PERGAMON

Molecular Immunology 35 (1998) 469–478

Molecular Immunology

Cloning and immunological characterization of the allergen Hel a 2 (profilin) from sunflower pollen

J. A. Asturias*, M. C. Arilla, N. Gómez-Bayón, M. Aguirre, A. Martínez, R. Palacios, J. Martínez

R&D Department, IFIDESA-ARISTEGUI, Alameda Urquijo, 27, 48008-Bilbao, Spain

Received 2 January 1998; accepted 1 April 1998

Abstract

Sunflower (*Helianthus annuus*) sensitization is not always related with occupational allergy. We have isolated the allergen profilin (Hel a 2) from this Compositae plant, cloned and sequenced five cDNAs encoding for full-length or partial Hel a 2. Natural sunflower profilin reacted with specific IgE in the 121 sera tested, at a frequency of 30.5%. Expression of the cDNA encoding Hel a 2 in *Escherichia coli* and a simple purification procedure by poly-L-proline chromatography allowed immunological characterization of the recombinant allergen. Binding of monoclonal antibodies against sunflower profilin revealed that some epitopes responsible for antigen-specific IgG production were not present in the recombinant allergen. High cross-reactivity has been found between recombinant Hel a 2 and profilins from other Compositae plants and also from botanically distant plants.

Keywords: Profilin; Pan-allergen; Cloning; Bacterial expression; Recombinant allergen; Affinity chromatography; IgE binding; Monoclonal antibodies; *Helianthus annuus*

1. Introduction

The Compositae (Asteraceae) family is one of the most numerous among flowering plants and comprises over 20,000 species. This botanic family is distributed throughout the world and embraces genera of great allergologic interest. Some, such as short ragweed (*Ambrosia artemisiifolia*) and mugwort (*Artemisia vulgaris*) have been intensively studied as important etiologic agents of pollinosis and several ragweed and mugwort allergens have been cloned and characterized at the molecular level (Stewart, 1995). Fewer studies have been carried out on other Compositae plants such as sunflower (*Helianthus annuus*). Sunflower has great economic interest because of its uses in the food industry as raw material for margarine and cooking oil production, or for processing of its dried seeds. Due to this commercial importance, the sunflower crop is expanding through the world, with Russia, Argentina, the U.S.A., China, France and Spain as leading producers.

Allergy to sunflower pollen has been described in people involved in the processing of sunflower seeds, living close to plantations, or after eating sunflower pollen-contaminated honey (Bousquet et al., 1985; Birnbaum et al., 1989; Jiménez et al., 1994). Some cases of anaphylactic reactions after eating sunflower seeds have also been described (Axelsson et al., 1994; Hefle et al., 1997). Several sunflower allergens, with apparent molecular masses of 39, 32, 28.7 and 14.4 kDa, have been isolated and partially characterized from sunflower pollen (Fernández et al., 1993; de la Hoz et al., 1994; Jiménez et al., 1994) and sunflower seeds (Hefle et al., 1997). Cross-reactivity between sunflower and other Compositae pollen allergens, mainly those from mugwort, has been reported (Fernández et al., 1993; de la Hoz et al., 1994). In our previous studies (Jiménez et al., 1994), we detected a relevant allergen of 14 kDa in 28% of patient sera. Profilins are ubiquitous cytosolic actin-binding proteins with molecular masses of 12–15 kDa which play a crucial role in regulating the activity in the microfilament system and intracellular calcium levels (Carlsson et al., 1977; Goldschmidt-Clermont et al., 1991; Baatout, 1996) and are produced in high amounts in some biological pro-

* Corresponding author: Tel: +34-94-443 8000; fax: +34-94-443 8016; e-mail: im000001@sarenet.es

Abbreviations: IPTG, isopropyl-thio-β-galactoside; LB, Luria-Bertani medium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLP-Sepharose, poly-(L-proline)-CNBr activated Sepharose; PVDF, polyvinylene difluoride; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Hel a 2, sunflower profilin; mAbs, monoclonal antibodies; GST, glutathione-S-transferase.

S0161–5890/98 $19.00
PII: S0161-5890(98)00036-4

cesses such as pollen hydration. The profilin has been identified as an allergen in several plant species of trees, grasses and weeds pollens and in many fruits and vegetables (Valenta et al., 1992a). It is described as a panallergen (Valenta et al., 1992a,b), because it is recognized by 20% of all pollen-allergic patients (Valenta et al., 1991, 1992; Hirschwehr et al., 1992; van Ree et al., 1992).

The advent of molecular biology and its application to the study of allergens constitutes a great advance for the characterization of some important allergens. Recombinant products are expected to represent pure, well defined and reproducible compounds, which could facilitate the elucidation of the specific amino acid residues involved in recognition by B- and T-cells (Scheiner, 1992; Scheiner and Kraft, 1995; Stewart, 1995). In this study, we describe the cloning, expression and purification of the panallergen profilin from sunflower pollen and the immunological characterization of the recombinant allergen.

2. Material and methods

2.1. Material

100 g of pure *Helianthus annuus* pollen (locally collected from Córdoba, Spain) were extracted as previously described (Jiménez et al., 1994). 6.5 g of freeze-dried allergenic extract was obtained at the end of the process and stored freeze-dried at −20°C until used.

2.2. Chromatographic purification of sunflower profilin

The first step in the profilin purification was accomplished by affinity chromatography in PLP-Sepharose column, following the modified method of Lindberg described previously (Vallverdú et al., 1997). Proteins co-eluting with profilin were removed by high resolution ion exchange chromatography in the SMART System (Pharmacia Biotech, Uppsala, Sweden). An anionic exchange column (MonoQ PC 1.6/5) was loaded with 40 mg of PLP-purified profilin in 40 mM Tris-HCl, pH 8.0, using a 1 ml injection loop. Elution was carried out with a linear gradient from 0–1 M of NaCl. Afterwards, a gel filtration column, Superdex 75 PC 3.2/30 was used. Samples were applied at a concentration of 20 mg/ml in 0.05 M phosphate buffer, 0.15 M NaCl pH 7.0 (PBS) and injected with a loop of 20 μl. A flow rate of 40 μl/min was maintained during the whole chromatographic process. The column was calibrated with a set of proteins of known molecular mass. Determination of the protein concentration was performed according to Bradford (1976).

