

Characterization of recombinant *Mercurialis annua* major allergen Mer a 1 (profilin)

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Background: Two major allergens (Mer a 1A and Mer a 1B), tentatively identified as profilin, have been described in the euphorbiaceae, *Mercurialis annua*.

Objectives: We sought to clone and characterize these major allergens from *M. annua* pollen and to obtain the immunologically active and soluble recombinant allergen, which could then be used for diagnostic procedures and therapy.

Methods: Isolation of cDNA clones was performed by polymerase chain reaction amplification with degenerate primers. Expression in *Escherichia coli* BL21 (DE3) was carried out with a vector based in the T7 expression system, and the recombinant allergen was isolated by affinity chromatography on poly-(L-proline)-Sephacrose. Electrophoretic (sodium dodecylsulfate-polyacrylamide gel electrophoresis, isoelectric focusing, and 2-dimensional polyacrylamide gel electrophoresis) and immunochemical methods (Western blot and ELISA) were used for the characterization of the recombinant allergen.

Results: Two cDNA inserts coding for *M. annua* pollen profilin (Mer a 1) were cloned and sequenced. Full-length Mer a 1 cDNA was expressed in *E. coli* as nonfusion protein. The final yield of recombinant Mer a 1 from the culture media after a single purification step on poly-(L-proline)-Sephacrose was as much as 5 mg per liter. The reactivity of recombinant Mer a 1 with IgE antibodies present in sera from patients allergic to *M. annua*, *Olea europaea*, and *Ricinus communis* pollens was comparable to that of the natural counterparts, but latex profilin had no cross-reactivity with *M. annua* profilin. Recombinant Mer a 1 was shown to share B-epitopes with sunflower profilin.

Conclusion: This approach is suitable for the production of defined and purified recombinant allergens, which could allow more detailed immunologic characterization of these proteins and the development of much more accurate diagnostic measures and specific anti-allergic treatments. (J Allergy Clin Immunol 1998;101:363-70.)

Key words: cDNA cloning, euphorbiaceae, *Ricinus communis*, cross-reactivity, latex, olive tree pollen, sequencing, polymerase chain reaction, recombinant allergen, expression

Abbreviations used

BSA:	Bovine serum albumin
IPTG:	Isopropyl-thio- β -galactoside
mAb:	Monoclonal antibody
PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
pI:	Isoelectric point
PLP-Sepharose:	Poly-(L-proline)-CNBr activated Sepharose
PRP:	Proline-rich peptide
PVDF:	Polyvinylene difluoride
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

Up to 20% of the human population in developed countries suffers from IgE-mediated atopic diseases in any of their different manifestations (e.g., rhinitis, conjunctivitis, urticaria, angioedema, or asthma).¹ Mites and pollen are considered the main natural sources that cause allergy induced by inhalation. Among them, *Mercurialis annua* constitutes an important source of allergy in the Mediterranean areas of Spain and Italy.²⁻⁴ This weed belongs to the Euphorbiaceae family, which comprises more than 7000 species, including important sources of strong sensitizing allergens such as latex from the *Hevea brasiliensis* tree and seeds of castor bean (*Ricinus communis*). The flowering period of *M. annua* in Spain extends through winter and spring.² A high level (46.4%) of sensitization to this pollen, ranging from 28% to 54% of all patients allergic to pollen, has been reported in several areas of Spain.⁴

Two major allergens (Mer a 1A and Mer a 1B) with apparent molecular masses of 15.8 and 14.1 kd have been purified from *M. annua* and identified as profilins.⁴ Profilin is a cytoskeletal protein found in all eukaryotic cells, which interacts specifically with phospholipids⁵ and actin by modulating the ability of this protein to polymerize.⁶ Profilin probably links the remodeling of the actin cytoskeleton to the signal transduction pathway in response to extracellular stimuli.⁷ When pollen is hydrated, for example when it contacts a human mucosal membrane after inhalation, pollen tube formation is initiated, and large amounts of profilin are released. This protein, able to sensitize atopic patients, is called a panallergen because of the high cross-reactivity found in several species of tree, grass, and weed pollen and in many fruits and vegetables.⁸⁻¹³ The two allergens from *M. annua*, Mer a 1A and Mer a 1B,

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Supported in part by Grants No. 53-06-07 from the Plan Nacional de I+D (Farma III), No. 94-0299 from the CDTI (Ministerio de Industria y Energía, Spain), and No. 180A01 from the Programa PGTI (Departamento de Industria, Agricultura y Pesca, Basque Government).

Received Sept. 8, 1997; revised Oct. 17, 1997; accepted for publication Oct. 20, 1997.

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0091-6749/98 \$5.00 + 0 1/1/87165

ATG TCG TGG CAG ACG TAC GTC	T	GAC GAC CAT TTG ATG TGC GAC ATT GAT GGA CAA GGC CAG	T	60
M S W Q T Y V D-Y D H L M C D I D G Q G Q-L				21
CAT CTC GCC GCT GCT TCC ATT GTT GGC CAC GAC GGT AGC ATT TGG GCT CAG AGC GCC TCT	C			120
H L A A A S I V G H D G S I W A Q S A S				41
TTT CCT CAG TTG AAA CCA GAG GAG ATC ACC GGC ATT ATG AAG GAT TTT GAT GAG CCA GGT				180
F P Q L K P E E I T G I M K D F D E P G				61
CAT CTT GCT CCT ACA GGC TTG TAC ATT GCA GGC ACA AAA TAT ATG GTT ATC CAG GGC GAA				240
H L A P T G L Y I A G T K Y M V I Q G E				81
AGT GGC GCT GTA ATC CGT GGA AAG AAG GGA TCG GGA GGG ATC ACT ATA AAG AAA ACT GGC	T			300
S G A V I R G K K G S G G I T I K K T G				101
CAA GCT CTT GTG TTC GGA ATC TAC GAG GAG CCG GTA ACT CCG GGA CAG TGC AAC ATG GTG				360
Q A L V F G I Y E E P V T P G Q C N M V				121
GTT GAG AGG CTC GGT GAC TAC CTCATA GAA CAA GGC ATG TAA				402
V E R L G D Y L I E Q G M *				135

