

Cloning, expression and characterization of mugwort pollen allergen Art v 2, a pathogenesis-related protein from family group 1

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Abstract

Mugwort (*Artemisia vulgaris*) belongs to the Compositae family, and is one of the main causes of allergy in late summer and autumn. The aim of the study was to characterize the allergen Art v 2 from mugwort pollen. Skin prick tests, performed in 19 patients allergic to mugwort and 10 control patients, showed an Art v 2 sensitization prevalence of 58%, whereas none false-positives were detected among control patients. Art v 2 was purified by standard chromatography and binding to Concanavalin A column and had an apparent molecular mass of 33 and 20 kDa, calculated by gel permeation and SDS-PAGE under denaturing conditions, respectively, showing that the allergen is composed of two identical subunits. Art v 2-encoding cDNA was amplified by PCR using degenerate primers based on reported partial amino acid sequences. Cloned cDNA encoding Art v 2 contains 140 bp that codify for a polypeptide of 15.8 kDa, with a predicted pI value of 5.2, and one potential N-glycosylation site. Protein homology search demonstrated that Art v 2 share 55–42% identical residues with pathogenesis-related protein PR-1 of tomato, potato, rape, wheat and rice. Homology was also found to Ves v 5 (41% identical residues). Bacterial-expressed recombinant Art v 2 was recognized only by 21% of mugwort-allergic patients. In conclusion, Art v 2 from mugwort is the first weed pollen allergen that belongs to the pathogenesis-related protein PR-1 and its recombinant form could help molecular diagnosis of mugwort associated allergy.

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Keywords: *Artemisia vulgaris*; Art v 2; Recombinant allergen; Gene expression; Pathogenesis-related protein; PR-1

1. Introduction

Type I (IgE-mediated) allergy is a genetically determined clinical disorder that affects almost 25% of the population in development countries (Akdis and Blaser, 2000). *Artemisia vulgaris* (mugwort), included in the family Compositae or Asteraceae (one of the largest families of flowering plants), is a weed widely spread in temperate regions and subtropics of Europe, North America and Asia (Charpin and Surinyach, 1974). Its pollen is considered an important elicitor of type I allergy during late summer and the beginning of autumn (Spieksma et al., 1980). *A. vulgaris* pollen has allergenic structures that are recognized by cross-reactive IgE antibodies to different allergen sources and therefore, it is involved in several pollen-food syn-

dromes (Egger et al., 2006). Up to 15 allergic proteins with variable clinical importance have been described in *A. vulgaris* pollen extracts (Nilsen et al., 1991a). Art v 1 is a 24–28 kDa glycoprotein encoded by an open-reading frame of 108 amino acids with a defensin-like domain (Himly et al., 2003). Art v 1 prevalence varies depending on the studies, it has been described that between 50 and 90% of the mugwort-allergic patients had specific IgE to Art v 1 (Himly et al., 2003; Lombardero et al., 2004), and from 50 to 70% had positive skin prick test (SPT) to the same allergen (Lombardero et al., 2004). Art v 3 is a major allergen with an apparent molecular weight of 9.7 kDa. It belongs to the lipid-transfer-protein (LTP) family and its N-terminal amino acid sequence has been published (Díaz-Perales et al., 2000). Around 70% of mugwort sensitized patients had positive SPT and specific IgE to natural Art v 3 (Lombardero et al., 2004). Mugwort profilin (Art v 4), which is an important cross-reactive panallergen for patients suffering from multiple food and pollen allergies, reacted to IgE antibodies of 36% of mugwort-allergic patients (Wopfner et al., 2002). Art v 2 has been identified as a

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glycoprotein of about 35 kDa that consisted of at least six isoforms and reacted to 45% of sera from mugwort-allergic patients (Nilsen and Paulsen, 1990). Although the NH₂-terminal amino acid sequence and cyanogen bromide fragments of deglycosylated Art v 2 has been described (Nilsen et al., 1991b), its complete molecular structure had not been identified up to this work.

Pathogenesis-related proteins (PRs) are proteins encoded by the host plant but induced specifically in pathological or stress situations (Van Loon and Van Strien, 1999). Plant-derived allergens have been identified with sequence similarity to PR families 2–5, 8, 10 and 14 comprising β -1,3-glucanases, three types of chitinases, thaumatococcal-like proteins, Bet v 1-like proteins and lipid transfer proteins (Hoffmann-Sommergruber, 2002). Recently, a Bermuda grass pollen allergen, Cyn d 24, has been described as new member of PR-1 type proteins (Chow et al., 2005).

