

Structural Analysis of the Glycoprotein Allergen *Art v* II from the Pollen of Mugwort (*Artemisia vulgaris* L.)*

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The glycoprotein allergen *Art v* II, from the pollen of mugwort (*Artemisia vulgaris* L.) was treated with peptide:N-glycosidase F (PNGase F) to release asparagine-linked oligosaccharides. The oligosaccharides were isolated by gel permeation chromatography and their structures determined by 500-MHz ¹H NMR spectroscopy, fast atom bombardment-mass spectrometry, and high-pH anion-exchange chromatography. The high-mannose oligosaccharides Man₅GlcNAc₂, Man₆GlcNAc₂, Man₇GlcNAc₂, Man₈GlcNAc₂, and Man₉GlcNAc₂ were present in the ratios 2:49:19:24:6 and accounted for all the asparagine-linked oligosaccharides released from *Art v* II by PNGase F. The NH₂-terminal amino acid sequences of *Art v* II and of four peptides generated by cyanogen bromide (CNBr) cleavage of deglycosylated *Art v* II were determined. The first 30 amino acid residues of *Art v* II did not contain any potential N-glycosylation sites. One potential N-glycosylation site was identified in one of the CNBr fragments. The native protein conformation was shown by enzyme-linked immunosorbent assay inhibition assays to be essential for the binding of rabbit IgG to *Art v* II and for the binding of human IgE to the major IgE-binding epitope(s) in this allergen. At least one minor IgE-binding epitope still bound IgE after denaturation of the allergen. Removal of the high-mannose chains from denatured *Art v* II had no significant effect on the binding of human IgE to the minor IgE-binding epitope(s).

Pollen of mugwort (*Artemisia vulgaris* L.) is one of the main causes of allergic reactions in late summer in Europe (1). It has been demonstrated that extracts of mugwort pollen contain 8–11 different allergens of variable clinical importance (2–4). No data about the molecular structure of any of the allergens from mugwort pollen have been reported to date.

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Two mugwort pollen allergens,¹ *Art v* I and *Art v* II, have recently been isolated (5, 6). These purified allergens were found to react with IgE from 70 and 45%, respectively, of sera from mugwort allergic patients (5, 6). *Art v* II (previously denoted Ag7) has been identified as a concanavalin A-binding glycoprotein (7) that consists of at least 6 isoforms with pI values between 4.10 and 4.65 (6). The *M_r* of *Art v* II is approximately 35,000 and the allergen appears to be composed of two identical, or nearly identical, subunits linked together by disulfide bridge(s) (6). The isolated *Art v* II preparation had a protein to carbohydrate ratio of 10:1. Glycosyl composition analysis of *Art v* II revealed the presence of mannose, N-acetylglucosamine, glucose, and galactose in the ratios 13.4:3.2:1.3:1.0 (6). The glycosyl composition and the high affinity for concanavalin A suggest that at least part of the carbohydrate structure of *Art v* II consists of Asn-linked oligosaccharides of the high-mannose type.

In this study, the complete structural analysis of the Asn-linked oligosaccharide chains and partial amino acid sequence data of the glycoprotein allergen *Art v* II are reported. This is the first time that the complete structures of the Asn-linked oligosaccharides of a glycoprotein allergen from a plant have been determined. The IgE- and IgG-binding activity of *Art v* II after denaturation and denaturation followed by deglycosylation is also reported.

EXPERIMENTAL PROCEDURES

Materials—*Art v* II was purified from an aqueous phosphate-NaCl buffer extract of mugwort (*A. vulgaris* L.) pollen by a combination of preparative isoelectric focusing in Ultrodex granulated gel (pH 3.5–5), affinity chromatography on concanavalin A-Sepharose and HPLC² size exclusion on Ultropac TSK G2000SW as previously described (6). A human serum pool containing specific IgE against mugwort pollen allergens including *Art v* II (Mugwort serum pool) was prepared from equal volumes of sera from 64 individuals with positive reactions against mugwort pollen extract (RAST-class 3) in the radioallergosorbent test (RAST). A rabbit antiserum against *Art v* II (anti-*Art v* II) was prepared by immunization with immunoprecipitates of *Art v* II obtained in crossed immunoelectrophoresis of

¹ The nomenclature of the allergens referred to in this presentation follows the allergen nomenclature system recommended by the International Union of Immunological Societies (IUIS) ((1986) *Bull. WHO* 64, 767–770).

² The abbreviations used are: HPLC, high performance liquid chromatography; PNGase F, peptide:N-glycosidase F; Endo H, endo-β-N-acetylglucosaminidase H; WEFT, water-elimination Fourier transform; HPAE, high-pH anion exchange; PAD, pulsed amperometric detector; FAB-MS, fast atom bombardment-mass spectrometry; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

mugwort pollen extract, essentially as described by Harboe and Closs (8). ^{125}I -Anti-IgE (PhadebasRAST™) was obtained from Pharmacia LKB Biotechnology Inc. Rabbit anti-human IgE (ϵ -chain-specific) was obtained from Dakopatts (Glostrup, Denmark, Lot 036). Horseradish peroxidase-conjugated goat anti-rabbit IgG (γ -chain-specific) was obtained from Boehringer Mannheim (Lot 22024) or Cappel (Lot 019). PNGase F (N-glycanase™) and Endo H were obtained from Genzyme (Boston, MA). $\text{Man}_5\text{GlcNAc}_2$ and $\text{Man}_9\text{GlcNAc}_2$ oligosaccharide standards were obtained from Dionex (Sunnyvale, CA).

Endo H Treatment—A solution of Art v II (25 μg) in 0.1% SDS (12.5 μl) containing 20 mM 2-mercaptoethanol was heated for 4 min at 100 °C. A portion (10 μl) of this solution was mixed with 50 mM sodium citrate, pH 5.5 (24 μl), and treated with Endo H (20 milliunits in 10 μl) for 17 h at 37 °C. A control sample was prepared the same way without the addition of Endo H.

PNGase F Treatment—A solution of Art v II (1.9 mg) in 0.2% SDS (750 μl) containing 100 mM 2-mercaptoethanol was heated for 3 min at 100 °C. The solution was diluted with 100 mM NH_4HCO_3 (1.5 ml) and 100 mM EDTA (300 μl), and PNGase F (78 units in 310 μl of 50% glycerol) was added. The mixture was incubated for 13 h at 37 °C. A control sample (denatured Art v II) was prepared without the addition of PNGase F.

Gel Permeation Chromatography on Bio-Gel P-4—Oligosaccharides released by treatment of Art v II with PNGase F were purified by gel permeation chromatography on a column (1.5 $\times$ 47 cm) of Bio-Gel P-4 (minus 400 mesh) equilibrated and eluted with 0.3 M NH_4HCO_3 at a flow rate of 2.5 ml/h. Fractions (0.9 ml) were collected and assayed for protein by absorbance at 280 nm. The anthrone assay was used to assay the fractions for carbohydrate by absorbance at 620 nm (9) and for glycerol by absorbance at 520 nm. The (partially) deglycosylated Art v II eluted in the void volume and was recovered by lyophilization.

