

Original article

Molecular and immunological characterization of novel weed pollen pan-allergens

Background: Pan-allergens like profilins, calcium-binding proteins (CBPs), and nonspecific lipid transfer proteins have been suggested as possible specific markers for multiple pollen sensitizations, and could be used to predict cross-sensitization/poly-sensitization to several pollen allergens. Therefore, the purification and characterization of cross-reacting allergens in pollen is an extremely important task towards correct allergy diagnosis.

Methods: New pan-allergens were identified by screening a ragweed pollen cDNA library with sera of patients allergic to mugwort pollen. Resulting proteins were cloned, expressed, purified and characterized.

Results: We report complete cDNA sequences of two profilin isoforms (Amb a 8.01 and Amb a 8.02), two isoforms of a 2EF-hand CBP (Amb a 9.01 and Amb a 9.02), a new 3EF-hand CBP (Amb a 10) from ragweed pollen and a 2EF-hand CBP from mugwort (Art v 5). All these proteins were expressed in *Escherichia coli*, purified to homogeneity and characterized by biochemical and immunological means.

Conclusions: The identified proteins are novel pan-allergens and can be used as diagnostic markers for polysensitization and used in component-resolved diagnosis.

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Immediate-type allergies are ever increasing in industrialized countries over the last decades (1). Therefore, the most important task in allergy diagnosis and treatment should be the identification and characterization of allergens that are actually eliciting sensitization and causing the disease. So far a significant number of molecules from different sources have been identified for inducing hypersensitivity reactions. The high structural similarity between phylogenetically conserved allergenic proteins present in different, apparently unrelated, sources seems to play an important role in IgE-mediated poly-sensitization, which subsequently leads to immunological cross-reactivity and clinical syndromes (2, 3). Various studies demonstrated that more than 60% of the allergic patients were sensitized to pollen of more than one species (4). Extensive cross-reactivity within allergenic plants is likely to be caused by three families of widely distributed pan-allergens: the profilins, the calcium-binding proteins (CBPs), and the nonspecific lipid transfer proteins.

Allergen extracts used for diagnosis today consist of undefined mixtures of allergenic and nonallergenic molecules and can only be used to determine if a patient reacts to a particular allergen containing the source and for the interpretation of biological tests of IgE-binding, which is also biased by cross-reactivity. The use of panels of recombinant allergens in diagnosis could help to delineate the reactivity profile of a given allergic patient and determine whether a patient is co-sensitized against allergens from different sources or whether it cross-reacts with allergens that are present in these sources (5). Pan-allergens have been suggested as possible markers of multiple pollen sensitizations (4, 6), and could be further used to predict cross-sensitization/poly-sensitization to several pollen allergens. Therefore, the purification and characterization of cross-reacting allergens in pollen is an extremely important task towards correct allergy diagnosis.

The IgE-reactivity to pan-allergens from ragweed and mugwort, members of the same plant family *Compositae*,

ranges from 10% to 36% (7–9). Hence, it is crucial to include our newly described weed-pollen allergens in cross-reactivity studies.

Material and methods

Patient's sera

Mugwort and ragweed-allergic patients were preselected according to typical case history, positive skin prick test and radio-allergosorbent test classes >3.5. A serum pool ($n = 46$) was used to screen the ragweed pollen cDNA library. Sera from polysensitized patients ($n = 246$, Italy) as well as ragweed-allergic patients from Italy (Dr Riccardo Asero, Ambulatorio di Allergologia, Clinica San Carlo, Paderno Dugnano, Italy, $n = 42$) and Austria ($n = 90$) were selected for allergen characterization. Sera were stored at -20°C .

Purification of natural ragweed pollen profilin

Short ragweed pollen (*Ambrosia artemisiifolia*) (Allergon AB, Angelholm, Sweden) was extracted by suspending pollen in distilled water, shaking for 15 min at room temperature, followed by centrifugation. Natural profilin was purified from ragweed-pollen extract by affinity chromatography using a poly-(L-proline)-CNBr-activated sepharose column (10). The column was equilibrated with phosphate buffer saline (1× PBS), pH 7.4 containing 10 mM EDTA and 5 mM DTT. Ragweed pollen extract was loaded onto the column, followed by washing with 1× PBS, pH 7.4 containing 1.5 M urea and eluted with 1× PBS, pH 7.4 containing 6 M urea. Profilin-positive fractions were pooled, dialysed against distilled water followed by lyophilization.

cDNA library and cloning

Total RNA preparation from mugwort and ragweed pollen and *in vitro* phage packaging was performed as previously described (8). Ragweed pollen phage cDNA library was screened with a serum pool of Austrian weed pollen-allergic patients and bound-IgE was detected using ^{125}I -rabbit anti-human IgE (MALT Allergy system, IBL GmbH, Hamburg, Germany). Mugwort pollen phage cDNA library was screened using a labelled gene-specific Bet v 4 cDNA probe (11). Isolated phage clones were treated according to the *in vivo* excision protocol (Stratagene, La Jolla, CA, USA) followed by sequencing and sequence similarity was searched using BLAST (12). Novel sequences were designated according to the IUIS allergen nomenclature system (13) and submitted to the Genbank. Complementary DNAs corresponding to profilin (Amb a 8.01 and Amb a 8.02) and CBPs (Amb a 9.01, Amb a 9.02, Amb a 10, and Art v 5) were PCR amplified (Primers see Table 1) and subcloned into pHis-Parallel2 (14).

Expression and protein purification

Protein expression was performed as previously described (8). In brief, expression was induced by addition of 0.4 mM IPTG (isopropylthio- β -D-galactopyranoside) and the cells were harvested after 4 h of expression at 37°C , and then resuspended and lysed using freeze-thaw cycles. His-tagged proteins were purified by Ni-affinity chromatography. Pure fractions were pooled, his-tags were removed using rTEV protease (Invitrogen, Carlsbad, CA, USA), dialysed against distilled water, lyophilized and stored at -20°C .