In this study, we report for the first time the cloning and identification of Art v 2 from *A. vulgaris* pollen as member of the pathogenesis-related proteins PR-1. The allergen was expressed in *Escherichia coli* as a non-fusion protein and the allergenic reactivity of the recombinant protein compared with the natural counterpart in relation to their capacity to bind specific IgE and to elicit skin prick test (SPT).

2. Materials and methods

2.1. Patients

Clinical data of 19 patients (age 10–55 years; 9M/10F) used in this study are shown in Table 1. Patients showed immediate hypersensitivity to *A. vulgaris* according to a clinical history of seasonal or perennial rhinitis, positive SPT response to commercially available *A. vulgaris* extract (Bial-Aristegui, Bilbao,

Spain) and specific IgE serum level to *A. vulgaris* extract as determined by using the ImmunoCAP System (Phadia, Uppsala, Sweden). Ten control subjects, three no atopics, and seven with other no-related allergies (olive and grass pollen, mites and cat dander) were also included. All patients and control subjects provided oral and written informed consent.

2.2. Purification of natural allergens and experimental rabbit antisera

Fifteen grams of *A. vulgaris* pollen was extracted with 150 mL PBS for 2 h, centrifuged at 5000×g for 15 min and the pellet extracted again. The supernatants were dialyzed against 20 mM phosphate pH 7.0 and subjected to cationic exchange chromatography in a High Flow SP column (GE-Healthcare, Uppsala, Sweden) in order to purify Art v 1 as previously described (Schmid-Grendelmeier et al., 2003). Flow through fraction was adjusted to 1 M ammonium sulfate and applied to a Phenyl Sepharose 16/20 column (GE-Healthcare). Bound proteins were eluted with water and dialyzed, adjusted to 20 mM Tris pH 8.0; 0.5 M NaCl; 1 mM CaCl₂; 1 mM MnSO₄ and injected into a HiTrap Concanavalin A-Sepharose (GE-Healthcare). Bound glycoproteins were eluted with 0.5 M methyl- α -D-glucopyranoside and concentrated by ultrafiltration (Amicon Ultra 4, Millipore, Bedford, MA, USA). Final purification was achieved with a Superdex 75 gel permeation column fitted in a Smart system (GE-Healthcare). Art v 4 was purified following the modified method of Lindberg previously described (Vallverdú et al., 1997).

Polyclonal antibodies were obtained in New Zealand rabbits by immunizing with five boosts of 200 μ g of natural Art v 2 every 2 weeks, emulsified in complete Freund's adjuvant (Difco, Detroit, Michigan). Sera, collected 10 days after the last injection, were tested by ELISA and stored at –80 °C.

Table 1
Sensitization data of patients allergic to mugwort included in the study

Patient	Gender	Symptoms	Mugwort IgE reactivity (U/mL)	Minimal concentration of allergen with positive SPT (μ g/mL)			
				nArt v 2	rArt v 2	nArt v 1	nArt v 4
1	F	RC	91.81	5	–	5	50
2	F	RC, A	62.57	–	–	–	–
3	F	A	45.36	5	5	5	50
4	F	RC	54.59	100	–	50	–
5	F	R	79.47	–	–	5	5
6	M	RC, A	35.51	100	–	100	–
7	M	RC	71.59	–	–	5	–
8	M	R	52.57	–	–	–	5
9	M	RC	50.75	–	–	–	–
10	M	RC, A	43.13	100	–	–	–
11	M	RC	122.50	5	5	5	5
12	F	RC	48.37	50	50	5	5
13	F	RC, A	97.14	5	–	5	50
14	F	RC, A	73.95	–	–	5	–
15	F	RC	65.17	100	–	5	5
16	M	R	67.18	–	–	5	–
17	F	RC	46.90	–	–	50	–
18	M	RC	64.46	50	50	5	–
19	M	RC	49.86	5	–	–	5

(RC) Rhinoconjunctivitis, (A) asthma, (R) rhinitis, (–) no SPT response at any of the concentrations tested.

2.3. Protein identification by tandem electrospray mass spectra

Selected protein bands were excised manually from the gel and subjected to in-gel tryptic digestion according to Shevchenko et al. (1996). The supernatant was recovered and peptides were extracted twice: first, with 25 mM NH_4HCO_3 and acetonitrile, and then with 0.1% TFA and acetonitrile. The recovered supernatants and extracted peptides were pooled, dried in a SpeedVac, redissolved in 10 μL of 0.1% TFA and sonicated for 5 min. Tandem electrospray mass spectra were acquired using a Q-ToF micro mass spectrometer (Waters, Milford, MA, USA) interfaced with a CapLC capillary chromatograph (Waters). Obtained spectra were searched against a home-made database containing the complete Swiss-Prot database plus the deduced amino acid sequence from the cloned protein using VEMS 3.0 (Matthiesen et al., 2005).