FIG. 1. Complete nucleotide and amino acid–deduced (in bold) sequences of Mer a 1 (clone M2). Differences are shown only in clone M1 sequence, with corresponding amino acid change when it occurs. Primers used for cDNA amplification are underlined. The vertical arrow indicates the length of the C-truncated profilin. The nucleotide sequence of clone M2 has been submitted to the GeneBank/European Molecular Biology Laboratory (EMBL) Databases under accession number Y13271.

constitute the first cases in which profilin is considered a major allergen because they are detected as an IgE-binding component in more than 50% of the sera studied.⁴

The difficulties in extracting, purifying, and standardizing natural allergens make them less suited for diagnostic and therapeutic purposes than their recombinant counterparts. Therefore standardized recombinant allergens will play an important role for investigations into the molecular basis of the IgE response^{14,15} and the development of new diagnostic procedures and therapeutic strategies.^{16,17} In this study complete amino acid sequencing of a major allergen, Mer a 1, and its definitive identification as profilin were derived on the basis of the nucleotide sequence of a cDNA clone obtained from *M. annua* pollen mRNA. The IgE-binding properties of the purified recombinant Mer a 1 produced in *Escherichia coli* were compared with the natural Mer a 1 present in protein extracts from *M. annua* pollen and to other natural and recombinant plant profilins.

METHODS

Cloning of profilin cDNA by polymerase chain reaction (PCR) amplification and nucleotide sequence determination

Poly(A⁺)-enriched mRNA was isolated from 100 mg of *M. annua* pollen (collected in Barcelona, Spain) with the Quick Prep MicroRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden). First-strand cDNA was synthesized from 1 mg Poly(A⁺)-enriched mRNA by using a first-strand synthesis kit (Pharmacia Biotech) with random hexadeoxynucleotides as primers. Degenerate oligodeoxynucleotide primers (Genosys Biotechnologies Inc., Cambridge, U.K.) for cDNA amplification were designed according to previously reported sequences.^{11,18–21} The primers used were PF (5'-AGAGAATTCATATGTCGTGGCA[A/G][A/G]CGTACGT) for the N-terminus and PR1 (5'-AGAAA-GCTT[C/T]TACA[G/T]GCC[C/T]TGTTTC[G/A/T][A/G/T/C][G/A/C]AGGTA) or PR2 (AGAAAGCTTTTCATTAGAGGTAGTC-ACCGAGCTCTC) for the C-terminus. The slash between bases means that they were added at that particular position in equimolar amounts during the synthesis of the primer. *EcoRI*, *NdeI*,

and *HindIII* restriction sites are underlined. Five milliliters of the single-stranded cDNA were mixed with the described degenerate primers in standard PCR mixture and subjected to amplification with Taq DNA polymerase (Bioline, London, U.K.) in the following conditions: 5 cycles at 94° C (1 minute), 45° C (2 minutes), and 72° C (2 minutes); 30 cycles at 94° C (1 minute), 56° C (2 minutes), and 72° C (2 minutes); and a final incubation at 72° C (6 minutes). The predominant band was isolated from 2% agarose gel (Gene-Clean, Bio101, La Jolla, Calif.) and, after cutting with *EcoRI* and *HindIII*, cloned into pBluescript II SK (Stratagene, La Jolla, Calif.).

Nucleotide sequences were determined by using the ABI PRISM Dye Terminator Cycle Sequencing Ready reaction kit and run on an ABI 373A DNA sequencer (Perkin-Elmer, Foster City, Calif.). The analysis of the sequences was done with the GCG Program Package (Genetics Computer Group Inc., Madison, Wis.).

Expression and purification of recombinant profilin

Recombinant plasmids were cut with *NdeI* and *HindIII*, and the resulting fragment was cloned into the expression vector pKN172, in which the cloned cDNA is under the control of the ϕ 10 promoter from the T7 bacteriophage.²² Recombinant DNA techniques were performed according to standard methods.²³ Competent *E. coli* BL21 (DE3)²⁴ cells were transformed with the resulting recombinant plasmids. In these cells the T7 RNA polymerase, produced after induction of the lacUV promoter by isopropyl-thio- β -galactoside (IPTG), interacts specifically with the ϕ 10 promoter.²⁴ The cells were grown to an OD₆₀₀ of 0.6 in Luria-Bertani supplemented with 200 μ g/ml ampicillin. IPTG was then added to a final concentration of 0.6 mmol/L, and incubation was continued for another 3 hours. Cells were harvested by centrifugation, and the pellet was resuspended in 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 1 mmol/L ethylenediamine tetraacetic acid containing 100 mg/ml lysozyme and incubated for 15 minutes at 37° C. Nucleic acids were broken down by mild sonication (6 pulses of 10 seconds each). Insoluble material was removed by centrifugation, and profilin present in the supernatant was purified by poly-(L-proline)-CNBr-activated Sepharose (PLP-Sepharose) chromatography.²⁵ The column was washed with 10 volumes of phos-

