Molecular and Immunological Characterization of Profilin from Mugwort Pollen

Nicole Wopfner[1,a], Marika Willeroider[2,a],
Daniel Hebenstreit[1], Ronald van Ree[3],
Marja Aalbers[3], Peter Briza[1], Josef Thalhamer[2],
Christof Ebner[4], Klaus Richter[1] and
Fátima Ferreira[1,*]

[1] Institute of Genetics,
[2] Institute of Chemistry and Biochemistry, University of
Salzburg, A-5020 Salzburg, Austria
[3] Department of Immunopathology, Sanquin Research at
CLB, NL-1066 CX Amsterdam, The Netherlands
[4] Department of Pathophysiology, University of Vienna,
AKH-EBO-3Q, A-1090 Vienna, Austria

* Corresponding author

In late summer in Europe, pollen of mugwort is one of
the major sources of atopic allergens. No information
about the complete molecular structure of any mug-
wort allergen has been published so far. Here we re-
port the isolation and characterization of mugwort
pollen cDNA clones coding for two isoforms of the
panallergen profilin. Thirty-six percent of the mug-
wort-allergic patients tested displayed IgE antibodies
against natural and recombinant profilin, and no sig-
nificant differences were observed in the IgE-binding
properties of the isoforms. One profilin isoform was
purified to homogeneity and detailed structural
analysis indicated that the protein exists in solution
as dimers and tetramers stabilized by sulfydryl and/or
ionic interactions. Profilin monomers were detec-
table only after exposure of multimers to harsh dena-
turing conditions. Dimers and tetramers did not sig-
nificantly differ in their ability to bind serum IgE from
mugwort pollen-allergic patients. However, oligomer-
ic forms might have a higher allergenic potential than
monomers because larger molecules would have ad-
ditional epitopes for IgE-mediated histamine release.
Profilin isolated from mugwort pollen also formed
multimers. Thus, oligomerization is not an artifact re-
sulting from the recombinant production of the aller-
gen. Inhibition experiments showed extensive IgE
cross-reactivity of recombinant mugwort profilin and
profilin from various pollen and food extracts.
Key words: Allergen / cDNA cloning / IgE-binding /
Isoallergen / Isoform / Mugwort pollen / Oligomerization /
Profilin.

[a] These authors contributed equally to this work

Introduction

The family Compositae or Asteraceae is known to include
several allergenic plants, and among them Ambrosia (rag-
weed), Parthenium (feverfew), and Artemisia (mugwort)
are of major importance. Pollen of mugwort (Artemisia
vulgaris) is one of the main causes of allergic reactions in
late summer and autumn in Europe. It is widely spread,
but is predominantly found in the temperate and humid
zones of the Northern hemisphere and along the Mediter-
ranean basin (Futin et al., 1976). In central Europe, mug-
wort pollination occurs at the end of July and through Au-
gust whereas in the Mediterranean areas it is mainly
occurring in September and beginning of October
(Spieksma et al., 1980). Among patients suffering from
pollinosis, the incidence of allergic disease caused by
mugwort pollen is between 10 and 14% (Cornillon et al.,
1972; Spieksma et al., 1980).

Previous studies performing physico-chemical and
immunochemical characterization of crude and partially
purified extracts demonstrated that mugwort pollen
contains at least 9 different allergens (de la Hoz et al.,
1990; Nilsen and Paulsen, 1990; Nilsen et al., 1991).
Two mugwort allergens, designated Art v 1 and Art v 2,
were purified from pollen and shown to bind IgE from
70% and 45% of mugwort pollen-allergic patients, re-
spectively. In addition, the complete structure of the
Asn-linked oligosaccharide chains and a partial amino
acid sequence of Art v 2 were reported (Nilsen et al.,
1991). The partial amino acid sequence of Art v 2 shows
some similarities to a group of pathogenesis-related
proteins from Brassica (Hanfrey et al., 1996) and Ara-
bidopsis (Uknes et al., 1992). Art v 3, a cross-reactive
mugwort pollen allergen belonging to the family of lipid
transfer proteins (LTPs) was purified and the N-terminal
amino acid sequence determined (Diaz-Perales et al.,
2000). Poly(L-proline)-affinity chromatography (Valenta
et al., 1992) and IgE cross-inhibition experiments
(Hirschwehr et al., 1998) demonstrated that the plant
pan-allergen profilin is also present in mugwort pollen.
Nevertheless, no information about the complete mo-
lecular structure of any of the mugwort pollen allergens
has been reported so far.

In this study we report the complete cDNA sequences
of two isoforms of profilin from mugwort pollen. The iso-
forms were expressed in E. coli and the recombinant
non-fusion proteins tested with sera from allergic pa-
tients for their IgE-binding activity. One isoform was puri-
fied to homogeneity and its biochemical and immunolog-
ical properties were characterized.

Results

Cloning and Sequence Analysis of IgE-Positive Clones

A serum pool from 16 mugwort pollen-allergic patients was used for IgE immunoscreening of a mugwort pollen cDNA library. Forty-two IgE-positive plaques were isolated out of 400 000 plaques. The first IgE-positive clone sequenced was identified as profilin and it was subsequently used as a probe for hybridization with the remaining forty-one clones to identify other profilin clones. In total thirteen of the IgE-positive clones coded for profilin as determined by DNA hybridization. Six of the profilin clones (Av1-Av6) were selected for DNA sequence analysis. All clones were complete and contained 5' untranslated leaders of 11 to 50 bp. The 3'-noncoding regions of six clones differed in their length [Av1, 225 bp; Av3, 330 bp; Av4, 182 bp; Av5, 131 bp; and Av6, 114 bp, all followed by short poly(A⁺) tails], but had identical sequences in their overlapping regions. The 3'-noncoding region of clone Av2 (200 bp) did not show sequence similarities with the corresponding region of other profilin clones, except for two stretches of 12 bp and 19 bp. The cDNAs contained open reading frames of 402 nucleotides, thus coding for a profilin protein of 132 amino acids (excluding the starting methionine). The differences in the nucleotide sequences of five profilin clones (Av1, Av3, Av4, Av5 and Av6) did not result in amino acid exchanges. Clone Av2 differed from Av1 through 70 nucleotide exchanges, resulting in 17 amino acid substitu-
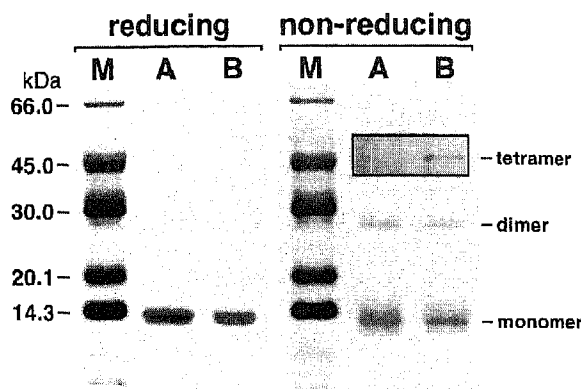
tions. We designated the protein encoded by clones Av1, Av3, Av4, Av5 and Av6 as Art v 4.01 and by clone Av2 as Art v 4.02, in accordance with the IUIS allergen nomenclature system (King et al., 1995). The deduced amino acid sequences of the Art v 4 isoallergens are shown in Figure 1.
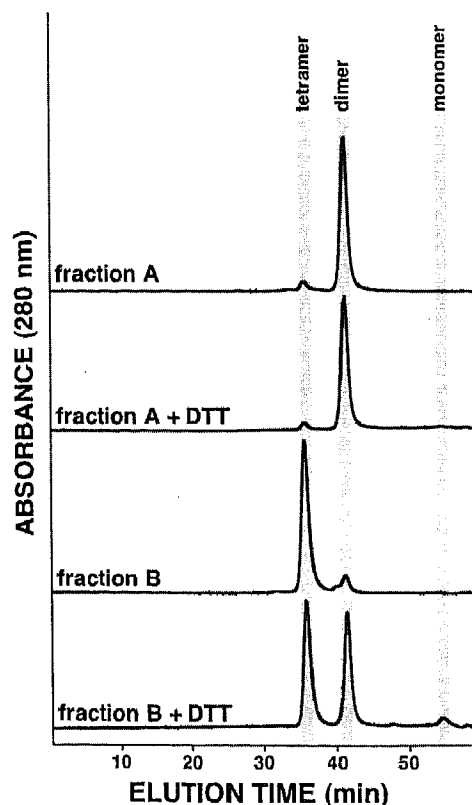