2.4. Amplification of Art v 2 cDNA by RT-PCR and nucleotide sequence determination

Quick Prep MicroRNA Purification Kit (GE-Healthcare) was used to isolate Poly(A⁺)-enriched RNA from *A. vulgaris* pollen. This mRNA was reverse transcribed to first-strand cDNA (3' and 5') with SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's recommendations. Degenerate primers were designed on the basis of reported partial amino acid fragments of Art v 2 (Nilsen et al., 1991b): NF, 5'-GAYGAYTAYGTNCAYGCNCAYAA-3', and BR, 5'-YTTTRTTRTTRTARTCRTARTCVA-3'. A PCR product of 232 bp was isolated, cloned into the pGEM vector (Promega, Madison, WI, USA) and sequenced (Secugen SL, Madrid, Spain). The obtained nucleotide sequence allowed design two non-degenerate primers: CBF, 5'-GCAGTCCAATTGTGGCTTGATGAGAGA-3' and NHR, 5'-AGGTTTCATACCTAATACTCTTCGGAT-3'. The 3'-cDNA used as template and the primers CBF and UPM (Universal primer A Mix, RACE Kit) served to amplify the nucleotide sequence codifying the carboxy-terminal end of protein. The nucleotide sequence of amino terminal end was amplified by using NHR and NUP (Nested Universal Primer; RACE Kit) primers and 5'-cDNA as template. On the basis of these previous partial sequences, two primers were designed to determine the full-length Art v 2 coding region: sense primer 5'-GCGGATCCCACGAAACGTACGGGGAGC-3', and anti-sense primer 5'-GCGAATTCCTAGTAAGGTTTCTGACCAAC-3' (*Bam*HI and *Eco*RI restriction sites are underlined). After sequencing, Art v 2-encoding cDNA was subcloned into the *Bam*HI/*Eco*RI sites of pKN172 expression vector (Way et al., 1990) and used to transform *E. coli* BL21(DE3) cells.

2.5. Production and purification of rArt v 2

A recombinant *E. coli* BL21(DE3) single colony was used to start a 50 mL overnight culture, afterwards it was diluted with fresh LB supplemented with 0.2 mg/ml ampicillin to a 0.2 OD₆₀₀ and grown until it reached 0.8. Protein produc-

tion was induced with 0.6 mM IPTG for 3 h at 37 °C. Bacteria were harvested by centrifugation and disrupted by lysozyme treatment (100 $\mu\text{g}/\text{mL}$, 30 min, 37 °C) and mild sonication. Insoluble material was collected by centrifugation, washed and extracted for 1 h at room temperature with 50 mM Tris pH 8.0, 0.1% 2-mercaptoethanol containing 6 M guanidine hydrochloride. Following centrifugation, the extract was dialyzed against 25 mM phosphate buffer, pH 7.0 containing 6 M urea and refolded by lowering the urea concentration by a half in consecutive steps (5 steps, 90 min each). Protein was concentrated by ultrafiltration and loaded into a Superdex 200 16/60 gel permeation column (GE-Healthcare). Fractions containing pure Art v 2 were identified by SDS-PAGE and pooled.

2.6. Electrophoresis and Western blotting

A. vulgaris pollen extracts and purified Art v 2 were separated by SDS-PAGE (12.5% polyacrilamide gels) under reducing and non-reducing conditions using Laemmli method (Laemmli, 1970). Protein bands were stained with Coomassie brilliant blue. For immunoblot studies, the separated proteins were transferred electrophoretically to PVDF (Hybond P, GE-Healthcare). After blocking, membranes were incubated at 4 °C overnight with antiserum against Art v 2 (diluted 1/50,000), washed and bound antibodies were detected with 1:10,000 horseradish peroxidase-conjugated anti-rabbit IgG (Dako, Copenhagen, Denmark). Afterwards, the blot was washed and developed by the ECL Western blotting detection system (GE-Healthcare). For IgE dot-blot inhibition experiments, 2 μg in 2 μL of PBS were dotted onto a nitrocellulose membrane (Hybond C+, GE-Healthcare). After drying, the membrane was blocked and incubated overnight at 4 °C with sera (diluted 1:3) from mugwort-pollen allergic patients previously incubated for 3 h at room temperature with buffer alone or 20 μg of nArt v 2, rArt v 2 and BSA. Afterwards, detection of IgE-binding was performed as described above.

