

Art v 1, the major allergen of mugwort pollen, is a modular glycoprotein with a defensin-like and a hydroxyproline-rich domain

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ABSTRACT

In late summer, pollen grains originating from *Compositae* weeds (e.g., mugwort, ragweed) are a major source of allergens worldwide. Here, we report the isolation of a cDNA clone coding for Art v 1, the major allergen of mugwort pollen. Sequence analysis showed that Art v 1 is a secreted allergen with an N-terminal cysteine-rich domain homologous to plant defensins and a C-terminal proline-rich region containing several (Ser/Ala)(Pro)₂₋₄ repeats. Structural analysis showed that some of the proline residues in the C-terminal domain of Art v 1 are posttranslationally modified by hydroxylation and O-glycosylation. The O-glycans are composed of 3 galactoses and 9–16 arabinoses linked to a hydroxyproline and represent a new type of plant O-glycan. A 3-D structural model of Art v 1 was generated showing a characteristic “head and tail” structure. Evaluation of the antibody binding properties of natural and recombinant Art v 1 produced in *Escherichia coli* revealed the involvement of the defensin fold and posttranslational modifications in the formation of epitopes recognized by IgE antibodies from allergic patients. However, posttranslational modifications did not influence T-cell recognition. Thus, recombinant nonglycosylated Art v 1 is a good starting template for engineering hypoallergenic vaccines for weed-pollen therapy.

Key words: IgE • type I allergy • O-glycan • epitope • T cell

Pollen grains originating from weeds, in particular those belonging to the family *Compositae* or *Asteracea*, are common sources of allergens worldwide. The most important wind-pollinated genera in this family are *Ambrosia* (ragweed), *Artemisia* (mugwort), and *Parthenium* (feverfew). Mugwort is widely spread throughout the European temperate climate zone, North America, and parts of Asia (1) and is one of the main causes of hay fever in late summer and autumn. Among patients suffering from pollinosis, the prevalence of sensitization caused by mugwort pollen is between 10 and 14% (2).

Previous studies using electrophoretic and immunoblotting techniques showed that mugwort pollen contains at least nine different allergenic proteins (3, 4). So far, Art v 2 and Art v 3 are the best characterized allergens from mugwort pollen. The N-terminal amino acid sequence of Art v 2 (3) showed some similarities to a group of pathogenesis-related proteins from *Brassica* (5). Moreover, the N-glycans of Art v 2 purified from pollen were shown to be of the oligomannosidic type and to play no significant role in IgE recognition (6). 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 was determined (7). Poly(l-proline)-affinity chromatography (8) and IgE cross-inhibition experiments (9) demonstrated that the plant pan-allergen profilin is also present in mugwort pollen. However, no information about the complete molecular structure of any of the mugwort pollen allergens has been reported so far.

Presently, it is widely accepted that recombinant allergens are promising tools for diagnosis and therapy of type I allergy. The value of these molecules for diagnosis has been evaluated in detail, and a panel of recombinant allergens is already available for routine diagnosis of certain inhalant allergies, including tree- (birch) and grass-pollen allergies (10, 11) but not for weed-pollen allergies. As a first step for the establishment of a recombinant-based diagnostic and therapy approach for weed-pollen allergies, we are presenting here a detailed characterization of Art v 1, the major mugwort pollen allergen. The cDNA clone coding for Art v 1 was isolated and used to produce the recombinant allergen in *E. coli*. Our results showed that Art v 1 is a two-domain protein with a defensin-like N-terminal part stabilized by disulfide bonds and a C-terminal proline-rich domain carrying a new type of plant O-glycan. In addition, the immunological properties of the purified recombinant protein were compared to those of Art v 1 isolated from mugwort pollen.

MATERIALS AND METHODS

Patients and sera

Approximately 600 patients allergic to mugwort pollen were selected according to typical case history, positive skin prick test, and radioallergosorbent test (RAST) classes >3.0. Sera from these patients were tested in IgE immunoblots with mugwort pollen extract and were used for all experiments described, except for the Art v 1-RAST assays. A serum pool from 13 mugwort pollen-allergic patients displaying IgE antibodies exclusively against Art v 1 was used to monitor the purification steps of natural Art v 1. A serum pool from 14 nonallergic (case history, skin prick test, RAST) healthy donors was used as control. Sera were stored at -20°C.

Construction and IgE immunoscreening of a mugwort pollen cDNA library

Total RNA was isolated from mature mugwort (*Artemisia vulgaris*) pollen (Allergon AB, Ängelholm, Sweden) as previously described (12), with minor modifications. Superscript II reverse transcriptase (Invitrogen, San Diego, CA) was used with 5 µg of poly(A)⁺-mRNA for the first strand synthesis. DNA polymerase I and RNase H were used for the second strand synthesis. The cDNA was methylated with EcoRI methylase, and EcoRI linkers were added. Afterward, the cDNA was digested with XhoI and EcoRI, size-selected on a Sepharose CL-4B column (Amersham Biosciences, Buckinghamshire, UK), and ligated to predigested λ ZAPII arms (Stratagene, La Jolla, CA). The library was amplified once in *E. coli* strain XL-1 blue and

screened with a serum pool from mugwort pollen-allergic patients. Bound IgE was detected using ^{125}I -rabbit anti-human IgE (Amersham Biosciences). cDNA inserts were sequenced according to the dideoxy chain termination method (13).

Expression constructs

An expression plasmid containing the Art v 1 cDNA was constructed in the vector pMW172B (a modified version of pMW172) (14), which is based on the original pET vectors (15). Using PCR, we amplified the Art v 1 cDNA part corresponding to the mature form of the protein and modified it by adding an NdeI site at the 5' end and an MluI site at the 3' end. The following primers were used: Art33-NdeI, 5' GAGAGACCATATGGCTGGTTCAAAGTTGTGTGA 3' (NdeI site is underlined), and Art33-MluI 5' GAGAGAACGCGTTTAGTGAGTGGACGGAGGAGG 3' (MluI site is underlined). The polymerase chain reaction (PCR) products were digested with NdeI and MluI and ligated to the respective sites of the modified pMW172B. The resulting pMW172B/Art v 1 construct was sequenced according to the Dye Terminator Cycle Sequencing protocol (Applied Biosystems, Foster City, CA).

Expression and purification of recombinant Art v 1 (rArt v 1)

For production of rArt v 1, competent *E. coli* strain BL21 DE3 (Stratagene, La Jolla, CA) was transformed with the expression plasmid pMW172B/Art v 1 and selected on LB plates containing 100 mg/l ampicillin. A single transformant colony was picked, and bacteria were grown in a 10-L Bioflow 3000 fermenter (New Brunswick, Edison, NJ) with LB medium containing 100 mg/l ampicillin to an A_{600} of 2.4. Isopropyl- β -D-galactopyranoside (IPTG) was then added to a final concentration of 0.4 mM, and incubation continued for 2.5 h. Bacterial cells were harvested by centrifugation, and pellets were disrupted by two cycles of freezing in liquid nitrogen, followed by thawing at 37°C. After centrifugation at 10,000g for 25 min at 4°C, solid NaCl and NaH_2PO_4 were added to the supernatant to final concentrations of 1 M and 0.5 M, respectively, at 4°C. After centrifugation at 10,000g for 25 min at 4°C, the supernatant was applied to a 2.6×40 cm Phenyl Sepharose CL-4B column (Amersham Biosciences). The column was washed with 0.8 M NaCl, 15 mM NaH_2PO_4 , and 10 mM acetic acid, adjusted to pH 4.5 with Tris-base, and proteins were eluted with a linear gradient to 25 mM Tris-HCl (pH 9.3) containing 8% (v/v) isopropanol. Fractions (10 ml) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and those containing rArt v 1 were pooled. After dialysis against 10 mM sodium acetate (pH 6.5) containing 0.5 mM EDTA and 2 mM β -mercaptoethanol, the protein solution was applied to a 1.6×20 cm SP Sepharose cation exchange column (Amersham Biosciences). The column was washed with 10 mM sodium phosphate (pH 6.8), 0.5 mM EDTA, 2 mM β -mercaptoethanol and eluted with a linear gradient to 0.3 M NaCl under the same buffer conditions. Pooled fractions containing rArt v 1 were concentrated, dialyzed against 1 mM sodium phosphate (pH 6.8), lyophilized, and stored at -20°C.

Protein extracts

Aqueous protein extracts of mugwort (*Artemisia vulgaris*) pollen (Allergon AB) were prepared by resuspending the grains in distilled water (0.1 g/ml) and shaking for 10 h at 4°C. Extracts

were centrifuged twice at 10,000g for 25 min at 4°C and filtered through 0.45-µm filters (Millipore, Bedford, MA).