A

B

Fig. 2 Homogeneity of Purified Recombinant Art v 4.01 Isoallergen.
(A) Two μg of Fractions A (lanes A) and B (lanes B) of purified recombinant Art v 4.01 (see Materials and Methods) were analyzed by SDS-PAGE under reducing (left panel) and non-reducing conditions (right panel). The inset in the right panel shows the corresponding region of a gel loaded with 10 μg protein/lane. Gels were stained with Coomassie Blue R-250. Lanes M, molecular mass markers. (B) Analytical HPLC size exclusion chromatography of recombinant profilin Fractions A and B using phosphate-buffered saline with or without DTT as reducing agent.

```
            1                                                 50
Art v 4.01  MSWQTYVDDHLMCDIEG-TGQHLTSAAIFGTDGTVWAKSASFPEFKPNEI
Art v 4.02  ................-......A...L.L........DK......E.M
Hel a 2     ....A...E.......-........L.L.......Q..K..Q...E.M
Mer a 1     ..............D.-Q....AA.S.V.H..SI..Q.....QL..E..
Bet v 2     .........E......D.-QASNSLAS..V.H..S...Q.S...Q...Q..
Ole e 2     ....A...........HEDHR..A...V.H..S...Q..T..Q...E.M
Api g 4     ....A..........EV..NP..T..A...I.H..S...Q.ST..QI..E..
Phl p 12    .........E....E...---H..A....L.H......Q..D..Q...E..

            51                                               100
Art v 4.01  DAIIKEFNEAGQLAPTGLFLGGAKYMVIQGEAGAVIRGKKGAGGICIKKT
Art v 4.02  KG..N....V.T..................L...................
Hel a 2     KG.....D...T.....M.IA......L...P..................
Mer a 1     TG.M.D.D.P.H......YIA.T.........S.........S...T....
Bet v 2     TG.M.D.E.P.H......H...I..................S...T....
Ole e 2     NG.MTD...P.H......H...T..................S...T....
Api g 4     AG.M.D.D.P.H......Y...........PN..........S..VT....
Phl p 12    TG.M.D.D.P.H.....M.VA..........P.R...........T....

            101                             134
Art v 4.01  GQAMVFGIYDEPVAPGQCNMVVERLGDYLLDQGM
Art v 4.02  .....M...................I........V..N.
Hel a 2     ....IM.......................E...
Mer a 1     ...L.....E...T...............IE...
Bet v 2     ...L.....E...T...............I...L
Ole e 2     ...L.....E...T...............VB...
Api g 4     ...L...V.....T.....VI........I...L
Phl p 12    ...L.V......MT...............VE...
```

Fig. 1 Amino Acid Sequence Comparison of Mugwort Profilin Isoallergens Art v 4.01 (Accession Number AJ421030) and Art v 4.02 (Accession Number AJ421031) with Different Plant Profilins.
The following sequences were obtained from the EMBL database (accession numbers given in parentheses): Hel a 2, Helianthus annuus pollen profilin (Y15210); Mer a 1, Mercurialis annua pollen allergen (Y13271); Bet v 2, Betula verrucosa pollen allergen (M65179); Ole e 2, Olea europea pollen allergen (Y12425); Api g 4, Apium graveolens allergen (AF129423); Phl p 12, Phleum pratense pollen allergen (X77583). Dots indicate identical amino acids.

Expression and Purification of Recombinant Mugwort Profilin

Non-fusion forms of two isoforms of mugwort pollen pro-filin (Art v 4.01 and Art v 4.02) were produced in *E. coli* as intracellular proteins. High levels of expression of recombinant proteins were obtained. To evaluate the IgE-binding activity of the recombinant profilin isoforms, im-munoblot experiments with bacterial lysates and mugwort pollen extract were performed using sera from 100 mugwort pollen-allergic patients (data not shown). Thirty-six percent of the mugwort pollen-allergic patients tested displayed IgE antibodies reacting with natural and recombinant profilin. All sera recognizing natural profilin also showed IgE reactivity with the recombinant iso-forms. However, no significant differences were observed on the IgE binding properties of the two profilin isoforms. The isoform Art v 4.01 was selected for purification and further biochemical and immunological characterization. The total yield of recombinant protein was approximately 25 mg/l bacterial culture. Interestingly, profilin eluted as two distinct peaks from the DEAE-Sepharose column, but both fractions appeared homogenous as judged by SDS-PAGE analysis and Coomassie Blue staining (Fig-ure 2A, left panel). These two peaks were termed fraction A and fraction B and were further analyzed separately.

Structural Characterization of Recombinant Mugwort Profilin

The primary structure of purified recombinant Art v 4.01 isoform was confirmed by MALDI-TOF mass spectrome-try (Figure 3A). The molecular mass of profilin in fractions A and B was determined at 14 077.0, which is in excellent agreement with the theoretical molecular mass of 14 076.1 based on the cDNA sequence. This result is con-sistent with the removal of the initiating methionine and the N-end rule (Bachmair *et al.*, 1986) and strongly sup-ports the notion that differences between fractions A and B are not due to modification(s) of the primary structure.

The homogeneity of the mugwort profilin preparations with respect to molecular mass species was examined by analytical gel filtration (Figure 2B). Recombinant profil-in in fractions A and B eluted as one major peak at 41.3 min and 35.8 min, respectively. Pre-treatment of the samples with DTT followed by HPLC analysis under re-ducing conditions caused the appearance of an addition-al peak in fraction B eluting at 41.3 min. Under similar conditions, no changes were observed in the elution pro-file of fraction A. Based on the elution times of molecular mass standards, we concluded that profilin in fraction A is present in solution mainly as stable dimers. On the oth-er hand, in fraction B mostly tetramers are found that can be converted to profilin dimers under non-denaturing re-ducing conditions. When samples were boiled in the presence of DTT and analyzed by HPLC, most of the dimers and tetramers in fractions A and B, respectively, were converted to monomers eluting at 58.6 min (data not shown).
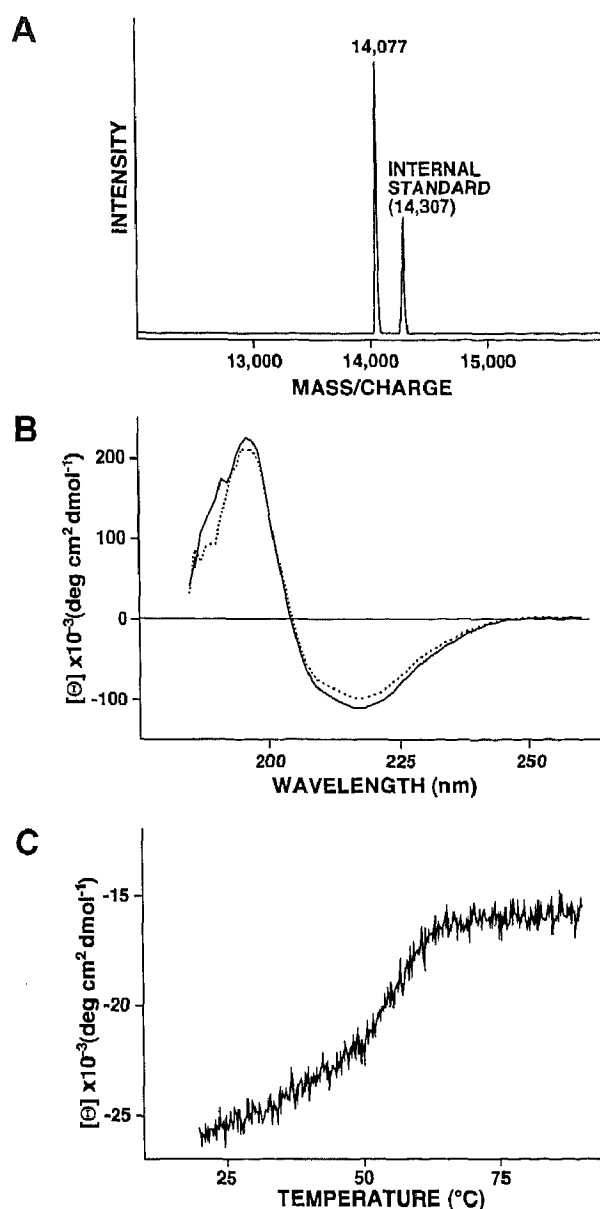

Fig. 3 Primary and Secondary Structure Analyses of Purified Recombinant Art v 4.01 Isoallergen.