2.7. Determination of specific IgE levels

Specific IgE levels to *A. vulgaris* pollen extract and Art v 2 purified allergens were evaluated by enzyme allergosorbent test (EAST). *A. vulgaris* extract (1500 $\mu\text{g}/\text{mL}$) and Art v 2 (100 $\mu\text{g}/\text{mL}$) was coupled to paper discs activated with cyanogen bromide as described by Ceska and Lundkvist (1972). Sera were added to the discs and incubated at 37 °C for 1 h. After washing, the discs were incubated 30 min at 37 °C with 50 μL of alkaline phosphatase-labelled mouse anti-human IgE and developed following the instructions of Hytec specific IgE EIA (Hycor Biomedical Inc., Kassel, Germany).

2.8. SPTs with *A. vulgaris* pollen extract and purified allergens

SPTs were performed with a commercial *A. vulgaris* pollen extract (Bial-Arístegui) and natural and recombinant purified allergens. The individual proteins were diluted in NaCl 0.9% while histamine hydrochloride 10 mg/mL and NaCl 0.9% served

as positive and negative controls. All skin tests were performed in duplicate with 20 μ L on the volar forearm and applied in two opposite directions with a prick needle (Morrow-Brown, Derby, UK). The elicited wheals areas were determined by computerized planimetry (AutoCAD 11, Autodesk Inc., San Rafael, CA, USA). A wheal surface area greater than 7 mm² was considered positive.

2.9. Statistical analysis

Statistical data analysis was performed by using the Wilcoxon test and the Spearman's rho correlation included in the SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA). A *p*-value of less than 0.05 was considered significant.

2.10. Protein modeling

Homology searches and multiple amino acid sequence alignments were performed using the BLAST and CLUSTAL networks, respectively (Altschul et al., 1997; Thompson et al., 1994). Signal peptide was predicted using SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>). Model building and analysis were performed using the SWISS-MODEL server (<http://www.expasy.ch/swissmod/SWISS-MODEL.html>) (Guex and Peitsch, 1997). The atomic coordinates of the tomato PR-1a (PDB Accession number: 1CFE) were used to build a 3D model of Art v 2. Prediction of solvent accessibility was performed using the Program PHDacc (Rost and Sander, 1994).

3. Results

3.1. Purification of Art v 2

Art v 2 was purified from mugwort pollen extract with a final yield of 35 μ g/gram of mugwort pollen. Purified nArt v 2 gave a single band with an apparent molecular mass of 19.2 kDa under reduced conditions and 34.2 kDa under no reduced conditions (Fig. 1). The apparent molecular mass on gel filtration chromatography of purified protein was 33 ± 3 kDa, suggesting that Art v 2 is composed of two mainly identical subunits linked by disulfide bridges. This result was according to previous observations of the dimeric structure of Art v 2 (Nilsen et al., 1991b). The Art v 2 content in 14 mugwort extracts, estimated by ELISA titration using purified Art v 2 and rabbit antiserum against Art v 2, showed high variation ranging from 0.1 to 3.2% of extract protein. The low yield (ca. 0.1% of protein) of nArt v 2 purification could be due to the high number of chromatographic steps required for purification and to the low and variable Art v 2 content in mugwort pollen extracts.

3.2. Cloning and characterization of primary structure of Art v 2

The cloned cDNA had 739 bases, including the poly(A+) tail and predicted an open reading frame of 164 amino acids in length. The cloned Art v 2 cDNA certainly encoded the purified allergen since it contained all peptide sequences previously

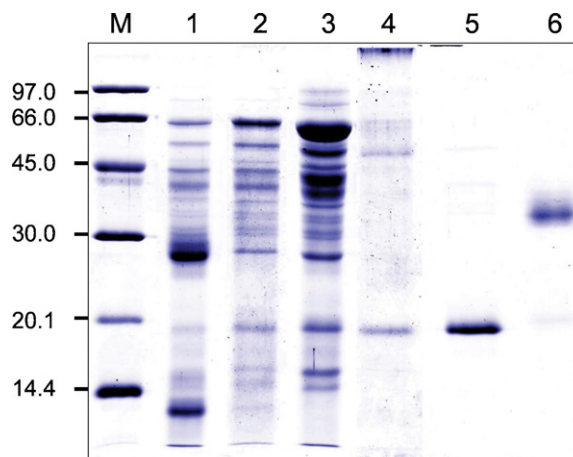


Fig. 1. Electrophoretic analysis of purified Art v 2. Coomassie-stained SDS-PAGE of fractions from the purification steps: (lane 1) mugwort raw extract, (lane 2) after hydrophobic interaction column, (lane 3) after ion exchange column, (lane 4) after Concanavalin A column, (lane 5) purified Art v 2 after gel permeation column and (lane 6) same as lane 5 run under non-denaturing conditions.

reported (Nilsen et al., 1991b), although two minor differences were detected: (Lys30–Leu and Asp67–Asn). The deduced amino acid sequence showed one potential *N*-glycosylation site and a calculated isoelectric point of 5.23 (Fig. 2A). A predicted molecular mass of 15770 Da, which agreed with the observed mass of 17500 Da, obtained after treatment with deglycosylating enzymes (Nilsen et al., 1991b). Signal peptide prediction result agreed to the experimental N-terminal sequence previously reported (Nilsen et al., 1991b). Accessibility prediction results showed that 68.6% of the residues presented a predicted accessibility of >16% of their surface. The identity of purified nArt v 2 was confirmed by tandem electrospray mass spectra, as the sequences obtained for MS-MS-derived peptides matched deduced sequence from cloned Art v 2 cDNA.

