.^{6,7} Because of this broad allergic incidence, profilins have been described as panallergens.^{7,8}

Precise in vivo and in vitro diagnostic procedures can be achieved with a defined panel of recombinant aller-

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The nucleotide sequences reported have been submitted to the GeneBank, European Nuclear Biology Laboratory, and DNA Data Bank of Japan, and DDBJ Nucleotide Sequences Databases under accession numbers Y12425 (OePRO1), Y12429 (OePRO2), Y12430 (OePRO3), Y12428 (OLE4), Y12426 (OLE5), and Y12427 (OLE6).

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1	ATGTCGTGGCAGGCGTACGTTGATGATCATTGATGTGTGACATTGAGGGCCATGAAGACCACCGCTCACCGCAGCTGCCATCGTCGGCCATGACGGTTCT	102
1	M S W Q A Y V D D H L M C D I E G H E D H R L T A A A I V G H D G S	34
	OePRO2 -G-	
	OePRO3 -G-	
	G	
103	GTTTGGGCTCAGAGCGCTACCTTCCTCAGTTCAGCCAGAGGAGATGAATGGAATTATGACAGATTCAATGAACCTGGTCATCTGGCACCAACAGGCCTA	204
35	V W A Q S A T F P Q F K P E E M N G I M T D F N E P G H L A P T G L	68
205	CATCTTGGAGGAACAAAGTATATGGTGTATCAAGGAGAGGCTGGAGCTGTCCGTGGAAGAAGGGATCTGGTGGTATTACCATTAAGAAGACTGGCCAA	306
69	H L G G T K Y M V I Q G E A G A V I R G K K G S G G I T I K K T G Q	102
	OePRO2 --C	
307	GCACGTAGTTTTTGGTATCTACGAGGAGCCTGTAACTCCAGGACAATGCAACATGGTGTGCGAGAGGTGGGAGACTACCTGGTCAACAAGGCATGTAA	405
103	A L V F G I Y E E P V T P G Q C N M V V E R L G D Y L V E Q G M *	135
	OePRO3 -C-	
	A	
	OePRO2 C-T	
	OePRO3 C-A	
	L	
	L	

FIG. 1. Microheterogeneity of olive profilin. Nucleotide (plain typeface) and deduced amino acid (bold typeface) sequences of olive profilins OePRO1, OePRO2, and OePRO3. Only different codons are shown in OePRO2 and OePRO3 sequences, with the corresponding amino acid change when it occurs. Dashes indicate identity of nucleotides with respect to the upper sequence.

gens.^{9,10} Characterization of the individual IgE-reacting pattern may consequently lead to the development of patient-tailored immunotherapy. In the present study we determined the sequence polymorphism of cDNAs encoding the olive tree pollen allergens Ole e 1 and profilin. In addition, immunologically active, soluble, recombinant allergens from *O. europaea* pollen have been produced in *E. coli*.

METHODS

Purification of natural profilin

Olive tree pollen (Allergon AB, Välinge, Sweden) was extracted as previously described¹¹ and stored lyophilized at -20° C until used. Profilin purification was accomplished by affinity chromatography in a poly-(L-proline)-CNBr activated Sepharose (PLP-Sepharose) column.¹² Briefly, the gel was prepared by coupling 30 mg poly-(L-proline) (INC Biochemicals, Aurora, Ohio) to 2 gm CNBr-activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden). The column was washed with 10 volumes of phosphate-buffered saline (PBS), then washed with 4 volumes of 2 mol/L urea in PBS. Finally, the profilin was eluted with 6 mol/L urea in PBS.

Isolation of poly(A⁺)-mRNA and first-strand cDNA synthesis

Poly(A⁺)-enriched messenger ribonucleic acid (RNA) was isolated from 100 mg of olive tree pollen by use of the Quick Prep MicroRNA Purification Kit (Pharmacia Biotech). mRNA (1 mg) was reverse transcribed by use of a first-strand synthesis kit (Pharmacia Biotech) and random hexadeoxynucleotides as primers.

