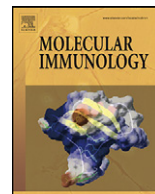




Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm



Immune recognition of novel isoforms and domains of the mugwort pollen major allergen Art v 1

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ARTICLE INFO

Article history:

Received 11 September 2008

Accepted 14 October 2008

Available online xxx

Keywords:

Art v 1 allergen

Glycoallergen

Isoforms

Mugwort allergy

Pollinosis

IgE binding

ABSTRACT

Allergen isoforms can differ in their IgE and T cell recognition patterns, and thus might have an impact on the selection of candidates for molecule-based diagnostic and therapeutic approaches. The present study aimed at the identification and characterization of isoforms of Art v 1, the mugwort pollen major allergen. In addition, single Art v 1 domains were physicochemically and immunologically characterized. For this purpose, the Art v 1 cDNA was radiolabeled and used to screen a mugwort pollen cDNA library. Positive clones were sequenced and used for the production of recombinant proteins in *Escherichia coli* using the pHis-Parallel2 vector. Protein purification was performed by affinity- and ion exchange chromatography. Antibody binding to the recombinant proteins was determined by immunoblot, ELISA, cross-inhibition experiments, and mediator release assays. We could identify 7 Art v 1 isoforms differing in 1–6 amino acid residues. Interestingly, all amino acid variations were restricted to the proline domain carrying the molecule's post-translational modifications. No significant difference in IgG or IgE reactivity could be observed between Art v 1 isoforms and the defensin domain produced in *E. coli*. When expressed in *E. coli*, the proline domain was not recognized by Art v 1-specific antibodies. Our results demonstrated that the relevant IgE epitopes of Art v 1 are located on the defensin domain and suggest the involvement of carbohydrates in the allergenicity of natural Art v 1. Plant-based expression systems could help to reveal possibly different glycosylation patterns and IgE binding properties of Art v 1 isoforms. These findings have direct implications on the development of novel tools for mugwort pollen allergy diagnosis and therapy.

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1. Introduction

The herb mugwort (*Artemisia vulgaris*), belonging to the botanical family of Asteraceae, is widely spread in the temperate and humid climate zones of the Northern Hemisphere, and thus represents one of the main causes of late summer pollinosis in Europe, Northern America, and parts of Asia. More than 95% of mugwort pollen-allergic patients are sensitized to the major allergen Art v 1, a modular glycoprotein composed of two domains (Himly et al., 2003). The globular amino terminal domain displays a high homology to plant defensins. The carboxy terminal domain rich in proline residues shows an extended left-handed helical shape and carries the molecule's post-translational modifications, i.e. hydroxylation of proline residues and a novel type of plant O-glycosylation

(Leonard et al., 2005). Due to the presence of several alleles or families of related genes, pollen allergens can be polymorphic, i.e. they exist as isoallergens, isoforms, or isoallergenic variants (Lagares et al., 2002). Indeed, sequence polymorphisms have been described for many major allergens, e.g. Amb a 1 (Griffith et al., 1991), Par j 1 (Duro et al., 1997), Cor a 1 (Breiteneder et al., 1993), Bet v 1 (Swoboda et al., 1995), Ole e 1 (Gonzalez et al., 2002), group 1 and 5 grass pollen allergens (Au et al., 2002; Chang et al., 1999; Muller et al., 1998; Wurtzen et al., 1999), Mal d 1 (Helsper et al., 2002), Api g 1 (Hoffmann-Sommergruber et al., 2000), group 1 and 2 house dust mite allergens (Smith et al., 2001a,b), Bos d 2 (Rautiainen et al., 2001), and Hev b 7 (Sowka et al., 1999). However, despite its importance as allergen no information is available on naturally occurring isoforms of Art v 1.