Protein homology search using BLAST software demonstrated that Art v 2 shared 54.7, 53.7, 49.6, 48.9, 46.0 and 45.3% identical residues with pathogenesis-related protein PR-1 of tomato, potato, bell pepper, rape, barley and maize, respectively (Fig. 2C). A sequence identity of 44% was found with the allergen Cyn d 24 from Bermuda grass pollen. Art v 2 and all these proteins have six conserved Cys residues that should be involved in the tertiary structure stability. Homology modeling of the three-dimensional structure of Art v 2 yielded a conformation that was highly similar to tomato PR-1a structure, with the exception that in Art v 2 model α -helix II is composed of two smaller α -helices (Van Loon and Van Strien, 1999). This protein folds into an α – β – α sandwich structure with five α -helices and four β -strands arranged antiparallel and compacted by three disulfide bridges (Fig. 2B).

3.3. Expression in *E. coli* and purification of rArt v 2

A non-fusion form of mature Art v 2 was expressed in *E. coli* BL21 (DE3) using the expression vector pKN172 based on the phage T7 system. Rupture and fractionation of the cells showed that the expressed protein resided entirely in the insoluble

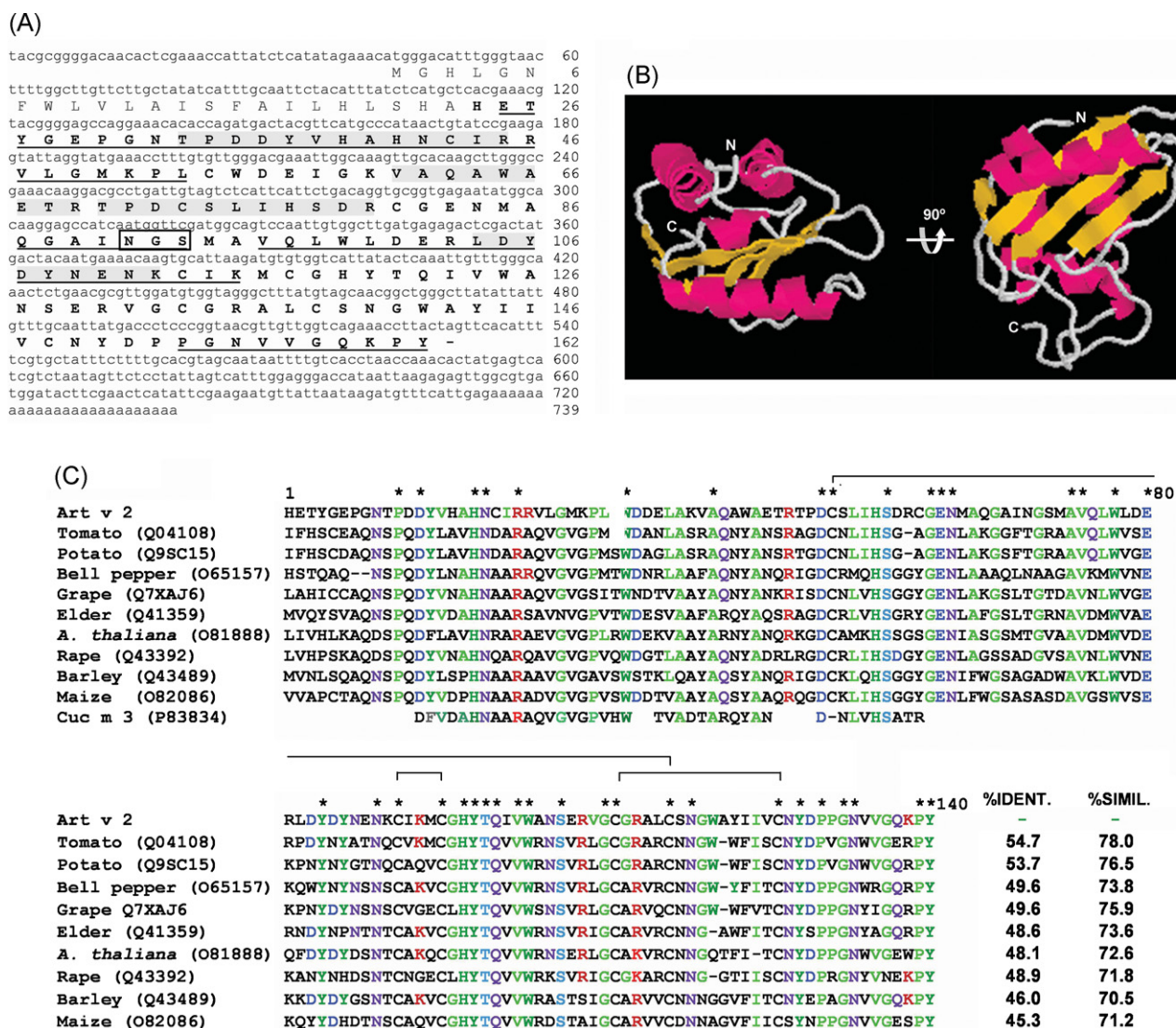


Fig. 2. cDNA sequence and sequence alignment of Art v 2. (A) Nucleotide and deduced amino acid sequence of Art v 2. N-terminal and previous reported amino acid sequences (Nilsen et al., 1991b) are underlined. Glycosylation site is boxed. Amino acid sequences identified by tandem electrospray mass spectra are shaded. The nucleotide sequence for Art v 2 has been deposited in the GenBank database under accession number AM279693. (B) Ribbon representation of the molecular modeling of a Art v 2 based on 3D structure of tomato PR-1a (Van Loon and Van Strien, 1999). (C) Comparison of the amino acid sequence of Art v 2 with those of various PR-1. Dashes are introduced for optimal alignment. Asterisks showed conserved residues and putative disulphide bridges were marked.