Table 1. Sense (forward) and anti-sense (reverse) primer with underlined restrictions sites

Amb a 8.01 fwd-BamH I	5'-GAGAGGATCCTCGTGGCAGACTTATGTGGATG-3'
Amb a 8.01 rev-Xho I	5'-GAGACTCGAGTTACATGCCTTGATCCACAAGATA-3'
Amb a 8.02 fwd-BamH I	5'-GAGAGGATCCTCGTGGCAAACCTACGTGGATG-3'
Amb a 8.02 rev-Xho I	5'-GAGACTCGAGTCACATGCCCTGATCCACGAG-3'
Amb a 9.01 fwd-Nco I	5'-GAGACCATGGCAGAGGAAGAAGACAA-3'
Amb a 9.01 rev-Xho I	5'-GAGACTCGAGTTAGAATATCTTGCCAAACGTCT-3'
Amb a 9.02 fwd-Nco I	5'-GAGACCATGGCAGAGGACGAAGACAAGGC-3'
Amb a 9.02 rev-Xho I	5'-GAGACTCGAGTTAGAATATCTTGCCAAACGTCT-3'
Amb a 10 fwd-Nco I	5'-GAGACCATGGCGCCAGAAAATAACAAACATTCA-3'
Amb a 10 rev-Xho I	5'-GAGACTCGAGTCACATTGCACCATCACCTCCCTTC-3'
Art v 5 fwd-Nco I	5'-GAGACCATGGCTGATGAAGACAAG-3'
Art v 5 rev-Xho I	5'-GAGACTCGAGTTAGAAGATTTTCCAAC-3'

SDS-PAGE and immunoblots

Proteins were analysed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (15) and Coomassie brilliant-blue staining. For immunoblot analyses, proteins were separated and electroblotted (16) onto nitrocellulose membranes (Protran, Schleicher & Schuell, Germany). New Zealand White rabbits were immunized with purified natural *Lolium perenne* profilin, or purified recombinant Phl p 7, a calcium-binding allergen from timothy grass (*Phleum pratense*) pollen as previously described (8). Rabbit antisera were diluted 1 : 250 000 (anti-*Lolium* pollen profilin) or 1 : 50 000 (anti-Phl p 7) in 25 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.5% (v/v) Tween 20, 0.5% (w/v) bovine serum albumin, 0.05% (w/v) sodium azide, and detection was performed using alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories Inc., PA, USA).

ELISA, cross-inhibition and dot-blot experiments

To detect whether the polysensitized sera reacted with profilins and CBPs from weed pollen, the IgE reactivity to recombinant allergens was measured using dot-blot analyses and ELISA (17). For dot-blot experiments, these included: (i) profilins from ragweed (rAmb a 8.01, rAmb a 8.02), mugwort (rArt v 4) (8) and birch (rBet v 2, obtained from Biomay AG, Vienna, Austria) and (ii) CBPs from ragweed (rAmb a 9.01, rAmb a 9.02, and Amb a 10), mugwort (rArt v 5), birch (rBet v 4) (11) and from grass pollen (rPhl p 7). For ELISA only rAmb a 8.02, rAmb a 9.02, rAmb a 10, rArt v 4 and rArt v 5 were used.

For cross-inhibition experiments either purified nAmb a 8 or rPhl p 7 was coated to ELISA plates for profilin and CBP inhibition experiments respectively. In each case, five sera were used, diluted 1 : 5 and preincubated with increasing amounts of inhibitory proteins. Inhibition was performed at protein concentrations of 0.000, 0.002, 0.020, 0.200, 2.000 and 20.000 $\mu\text{g}/\text{ml}$ using nAmb a 8, rAmb a 8 and rArt v 4 for profilin cross-reactivity tests and rPhl p 7, rAmb a 9, rAmb a 10 and rArt v 5 for CBP cross-inhibition. Proteins were incubated with patients' IgE at 4°C overnight. Values at protein concentration 0.000 $\mu\text{g}/\text{ml}$ were used for data normalization.

Mediator release from rat basophilic leukaemia cells

Rat basophil degranulation assays were performed as previously described (18). Rat basophilic leukaemia (RBL)-2H3 cells transfected with human high-affinity IgE receptor (Fc ϵ RI) were passively sensitized with serum IgE from patients allergic to pan-allergens

profilin and CBPs. Degranulation was triggered by addition of serial dilutions of nAmb a 8 or rAmb a 8 or different recombinant CBPs (rAmb a 9, rAmb a 10 and rArt v 5). β -Hexosaminidase release into the supernatant was measured by enzymatic cleavage of the fluorogenic substrate 4-methyl umbelliferyl-*N*-acetyl- β -glucosaminide and expressed as the percentage of total enzyme content obtained by lysing the cells with Triton X-100.

Mass spectrometry

Tryptic peptides of the nAmb a 8 were separated by Capillary HPLC and analysed by ElectroSpray tandem mass spectrometry (ESI-MS/MS) using a quadrupole-time-of-flight (Q-TOF) mass analyser (Waters; Milford, MA, USA). Peptide mass and sequence were established from MS data and MS/MS fragmentation patterns of the peptide. Analysis of intact proteins was performed by direct infusion into the ESI-Q-TOF mass spectrometer. Molecular weight (theoretical values including cloning artifact): rAmb a 8.02: 14,544.0 (14,543.6), rAmb a 9.02: 9,421.0 (9,422.2); rAmb a 10: 17,928.0 (17,926.7) and rArt v 5: 9,124.0 (9,126.0) respectively.

Circular dichroism

Circular dichroism (CD) spectra were recorded at 20°C in 5 mM sodium phosphate pH 8 using a JASCO J-815 spectropolarimeter (Jasco, Tokyo, Japan). These data were baseline-corrected and presented as mean residue molar ellipticity $[\Theta]_{MRW}$ at a given wavelength. Spectra of CBPs were recorded both in the presence and absence of Ca^{2+} . To remove protein-bound calcium, samples were incubated with Chelex-100 (Sigma-Aldrich, St Louis, MO, USA) and mock-titrated with buffer or incubated with a buffer containing 10-fold molar excess of Ca^{2+} .