Gel Permeation Chromatography on Bio-Gel P-10—The (partially) deglycosylated Art v II was applied to a column (1.5 $\times$ 46 cm) of Bio-Gel P-10 (200–400 mesh) equilibrated and eluted with 0.3 M NH_4HCO_3 at a flow rate of 5 ml/h. Fractions (0.9 ml) were collected and assayed for protein and carbohydrate.

500 MHz ^1H NMR Spectroscopy—Oligosaccharides released from Art v II by PNGase F and purified by chromatography on Bio-Gel P-4 were analyzed by ^1H NMR spectroscopy at 500 MHz. The oligosaccharide sample (about 150 μg) was repeatedly dissolved in $^2\text{H}_2\text{O}$ (Aldrich; 99.98 atom % ^2H) at room temperature and pH 5.5, with intermediate lyophilization. Prior to ^1H NMR spectroscopic analysis, the sample was redissolved in 0.5 ml of $^2\text{H}_2\text{O}$ (Cambridge Isotope Laboratories, 99.996 atom % ^2H) and transferred into a 5-mm NMR tube (Wilmad; 535-PP). 500 MHz ^1H NMR spectroscopy was performed on a Bruker AM-500 spectrometer interfaced with an Aspect-3000 computer. The residual HO^2H resonance was suppressed with a WFT pulse sequence that included a composite nonselective π -pulse (10). The probe temperature was kept at 27 °C. Further experimental details have been described (11, 12). Chemical shifts (δ) are expressed in parts per million downfield from internal sodium 4,4-dimethyl-4-silapentane 1-sulfonate, measured by reference to internal acetone (δ 2.225 in $^2\text{H}_2\text{O}$ at 27 °C) or free acetate (δ 1.908 in $^2\text{H}_2\text{O}$ at pH 6–8 and 27 °C) with an accuracy of 0.002 ppm.

High-pH Anion-exchange HPLC (HPAE-PAD)—Oligosaccharides released from Art v II by PNGase F treatment and purified by chromatography on Bio-Gel P-4 were analyzed by HPAE chromatography using a nonmetallic HPLC system (Dionex BioLC, Dionex Corp., Sunnyvale, CA) equipped with a polymeric pellicular anion exchange column (Carbopac PA-1, 0.4 $\times$ 25 cm) and a pulsed amperometric detector (PAD II Dionex). The column was equilibrated and eluted with 100 mM NaOH containing 37.5 mM sodium acetate at a flow rate of 1 ml/min. Carbohydrate was detected by pulsed amperometry, using detector potentials of -0.05 , $+0.6$, and -0.6 volts with a duration of 480, 180, and 60 ms, respectively. Detection was facilitated by the post-column addition of 0.3 M NaOH.

FAB-MS—Oligosaccharides released from Art v II were per-O-acetylated as described by Dell and Tiller (13) and analyzed by FAB-MS on a VG ZAB-SE mass spectrometer. FAB was performed with 6 keV xenon atoms (10 mA emission current). Samples were dissolved in methanol and mixed with thio glycerol and sodium acetate on the probe tip of the mass spectrometer. Data were acquired over a mass range of 1000–3400 amu.

Glycosyl Composition Analysis—The glycosyl residue composition of Art v II was determined by gas-liquid chromatography after methanolysis (1 M HCl in anhydrous methanol, 80 °C, 16 h), re-N-acetylation (pyridine:acetic anhydride:anhydrous methanol (1:1:5),

20 °C, 16 h) and trimethylsilylation (TRI-Sil (Pierce Chemical Co.), 80 °C, 20 min) of the methyl glycosides (14, 15). Gas-liquid chromatography was performed on a DB-1 fused silica capillary column (30 m $\times$ 0.25 mm inner diameter) with He as carrier gas using the following temperature program: 140–220 °C at 2.0 °C/min, 220–260 °C at 10.0 °C/min, and 260 °C for 5 min. The chromatograph (HP 5880A) was fitted with a flame ionization detector and a split injector (split ratio 1:50).

SDS-PAGE—SDS-PAGE of Art v II before and after deglycosylation with Endo H and PNGase F was performed using a Pharmacia PhastSystem (Pharmacia, Uppsala, Sweden) with PhastGel Homogenous 20 (7.5 %T, 3 %C in the stacking gel, 20 %T, 2 %C in the separation gel) according to the manufacturer's instructions. Samples were pretreated by heating at 100 °C for 3–5 min with the sample buffer of Laemmli (16). Samples containing 230–350 ng of protein were applied and electrophoresis was performed at 10 mA for 97 V-h. The Low Molecular Weight Electrophoresis Calibration Kit (Pharmacia) was used for molecular weight determination, and the gels were stained with PhastGel Silver Kit.

CNBr Cleavage of Deglycosylated Art v II—Art v II deglycosylated with PNGase F and purified by gel permeation chromatography on Bio-Gel P-4 and Bio-Gel P-10 (~12 nmol of protein) was cleaved with CNBr (17). The resulting fragments were separated by reverse phase HPLC on a Vydac C_{18} column (250 $\times$ 4.6 mm). The column was eluted with an eluent system composed of solvent A, aqueous 0.1% trifluoroacetic acid, and solvent B, 70% acetonitrile in solvent A, using a linear gradient of 0–80% B in 45 min.

NH_2 -terminal Amino Acid Sequence Analysis— NH_2 -terminal amino acid sequence analysis of Art v II (0.5–1.0 nmol of protein) and CNBr fragments of Art v II (0.04–2.0 nmol of polypeptide) was performed on an Applied Biosystems (Foster City, CA) 477A protein sequenator. The phenylthiohydantoin amino acid derivatives were determined on-line with the aid of an Applied Biosystems 120 phenylthiohydantoin analyzer.

ELISA Inhibition—The relative ability of native, denatured, and PNGase F-treated Art v II to bind human IgE from mugwort serum pool was examined by ELISA inhibition. The denatured Art v II was prepared as described under PNGase F treatment without the addition of PNGase F. Immunoplates (Nunc Maxisorp) were coated with native or denatured Art v II (45 ng/well in 100 μl of PBS) for 16 h at 4 °C. The wells were washed with PBS (210 μl) containing 0.1% Tween 20 (PBS/Tween) and then blocked with a solution of PBS/Tween (210 μl) containing 1% bovine serum albumin for 4 h at 4 °C. The coated wells were incubated for 16 h with solutions (100 μl) containing the appropriate antigens (1.9–200 ng of native Art v II, 3.8–200 ng of denatured Art v II or 30 ng of PNGase F-treated Art v II) and Mugwort serum pool (1:4 dilution). The plates were washed two times, incubated for 8 h with rabbit anti-human IgE (100 μl , 1:1,000), washed three times and incubated overnight at 4 °C with horseradish peroxidase-conjugated anti-rabbit IgG (100 μl , 1:10,000) (Boehringer Mannheim). After washing the plates three times, peroxidase activity was detected by incubation with 0.1 M citrate phosphate buffer, pH 6.0 (100 μl) containing 0.01% hydrogen peroxide and 1 mg/ml o-phenylenediamine. The reaction was terminated after 20 min by addition of 0.33 M citric acid (50 μl) and the absorbance at 490 nm determined. All antigens, serum and antisera were diluted in PBS/Tween containing 0.5% bovine serum albumin and the incubations were performed at room temperature unless otherwise stated.