ble fraction. After solubilization with guanidine hydrochloride and refolding, pure rArt v 2 was obtained by permeation chromatography as aggregates of ca. 80 kDa with a final yield of 16 mg/L bacterial culture. The recombinant allergen had an apparent molecular mass of 17.1 kDa (Fig. 3A) comparable with that of its natural counterpart after treatment with deglycosylation enzymes (Nilsen et al., 1991b). Natural and recombinant Art v 2 were subjected to immunoblotting analysis with a rabbit antisera against Art v 2 showing similar IgG-epitopes (Fig. 3A).

3.4. Antibody recognition of Art v 2

Determination of Art v 2-specific IgE levels showed that 63.2% (12 out of 19) sera from mugwort-allergic patients reacted with nArt v 2, while 10.5% (2/19) reacted with the

recombinant counterpart. Immunoblotting analysis revealed that the purified allergen Art v 2 (in both natural and recombinant forms) was not detected by IgE from patient sera after transferring to PVDF from either denaturing or non-denaturing SDS-PAGE gels. IgE-reactivity to Art v 2 was only found after dot-blot experiments (Fig. 3B). The IgE binding capacity of natural and recombinant forms of Art v 2 was compared by dot-blot inhibition experiments. Pre-incubation treatment of serum samples with nArt v 2 completely abolished signal to the bound proteins. However, different degree of nArt v 2 inhibition was found after pre-incubation of some patients sera with rArt v 2 (Fig. 3B). BSA, used as control protein for unspecific inhibition, had no visible effect on IgE binding to extract proteins. These results demonstrated that the recombinant allergen shared epitopes with the natural counterpart.