Results

Analyses of IgE-positive clones

To obtain information about new cross-reacting ragweed pollen allergens, the cDNA library was screened with a serum pool of weed-allergic patients ($n = 46$) displaying IgE antibodies to a broad spectrum of proteins. Out of > 700 000 plaques, 30 IgE-positive clones were isolated and sequenced. Six IgE-positive clones contained a 133-residue protein. Two isoforms, with 11 amino acid substitutions were obtained, identified as profilins and designated Amb a 8.01 and Amb a 8.02 (GenBank accession no.: AY894660 and AY894661, Fig. 1A). Thereafter, 22/30 IgE-positive plaques were coded for a protein with 71% sequence identity to Bet v 4, a 2EF-hand calcium-binding allergen from birch (11). All the 22 isolated clones contained full-length proteins of 82 residues and revealed nine isoforms with one to four amino acid substitutions. On account of the high sequence similarity, two most prominent isoforms were selected for further study. Two isoforms designated as Amb a 9.01 and Amb a 9.02 (GenBank Accession no.: AY894657 and AY894658, Fig. 1B) were selected for further analyses. It was observed that two of the 30 isolated clones contained a 160 residue protein with 55% sequence identity to Ole e 8 from *Olea europaea* (19)

containing three EF-hand sequence motifs. The protein was designated Amb a 10 (GenBank accession no.: AY894659, Fig. 1B). On screening the mugwort pollen cDNA library, a clone was isolated with 82 residues coding for a 2EF-hand CBP with 68% sequence identity to Bet v 4 (Fig. 1B) and designated Art v 5 (GenBank accession no.: AY904434).

Purification and cross-reactivity of weed pan-allergens

To evaluate the IgE-binding activity of recombinant CBPs and profilin isoforms, dot-blot experiments with purified proteins were performed using the sera from polysensitized Italian patients. No differences were observed in the IgE-binding frequency of the two CBP and profilin isoforms. Amb a 8.02 and Amb a 9.02 were selected for further biochemical and immunological characterization.

On SDS-PAGE ragweed profilins (Amb a 8) migrate at 14kDa, the 2EF-hand CBPs Amb a 9 and Art v 5 at approximately 9kDa and Amb a 10 at 17kDa (Fig. 2, left panel). Mass-spectrometry analysis confirmed the expected molecular weights for the recombinant proteins. Identity of natural Amb a 8 isoforms was confirmed by peptide mass fingerprinting, which revealed acetylation (data not shown).

To detect cross-reactivity, blotted proteins were incubated with polyclonal rabbit sera. As shown in Fig. 2A, after incubating membrane-blotted profilins from mugwort and ragweed with *Lolium* antiserum, a single reactive band migrating at 14 kDa could be confirmed. The Phl p 7 antiserum reacted with rBet v 4 and rAmb a 10 but not with the 2EF-hand CBPs (Fig. 2B). His-tagged rPhl p 7 was used as a positive control, where the formation of dimers was detected even under reducing conditions (right panel, Fig. 2B).

Quantitative evaluation of IgE cross-reactivity was performed with dose-related inhibition experiments using the five sera, previously shown to react with CBPs and profilin in ELISA studies. Recombinant CBPs from ragweed and mugwort inhibited immobilized rPhl p 7 between 25% and 50% (Fig. 2C). Cross-inhibition experiments using profilin demonstrated that natural and recombinant profilins were able to inhibit IgE-binding. Complete inhibition was observed for nAmb a 8, the recombinant proteins achieved an inhibition of about 90%, as shown in Fig. 2D.

Structural analyses

The CD-spectra of recombinant and natural Amb a 8.02 were almost identical in overall secondary-structure content and revealed a well-structured protein, showing typical bands for the profilin-mixed α - β structures (Fig. 3D). All CBPs were measured both in the presence and absence of calcium, and denaturing studies were performed under identical conditions. The spectra