(A) MALDI-TOF mass spectrometry analysis of recombinant Art v 4.01 isoallergen using chicken egg white lysozyme as internal mass standard. (B) Far-ultraviolet circular dichroism spectra of recombinant Art v 4.01 fractions A (solid line) and B (dashed line). (C) Thermal unfolding of purified recombinant Art v 4.01 fraction B. Ellipticity was monitored at 222 nm. The melting point was estimated as 55 °C.

Oligomeric forms of profilin were also detected by SDS-PAGE analysis (Figure 2A, right panel). As expected, only the monomeric form of profilin migrating at 14 kDa was observed in fractions A and B under denaturing and reducing conditions. Under denaturing non-reducing conditions, both monomeric (14 kDa) and dimeric (28 kDa) forms were observed in fraction A, whereas in fraction B monomers, dimers, and tetramers were ob-served. To investigate whether natural profilin also forms oligomers, the allergen was purified from mugwort pollen

and analyzed by SDS-PAGE under denaturing reducing and non-reducing conditions and immunoblotting with a rabbit anti-profilin serum. Under reducing conditions, a single immunoreactive band migrating at 14 kDa was detected in mugwort pollen as well as in other pollens (Figure 4A). Under non-reducing conditions, monomeric, dimeric, trimeric, and tetrameric forms of natural mugwort profilin reacting with the rabbit antiserum were observed (Figure 4B). Negative controls in Figure 4A and B demonstrate the specificity of the rabbit antiserum and of the detection system used for the immunoblot assay.

The far-ultraviolet circular dichroism spectra of purified Art v 4.01 fractions A and B were characteristic for a well-structured protein, showing the typical bands for α helices (Figure 3B). The spectra showed a broad minimum at 217 nm and a maximum at 196 nm. The overall sec-

ondary structure content of the two fractions A and B was very similar. Figure 3C shows the thermal unfolding of profilin fraction B monitored as change in ellipticity at 222 nm and expressed as mean residue ellipticity (Θ). The melting point was calculated as 55 °C. After cooling from 90 °C to 20 °C, the far-UV spectra showed that mugwort profilin does not refold after thermal denaturation (data not shown).

Antibody Binding to Purified Recombinant Mugwort Profilin

IgE binding to purified profilin in Fractions A and B was investigated by immunoblot, ELISA, and RAST inhibition using sera from mugwort pollen-allergic patients reacting to profilin. Figure 5 (left panels) shows the IgE reactivity
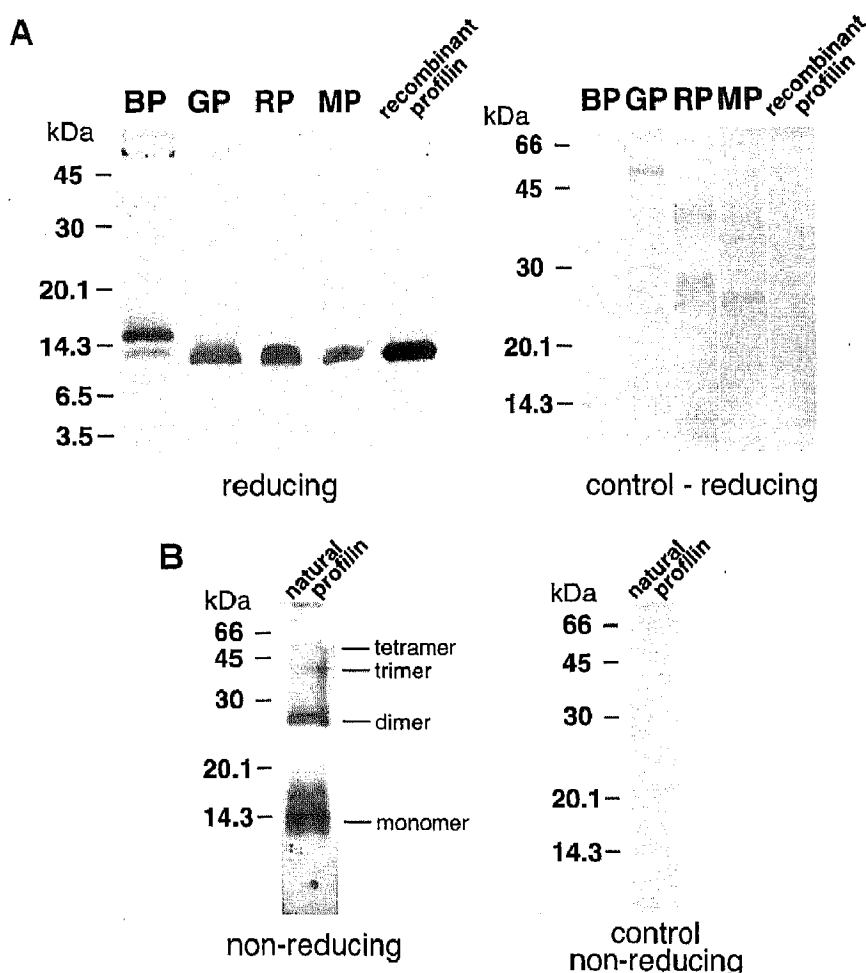

Fig. 4   Natural Mugwort Pollen Profilin Forms Oligomers.
(A) 1.5 μg of purified recombinant mugwort profilin (isoallergen Art v 4.01) and 10 μl of extracts from birch- (BP), grass- (GP), ragweed- (RP), and mugwort- (MP) pollen were separated by 15% SDS-PAGE under reducing conditions and transferred to nitrocellulose. After incubation with rabbit anti-*Lolium perenne* pollen profilin antiserum, bound antibodies were detected with alkaline phosphatase-conjugated anti-rabbit IgG antibodies (left panel). A single reactive band migrating at 14 kDa and corresponding to profilin monomer was detected in mugwort and other pollens. Negative controls leaving out the primary antibody incubation step (right panel) showing faint bands at higher molecular weight ranges after being developed for longer periods of time. (B) 1.5 μg of purified natural mugwort profilin was separated by 15% SDS-PAGE under non-reducing conditions, transferred to nitrocellulose, and incubated with rabbit anti-*Lolium perenne* pollen profilin antiserum (left panel). Bound antibodies were detected with alkaline phosphatase-conjugated anti-rabbit IgG antibodies. Monomers, dimers, trimers, and tetramers of natural profilin reacted with the rabbit anti-profilin serum. Negative control leaving out the primary antibody incubation step (right panel).
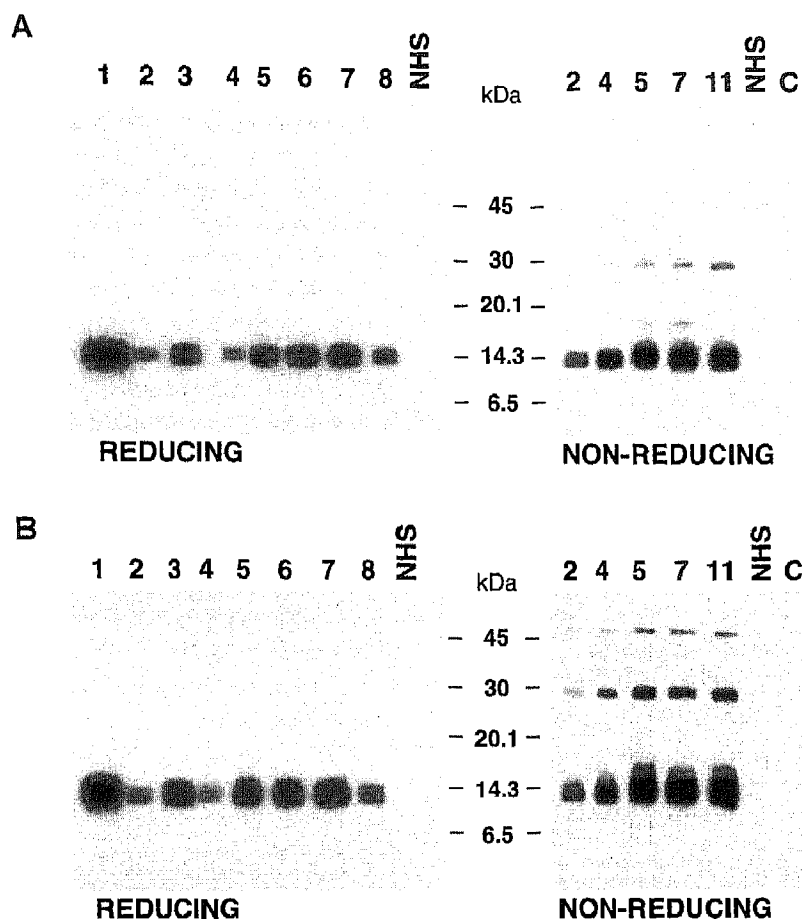
Fig. 5   IgE Immunoblot Analyses of Purified Recombinant Art v 4.01 Isoallergen.
(A) Purified Art v 4.01 Fraction A (9 µg/cm gel) was electrophoresed by 15% SDS-PAGE under reducing and non-reducing conditions, transferred to nitrocellulose, and developed with serum IgE from mugwort pollen-allergic patients (1–8) or with a serum pool from healthy non-allergic individuals (NHS). (B) Purified Art v 4.01 fraction B (10 µg/cm gel) was separated by 15% SDS-PAGE under reducing and non-reducing conditions, transferred to nitrocellulose, and developed with serum IgE from five mugwort pollen-allergic patients. Sera used for the experiments in panel B were obtained from the same allergic individuals, as indicated in panel (A). Negative controls included incubation with a serum pool from healthy non-allergic individuals (NHS) and leaving out the serum incubation step (lanes 'C').