Purification of natural Art v 1 (nArt v 1)

nArt v 1 was purified from aqueous extracts of mugwort pollen by cation exchange chromatography, using a CM Sepharose CL-6B (Amersham Biosciences) column (1.5×20 cm) equilibrated with 20 mM sodium phosphate buffer (pH 6.8). After it was washed, bound protein was eluted with a linear gradient to 0.3 M NaCl under the same buffer conditions. Fractions (3 ml) showing absorbance at 280 nm were analyzed by SDS-PAGE, and those containing nArt v 1 were pooled and dialyzed against phosphate-buffered saline (PBS). After concentration to ~5 ml, the protein solution was separated by size-exclusion chromatography on a 2.5×120 cm Sephacryl S-100 HR column (Amersham Biosciences). UV absorbance was monitored at 280 nm. Fractions containing nArt v 1 were pooled and dialyzed against distilled water. Aliquots were dried under vacuum and stored at -20°C.

SDS-PAGE and immunoblots

Crude pollen extracts, bacterial lysates, and purified allergens were analyzed by SDS-PAGE according to the method of Laemmli (16), using 15% acrylamide gels. Proteins were visualized by staining with Coomassie brilliant blue R-250. For IgE immunoblot analysis, proteins were separated by 15% acrylamide gels and electroblotted (17) onto nitrocellulose Protran membranes (Schleicher & Schuell, Dassel, Germany). Sera from allergic patients were diluted 1:10 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, and 0.05% (w/v) sodium azide (blocking buffer). Bound IgE was detected using ¹²⁵I-rabbit anti-human IgE (Pharmacia Diagnostics, Uppsala, Sweden). Autoradiography was performed at -70°C with intensifying screens. Alternatively, blots were exposed for 2–12 h to an imaging plate and analyzed using a Bas-1800 II scanner (Fujifilm, Tokyo, Japan).

N-terminal sequence analysis

Mugwort pollen proteins were separated by 15% SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore). Bands corresponding to Art v 1 were excised, and proteins were eluted by incubation in aqueous 40% (v/v) acetonitrile and 30% (v/v) trifluoroacetic acid for 1 h at room temperature. Samples were vacuum dried, resuspended in water, and sequenced with the HP G1005A protein sequencing system (Agilent Technologies, Palo Alto, CA).

Amino acid analysis

Amino acid composition of nArt v 1 and rArt v 1 was determined using the Pico Tag method (Waters, Milford, MA). Phenylthiocarbamyl amino acid derivatives were analyzed by reverse phase high-performance liquid chromatography (RP-HPLC) (HP 1100, Agilent Technologies, Palo Alto, CA), using a 3.9×3000 mm Nova-Pak C18 column (Waters).

Reduction and alkylation of nArt v 1 and rArt v 1

Samples of 20 nmol nArt v 1 and rArt v 1 (corresponding to 160 nmol of cysteines for each sample) were dried under vacuum and dissolved in 150 μ l 0.3 M Tris-HCl (pH 8.0), 8 M guanidinium-HCl, 1.3 M EDTA. DTT was added in a 50-fold molar excess (7.5 μ mol), and samples were incubated for 4 h at 50°C. Afterward, 15 μ mol iodoacetamide were added and alkylation was performed for 1 h at 37°C. The reaction was stopped by adding 7.5 μ mol DTT. Samples were then extensively dialyzed against distilled water and vacuum dried.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry

For determining the intact molecular mass of nArt v 1, we applied 0.5 μ l (~2.5 μ g) of purified protein and 0.5 μ l of 20 mg/ml gentisic acid in water (matrix) to the target slide, with intermittent drying in an airstream. rArt v 1 was measured in the presence of 100 mM DTT with 20 mg/ml gentisic acid in 0.1% (v/v) trifluoroacetic acid as a matrix. Samples were analyzed with the Kompact MALDI-TOF IV mass spectrometer (Shimadzu, Kyoto, Japan) in the linear flight mode. For calibrating the instrument, molecular peaks of myoglobin and gentisic acid were used.

Sugar analysis

Samples of mugwort pollen extract and purified nArt v 1 and rArt v 1 proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Sugar detection was then performed using periodic acid Schiff (PAS)-staining. For a more detailed analysis, nArt v 1 samples were hydrolyzed with 4 M trifluoroacetic acid at 100°C for either 1 or 4 h and dried under vacuum. Samples were then derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) and analyzed by RP-HPLC as described previously (18). To discriminate between arabinose and xylose, we also performed RP-HPLC at pH 6.9 (19). In addition, monosaccharides were analyzed as alditol acetates by gas chromatography with a mass spectrometry detector (GC/MS) as previously described (20). Alkaline degradation of ~1 mg of nArt v 1 was performed by incubation with 0.1 ml of 0.44 M Ba(OH)₂ for 6 h at 100°C (21). The sample was subsequently neutralized by an excess volume of 1 M acetic acid and subjected to gel filtration using a 1×50 cm Sephadex G50 superfine column (Amersham Biosciences) equilibrated with 0.16 M acetic acid. Fractions were analyzed for carbohydrate content according to the method of Winzler (22). Positive fractions were pooled, concentrated under vacuum, and subjected to MALDI-TOF mass spectrometry, using a Dynamo mass spectrometer (Thermo Electron, Waltham, MA) in the delayed extraction mode and 2,5-dihydroxybenzoic acid as a matrix (23). The instrument was externally calibrated against partially hydrolyzed dextran. For reference, similar experiments were performed with potato lectin (Sigma-Aldrich, St. Louis, MO).

Animal immunizations and antibody purification

New Zealand white rabbits were separately immunized with 50 μ g of either purified nArt v 1 or rArt v 1. As an adjuvant, Montanide ISA 50 (Seppic, Paris, France) was used. Rabbits were boosted at 4-wk intervals. Immune reactivity was monitored by radioimmunoassay with Sepharose-coupled purified nArt v 1 and rArt v 1, respectively. To this end, 100 μ g Art v 1 was coupled to 100 mg CNBr-activated Sepharose 4B (Amersham Biosciences). Serum dilutions

were incubated overnight with 0.5 mg Sepharose-coupled Art v 1 in a final volume of 300 μ l PBS/0.3% bovine serum albumin (BSA)/0.1% Tween 20. After washing five times with PBS/0.1% Tween, we added radiolabeled sheep antibodies against rabbit IgG (CLB, Amsterdam, The Netherlands). After overnight incubation and four washings, we measured bound radioactivity on a γ -counter. One week after the second and third boost, rabbits were plasmapheresed three times a week. Plasma was recalcified to allow clotting, and all six serum samples were pooled. After the fourth boost, rabbits were bled, and the obtained serum was added to the pool. Art v 1-specific antibodies were affinity purified from immune sera by using rArt v 1 coupled to Affigel beads (Bio-Rad, Hercules, CA) as stationary phase. Purified antibodies were then used for immunoblotting experiments.

ELISA cross-inhibition experiments

Nunc-Immuno MaxiSorp microtiter plates (Nunc, Roskilde, Denmark) were coated with 0.5 μ g of nArt v 1 and rArt v 1, respectively, for 6 h at room temperature. Remaining binding sites were saturated by incubation with 110 μ l of PBS, 1% (w/v) human serum albumin, and 0.05% Tween 20 (buffer A) overnight at 4°C. The plates were then incubated with a serum pool (diluted 1:4 in buffer A) of nine patients reactive with both nArt v 1 and rArt v 1, as determined by IgE immunoblots. The serum pool was preincubated overnight at 4°C with 20.0, 10.0, 5.0, 2.5, 1.25, 0.6, 0.3, and 0 μ g/ml of nArt v 1, rArt v 1, or reduced-alkylated rArt v 1 preparations. After washing, the plates were incubated with 1:1000 diluted mouse anti-human IgE (Pharmacia Diagnostics, Uppsala, Sweden) overnight at 4°C and with horseradish peroxidase-conjugated sheep anti-mouse Ig (Amersham Biosciences) diluted 1:2000 for 1 h at 37°C and 1 h at 4°C. After incubation with the substrate 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) for 30 min, the reaction was stopped with 0.32% NaF, and absorption was measured at 405 nm by using a Dynatech MR 600 ELISA reader (Dynex Technologies, Chantilly, VA). Adequate controls were performed for each incubation step of the ELISA, and normal human serum assured the specificity of the IgE detection system.

