

ISOLATION AND CHARACTERIZATION OF A GLYCOPROTEIN ALLERGEN, *ART V* II, FROM POLLEN OF MUGWORT (*ARTEMISIA VULGARIS* L.)

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Abstract—A glycoprotein allergen, *Art v* II, was isolated from pollen of mugwort by two different isolation procedures. *Art v* II-A was isolated by a combination of ion-exchange chromatography on DEAE-Sephacrose, affinity chromatography on Con A-Sephacrose and ion-exchange chromatography on Mono P. *Art v* II-B was isolated by a combination of preparative IEF in Ultrodex granulated gel, affinity chromatography on Con A-Sephacrose and HPLC size exclusion on Ultropac TSK G2000SW. *Art v* II-A and *Art v* II-B were shown to be antigenically identical with the allergen we have formerly denoted Ag7. The MW of *Art v* II A/B was determined to be 34,000–38,000 by HPLC size exclusion, and 35,000 and 20,000 by SDS-PAGE under non-reducing and reducing conditions, respectively. *Art v* II was found to consist of 6(7) isoforms with pI 4.10, 4.20 (major component), 4.35, 4.45, 4.55, 4.65, (4.80). The glycoprotein allergen had a protein to carbohydrate ratio of 10:1 and the carbohydrate part contained mannose (70.7%), *N*-acetyl-glucosamine (17.0%), glucose (7.0%) and galactose (5.3%). In R(R)IE the purified allergen bound IgE from 5 (33%) of 15 sera from patients with clinical allergy against mugwort pollen and from 13 (52%) of 25 sera from patients selected only on the basis of a RAST-class 4 against mugwort pollen.

INTRODUCTION

Pollen of mugwort (*Artemisia vulgaris* L.) is one of the main causes of allergic reactions in late summer in Europe (Charpin *et al.*, 1974; Spieksma *et al.*, 1980). Physicochemical and immunochemical characterization of crude and partly purified extracts of mugwort pollen have been performed by several investigators (Ipsen *et al.*, 1985; Smestad Paulsen *et al.*, 1985; Nilsen and Smestad Paulsen, 1986; Dreborg *et al.*, 1987; Diener *et al.*, 1988), and it has been demonstrated that crude extracts of mugwort pollen contain 8–11 different allergens, most of which migrate towards the anode in CIE. Different groups have found that mugwort pollen extracts contain 1–2 major allergens (Ipsen *et al.*, 1985; Dreborg *et al.*,

1987; Diener *et al.*, 1988), but whether the major allergens identified by the different groups are identical remains to be established.

The allergens in mugwort pollen have mainly been identified by their antigenic and allergenic activity in CIE/CRIE and limited information about physicochemical parameters have been given for only a few of the allergens (Nilsen and Smestad Paulsen, 1986; Dreborg *et al.*, 1987). Recently one allergen, denoted *Art v* I, was isolated from mugwort pollen by a combination of ammonium sulphate precipitation, gel filtration on Ultrogel AcA 44 and HPLC size exclusion (de la Hoz *et al.*, 1989). This allergen had a pI of 4.4, and the MW was determined to be 47,000 by HPLC size exclusion and 60,000 by SDS-PAGE. *Art v* I was judged to be a clinically relevant allergen since at least 70% of sera from mugwort allergic patients contained specific IgE against it and a similar percentage of the patients showed specific skin reactivity when the purified *Art v* I was used for skin prick test.

We have previously identified a glycoprotein allergen, Ag7, in mugwort pollen extract by its ability to bind to the lectin Con A in crossed immunoaffinoelectrophoresis (Nilsen and Smestad Paulsen, 1986). This allergen was relatively heat-resistant and had a pI of 4.3 as demonstrated by crossed immuno-IEF. CRIE analysis of mugwort pollen extract with a panel of 26 sera with RAST-class 4 against mugwort pollen indicated that 24 (92%) of the sera contained specific IgE against Ag7.

In this study we report the isolation and characterization of the glycoprotein allergen Ag7. The isolated

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Abbreviations: Con A, concanavalin A; Ag, antigen; IgE, immunoglobulin E; RAST, radioallergosorbent test; CIE, crossed immuno-electrophoresis; CRIE, crossed radioimmuno-electrophoresis; FRIE, fused rocket immuno-electrophoresis; FR(R)IE, fused rocket (radio)-immuno-electrophoresis; RIE, rocket immuno-electrophoresis; R(R)IE, rocket (radio)immuno-electrophoresis; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; %T, polyacrylamide gel concentration defined as percentage total monomer (acrylamide + bisacrylamide); %C, percentage bis-acrylamide (as percentage of total monomer); MW, molecular weight; pI, isoelectric point; IEF, isoelectric focusing; GLC, gas liquid chromatography; TMSi, trimethylsilyl; TCA, trichloroacetic acid; α -MeGlcP, α -methyl-D-glucopyranoside; α -MeManp, α -methyl-D-mannopyranoside; Tris, tris(hydroxymethyl)-aminomethane.

glycoprotein allergen seems to be different from the previously isolated *Art v* I (de la Hoz *et al.*, 1989) and we suggest that it is designated *Art v* II according to the allergen nomenclature system recommended by the International Union of Immunological Societies (IUIS) (Marsh *et al.*, 1986).