2.3. Peptide sequencing

Purified profilin was electrophoresed on 12.5% SDS–PAGE and after Coomassie blue staining, gel slices containing profilin were sent to W.M. Keck Foundation (Biotechnology Resource Laboratory, Yale University, New Haven, CT, U.S.A.) for amino acid sequencing. Initial sequencing indicated that the amino terminal end of the protein was blocked. In order to obtain protein sequencing data, in-gel enzymatic digestion was carried out as described by Williams and Stone (1995). Basically, this procedure involves perfusing in an approximately 1 : 5 (enzyme weight: substrate weight) ratio of modified trypsin or lysyl endopeptidase for 24 h at 37°C. The resulting peptides were separated by reverse phase on a Hewlett Packard 1090 HPLC equipped with a Vydac C-18 (5 micron particle size, 300 A pore size) column. Sequencing of the separated peptides was carried out on ABI 470A or 477 Sequencers (Perkin-Elmer, Foster City, CA, U.S.A.).

2.4. Cloning of sunflower pollen profilin cDNA

Poly(A^+) mRNA was isolated from 100 mg of *Helianthus annuus* pollen using the Quick Prep MicroRNA Purification Kit (Pharmacia Biotech). 1 μg poly(A^+)-enriched mRNA was reverse transcribed by using a First-Strand cDNA Synthesis kit (Pharmacia Biotech) and random hexadeoxynucleotides as primers. Degenerate oligodeoxynucleotide primers (Genosys Biotechnologies Inc., Cambridge, U.K.) for cDNA amplification were designed according to previously reported sequences (Valenta et al., 1991; Staiger et al., 1993; Rihs et al., 1994; Valenta et al., 1994; Mittermann et al., 1995). The primers used were: PF: 5′-AGAGAATTCCATATGTCGTGG CA(A/G)(A/G)CGTACGT (for the N-terminus) and PR1: 5′-AGAAAGCTT(C/T)TACA(G/T)GCC(C/T) TGTTC(G/A/T)A(G/T/C)(G/A/C)AGGTA or PR2: AGAAAGCTTTCATTAGAGGTAGTCACCGAG CCTCTC (for the C-terminus). *Eco*RI, *Nde*I and *Hin*dIII restriction sites, respectively, are underlined. Profilin-encoding cDNA was amplified by PCR using 5 μl of the products of the first-strand cDNA synthesis and the described degenerate primers, under the following conditions: 5 cycles at 94°C (1 min), 45°C (2 min) and 72°C (2 min), followed for 30 cycles at 94°C (1 min), 56°C (2 min) and 72°C (2 min) and a final incubation at 72°C (6 min). The predominant band was isolated from 2% agarose gels (GeneClean, Bio101, La Jolla, CA, U.S.A.) and after cutting with *Nde*I and *Hin*dIII, cloned into the expression vector pKN172 (Way et al., 1990). Recombinant DNA techniques were performed following standard methods (Sambrook et al., 1989).

2.5. Nucleotide sequence determination

Nucleotide sequences were determined using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and analysed on an ABI 373A DNA sequencer

(both, Perkin-Elmer). The analysis of the sequences was done using the GCG Program Package (Genetics Computer Group Inc., Madison, WI, U.S.A.).

2.6. Production of recombinant profilin

E. coli BL21 (DE3) (Studier and Moffatt, 1986) cells containing the pKN172-derived recombinant plasmids were grown in LB with 200 μg/ml ampicillin. When cultures reached an OD_{600} of 0.6, 0.6 mM IPTG was added to the culture and incubation continued for another 3 h. Cells were harvested by centrifugation and the pellet, resuspended in 0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl, was lysed by lysozyme treatment (100 mg/ml, 15 min, 37°C) and mild sonication. Insoluble material was removed by centrifugation and profilin in the supernatant was purified by PLP-Sepharose chromatography as described above.

2.7. Construction of truncated sunflower profilins

For the amino-terminal half of the sunflower profilin, the PCR product from the amplification using PF1 and PR1 primers was digested with *Eco*RI; a 240 bp fragment from the N-terminal was isolated from agarose gels. For the carboxyl-terminal half, plasmid pHaPRO1 was digested with *Eco*RI and a 110 bp fragment was isolated. Both fragments were subcloned into the *Eco*RI site of a pGEX-4T-1 expression vector (Pharmacia Biotech) and correct orientation of the insert was checked by restriction analyses. These constructions encode fusion proteins (GST-N-term or GST-C-term) consisting of glutathione *S*-transferase from *Schistosoma japonicum* followed by the truncated profilin. The production of the fusion proteins was carried out as previously described (Asturias et al., 1997b).

2.8. Agarose-IEF

Isoelectric focusing was performed on Isogel Agarose plates (FMC Bio Products, Rockland, MN, U.S.A.), pH 3 to 10, using 20 μg of protein per lane and following the manufacturer recommendations. The resulting electrophoretic patterns were evaluated by image analysis in the Bio-Image System (Millipore Corp., Bedford, MA, U.S.A.).

2.9. Immunoblotting

Proteins were analysed by SDS–PAGE under reducing conditions (Laemmli, 1970) and visualized by Coomassie Blue R250 staining. Separated protein bands were electrophoretically transferred to PVDF (Immobilon-P membranes, Millipore) (Towbin et al., 1979). After blocking for 1 h with 8.8% defatted dry milk in Tris-buffered saline (TBS), membranes were incubated overnight at 4°C with allergic patients sera, or at 37°C for 60 min with rabbit polyclonal serum against sunflower profilin (diluted 1 : 3000). IgG or IgE-peroxidase conjugates were detected by the addition of 3 ml of freshly prepared 0.06% w/v of 4-chloro-1-naphtol solution (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and 0.01% H_2O_2 in TBS.

2.10. Antisera and mAbs

For the preparation of experimental immunosera, immunization was performed in adult male white New Zealand rabbits. Monthly injections of 150 μg of purified sunflower profilin with Freud's complete adjuvant were given subcutaneously and after 3 injections, the animals were bled and the serum collected. mAbs 3H8 and 3G4 were produced as previously described (Arilla et al., 1997). Sera of sunflower-allergic patients had been previously characterized by radioallergosorbent test (RAST) and immunoblotting with a standardized sunflower extract (Jiménez et al., 1994). Sera showing a RAST class greater than 2 were selected.

2.11. Inhibition assays

For ELISA inhibition assays, microtiter plates were coated with 200 ng/well of natural sunflower profilin in 0.1 M bicarbonate buffer pH 9.6 and saturated with blocking buffer (PBS supplemented with 0.05% Tween 20 and 1% BSA). 100 μl aliquots of profilin antisera or a serum pool from sunflower-allergic patients (1/6000 or 1/2 dilution in blocking buffer, respectively) were incubated for 16 h at 4°C with different concentrations of the inhibitor, added to the coated plate and incubated for 1 h at 37°C. Detection was carried out as previously described (Jiménez et al., 1994). For immunoblot-inhibition studies, 0.5 ml of 1/2 diluted serum pool from sunflower-allergic patients was incubated with 10 μg purified recombinant profilin or 10 μg BSA (negative control) overnight at 4°C. The preadsorbed serum was then used for immunoblot experiments as described above. Recombinant profilins from *Cynodon dactylon*, *Mercurialis annua* and *Olea europaea* were obtained as previously described (Asturias et al., 1997b; Asturias et al., 1997c; Vallverdú et al., 1998).