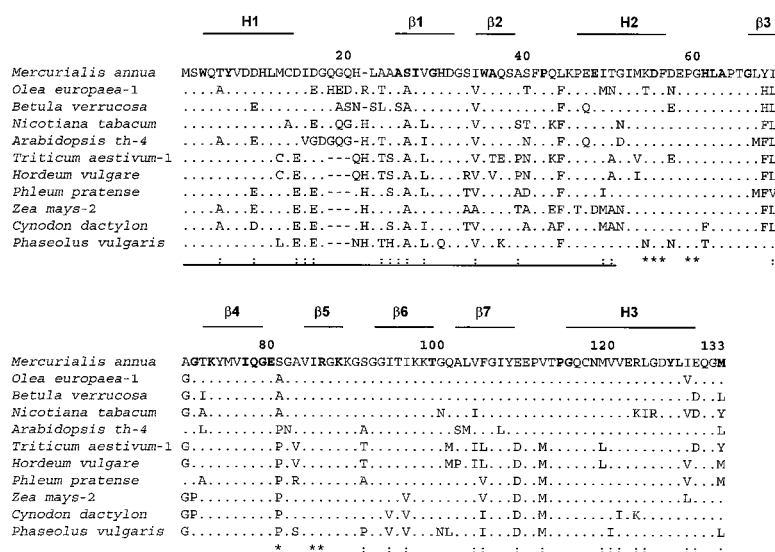


FIG. 2. Comparison of Mer a 1 with different plant profilins: *Olea europaea* profilin 1 (Y12425), *Betula verrucosa* (M65179), *Nicotiana tabacum* (X82120), *Arabidopsis thaliana* profilin 4 (U43324), *Triticum aestivum* profilin 1 (X89825), *Hordeum vulgare* (U49505), *Phleum pratense* (X77583), *Zea mays* profilin 2 (X73280), *Cynodon dactylon* (Y08390), and *Phaseolus vulgaris* (X81982). Points indicate identity of residues with respect to upper sequence. Structural and allergenic data obtained from birch and *Arabidopsis* profilins^{15,36} are shown for comparison; secondary structure is represented in upper part of sequence. Important residues involved in conformational stability are in bold, and areas covering experimentally determined sequential IgE-reactive epitopes are underlined. Residues marked with asterisks are involved in the plant-specific binding pocket.³⁶ Two points (:) indicate similar amino acids (A, S, T; D, E, N, Q; R, K; L, M, V; and F, Y, W).

incubated overnight at 4°C with sera from allergic patients or at 37°C for 60 minutes with rabbit polyclonal serum against sunflower profilin (diluted 1:3000). Immunochemical staining was performed as previously described.³² Image analysis was performed with the Bio-Image System (Millipore). Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) of lyophilized protein extracts or purified profilin was performed as described by O'Farrell.³³ Isoelectric focusing was performed on Isogel Agarose plates (pH 3 to 10) (FMC Bio Products, Rockland, Minn.) with 20 µg of protein per lane according to the manufacturer recommendations. Determination of glycoproteins on PVDF membranes was performed with the DIG Glycan detection kit (Boehringer Mannheim, Mannheim, Germany). The 15.3 kd protein was isolated by electroelution. Briefly, after SDS-PAGE, the gel was slightly stained and the 15.3 kd band was cut, minced, placed in an elution tube, and eluted with a BioRad model 422 electroeluter at 10 mA/tube for 4 hours.

Inhibition assays

For ELISA inhibition assays, microtiter plates (A/S Nunc, Roskilde, Denmark) were coated with 200 ng/well natural *M. annua* profilin in 0.1 mol/L bicarbonate buffer (pH 9.6) and blocked with blocking buffer (PBS supplemented with 0.05% Tween 20 and 1% bovine serum albumin [BSA]). Aliquots (100 μ l) of rabbit profilin antisera (diluted 1:7000), previously incubated with different concentrations of natural and recombinant profilins, were subsequently added and incubated for 1 hour at 37°C.

For immunoblot inhibition studies, 1 ml of sera from patients allergic to *M. annua* pollen was incubated overnight at 4° C with 20 µg purified inhibitor or BSA (negative control). The preadsorbed serum was then used for immunoblot experiments.

Determination of the protein concentration was performed according to the method of Bradford.²⁹ Proteins were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions³⁰ and visualized by Coomassie Brilliant Blue R-250 or silver staining (BioRad, Hercules, Calif.). For immunoblotting experiments, separated proteins after electrophoresis were electrophoretically transferred to polyvinylene difluoride (PVDF) membranes (Immobilon-P; Millipore Corp., Bedford, Mass.).³¹ Membranes were

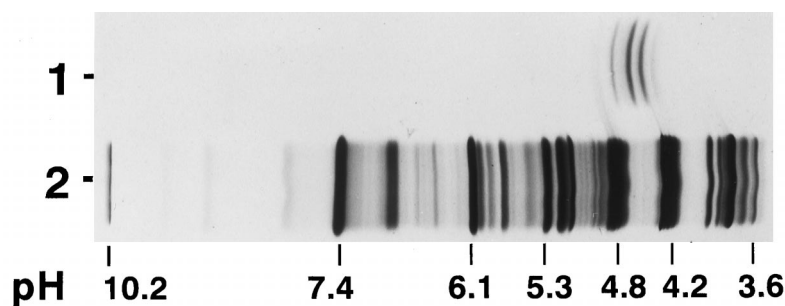


FIG. 3. Isoelectric focusing of *M. annua*-purified profilin. Lane 1, *M. annua* profilin; lane 2, pI markers.