PCR-based cloning and sequencing of olive allergen cDNA

Degenerate oligodeoxynucleotide primer sequences (Genosys Biotechnologies Inc., Cambridge, U.K.) for olive profilin cloning were designed according to previous reported sequences^{6,13-16}: 5'-AGAGAATTCCATATGTCGTGGCA(A/G)(A/G)CGTACGT-3' and 5'-AGAAAGCTT(C/T)TACA(G/T)GCC(C/T)GTGTC(G/A/T)A(G/T/C)(G/A/C)AGGTA-3' (where the slash between bases means that they were added at that

particular position in equimolecular amounts during the synthesis of the primer). Products of the first-strand cDNA synthesis (5 µl) and primers were added to a polymerase chain reaction (PCR) standard mixture. After a denaturation step at 94° C for 4 minutes, the sample was subjected to 5 cycles at 94° C (1 minute), 45° C (2 minutes), and 72° C (2 minutes), followed by 30 cycles at 94° C (1 minutes), 56° C (2 minutes), and 72° C (2 minutes), and a final incubation at 72° C (6 minutes). The predominant band was isolated from the gel (GeneClean, Bio101, La Jolla, Calif.), and after cutting with *NdeI* and *HindIII* it was cloned into the expression vector pKN172.¹⁷ Ole e 1 cDNA was amplified from products of the first-strand cDNA synthesis (5 µl) by the use of primers designed according to a previously reported sequence⁴: 5'-AGAGAATTCCATATGAGGATGTTCCGCAACCT-3' and 5'-AGAAAGCTTCATTACATGTTGGGCGGGTACAT-3'. Amplification conditions were a denaturation step at 94° C for 4 minutes, followed by 35 cycles each at 94° C (1 minute), 45° C (2 minutes), and 72° C (2 minutes), with a final incubation at 72° C (6 minutes). The predominant band was isolated from the gel, and after cutting with *EcoRI* and *HindIII* it was cloned into pBlue-script-II SK (Stratagene, La Jolla, Calif.). Recombinant deoxyribonucleic acid (DNA) techniques were performed by standard methods.¹⁸

Nucleotide sequences were determined by the dideoxy chain termination method¹⁹ according to standard protocols for Taq DNA-polymerase-initiated cycle sequencing reactions with fluorescence-labeled dideoxynucleotide termination (Applied Biosystems Inc., Foster City, Calif.). The sequencing reactions were analyzed on an ABI 373A DNA sequencer (Applied Biosystems Inc.). Commercial or vector-specific primers were used for sequencing both strands. Sequences were analyzed by the GCG Program Package (Genetics Computer Group Inc., Madison, Wis.).

Production and purification of recombinant olive profilin

Competent *E. coli* BL21 (DE3)²⁰ cells were transformed with the resulting recombinant plasmids. The cells were grown in Luria-Bertani medium supplemented with 0.2 mg/ml ampicillin to an absorbance of 0.6 at 600 nm. Isopropyl-thio-β-galactoside (IPTG) was then added to the culture to a final concentration

of 0.6 mmol/L, and incubation continued for another 3 hours. Cells were harvested by centrifugation, and the pellet (resuspended in 50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl) was disrupted by sonication. Insoluble material was removed by centrifugation at 20,000 g for 30 minutes. Profilin in the supernatant was purified by PLP-Sepharose chromatography as described above. Alternatively, cells were resuspended in 10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) containing 0.1 mg/ml lysozyme, and incubated for 15 minutes at 37° C. Dithiothreitol and sarkosyl were added to final concentrations of 5 mmol/L and 1.5%, respectively. After vortexing for 5 seconds, cells were mildly sonicated and the lysate was clarified by centrifugation. The supernatant, after the addition of Triton X-100 (final concentration of 2%), was purified by PLP-Sepharose chromatography as described above. Fractions containing protein were pooled, dialyzed overnight against water, lyophilized, and dissolved in PBS.

Construction of Ole e 1 expression plasmid

After sequencing, cDNA clone OLE5 was subcloned into the *EcoRI/XhoI* sites of a pGEX-4T-1 expression vector (Pharmacia Biotech). The recombinant protein was produced after transforming *E. coli* BL21 competent cells with the resulting plasmid, called pIO35. This construction encodes a fusion protein (GST-Ole e 1) consisting of glutathione S-transferase (GST) from *Schistosoma japonicum* and a 6-amino acid peptide spanning the thrombin cleavage site, followed by the Ole e 1 polypeptide.