The relative ability of native, denatured, and PNGase F-treated Art v II to bind rabbit IgG from anti-Art v II was examined using the procedure described above with the following modifications: the immunoplate was coated with native Art v II (2 ng/well). After blocking, the coated wells were incubated with solutions (100 μl) of the appropriate antigens (0.02–10 ng of native Art v II, 10 ng of denatured Art v II, or 10 ng of deglycosylated Art v II) and anti-Art v II (1:800), followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Cappel) (1:10,000). Peroxidase activity was detected as described above.

Rocket Immuno-electrophoresis—Rocket immuno-electrophoresis was performed essentially as described by Axelsen and Bock (18).

RESULTS

Deglycosylation of Art v II with PNGase F and Endo H—Glycosyl composition analysis of Art v II established the presence of mannose, N-acetylglucosamine, glucose, and galactose in the ratios 13.4:3.2:1.3:1.0 (6). These results sug-

gested that Art v II contains Asn-linked oligosaccharides. The conditions for the release of these oligosaccharide chains were investigated by treating Art v II with Endo H or PNGase F. Endo H releases high-mannose oligosaccharides and some hybrid-type oligosaccharides (19), whereas PNGase F releases all classes of Asn-linked oligosaccharides (20). The extent of enzymatic deglycosylation was monitored by SDS-PAGE (Fig. 1), and it was found that Art v II in its native conformation could not be deglycosylated with Endo H or PNGase F. Endo H released oligosaccharides from Art v II after denaturation with 0.1% SDS containing 20 mM 2-mercaptoethanol. The deglycosylation of Art v II with PNGase F required denaturation of the allergen with 0.2% SDS containing 100 mM mercaptoethanol. The addition of the non-ionic detergent, Nonidet P-40, to protect the PNGase F from the inhibitory effect of SDS (20), was not necessary.

The subunit molecular weight of Art v II, as judged by SDS-PAGE, decreased from 20,000 to 17,500 after treatment with Endo H or PNGase F. This suggests that each subunit of the glycoprotein contains at least one Asn-linked oligosaccharide chain.

Preparative scale deglycosylation of Art v II (1.9 mg) was performed with PNGase F to ensure that all the Asn-linked oligosaccharide chains were released. The PNGase F digestion was judged to be complete after incubation for 13 h since only one band with apparent molecular weight 17,500 was observed on SDS-PAGE (see Fig. 1).

Isolation of Asn-linked Oligosaccharides—The products obtained by PNGase F treatment of Art v II were fractionated on a column of Bio-Gel P-4. Material containing carbohydrate eluted from the column in two peaks (Fig. 2) and was well separated from glycerol. Glycerol is present in commercial preparations of PNGase F. The void volume of the column (fractions 23–28, Pool A) contained 30% of the carbohydrate and >90% of the deglycosylated allergen. The remaining 70% of the carbohydrate eluted in a broad, partially included peak (fractions 42–56, Pool B). A second protein peak (fractions 83–95, Pool C) was detected after the included volume of the column and was shown by SDS-PAGE and amino acid analysis to account for <10% of the deglycosylated allergen. The elution of part of the protein after the column included volume is probably due to the effects of denaturing Art v II with SDS and mercaptoethanol. A similar elution profile was obtained when RNase A was denatured with SDS and mercaptoethanol and fractionated on the Bio-Gel P-4 column.

Characterization of Carbohydrate in Pool A—Glycosyl composition analysis of Pool A showed the presence of mannose, galactose, arabinose, and glucose in the ratios 6.4:1.6:1.0:1.0. Only traces of N-acetylglucosamine and N-acetylgalactosa-

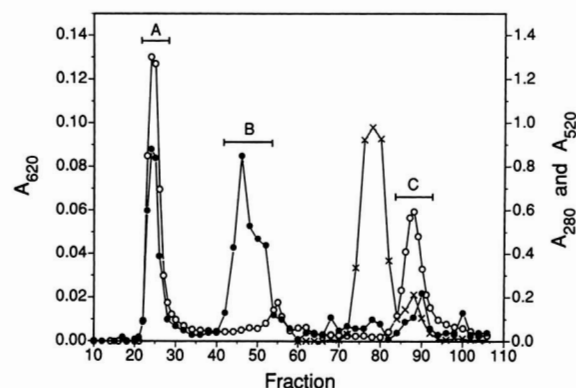


FIG. 2. Chromatography of Art v II after treatment with PNGase F on Bio-Gel P-4. The column (1.5 × 47 cm) was equilibrated and eluted with 0.3 M NH_4HCO_3 . Fractions were assayed for protein by absorbance at 280 nm (○). The carbohydrate (A_{420} ; ●) and glycerol (A_{520} ; ×) content of the fractions were determined by the anthrone assay. Pools A, B, and C were collected as indicated.

mine were detected. This monosaccharide composition is consistent with a structure that would not be released from the glycoprotein by PNGase F. To determine if this carbohydrate was linked to the protein, Pool A was fractionated on a Bio-Gel P-10 column. The carbohydrate and protein were partially separated which indicates that the carbohydrate present in Pool A may arise from a contaminating polysaccharide or a glycoprotein with a relatively high carbohydrate:protein ratio. Further studies are needed to establish if Art v II contains carbohydrate that is not released by PNGase F. The protein eluted from the Bio-Gel P-10 column was pooled and used for amino acid sequence analysis (see below).

Characterization of Oligosaccharide Pool B—The ^1H NMR spectrum of Pool B (Fig. 3) and the chemical shifts of the signals of the structural reporter groups (Table I) showed the presence of a mixture of high-mannose oligosaccharides differing in the number of mannose residues. Comparison of these data with the ^1H NMR spectra of other N-type oligosaccharides and glycopeptides (21, 22) indicates that the most abundant among the high-mannose oligosaccharides has the structure $\text{Man}_6\text{GlcNAc}_2$ (see Table II, structure B2).

The treatment of Art v II with PNGase F releases high-mannose oligosaccharides with an intact chitobiose core. Therefore, the H-1 signals of the free reducing GlcNAc-1 residue are observed in the ^1H NMR spectrum of Pool B (Fig. 3). These H-1 signals have been assigned in the ^1H NMR spectra of complex Asn-linked oligosaccharides (23), but not in the ^1H NMR spectra of the high-mannose type. High-mannose oligosaccharides are usually released from glycoproteins with Endo H, which cleaves the glycosidic linkage between the two GlcNAc residues. Thus, the H-1 signals of the GlcNAc-1 residue are not present in the ^1H NMR spectra of high-mannose oligosaccharides released by Endo H.

The ^1H NMR spectrum of Pool B contained a signal at δ 5.189 that was assigned to H-1 of the α -anomer of GlcNAc-1. The H-1 signal of the β -anomer of GlcNAc-1 is near δ 4.69 and is obscured by the signal from the residual HO^2H . The signal from HO^2H was suppressed using a WEFT pulse sequence (10) and the ^1H NMR spectrum of Pool B was then found to contain a signal at δ 4.698 which was assigned to the H-1 signal of the β -anomer of GlcNAc-1. The intensity ratio of the H-1 signals for the α - and β -anomer of GlcNAc-1 was approximately 2:1. A similar ratio has been found for the H-1 signals of GlcNAc-1 in complex-type oligosaccharides (23). Also, the N-acetyl signal of GlcNAc-1 is observed at the same position (δ 2.039) as for complex type oligosaccharides (23).