RESULTS

Amplification, cloning, and sequencing of *M. annua* profilin cDNA

PCR after synthesis of the first cDNA strand with reverse transcriptase (RT-PCR) carried out on *M. annua* pollen mRNA with PF and PR1 primers produced a single fragment of approximately 400 bp. After cloning of this PCR product in pBluescript, two clones (M1 and M2) were sequenced (Fig. 1). Four nucleotide differences were found; two of them produced changes in the amino acid sequence (Asp8→Tyr and Gln20→Leu), and the other two changes (ATT⁸¹→ATC and ATA²⁸⁸→ATT) produced silent mutations. Sequences encoded polypeptides of 132 amino acids with predicted molecular masses of 14,270 and 14,300 d and isoelectric point (pI) values of 5.02 and 4.87 for M1 and M2 clones, respectively. No sequences containing putative N-glycosylation sites (NXT/S) were found, but a protein kinase phosphorylation site (S/TXR/K) was found at residue 95. Comparison of the deduced amino acid sequence of the *M. annua* profilin revealed high homology with other described plant profilins (Fig. 2). Pairwise alignments indicated the highest homology with profilins from *O. europaea* and *Betula verrucosa* with 86% and 85% identical amino acids, respectively, and a 99% identity in the C-terminus of the molecule (residues 71 to 133). The lowest homology among plant profilins was found with gramine profilins from *Triticum aestivum* and *Cynodon dactylon* with 78% identical amino acids. The sequence homology fell down to ≈25% identical amino acids when *M. annua* profilin was compared with profilins of mammalian origin. *M. annua* profilin was named Mer a 1 in accordance with the International Union of Immunological Societies (IUIS) Allergen Nomenclature Subcommittee.³⁴

Purification of natural *M. annua* profilin

Natural profilin isolated from *M. annua* extracts by PLP-Sepharose affinity chromatography is composed of a doublet band with apparent molecular masses of 15.3 and 14.1 kd with a relative ratio of 80% and 20%, respectively. The final yields obtained were 12.5 mg purified protein per 100 gm *M. annua* pollen. Isoelectric focusing analysis of purified profilin showed 4 bands with isoelectric point values of 4.9, 4.7, 4.5, and 4.4 (Fig. 3). The bands with lower pI values corre-

sponded to the 15.3 kd profilin, and the bands with higher pI values corresponded to the 14.1 kd profilin, as deduced after 2D PAGE analysis (data not shown). The glycoprotein detection assay carried out on purified profilin revealed that none of the two isoforms were glycosylated.

Expression of *M. annua* profilin and purification of recombinant protein

To express the *M. annua* profilin in *E. coli*, the M2 cDNA clone was inserted into pKN172. This clone was selected because it contains an Asp8 that is conserved in all plant profilin sequences and is not present in clone M1. A nonfusion form of *M. annua* profilin was expressed in *E. coli* BL21 (DE3) after induction with IPTG. The yield of recombinant Mer a 1 was between 40 and 45 mg/L bacterial culture. This yield represented approximately 20% of total *E. coli* proteins, with more than 90% of the expressed protein in soluble form. The soluble material underwent chromatography over a PLP-Sepharose affinity column. After elution with 6 mol/L urea in PBS, rMer a 1 showed a single band with an apparent molecular mass of 15.3 kd, as determined by Coomassie Brilliant Blue-stained SDS-PAGE (Fig. 4, A). Image analysis of the gels showed more than 95% purity of the 6 mol/L eluted profilin. The final yield of purified recombinant profilin was between 5 and 10 mg/L of bacterial culture. A truncated *M. annua* profilin, which lacked five residues from the C-terminal (Fig. 2), was obtained by PCR amplification of the M2 clone by using PF and PR2 primers and expressed in *E. coli*. The modified profilin could not be purified by affinity chromatography because this truncated protein was unable to bind to PLP-Sepharose.

Immunologic characterization of recombinant profilin

After Western blotting and immunostaining, recombinant and natural Mer a 1 were recognized by a rabbit anti-serum obtained against sunflower profilin (Fig. 4, B), whereas rabbit preimmune serum gave no signal. Most mAbs (7B12, 5F2, 5B7, 10A4, 11F11, and 3G4) raised against sunflower profilin bound to recombinant Mer a 1, as well as to the natural one (Fig. 4, C). 8F1 mAb bound only to the natural profilin, and two mAbs