Production and purification of recombinant Ole e 1

Overnight cultures of *E. coli* BL 21 cells containing pIO35 were diluted to an $OD_{600} = 0.2$ in Luria-Bertani medium containing 0.1 mg/ml ampicillin. Cells were grown up to an absorbance of 0.6 nm and induced with 0.6 mmol/L IPTG. After incubation for 3 hours, cells were harvested by centrifugation at 4000 g for 10 minutes at 4° C. The pellet was resuspended in 50 mmol/L Tris-HCl pH 8.0, 20 mmol/L NaCl containing 0.1 mg/ml lysozyme and incubated for 20 minutes at 37° C. Triton X-100 and dithiothreitol were added to a final concentration of 1% and 5 mmol/L, respectively, and the lysate was centrifuged at 22,000 g for 20 minutes at 4° C. The precipitated material was resuspended in 0.1 mol/L glycine/NaOH at pH 9.0 containing 8 mol/L urea, and passed through a Sephadex G-25 column (Pharmacia Biotech) equilibrated in 0.1 mol/L glycine/NaOH at pH 9.0. The eluted protein was stored overnight at 4° C for refolding. The fusion protein was bound overnight at 4° C to 1 ml of glutathione-Sepharose 4B (Pharmacia Biotech) equilibrated in PBS and 1% Triton X-100. After packing and washing cells with PBS, fusion protein was eluted with 10 mmol/L reduced glutathione in 50 mmol/L Tris-HCl at pH 8.0. Fractions containing GST-OLE4 fusion protein were dialyzed against 10 mmol/L Tris-HCl at pH 8.0 and lyophilized. The fusion protein in 50 mmol/L Tris-HCl at pH 8.0 and 5 mmol/L EDTA was digested with 10 units of bovine thrombin per milligram of protein at room temperature for 16 hours. The recombinant allergen was separated from GST on a second glutathione-Sepharose column. Alternatively, the GST-OLE4 fusion protein was cleaved in the column with thrombin and the recombinant Ole e 1 was eluted.

Electrophoresis and immunoblotting

Proteins were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to

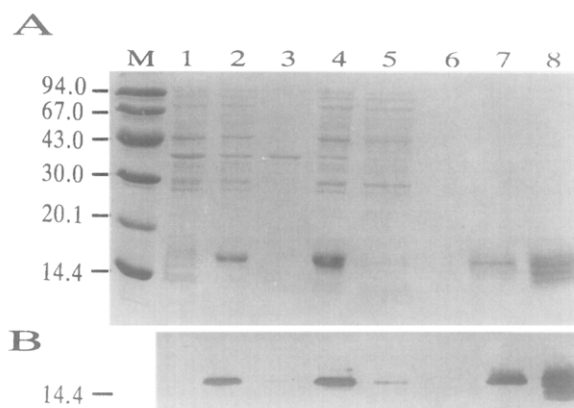


FIG. 2. Purification of recombinant olive profilin expressed in *E. coli*. **A**, Results of 12% SDS-PAGE analysis of the purification procedure. **B**, Western blotting of material in **A** with rabbit anti-profilin sera. Lane *M*, Molecular mass markers; lanes 1 and 2, total cell lysate from *E. coli* BL21 (DE3) containing pKN172 alone and pKN172 with OePRO1 insert, respectively; lanes 3 and 4, insoluble and soluble fractions of sample in lane 2, respectively; lane 5, PLP-Sepharose column nonretained fractions pool; lane 6, 2 mol/L urea-washed fractions pool; lane 7, 6 mol/L urea elution of profilin from PLP-Sepharose column; lane 8, natural olive profilin.