3. Results

3.1. Isolation and characterization of natural sunflower profilin

Purification of profilin from sunflower pollen by affinity chromatography on PLP-Sepharose column proved highly effective, as evaluated by SDS–PAGE (Fig. 3, lane 6). The molecular mass of the protein band, estimated by

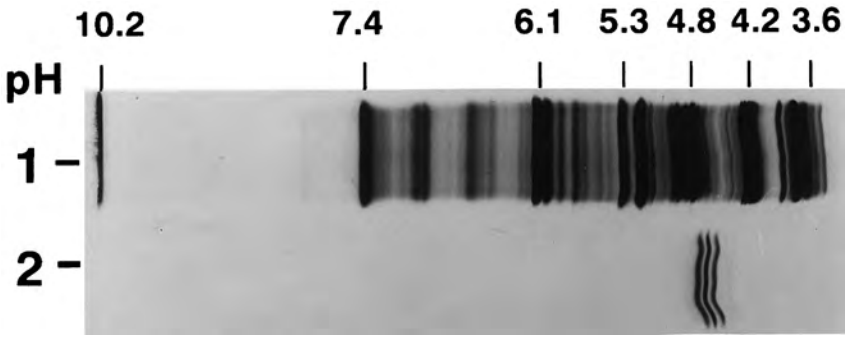

Fig. 1. Agarose IEF (3.0–10.0 pH range) of profilin obtained after gel filtration chromatography (lane 2). Lane 1: indicates the lane in which pI markers were electrofocused.

this technique, was 14.7 kDa. Further purification of the 6 M urea-eluted fraction from PLP-Sepharose was achieved by means of ionic exchange chromatography in the SMART System. This procedure provided a pure profilin sample which eluted as a symmetric peak at 0.25 M NaCl. The presence of three isoforms could be detected by Agarose-IEF with pIs of 4.41, 4.46 and 4.50 (Fig. 1). The amino acid sequence of the six peptides obtained after in-gel enzymatic digestion of sunflower profilin is shown in Fig. 2B. *H. annuus* (sunflower) pollen profilin was named Hel a 2 in accordance with the IUIS/WHO Allergen Subcommittee (King et al., 1995).

3.2. Amplification, cloning and sequencing of cDNA coding for sunflower profilins

Amplification of sunflower pollen cDNA using PF/PR1 or PF/PR2 primer pairs produced a main fragment of approximately 400 bp. After cloning these PCR products in the pKN172 expression vector, five clones were sequenced, corresponding to full-length (HaPRO1) or incomplete (HaPRO3-6) profilin sequences (Fig. 2A). HaPRO1 encoded a polypeptide of 133 amino acids with a predicted molecular mass of 14,285 Da and a pI value of 4.94. Microheterogeneity was found among the sequences obtained after cDNA cloning or peptide sequencing (Fig. 2B). Comparison of its deduced amino acid sequence using the BLAST program showed high homology with other plant profilins. The highest homology (82% identity) was found with profilin 2 from *Phleum pratense* (Bermuda grass) and the lowest homology among plant profilins was found with *Arabidopsis thaliana* profilin 1 (71% identity). Less homology was found with non-plant profilins such as profilins from the mold *Dictyostelium discoideum* (46% identity), the fruitfly (43%), the protist *Acanthamoeba castellanii* (40%) and the yeast *Schizosaccharomyces pombe* (35%). Vertebrate profilins revealed an average of 30% of identity.

3.3. Expression and purification of profilins

The expression of full-length profilin region was carried out using the T7 system in which the gene of interest is expressed under the control of the ϕ10 promoter specifically activated by the T7 RNA polymerase produced from the *lac* promoter. A non-fusion form of profilin was expressed in *E. coli* BL21 (DE3). The soluble material was chromatographed over a PLP-Sepharose affinity column. After elution with 6 M urea in PBS, rHel a 2 showed a single band with an apparent molecular mass of 14.1 kDa, as determined by Coomassie Brillant Blue-stained SDS–PAGE (Fig. 3).

Taking advantage of the *Eco*RI restriction site located in frame at the middle of the Hel a 2 sequence (position 162), two truncated sunflower pollen profilins were constructed as fusion proteins with GST (Fig. 2B) and expressed in *E. coli*. Another truncated sunflower pollen profilin, that lacked five residues from the C-terminal (Fig 2), was obtained by PCR amplification of HaPRO1, using PF and PR2 primers. After expression in *E. coli* BL21 (DE3), the modified profilin could not be purified by affinity chromatography because it was unable to bind to PLP-Sepharose.

3.4. Allergenic capacity of sunflower recombinant profilin

Sera from sunflower pollen-allergic patients were tested for IgE reactivity to crude pollen extracts and purified recombinant profilin after SDS–PAGE. The frequency of the IgE binding to Hel a 2 was 30.5% of 121 sunflower-allergic patients (data not shown). Representative results from selected sunflower-allergic patients are shown in Fig. 4. The majority of the selected patients showed specific IgE reaction with both the natural and recombinant sunflower profilins indicating the presence of allergenic epitopes in the recombinant protein.