patterns of eight patients with profilin in fractions A and B using the immunoblot technique. No major differences were observed in the IgE binding activity of the two fractions after being subjected to the denaturing conditions used for SDS-PAGE/blotting analysis. Interestingly, under denaturing non-reducing conditions, oligomeric forms of profilin in fractions A (dimers) and B (dimers and tetramers) also showed IgE reactivity (Figure 5, right panels).

In ELISA experiments no significant differences in the IgE recognition of fractions A and B were detected, except for patient 2 showing a slightly decreased reactivity with fraction B (Figure 6A). Therefore, fraction A was subsequently used in the cross-inhibition experiments.

IgE cross-reactivity of two sera with clear anti-profilin activity (P52 and P200) was examined using RAST inhibition with recombinant mugwort profilin (fraction A) coupled to Sepharose. As inhibitors extracts from pollen (birch and rye grass) and from food plants (potato, soy, celery, and carrot) were used. Although a quantitative

comparison is difficult to be established, partly because of the quality of extracts, the results clearly show that there is broad cross-reactivity (Figure 6B). The cross-reactivity of mugwort profilin was evaluated in another set of RAST inhibition experiments using the same two P52 and P200 sera and recombinant mugwort profilin as inhibitor (Figure 6C). The results, given in Figure 6C, also showed that mugwort profilin inhibits IgE binding to other extracts coupled to Sepharose, in most cases between 40% and 80%. Grass pollen is hardly inhibited. Both sera showed considerable levels of inhibition (P52: 75.9%, P200: 58.7%) for birch pollen. For a more quantitative evaluation of IgE cross-reactivity, dose-related RAST inhibition experiments using P52 and P200 sera and recombinant mugwort profilin as inhibitor (Figure 6D) were also performed. Based on these experiments, the amount of recombinant profilin required to give 50% inhibition of IgE to immobilized pollen and food extracts was determined as following: for patient P52, 16, 20, 20, and 40 µg of rArt v 4/ml gave 50% inhibition of IgE binding to

extracts from carrot, celery, peanut, and birch, respectively. For patient P200, 2.9, 2.8, 2.5, and 200 µg of rArt v 4/ml resulted in 50% inhibition of IgE binding to extracts from carrot, celery, peanut, and birch, respectively.

Discussion

Profilins are one of the most abundant actin monomer binding proteins, forming a 1:1 complex with G-actin. In addition to actin, profilins also interact with poly-L-proline (PLP) and proline-rich proteins, membrane polyphosphoinositides, phosphatidylinositol-3-kinase, annexin, and several multiprotein complexes that are implicated in the regulation of actin nucleation and endocytosis (for a review see Schlüter et al., 1997). In plants, profilins were first identified as a birch pollen allergen (Valenta et al., 1991) and as an important cross-reactive panallergen for patients suffering from multiple food and pollen allergies (Valenta et al., 1992; van Ree et al., 1992). Since then, profilin has been shown to be present in plants as large multigene families, and many cells or tissues can express multiple isoforms of profilin.

In this study, we presented the complete cDNA sequences of profilin isoforms from mugwort pollen. This is the first report of complete sequences of a mugwort pollen allergen. The two predicted profilin isoforms had calculated molecular masses and pI values of 14 076.0 Da/4.74 (Art v 4.01) and 14 104.1 Da/4.74 (Art v 4.02). Comparison of the deduced amino acid sequence of mugwort pollen profilin revealed high sequence identity with other plant profilin sequences. Pairwise alignments of Art v 4.01 and Art v 4.02 indicated the highest sequence identity with profilins from sunflower (84% and 86% identities; Asturias et al., 1998), olive (77%; Asturias et al., 1997b), Mercurialis annua (75% and 72%; Vallverdu et al., 1998), and tomato (75% and 73%, accession number AJ417553). The lowest homology among profilins from plant origin was found with peanut (68% and 67%; Kleber-Janke et al., 1999) and hazelnut (67% and 69%, acc. no. AF327623).