Laemmli²¹ in 12.5% polyacrylamide gels under reducing conditions. Proteins were visualized by Coomassie Brilliant Blue R-250 staining or electrophoretically transferred to polyvinylidene difluoride (PVDF, Immobilon-P membranes, Millipore Corp., Bedford, Mass.).²² For IgE binding analysis, membranes were incubated overnight at 4° C with sera from patients allergic to olive. Alternatively, the membranes were incubated for 60 minutes at 37° C with rabbit polyclonal serum against olive pollen extract or sunflower purified profilin, diluted 1:1000 or 1:3000, respectively. Immunochemical staining was performed as previously described.¹¹

Inhibition experiments

For ELISA inhibition assays, microtiter plates (A/S Nunc, Roskilde, Denmark) were coated with 5 µg/well of olive pollen extract in 0.1 mol/L bicarbonate buffer at pH 9.6 and blocked with blocking buffer (PBS supplemented with 0.05% Tween 20 and 1% bovine serum albumin [BSA]). The 0.1 ml aliquots of a serum pool (1:4 diluted in blocking buffer) from patients allergic to olive pollen were previously incubated with different concentrations of the inhibitor and were subsequently used for ELISA experiments. For immunoblot inhibition studies, 1 ml of 1:2 diluted pool of sera from patients allergic to olive pollen was incubated overnight at 4° C with 10 µg of purified proteins, 10 µg BSA (negative control), or 0.5 mg of olive pollen extract. The preadsorbed serum was then used for immunoblot experiments.

RESULTS

Amplification of olive profilin cDNA and sequence analysis

PCR amplification of cDNAs with the use of degenerate primers produced two main fragments of approximately 400 and 310 bp. The largest one, corresponding in size with the full-length profilin gene, was subcloned in the expression vector pKN172. Three full-length

	1	25	50	75
Ole e 1	MEDVPQPPVSQFHIQGQVYCDTCRAGFITELSEFIPGASLRLQCKDKENGDTVTFTEVGYTRAEGLYSMLVERDHK			
	I	PSR	V R I K S	I
OLE4			V.....I.....	
OLE5		SR.....	V...RE...I...I.....	
OLE6			V.....I.....	
	100	125	146	
Ole e 1	NEFCEITLISSGRKDCNEIPTEGWAKPSLKFKLNTVNGTTRTVNPLGFFKKEALPKCAQVYNKLGMYPPNM			
		I D	Y	
OLE4			R.Y.....	
OLE5		D...I.....		
OLE6		S.....	Y.....	

FIG. 3. Comparison of deduced amino acid sequences of cDNAs encoding Ole e 1 with that obtained by Edman degradation of natural Ole e 1.³ Points indicate identity with respect to the main sequence of Ole e 1.

cDNAs, OePRO1, OePRO2, and OePRO3, were obtained by this method. All of them had a length of 405 bp and encoded a polypeptide of 134 amino acids with a predicted average molecular mass of 14.4 kDa and an average isoelectric point value of 5.1. High identity (>98%) was found between the sequences (Fig. 1). Heterogeneity appears at four positions of the primary structure: Asp²⁰→Gly, Val¹²²→Ala, Val¹³⁰→Leu, and Met¹³⁴→Leu. Comparison of the amino acid sequences of the olive profilins revealed high identity to other described plant profilins.^{6, 13-16, 23} Eighteen sequences of plant profilin, belonging to eight different species, were deposited in the European Molecular Biology Laboratory Data Bank (December 1996). Given that these plant profilins share more than 60% identity, clustering was done between sequences with more than 85% identity, revealing eight different groups. Similarities among representative proteins of these groups were apparent throughout the entire sequence, and several regions of high sequence conservation, mainly located in the carbon terminal of the protein, were obvious in the alignment (data not shown). Pairwise alignments indicated 73% to 86% identical amino acids, with the highest identity with profilin from the tree *Betula verrucosa*, and to a lesser extent with those of grass species.

Expression and purification of olive profilins

The T7 system, in which the gene of interest is expressed under the control of $\Phi 10$ promoter, was used. A nonfusion form of olive profilin (OePRO1) was expressed in *E. coli* BL21 (DE3) as an intracellular protein with an estimated molecular mass of 17.2 kDa (Fig. 2). The yield of recombinant profilin produced according to the described conditions (cell density at an OD₆₀₀ = 0.6, 0.6 mmol/L IPTG, and 3-hour expression) was about 70 mg/L of cell culture, with more than 95% in soluble form. No appreciable increase in yield was found at higher cell densities, IPTG concentrations, or

longer expression times. The soluble material was chromatographed over a PLP-Sepharose affinity column. After elution with 6 mol/L urea in PBS, the profilin preparation (about 20 to 30 mg/L of bacterial culture) was homogeneous, as determined by SDS-PAGE (Fig. 2, A). Two more recombinant olive profilins (OePRO2 and OePRO3) were expressed in similar conditions but in both cases only 50% to 60% of the expressed protein was in soluble form. Complete solubilization was carried out with the use of sarkosyl, and binding to the PLP-Sepharose column was increased by adding 2% Triton X-100 to the sample. Passing the sarkosyl-lysed extract without adding Triton X-100 reduced drastically binding efficiency.