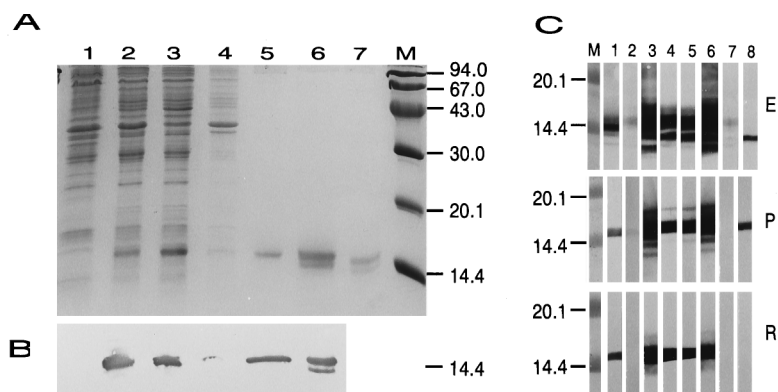


FIG. 4. Purification of recombinant Mer a 1 expressed in *E. coli*. **A**, Twenty percent SDS-PAGE analysis of samples from the purification steps. **B**, Western blotting of **A** with rabbit anti-profilin serum. Lane M, molecular mass markers; lanes 1 and 2, total cell lysate from *E. coli* BL21 (DE3) containing pKN172 alone and pKN172 with Mer a 1 insert (clone M2), respectively; lanes 3 and 4, soluble and insoluble fractions of sample on lane 2, respectively; lane 5, purified recombinant Mer a 1 (2 μ g); lanes 6 and 7, natural *M. annua* profilin (10 and 2 μ g, respectively). **C**, mAb-reactivity to *M. annua* (E) and purified natural (P) and recombinant (R) Mer a 1. Lane M, molecular mass markers; lane 1, 7B12 mAb; lane 2, 6D2 mAb; lane 3, 5F2 mAb; lane 4, 10A4 mAb; lane 5, 11F11 mAb; lane 6, 3G4 mAb; lane 7, 1C12 mAb; and lane 8, 8F1 mAb.

(6D2 and 1C12) bound very weakly to *M. annua* profilin. These data demonstrate the existence of some IgG-binding sites shared by natural and recombinant *M. annua* profilin and sunflower pollen profilin.

For ELISA inhibition experiments, microtiter plates were coated with natural *M. annua* profilin and incubated with sunflower profilin-specific IgG antibodies previously preincubated with different concentrations of natural and recombinant profilins from different sources. Recombinant Mer a 1 or the electroeluted band of 15.3 kd from *M. annua* were unable to inhibit rabbit IgG binding to immobilized *M. annua* profilin at a similar level than natural *M. annua* profilin (Fig. 5, A). Similar behavior was found when recombinant and natural profilin from *O. europaea* (olive tree) pollen were used. In contrast, recombinant and natural profilin from *C. dactylon* (Bermuda grass) pollen were able to inhibit IgG-binding at similar levels (Fig. 5, B).

Selected profilin-reacting sera from individuals allergic to *M. annua* were tested for IgE reactivity to *M. annua* extract and recombinant Mer a 1. All patient sera had comparable IgE reactivity to natural and recombinant allergens, indicating the presence of allergenic epitopes in the expressed protein (Fig. 6, A and B). No IgE binding to the recombinant Mer a 1 was observed in a nonprofilin-reacting serum from a patient allergic to *M. annua* pollen (Fig. 6, A, lane 3). In some cases the reactivity of the natural profilin was difficult to detect because of under-representation of this protein in the allergenic extract, but it was clear in the recombinant allergen (Fig. 6, A, lanes 7 and 9).

In immunoblot-inhibition assays, preincubation of a profilin-reacting human serum with purified recombinant Mer a 1 (20 μ g/ml) completely inhibited IgE binding to the doublet band of natural *M. annua* profilin, whereas preincubation with BSA did not (Fig. 6, C). Similar experiments with sera pools from eleven patients

allergic to olive tree pollen and six patients allergic to *R. communis* pollen showed a complete inhibition of the IgE binding to olive tree and *R. communis* pollen profilins, respectively, when sera were preincubated with natural and recombinant Mer a 1. In the latter case, a strong depletion of other IgE-binding bands was observed, which could be related with an unusual aggregation of the profilin in this extract. In contrast, preincubation of a serum pool from six patients allergic to latex with natural and recombinant Mer a 1 had no effect on the binding to the triplet band of natural latex profilin, although a slight depletion of a 32 kd band was detected (Fig. 6, C). This result showed that most of the allergenic determinants of *M. annua*, *R. communis*, and olive tree pollen profilins are present in the recombinant Mer a 1, but not in the profilin from *H. brasiliensis* (latex tree).

DISCUSSION

Cloning and production of recombinant allergens in heterologous expression systems may provide defined proteins for diagnostic, therapeutic, and experimental purposes. A simple and efficient method has been used to clone cDNA molecules encoding the translated region of a major allergen (Mer a 1) from *M. annua* that corresponds to profilin and to express this recombinant allergen in *E. coli*. *M. annua* is the first reported case where profilin is considered a major allergen, reacting with specific IgE in 59% of the sera tested.⁴ To date, the frequency of IgE reactivity to profilins has been considered to be 20% of all patients allergic to pollen,^{8,11} although recent results indicated a geographic variability of profilin incidence among patients allergic to birch, ranging from 7% in a Swedish group to 38% in an Italian group.³⁵

Comparisons among all the eukaryotic profilins revealed little overall amino acid sequence conservation, but recent x-ray crystal structure determination of plant

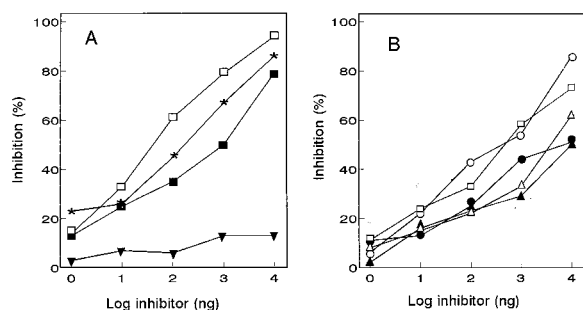


FIG. 5. **A**, Competitive inhibition of binding of sunflower profilin rabbit IgG antibodies to solid-phase natural *M. annua* profilin by natural (open squares) or recombinant (filled squares) profilins from *M. annua* and the 15.3 kd profilin isoform isolated by electroelution (*). Control experiments were performed with BSA (filled inverted triangles). **B**, Competitive inhibition of binding of sunflower profilin rabbit IgG antibodies to solid-phase natural *M. annua* profilin by natural (open squares, circles, and triangles) or recombinant (filled circles and triangles) profilins from *M. annua* (open squares), *Olea europaea* (filled and open circles), and *Cynodon dactylon* (filled and open triangles).