Different profilin isoforms in the same plant have been previously reported in wheat (Rihs et al., 1994), tobacco pollen (Mittermann et al., 1995), Arabidopsis (Huang et al., 1996), timothy grass pollen (Asturias et al., 1997a), olive pollen (Asturias et al., 1997b), maize (Staiger et al., 1993), bean (Guillen et al., 1999), and soybean (Rihs et al., 1999). However, the significance of distinct profilin isoforms for cellular function is not yet well understood. In this context, pollen is of particular interest because several isoforms are expressed in this tissue and it is not clear whether they are functionally redundant or perform specific functions. Analysis of native and recombinant profilin isoforms from maize pollen demonstrated the existence of two classes of functionally distinct isoforms (Kovar et al., 2000). Class II profilins have a higher affinity for PLP, sequester more G-actin than do class I profilins and are able to more rapidly disrupt cellular architecture when microinjected in Tradescantia virginiana stamen
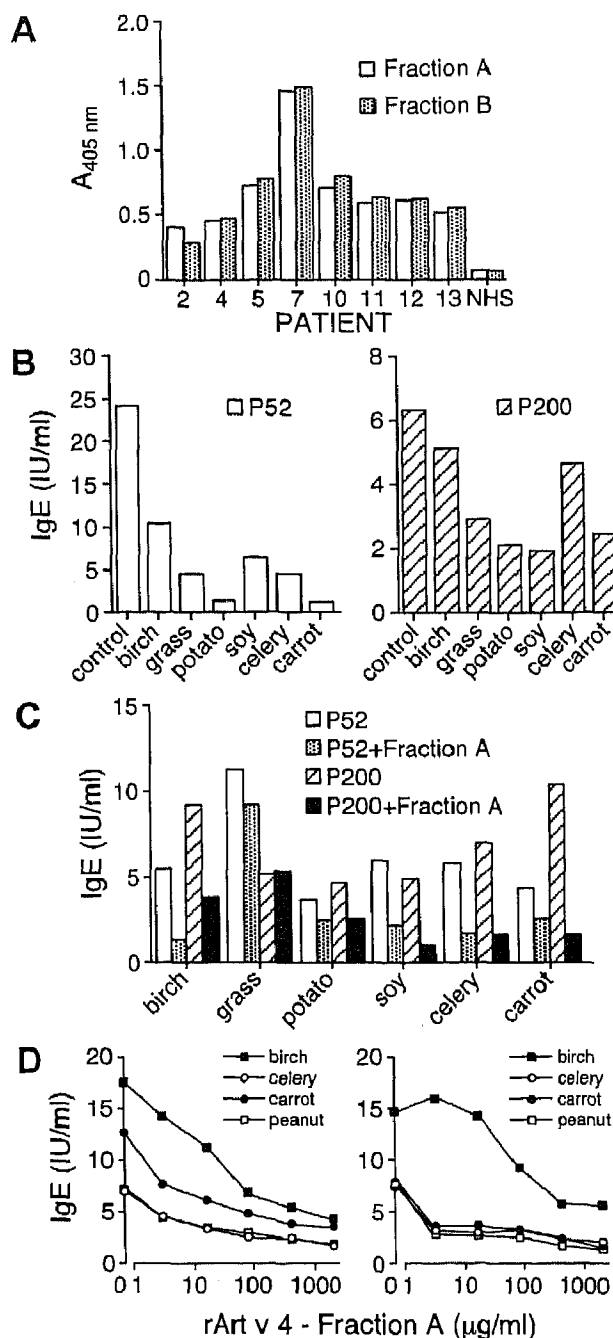
Fig. 6   IgE Binding Activity of Dimers and Tetramers of Purified Recombinant Art v 4.01 Isoallergen and Cross-Reactivity with Natural Profilin in Various Allergenic Sources.
(A) IgE-binding to dimers (fraction A) and tetramers (fraction B) tested in an ELISA assay using sera from mugwort pollen-allergic patients or a serum pool from healthy non-allergic individuals (NHS) as negative control. Sera used for the ELISA experiments were obtained from the same allergic individuals, as indicated in Figure 5. (B) RAST (control bars) and RAST inhibition experiments using recombinant mugwort profilin (fraction A) coupled to Sepharose and pollen or food extracts as inhibitors. Sera from two patients (P52 and P200) displaying IgE against profilin were used. (C) RAST (P52 and P200 bars) and RAST inhibition using pollen or food extracts coupled to Sepharose and mugwort profilin fraction A as inhibitor. Sera from two patients (P52 and P200) displaying IgE against profilin were used. (D) Dose-related RAST inhibition curves using pollen or food extracts coupled to Sepharose and increasing concentrations (3.2, 16, 80, 400, and 2000 µg/ml) of recombinant mugwort profilin (fraction A) as inhibitor.

hair cells. Native and recombinant profilin isoforms isolated form different eukaryotic cells have also been tested for their ability to interact with G-actin, PLP or proline-rich peptides, and phosphoinositide lipids, the three general profilin ligands. Taken together, the results from these studies indicate that profilins from different organisms as well as individual isoforms from the same organism do not share identical biochemical properties. However, sequence analysis of profilins revealed no obvious patterns of substitutions of the residues involved in ligand binding that would explain differences in the biochemical properties of profilins from different species or of profilin isoforms in one organism. Therefore, predictions of biochemical properties based upon amino acid sequences of the profilin isoforms from mugwort pollen would not be reliable and only a detailed biochemical analysis could establish their biological activities.

Concerning the recognition of profilin isoforms from mugwort pollen by IgE antibodies from allergic patients, no significant differences in their IgE reactivity were observed. Although the allergenicity of different profilin isoforms from various allergenic sources has not been extensively investigated, other studies also did not report major differences in IgE recognition of individual isoforms (Mittermann et al., 1995; Asturias et al., 1997a,b).

A detailed structural analysis of recombinant mugwort profilin indicated that the protein exists in solution as dimers and tetramers of the same chemical species. Our experiments also showed that oligomerization occurs with natural profilin isolated from mugwort pollen. Thus, the observed multimerization effect is not an artifact resulting from the recombinant production and purification of mugwort profilin. Previously human profilin purified from platelets (Babich et al., 1996) was also shown to exist in solution as tetramers and dimers. The two-step purification method used here allowed us to separate dimers from tetramers and analyze them separately. Monomers were just observed after subjecting dimers and tetramers to the harsh SDS-PAGE conditions (SDS, reducing agent and sample boiling) or similar (reducing agent and sample boiling). Taken together, our results indicate that profilin dimers and tetramers are stabilized in solution by sulphydryl and/or ionic interactions.

The biochemical and immunologic significance of profilin multimerization have not been fully investigated and only limited data is available. In a protein overlay assay, human profilin tetramers were shown to be the relevant form for high-affinity binding to actin (Babich et al., 1996). Recombinant maize pollen profilin (Psaradellis et al., 2000) was also shown to form multimers. The authors used a dot filtration immunoblot assay to determine the IgE-reactivity of maize profilin subjected to reducing agents plus boiling or non-reducing conditions, which favored the predominance of monomers and multimers, respectively, and found that multimers bind more IgE antibodies than monomers. In the present study, we clearly showed that recombinant mugwort profilin exists in solution as stable dimers and tetramers and that monomers

are detectable only after exposure of multimers to denaturing conditions. Our results also demonstrated that dimers and tetramers do not significantly differ in their ability to bind serum IgE from mugwort pollen-allergic patients. However, it is possible that oligomeric forms of profilin would be more allergenic than monomers because larger molecules very likely would have additional epitopes for IgE-mediated histamine release from basophils and mast cells. This possibility is presently being investigated in our laboratory.

Recombinant profilin was also used in RAST inhibition experiments to detect cross-reactive IgE antibodies in sera from allergic patients. From the clinical point of view, it is important to identify the patterns of IgE cross-reactivity as they often reflect the pattern of clinical sensitization (Aalberse et al., 2001). Although individual differences were evident, in general mugwort profilin strongly inhibited IgE-binding to profilin in extracts from birch pollen, celery, carrot, soy and peanut. In contrast, grass pollen profilin showed weak cross-reactivity with mugwort profilin. These results are in accordance with the observation that profilin is involved in the birch-celery-mugwort-spice cross-reactivity syndrome (Wüthrich and Hofer, 1984; Wüthrich et al., 1992; Bauer et al., 1996) and demonstrate that recombinant mugwort profilin shares epitopes with natural profilin in various sources of atopic allergens.

In conclusion, our studies revealed that recombinant mugwort profilin forms stable multimers in solution, which are recognized by IgE antibodies from mugwort pollen-allergic patients using different techniques like immunoblotting, ELISA, and RAST. This multimerization effect is also observed with natural profilin purified from mugwort pollen. Thus, recombinant mugwort profilin represents a useful tool for a more accurate diagnostic of patients suffering from weed pollen allergy and associated food allergies.

Materials and Methods

Patients and Sera

One hundred patients allergic to mugwort pollen were selected according to typical case history, positive skin prick test, and RAST (radioallergosorbent test) classes >3.0. Sera from two patients (P52 and P200) with clear anti-profilin reactivity (P52: 25.3 IU/ml; P200: 10.5 IU/ml) were used for RAST and RAST inhibition experiments. A serum pool from non-allergic (case history, skin prick test, RAST) healthy donors (NHS, 14 donors) was used as control. Sera were stored at −20°C.

Rabbit Antiserum

New Zealand white rabbits were immunized with 100 μg/ml purified natural Lolium perenne profilin. As an adjuvant Montanide ISA 50 (Seppic, Paris, France) was used. Rabbits were boostered at 4 week intervals. Immune reactivity was monitored by RIA with Sepharose-coupled purified natural profilin. To this end 100 μg profilin was coupled to 100 mg CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Swe-

den). Serum dilutions were incubated overnight with 0.5 mg Sepharose-coupled profilin in a final volume of 300 µl PBS/0.3% BSA/0.1% Tween 20. After washing 5 times with PBS/0.1% Tween, radiolabeled sheep antibodies against rabbit IgG (CLB, Amsterdam, The Netherlands) were added. After overnight incubation and 4 washing steps bound radioactivity was measured on a γ-counter. One week after the second and third booster, rabbits were plasmapheresed 3 times a week. Plasma was recalcified to allow clotting, and all six serum samples were pooled. After the fourth booster, rabbits were bled and the obtained serum was added to the pool.