RAST

From sera submitted for routine allergy diagnostics, a panel of 55 was selected based on positive RAST to *Artemisia vulgaris* extract. These sera were tested in a RAST with Sepharose-coupled nArt v 1 and rArt v 1. RAST was performed as described previously (24). In brief, sera (50 μ l) were incubated overnight with 0.5 mg of Sepharose-coupled allergen in a final volume of 300 μ l PBS, 0.3% BSA, 0.1% Tween-20 (PBS-AT). After removing unbound serum components, we added radiolabeled sheep antibodies (CLB) raised against human IgE (in 500 μ l PBS-AT plus 4.5% bovine and 0.5% sheep serum (v/v): PBS-AT+BS) (~20,000 cpm/test). After overnight incubation and washing, we measured bound radioactivity. Results were expressed as international units IgE 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 house dust mite allergen Der p 2 and Sepharose-coupled mite extract (25). A result of ≥ 0.3 IU/ml was regarded as positive. Negative results were given a value of 0.1 IU/ml (1 IU = 2.4 ng IgE).

Allergen-specific T-cell responses

For primary in vitro responses, peripheral blood mononuclear cells (PBMCs) were obtained from patients allergic to mugwort pollen. PBMCs (10^5) were cultured in triplicate and stimulated with 20, 10, 5, and 2.5 $\mu\text{g/ml}$ Art v 1 for 6 days. T-cell proliferation was then assessed by incorporation of [^3H -TdR] as described previously (26). Allergen-specific T-cell lines (TCLs) were established as previously described (26). In brief, 1.5×10^6 PBMCs were stimulated with optimal concentrations of nArt v 1 and rArt v 1 as initial stimulus. After 5 days, suboptimal doses of human rIL-2 (10 U/ml, Boehringer Mannheim, Mannheim, Germany) were added, and cultures were continued for 7 days. The proliferative responses of these TCLs to either nArt v 1 or rArt v1 (5×10^4 T cells in the presence of 1×10^5 autologous irradiated PBMCs with or without 2.5 $\mu\text{g/ml}$ allergen) were tested using [^3H -TdR] incorporation. Stimulation indices (ratio of cpm obtained in cultures stimulated with and without allergen) were determined. Correlation was calculated using Pearson statistics.

3-D structural modeling

Sequence similarity search was performed using PSI-Blast version 2.1 (27) vs. domain-based sequence database of proteins with known 3-D structure. The ProFIT program (Proceryon BioSciences GmbH, Salzburg, Austria) was used for fold recognition calculation (28). The 3-D structural models were generated by the threading procedure, which aligns the query sequence with the template structure according to pair and surface mean potentials. The structural models were evaluated according to their Z-scores. Data analysis and interpretation were performed using ProHIT package facilities. The electrostatic surface potential of N-terminal domain of Art v 1 was calculated with the program MOLMOL (29). The partial charges on all atoms were incorporated. We used a dielectric constant of 80 for the solvent and 2 for the molecule and an ionic strength of 0.01 M for the solvent. Multiple sequence alignments were generated with ClustalX.

RESULTS

Cloning and sequence analysis of Art v 1

IgE immunoblots of aqueous extracts of mugwort pollen, using sera from ~600 mugwort pollen-allergic patients revealed that >95% of the patients tested reacted with a double-band protein migrating with an apparent molecular mass of 24–28 kDa. The double-band protein was purified to homogeneity from aqueous mugwort pollen extract by a combination of cation exchange and size exclusion chromatography. The purified protein was subjected to Edman degradation, and both bands yielded identical N-terminal sequences. To obtain more information about this allergen, a serum pool from mugwort pollen-allergic patients was used for immunoscreening of a mugwort pollen cDNA library. Forty-two IgE-positive plaques were isolated out of 900,000 plaques, and their inserts were sequenced. Computer analysis, using a method developed by Nielsen et al. (30), predicted that the derived protein sequence of one cDNA clone (Av33, open reading frame of 399 bp) contained an N-terminal hydrophobic signal sequence typical for a protein destined to be secreted through the endoplasmic reticulum and the Golgi apparatus. The sequence predicted by this computer algorithm after the start of the mature form of the protein was identical to the 15 amino acids determined by N-terminal sequence analysis of the 24- to 28-

kDa double-band allergen. We designated the protein encoded by clone Av33 as Art v 1, in accordance with the International Union of the Immunological Societies allergen nomenclature system (31). Previously, an allergenic protein of 47 kDa was isolated from mugwort pollen and designated Art v 1 (4). Because no sequence information became available, this 47-kDa protein is no longer designated Art v 1, according to the IUIS allergen nomenclature subcommittee. The deduced amino acid sequence of Art v 1 is shown in [Figure 1A](#).

When mature Art v 1 was compared with sequences in the database, similarities were found to anther-specific protein SF18 (54% identity) from sunflower (*Helianthus annuus*) (32) and to plant defensins. In addition, the allergen Par h 1 from feverfew (*Parthenium hysterophorus*) pollen (33), a *Compositae* weed growing in India, the southern United States, and western Australia, also showed sequence similarities to Art v 1. However, the complete sequence of Par h 1 is not available. Similar to the protein SF18 from sunflower and Par h 1 from feverfew, Art v 1 was found to consist of two domains: 1) a cysteine-rich N-terminus (amino acids 1–55), which shows high sequence similarities to antifungal proteins belonging to the plant defensin family (34, 35), and 2) a C-terminal (amino acids 56–108) proline-rich domain containing several (Ser/Ala)(Pro)₂₋₄ extensin-like repeats (36). However, the Art v 1 C-terminal domain does not show a resemblance to extensins and extensin-like proteins except for the high proline content and the occurrence of two Ser(Pro)₄ repeats. Similar to extensins, the determined amino acid composition of natural Art v 1 (data not shown) indicated that it is rich in Hyp/Pro (14.8%/19.4%). However, the hydroxyproline-rich domain of Art v 1 has no Tyr and Lys, compared with 14.4% and 12.2%, respectively, in tobacco extensin. Tyrosine residues in extensins are closely associated with the Ser(Pro)₄ motifs and are proposed to form isodityrosine linkages via intramolecular crosslinking (36), rendering them highly insoluble in the cell wall matrix.

Structure analysis of nArt v 1 and rArt v 1

The region of the cDNA corresponding to mature Art v 1 was expressed in *E. coli* as a nonfusion protein. The recombinant protein was purified to apparent homogeneity ([Fig. 2A](#)), with a total yield of ~6 mg rArt v 1/l bacterial culture. The integrity of the primary structure of rArt v 1 was determined by MALDI-TOF mass spectrometry ([Fig. 3A](#)). The molecular mass of the intact protein was measured as 10,802±8 Da, which is in good agreement with the theoretical molecular weight of 10,800 based on the cDNA sequence. However, MALDI-TOF mass spectrometry analysis ([Fig. 3B](#)) of purified nArt v 1 ([Fig. 2A](#)) revealed that the protein is quite heterogeneous in size. Two major series of peaks with molecular weights ranging from 12,916 to 13,451 and from 14,053 to 16,313, respectively, were detected corresponding to the double-band pattern observed after SDS-PAGE of nArt v 1. This significant discrepancy between the mass spectra pattern and the molecular masses of rArt v 1 and nArt v 1 suggest the protein might be posttranslationally modified. Therefore, we performed experiments for sugar detection on purified nArt v 1. PAS staining ([Fig. 2B](#)) and DIG glycan/protein double labeling showed that the preparation consisted of glycoprotein. In addition, amino acid analysis of purified nArt v 1 showed that proline residues are modified with the result that in the mature protein 76% of these residues are present as hydroxyproline (data not shown). Thus, the mass difference between natural and recombinant protein produced in *E. coli* can be explained by extensive posttranslational modification of nArt v 1.

To find out more about the posttranslational modifications of Art v 1, we performed a detailed glycan analysis. Using a series of plant lectins with distinct sugar specificities, no binding to nArt v 1 was observed (data not shown), indicating that neither the usual N-glycans nor O-glycans of the mammalian mucin type were present. Monosaccharide analysis by GC/MS and RP-HPLC showed that nArt v 1 contained galactose and arabinose in a ratio of 1:5.5 (data not shown). After alkaline degradation of nArt v 1, using Ba(OH)₂, and fractionation by gel filtration, the resulting glycopeptide fractions were pooled and subjected to monosaccharide and amino acid analyses. Again, galactose and arabinose were detected in a ratio of 1:5.5. In addition, the glycopeptide fractions contained hydroxyproline (data not shown). In contrast to carbohydrate chains linked to serine or threonine, a hydroxyproline-linked glycan is stable to alkaline degradation and can be isolated as Hyp-glycan (21). Therefore, we concluded that the Art v 1 glycans were attached to hydroxyproline residues in the C-terminal domain of the protein.