profilins has demonstrated that these profilins share a common tertiary structure with the mammalian profilins.^{15,36} Sequence comparisons showed that the important residues involved in the stability of profilin conformation are conserved in Mer a 1, as well as in other plant profilins (Fig. 2). A plant-specific site considered the most likely conformational immunogenic site of plant profilins³⁶ is also present in Mer a 1 (Fig. 2). In addition, three sequential IgE-reactive epitopes, the N- and C-terminal α -helices and a segment from residue 30 to 50, have been found with sera from patients allergic to profilin.¹⁵ The segment from residue 30 to 50 is very conserved in Mer a 1, as well as in profilin from *O. europaea*, but less homology was found with other plant profilins. The two α -helical IgE-reactive epitopes are almost identical in Mer a 1 and other plant profilins, suggesting the existence of conserved IgE-binding epitopes that could explain the high cross-sensitizing towards pollen profilins from trees, weeds, and grasses and profilins from many fruits and vegetables.^{8,9,11-13} These two α -helices contribute to the hydrophobic interface involved in the proline-rich peptide (PRP) interaction in human profilin.³⁷ Recently, Fedorov et al.¹⁵ have suggested a different binding mode for PRP binding on plant profilins on the basis of the different orientation of the N-terminal α -helix with respect to the C-terminal α -helix in birch profilin. Analysis of the PRP-binding site of the profilin-1 crystal structure from *Arabidopsis thaliana*³⁶ and the incapacity of the C-truncated profilin from *M. annua* to bind PLP-Sepharose (shown in this work) suggested, in contrast, a common PRP-binding mode among profilins.

Profilin from *M. annua* pollen is present in two isoforms with different electrophoretic behavior (15.3 and 14.1 kd). The existence of profilin isoforms with different electrophoretic migration has been previously described in profilins from olive tree pollen,²⁷ *H. brasil-*

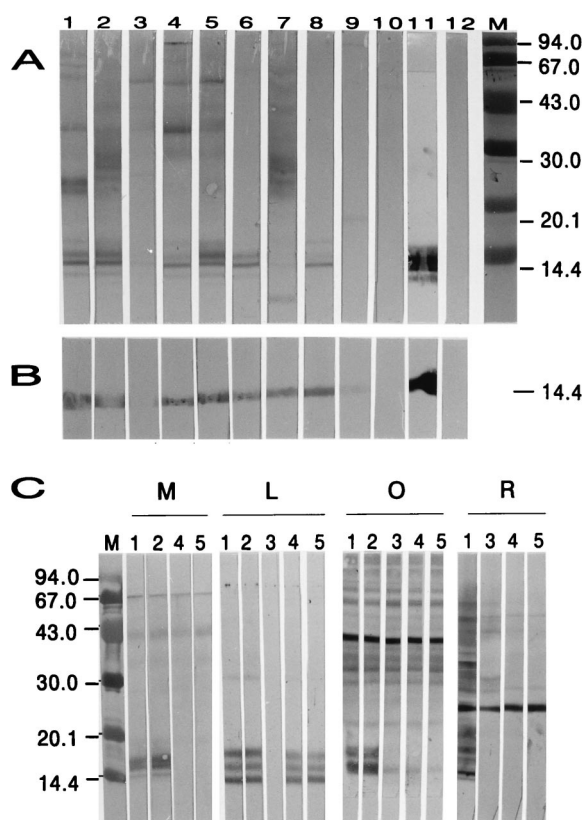


FIG. 6. Immunologic characterization of Mer a 1. IgE reactivity of sera from patients allergic to *M. annua* with *M. annua* extract (**A**) and purified recombinant Mer a 1 (**B**). Proteins transferred onto PVDF membranes were probed with sera from patients allergic to *M. annua* (lanes 1 through 9), serum pool from healthy individuals (lane 10), rabbit anti-profilin serum (lane 11), and buffer alone (lane 12). Molecular mass markers (lane M) were stained with Amido Black B. **C**, Mer a 1 immunoblot-inhibition. Protein extract from *M. annua* (M), *O. europaea* (O), and *R. communis* pollen (R) or purified latex profilin (L) were separated by SDS-PAGE and transferred onto PVDF membranes. Inhibition was performed by preincubating serum pools (L, O, and R) or serum (M) from patients sensitive to the corresponding extract with: lane 1, PBS buffer; lane 2, BSA; lane 3, natural profilin from latex (L), *O. europaea* pollen (O), or *R. comunis* pollen (R); lane 4, natural profilin from *M. annua* pollen; and lane 5, recombinant Mer a 1.