Pollen Extracts

Aqueous extracts of Timothy grass (*Phleum pratense*), mugwort (*Artemisia vulgaris*), birch (*Betula verrucosa*) and ragweed (*Ambrosia artemisiifolia*) pollen (Allergon AB, Ängelholm, Sweden) were prepared by resuspending 150 mg of pollen in one ml of distilled water and shaking at room temperature for 15 minutes. After centrifugation at 14 000 *g*, the supernatants were collected and stored at −20 °C until use.

RNA Preparation

Total RNA was isolated from mature mugwort (*Artemisia vulgaris*) pollen (Allergon AB). Pollen grains (5 grams) were ground in a porcelain mortar under liquid nitrogen, which was then allowed to boil off, and the frozen powder was suspended in 4.2 M guanidinium thiocyanate, 50 mM BES, pH 7.2, containing 5 mM EDTA and 1% (v/v) β-mercaptoethanol. After extraction with phenol-chloroform-isoamyl alcohol (25:25:1) and centrifugation, the aqueous phase was transferred to fresh tubes and 1/10 volume of 2 M sodium acetate, pH 4.4 plus 2/10 volume of water were added. A second extraction with phenol-chloroform (3:1) was performed to remove DNA (Chomczynski and Sacchi, 1987) and the RNA in the aqueous phase was precipitated with ethanol. After centrifugation, the pellet was washed with 80% ethanol, dried, and dissolved in 50 mM HEPES, pH 7.5, 5 mM EDTA, 1% (w/v) SDS, 100 µg/ml proteinase K. After incubation at 37 °C for 20 min, 3 M NaCl was added to a final concentration of 300 mM and the solution extracted once with phenol-chloroform–isoamyl alcohol and once with chloroform. The RNA was precipitated with ethanol, centrifuged, and the pellet dissolved in a small volume of 6 M urea, 50 mM BES, pH 7.0, 5 mM EDTA. Afterwards, 1/2 volume of 9 M LiCl was added and the RNA precipitated overnight at −20 °C. After centrifugation, the pellet was washed with 80% ethanol, dried, and dissolved in 20 mM BES, pH 7.0 containing 1 mM EDTA. Poly(A)⁺ was selected using oligod(T)-magnetizable beads (Serotec, Oxford, UK) according to the manufacturer's instructions.

Construction and IgE Immunoscreening of a Mugwort Pollen cDNA Library

Poly(A)⁺-enriched RNA (5 µg) was used for first strand synthesis in a 30 µl reaction volume. Two µl of Superscript II reverse transcriptase (Life Technologies Inc., Rockville, MD, USA) were added and the reaction were incubated for 10 min at room temperature followed by 45 min at 42 °C. Afterwards, the reaction tube was placed on ice and 200 µl of second strand buffer containing 0.25 mM dNTPs and 0.1 mM NAD, as well as 50 U DNA polymerase I, 2 U *E. coli* ligase, and 1 U RNase H were added (Okayama *et al.*, 1987). The second strand synthesis was performed at 14–15 °C for 2 hours. Thereafter, 10 U T4 DNA polymerase were added and the incubation continued for 20 min. The cDNA was methylated with *Eco*RI methyltransferase and afterwards *Eco*RI linkers were added. The cDNA was then digest-

ed with *Xho*I and *Eco*RI, size-selected on a Sepharose CL-4B column (Amersham Biosciences, Uppsala, Sweden), and ligated to the pre-digested lambda ZAPII arms (Stratagene, La Jolla, USA). After *in vitro* packaging with the Gigapack Cloning Kit (Stratagene), the library was amplified once in *E. coli* strain XL-1 blue. The amplified library was screened with a serum pool from mugwort pollen-allergic patients. Bound IgE was detected using [¹²⁵I]-rabbit anti-human IgE (Pharmacia Diagnostics AB, Uppsala, Sweden). Isolated immunopositive phage clones were treated according to the *in vivo* excision protocol for subcloning into pBluescript SK⁺ (Stratagene). cDNA inserts were sequenced according to the dideoxy chain termination method (Sanger *et al.*, 1977).

Cloning of Mugwort Profilin as Non-Fusion Protein in pMW172 and Expression in *E. coli*

Expression plasmids containing the profilin cDNAs clones Av1 and Av2 coding for isoforms Art v 4.01 and Art v 4.02, respectively, were constructed in the vector pMW172B. For this purpose, the original pMW172 (Way *et al.*, 1990), which is based on the original pET vectors (Studier *et al.*, 1990), was modified. A polylinker containing sites for *Hind*III, *Sac*II, *Not*I, *Bst*WI, *Rsr*II, *Pst*I, *Xho*I, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Bst*XI, *Sfi*I, *Mlu*I, and *Eco*RI was introduced into an *Hind*III-*Eco*RI digested pMW172. The complete coding sequences of two profilin isoforms were modified by adding an *Nde*I site at the 5'-end and an *Mlu*I site at the 3'-end by PCR. The following primers were used:

Av1-Ndel   5'GAGAGA<u>CATATG</u>TCGTGGCAAACCTATGTT3', *Nde*I site is underlined; Av1-MluI 5'GAGAGA<u>ACGCGT</u>CTACAT-CCCCTGATCGAGTAG3', *Mlu*I site is underlined; Av2-Ndel 5'GAGAGA<u>CATATG</u>TCGTGGCAAACTTACGTTG3', *Nde*I site is underlined; Av2-MluI 5'GAGAGA<u>ACGCGT</u>CTACATGTTCTGAT-CAACGAG3', *Mlu*I site is underlined. The PCR products were digested with *Nde*I and *Mlu*I, ligated to the respective sites of the modified pMW172 expression vector, and sequenced according to the Dye Terminator Cycle Sequencing protocol (Applied Biosystems, USA).

For expression of the pMW172B/Art v 4.01 and pMW172B/Art v 4.02 plasmids, competent *E. coli* strain BL21(DE3) was transformed and selected on plates containing 100 mg/liter ampicillin (Studier *et al.*, 1990). A single transformant bacterial colony was picked and grown to an A_{600} of 1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and incubation continued for 6 h at 37 °C. Bacterial cells were harvested by centrifugation and pellets resuspended in 50 mM Tris-HCl, pH 7.5, containing 220 mM NaCl (buffer A). The cells were then disrupted by freezing in liquid nitrogen, followed by thawing at 37 °C. This step was repeated twice. Both profilin isoforms were recovered in the pellet as inclusion bodies after low speed centrifugation of the crude bacterial lysates. Insoluble inclusion bodies were solubilized in buffer A containing 6 M urea and dialyzed at 4 °C against buffer A. After dialysis, the extracts were used for immunoblot experiments.

Purification of Recombinant and Natural Mugwort Profilin

A single colony of *E. coli* cells containing the pMW172B/Art v 4.01 plasmid was used to inoculate 300 ml LB medium containing 0.1 mg/ml ampicillin and 2 mM $MgCl_2$. After incubating overnight, the cells were diluted in 9 liters of pre-warmed medium of the same composition. The cultures were grown to an A_{600} of 1.0 and induced with 0.4 mM IPTG. After incubation for 5 hours, cells were harvested by centrifugation. The pellet was resuspended in 50 mM Tris-HCl pH 7.4, 0.1% v/v Triton X-100, 1 mM ethylenediamine tetraacetic acid, 1 mM β-mercaptoethanol. The cells were disrupted by freezing and thawing at