Using a pool of sera from profilin-reacting patients, IgE inhibition experiments, both with native (ELISA-inhibition) and denatured/reduced (immunoblot-inhibition) proteins, were carried out in order to compare antibody-binding properties. The inhibition curves for the natural and recombinant allergens (Fig. 5B) showed that most of the allergenic determinants are present in rHel a 2. Similar results were obtained when denatured

A

```
                                                                               52
HaPRO1  ATG TCG TGG CAG GCG TAC GTC GAT GAA CAC TTG ATG TGC GAC ATT GAA GGT
HaPRO4  ... ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO6  ... ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO3  ... ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO5  ... ... ... ... A.. ... ... ... ... ... ... ... ... ... ... ... ...

                                                                              102
HaPRO1  ACT GGC CAG CAT CTC ACA TCT GCC GCT ATT CTC GGT CTC GAC GGT ACT GTT
HaPRO4  ... ... ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO6  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO3  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO5  ... ... ... ... ... ... C.. ... ... ... ... ... ... ... ... ... ..A

                                                                              153
HaPRO1  TGG GCT CAG AGC GCT AAG TTT CCA CAG TTC AAA CCC GAG GAG ATG AAA GCC
HaPRO4  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO6  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO3  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO5  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                              204
HaPRO1  ATC ATT AAG GAA TTC GAC GAA GCT GGT ACT CTT GCT CCA ACA GGT ATG TTC
HaPRO4  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO6  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO3  ... ... ... .G. ... ... ... ... ... ... ... ... ... ... ... ... ..T
HaPRO5  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                              255
HaPRO1  ATC GCG GGT GCA AAA TAT ATG GTA CTC CAA GGA GAG CCC GGA GCT GTT ATC
HaPRO4  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO6  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO3  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO5  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                              306
HaPRO1  CGT GGA AAG AAG GGA GCT GGA GGA ATA TGC ATC AAG AAA ACG GGC CAA GCT
HaPRO4  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO6  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO3  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO5  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                              357
HaPRO1  ATG ATT ATG GGA ATT TAC GAC GAG CCC GTT GCT CCT GGA CAA TGC AAC ATG
HaPRO4  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO6  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ..T
HaPRO3  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
HaPRO5  ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

                                                                  402
HaPRO1  GTT GTT GAG AGG CTT GGC GAT TAC CTG CTC GAA CAA GGC ATG TAA
HaPRO4  ... ... ... ... ..C ..T ..C ... ..C
HaPRO6  ... ... ... ... ..C ..T ..C ... ..C
HaPRO3  ... ... ... ... ..C ..T ..C ... ..C
HaPRO5  ... ... ... ... ... ... ... ... ...
```

Fig. 2. Sunflower profilin sequence polymorphism. Comparison of **(A)** nucleotide and **(B)** deduced amino acid sequences of Hel a 2 (clone HaPRO1). Peptides sequences are shown in B under the deduced amino acid sequences. Primer sequences were underlined and *Eco*RI restriction site was boxed. Identical residues are indicated by points in the deduced amino acid sequences and by asterisks in the peptides. Arrow indicate the length of the truncated profilins.

/reduced proteins were used in immunoblot-inhibition assays (Fig. 6). IgE reactivity to the 14 kDa band, corresponding to profilin, disappeared when the sera pool was inhibited with 20 μg/ml of natural and recombinant sunflower profilin. IgE-binding to a 24 kDa band was also inhibited by both natural and recombinant profilin.

```
B
          1          .          .          .          .          50
HaPRO1    MSWQAYVDEHLMCDIEGTGQHLTSAAILGLDGTVWAQSAKFPQFKPEEMK
HaPRO4    ..................H.............................
HaPRO6    ..................................................
HaPRO3    ..................................................
HaPRO5    ....................P.............................
                                                **********

          51 ↓       .          .          .          .      100
HaPRO1    GIIKEFDEAGTLAPTGMFIAGAKYMVLQGEPGAVIRGKKGAGGICIKKTG
HaPRO4    ..................................................
HaPRO6    ..................................................
HaPRO3    ....G.............................................
HaPRO5    ..................................................
           ***G**x*x****x*        *************
               *Q**********T*xGQ*
               **AP*H**W*         ***I*********

          101        .          .         ↓.133
HaPRO1    QAMIMGIYDEPVAPGQCNMVVERLGDYLLEQGM
HaPRO4    ...........................
HaPRO6    ..................I.......
HaPRO3    ...........................
HaPRO5    ...........................
```

Fig. 2.*Continued.*

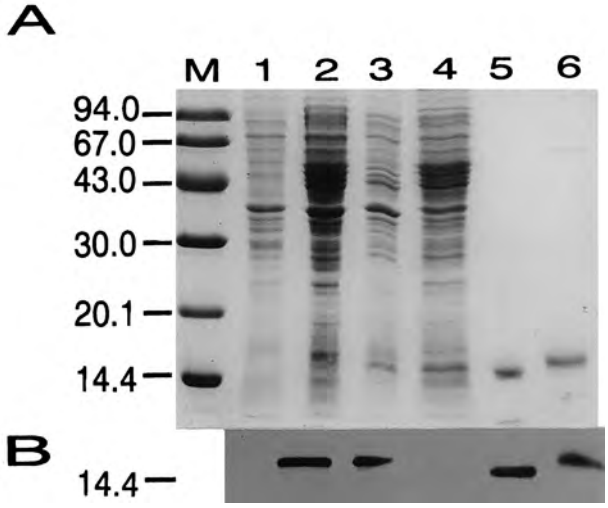

Fig. 3. Purification of recombinant Hel a 2 expressed in *E. coli*. **(A)** 12.5% SDS–PAGE analysis of samples from the purification steps. **(B)** Western blotting of A using rabbit anti-profilin serum. Lane M, molecular mass markers; lanes 1 and 2, total cell lysate from *E. coli* BL21 (DE3) containing pKN172 alone and pKN172 with HaPRO1 insert, respectively; lanes 3 and 4, soluble and insoluble fractions of sample on lane 2, respectively; lane 5, purified recombinant Hel a 2; lane 6, natural *H. annuus* profilin.

3.5. Cross-reactivity between plant profilins

In order to compare epitope similarity between profilins from other Compositae plants, recombinant sunflower profilin was used to inhibit specific IgE binding to mugwort and short ragweed profilins from protein extracts. Western-inhibition of IgE binding to blotted proteins from sunflower extract were performed in order to compare cross-reactivity among plant profilins (Fig. 6). Incubation of the serum pool from sunflower allergic patients with recombinant profilins from Bermuda grass (*Cynodon dactylon*), olive tree (*Olea europaea*) and *Mercurialis annua* pollen produced inhibition of the specific IgE binding to the 14–24 kDa bands, similar to that observed when recombinant Hel a 2 was used as inhibitor. In ELISA-inhibition experiments, inhibition patterns similar to those obtained with rHel a 2 were observed using different concentrations of heterologous recombinant profilin as inhibitors (Fig. 7). The only exception was olive tree pollen profilin which produced similar inhibition only at high concentration.