Polymorphisms can have important effects on T cell and B cell recognition. For example, the Bet v 1 isoforms Bet v 1d, -g, and -l were found to be potent in T cell stimulation but low in IgE binding capacity (Arquint et al., 1999; Ferreira et al., 1996). Differences in T

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cell reactivity of isoforms have also been reported for other allergens, such as Cor a 1, Phl p 5, and Der p 2. These data should be taken into account for the development of new strategies for allergy diagnosis and therapy. The use of isoforms with low or total absence of IgE binding capacity but retaining T cell responsiveness could represent a safer alternative to conventional specific immunotherapy for allergic diseases. In addition, the standardization of allergenic extracts for diagnostic purposes can be affected by variable isoform proportions in allergenic sources of different origins. In this respect, Lowenstein et al. (1995) showed that individual birch trees from the same region produce various subsets of isoallergens, emphasizing the importance of careful selection of isoforms for diagnosis or therapy.

We have investigated the sequence variability of Art v 1 by screening a mugwort pollen cDNA library. Novel Art v 1 isoforms and domains were identified and subsequently produced in *Escherichia coli*. They were characterized physicochemically and immunochemically. Most importantly, their recognition by a variety of reagents, i.e. polyclonal and monoclonal antibodies as well as IgE antibodies from mugwort-sensitized patients was addressed.

2. Materials and methods

2.1. Patients and sera

Mugwort pollen-allergic patients from Vienna (Austria) were selected on the basis of typical case history, positive *in vivo* skin prick test and *in vitro* IgE detection (CAP system, Phadia AB, Uppsala, Sweden). Patients with IgE values of greater than class 3 and immunoreactivity to the recombinant major mugwort pollen allergen (rArt v 1) were selected. Experiments with blood samples from pollen-allergic patients were approved by the Ethics Committee of the Medical University and General Hospital of Vienna (no. EK497/2005). Informed written consent was obtained from all subjects included in the study.

2.2. Construction and IgE immunoscreening of mugwort pollen cDNA library

Construction and IgE immunoscreening of a mugwort pollen cDNA library was performed as previously described (Himly et al., 2003). Radioactive labeling of Art v 1.0101 (GenBank accession no. AF493943) was done according to the random priming method (Feinberg and Vogelstein, 1983). A ³²P-labeled cDNA probe was used to re-screen the λ ZAP II cDNA library and λ phages containing homologous DNA inserts were isolated using the hybridization technique (Benton and Davis, 1977). Isolated immunopositive phage clones were treated according to the *in vivo* excision protocol for subcloning into pBluescript SK⁺ phagemid (Stratagene, La Jolla, CA). Constructs obtained were sequenced using an ABI PrismTM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.3. Cloning of Art v 1 isoforms and domains

For expression of recombinant hexahistidine fusion proteins in *E. coli*, cDNAs coding for rArt v 1.0101, Art v 1 isoforms, and single domains were cloned into the pHis-Parallel2 vector (Sheffield et al., 1999). Fragments corresponding to the mature sequences were amplified with flanking 5' *Xho*I (*Eco*RI for the proline domain) and 3' *Nco*I restriction sites using the following oligonucleotides: 5'-GAG AGA CCA TGG CTG GTT CAA AGC TGT GTG AAA AG-3', 5'-GAG AGA CTC GAG TTA GTG AGG GGA CGG AGG AGG-3', 5'-GAG AGA CTC GAG TTA GTG AAT GGA CGG AGG AGG-3', 5'-GAG AGA CTC GAG TTA GTG ACC GGA CGG AGG AGG-3', 5'-GAG AGA CCA TGG CTG GTT CAA AGT TGT GTG AAA AG-3', 5'-GAG AGA CTC GAG TTA CGA TTT GGA

ACA GTC AAA GTA-3', 5'-GAG AGA CCA TGG CCT CCT GGA GCA ACA CCA GCG-3', and 5'-GAG AGA CTC GAG TTA GTG AGT GGA CGG AGG AGG-3'. Amplificates were digested and ligated into the respective sites of the pHis-Parallel2 vector.