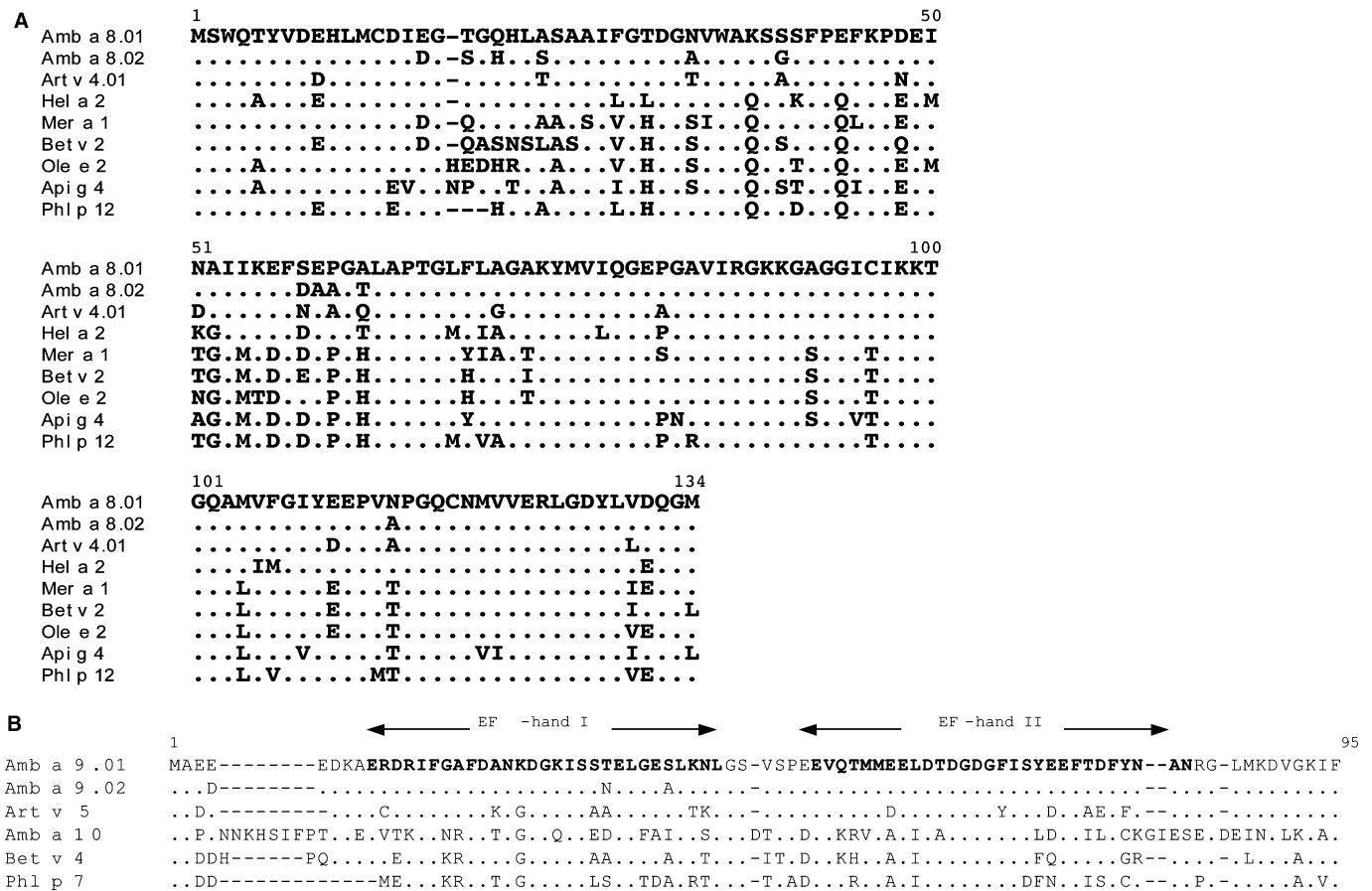


Figure 1. Sequence alignments of weed pan-allergens. (A) Comparison of ragweed profilin isoforms Amb a 8.01 and Amb a 8.02 (AY894660 and AY894661) with other plant profilins. Art v 4.01, *Artemisia vulgaris* pollen profilin (AJ421030); Hel a 2, *Helianthus annuus* pollen profilin (Y15210); Mer a 1, *Mercurialis 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, *P. pratense* pollen allergen (X77583). (B) Comparison of calcium-binding allergens with 2EF-hand motifs from ragweed, Amb a 9.01 and Amb a 9.02 (AY894657 and AY894658), mugwort, Art v 5 (AY904434), birch, Bet v 4 (X87153) and grass pollen, Phl p 7 (Y17835), and a CBP with 3EF-hands from ragweed, Amb a 10 (AY894659). Residues involved in the two EF-hand sequence motifs known from Bet v 4 are shown in bold and are indicated with a solid line. Amb a 10 sequence from 96–160 not shown. Dots indicate identical amino acids.

recorded at 20°C indicated that the proteins contain a considerable amount of alpha-helical secondary structure. Circular dichroism-spectra show a similar shape both in the presence and absence of calcium and reveal an increase of helicity with calcium (Fig. 3A–C). Thermal stability of recombinant Art v 5 and Amb a 9 depended on the presence of calcium. Circular dichroism-spectra of renaturation curves revealed a similar refolding behavior of rArt v 5 and rAmb a 9 from 95 to 20°C with calcium. Recombinant Amb a 9 refolded slightly better under calcium depletion. In the case of rAmb a 10, no difference in the renaturation behaviour was observed (data not shown).

IgE antibody binding to CBPs and profilin

Binding of recombinant proteins to human IgE was evaluated by dot-blot. From the tested pan-allergens,

birch pollen profilin (Bet v 2) was best recognized with an IgE-binding prevalence of 30% followed by mugwort and ragweed profilin (26%). No difference could be observed in the recognition pattern of rAmb a 8 and rArt v 4. Recombinant Phl p 7 reacted with 12% of the patients tested, rArt v 5 and rAmb a 9 demonstrated a reactivity of 11.4%. About 10% of all patients tested, displayed IgE antibodies reacting with rBet v 4 and rAmb a 10. To a greater or lesser extent, all tested CBPs showed about the same ability to bind human IgE (Fig. 4A).

In ELISA, proteins were tested with a panel of weed-allergic patients (Italy and Austria). Furthermore, the reactivity of natural and recombinant Amb a 8 was compared using preselected profilin-allergic patients. Our data suggest that sensitization to profilin is more abundant in the Austrian than in the Italian weed-allergic population (Fig. 4B) and no major difference in