Natural profilin (4.5 mg of protein) was isolated from olive pollen extracts (300 mg of protein) by PLP-Sepharose affinity chromatography. The isolated proteins revealed an electrophoretic pattern composed of four bands, all of them immunoreactive, with molecular masses of 17.8, 17.0, 16.0, and 15.2 kDa (Fig. 2).

Amplification of Ole e 1 cDNA and sequence analysis

Primers corresponding to the 5' and 3' ends of the Ole e 1 sequence⁴ were used for cloning by PCR amplification of different isoforms of this allergen. A single band (440 bp) obtained by PCR was cloned into pBluescript II SK, and three clones were sequenced. The cDNA fragment corresponded to the full-length sequences encoding polypeptides with an average molecular mass of 16.5 kDa. The average isoelectric point value (5.9) coincided with the range previously determined for the allergen.^{3, 24, 25} Deduced amino acid sequences for the Ole e 1 variants were compared with the sequences obtained by Edman degradation of Ole e 1³ (Fig. 3). Heterogeneity was found at 13 positions in the amino acid sequences, but with a high proportion of conservative changes. OLE4 and OLE6 clones have only one nonconservative change in relation to natural purified Ole e 1

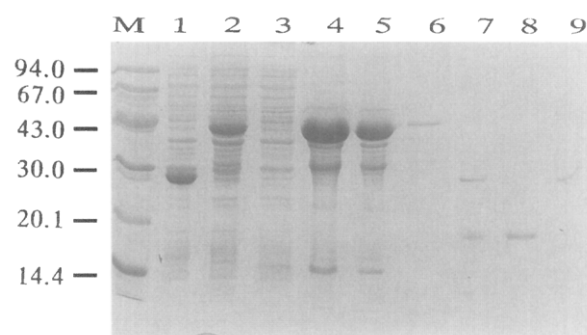


FIG. 4. Twelve percent SDS-PAGE analysis of the purification of recombinant Ole e 1 expressed in *E. coli*. Lane M, Molecular mass markers; lanes 1 and 2, total cell lysate from *E. coli* BL21 containing pGEX-4T-1 and pIO35 (expressing OLE5 insert), respectively; lanes 3 and 4, soluble and insoluble fractions of sample on lane 2, respectively; lane 5, eluted proteins from the G-25 column; lane 6, eluted proteins from the agarose-glutathione column; lane 7, sample from lane 6 after thrombin digestion; lane 8, recombinant Ole e 1; lane 9, GST eluted from the agarose-glutathione column after thrombin digestion.

(Gly¹²⁰→Arg and Asn¹⁰⁸→Ser, respectively), whereas OLE5 presented two nonconservative changes (Asn⁹¹→Asp and Thr⁹⁵→Ile).

According to the International Union of Immunological Societies nomenclature for allergens,²⁶ the proteins encoded by OLE4 and OLE6 are variants of Ole e 1.0101 (the main sequence obtained by Edman degradation³) because they have only minor sequences variations, and therefore they should be called Ole e 1.0102 and Ole e 1.0103, respectively. The protein encoded by OLE5 (<95% sequence identity to Ole e 1.0101) is an isoallergen and should be called Ole e 1.05, following isoform denominations described previously.⁴

Expression and purification of recombinant Ole e 1

The *EcoRI/XhoI* fragment containing cDNA clone OLE5 was subcloned and expressed in the pGEX-4T-1 plasmid. The expression of the insert cDNA fragment generated a fusion protein, GST-OLE5, with an estimated molecular mass of 44 kDa (Fig. 4); mass was calculated by adding the molecular mass of 26 kDa for GST and 18 kDa for Ole e 1. The fusion protein was present at concentrations of 70 to 80 mg/L of culture and represented 30% to 40% of total protein. After lysis, all the recombinant fusion protein appeared as insoluble material, and was solubilized in 8 mol/L urea. After chromatography through Sephadex G-25 columns and overnight renaturation, the soluble material was incubated with glutathione-Sepharose. Digestion with thrombin was performed before or after elution from the glutathione-Sepharose column. A purity of more than 95% was achieved as shown by SDS-PAGE (Fig. 4). The final yield of soluble purified recombinant Ole e 1 was 5 to 10 mg/L of bacterial culture. This low yield could be due to incomplete or incorrect refolding of the protein after denaturation.