ensis (latex tree),³⁸ and sunflower pollen (A. Martínez, manuscript in preparation). These heterogeneities have been explained on the basis of glycosylation or proteolytic processes.³⁹ The former possibility is not convincing because Mer a 1 is not glycosylated, as has been demonstrated in this work by the absence of glycosylation motifs in the amino acid chain and the negative result after glycoprotein detection assay. The possibility of a proteolytic attack is not likely because profilin isoforms could be purified by PLP-Sepharose, and residues involved in poly-(L-proline)-binding are located at the N- and C-terminus of the profilin molecule, as has been demonstrated previously³⁶ and in this work. Nevertheless, degradation after purification cannot be ruled out completely. Different composition in charged or modi-

fied amino acids of the isoforms might explain the different electrophoretic migration, as has been suggested for grass group I allergens.⁴⁰ The existence of different amino acid composition between both profilin isoforms from *M. annua* could also be responsible for the impossibility of recombinant Mer a 1 or the 15.3 kd electroeluted profilin to inhibit IgG binding at the same level as natural *M. annua* profilin. Few amino acid changes are sufficient to provoke differences in the IgG-epitope interaction. This has been demonstrated in birch profilin in which the change of Gln-47 abolishes the binding of mAb 4A6.¹⁴ Recently, De Corte et al.⁴¹ have demonstrated phosphorylation of gelsoline and other related actin-binding proteins by a tyrosine kinase. A protein kinase site was found at residue 95 of Mer a 1, therefore phosphorylation could also be responsible for the Mer a 1 pattern obtained by isoelectric focusing.

Inhibition experiments with rMer a 1 showed the existence of similar allergenic epitopes between *M. annua* profilin and profilins from *R. communis* (another euphorbiacea) and olive tree pollen. In contrast, no cross-reactivity was found to the profilin from another euphorbiacea, *H. brasiliensis*. This constitutes the first reported case where profilins from members of the same taxonomic family share no IgE epitopes. Previous results demonstrated that only a minority of IgE epitopes are noncrossreactive in profilins from tobacco, birch, and timothy grass.⁴² The surprising result reported here showed that in spite of the high structural identity shared by plant profilins, they could have different IgE epitopes. Experiments on cross-reactivity and structural analysis of recombinant latex profilin are now in progress to explain this point because of the importance of latex-allergy among spina bifida patients and health-care workers.

In conclusion, these results show that this heterologous expression system and the purification procedure are suitable for the production of well-characterized and purified recombinant allergens, which could allow more detailed immunologic characterization of these proteins and the development of much more accurate diagnostic measures and specific antiallergic treatments.

We thank the Servicio de Secuenciación Automática from CIB-CSIC (Spain) for sequencing facilities and Dr. Borja Bartolomé for providing *R. communis* pollen profilin.

REFERENCES

- Miyamoto T. In: Godard P, Bousquet J, Michel FB, editors. Advances in allergology and clinical immunology. Carnforth (UK): The Parthenon Publishing Group-Carnforth; 1991. p. 343-7.
- García-Ortega P, Martínez J, Martínez A, Palacios R, Belmonte J, Richart C. *Mercurialis annua* pollen: a new source of allergic sensitization and respiratory disease. J Allergy Clin Immunol 1992; 89:987-93.
- Ariano R, Panzani RC, Falagiani P, Chiappella M, Augeri G. Respiratory allergy to the pollen of *Mercurialis annua* (euphorbiaceae). Ann Allergy 1993;70:249-54.
- Vallverdú A, García-Ortega P, Martínez J, Martínez A, Esteban MI, Molina de M, et al. *Mercurialis annua*: characterization of main allergens and cross-reactivity with other species. Int Arch Allergy Immunol 1997;112:356-64.
- Goldschmidt-Clermont PJ, Kim J, Machesky LM, Rhee S, Pollard TD. Regulation of phospholipase C-1 by profilin and tyrosine phosphorylation. Science 1991;251:1231-3.
- Carlsson I, Nyström LE, Sundkvist F, Markey F, Lindberg U. Actin polymerization is influenced by profilin, a low molecular weight protein in non-muscle cells. J Mol Biol 1977;115:465-83.
- Baatout S. Profilin: an update. Eur J Clin Chem Clin Biochem 1996;34:575-7.
- Valenta R, Duchêne M, Ebner C, Valent P, Sillaber C, Deviller P, et al. Profilins constitute a novel family of functional plant pan-allergens. J Exp Med 1992;175:377-85.
- Valenta R, Duchêne M, Vrtala S, Valent P, Sillaber C, Ferreira F, et al. Profilin, a novel plant pan-allergen. Int Arch Allergy Immunol 1992;99:271-3.
- van Ree R, Fernández-Rivas M, Cuevas M, van Wijngaarden M, Aalberse R. Pollen-related allergy to peach and apple: an important role for profilin. J Allergy Clin Immunol 1995;95:726-34.
- Valenta R, Duchêne M, Pettenburger K, Sillaber C, Valent P, Bettelheim P, et al. Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. Science 1991; 253:557-60.
- Hirschwehr R, Valenta R, Ebner C, Ferreira F, Sperr W, Valent P, et al. Identification of common allergenic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. J Allergy Clin Immunol 1992;90:927-36.
- van Ree R, Voitenko V, van Leeuwen W, Aalberse R. Profilin is a cross-reactive allergen in pollen and vegetable foods. Int Arch Allergy Immunol 1992;98:97-104.
- Wiedemann P, Giehl K, Almo SC, Girvin M, Steinberger P, et al. Molecular and structural analysis of a continuous birch profilin epitope defined by a monoclonal antibody. J Biol Chem 1996;271:29915-21.
- Fedorov AA, Ball T, Mahoney NM, Valenta R, Almo SC. The molecular basis for allergen cross-reactivity: crystal structure and IgE-epitope mapping of birch pollen profilin. Structure 1997;5:33-45.
- Laffer S, Vrtala S, Duchêne M, van Ree R, Kraft D, Scheiner O, et al. IgE-binding capacity of recombinant timothy grass (*Phleum pratense*) pollen allergens. J Allergy Clin Immunol 1994;94:88-94.
- Pauli G, Oster JP, Deviller P, Heiss S, Bessot JC, Susani M, et al. Skin testing with recombinant allergens rBet v 1 and birch profilin, rBet v 2: diagnostic value for birch pollen and associated allergies. J Allergy Clin Immunol 1996;97:1100-9.
- Rihs H, Rozynek P, May-Taube K, Welticke B, Baur X. Polymerase chain reaction based cDNA cloning of wheat profilin: a potential plant allergen. Int Arch Allergy Immunol 1994;105:190-4.
- Valenta R, Ball T, Vrtala S, Duchêne M, Kraft D, Scheiner O. cDNA cloning and expression of timothy grass (*Phleum pratense*) pollen profilin in *Escherichia coli*: comparison with birch pollen profilin. Biochem Biophys Res Commun 1994;199:106-18.
- Mittermann I, Swoboda I, Pierson E, Eller N, Kraft D, Valenta R, et al. Molecular cloning and characterization of profilin from tobacco (*Nicotiana tabacum*): increased profilin expression during pollen maturation. Plant Mol Biol 1995;27:137-46.
- Staiger C, Goodbody K, Hussey P, Valenta R, Drobak B, Lloyd C. The profilin multigene family of maize: differential expression of three isoforms. Plant J 1993;4:631-41.
- Way M, Pope B, Gooch J, Hawkins M, Weeds AG. Identification of a region in segment 1 of gelsolin critical for actin binding. EMBO J 1990;9:4103-9.
- Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory; 1989.
- Studier FM, Moffatt BA. Use of bacteriophage T7 polymerase to direct selective high-level expression of cloned genes. J Mol Biol 1986;189:113-30.
- Lindberg U, Schutt C, Hellsten E, Tjäder A, Hult T. The use of poly(L-proline)-Sephacrose in the isolation of profilin and profilactin complexes. Biochim Biophys Acta 1988;967:391-400.
- Nieto A, Mazón A, Montoro J, Boquete M, Estornell F, Martínez A,