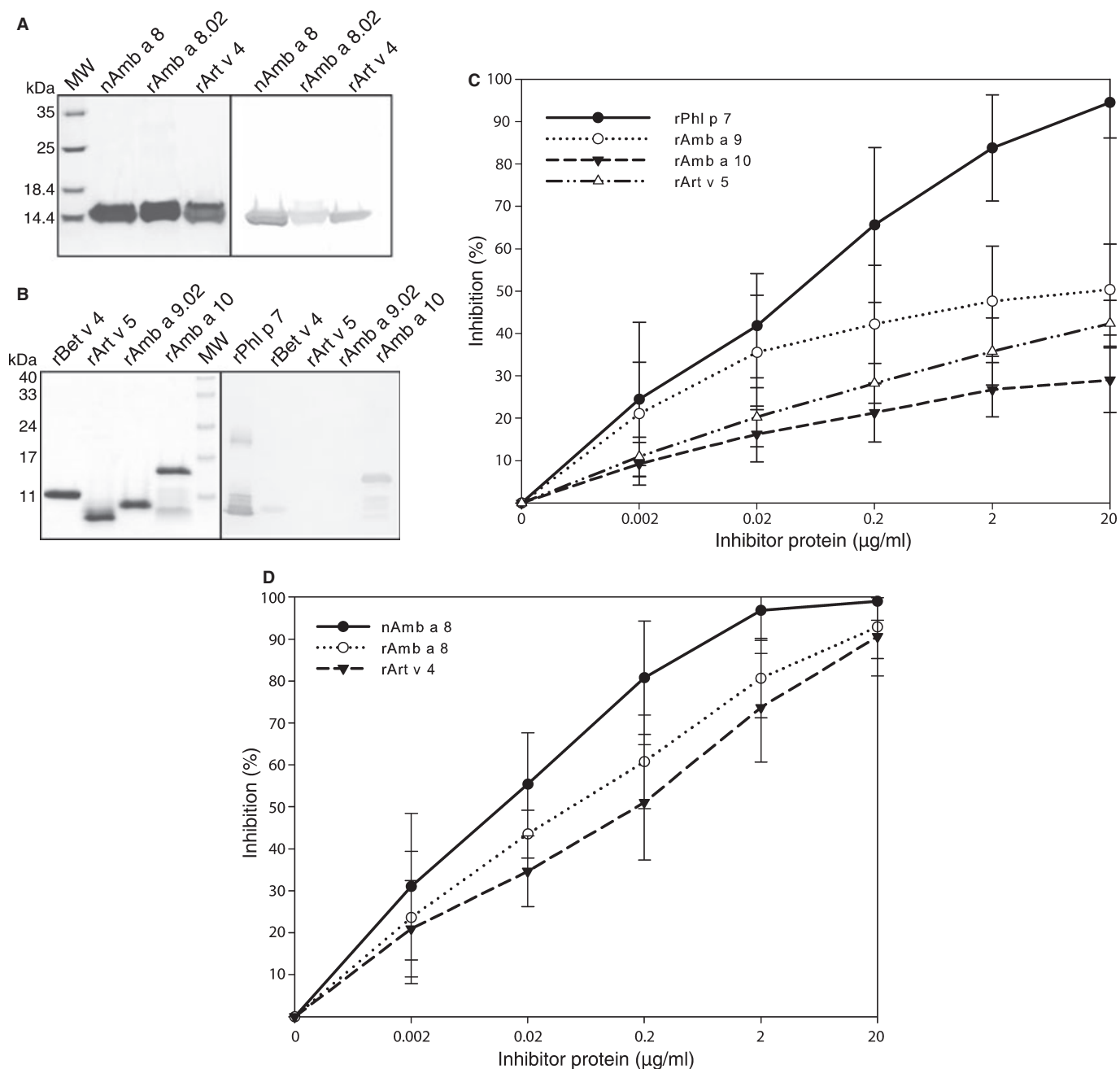


Figure 2. Purified allergens, immunoblotting and cross-inhibition. (A) Purified natural profilin from ragweed pollen and recombinant Amb a 8.02 and Art v 4 were separated by SDS-PAGE followed by either Coomassie brilliant blue staining (left panel) or were nitrocellulose-blotted and incubated with rabbit anti-*Lolium* pollen profilin antibody (right panel). (B) Purity of recombinant calcium-binding proteins from birch (Bet v 4), mugwort (Art v 5) and ragweed (Amb a 9 and Amb a 10) was demonstrated by SDS-PAGE and Coomassie staining (left panel). Nitrocellulose-blotted proteins were further analysed with rabbit Phl p 7 antiserum (right panel). Recombinant Phl p 7 was used as positive control. Recombinant Phl p 7 (C) and nAmb a 8 (D) were coated on ELISA plates. Cross-inhibition was performed using serum of pan-allergic Italian patients and increasing concentrations (0.002–20 $\mu\text{g/ml}$) of inhibitory proteins. Inhibition values of all proteins tested with five individual patients were combined and mean values $\pm$ SD are shown in the plot.

IgE-binding could be observed between natural and recombinant Amb a 8 as well as for rArt v 4 (Fig. 4C).

In the Austrian group, sensitization to CBPs was as expected around 10% and no major difference in IgE-

recognition was observed between CBP-allergens from mugwort and ragweed. Surprisingly the IgE reactivity of the Italian group was much higher and ranged from 21% to 28% (Fig. 4B). Although, it is worth mentioning that