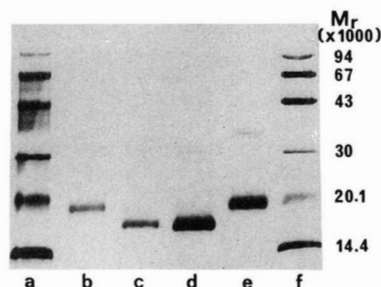


FIG. 1. SDS-PAGE of Art v II before and after treatment with PNGase F and Endo H. Lanes a and f, molecular weight references; lanes b and c, Art v II before (b) and after (c) treatment with Endo H for 17 h; lanes d and e, Art v II before (e) and after (d) treatment with PNGase F for 13 h. Electrophoresis and detection conditions are described under "Experimental Procedures."

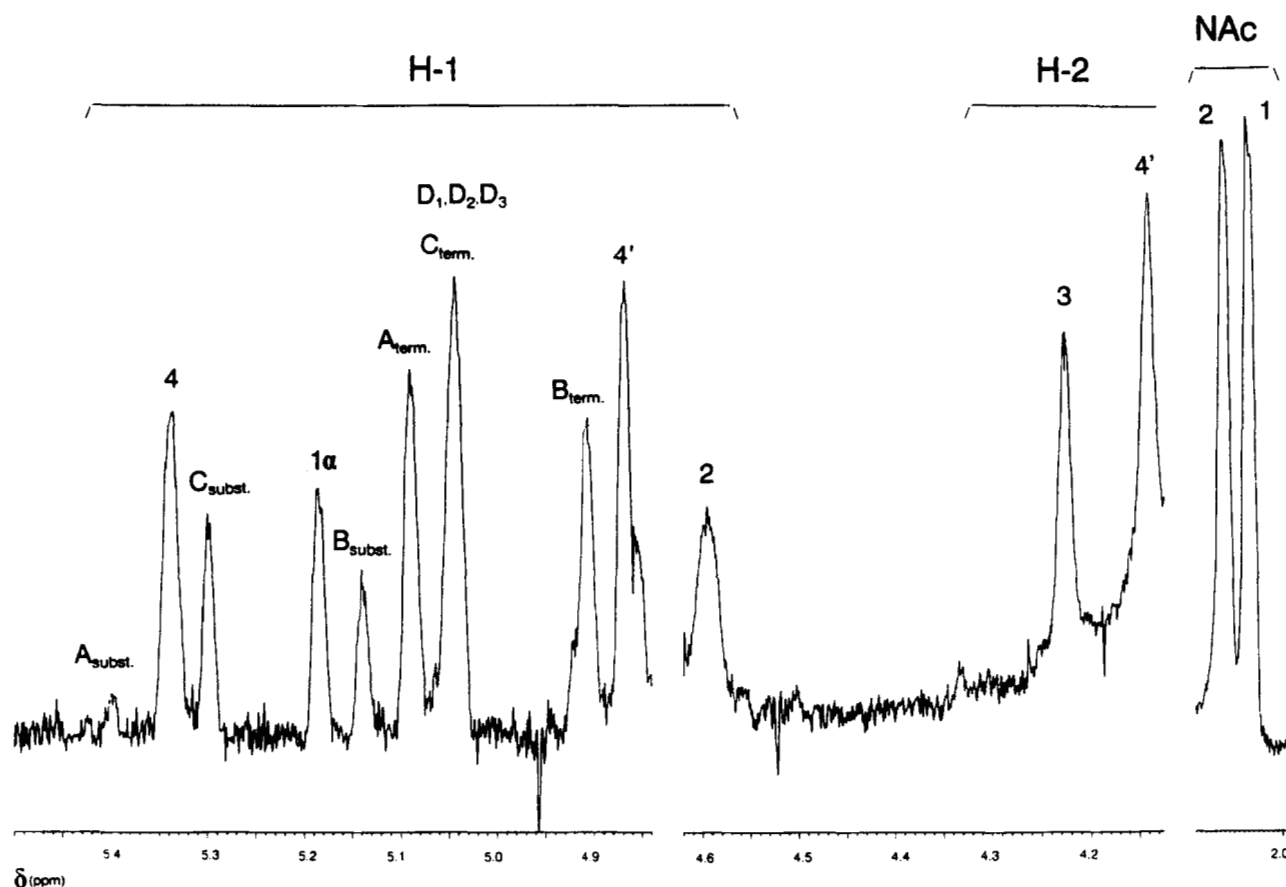
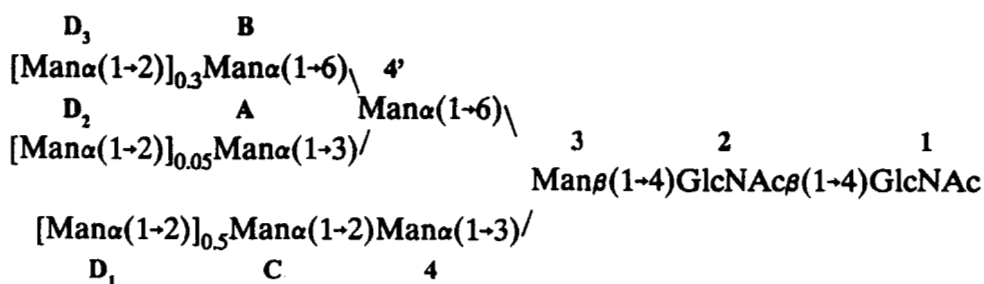


FIG. 3. Structural reporter-group regions of the 500 MHz ^1H NMR spectrum ($^2\text{H}_2\text{O}$; p^2H 5.5, 27 $^\circ\text{C}$) of oligosaccharide Pool B. The bold numbers in the spectrum refer to the corresponding monosaccharide residues in the structure shown at the top. The relative intensity scale of the *N*-acetyl- CH_3 region differs from that of the other parts of the spectrum.

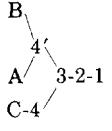
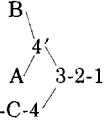
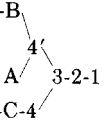
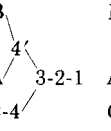
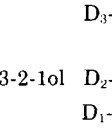
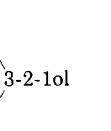
The effect of the anomerization of GlcNAc-1 on the ^1H NMR spectra of oligosaccharides with intact chitobiose cores appears to be restricted to GlcNAc-1 itself. In contrast, the anomerization of oligosaccharides containing a single GlcNAc residue (GlcNAc-2, see scheme in Fig. 3) considerably affects the chemical shift values of H-1 of Man-A, H-2 of Man-3, and of H-1 and the NAc CH_3 protons of GlcNAc-2 (see *e.g.* Ref. 23).