Interestingly, the Art v 1 Hyp-glycans eluted much earlier from the gel filtration column than alkaline degradation products derived from potato lectin, indicating a much higher apparent mass and possibly differences in their structures. MALDI-TOF mass spectrometry of Art v 1 Hyp-glycans showed a very broad distribution of peaks with molecular masses ranging from ~1,400 to 3,800 ([Fig. 3C](#)). The mass peaks essentially comprised a series of compounds, each differing by 132.1 mass units, which corresponds to one arabinosyl residue. The most intense peaks, with molecular masses ranging from 1,566 to 3,149, were consistent with arabinogalactosyl chains composed of 3 galactoses and 7–19 arabinoses linked to a hydroxyproline residue ([Table 1](#)). By adding one or two arabinogalactosyl chains to the Art v 1 polypeptide (10,800 Da) plus 256 mass units for hydroxylation of 16 proline residues, the resulting proteins would have theoretical molecular masses between 12,174 and 18,372. From the 24 mass signals obtained with nArt v 1, 15 could be easily matched with calculated molecular weights of Art v 1 proteins with one or two arabinogalactosyl chains ([Table 2](#)), with 0.001–0.08% accuracy. These assignments corresponded to the major series of peaks with molecular masses ranging from 14,053 to 16,313. Thus, the heterogeneous pattern of nArt v 1 can be at least partially explained by the polypeptide sequence presented here, which is posttranslationally modified with one or two arabinogalactosyl O-glycans composed of 3 galactoses plus 9–16 or 21–23 arabinose residues (see [Fig. 3](#) and [Table 2](#)). However, the mass signals ranging from 12,916 to 13,451 could not be exactly matched by any of the calculated masses taking the sequence presented here with 16 hydroxyproline residues. One possible explanation for the failure in the assignment of all mass signals could be that Art v 1 exists in nature as a mixture of isoforms, as described for several other pollen allergens. In this way, sequence variations could give rise to a group of molecules with differences in the molecular masses of the polypeptide backbone and of posttranslational modifications that would exactly correspond to the nine nonassigned mass signals obtained with nArt v 1.

Antibody binding properties of nArt v 1 and rArt v 1 proteins

The IgE-binding properties of purified nArt v 1 and rArt v 1 were initially evaluated in vitro by using the immunoblot technique and sera from 150 mugwort pollen-allergic patients (data not shown). Approximately 50% of the patients reacting with nArt v 1 also recognized rArt v 1 produced in *E. coli*. However, a significant portion of the Art v 1-allergic patients showed very weak or no IgE reactivity with rArt v 1, suggesting that posttranslational modifications might be

important in the formation of epitopes recognized by IgE antibodies. [Figure 4](#) shows the IgE-binding patterns of 13 mugwort pollen-allergic patients tested with mugwort pollen extract ([Fig. 4A](#)), and purified nArt v 1 ([Fig. 4B](#)) and rArt v 1 ([Fig. 4C](#)) under reducing and nonreducing SDS-PAGE conditions. These sera were from the same patients that we obtained PBMCs for establishing Art v 1-specific TCLs. Under nonreducing conditions, rArt v 1 showed a distinct pattern suggestive of multimerization. No major differences were observed in the IgE recognition of nArt v 1 under reducing or nonreducing conditions.

To evaluate the antibody binding activity of Art v 1 proteins under nondenaturing conditions, we performed RAST and ELISA inhibition experiments. Using sera from 55 mugwort pollen-sensitized patients, we found that 90% reacted with nArt v 1, whereas only 49% recognized rArt v 1. Among the nArt v 1-positive sera, two subgroups were identified ([Fig. 5A](#)), one with similar IgE recognition of natural and recombinant (14/49, 29%) and another with significantly lower or no reactivity to rArt v 1 (35/49, 71%).

The ability of rArt v 1 to inhibit binding of IgE to nArt v 1 was determined by ELISA, using a serum pool of patients reactive in immunoblots with both rArt v 1 and nArt v 1. As can be seen in [Figure 5C](#), IgE reactivity of rArt v 1 was totally abolished by preincubation of the serum pool with nArt v 1 and rArt v 1 proteins. However, pre-incubation of sera with rArt v 1 caused only a partial inhibition of IgE binding to nArt v 1 ([Fig. 5B](#)). At the highest concentration of rArt v 1 used (20 µg/ml), only 30% inhibition of IgE reactivity to nArt v 1 was observed. No inhibition of binding to either nArt v 1 or rArt v 1 was observed upon preincubation with reduced and alkylated rArt v 1 ([Fig. 5B](#), [5C](#)).

A rabbit serum raised against purified nArt v 1 strongly reacted with rArt v 1 ([Fig. 2C](#)). Similarly, a rabbit serum raised against purified rArt v 1 also recognized nArt v 1 ([Fig. 2D](#)).

Allergen-specific T-cell responses

The T-cell proliferation in response to nArt v 1 and rArt v 1 was assessed in PBMCs and in allergen-specific TCLs derived from mugwort pollen-allergic individuals. In PBMCs, comparable primary *in vitro* responses were induced with nArt v 1 or rArt v 1 ([Fig. 5D](#)). This was indicated by similar stimulation indices (mean $\pm$ SD: 2.62 ± 1.16 and 2.36 ± 0.92 , respectively; $n=20$) and a correlation of 0.836 ($P<0.001$). Allergen-specific TCLs were established with nArt v 1 or rArt v 1 as the initial stimulus. Subsequently, secondary *in vitro* responses of these TCLs ($n=12$ each) to either nArt v 1 or rArt v 1 were tested. Both antigens induced comparable proliferative responses in the TCLs ([Fig. 5E](#)) obtained with nArt v 1 (3.64 ± 1.45 and 4.24 ± 2.26) with a correlation of 0.61 ($P=0.037$) or rArt v 1 (2.99 ± 1.65 and 4.17 ± 3.61) with a correlation of 0.75 ($P=0.005$).

Structural modeling

Sequence similarity searches revealed significant homology of the N-terminal region of Art v 1 to plant defensins. Plant defensins adopt a compact globular fold known as cysteine-stabilized α/β -motif. This is a common motif characteristic for some scorpion neurotoxins as well as plant and insect defensins. The fold comprises an α -helix packed on a three-stranded β -sheet and is stabilized via four disulfide bridges (34, 35). All eight cysteine residues involved in disulfide

bridge formation appear to be strictly conserved among all plant defensins as well as in the Art v 1 sequence. The antifungal protein 1 from *Aesculus hippocastanum* (Ah-AMP1), a plant defensin with known fold (37), shares significant (62%) sequence similarity to the N-terminal domain of Art v 1, and it was used as a template for model generation of the N-terminal domain of Art v 1 ([Fig. 1B](#)). The N-terminal domain consists of an α -helix ranging from Lys21 to Glu28 and a triple-stranded β -sheet (β 1 from Cys6 to Thr9, β 2 from His34 to Glu41, and β 3 from Lys44 to Asp52).

The electrostatic surface potential of Art v 1 and of five other defensins with different biological activities was calculated with the program MOLMOL. Each protein is presented in two orientations obtained by a rotation of 180° around the vertical axis ([Fig. 1C](#)). Comparison of the surface charge distribution of the Art v 1 defensin domain and other defensins shows striking differences. Scorpion (1agt) and insect (1ica) defensins have positive charges located all over the surface, with a single negatively charged patch. In contrast, the scorpion defensin with sodium channel blocking activity (1snb) shows a predominance of negative patches. In both Art v 1 and horse chestnut AMP1 defensin (1bk8), the positive charges are alternated with negative charges and evenly distributed over the molecule. However, the electrostatic surface potential of the N-terminal domain of Art v 1 definitely has a more basic nature than that of AMP1. Interestingly, the side chains of positively charged residues of the Art v 1 defensin domain form multiple protrusions distributed all over the molecule, a feature not observed for other defensins.

In general, the Art v 1 C-terminal domain can be described as a Pro-rich domain ([Fig. 1A](#)). The main characteristic is the presence of several consecutive repeats of the (Ser/Ala)(Pro)₂₋₄ and (Ala/Asp)(Gly)₂ types ([Fig. 1A](#)). The C-terminal region of Art v 1 was also used for fold similarity searches. Using the ProFIT program to calculate fold recognition, we predicted that the C-terminal part would adopt an extended left-helical structure similar to that of collagen. Finally, the N- and C-terminal domains of Art v 1 were connected, and the final model was evaluated. The complete 3-D model of Art v 1 resembles a characteristic "head and tail" structure ([Fig. 1D](#)).