2.4. Expression and purification of Art v 1 isoforms and domains

Natural Art v 1 (nArt v 1) was purified as previously reported (Himly et al., 2003). *Escherichia coli* BL21CodonPlus(DE3)+RIL (Stratagene, La Jolla, CA) were transformed with Art v 1 isoforms and domain constructs. For lab scale production of recombinant proteins, a modified protocol for expression under osmotic stress conditions was used (Barth et al., 2000). Briefly, pre-cultures were grown overnight at 30 °C in Super Broth medium containing 100 mg/l ampicillin. Cultures were diluted 1:30 in fresh medium and grown to an OD₆₀₀ of 1.6–1.8. Hyperosmotic stress was induced by addition of 0.5 M sorbitol, 4% NaCl, and 10 mM betaine. After another hour of incubation at 30 °C, protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside and the culture was grown overnight at 16 °C. By contrast, Art v 1 domains were expressed for 3 h at 37 °C. Cells were harvested and resuspended in 50 mM sodium phosphate pH 8, 500 mM NaCl, and 10 mM imidazole. Cell lysis was performed by a 45-min incubation with 0.25 mg/ml lysozyme at room temperature (RT) followed by 3 freeze-thaw cycles at –196 and 37 °C, respectively. DNA was digested with 5 µg/ml *DNase*I for 15 min at RT. Cellular debris was pelleted at 20,000 × g and 4 °C. The supernatant containing hexahistidine-tagged recombinant fusion proteins was applied to immobilized metal chelate affinity chromatography using a 5 ml Ni²⁺ charged HiTrapTM Chelating HP column (GE Healthcare, Chalfont St. Giles, UK). All chromatography steps were performed using the ÄKTA prime system (GE Healthcare, Chalfont St. Giles, UK). Bound protein was eluted with a linear gradient to 50 mM sodium phosphate pH 8, 500 mM NaCl, and 400 mM imidazole. Collected fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Recombinant tobacco etch viral (rTEV)-protease (Invitrogen, Carlsbad, CA) cleavage was performed according to the manufacturer's advice. Proteins were dialyzed against 20 mM sodium acetate pH 5 and applied to cation exchange chromatography using a 1 ml HiTrap CM FF column (GE Healthcare, Chalfont St. Giles, UK). Elution of bound protein was performed with a linear gradient to 0.6 M NaCl under the same buffer conditions. Fractions containing pure recombinant protein were dialyzed against dH₂O, lyophilized, and stored at –20 °C. The proline domain was purified under denaturing conditions of 6 M urea which was subsequently removed by stepwise dialysis.

2.5. Physicochemical protein characterization

Protein purity was analyzed by SDS-PAGE and Coomassie Brilliant Blue R250- or Silver-Staining (Invitrogen, Carlsbad, CA). Amino acid compositions and exact protein concentrations of allergens were determined using the Pico Tag method (Waters Corp., Milford, MA). Circular dichroism spectra of the proteins were recorded in 5 mM sodium phosphate pH 7.4 with a JASCO J-810 spectropolarimeter (Jasco, Tokyo, Japan). In addition, mass spectra of proteins were acquired using electrospray ionization coupled to a Quadrupole Time of Flight Mass Spectrometer (Waters, Milford, MA).

2.6. Immunological protein characterization

IgE immunoblotting was performed as described elsewhere (Himly et al., 2003) using sera from 24 mugwort-allergic patients. For IgG and IgM immunoblots, a polyclonal rabbit serum raised

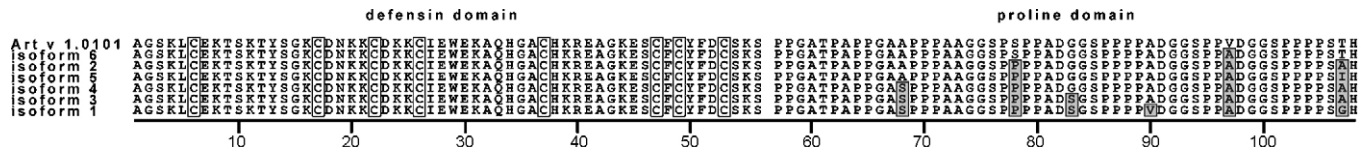


Fig. 1. Sequence alignment of Art v 1 isoforms. Amino acids corresponding to the mature protein sequence differ in one (isoform 6), three (isoforms 2 and 5), four (isoform 4), five (isoform 3) and six (isoform 1) amino acid residues compared to Art v 1.0101 (grey boxes). Amino acid variations are exclusively restricted to the carboxy terminal proline domain. Conserved cysteine residues in the amino terminal defensin domain presumably involved in disulfide bond formation are shown in light grey boxes.