The chemical shifts of the signals from the structural reporter groups in the ^1H NMR spectra of $\text{Man}_6\text{GlcNAc-GlcNAcol}$ (21) (see Table I) and $\text{Man}_6\text{GlcNAc}_2\text{-Asn}$ (22) allowed most of the signals of the structural reporter groups in the ^1H NMR spectrum of Pool B (Fig. 3, Table I) to be assigned. The signals at δ 4.597 and 2.064 were assigned to H-1 and the methyl protons of the *N*-acetyl group, respec-

tively, of GlcNAc-2 (Table I). The signal from H-2 of Man-3 occurs at δ 4.230 and the signals for H-1 of Man-4 and Man-4' occur at δ 5.340 and 4.870, respectively (Table I). The signals at δ 5.093, 4.909, and 5.046 were assigned to the H-1 signals of the terminal nonreducing mannosyl residues, Man-A, Man-B, and Man-C, respectively (Table I). The signal for the H-1 of Man-3 resonates at δ 4.765 and was hidden by the signal from HO²H.

The presence of signals at δ 5.301, 5.142, and 5.404, with non-integer intensity ratios, in the ^1H NMR spectrum of Pool B (Fig. 3) suggested that the Man-A, Man-B, and Man-C residues are, in part, substituted with other mannosyl residues. The signal at δ 5.301 was assigned to H-1 of a Man-C that is substituted at C-2 with Man-D₁ (see scheme in Fig. 3). The signal at δ 5.142 is typical for H-1 of a Man-B residue

TABLE I
¹H chemical shifts of structural-reporter groups of constituent monosaccharides for oligosaccharides released from allergen Art v II by PNGase F

Reporter group	Residue ^a	Chemical shift ^b (ppm) in ^c					
		Allergen Art v II				Reference compounds	
		M6	M7	M8	M9	M6-ol	M9-ol
							
H-1	GlcNAc-1α ^d	5.189	5.189	5.189	5.189	— ^e	—
	GlcNAc-1β ^d	4.698	4.698	4.698	4.698	—	—
	GlcNAc-2	4.597	4.597	4.597	4.597	4.628	4.630
	Man-3	4.765 ^f	4.765 ^f	4.765 ^f	4.765 ^f	4.77	4.77
	Man-4	5.340	5.340	5.340	5.340	5.348	5.333
	Man-C	5.046	5.301	5.301	5.301	5.047	5.307
	Man-D ₁	—	5.046	5.046	5.046	—	5.045
	Man-4'	4.870	4.870	4.870	4.870	4.871	4.867
	Man-A	5.093	5.093	5.093	5.404	5.092	5.400
	Man-D ₂	—	—	—	5.046	—	5.054
	Man-B	4.909	4.909	5.142	5.142	4.907	5.140
	Man-D ₃	—	—	5.046	5.046	—	5.038
	Man-3	4.230	4.230	4.230	4.230	4.236	4.232
H-2	Man-4'	4.143	4.143	4.143	4.143	4.146	4.153
	Man-3	4.230	4.230	4.230	4.230	4.236	4.232
NAc	GlcNAc-1	2.039	2.039	2.039	2.039	— ^e	— ^e
	GlcNAc-2	2.064	2.064	2.064	2.064	2.063	2.067

^a For numbering and lettering of monosaccharide residues, see Fig. 3.

^b Data were acquired at 500 MHz for neutral solutions of the compounds in ²H₂O at 27 °C.

^c For complete structures of the compounds, see Table II. Structures are schematically illustrated in the table-heading by using the number-and-letter representation for the constituting monosaccharides (see Fig. 3). The data for reference compounds M6-ol and M9-ol were taken from Ref. 21.

^d The only structural reporter group whose chemical shift shows the anomerization effect is H-1 of GlcNAc-1 (see text).

^e In M6-ol and M9-ol, GlcNAc-1 is present as GlcNAc-ol.

^f Signal hidden under the residual HO²H peak at 27 °C, even when the solvent signal was being suppressed by WEFT.

which is substituted at C-2 with Man-D₃ (see scheme in Fig. 3). The low intensity signal at δ 5.404 indicated that some of the Man-A residues were substituted with Man-D₂. Integration of the ¹H NMR spectrum of Pool B indicated that approximately 50% of the Man-C residues were substituted with Man-D₁, about 30% of the Man-B residues were substituted with Man-D₃ and less than 5% of the Man-A residues were substituted with Man-D₂. This was deduced from the ratios of the intensity of the anomeric signals at δ 5.340 (Man-4 in all components), 5.301 (Man-C-substituted), 5.142 (Man-B-substituted) and 5.404 (Man-A-substituted).

The ratios of the H-1 signals in the ¹H NMR spectrum of Pool B can be explained by the presence of Man₆GlcNAc₂ as the major component of the mixture. In addition, Pool B contains one or more isomers of Man₇GlcNAc₂ (one being Man₆GlcNAc₂ substituted with Man-D₁ and the other one having additional Man-D₃), possibly a Man₈GlcNAc₂ which is Man₆GlcNAc₂ substituted with both Man-D₁ and Man-D₃, and perhaps a very small amount of Man₉GlcNAc₂ (see scheme in Fig. 3). The presence of hybrid-type structures in Pool B was ruled out chiefly because no galactosyl H-1 signals were observed around δ 4.47 ppm (Fig. 3).

To verify the molecular mass of the high-mannose oligosaccharides present in Pool B, a portion of the oligosaccharides was O-acetylated and analyzed by FAB-MS. The FAB mass spectrum of the O-acetylated high-mannose oligosaccharides derived from Pool B contained ions at m/z 2388, 2345, and 2303 which correspond to (M + Na - Ac)⁺, (M + Na - 2Ac)⁺, and (M + Na - 3Ac)⁺, respectively, from per-O-

acetylated Man₆GlcNAc₂. The FAB mass spectrum also contained ions at m/z 2099, 2057, and 2015, m/z 2676, 2633, and 2591, and m/z 2963, 2922, and 2879 which correspond to the pseudomolecular ions (M + Na)⁺ of the O-acetylated derivatives of Man₆GlcNAc₂, Man₇GlcNAc₂ and Man₈GlcNAc₂ that have lost one, two or three O-acetyl groups, respectively. The corresponding ions for O-acetylated Man₉GlcNAc₂ were very weak.

Analysis of the high-mannose components in Pool B by HPAE-PAD showed the presence of three major peaks (B2, B3, and B4) and two minor peaks (B1 and B5, see Fig. 4). The relative abundances of the components B1 through B5 were about 2:49:19:24:6. The positions of elution of the first (B1) and last minor peak (B5) did not correspond to the positions of elution of authentic samples of Man₆GlcNAc₂ and Man₉GlcNAc₂ (see open arrows in Fig. 4). The components in Pool B eluted earlier than the two oligosaccharide standards and the peaks were also unusually broad. However, when Pool B and the Man₆GlcNAc₂ and Man₉GlcNAc₂ standards were co-injected, the minor peaks, B1 and B5, co-eluted with the oligosaccharide standards. It is probable that the presence of EDTA in Pool B affected the separation of the high-mannose oligosaccharides by HPAE-PAD. The EDTA was introduced to the sample during treatment with PNGase F. The presence of EDTA was confirmed by the ¹H NMR spectrum of Pool B which showed intense signals at δ 3.68 and 3.89 (data not shown). No Man₆GlcNAc₂, Man₇GlcNAc₂, or Man₈GlcNAc₂ oligosaccharide standards were available for co-injection with Pool B, but on the basis of the NMR, FAB-