DISCUSSION

The Art v 1 molecule

Here, we have described Art v 1, the major allergenic protein from mugwort pollen. Mature Art v 1 has 108 amino acids, a calculated molecular mass of 10,800.02, and a theoretical pI of 8.17. The deduced amino acid sequence indicates that this protein consists of two modules: a 55-amino acid-long N-terminal defensin domain and a 53-amino acid-long C-terminal proline-rich domain. Defensins are widely distributed cysteine-rich antimicrobial peptides, which differ in length, number of cysteine residues, disulfide bridges, and folding pattern. For example, insect (34–43 amino acids) and mammalian (29–34 amino acids) defensins have three intramolecular disulfide bridges, whereas plant defensins (45–55 amino acids) have four (34). Defensins possess antimicrobial activity against fungi and/or bacteria and can be classified into five groups according to their biological activity and similarity in the amino acid sequence. For example, Group I proteins (e.g., pea, radish, horse chestnut defensins) have antifungal activity and group II proteins (e.g., wheat defensin) do not (38-41). Furthermore, groups III and IV (e.g., scorpion

neurotoxins) represent sodium and potassium channel blockers (42, 43), whereas proteins belonging to group V (e.g., flesh fly defensin) have antibacterial activity (44).

Despite differences in their biological activity, plant defensins share the same cysteine-stabilized $\alpha\beta$ -motif. This motif was first recognized by Kobayashi et al. (45) in the structures of a series of small neurotoxins isolated from scorpion and honeybee venom, which featured an α -helical fragment connected by two disulfide bonds to a β -strand. Later, the term “cysteine-stabilized $\alpha\beta$ -motif” was proposed for this particular fold (44). Amino acid sequence comparison of 14 representative members of the plant defensin family showed that residues conserved in all sequences are restricted to the eight cysteines, two glycines at positions 15 and 35, an aromatic residue at position 13, and a glutamic acid at position 30 (numbering relative to Art v 1) (34). These consensus residues are also conserved in the defensin domain of the Art v 1 sequence ([Fig. 1A](#)). Therefore, we undertook the molecular modeling of the Art v 1 defensin domain to gain more insight on the molecular structure and possible biologic activity of this allergen. We used as a template the 3-D structure of the horse chestnut defensin Ah-AMP1, which was determined by 2-D ^1H nuclear magnetic resonance (37).

Analyses of the electrostatic surface potential of three-dimensionally similar defensins revealed a similar pattern of charge distribution among sodium channel and potassium channel inhibitors (38). Although no correlation between surface charge distribution and the presence of antifungal activity could be established among plant defensins (38), the defensin domain of Art v 1 and the antifungal protein Ah-AMP1 have similar surface charge distributions. In addition, amino acid residues Thr12, Ser14, Phe51, Trp29, and Phe48 as well as Lys39, Arg40, His/Lys44, and Gln/Lys8 (numbering relative to Art v 1), predicted to be important for the potent antifungal activity and specificity of Ah-AMP1 (37), are also conserved in the Art v 1 molecule. Thus, the overall features of the defensin domain suggest that the Art v 1 molecule might have antifungal activity. However, for a definitive classification of Art v 1 in the group I defensin, assays to verify the antifungal activity should be performed.

The C-terminal proline-rich domain was predicted to adopt an extended left-helical structure resembling that of collagen. Thus, the complete Art v 1 model exhibits a compact globular “head” and an extended “tail.” This extended type of structure predicted for the proline-rich domain is suited to accommodate large sugar chains and fits to our findings showing that Art v 1 contains O-glycans of ~3,500–5,200 Da attached to hydroxyproline residues. Two types of plant O-glycoproteins have been described so far. The first group includes the hydroxyproline-rich glycoproteins, or extensins (e.g., potato lectin), which contain serine and hydroxyproline-rich regions. In this case, two types of O-glycans are known, one composed of a single galactose linked to serine residues and the other of small arabinosyl chains directly linked to hydroxyproline residues and composed of two to three arabinoses with $\alpha 1,2$ - and $\alpha 1,3$ -furanosidic linkages (46). The second group is the arabinogalactan proteins, in which the O-glycans are long galactan chains probably linked to hydroxyproline and with a few galactose residues substituted by arabinoses (47). The gel filtration elution patterns of alkaline-digested Art v 1, mass spectrometry measurements, and monosaccharide analysis indicated that the Art v 1-derived glycans represent a new class of plant O-glycans. This new type of O-arabinogalactosyl chain is composed of 3 galactoses and 9–16 arabinoses linked to a hydroxyproline residue.

Recombinant Art v 1 for diagnostic and therapy of weed-pollen allergy

The IgE-binding properties of nArt v 1 and rArt v 1 were evaluated in immunoblots, RAST, and ELISA inhibition experiments. Two subgroups of patients were identified: one with similar IgE recognition of natural and recombinant proteins and another with significantly lower or no reactivity to rArt v 1. In ELISA inhibition, rArt v 1 caused only a partial inhibition of IgE binding to nArt v 1. The lower IgE binding activity of rArt v 1 produced in *E. coli* could be due to incorrect folding of the protein. However, this possibility seems less likely because in RAST experiments several patients showed similar IgE binding to both natural and recombinant proteins. Furthermore, no differences in the circular dichroism spectra of both protein preparations were observed (data not shown). Another possible explanation for the decreased IgE binding of rArt v 1 is that posttranslational modifications might be directly or indirectly involved in the formation of the epitopes recognized by some allergic patients. No inhibition of binding to either nArt v 1 or rArt v 1 was observed upon preincubation with reduced and alkylated rArt v 1, indicating that the cysteine-stabilized defensin domain also plays a role in IgE recognition of the allergen.

In contrast, T-cell proliferation in response to nArt v 1 and rArt v 1 assessed in PBMCs and in allergen-specific TCLs derived from mugwort pollen-allergic patients indicated that posttranslational modifications are not relevant for T-cell recognition. In PBMCs, comparable primary *in vitro* responses were induced with nArt v 1 and rArt v 1. Allergen-specific TCLs established with either nArt v 1 or rArt v 1 as initial stimulus showed comparable proliferative responses after stimulation with both antigens.

Taken together, our results clearly demonstrate that although rArt v 1 produced in *E. coli* lacks all posttranslational modifications shown to be present in nArt v 1, both protein preparations share common epitopes recognized by human IgE and T lymphocytes as well as by rabbit IgG antibodies. In principle, rArt v 1 produced in *E. coli* cannot be considered a suitable reagent for diagnosis of weed-pollen allergies because the sera of only ~30–50% of Art v 1-allergic patients recognize this molecule. Plant-based expression systems might be the choice for the production of glycosylated Art v 1 for diagnostic purposes. However, its IgE and T-cell activation properties make recombinant nonglycosylated Art v 1 a good starting template for engineering hypoallergenic variants of this major allergen to be used in safer forms of specific immunotherapy. During immunotherapy, increasing doses of natural allergen preparations are given on a regular basis, and for the patient, the beneficial effects depend on the amount of allergen used (48). Because treatment involves injecting allergens into a sensitized organism, the occurrence of typical allergic symptoms is frequent, with risks increasing with the concentration of allergen applied per injection. These problems could be overcome with the use of genetically engineered allergens (49–51) with reduced IgE binding epitopes (hypoallergens), but preserved structural motifs necessary for T-cell recognition (T-cell epitopes) and for the induction of IgG antibodies reactive with the natural allergen (blocking antibodies). The uptake of allergens by antigen-presenting cells (APCs) is mediated and facilitated by the interaction of the allergen with specific IgE (52, 53) and leads to higher Th2 cytokines and IgE production (54). Engineered allergens lacking IgE binding might avoid these pathways and preferentially target APCs that utilize phagocytosis or pinocytosis for antigen uptake (e.g., monocytes, macrophages, and dendritic cells). This in turn induces a balanced Th0- or Th1-like cytokine production by T cells as well as low IgE and high IgG production by B cells. The presence of intact T-cell epitopes on

hypoallergens would also target T cells, allowing administration of higher doses to induce tolerance of allergen-specific T cells and alteration of cytokine production toward a Th1-like pattern. Based on the results presented here, hypoallergenic forms of Art v 1 for specific immunotherapy could be engineered by targeting the disulfide bonds that stabilize the defensin domain and by production of the nonglycosylated mutant protein in *E. coli*.

Finally, plant defensins are being considered as valuable weapons against pests (34, 35). A recent publication showed effective fungal control in potato by expression of a plant defensin peptide (55). Our results for the first time associate the plant defensin fold with the induction of allergic responses in humans and therefore raise concerns for the use of defensins in genetically modified crops.