against rArt v 1.0101 (Himly et al., 2003) and a monoclonal anti-Art v 1 antibody produced by genetic immunization (Bauer et al., 2003) were used at a dilution of 1:50 in 25 mM Tris–HCl pH 7.5, 0.5% (w/v) BSA, 150 mM NaCl, 0.5% (v/v) Tween-20, and 0.05% (w/v) Na₂S₂O₃. Bound rabbit IgG and murine IgM were detected by goat anti-rabbit IgG or rabbit anti-mouse IgM alkaline phosphatase (AP) conjugates (Jackson ImmunoResearch Laboratories, West Grove, PA). Detection was performed using nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrates.

Allergens were tested in ELISA using plasma from 11 mugwort-allergic patients as previously published (Wallner et al., 2007b). For cross-inhibition ELISA, five representative sera were pre-incubated with serial dilutions of selected allergens.

In vitro mediator release assays were performed as previously reported (Vogel et al., 2005; Wallner et al., 2007b) using six Art v 1-reactive patients' sera. Briefly, RBL-2H3 cells transfected with human high-affinity IgE receptor (FcεRI) were passively sensitized with serum IgE from mugwort pollen-allergic patients. After washing, degranulation was triggered by addition of serial dilutions of the proteins. β-Hexosaminidase release into the supernatant was measured by enzymatic cleavage of the fluorogenic substrate 4-methyl umbelliferyl-*N*-acetyl-β-D-glucosaminide and expressed as percentage of total enzyme content of Triton X100-treated cells.

3. Results

3.1. Molecular cloning of Art v 1 isoforms and domains

Screening of the λ ZAP II cDNA library using ³²P-labelled Art v 1.0101 cDNA resulted in the identification of 25 Art v 1 clones. Nucleotide sequence alignment showed differences in the 3'-untranslated region as well as in the open reading frames. On the protein level, seven Art v 1 isoforms have been identified differing in 1–6 amino acid residues (Fig. 1). Interestingly, all amino acid variations of the isoforms were restricted to the carboxy terminal proline domain.

3.2. Protein expression and purification

Art v 1 isoforms as well as defensin- and proline domains were expressed as hexahistidine-tagged proteins in *E. coli* BL21CodonPlus(DE3)+RIL. Using recombinant TEV protease, the fusion tag was proteolytically removed yielding 1–2 mg protein per liter bacterial culture.

3.3. Physicochemical protein characterization

Protein preparations were analyzed by SDS-PAGE and silver staining and displayed apparent purity of >98% (Fig. 2). Experimental data obtained by amino acid analysis and mass spectrometry are in good correlation with theoretical amino acid compositions and molecular masses of the proteins. Further, proteolytic cleavage and sequence integrity defined by the absence of amino acid modifications or alterations could be assessed. CD spectroscopy revealed

spectra typically obtained for polyproline helices, which is in accordance with previous findings (Himly et al., 2003), and no differences between the isoforms were found (data not shown).

3.4. Immunological characterization

Polyclonal rabbit and monoclonal mouse antibodies raised against Art v 1 were used to assess the conservation and distribution of epitopes among Art v 1 isoforms and domains. Immunoblot analysis showed no significant differences in rabbit and murine antibody binding to Art v 1 isoforms and the defensin domain. Similar results were obtained using serum IgE from mugwort pollen-allergic patients. By contrast, the proline domain neither showed reactivity with patients' IgE nor with murine and rabbit antibodies (data not shown).

Human IgE binding properties of isoforms and domains were further investigated under non-denaturing conditions by ELISA. As observed in immunoblots, isoforms and defensin domain showed similar IgE-binding capacity, whereas the proline domain displayed no antibody reactivity (Fig. 3).