3.6. Antigenic capacity of sunflower recombinant profilin

IgG antibody reactivity of natural and recombinant sunflower profilins was compared by ELISA-inhibition experiments. The recombinant allergen was not able to inhibit rabbit IgG binding to immobilized Hel a 2 to the same level that the natural counterpart was (Fig. 5A), which indicates that some antigenic determinants are not present in the recombinant form. In order to compare in more detail the antigenic structure of recombinant and natural Hel a 2, inhibition experiments with two mAbs (3G4 and 3H8) were performed (Fig. 8). In Western-

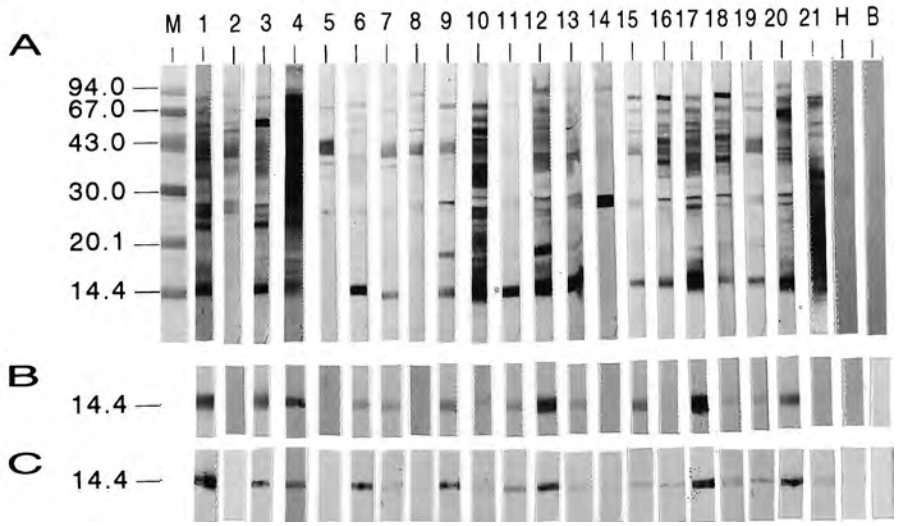

Fig. 4. Immunological characterization of Hel a 2. IgE reactivity of sera from sunflower-allergic patients with *H. annuus* extract **(A)** and purified natural **(B)** and recombinant Hel a 2 **(C)**. Proteins transferred onto PVDF-membranes were probed with sera from sunflower-allergic patients (lanes 1–21), serum pool from healthy individuals (lane H) and buffer alone (lane B). Molecular mass markers (lane M) were stained with Amido Black B.

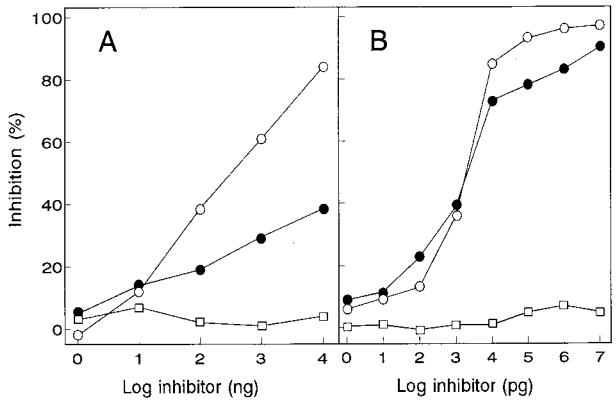

Fig. 5. Competitive inhibition of the binding of Hel a 2-specific polyclonal antibodies to solid-phase natural Hel a 2 by natural (○) or recombinant (●) Hel a 2. **(A)** ELISA-inhibition of rabbit IgG binding to Hel a 2-coated well. **(B)** ELISA-inhibition of human IgE binding to Hel a 2-coated well. Control experiments were performed with BSA (□).

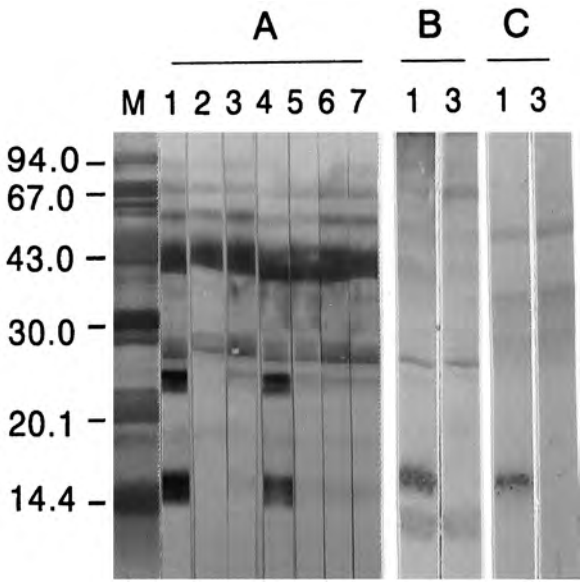

Fig. 6. Hel a 2 immunoblot-inhibition. Protein extracts from sunflower **(A)**, mugwort **(B)** and short ragweed **(C)** were separated by SDS–PAGE and transferred onto PVDF-membranes. Inhibition was performed by pre-incubating patients serum pools with: lane 1, PBS buffer; lane 2, natural sunflower profilin; lane 3, recombinant sunflower profilin; lane 4, BSA; lanes 5–7, recombinant profilins from *Olea europaea*, *Mercurialis annua*, and *Cynodon dactylon*, respectively.

blot experiments using fusion proteins with the amino o carboxy-part of Hel a 2, mAb 3G4 bound preferentially to the amino-part of the Hel a 2 and 3H8 to the carboxy-part (data not shown). In ELISA-inhibition experiments, total inhibition of the binding of the 3G4 antibodies to the solid phase-linked nHel a 2 was obtained using the recombinant protein or the GST-fused amino half of the protein as inhibitor. The GST-C-term protein had no effect in the IgG binding. These results indicated that the mAb 3G4 bound to an epitope located in the first 54 amino acids of the protein. On the other hand, binding of the 3H8 antibody was not completely inhibited by recombinant Hel a 2 or by the carboxy-half (50 and 32% of the inhibition obtained by natural Hel a 2, respectively). The amino-half of rHel a 2 had no inhibitory effect in the binding of this monoclonal antibody. The protein used for the fusion of profilin fragments, GST, had no effect in the binding of both mAbs tested.