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Table 1**Theoretical and experimental masses of hydroxyproline-glycans of nArt v 1**

nArt v 1 Glycopeptides					Arabinogalactosyl	
Composition (No. of residues)			[M + Na] ⁺ m/z			
Hyp	Gal	Ara	Calculated	Experimental	Type	[M + H] ⁺ m/z
1	2	6	1271.1	1271.1	1	1136.0
1	3	5	1301.1	1301.5	2	1166.0
1	2	7	1403.2	1403.7	3	1268.1
1	3	6	1433.2	1433.5	4	1298.1
1	2	8	1535.3	1535.9	5	1400.2
1	3	7	1565.4	1566.1	6	1430.3
1	2	9	1667.5	1667.3	7	1532.4
1	3	8	1697.5	1697.7	8	1562.4
1	2	10	1799.6	1799.7	9	1664.5
1	3	9	1829.6	1829.5	10	1694.5
1	2	11	1931.7	1932.3	11	1796.6
1	3	10	1961.7	1961.5	12	1826.6
1	2	12	2063.8	2063.7	13	1928.7
1	3	11	2093.8	2093.7	14	1958.7
1	3	12	2225.9	2225.5	15	2090.8
1	3	13	2358.1	2357.6	16	2223.0
1	3	14	2490.2	2489.4	17	2355.1
1	3	15	2622.3	2621.7	18	2487.2
1	3	16	2754.4	2753.6	19	2619.3
1	3	17	2886.5	2885.6	20	2751.4
1	3	18	3018.6	3017.5	21	2883.4
1	3	19	3150.8	3149.0	22	3015.7
1	3	20	3282.9	3281.0	23	3154.8
1	3	21	3415.0	3413.3	24	3279.9
1	3	22	3547.1	3545.3	25	3412.0
1	3	23	3679.2	3677.9	26	3544.1
1	3	24	3811.3	3809.3	27	3676.2

Table 2**Theoretical and experimental masses of nArt v 1**

Arabinogalactosyl		nArt v 1 [M + H] ⁺ (m/z)		
No. chains	Type ^a	Calculated	Measured	Δmass
1 chain	10	12,734		
	12	12,866		
	14	12,998		
	15	13,130		
	16	13,262		
	17	13,394		
	22	14,055	14,053	− 2
	23	14,187	14,178	− 9
	24	14,319	14,304	− 15
	25	14,451	14,446	− 5
	26	14,583	14,578	− 5
2 identical chains	10	14,450	14,446	4
	12	14,714	14,714	0
	14	14,978	14,978	0
	15	15,243	15,249	+ 6
	16	15,507	15,510	+ 3
	17	15,771	15,784	+ 13
	18	16,035	16,042	+ 7
	19	16,300	16,313	+ 13
2 different chains	12 + 14	14,847	14,846	− 1
	12 + 15	14,979	14,978	− 1
	12 + 16; 14 + 15	15,111	15,113	+ 2
	12 + 17; 14 + 16	15,243	15,249	+ 6
	12 + 18	15,375	15,384	+ 9
	14 + 17	15,375	15,384	+ 9
	12 + 19; 14 + 18	15,507	15,510	+ 3
	14 + 19; 15 + 18; 16 + 17	15,639	15,649	+ 10
	15 + 19; 16 + 18	15,771	15,784	+ 13
	16 + 19; 17 + 18	15,903	15,913	+ 10
	17 + 19	16,035	16,042	+ 7
	18 + 19	16,168	16,178	+ 10

^aThe numbers refer to the arabinogalactosyl chains given in **Table 1**.

Fig. 1

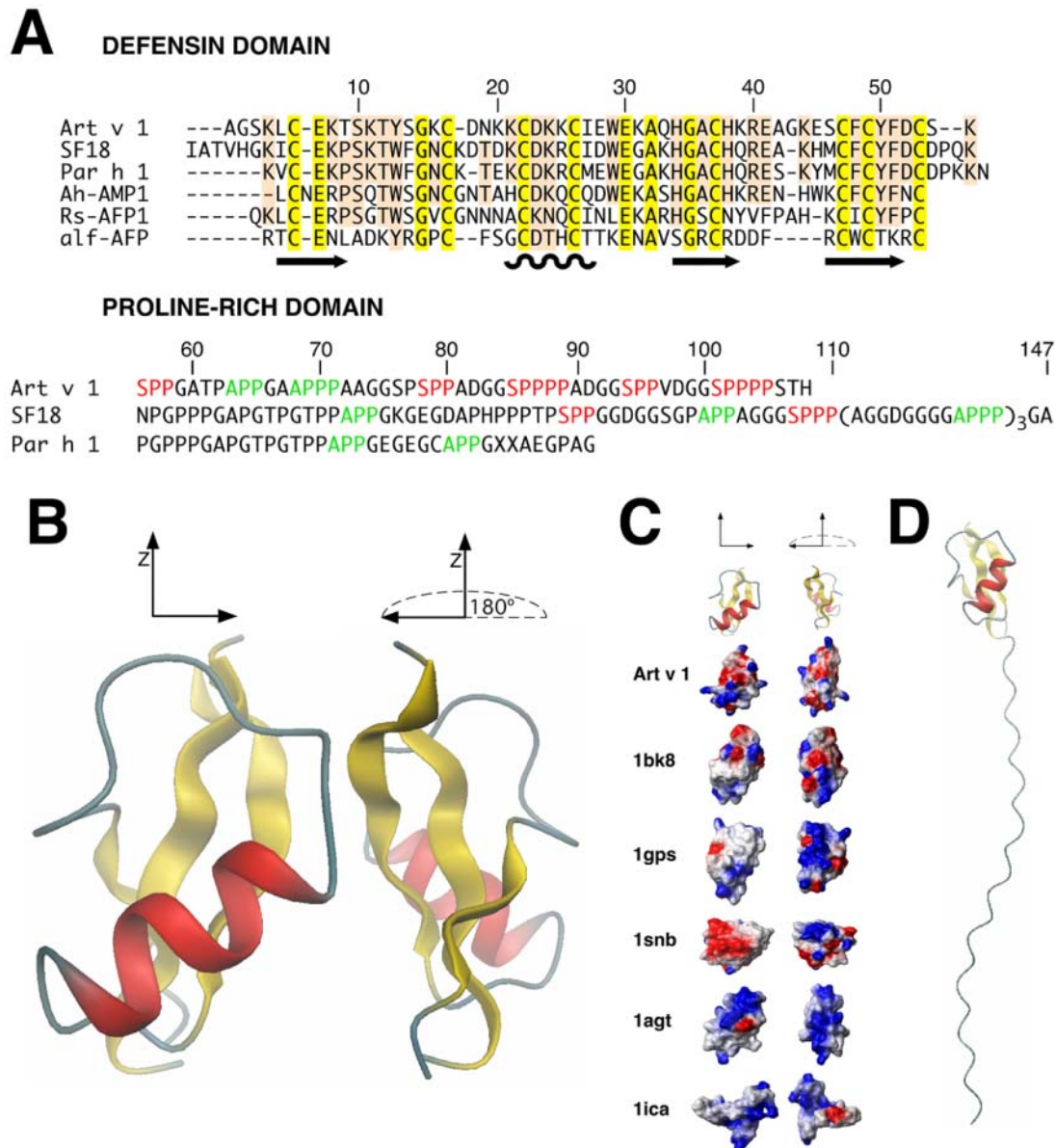


Figure 1. Sequence alignment and 3-D structural modeling of Art v 1. **A)** Amino acid sequence comparison of the two domains of Art v 1 (GenBank accession no. AF493943), with plant defensins and proteins containing defensin and proline-rich domains. The following sequences were obtained from databases (accession numbers given in parentheses), except for Par h 1: SF18 (X53375), anther-specific cDNA from sunflower (*Helianthus annuus*); Par h 1 (partial amino acid sequence; see ref 33), pollen allergen from feverfew (*Parthenium hysterophorus*); Ah-AMP1 (PDB 1bk8) defensin, antimicrobial protein-1 from horse chestnut (*Aesculus hippocastanum*); Rs-AFP1 (P30225), antifungal protein-1 from radish (*Raphanus sativus*) seeds; and alf-AFP defensin (AF319468), antifungal peptide from alfalfa (*Medicago sativa*) seeds. Arrows and wave indicate the location in the defensin domain of β -strands and one α -helix, respectively. **B)** Calculated model of the defensin domain of Art v 1, using the Ah-AMP1 (PDB 1bk8) defensin as a template. Two views representing a rotation of 180° around the vertical (z) axis showing the ribbon representation of secondary structure elements with β -strands in yellow and the α -helix in red. **C)** Electrostatic surface potential of Art v 1 and defensins (identified by their PDB code) with different biologic activities: antifungal activity (1bk8), without antifungal activity (1gps), sodium channel blocker (1snb), potassium channel blocker (1agt), and antibacterial activity (1ica). Electrostatic potential is indicated in red (negative charge), white (neutral), and blue (positive charge) and was calculated using the same parameters for all proteins with the program MOLMOL. Each protein is represented by two surface plots, corresponding to a rotation of 180° around the vertical (z) axis. **D)** A ribbon representation of the complete Art v 1 model showing β -strands in yellow and the α -helix in red.