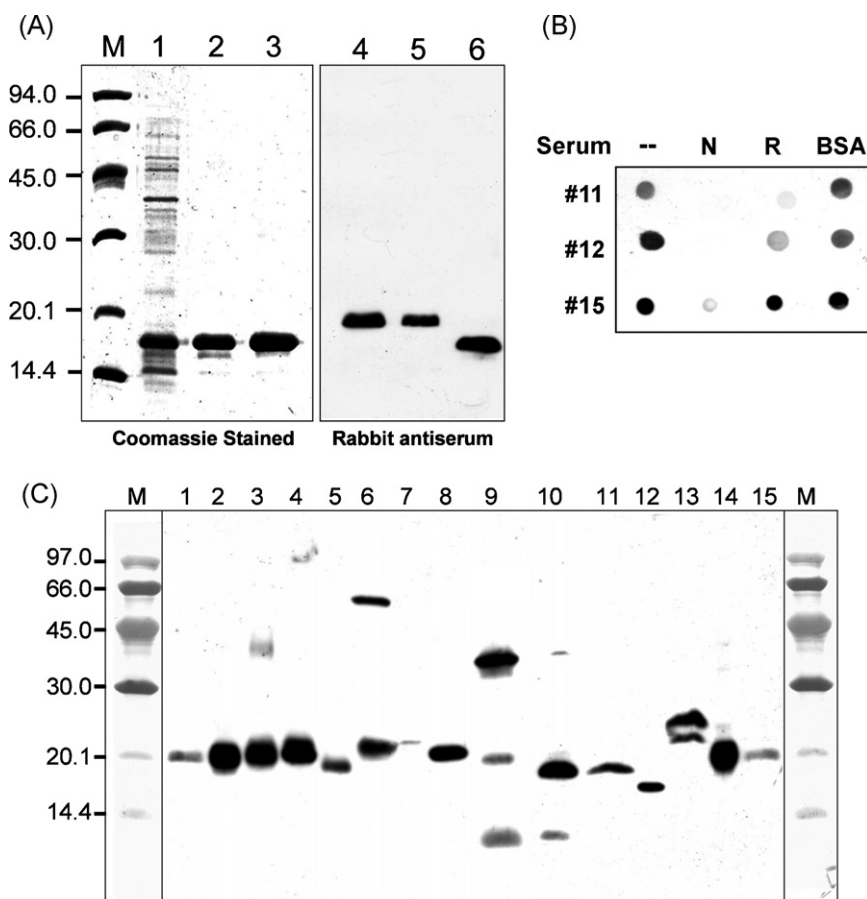


Fig. 3. (A) Electrophoretic analysis of purified rArt v 2: Coomassie-stained SDS-PAGE of *E. coli* BL21(DE3) extract expressing Art v 2 (lane 1), inclusion bodies of rArt v 2 (lane 2) and purified rArt v 2 (lane 3). Western blotting with a rabbit antiserum against Art v 2 of mugwort pollen extract (lane 4), purified nArt v 2 (lane 5), purified nArt v 2 (lane 6). (B) Dot-blot inhibition experiments. IgE binding of sera (diluted 1:3) from mugwort-pollen allergic patients to blotted nArt v 2 (2 µg) after inhibition with buffer alone (–) or 20 µg of nArt v 2, rArt v 2 and BSA. (C) Western blotting incubated with antiserum against Art v 2 (diluted 1:50,000): rArt v 2 (lanes 1 and 15) and pollen extracts from mugwort (lanes 2 and 14), ox-eye daisy (*Chrysanthemum leucanthemum*) (lane 3), golden rod (*Solidago aurea*) (lane 4), daisy (*Bellis* sp.) (lane 5), sunflower (lane 6), ragweed (*Ambrosia artemisifolia*) (lane 7), Bermuda grass (*Cynodon dactylon*) (lane 8), camomile (*Matricaria chamomila*) (lane 9), globe artichoke (*Cynara scolymus*) (lane 10), cardoon (*Cynara cardunculus*) (lane 11), cauliflower (lane 12) and tomato (lane 13).

Table 1 displays the results of SPT carried out with mugwort pollen whole extract and purified natural mugwort allergens Art v 1, Art v 2 and Art v 4 and *E. coli*-expressed Art v 2. The prevalence of nArt v 2 sensitization was 57.9% (11 out of 19) at 100 g/ml and 36.8% (7/19) at 50 g/ml, whereas none false-positive was detected among control patients. For rArt v 2, positive skin prick test was found only in 4 out of 19 mugwort-allergic patients (21.0%). Prevalences of 73.7% (14/19) and 47.3% (9/19) were found for Art v 1 and Art v 4 at 100 g/ml, respectively.

3.5. Presence of Art v 2-like proteins in other pollen species and plant-derived foods

The expression of Art v 2-like proteins in different pollens and plant-derived foods was studied by Western blotting using specific rabbit antiserum against nArt v 2. Specific IgG-reactive bands of 18.6–21.4 kDa were detected in pollen extracts from Compositae plants and Bermuda grass (Fig. 3C). Extracts from cardoon and globe artichoke, both plants from the Compositae family, also gave a reactive band at 18.0 kDa

with minor bands at 12.6 and 37.0 kDa. Plants belonging to phylogenetically distant families, such as Brassicaceae and Solanaceae, also contained Art v 2-like proteins of 16.7 and 22–25 kDa.

4. Discussion

Pollen grains of mugwort are an important cause of pollinosis during late summer and fall in temperate and humid areas. They contain a number of allergenic molecules, several of which have been identified and characterized in the recent past (Díaz-Perales et al., 2000; Himly et al., 2003; Wopfner et al., 2002, 2005). In this work, we report the identification, cloning based on reported partial amino acid sequences and recombinant production of Art v 2. This allergen belongs to the scarcely represented PR-1 type proteins.