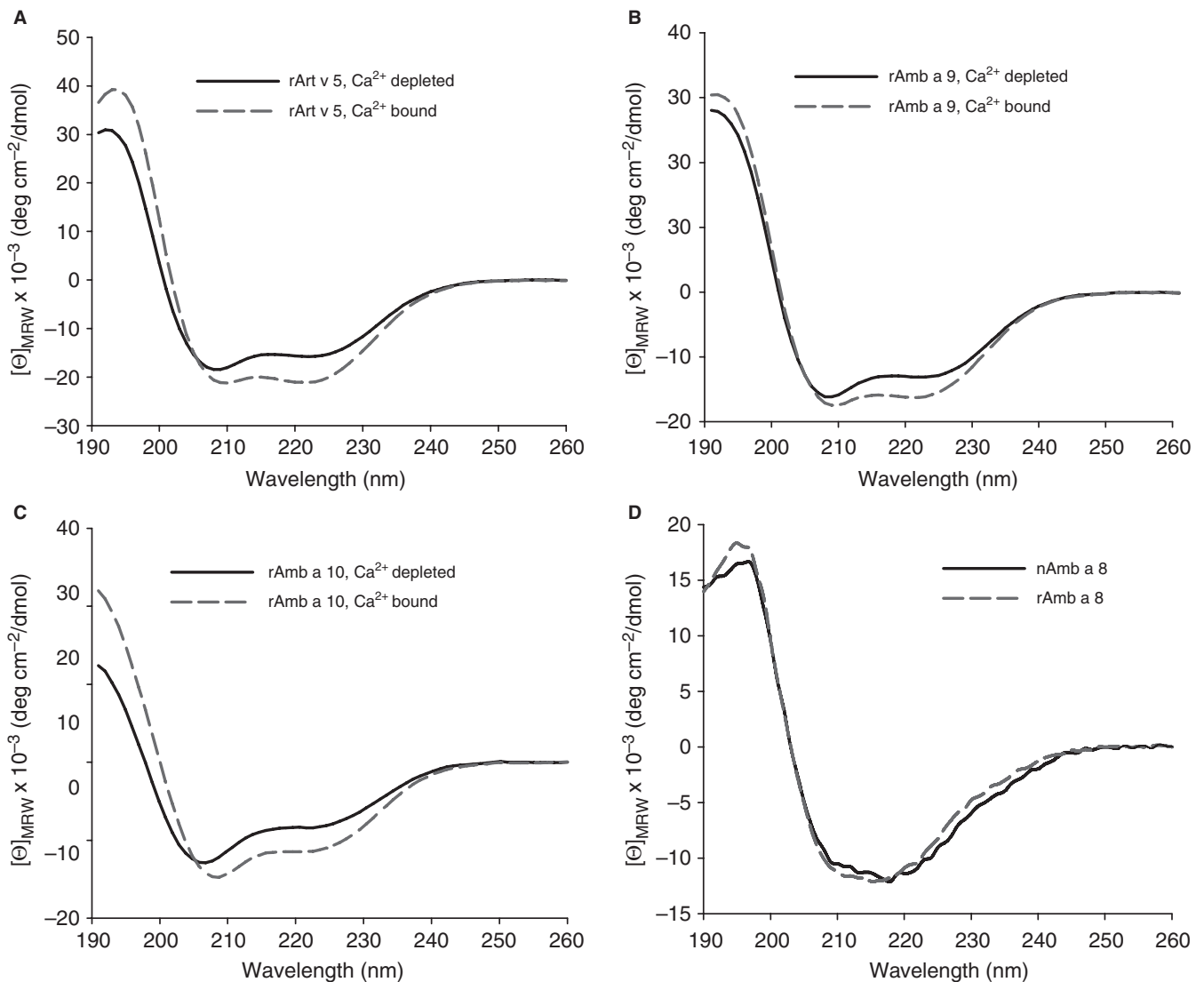


Figure 3. Structural analyses of purified weed allergens. Secondary structure contents of recombinant calcium binding allergens from mugwort (A) or ragweed pollen (B, C) as well as natural and recombinant ragweed pollen profilin (D) were determined by means of circular dichroism (CD). CD-spectra of calcium binding allergens were obtained in the presence or absence of protein-bound Ca^{2+} .

the general reactivity of patients from Italy against the CBPs was weaker compared to Austria, all CBPs had in common the calcium that was necessary for IgE-binding. Few patients were detected who had higher readings after depletion of calcium.

Furthermore, the allergenic potential of all allergens was determined by degranulation of RBL cells. Dose-dependent maximal mediator release was determined to be 8–20% for all tested patients and proteins in this study (Fig. 5). As shown in Fig. 5A the maximum mediator release for Amb a 8 was around 8–14% at concentrations of 0.1–1.0 ng/ml. Natural Amb a 8 induced the highest release values, whereas rAmb a 8 was only able to trigger comparable responses in one patient. In general, release curves for nAmb a 8 and rAmb a 8 were similar, but maximum values deviated by 50–85%. Compared to

Amb a 8, recombinant CBPs showed different response curves regarding the onset of mediator release (up to 20%) and protein concentration (1–100 ng/ml), necessary for maximal mediator release (Fig. 5B). The 2 EF-hand CBPs did show comparable activity, whereas rAmb a 10 was less potent in the RBL-assay.

Discussion

In this study, we present the discovery, cloning and characterization of novel cross-reacting pan-allergens from ragweed and mugwort pollen. On screening a ragweed cDNA library using IgE from weed-allergic patients, we obtained complete cDNA sequences coding for two new profilins (Amb a 8.01 and 8.02) and two

novel CBPs (Amb a 9 and Amb a 10). A new polcalcin from mugwort (Art v 5) was obtained by hybridization of a mugwort cDNA library with Bet v 4.

Although the ragweed pollen cDNA library was screened with a serum pool of allergic patients, none of the major ragweed-pollen allergens like Amb a 1, Amb a 2 or Amb a 6 were found. This can be explained by the

fact, that the sera used for screening were from patients mainly sensitized to mugwort pollen that do not contain IgEs against major allergens from ragweed.

Profilin

The two profilin isolated isoforms differ by 11/133 amino acid substitutions (91% sequence identity). Sequence searches with Amb a 8.01 revealed profilins from mugwort pollen, Art v 4 (89% identity), sunflower, Hel a 2 (81% identity) and timothy grass, Phl p 12 (79% identity) (8, 20, 21). Interestingly, a previously published Amb a 8 sequence from Tao and He (22), obtained from PCR cloning approach, revealed a sequence identity of only 79% to Amb a 8.01 indicating a new Amb a 8 homologue from ragweed. Immunoblots using rabbit antiserum indicated cross-reactivity of grass pollen and ragweed profilins. This cross-reactivity was already described for the natural profilin in ragweed pollen extract (8) and could be confirmed with purified proteins, from natural and recombinant sources. For IgE screening, sera from polysensitized Italian patients were used. These patients were mainly sensitized to tree but also to grass pollen. Nevertheless 26% of the tested patients showed IgE-binding to rAmb a 8 as well as rArt v 4. Bet v 2 from birch pollen showed the highest reactivity as patients from that region were mainly exposed to birch or other tree pollen. Another interesting point was that the reactivity to weed pollen profilins was much lower in Italian (20%) patients when compared to Austrian ones (45–50%).