TABLE II

Structures of the Asn-linked oligosaccharides obtained from Art v II

Oligosaccharide	Structure	Relative amount %
B1	$ \begin{array}{c} \text{Man } \alpha(1 \rightarrow 6) \\ \diagup \quad \diagdown \\ \text{Man } \alpha(1 \rightarrow 3) \quad \text{Man } \alpha(1 \rightarrow 6) \\ \diagdown \quad \diagup \\ \text{Man } \alpha(1 \rightarrow 3) \quad \text{Man } \beta(1 \rightarrow 4) \text{ GlcNAc } \beta(1 \rightarrow 4) \text{ GlcNAc} \end{array} $	2
B2	$ \begin{array}{c} \text{Man } \alpha(1 \rightarrow 6) \\ \diagup \quad \diagdown \\ \text{Man } \alpha(1 \rightarrow 3) \quad \text{Man } \alpha(1 \rightarrow 6) \\ \diagdown \quad \diagup \\ \text{Man } \alpha(1 \rightarrow 2) \text{ Man } \alpha(1 \rightarrow 3) \quad \text{Man } \beta(1 \rightarrow 4) \text{ GlcNAc } \beta(1 \rightarrow 4) \text{ GlcNAc} \end{array} $	49
B3	$ \begin{array}{c} \text{Man } \alpha(1 \rightarrow 6) \\ \diagup \quad \diagdown \\ \text{Man } \alpha(1 \rightarrow 3) \quad \text{Man } \alpha(1 \rightarrow 6) \\ \diagdown \quad \diagup \\ \text{Man } \alpha(1 \rightarrow 2) \text{ Man } \alpha(1 \rightarrow 2) \text{ Man } \alpha(1 \rightarrow 3) \quad \text{Man } \beta(1 \rightarrow 4) \text{ GlcNAc } \beta(1 \rightarrow 4) \text{ GlcNAc} \end{array} $	19
B4	$ \begin{array}{c} \text{Man } \alpha(1 \rightarrow 2) \text{ Man } \alpha(1 \rightarrow 6) \\ \diagup \quad \diagdown \\ \text{Man } \alpha(1 \rightarrow 3) \quad \text{Man } \alpha(1 \rightarrow 6) \\ \diagdown \quad \diagup \\ \text{Man } \alpha(1 \rightarrow 2) \text{ Man } \alpha(1 \rightarrow 2) \text{ Man } \alpha(1 \rightarrow 3) \quad \text{Man } \beta(1 \rightarrow 4) \text{ GlcNAc } \beta(1 \rightarrow 4) \text{ GlcNAc} \end{array} $	24
B5	$ \begin{array}{c} \text{D}_3 \quad \text{B} \\ \text{Man } \alpha(1 \rightarrow 2) \text{ Man } \alpha(1 \rightarrow 6) \quad 4' \\ \diagup \quad \diagdown \\ \text{D}_2 \quad \text{A} \quad \text{Man } \alpha(1 \rightarrow 6) \quad 3 \quad 2 \quad 1 \\ \text{Man } \alpha(1 \rightarrow 2) \text{ Man } \alpha(1 \rightarrow 3) \quad \text{Man } \beta(1 \rightarrow 4) \text{ GlcNAc } \beta(1 \rightarrow 4) \text{ GlcNAc} \\ \diagdown \quad \diagup \\ \text{D}_1 \quad \text{C} \quad \text{Man } \alpha(1 \rightarrow 2) \text{ Man } \alpha(1 \rightarrow 2) \text{ Man } \alpha(1 \rightarrow 3) \quad 4 \end{array} $	6

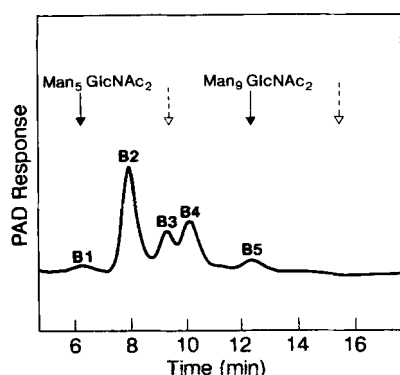


FIG. 4. Separation of the Art v II oligosaccharide Pool B by high-pH anion-exchange chromatography. Open arrows, elution positions of $\text{Man}_5\text{GlcNAc}_2$ standard (left arrow) and $\text{Man}_9\text{GlcNAc}_2$ standard (right arrow) when the standards were injected alone. Solid arrows, elution positions of $\text{Man}_5\text{GlcNAc}_2$ standard and $\text{Man}_9\text{GlcNAc}_2$ standard when co-injected with Pool B. The chromatography and detection conditions are described under "Experimental Procedures."

MS, and HPAE-PAD results we conclude that Pool B contains a mixture of five high-mannose oligosaccharides as summarized in Table II.

Amino Acid Sequence Analysis of Art v II—In an attempt to determine the site of *N*-glycosylation in the amino acid sequence of Art v II, native Art v II was subjected to NH_2 -terminal amino acid sequence analysis. The sequence ob-

tained after 30 cycles is given in Fig. 5A. No potential *N*-glycosylation site was revealed within this NH_2 -terminal region. Further sequencing after position 30 gave amino acid derivatives in low yields, which may indicate that the region following residue 30 contains labile peptide bonds or glycosylated amino acid residues. In addition to the main sequence given in Fig. 5A, the NH_2 -terminal sequence analysis revealed the presence of a minor sequence (accounting for 10–30% of the total protein) that from position 5–26 was identical with the sequence from position 6–27 in the main sequence. The amino acid residues in positions 1–4 of this minor sequence were not identified. These results show that a minor fraction of the Art v II-molecules lacks one of the four first residues in the amino acid sequence. The NH_2 -terminal amino acid analysis also showed that small amounts of contaminating protein constituting no more than 10% of the total protein was present in the Art v II-preparation.

Additional information about the amino acid sequence of Art v II was obtained using the deglycosylated Art v II obtained after fractionation of Pool A on Bio-Gel P-10. Since the results from Bio-Gel P-10 chromatography (see above) indicated that Pool A possibly contained small amounts of a contaminating glycoprotein which eluted from the column slightly later than deglycosylated Art v II, only the fractions eluted before the main carbohydrate peak were pooled and used for the sequencing experiments.

The deglycosylated Art v II was cleaved by CNBr since amino acid composition of native Art v II indicated that each Art v II subunit contained 2–3 methionine residues (6). As shown in Fig. 5A, 1 methionine residue was placed in position 27 and cleavage by CNBr should thus at least give the two fragments denoted CB-1 and CB-2 in Fig. 5A. The CNBr fragments of deglycosylated Art v II were purified by reversed phase HPLC. Several of the HPLC peaks were found to contain mixtures of peptides, however, four peptides denoted CB-1, CB-A, CB-B, and CB-C were sequenced (Fig. 5B). The yields of these four peptides were consistent with expected yields of peptides generated from Art v II. The amino acid composition of the CNBr fragments was not determined due to lack of material.