A number of identified plant-derived allergens have been classified into defined groups of plant proteins, such as pathogenesis-related proteins (PR-proteins), seed storage proteins and structural proteins (Radauer and Breiteneder, 2006). Many plant allergens from food and pollen have been found to

be PR-proteins and these have grouped into one of 14 families, being PR-protein families 2–5, 8, 10 and 14 the most represented (Breiteneder and Ebner, 2000; Hoffmann-Sommergruber, 2002). Art v 2 share 55–44% identical residues to PR-1 proteins containing the conserved six Cys residues involved in three disulphide bridges. By homology modelling, it showed a compact α – β – α sandwich structure with five α -helices and four β -strands arranged antiparallel similar to one described for tomato PR-1a (Van Loon and Van Strien, 1999). This protein family is widely distributed in monocotyledons and dicotyledons species (Van Loon and Van Strien, 1999). Art v 2, as other PR-1 protein family members, showed homology (41% of sequence identity) with group 5 of major allergens from vespid and fire ant venoms, such as Ves v 5, Vesp c 5, Dol m 5 and Sol i 3 (Henriksen et al., 2001). Amino acid sequence comparisons and IgG cross-reactivity experiments using specific rabbit antiserum against Art v 2 have demonstrated the existence of Art v 2-like proteins in several plant food sources which could act as allergens. To date, only a major allergen from Bermuda grass pollen, Cyn d 24, has been identified as a pollen allergen belonging to the PR-1 protein family (Chow et al., 2005). Furthermore, partial amino acid sequences of a food allergen from melon, Cuc m 3, showed that this allergen could be also a member of this protein family (Asensio et al., 2004). According to these results, Art v 2-like proteins could play an important role on pollen-food syndromes associated with mugwort pollinosis (Egger et al., 2006).

Native protein conformation of Art v 2 was required for binding of human IgE since no IgE binding activity could detect after transfer from SDS-PAGE gels, and *in vitro* IgE reactivity could be only found by dot-blot and EAST (Fig. 3B) or rocket-radio-immuno-electrophoresis (Nilsen et al., 1991a; Nilsen and Paulsen, 1990). rArt v 2 conserved some of the IgE and IgG epitopes of its natural counterpart (Fig. 3A and B). However, the lower SPT reactivity of the bacterial-expressed rArt v 2 compared to the natural allergen and the partial inhibition of the nArt v 2 obtained by rArt v 2 suggested that the refolding step after solubilization of rArt v 2 inclusion bodies yielded an improper folded protein. The high-mannose oligosaccharides of Art v 2 has been reported to have no significant effect on the binding of IgE to the allergen but it could be an stabilizing element of the three-dimensional conformation (Nilsen et al., 1991b). Overproduction of foreign proteins in the *E. coli* cytoplasm often goes together with misfolding of the three-dimensional structure of the recombinant proteins and the absent of these Art v 2 carbohydrate structures from the bacterial-expressed rArt v 2 could be responsible of the misfolding of this recombinant allergen. Further search should be made in this direction, although the fact that Art v 2, prepared in our laboratory, could represent only one of the various isoforms occurring naturally and hence possess only some of the IgE epitopes present in nArt v 2 should not be ruled out.

PR-1 type proteins, as well as other PR proteins, can be induced by stress, such as pathogen infection and their quantitative presence in pollen can vary with growing, harvesting and storage conditions. This could be the reason for the high variability of the Art v 2 content found in several mugwort pollen

extract batches. Nevertheless, although antifungal activity has been demonstrated for tomato and tobacco PR-1 (Niderman et al., 1995), the function of PR-1 type proteins remains essentially unknown (Van Loon and Van Strien, 1999).

Cross-reactivity of mugwort and relevant composite pollen, such as ragweed has been attributed to allergen families with restricted distribution and panallergens (profilins, polcalcins and nsLTP). Among the first group, three families have been mentioned: pectate lyases, Amb a five homologous proteins and defensin-like Art v 1 (Wopfner et al., 2005). According to our results, a further protein, from the PR-1 group, should be added to the list of allergens accounting for this cross-reactions.

Comparative analysis of component-based allergen-microarrays and whole extract-based immunoCAP using Art v 1 and mugwort extract, respectively, showed lower sensitivity for the former indicating that alternative or additional components for detecting mugwort allergy are required (Wöhrle et al., 2006). Recently, component resolved diagnosis in mugwort sensitization using purified allergens Art v 1, Art v 3 and Art v 60 kDa showed that all of these allergens have a similar prevalence in SPT around 75% (Lombardero et al., 2004). The existence of one additional characterized allergen from mugwort could help molecular diagnosis of pollen-food syndromes and mugwort pollen sensitization.

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