Two representative isoforms, i.e. clones 1 and 2 differing from Art v 1.0101 by 3 (isoform 2) and the maximum number of 6 (isoform 1) amino acids, were selected for additional experiments to evaluate IgE cross-reactivity and mediator release capacity. In cross-inhibition ELISA, IgE reactivity to coated nArt v 1 could be inhibited to 98% by addition of nArt v 1, and up to 80% by addition of recombinant Art v 1 isoforms (Fig. 4(A)). The proline domain displayed no inhibitory capacity at low-protein concentrations and was only able to inhibit IgE reactivity to 33% at the highest protein concentration used. On the other hand, IgE reactivity to coated rArt v 1.0101 could be totally abolished by nArt v 1, recombinant Art v 1 isoforms, and defensin domain at low concentrations (Fig. 4(B)). The proline domain showed some inhibitory capacity (up to 61%) when recombinant Art v 1.0101 was used as the coating antigen, but only at high concentrations.

The allergenic potential of Art v 1 isoforms and domains was determined in mediator release assays using rat basophil leukemia

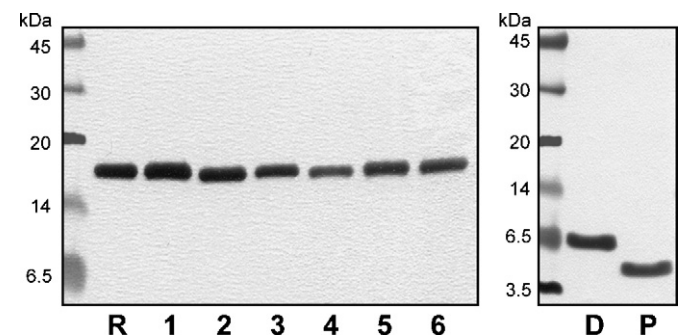


Fig. 2. Purified Art v 1 molecules and domains. Proteins were separated by 17% SDS-PAGE and visualized by silver staining. Notably, rArt v 1.010 (R) and isoforms (denoted 1–6) migrated at an apparent molecular weight of 18 kDa; defensin (D) and proline (P) domains at 6.5 and 5 kDa, respectively.

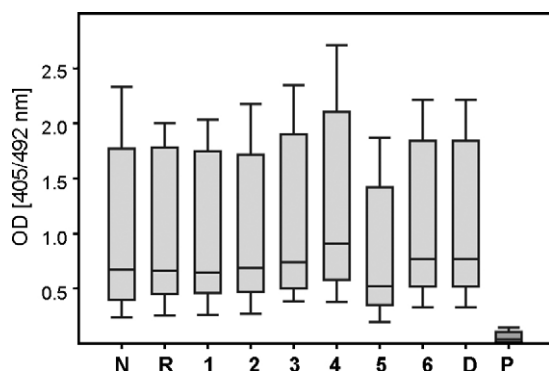


Fig. 3. Immunoreactivity of Art v 1 isoforms and domains. IgE reactivity of nArt v 1 (N), rArt v 1.0101 (R), six isoforms (1–6), defensin (D) and proline (P) domains was assessed in ELISA experiments using sera from 11 mugwort-allergic individuals. Box plots show the median as solid line, 25th and 75th percentiles as horizontal boxes, and whiskers indicate the 5th and 95th percentile. A significant difference in IgE binding capacity was observed for the Art v 1 proline domain ($P < 0.001$).

cells transfected with the high-affinity receptor for human IgE. Similar release curves were observed for Art v 1 isoforms and domains whereas no biological activity was found for the proline domain (Fig. 5).

4. Discussion

Isoforms are usually discovered and described by microarrays, proteomics, and cDNA libraries. In this study we identified six new Art v 1 isoforms displaying 94–99% identity to the published Art v 1.0101-deduced amino acid sequence by screening a mugwort pollen cDNA library. Physicochemical characterization of purified recombinant isoforms did not show striking differences in comparison to the previously reported analysis of the Art v 1.0101 isoform (Himly et al., 2003). The vector system, bacterial strain, and culture conditions used in this study allowed the recombinant production of correctly folded Art v 1 isoforms, as demonstrated by their capacity to bind IgE antibodies. IgE recognition of recombinant Art v 1 was shown to be completely dependent on correct disulfide bond formation stabilizing the defensin domain (Himly et al., 2003).