The amino acid sequence of CB-1 (Fig. 5B) confirmed 17 of the first 21 residues of the NH_2 -terminal sequence of Art v II. The residues in positions 1, 12, and 16 were not identified in CB-1. The residue in position 18 of CB-1 was tentatively assigned as alanine, whereas NH_2 -terminal sequencing of the native Art v II (Fig. 5A) gave histidine in this position. The peptide CB-C was sequenced for 17 cycles (Fig. 5B), but no amino acid residues could be detected after tyrosine in position 11, indicating that this peptide contained tyrosine as the COOH-terminal amino acid residue. The absence of homoserine in the COOH-terminal end of CB-C indicates that this peptide originates from the COOH-terminal end of Art v II. The peptide CB-A was also sequenced to the end as shown by the presence of homoserine in position 9 (Fig. 5B). CB-A contained the sequence Asp-Gly-Ser (Fig. 5B). The CNBr fragments were prepared from Art v II that had been deglycosylated with PNGase F, which converts the *N*-glycosylated asparagine residue to aspartic acid (24). The aspartic acid (asparagine) residue in position 6 of CB-A may thus be the site of attachment of the Asn-linked oligosaccharides characterized in this study. The possibility of an additional potential *N*-glycosylation site at the aspartic acid residue in position 7 of CB-B (Fig. 5B) cannot be excluded because the amino acid residue in position 9 was not identified. The CB-2 peptide which was expected to be generated by CNBr cleavage of the bond between Met-27 and the unknown amino acid residue

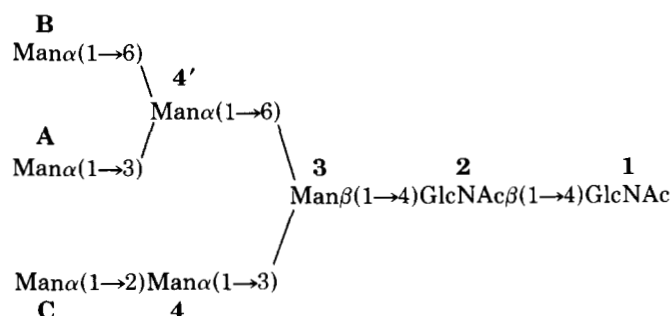
The IgE-binding activity of native *Art v* II, denatured *Art v* II and PNGase F-treated *Art v* II was compared in two ELISA inhibition assays, one in which the wells were coated with native *Art v* II and another where the wells were coated with denatured *Art v* II (Table III). When denatured *Art v* II was used as an inhibitor of the binding between human IgE and native *Art v* II, its inhibitory activity was only 30% of that of native *Art v* II. Conversely, when native *Art v* II was used as an inhibitor of the binding between human IgE and denatured *Art v* II, its inhibitory activity was 93% of that of denatured *Art v* II. These results indicate that native *Art v* II contains at least two IgE-binding epitopes. At least one IgE epitope, responsible for binding of about 70% of the *Art v* II-specific IgE in Mugwort serum pool, is destroyed when the allergen is denatured with SDS and mercaptoethanol. Another IgE epitope, that is responsible for binding of the remaining 30% of the *Art v* II-specific IgE in Mugwort serum pool, is resistant to the denaturation of the allergen. The IgE-binding activity of PNGase F-treated *Art v* II was 20% of that of native *Art v* II and 94% of that of denatured *Art v* II (Table III). These results indicate that removal of the high-mannose chains after denaturation of the allergen had only a minor

effect on the remaining IgE-binding ability of the allergen.

ELISA inhibition using a rabbit antiserum containing specific IgG against Art v II (anti-Art v II), demonstrated that the IgG-binding activity of both denatured and PNGase F-treated Art v II was reduced to less than 1% of the IgG-binding activity of the native allergen (Table III). This result was confirmed by rocket immunoelectrophoresis where denatured and PNGase F-treated Art v II did not give any immunoprecipitates in the antibody containing gel (result not shown).

DISCUSSION

Asparagine-linked Oligosaccharides of Art v II—A combination of ^1H NMR spectroscopy, FAB-MS, and HPAE-PAD established that the oligosaccharides released by treatment of the glycoprotein allergen Art v II with PNGase F were composed of 2 GlcNAc residues and between 5 and 9 mannose residues (Table II). Analysis of the oligosaccharides by FAB-MS and HPAE-PAD (Fig. 4) showed $\text{Man}_5\text{GlcNAc}_2$ (B1), $\text{Man}_6\text{GlcNAc}_2$ (B2), $\text{Man}_7\text{GlcNAc}_2$ (B3), $\text{Man}_8\text{GlcNAc}_2$ (B4), and $\text{Man}_9\text{GlcNAc}_2$ (B5) to be present in the ratios 2:49:19:24:6, respectively. The ^1H NMR spectrum of Pool B (Fig. 3) showed that 50% or less of the Man-A, Man-B, and Man-C residues (see B3–B5, Table II) were substituted by Man-D₂, Man-D₃, and Man-D₁, respectively. Therefore $\text{Man}_6\text{GlcNAc}_2$ devoid of Man-D residues:



accounts for 50% of the high-mannose oligosaccharides. The oligosaccharide $\text{Man}_5\text{GlcNAc}_2$ was present in amounts too small (2% of the mixture) to be detected by ^1H NMR. Integration of the anomeric signals in the ^1H NMR spectrum of Pool B showed that 5% of the Man-A residues were substituted with Man-D₂ and established that $\text{Man}_9\text{GlcNAc}_2$ (see B5, Table II) accounted for approximately 5% of the high-mannose oligosaccharides. Approximately 30% of the Man-B residues were substituted with Man-D₃ (25% $\text{Man}_8\text{GlcNAc}_2$ and 5% $\text{Man}_9\text{GlcNAc}_2$). Therefore, $\text{Man}_6\text{GlcNAc}_2$ that is substituted with Man-D₁ and Man-D₃ ($\text{Man}_8\text{GlcNAc}_2$) accounts for 25% of the oligosaccharides in Pool B (see B4, Table II). Approximately 50% of the Man-C residues were substituted with Man-D₁ (20% $\text{Man}_7\text{GlcNAc}_2$, 25% $\text{Man}_8\text{GlcNAc}_2$, and 5% $\text{Man}_9\text{GlcNAc}_2$) which established that a single $\text{Man}_7\text{GlcNAc}_2$ isomer in which Man-D₁ is attached to $\text{Man}_6\text{GlcNAc}_2$ accounted for 20% of the high-mannose oligosaccharides (see B3, Table II). The $\text{Man}_7\text{GlcNAc}_2$ isomer in which Man-D₃ is attached to $\text{Man}_6\text{GlcNAc}_2$ may be present in amounts too small (<5%) to be detected by ^1H NMR. Our data strongly suggest that only a single isomer of $\text{Man}_7\text{GlcNAc}_2$ and $\text{Man}_8\text{GlcNAc}_2$ are attached to asparagine residues of Art v II. Further studies are necessary to establish if Art v II contains one or more O-linked carbohydrate chains.