Fig. 2

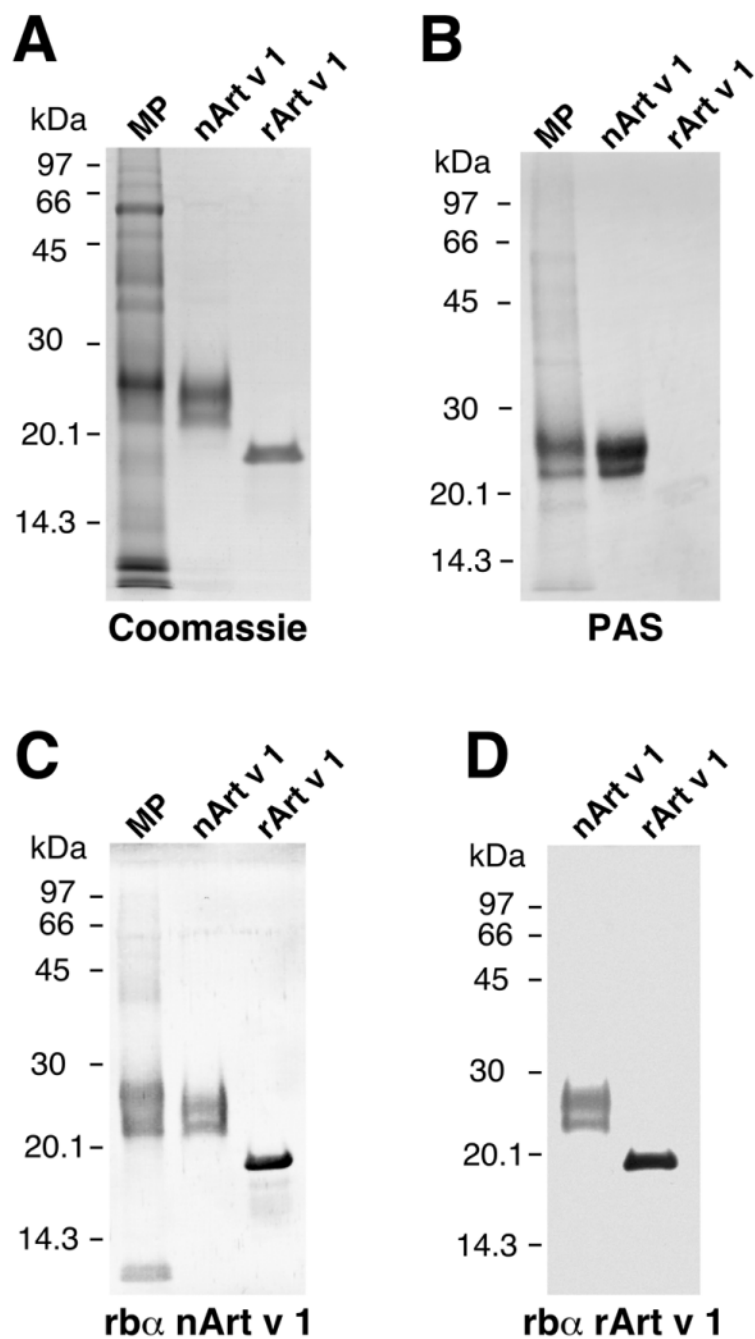


Figure 2. Characteristics of purified natural and rArt v 1 produced in *Escherichia coli*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of mugwort pollen extract (MP lanes), purified nArt v 1, and rArt v 1 followed by Coomassie staining (**A**), periodic acid Schiff staining (**B**), and immunoblotting using rabbit anti-nArt v 1 (**C**) or rabbit anti-rArt v 1 antibodies (**D**).

Fig. 3

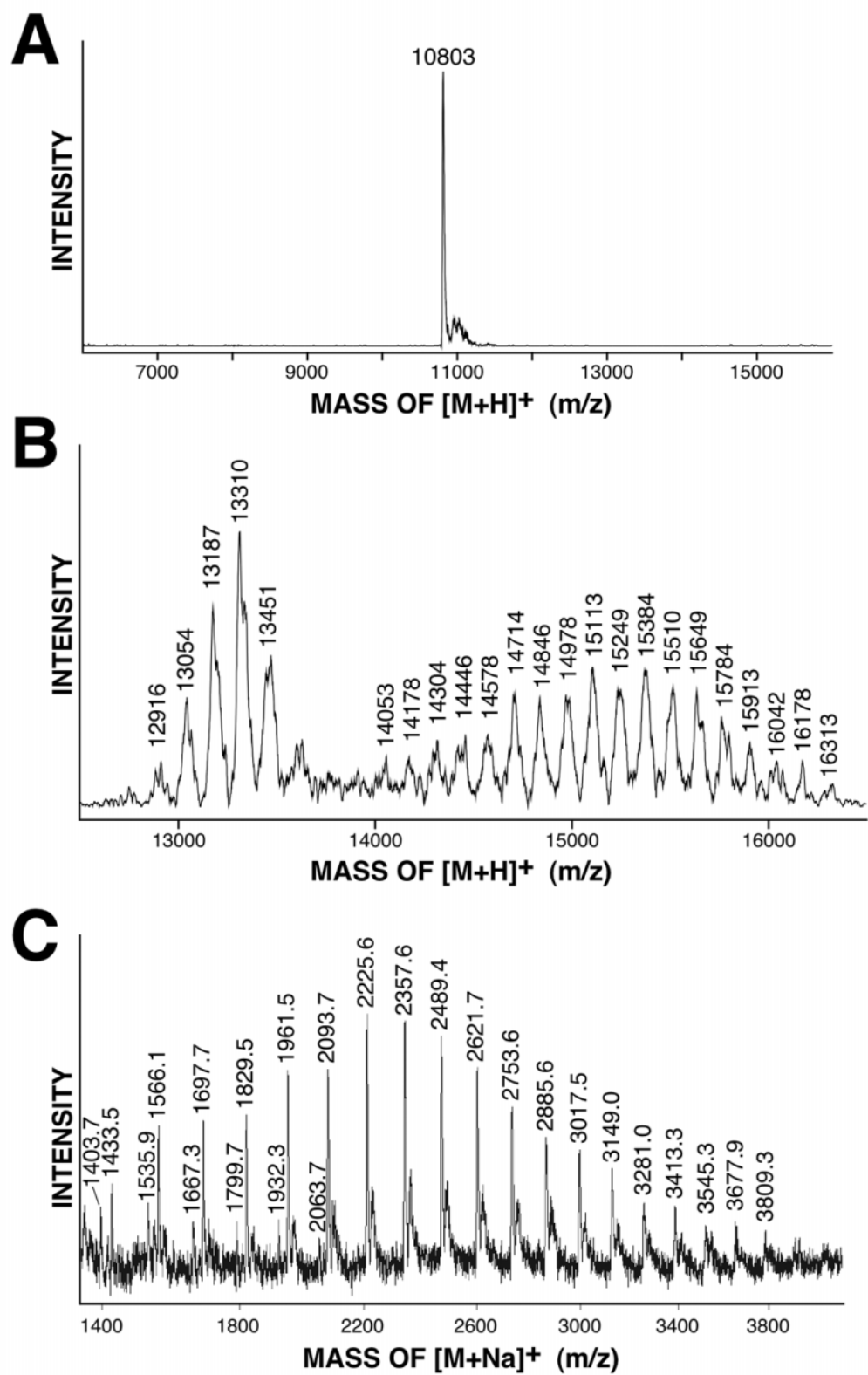


Figure 3. Mass spectrometry analyses of Art v 1 glycans. MALDI-TOF mass spectrometry analysis of purified rArt v 1 produced in *Escherichia coli* (A), purified nArt v 1 (B), and Hyp-glycans (C) obtained after alkaline degradation of Art v 1 and fractionation by gel filtration.