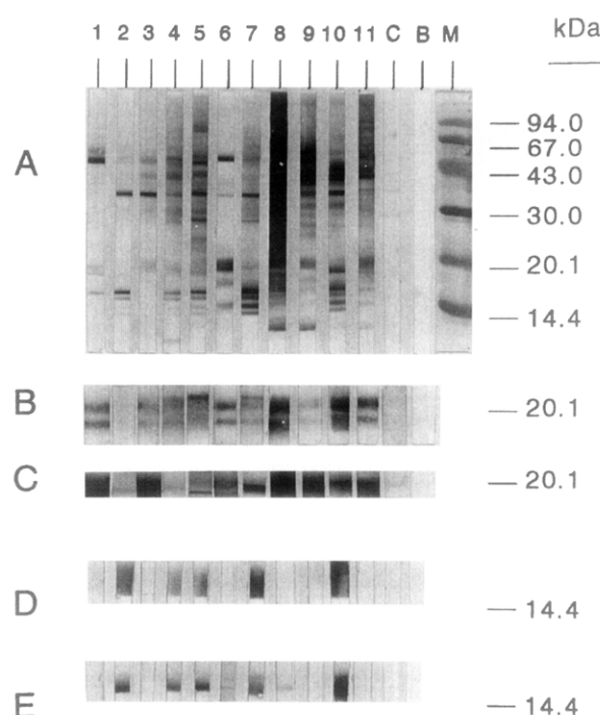


FIG. 5. Immunoblot comparison of the IgE reactivity of patients allergic to olive pollen with natural and recombinant olive allergens. A, Olive pollen extract; B, natural Ole e 1; C, recombinant Ole e 1; D, natural olive profilin; E, recombinant olive profilin. Proteins transferred onto PVDF membranes were probed with serum samples from patients allergic to olive pollen (lanes 1-9, 11), rabbit antisera against olive pollen extract (lane 10 in A, B, and C), or against sunflower profilin (lane 10 in D and E), with serum pooled from healthy individuals (lane C), and with buffer (lane B).

Immunological characterization of recombinant olive allergens

Cellular extracts from recombinants expressing olive profilin as well as purified recombinant profilin were used for immunological assays. After Western blotting and immunostaining, recombinant and natural profilin were recognized by a rabbit anti-serum obtained against sunflower profilin (Fig. 2, B). Five monoclonal antibodies raised against sunflower purified profilin (M.C. Arilla, manuscript in preparation) bound to the three recombinant olive profilins as well as to natural olive profilin (data not shown). Recombinant Ole e 1, either as GST-OLE5 fusion protein or purified allergen after thrombin digestion, was recognized by a rabbit anti-serum raised against olive pollen extract, whereas control GST protein was not recognized. These data demonstrate the existence of B cell epitopes shared by natural and recombinant olive pollen allergens, and common antigenic determinants between olive tree and sunflower pollen profilins.

Serum samples from 10 patients allergic to olive pollen were tested for IgE reactivity to olive pollen proteins and natural and recombinant olive allergens electroblotted after SDS-PAGE (Fig. 5). Natural Ole e 1, purified by electroelution from a preparative gel,