37°C. An Ultraturax was used to homogenize the viscous lysate. The freeze- and thaw-step was repeated twice. During the last homogenizing step, NaCl was added to the lysate to a final concentration of 150 mM. Cell debris were removed by centrifugation for 45 min at 20 850 g (Sorvall RC26 Plus centrifuge, SS-34 rotor). Profilin in the supernatant was enriched by fractionated ammonium sulfate precipitation. Briefly, ammonium sulfate was added to the supernatant to a final concentration of 35% (w/v) followed by centrifugation (30 min, 17 520 g, Sorvall SS-34 rotor) to remove contaminating proteins. Profilin was precipitated by adding ammonium sulfate to a final concentration of 55% followed by centrifugation for 30 min at 17520 g. The pellet was resuspended in distilled water, 0.1% (v/v) Triton X-100 and subjected to affinity chromatography in a poly-(L-proline)-CNBr-activated Sepharose (PLP-Sepharose) column (Lindberg et al., 1988). The column was first equilibrated with buffer A (20 mM imidazole, pH 7.4, 2 mM β-mercaptoethanol, 150 mM NaCl), then the profilin solution was applied and the column washed with several bed volumes of buffer A. Elution was accomplished by establishing a linear gradient of increasing buffer B (20 mM imidazole, pH 7.4, 2 mM β-mercaptoethanol, 150 mM NaCl, 50% dimethyl sulfoxide). The fractions containing profilin were pooled and further purified using a DEAE Sepharose column. The column was equilibrated in 20 mM imidazole, pH 7.4, 2 mM β-mercaptoethanol, 0.1 mM ethylenediamine tetraacetic acid and recombinant profilin eluted with a NaCl gradient. Fractions containing profilin were pooled and dialyzed against 10 mM phosphate, pH 7.4, 1 mM β-mercaptoethanol.

Natural profilin was purified from mugwort pollen extract by affinity chromatography in a poly-(L-proline)-CNBr-activated Sepharose (PLP-Sepharose) column (Lindberg et al., 1988). The column was first equilibrated with 25 mM imidazole, pH 7.4, containing 150 mM NaCl. Mugwort pollen extract (15 ml) was then applied to the column. After washing with 4 bed-volumes of imidazole buffer containing 2 M urea, profilin was eluted with imidazole buffer containing 6 M urea. Fractions containing profilin were pooled and dialyzed against 5 mM phosphate, pH 7.4.

SDS-PAGE and Immunoblotting

Pollen extracts, E. coli lysates of profilin isoforms, and purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970), using either 15% acrylamide gels or gradient (from 7.5% to 20%) gels. For reducing and non-reducing gels, samples were incubated at 95°C and 37°C with sample buffer with and without β-mercaptoethanol, respectively.

Proteins were visualized by staining with Coomassie Brilliant Blue R-250. For immunoblot analysis, proteins were separated by SDS-PAGE and electroblotted (Towbin et al., 1979) onto nitrocellulose membranes. IgE immunoblots were performed as described previously (Jarolim et al., 1989) using [125I]-rabbit + mouse anti-human IgE (Pharmacia). E. coli lysates harboring the plasmid without an insert were used as a control. Autoradiography was performed at −70°C for 12–48 h with intensifying screens. Immunoblots using rabbit anti-Lolium perenne pollen profilin (diluted 1:150 000 – 500 000) serum were performed using alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA).

MALDI-TOF Mass Spectrometry

For determining intact molecular masses, we applied 0.5 µl (approximately 0.5 µg) of purified recombinant profilin samples, 0.5 µl protein calibration (chicken egg white lysozyme, average molecular weight [M + H]+ 14 307) and 0.5 µl of a saturated solution of sinapinic acid in 40% (v/v) aqueous acetonitrile, 0.1% (v/v) trifluoroacetic acid to the target slide with intermittent drying in an airstream. Samples were analyzed with the Kompact Maldi IV mass spectrometer (Kratos Analytical, Manchester, UK) in the linear flight mode. For calibrating the instrument, we used the molecular peaks of chicken egg white lysozyme and sinapinic acid.

Size Exclusion HPLC

Purified profilin samples were analyzed by size exclusion chromatography using a HP 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). After equilibration with phosphate-buffered saline, a G-SW 2000 column (Tosohaas, pore diameter 10 µm) was loaded with 20 µg of purified recombinant mugwort profilin, at a flow rate of 0.2 ml/min. UV absorbance was monitored at 280 and 214 nm. In separate runs, the retention times of BSA (M_r 67 000), ovalbumin (M_r 43 000), chymotrypsinogen (M_r 25 000), and ribonuclease A (M_r 13 700) were used as molecular weight standards. In one set of experiments, samples of purified recombinant profilin were analyzed under reducing conditions using phosphate-buffered saline containing 10 mM DTT. In another set of experiments samples were pre-treated with DTT and boiled for 15 min before HPLC analysis.

Circular Dichroism

Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) fitted with an RTE-III temperature control system (NesLab). Far-UV CD spectra from 185 to 260 nm were recorded at 20°C in a 0.1 cm path length quartz cuvette at a protein concentration of 28 µM. The measurements were performed at a scanning speed of 100 nm/min, a response time of 1 s and a data pitch of 1 nm. Each spectrum represents an average of seven scans. The samples were buffered in 10 mM phosphate, pH 7.4, 1 mM β-mercaptoethanol. Thermal denaturation of mugwort profilin was monitored by recording the ellipticity during temperature increase (2°C/min) at 220 nm. Reversibility of the unfolding process was monitored by measuring the restoration of the CD signal after cooling from 90°C to the starting temperature (20°C).

ELISA

Microtiter plates (Nunc Immuno, Maxi Sorp, Roskilde, Denmark) were coated overnight at 4°C with 2.5 µg per well of purified recombinant profilin in 50 µl phosphate-buffered saline. Remaining binding sites were saturated by incubation with 100 µl of phosphate-buffered saline, 1% (w/v) human serum albumin, 0.05% (v/v) Tween (buffer A) for 5–8 h at room temperature. After washing four times with phosphate-buffered saline, 0.05% (v/v) Tween, plates were incubated overnight at 4°C with sera from mugwort pollen-allergic patients (diluted 1:25, 50, 100, and 200 in phosphate-buffered saline, final volume of 50 µl/well). After washing, the plates were incubated (5 h at room temperature and then overnight at 4°C) with alkaline phosphatase-conjugated equine anti-human IgE antibodies (Allercoat 6 Conjugate Kit, Bio-Rad, Hercules, CA, USA) and developed with the substrate p-nitrophenyl phosphate. The optical density was measured at 405 nm using the Anthos ELISA reader (Anthos Labtec Instruments, Wals, Austria). Adequate controls were performed for each incubation step of the ELISA assay and a serum from a non-allergic individual assured the specificity of the IgE-detection system.

RAST and RAST Inhibition

RAST was performed as described previously (Aalberse et al., 1981). Food and pollen extracts were prepared as previously de-

scribed (van Ree *et al.*, 1992). Briefly, serum (50 µl) was incubated overnight with of Sepharose-coupled pollen or food extract (0.5 mg or 1.5 mg of Sepharose), respectively, in a final volume of 300 µl of PBS with 0.3% BSA and 1% Tween-20 (PBS-AT). After washing away unbound serum components, radiolabeled sheep antibodies directed to human IgE in 500 µl of PBS-AT plus 4.5% bovine and 0.5% (v/v) sheep serum were added. After overnight incubation and washing, bound radioactivity was measured. Results were expressed as international units per ml (IU/ml). Calculation was performed by means of a standard curve that was obtained by RAST with a dilution series of a chimeric monoclonal IgE antibody against the major housedust mite allergen Der p 2 and Sepharose-coupled recombinant Der p 2 (Schuurman *et al.*, 1997). One IU is 2.4 ng of IgE.

For RAST inhibition, serum was pre-incubated for 1 h with inhibitor (50 µl) prior to addition of Sepharose-coupled allergen. Pollen and food extracts added as inhibitor were used at the following protein concentrations: potato 0.70 mg/ml, soy 1.4 mg/ml, celery 0.8 mg/ml, carrot 0.5 mg/ml, grass 0.9 mg/ml, birch 1.2 mg/ml. Recombinant mugwort profilin was used at 1 mg protein/ml. In dose-related RAST inhibition experiments, recombinant mugwort profilin was used at 2000, 400, 80, 16 and 3.2 µg/ml. For the uninhibited value, serum was pre-incubated with PBS-AT. Subsequent steps were identical to those described for RAST.

Acknowledgements

References

Aalberse, R.C., Koshte, V., and Clemens, J.G. (1981). Immunoglobulin E antibodies that crossreact with vegetable foods, pollen, and Hymenoptera venom. J. Allergy Clin. Immunol. *68*, 356–364.