4. Discussion

Type I allergy to pollen from members of the Compositae family is very common, particularly to pollen

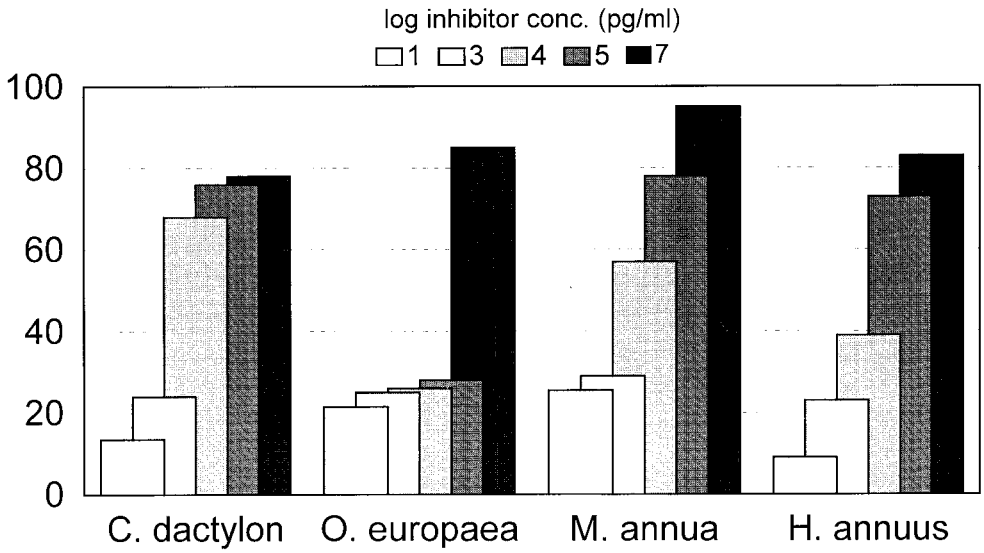

Fig. 7. Competitive inhibition of the binding of Hel a 2-specific human IgE antibodies to solid-phase natural Hel a 2 by different concentrations of recombinant profilins from *H. annuus*, *M. annua*, *C. dactylon* and *O. europaea*.

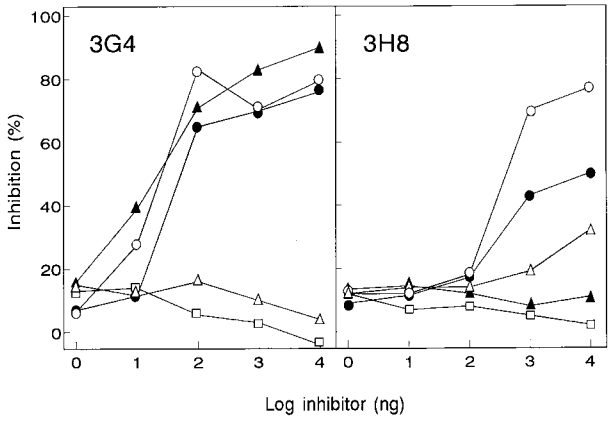

Fig. 8. Competitive inhibition of the binding of sunflower profilin mAbs 3G4 and 3H8 to solid-phase natural Hel a 2 by different concentrations of natural (○) and recombinant (●) Hel a 2, GST-N-term (▲) and GST-C-term (△). Control experiments were performed with BSA (□).

from anemophilous species such as short ragweed and mugwort. Less frequently, some entomophilous Compositae pollens, such as sunflower pollen, can also produce allergic symptoms, but mainly in people living around sunflower plantations (Fernández et al., 1993; de la Hoz et al., 1994; Jiménez et al., 1994). Cases of food allergy after eating sunflower seeds, sunflower oil and honey contaminated with sunflower pollen have been reported (Bousquet et al., 1985; Birnbaum et al., 1989; Hefle et al., 1997). These reports highlighted the importance of sunflower as an allergic plant not only related with occupational allergy (Jiménez et al., 1994). Allergens of similar molecular masses have been detected in both sunflower pollen and seeds and some of them have been isolated (Fernández et al., 1993; de la Hoz et al., 1994; Jiménez et al., 1994; Hefle et al., 1997). The allergen Hel a 1, with an apparent molecular mass of 32–34 kDa and an incidence among sunflower allergic-patients of 57%, has been isolated by two groups (de la Hoz et al., 1994; Jiménez et al., 1994). Purified sunflower profilin, as described herein, has a pI of 4.4–4.5 and an apparent molecular mass of 14.7 kDa. Immunoblot-inhibition experiments revealed that profilin from different origins inhibited IgE binding to the 14 kDa but also to a 24 kDa band. A sunflower pollen allergen with a pI identical of that of profilin (4.4) and molecular masses of 24 kDa, determined by size exclusion HPLC and 14 kDa, by SDS–PAGE, has been isolated (de la Hoz et al., 1994) and authors suggested that the native allergen was composed of two identical subunits. The possibility that the 24 kDa band corresponded to a profilin aggregate could not be ruled out since polymerization of profilins through disulfide bonds has been recently reported (Babich et al., 1996; Vrtala et al., 1996). Babich et al. (1996) postulated that the degree of aggregation observed by immunoblotting could be influenced by the protein isolation method or the screening techniques that select antibodies against profilin. Another possibility could be that two different proteins, of 14 and 24 kDa, shared common epitopes. Amino terminal sequencing data of the 24 kDa isolated protein could help to answer this question.

Different profilin isoforms, associated to plant polymorphisms, or even existence of different profilins in the same plant have been reported in tabacum (Mittermann et al., 1996), maize (Staiger et al., 1993), wheat (Rihs et al., 1994), *Arabidopsis* (Christensen et al., 1996), *Phleum pratense* (Asturias et al., 1997a) and olive tree (Asturias et al., 1997b) and it is explained on the basis of the important function that profilin plays in regulating the eukaryotic cytoskeleton (Baatout, 1996). The highest degree of allergen polymorphism described to date was

found in birch, where 13 isoforms of the pathogenesis-related allergen Bet v 1 have been isolated (Swoboda et al., 1995).

ELISA-inhibition experiments showed that antigenicity of the recombinant profilin was not identical to that of the natural counterpart, because the former was not able to inhibit IgG binding to the same level that the natural was. We suppose that rHel a 2, prepared in our laboratory, is one of the various isoform sunflower profilins so it only has some of the different IgG epitopes present in natural profilin. Since the antibodies of the rabbit profilin antiserum were produced against all the different IgG epitopes found in the sunflower profilin isoforms, only some of these antibodies are inhibited by rHel a 2. The epitope recognized by mAb 3G4 was present in both the recombinant and the truncated amino-end sunflower profilins. The conformation of the truncated amino-end sunflower profilin fused to GST was probably different of that of the complete protein therefore the epitope recognized by 3G4 was independent of the conformation and could be a sequential one. In contrast, other epitopes, such as that recognized by mAb 3H8, was not present in the rHel a 2 since the recombinant profilin was not able to inhibit IgG binding to the same level that natural Hel a 2 did. Few amino acid changes are sufficient to provoke differences in the IgG-epitope interaction as it has been demonstrated in birch profilin (Wiedemann et al., 1996). Nevertheless the determinants recognized by 3H8 could have some additional conformational component because the carboxy-half of the rHel a 2 produced even less inhibition than the whole rHel a 2. The folding of plant profilins is mainly stabilized by seven-stranded β-sheets and three α-helices (Fedorov et al., 1997; Thorn et al., 1997). Cutting Hel a 2 into two halves would undoubtedly disrupt contacts between secondary structure elements (Thorn et al., 1997) and strongly compromise the protein stability, as it has been reported for Bet v 1 (Vrtala et al., 1997).