Fig. 4

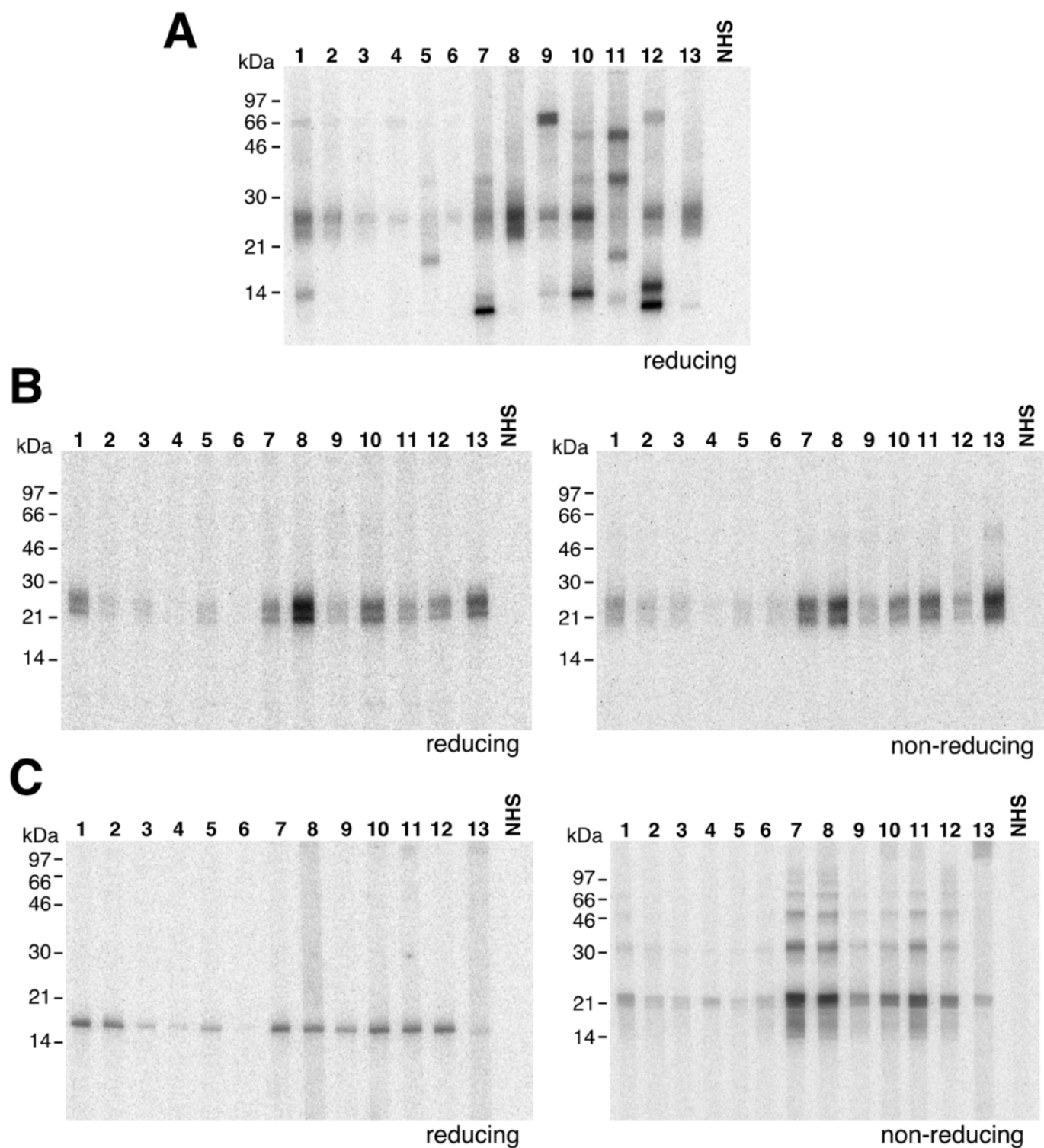


Figure 4. IgE binding of natural and rArt v 1. Sera from 13 mugwort pollen-allergic patients (lanes 1–13) were tested in immunoblots for reactivity with mugwort pollen extract (**A**), purified nArt v 1 (**B**), and purified rArt v 1 (**C**) after SDS-PAGE under reducing and nonreducing conditions. NHS, pool of normal human sera.

Fig. 5

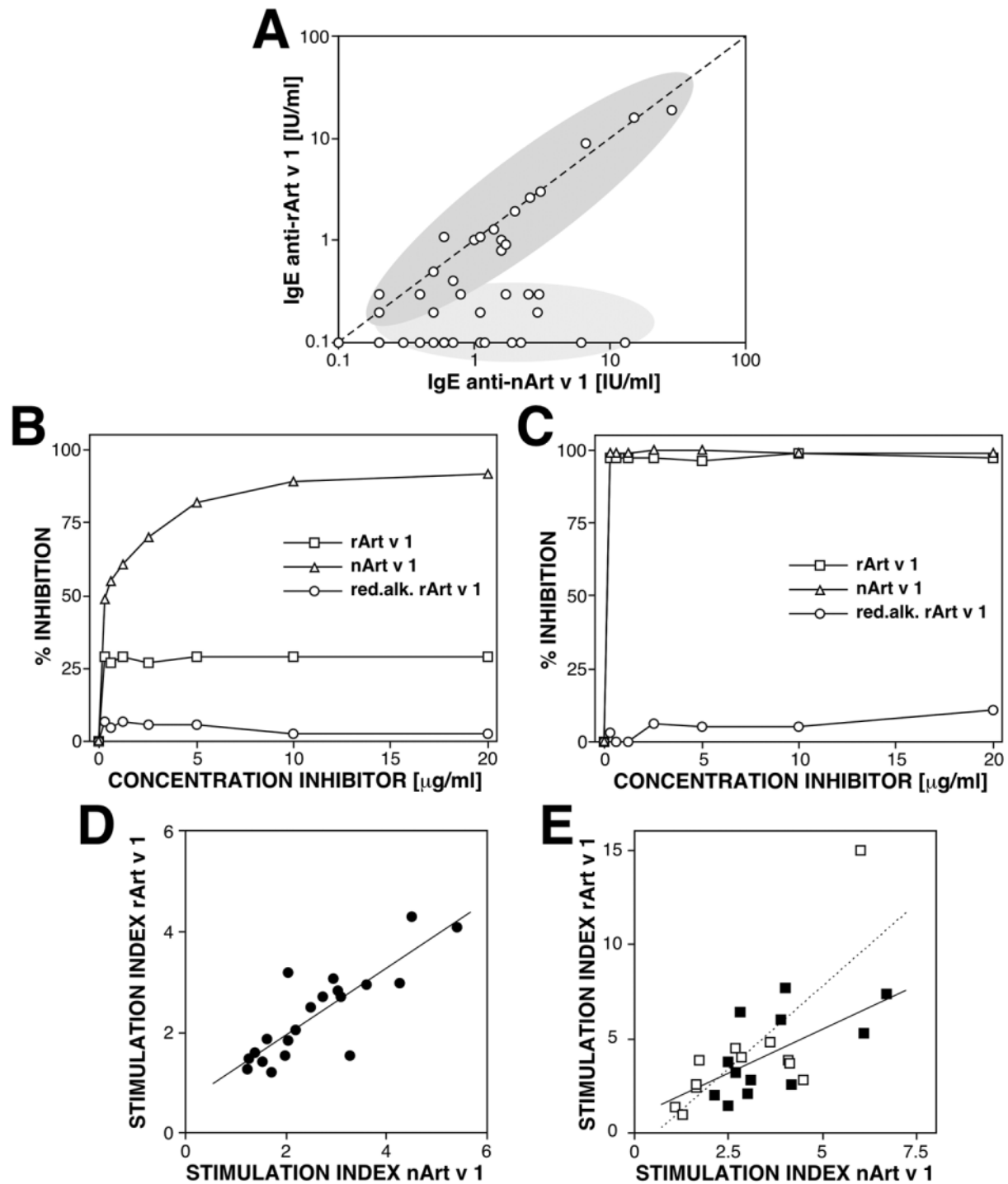


Figure 5. Human IgE and T-lymphocyte recognition of Art v 1. **A)** Comparison of IgE reactivity in radioallergosorbent test experiments, using purified nArt v 1 and rArt v 1 coupled to Sepharose and sera from mugwort pollen-sensitized patients. **B** and **C)** ELISA inhibition experiments, using a serum pool obtained from mugwort pollen-allergic patients. IgE binding to immobilized nArt v 1 (**B**) and rArt v 1 (**C**) after preabsorption of the serum pool, with increasing concentrations of rArt v 1, nArt v 1, and reduced-alkylated rArt v 1. **D** and **E)** Comparison of T-cell responses to nArt v 1 and rArt v 1. Peripheral blood mononuclear cells ($n=20$) (**D**) or allergen-induced T-cell lines (TCL) ($n=13$) (**E**) obtained from mugwort pollen-allergic patients were stimulated with nArt v 1 (x-axis) or rArt v 1 (y-axis). Stimulation indices are shown. Filled squares denote TCL obtained with nArt v 1; open squares, TCL obtained with rArt v 1. IgE immunoblots with sera from these patients are shown in **Figure 4**.