- et al. Pattern of latex sensitization in children with spina bifida [abstract]. *J Allergy Clin Immunol* 1997;99:S157.
27. Asturias JA, Arilla MC, Gómez-Bayón N, Martínez J, Martínez A, Palacios R. Cloning and expression of the panallergen profilin and the major allergen (Ole e 1) from olive tree pollen. *J Allergy Clin Immunol* 1997;100:365-72.
 28. Arilla MC, Asturias JA, Gómez-Bayón N, Martínez A, Martínez J, Palacios R. Production and characterization of profilin monoclonal antibodies. *Allergol Immunopathol* 1997;25:145-51.
 29. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-54.
 30. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* 1970;227:680-5.
 31. Towbin H, Staehelin I, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;76:4350-4.
 32. Jiménez A, Moreno C, Martínez J, Martínez A, Bartolomé B, Guerra F, et al. Sensitization to sunflower pollen: Only an occupational allergy? *Int Arch Allergy Immunol* 1994;105:297-307.
 33. O'Farrell PH. High resolution two-dimensional (2-D) electrophoresis of proteins. *J Biol Chem* 1975;250:4007-21.
 34. King T, Hoffman D, Lowenstein H, Marsh D, Platts-Mills T, Thomas W. Allergen nomenclature. *Int Arch Allergy Immunol* 1994;105:224-33.
 35. Elfman L, Svensson M, Lidholm J, Pauli G, Valenta R. Different profiles in specific IgE to rBet v 1 and rBet v 2 in patients allergic to birch pollen from six countries. *Int Arch Allergy Immunol* 1997;113:249-51.
 36. Thorn KS, Christensen HEM, Shigeta R, Huddler D, Shalaby L, Lindberg U, et al. The crystal structure of a major allergen from plants. *Structure* 1997;5:19-32.
 37. Metzler WJ, Bell AJ, Ernst E, Lavoie TB, Mueller L. Identification of the poly-L-proline-binding site on human profilin. *J Biol Chem* 1994;269:4620-5.
 38. Vallier P, Balland S, Harf R, Valenta R, Deviller P. Identification of profilin as an IgE-binding component in latex from *Hevea brasiliensis*: clinical implications. *Clin Exp Allergy* 1995;25:332-9.
 39. Vallier P, Dechamp C, Valenta R, Vial O, Deviller P. Purification and characterization of an allergen from celery immunochemically related to an allergen present in several other plant species. Identification as a profilin. *Clin Exp Allergy* 1992;22:774-82.
 40. Petersen A, Grobe K, Lindner B, Schlaak M, Becker WM. Comparison of natural and recombinant isoforms of grass pollen allergens. *Electrophoresis* 1997;18:819-25.
 41. de Corte V, Gettemans J, Vandekerckhove J. Phosphatidylinositol 4,5-bisphosphate specifically stimulates PP60 (c-src) catalyzed phosphorylation of gelsoline and related actin-binding proteins. *FEBS Lett* 1997;401:191-6.
 42. Vrtala S, Wiedemann P, Mittermann I, Eichler H-G, Sperr WR, Valent P, et al. High-level expression in *Escherichia coli* and purification of recombinant plant profilins: comparison of IgE-binding capacity and allergenic activity. *Biochem Biophys Res Commun* 1996;226:42-50.