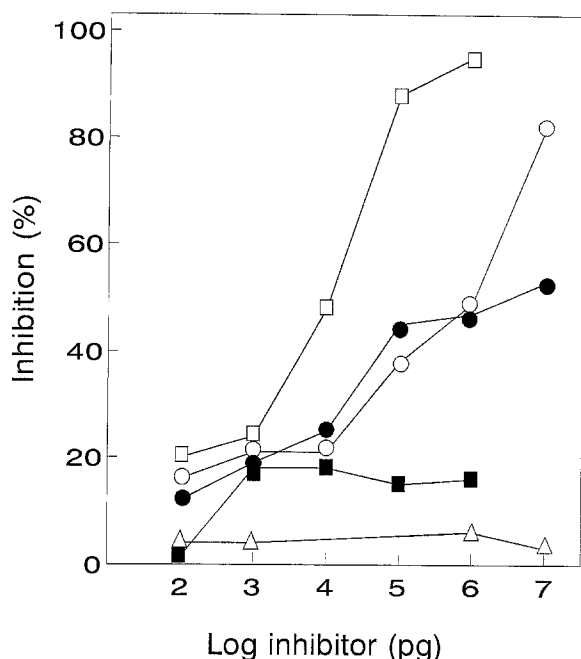


FIG. 6. Competitive inhibition of the binding of IgE antibodies from patients allergic to olive pollen to solid-phase olive pollen extract by olive pollen extract (□), by natural (○) and recombinant (●) Ole e 1 allergen, and by recombinant olive pollen profilin (■). Control experiments were performed with BSA (△).

showed the typical 18 to 20 kDa doublet. All patient sera had comparable IgE reactivity to natural purified allergens (Fig. 5, B and D) and to recombinant allergens (Fig. 5, C and E), indicating the presence of allergenic epitopes in the expressed proteins. Profilin (15 to 17 kDa) was difficult to identify on blots containing crude olive pollen extracts, but it was clearly detected in natural and recombinant profilins blots. The six extra residues, introduced as a consequence of the cloning at the amino terminus of Ole e 1 (Gly-Ser-Pro-Glu-Phe-His), did not change the immunological characteristics of this protein.

Inhibition experiments

For ELISA inhibition experiments, microtiter plates from 21 serum samples from patients allergic to olive were previously preincubated with increasing concentrations of different natural and recombinant olive allergens (Fig. 6). Pretreatment with olive extract abolished IgE binding to olive extract, whereas recombinant OePRO1 preincubation showed low reduction of IgE binding capacity (<20% inhibition). Pretreatment with Ole e 1, both in natural and recombinant forms, showed similar inhibition of IgE binding, but the inhibition was 50% higher when high concentrations of natural Ole e 1 were used.

Similar results were found when olive extracts were blotted onto PVDF membranes after electrophoresis (data not shown). Preincubation of pooled sera with olive extract and BSA served as positive and negative

controls of inhibition, respectively. Pretreatment with recombinant OePRO1 revealed the disappearance of a faint band of 15 to 17 kDa corresponding to olive profilin. Complete abolishment of IgE binding capacity to the Ole e 1 doublet (18 to 20 kDa) was obtained when the sera pool was incubated with natural and recombinant Ole e 1 proteins. In addition, preincubation with natural Ole e 1 resulted in a decrease of IgE binding to an important number of olive tree pollen proteins, which recombinant Ole e 1 did not produce.

DISCUSSION

Great interest exists in the reproducible preparation of pure allergens for diagnostic, therapeutic, and experimental purposes. However, obtaining reproducible batches of allergen from crude extracts derived from natural sources is rather difficult, and some extracts contain insufficient amounts of allergens or partly degraded allergens, such as profilin.²⁷ Recombinant allergens could help to produce pure allergens as well as to find modified molecules that, while maintaining antigenic determinants (i.e., the capacity to induce production of specific antibodies), lack allergenic determinants (i.e., incapacity to bind to mast cells, and thus produce allergic symptoms). The incidence of allergy to the major allergen Ole e 1 and the panallergen profilin in 120 patients allergic to olive pollen from Southern and Eastern Spain was 54% and 25%, respectively (A. Martínez, unpublished results). Due to the importance of these two allergens, a simple and efficient method has been used to clone two olive pollen cDNA molecules, encoding the translated regions of Ole e 1 and profilin from *O. europaea*, and to obtain a high level of expression of these recombinant allergens in *E. coli*.