Both immunological assays (ELISA and immunoblotting-inhibition) carried out on rHel a 2 showed that its allergenicity is very similar to that of the natural sunflower allergen. Results reported in this article indicated similar allergenicity but different antigenicity between natural and recombinant sunflower profilins. This would suggest the epitopes responsible for antigen-specific IgE antibodies production (allergenic epitopes) may differ from those promoting antigen-specific IgG antibodies (antigenic epitopes). Recent evidence suggests that there is a preferential variable heavy-chain usage in IgE synthesis and that IgE isotype switching progresses directly from IgM to IgE in human B lymphocytes (van der Stoep et al., 1994).

Recombinant Hel a 2 was also able to inhibit IgE binding to profilin from two important Compositae plants, namely short ragweed and mugwort. Cross-reactivity between sunflower pollen and other pollens from the Compositae family has been previously described (Fernández et al., 1993; Jiménez et al., 1994). Cross-inhibition experiments with profilin support the assumption that Hel a 2 shares epitopes with profilins from different phylogenetic origins such as the gramminea *C. dactylon*, the olive tree and the euphorbiacea, *M. annua*. The identification of common epitopes among plant profilins needs to be undertaken for the development of a potential immunotherapy based on profilin pan-allergenic epitopes.

Acknowledgements

The authors would like to thank Dr C. Moreno (Servicio de Alergia, Hospital Reina Sofia, Córdoba, Spain) for kindly providing sera from sunflower allergic patients and the Servicio de Secuenciación Automática (CIB-CSIC, Madrid, Spain) for sequencing facilities. M.A. is indebted to the Departamento de Educación (Basque Government) for a postdoctoral fellowship. This work was 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 Energia, Spain) and No. 346B04 from the Programa INTEK (Departamento de Industria, Agricultura y Pesca, Basque Government).

References

Arilla, M.C., Asturias, J.A., Gómez-Bayón, N., Martínez, A., Martínez, J., Palacios, R., 1997. Production and characterization of profilin monoclonal antibodies. Allergol. Et Immunopathol. 25, 145–151.

Asturias, J.A., Arilla, M.C., Bartolomé, B., Martínez, A., Martínez, J., Palacios, R., 1997a. Sequence polymorphism and structural analysis of timothy grass pollen profilin allergen (Phl p 11). Biochim. Biophys. Acta 1352, 253–257.

Asturias, J.A., Arilla, M.C., Gómez-Bayón, N., Martínez, J., Martínez, A., Palacios, R., 1997b. Cloning and expression of the panallergen profilin and the major allergen (Ole e 1) from olive tree pollen. J. Allergy Clin. Immunol. 100, 365–372.

Asturias, J.A., Arilla, M.C., Gómez-Bayón, N., Martínez, J., Martínez, A., Palacios, R., 1997c. Cloning and high level expression of *Cynodon dactylon* (Bermuda grass) pollen profilin (Cyn d 12) in *Escherichia coli*: purification and characterization of the allergen. Clin. Exp. Allergy 27, 1307–1313.

Axelsson, I.G.K., Ihre, E., Zetterstöm, O., 1994. Anaphylactic reactions to sunflower seed. Allergy 49, 517–520.

Baatout, S., 1996. Profilin: An update. Eur. J. Clin. Chem. Clin. Biochem. 34, 575–577.

Babich, M., Foti, L.R.P., Sykaluk, L.L., Clark, C.R., 1996. Profilin forms tetramers that bind to G-actin. Biochem. Biophys. Res. Commun. 218, 125–131.

Birnbaum, J., Tafforeau, M., Vervloet, D., Charpin, J., Charpin, D., 1989. Allergy to sunflower honey associated with allergy to celery. Clin. Experiment. Allergy 19, 229–230.

Bousquet, J., Dhivert, H., Clanzel, A.M., Hewit, B., Michel, F.B., 1985. Occupational allergy to sunflower pollen. J. Allergy Clin. Immunol. 75, 70–74.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Carlsson, I., Nyström, L.E., Sundkvist, F., Markey, F., Lindberg, U., 1977. Actin polymerization is influenced by profilin, a low molecular weight protein in non-muscle cells. J. Mol. Biol. 115, 465–483.

Christensen, H.E.M., Ramachandran, S., Tan, C.T., Surana, U., Dong, C.H., Chua, N.H., 1996. *Arabidopsis* profilins are functionally similar to yeast profilins: Identification of a vascular bundle-specific profilin and a pollen-specific profilin. Plant J. 10, 269–279.

de la Hoz, F., Melero, J.A., González, R., Carreira, J., 1994. Isolation and partial characterization of allergens from *Helianthus annuus* (sunflower) pollen. Allergy 49, 848–854.

Fedorov, A.A., Ball, T., Mahoney, N.M., Valenta, R., Almo, S.C., 1997. The molecular basis for allergen cross-reactivity: Crystal structure and IgE-epitope mapping of birch pollen profilin. Structure 5, 33–45.

Fernández, C., Esteban, M.M., Fiandor, A., Pascual, C., López, Serrano, C., Martínez, Alzamora, F., Díaz Pena, J.M., Ojeda Casas, J.A., 1993. Analysis of cross-reactivity between sunflower pollen and other pollens of the compositae family. J. Allergy Clin. Immunol. 92, 660–667.

Goldschmidt-Clermont, P.J., Kim, J., Machesky, L.M., Rhee, S., Pollard, T.D., 1991. Regulation of phospholipase Cγ1 by profilin and tyrosine phosphorylation. Science 251, 1231–1233.

Hefle, S.L., Nordlee, J.A., Taylor, S.L., 1997. Identification and characterization of sunflower seed allergens. J. Allergy Clin. Immunol. 99, 142 (Abstract).

Hirschwehr, R., Valenta, R., Ebner, C., Ferreira, F., Sperr, W., Valent, P., Rohac, M., Rumpold, H., Scheiner, O., Kraft, D., 1992. 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. 90, 927–936.

Jiménez, A., Moreno, C., Martínez, J., Martínez, A., Bartolomé, B., Guerra, F., Palacios, R., 1994. Sensitization to sunflower pollen: only an occupational allergy? Int. Arch. Allergy Immunol. 105, 297–307.