Sequence analysis showed that all amino acid variations were restricted to the carboxy terminal proline domain of the mature Art v 1 isoforms. However, these amino acid exchanges did not influence binding of Art v 1-specific monoclonal and polyclonal

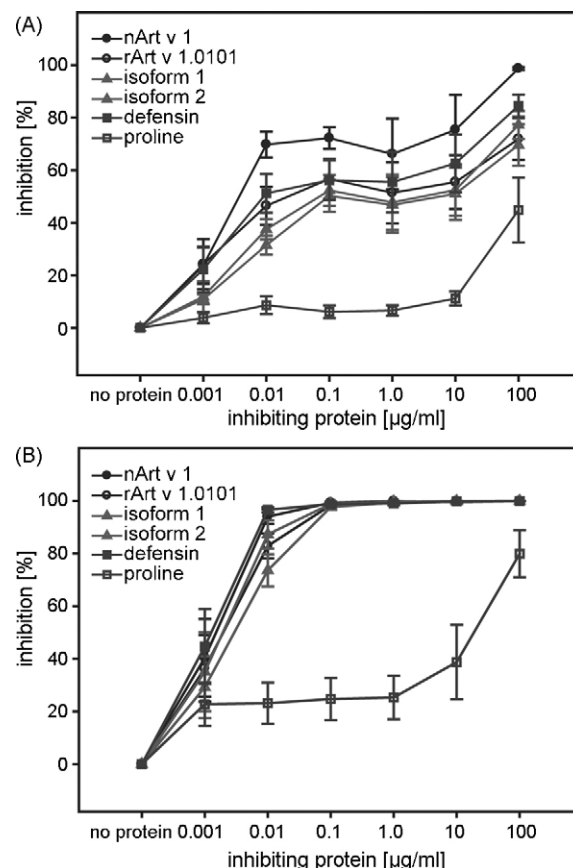


Fig. 4. Inhibitory capacity of Art v 1 isoforms and domains. Cross-inhibition ELISA experiments were performed with nArt v 1 (A) and rArt v 1 (B) as the solid phase, respectively, using sera from mugwort-allergic patients ($n = 5$). Dose-dependent IgE inhibition was determined after serum pre-incubation with nArt v 1, rArt v 1, isoforms 1 and 2, and domains.

antibodies, as well as IgE from mugwort pollen-sensitized patients. By expressing defensin and proline domains individually, we were able to show that the defensin domain contains all relevant B cell epitopes of the Art v 1 polypeptide. Together, these results suggest that the immunoreactivity of Art v 1 isoforms mostly depends on the cysteine-stabilized defensin domain.

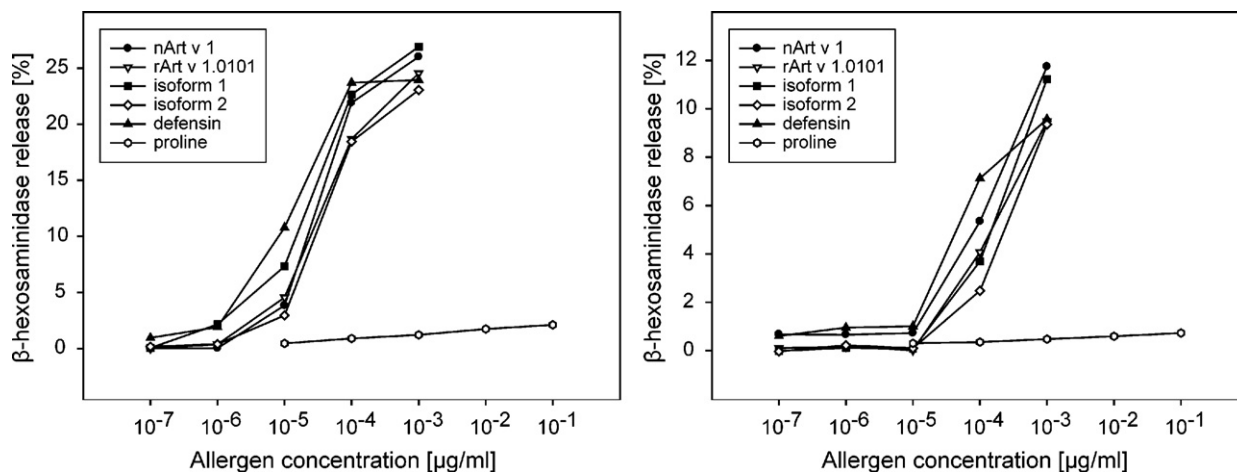


Fig. 5. Biological activity of Art v 1 proteins. Release curves using RBL-2H3 cells for two representative patients are shown. Dose-dependent mediator release was tested using allergen concentrations ranging from 10^{-7} to 10^{-3} and 10^{-5} to 10^{-1} for defensin harboring proteins and proline domain, respectively.