Three cDNA clones of each olive allergen have been isolated and sequenced. The microheterogeneity observed in both Ole e 1 and profilin is not surprising, because the olive pollen used in this study for mRNA extraction was obtained from a variety of different trees. The existence of different profilin isoforms in the same plant, shown previously in tobacco,²⁸ corn,²⁹ wheat,¹³ *Arabidopsis*,³⁰ and *Phleum pratense* (J. Asturias, submitted for publication), could even be explained on the basis of the important function that profilin plays in regulating the eukaryotic cytoskeleton.³¹ Although the frequency of nucleotide exchanges is much higher than the expected error rate of the Taq polymerase ($1/10^{-5}$ nucleotides per cycle³²) and the number of silent exchanges is far from the statistical probability, the possibility that PCR introduced mutations cannot be completely ruled out. A high degree of polymorphism has been described in plant pollen allergens from different sources: grass, Poa p 9³³; ragweed, Amb a 1³⁴; and tree, Bet v 1.³⁵ Moreover, four recombinant isoforms of Cor a 1 from hazel pollen displayed different antigenic and allergenic properties due to a few amino acid differences.³⁶ Whether the polymorphism found in olive pollen allergens is responsible for different immunological behaviors remains to be investigated in detail, but different

profilin isoform reactivity patterns were observed among patients allergic to olive (Fig. 5 and A. Martínez, unpublished results).

The olive profilin cloned in this work is the first profilin sequence described from a tree not belonging to the Fagales family. Although a high identity was found between plant profilins, olive profilins showed higher homology to birch profilin, *Betula verrucosa*, from the Fagales family. The alignment of the primary sequences and the recently reported three-dimensional structure of the birch profilin³⁷ could allow the identification of conserved segments, which may constitute the first candidates for peptide immunotherapy with allergen-derived T cell and B cell epitopes. This new strategy for the treatment of allergic diseases based on synthetic peptides resembling T and B cell epitopes has been recently reviewed.³⁸ Olive recombinant profilins were produced as nonfusion proteins (about 70 mg/L), but whereas all of the OePRO1 protein was in soluble form, 40% to 50% of the OePRO2 and OePRO3 proteins were found in inclusion bodies. Treatment of inclusion bodies with sarkosyl produced complete solubilization of profilins, but subsequent binding to poly-L-proline could only be achieved in the presence of Triton X-100. Nonionic detergents are reported to be capable of sequestering sarkosyl by forming micelles with it.³⁹ The final yield of purified profilin production (20 to 30 mg/L) is much higher than the profilin yield obtained from natural sources. Our results showed that these heterologous expression systems and purification procedures are suitable for the production of large amounts of pure allergen, which could then be used to characterize allergenic epitopes recognized by T and B cells and, finally, for diagnostic and therapeutic purposes.

The Western blotting and ELISA techniques we used allow the study of both sequential and conformational antigenic determinants. All immunochemical assays performed on recombinant olive profilin showed that its antigenicity and allergenicity are very similar to those of the natural purified allergen. Recombinant Ole e 1 inhibited completely IgE binding to Ole e 1, but was unable to produce the same degree of inhibition of natural Ole e 1 allergen in ELISA inhibitions experiments (Fig. 6), and the IgE binding pattern in immunoblot inhibition was also different (data not shown). Cross-reactivity between Ole e 1 and unrelated glycoproteins due to an epitope in the glycan moiety of this allergen has been recently reported.⁴⁰ The absence of this epitope in recombinant Ole e 1 could be responsible for the lack of complete inhibition of IgE antibodies against Ole e 1. Other possibilities, such as high polymorphism found in Ole e 1^{3,4,41} or incorrect folding of recombinant Ole e 1 expressed in *E. coli*, cannot be ruled out. Gene expression of different Ole e 1 isoforms in heterologous expression systems that result in high yields and are similar to plant cells systems (i.e., *Pichia pastoris* yeasts⁴² or insect cells⁴³) may solve this problem.

The sequence information reported here is essential for identifying B and T cell epitopes, and suggests the

possible importance of sequence polymorphism of allergens. The recombinant allergens presented here will provide pure protein for diagnostic purposes, for structural and immunologic studies, and potentially, when the expression of the glycosylated protein is improved, for allergen immunotherapy.

Note added in proof. The profilin from olive tree pollen (*Olea europaea*) has been named Ole e 2 in accordance with the International Union of Immunological Societies (IUIS) nomenclature.

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