As expected, no difference was observed in IgE-binding activity between Amb a 8 isoforms. Furthermore, IgE cross-reactivity of mugwort and ragweed profilin was comparable, which could be explained by their strong sequence identity. Both reacted with the same patients and even the IgE-binding activity, *in vivo* and *in vitro* was comparable. As the natural ragweed profilin was purified under denaturing conditions, a correct refolding was verified. The results demonstrated that recombinant and natural ragweed profilin have the same structural and immunological properties. Previously, similar results were obtained with recombinant profilins rArt v 4, rBet v 2,

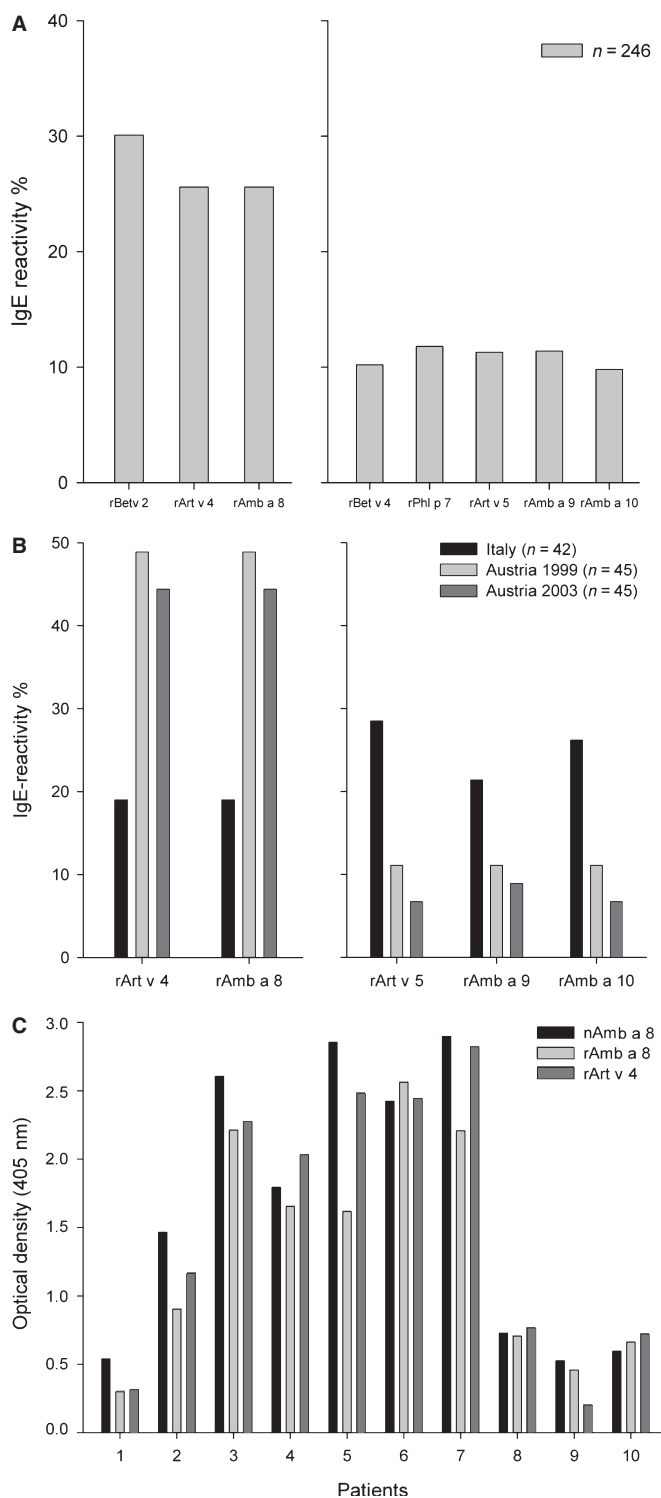


Figure 4. IgE-binding evaluation of profilin and CBPs. (A) Purified recombinant His-tagged proteins (exceptions: Bet v 2 and Art v 4) were dotted onto nitrocellulose membranes and exposed to sera from polysensitized patients from Italy (n = 246). Bound IgE was detected by radioactively labelled anti-human IgE. (B) Sera from Italian and Austrian patients were used to test the IgE-reactivity of purified recombinant profilins and calcium-binding proteins from mugwort (Art v 4 and Art v 5) and ragweed (Amb a 8, Amb a 9 and Amb a 10). (C) Comparison of specific IgE levels to natural Amb a 8 and recombinant Amb a 8 and Art v 4 using 10 preselected sera from weed pollen-allergic patients from Austria.

rCap a 2 and rHev b 8 (23). According to these findings, the ability to bind human IgE does not differ between natural and recombinant profilins. This is in contrast to the data presented by Wensing et al. (24) who reported a finding of lower IgE-binding for rBet v 2 when compared to natural Bet v 2 and Lol p 12.

As the sequences from profilins are highly conserved, strong cross-reactivity was observed even between profilins from distant plant species. In the case of weed-pollen profilins, we have shown that either rArt v 4 or rAmb a 8 could be used as a diagnostic marker for profilin sensitization.

Calcium-binding proteins

The 2EF-hand CBPs isolated from mugwort and ragweed encode two homologous proteins designated as Art v 5 and Amb a 9 with a sequence identity of 82%. BLAST searches of rAmb a 9 and rArt v 5 revealed the polcalcins CBP from birch Bet v 4 (71% and 68%), Ole e 3 from olive (71% and 65%) and Aln g 4 from alder (68% and 65%), respectively (11, 25, 26). Interestingly, Amb a 10, a 3EF-hand CBP from ragweed pollen, showed highest sequence similarities to Ole e 8 and Jun o 4, which are the 4EF-hand CBPs from olive and juniper respectively. It is unclear why the 3EF-hand CBPs like Bet v 3 (29% identity) showed lower sequence identities.