Despite the lack of influence of the sequence variations on antibody recognition, particular amino acid exchanges might affect the glycosylation pattern of Art v 1. We have recently shown that the proline domain carries two types of glycosylations: first, a large hydroxyproline-linked arabinogalactan composed of a short 1,6-galactan core substituted by a variable number (5–28) of α -arabinofuranose residues, which in turn forms branched side chains with 5-, 2,5-, 3,5- and 2,3,5-substituted arabinoses. This new type of plant polysaccharide has been tentatively named type III arabinogalactan. The second type of glycosylation consists of single but adjacent β -arabinofuranoses attached to hydroxyprolines (Leonard et al., 2005). The possibility of isoform-specific glycosylation patterns should be investigated in the future using plant-based expression systems.

The role of post-translational modifications of the proline domain on antibody binding to Art v 1 has been investigated (Leonard et al., 2005). The type III arabinogalactan was no target for patients' IgE, whereas the hydroxyproline-linked β -arabinoses seem to constitute additional epitopes for a small subgroup of patients. However, the clinical relevance of carbohydrate-specific IgE is still a matter of debate (Aalberse et al., 2001; Ebo et al., 2004; Mari, 2002; van der Veen et al., 1997; van Ree, 2002).

The results presented here have direct implications for diagnosis and therapy of mugwort pollen allergy. Presently, routine diagnosis and specific immunotherapy (SIT) of allergic diseases is performed with extracts prepared from the allergenic source. These extracts are difficult to standardize regarding their allergen content: several allergens might be under-represented due to degradation, other non-allergenic components are present and there might be even contamination with allergens from other sources. Systems for recombinant production of allergens and techniques for gene manipulation offer unique tools for the development of novel molecule-based products to be used in diagnosis and SIT, which addresses the problems resulting from the use of extracts. Pure and standardized recombinant allergens or cocktails prepared thereof, containing most of the IgE-binding epitopes of an allergen source, can be formulated and might eventually replace natural extracts. The use of recombinant allergens in diagnosis allows exact identification of the molecules causing the allergic reaction, i.e. a clear association between disease manifestation and the IgE-mediated immune reactions (Ferreira et al., 2006; Mothes et al., 2006; Wallner et al., 2007a). Recombinant Art v 1 isoforms produced in plant-based expression systems might represent suitable tools for mugwort pollen allergy diagnosis encompassing patients reacting to the molecule's carbohydrate moiety. The selection of isoform(s) appropriate for diagnosis, i.e. mimicking the natural epitopes composed of polypeptide and carbohydrate residues, should be possible by using plant-based production systems.

For SIT, the selected recombinant allergens can be modified to reduce the risk of IgE-mediated side effects. Modification of allergens for SIT should aim at the production of molecules with reduced IgE binding epitopes (hypoallergens), while preserving structural motifs necessary for T cell recognition (T cell epitopes) and for induction of IgG antibodies reacting with the natural allergen (blocking antibodies) (Wallner et al., 2007a). T cell recognition of Art v 1 is restricted to a single immunodominant epitope located between amino acids 25–36 (Art v 1_{25–36}) in the defensin domain (Jahn-Schmid et al., 2002; Jahn-Schmid et al., 2005), which is, as we have shown here, conserved in all isoforms. Art v 1_{25–36} is recognized by more than 95% of Art v 1-sensitized patients and 76% ($n=51$) react exclusively with this epitope. Therefore, the highly conserved Art v 1 defensin domain represents a suitable candidate for the generation of hypoallergenic variants lacking relevant B cell epitopes for safer forms of specific immunotherapy.

Acknowledgements

This work was supported by the Joint Research Project S88 (Projects S8802 and S8808) of the Austrian Science Funds "FWF".

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