King, T.P., Hoffman, D., Lowenstein, H., Marsh, D.G., Platts-Mills, T., Thomas, W., 1995. Allergen Nomenclature. J. Allergy Clin. Immunol. 96, 5–14.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 277, 680–685.

Mittermann, I., Swoboda, I., Pierson, E., Eller, N., Kraft, D., Valenta, R., Heberle-Bors, E., 1995. Molecular cloning and characterization of profilin from tobacco (*Nicotiana tabacum*) increased profilin expression during pollen maturation. Plant Mol. Biol. 27, 137–146.

Mittermann, I., Heiss, S., Kraft, D., Valenta, R., Heberle-Bors, E., 1996. Molecular characterization of profilin isoforms from tobacco (*Nicotiana tabacum*) pollen. Sex Plant Reprod. 9, 133–139.

Rihs, H., Rozynek, P., May-Taube, K., Welticke, B., Baur, X., 1994. Polymerase chain reaction based cDNA cloning of wheat profilin: a potential plant allergen. Int. Arch. Allergy Immunol. 105, 190–194.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Scheiner, O., 1992. Recombinant allergens: biological, immunological and practical aspects. Int. Arch. Allergy Immunol. 98, 93–96.

Scheiner, O., Kraft, D., 1995. Basic and practical aspects or recombinant allergens. Allergy 50, 384–391.

Staiger, C., Goodbody, K., Hussey, P., Valenta, R., Drobak, B., Lloyd, C., 1993. The profilin multigene family of maize: differential expression of three isoforms. Plant J. 4, 631–641.

Stewart, G., 1995. The molecular biology of allergens. In: Busse W., Holgate S. (Eds), Asthma and Rhinitis. Backwell Scientifics Publications, Boston, p. 898.

Studier, F.M., Moffatt, B.A., 1986. Use of bacteriophage T7 polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 189, 113–130.

Swoboda, I., Jilek, A., Ferreira, F., Engel, E., Hoffmann-Sommergruber, K., Scheiner, O., Kraft, D., Breiteneder, H., Pittenauer, E., Schmid, E., Vicente, O., Heberle-Bors, E., Ahorns, H., Breitenbach, M., 1995. Isoforms of Bet v 1, the major birch pollen allergen, analysed by liquid chromatography, mass spectrometry and cDNA cloning. J. Biol. Chem. 270, 2607–2613.

Thorn, K.S., Christensen, H.E.M., Shigeta, R., Huddler, D., Shalaby, L., Lindberg, U., Chua, N.H., Schutt, C.E., 1997. The crystal structure of a major allergen from plants. Structure 5, 19–32.

Towbin, H., Staehelin, I., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76, 4350–4354.

Valenta, R., Duchêne, M., Pettenburger, K., Sillaber, C., Valent, P., Bettelheim, P., Breitenbach, M., Rumpold, H., Kraft, D., Scheiner, O., 1991. Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. Science 253, 557–560.

Valenta, R., Duchêne, M., Ebner, C., Valent, P., Sillaber, C., Deviller, P., Ferreira, F., Tejkl, M., Edelmann, H., Kraft, D., Scheiner, O., 1992a. Profilins constitute a novel family of functional plant pan-allergens. J. Exp. Med. 175, 377–385.

Valenta, R., Duchêne, M., Vrtala, S., Valent, P., Sillaber, C., Ferreira, F., Tejkl, M., Hirschwehr, R., Ebner, C., Kraft, D., Scheiner, O., 1992b. Profilin, a novel plant pan-allergen. Int. Arch. Allergy Immunol. 99, 271–273.

Valenta, R., Ball, T., Vrtala, S., Duchêne, M., Kraft, D., Scheiner, O., 1994. cDNA cloning and expression of timothy grass (*Phleum pratense*) pollen profilin in *Escherichia coli*: comparison with birch pollen profilin. Biochem. Biophys. Res. Commun. 199, 106–118.

Vallverdú, A., García-Ortega, P., Martínez, J., Martínez, A., Esteban, M.I., Molina de, M., Fernández-Távora, L., Fernández, J., Bartolomé, B., Palacios, R., 1997. *Mercurialis annua*: characterization of main allergens and cross-reactivity with other species. Int. Arch. Allergy Immunol. 112, 356–364.

Vallverdú, A., Asturias, J.A., Arilla, M.C., Gómez-Bayón, N., Martínez, A., Martínez, J., Palacios, R., 1998. Characterization of recombinant *Mercurialis annua* major allergen Mer a 1 (profilin). J. Allergy Clin. Immunol. 101,363–370.

van der Stoep N., Korver W., Logtenberg T., 1994. In vivo and in vitro IgE isotype switching in human B lymphocytes: evidence for a predominantly direct IgM to IgE class switch program. Eur. J. Immunol. 24, 1307–1311.

van Ree, R., Voitenko, V., van Leeuwen, W., Aalberse, R., 1992. Profilin is a cross-reactive allergen in pollen and vegetable foods. Int. Arch. Allergy Immunol. 98, 97–104.

Vrtala, S., Mayer, P., Ferreira, F., Susani, M., Sehon, A.H., Kraft, D., Valenta, R., 1996. Induction of IgE antibodies' in mice and rhesus monkeys with recombinant birch pollen allergens: Different allergenicity of Bet v 1 and Bet v 2. J. Allergy Clin. Immunol. 98, 913–921.

Vrtala, S., Hirtenlehner, K., Vangelista, L., Pastore, A., Eichler, H.G., Sperr, W.R., Valent, P., Ebner, C., Kraft, D., Valenta, R., 1997. Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments. Candidates for a novel form of specific immunotherapy. J. Clin. Invest. 99, 1673–1681.

Way, M., Pope, B., Gooch, J., Hawkins, M., Weeds, A.G., 1990. Identification of a region in segment 1 of gelsolin critical for actin binding. EMBO J. 9, 4103–4109.

Wiedemann, P., Giehl, K., Almo, S.C., Fedorov, A.A., Girvin, M., Steinberger, P., Rudiger, M., Ortner, M., Sippl, M., Dolecek, C., Kraft, D., Jockusch, B., Valenta, R., 1996. Molecular and structural analysis of a continuous birch profilin epitope defined by a monoclonal antibody. J. Biol. Chem. 271, 29915–29921.

Williams, K.R., Stone, K.L., 1995. In gel digestion of SDS–PAGE separated proteins: observations from internal sequencing of 25 proteins. In: Crabb, J.W. (Eds), Techniques in Protein Chemistry VI. Academic Press, San Diego, CA, p. 143.