Comparison of the Asn-linked Oligosaccharides of Art v II, Other Allergens and Plant Glycoproteins—The glycoprotein allergen, Art v II, from the pollen of mugwort has been shown to contain Asn-linked oligosaccharides of the high-mannose

type (Table II). Several allergens isolated from other plants contain carbohydrate (26), but a complete structural characterization of the oligosaccharides has not been reported. Ovomucoid (Gal d I), ovalbumin (Gal d II), and ovotransferrin (Gal d III) from hen egg white and phospholipase A₂ (Api m I) from bee venom are glycoprotein allergens that contain Asn-linked oligosaccharides. Ovalbumin contains a mixture of high-mannose oligosaccharides ($\text{Man}_{4-7}\text{GlcNAc}_2$) in addition to hybrid-type oligosaccharides, whereas ovomucoid and ovotransferrin contain mixtures of multiantennary complex type oligosaccharides (Ref. 27 and references therein). Phospholipase A₂ has been reported to contain unusual "high-mannose" oligosaccharides in which the $\text{Man}_{4-5}\text{GlcNAc}_2$ core is extended with nonreducing terminal N-acetylglucosamine and fucose residues (28). These limited data indicate that there are a variety of oligosaccharide structures that are attached to the asparagine residues of glycoprotein allergens from plants and animals.

High-mannose oligosaccharides linked to asparagine residues have been found in numerous glycoproteins from mammalian cells (29). In contrast, the number of plant glycoproteins that have been unambiguously shown to contain Asn-linked high-mannose oligosaccharides is small (30). The $\text{Man}_{6-8}\text{GlcNAc}_2$ isomers found in Art v II are identical to the predominant $\text{Man}_{6-8}\text{GlcNAc}_2$ isomers found in kidney bean glycoprotein II (phaseolin) and 7S soybean storage protein (31, 32). The predominant $\text{Man}_7\text{GlcNAc}_2$ isomer found in these plant glycoproteins is substituted with Man-D₁ on Man-C (see scheme in Fig. 3 and Table II). In contrast, the predominant $\text{Man}_7\text{GlcNAc}_2$ isomer usually found in animal glycoproteins is the one in which Man-B is substituted with Man-D₃ (33). The limited data available suggest that although the biosynthesis of Asn-linked high-mannose oligosaccharides of mammalian and plant glycoproteins is similar, the enzymatic processing of the $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ precursor may differ (34). The finding that the predominant $\text{Man}_7\text{GlcNAc}_2$ isomer present in plant glycoproteins are different from the predominant $\text{Man}_7\text{GlcNAc}_2$ isomer found in animal glycoproteins may perhaps reflect such differences in the processing.

Amino Acid Sequence of Art v II—The NH₂-terminal sequences of Art v II and of four peptides generated by CNBr-cleavage of deglycosylated Art v II were determined in an attempt to identify potential N-glycosylation sites. The sequence Asp-Gly-Ser was located at positions 6–8 of the CNBr-cleavage product CB-A (Fig. 5B) and represents a potential N-glycosylation site. However, the location of this potential N-glycosylation site within the intact glycoprotein remains to be established. The peptides sequenced accounted for 71 of the approximately 150 amino acid residues each subunit of Art v II is composed of (6). Further studies are needed to determine the entire amino acid sequence of Art v II and the location of the high-mannose oligosaccharides.

The NH₂-terminal sequence of Art v II and the sequence of the peptide CB-C which probably originates from the COOH-terminal end of Art v II have some characteristic features. Of the 9 proline residues present in each Art v II subunit (6), 3 were positioned within the first 29 residues from the NH₂-terminal end and 2–3 were found within the first 11 residues from the COOH-terminal end. Thus, it seems that more than half of the proline residues are clustered within the NH₂-terminal and COOH-terminal regions of this allergen. These terminal regions also contain pairs of identical amino acid residues, i.e. Asp-Asp in positions 12–13, Asn-Asn in position 19–20, and Arg-Arg in position 22–23 of the NH₂-terminal sequence and Pro-Pro, Val-Val in positions 1–2 and 5–6, respectively, of peptide CB-C. It is not known if these struc-

tural features are related to the biological activity of this glycoprotein. Comparison of the amino acid sequence data of Art v II with amino acid sequence data of other allergens from a variety of sources showed significant similarity only between a hexapeptide sequence present in position 17–22 of the NH₂-terminal sequence of Fel d I (25) and the hexapeptide sequence in positions 10–15 of the NH₂-terminal sequence of Art v II. Since the function of Art v II and Fel d I has not been determined, the functional significance of this similarity is not known.

Art v II has earlier been found to consist of at least 6 isoforms with pI values in the region 4.10–4.65 (6). Since the carbohydrate part of Art v II contained only neutral monosaccharides, the pI heterogeneity was assumed to arise from (micro)heterogeneities in the protein part of the molecule. NH₂-terminal sequence analysis of native Art v II revealed that 10–30% of the molecules lacked one of the first four amino acid residues in the sequence. This may at least partly explain the pI heterogeneity of this allergen. No other major heterogeneities were observed in the amino acid sequence of the peptides sequenced, but may be present in parts of the sequence not determined here. Minor differences caused by partial deamidation of the amino acid residues asparagine and glutamine may also contribute to the pI heterogeneity of Art v II.

Antibody-binding Activity of Art v II after Denaturation and Treatment with PNGase F—The contribution of the Asn-linked high-mannose oligosaccharides of Art v II to the immunogenicity of the allergen was assessed by determining the relative IgE- and IgG-binding activities of the native allergen, denatured allergen, and denatured and deglycosylated allergen (Table III). The IgG-binding activity of Art v II was completely destroyed after the glycoprotein was denatured with SDS and mercaptoethanol. This shows that the native three-dimensional protein conformation of Art v II is essential for the binding of rabbit IgG indicating that at least part of the IgG epitope resides in the protein part of the molecule. The glycoprotein allergen could not be deglycosylated without prior denaturation with SDS and mercaptoethanol. Therefore, the effect of enzymatic deglycosylation of Art v II on the IgG-binding activity of this allergen could not be determined.

The IgE-binding activity of Art v II decreased by 70% when the allergen was denatured with SDS and mercaptoethanol (Table III) showing that the native three-dimensional conformation of Art v II is required for binding of human IgE to the major IgE-binding epitope(s). The IgE-binding epitope(s) of Art v II that are resistant to denaturation with SDS and mercaptoethanol were not significantly affected by the enzymatic deglycosylation of the allergen (Table III).

Our results suggest that the Asn-linked high-mannose oligosaccharides of Art v II have no direct effect on the binding of IgE to the allergen. A possible role of these oligosaccharides either as part of the dominant IgE-binding epitope(s) or as a stabilizing element of the three-dimensional protein conformation necessary for binding of human IgE to the dominant IgE epitope(s) can, however, not be ruled out. The data obtained for the IgE-binding activity of Art v II were obtained using a pool of sera from 64 individuals who were allergic to mugwort pollen. Studies with the glycoprotein allergen Api m I (phospholipase A₂) from bee venom have shown that indi-

vidual patients may recognize different IgE-binding epitopes (35). It is therefore possible that the IgE-binding epitope(s) recognized by some mugwort allergic individuals may include the Asn-linked high-mannose oligosaccharides of Art v II. The biological role(s) of the carbohydrate portions of glycoprotein allergens will only be determined when the Asn-linked oligosaccharide chains can be enzymatically removed from the native glycoprotein.

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