Immunological tests revealed that although the sequence identity of Phl p 7 with the homologous polcalcins Art v 5 and Amb a 9 was around 60%, no reactivity could be observed when using rabbit anti Phl p 7 serum, and on the IgE level only an inhibition of 40–50% could be observed. By contrast, Amb a 10 did show cross-reactivity using the rabbit antiserum but showed a lower level of inhibition in the IgE cross-reactivity studies. It seems that the similarities of the different CBPs are mainly restricted to the Ca^{2+} -binding motifs. Apparently, Amb a 10 has a higher similarity to Phl p 7 in the regions for the polyclonal rabbit antiserum than the other 2EF-hand allergens.

Approximately 10% of pollen-allergic patients react with CBPs from various sources like trees, grasses or weeds (17, 27). Furthermore, in a hierarchy of IgE cross-reactivity it was established that recombinant Bet v 3 (3 EF-hands) contained the least cross-reactive IgE epitopes when compared with rPhl p7, rAln g 4 (polcalcin) from alder and rJun o 4 (4 EF-hand motifs) from juniper (6, 28). This was also confirmed in this study, where the IgE reactivity of the polysensitized patients was found to be around 10% and Phl p 7 was the best-recognized CBP. Surprisingly, a completely different picture emerged with

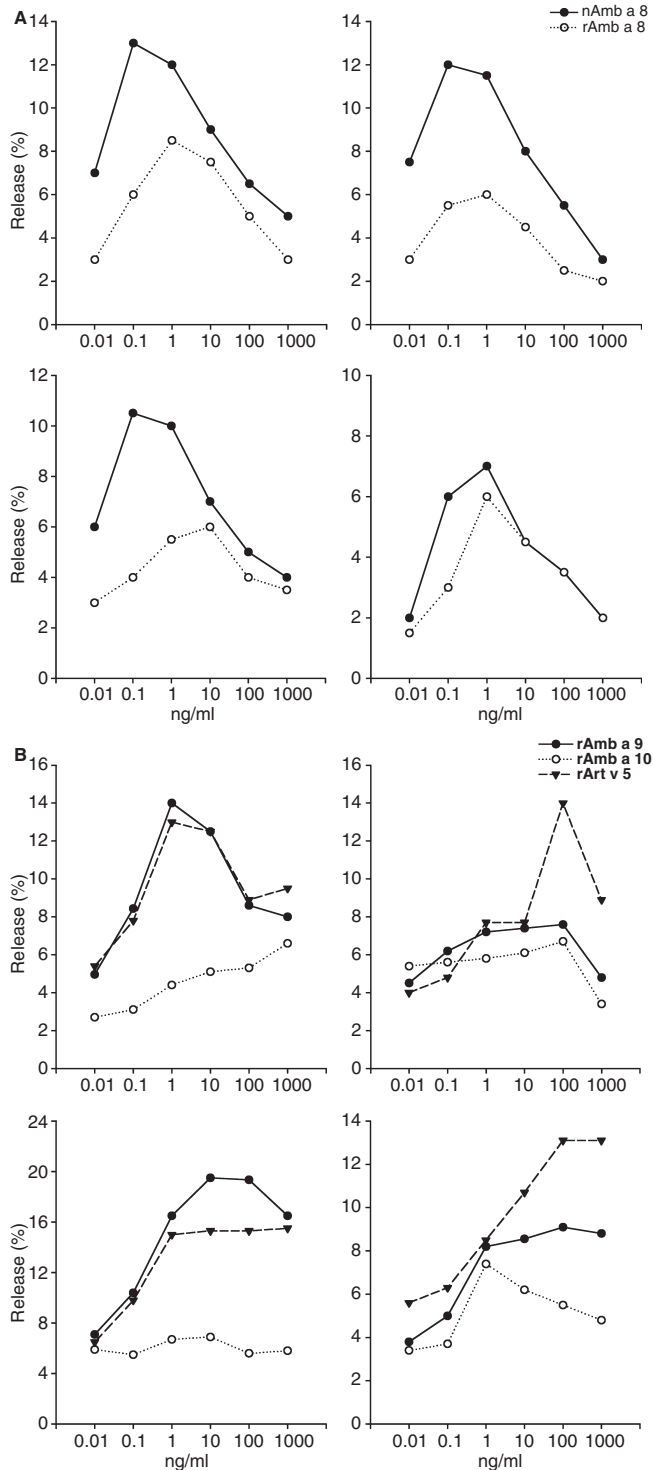


Figure 5. Mediator-release assay of pan-allergens. Induction of β -hexosaminidase release from RBL cell line expressing the human Fc ϵ RI. Rat basophil leukaemia cells were passively sensitized with IgE antibodies of four multiple sensitized patients from Italy. Allergen dependent mediator release was triggered by serial dilutions of either natural or recombinant Amb a 8 (A) or rAmb a 9, rAmb a 10 and rArt v 5 (B). Degranulation was measured by β -hexosaminidase release in the supernatant and is shown as percentage of degranulation achieved by total release of mediator through cell lysis.

the group of weed-allergic patients, when the reactivity to CBPs was much higher among the Italian group. Therefore, it is difficult to predict cross-reactivity within this group of allergens. So it may be that the positive IgE results do not only reflect cross-reactivity but also real sensitization to ragweed in this group of patients.

In general, the biological activity of the pan-allergens under study was surprisingly low for most of the tested patients. Considering these data, the newly described pan-allergens could be preliminarily classified as minor allergens. However, on account of the small lot of sera, more patients need to be tested because the diversity in the amount of mediator-release could be because of the different levels of specific serum IgE and polysensitization of the patients. Nevertheless, it needs to be considered that the severity of clinical symptoms caused by minor pollen allergens does not necessarily correlate with the

amount of specific IgE, cross-linking capacity and mediator-release.

In conclusion, further studies using well-characterized recombinant allergens of the CBP and profilin family are necessary to understand more thoroughly and predict sensitization, IgE cross-reactivity and clinical relevance. These pan-allergens should be considered as diagnostic markers for polysensitization and used in component-resolved diagnosis.

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