Aalberse, R.C., Akkerdaas, J., and van Ree, R. (2001). Cross-reactivity of IgE antibodies to allergens. Allergy *56*, 478–490.

Asturias, J.A., Arilla, M.C., Bartolome, B., Martinez, J., Martinez, A., and 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., Gomez-Bayon, N., Martinez, J., Martinez, A., and 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., Gomez-Bayon, N., Aguirre, M., Martinez, A., Palacios, R., and Martinez, J. (1998). Cloning and immunological characterization of the allergen Hel a 2 (profilin) from sunflower pollen. Mol. Immunol. *35*, 469–478.

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

Bachmair, A., Finley, D., and Varshavsky, A. (1986). *In vivo* half-life of a protein is a function of its amino-terminal residue. Science *234*, 179–186.

Bauer, L., Ebner, C., Hirschwehr, R., Wuthrich, B., Pichler, C., Fritsch, R., Scheiner, O., and Kraft, D. (1996). IgE cross-reactivity between birch pollen, mugwort pollen and celery is due

to at least three distinct cross-reacting allergens: immunoblot investigation of the birch-mugwort-celery syndrome. Clin. Exp. Allergy *26*, 1161–1170.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156–159.

Cornillon, J., Bernard, J.P., Gueho, E., and Touraine, R. (1972). [Estivo-autumnal pollinosis]. Rev. Fr. Allergol. *12*, 313–322.

de la Hoz, F., Polo, F., Moscoso del Prado, J., Selles, J.G., Lombardero, M., and Carreira, J. (1990). Purification of Art v I, a relevant allergen of Artemisia vulgaris pollen. Mol. Immunol. *27*, 651–657.

Diaz-Perales, A., Lombardero, M., Sanchez-Monge, R., Garcia-Selles, F.J., Pernas, M., Fernandez-Rivas, M., Barber, D., and Salcedo, G. (2000). Lipid-transfer proteins as potential plant panallergens: cross-reactivity among proteins of *Artemisia* pollen, Castanea nut and Rosaceae fruits, with different IgE-binding capacities. Clin. Exp. Allergy *30*, 1403–1410.

Futin, F.G., Person, K., and Gutermann, W. (1976). Artemisia. In: Flora Europea, Vol. 4B. F. G. Fritin, V. Heywood, N. A. Burges, D. M. Moore, S. M. Walters, and D. A. Webb, eds. (Cambridge, UK: Cambridge University Press), pp. 178–186.

Guillen, G., Valdes-Lopez, V., Noguez, R., Olivares, J., Rodriguez-Zapata, L.C., Perez, H., Vidali, L., Villanueva, M.A., and Sanchez, F. (1999). Profilin in *Phaseolus vulgaris* is encoded by two genes (only one expressed in root nodules) but multiple isoforms are generated *in vivo* by phosphorylation on tyrosine residues. Plant J. *19*, 497–508.

Hanfrey, C., Fife, M., and Buchanan-Wollaston, V. (1996). Leaf senescence in *Brassica napus*: expression of genes encoding pathogenesis-related proteins. Plant Mol. Biol. *30*, 597–609.

Hirschwehr, R., Heppner, C., Spitzauer, S., Sperr, W.R., Valent, P., Berger, U., Horak, F., Jager, S., Kraft, D., and Valenta, R. (1998). Identification of common allergenic structures in mugwort and ragweed pollen. J. Allergy Clin. Immunol. *101*, 196–206.

Huang, S., McDowell, J.M., Weise, M.J., and Meagher, R.B. (1996). The *Arabidopsis* profilin gene family. Evidence for an ancient split between constitutive and pollen-specific profilin genes. Plant Physiol. *111*, 115–126.

Jarolim, E., Rumpold, H., Endler, A.T., Ebner, H., Breitenbach, M., Scheiner, O., and Kraft, D. (1989). IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of *Betula verrucosa*. Allergy *44*, 385–395.

King, T.P., Hoffman, D., Lowenstein, H., Marsh, D.G., Platts-Mills, T.A., and Thomas, W. (1995). Allergen nomenclature. Allergy *50*, 765–774.

Kleber-Janke, T., Crameri, R., Appenzeller, U., Schlaak, M., and Becker, W.M. (1999). Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. Int. Arch. Allergy Immunol. *119*, 265–274.

Kovar, D.R., Drobak, B.K., and Staiger, C.J. (2000). Maize profilin isoforms are functionally distinct. Plant Cell *12*, 583–598.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Lindberg, U., Schutt, C.E., Hellsten, E., Tjader, A.C., and Hult, T. (1988). The use of poly(L-proline)-Sepharose in the isolation of profilin and profilactin complexes. Biochim. Biophys. Acta *967*, 391–400.

Mittermann, I., Swoboda, I., Pierson, E., Eller, N., Kraft, D., Valenta, R., and 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.

Nilsen, B.M., Grimsoen, A., and Paulsen, B.S. (1991). Identifica-

tion and characterization of important allergens from mugwort pollen by IEF, SDS-PAGE and immunoblotting. Mol. Immunol. 28, 733–742.

Nilsen, B.M., and Paulsen, B.S. (1990). Isolation and characterization of a glycoprotein allergen, Art v II, from pollen of mugwort (*Artemisia vulgaris* L.). Mol. Immunol. 27, 1047–1056.

Nilsen, B.M., Sletten, K., Paulsen, B.S., O'Neill, M., and van Halbeek, H. (1991). Structural analysis of the glycoprotein allergen Art v II from the pollen of mugwort (*Artemisia vulgaris* L.). J. Biol. Chem. 266, 2660–2668.

Okayama, H., Kawaichi, M., Brownstein, M., Lee, F., Yokota, T., and Arai, K. (1987). High-efficiency cloning of full-length cDNA; construction and screening of cDNA expression libraries for mammalian cells. Methods Enzymol. 154, 3–28.

Psaradellis, T., Kao, N.L., and Babich, M. (2000). Recombinant *Zea mays* profilin forms multimers with pan-allergenic potential. Allergology Int. 49, 27–35.

Rihs, H.P., Chen, Z., Rueff, F., Petersen, A., Rozynek, P., Heimann, H., and Baur, X. (1999). IgE binding of the recombinant allergen soybean profilin (rGly m 3) is mediated by conformational epitopes. J. Allergy Clin. Immunol. 104, 1293–1301.

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

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463–5467.

Schlüter, K., Jockusch, B.M., and Rothkegel, M. (1997). Profilins as regulators of actin dynamics. Biochim. Biophys. Acta 1359, 97–109.

Schuurman, J., Perdok, G.J., Lourens, T.E., Parren, P.W., Chapman, M.D., and Aalberse, R.C. (1997). Production of a mouse/human chimeric IgE monoclonal antibody to the house dust mite allergen Der p 2 and its use for the absolute quantification of allergen-specific IgE. J. Allergy Clin. Immunol. 99, 545–550.

Spieksma, F.T., Charpin, H., Nolard, N., and Stix, E. (1980). City spore concentrations in the European Economic Community (EEC). IV. Summer weed pollen (Rumex, Plantago, Chenopodiaceae, Artemisia), 1976 and 1977. Clin. Allergy 10, 319–329.

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

Studier, F.W., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185, 60–89.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. (1992). Acquired resistance in *Arabidopsis*. Plant Cell 4, 645–656.

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

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

Vallverdu, A., Asturias, J.A., Arilla, M.C., Gomez-Bayon, N., Martinez, A., Martinez, J., and Palacios, R. (1998). Characterization of recombinant *Mercurialis annua* major allergen Mer a 1 (profilin). J. Allergy Clin. Immunol. 101, 363–370.

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

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

Wüthrich, B., and Hofer, T. (1984). Nahrungsmittelallergie. Das "Sellerie-Beifuß-Gewürz-Syndrom". Dt. Med. Wochenschr. 25, 981–986.

Wüthrich, B., Stäger, P., and Johansson, S.G.O. (1992). RAST-spezifische IgE auf Gewürze bei Sensibilisierungen gegen Birke-Beifußpollen und Sellerie. Allergologie 11, 380–383.