MATERIALS AND METHODS

Pollen

Artemisia vulgaris pollen containing less than 0.5% (w/w) foreign pollen was obtained from Allergon AB (Engelholm, Sweden, batches no. 010182101 and 010184103).

Rabbit antibodies

Rabbit antibodies against mugwort pollen extract (anti-mugwort) were obtained from Pharmacia (Uppsala, Sweden, batch no. LJ 327520 840417 U.K.). Rabbit antibodies against the glycoprotein allergen *Art v* II (anti-*Art v* II) were prepared by immunization with immunoprecipitates of the allergen obtained by CIE essentially as described by Harboe and Closs (1983).

Human sera

A serum pool was prepared from equal volumes of sera from 64 individuals having RAST-class 3 against mugwort pollen.

Forty individual patient sera were also used in this study. Fifteen of these sera (clinical patient sera) were collected from patients with clinical mugwort allergy and was kindly provided by Dr A. Bryhni Frostad, Polyclinic of Allergology, Oslo, Norway. The patient inclusion criteria were: symptoms of allergy in the mugwort pollen season, positive skin prick test (mean weal diameter obtained with Spectralgen mugwort pollen extract, 100,000 BU (Nycomed AS, Oslo, Norway), equal to or larger than mean weal diameter obtained with histamine HCl 10 mg/ml) and RAST-class 2 or greater against mugwort pollen. The residual 25 patient sera (RAST-class 4 patient sera) were selected only on the basis of a RAST-class 4 against mugwort pollen.

Mugwort pollen extract

Mugwort pollen extract was prepared as previously described (Nilsen and Smestad Paulsen, 1986). The pollen (300 g) was extracted 1:20 (w/v) in phosphate-NaCl-buffer (20 mM phosphate, 55 mM NaCl, 2% *n*-butanol, pH 7.2) containing 5 mM thiourea, for 1 hr at 4°C. After removal of the pollen by filtration, the filtrate was dialyzed (MW cut off 3500) for a total of 90 hr against 10 changes of distilled water (containing 5 mM thiourea the first 24 hr) and lyophilized. The lyophilized extract (21.9 g) was stored at -20°C.

Isolation procedure

Lyophilized mugwort pollen extract (1.0–1.5 g) was fractionated by preparative IEF in granulated gel

(11 × 23 × 0.4 cm) containing 40% (w/v) Ultrodex (LKB, Bromma, Sweden) and 5% (v/v) Ampholine (pH 3.5–5) (LKB), using the LKB 2117 Multiphor according to the manufacturer's instructions. The extract was focused at 8 W for 16–17 hr with 1 M NaOH as the cathodic and 1 M H₃PO₄ as the anodic electrode solutions. After focusing, 30 gel sections (0.7 cm) were each eluted with 5 ml distilled water and pH in the fractions was measured. The fractions were assayed for protein by absorbance at 280 nm, for carbohydrate by the phenol sulphuric acid test (Dubois *et al.*, 1956) and for *Art v* II by FRIE and FRIE with Con A-Sepharose (Pharmacia) in the intermediate gel (see Immunoelectrophoretic methods). This procedure was performed seven times with a total of 8.0 g mugwort pollen extract and the fractions containing *Art v* II were pooled, dialyzed (MW cut off 3500) against three changes of distilled water and three changes of 50 mM NH₄HCO₃ (to dissolve material that precipitated in the dialysis bag) before lyophilization (IEF-pool).

The lyophilized IEF-pool was redissolved in Tris-buffer (20 mM Tris, 1 mM MnSO₄, 1 mM CaCl₂, 0.02% NaN₃, pH 7.4) and subjected to affinity chromatography at room temperature on a Con A-Sepharose column (2.5 (i.d.) × 2.0 cm) at a flow rate of 30 ml/hr. Unbound and non-specifically bound components were eluted with 100 ml Tris-buffer, 100 ml 1.0 M NaCl in Tris-buffer and 100 ml 20% ethylene glycol in Tris-buffer. After re-equilibration of the column with 40 ml Tris-buffer, specifically bound components were eluted with the following eluents in Tris-buffer: (1) 60 ml 0.2 M α -MeGlcp, (2) 50 ml 0.2 M α -MeManp, (3) 90 ml 0.5 M α -MeManp and (4) 55 ml 0.5 M α -MeManp, 1.0 M NaCl. The flow was stopped for 45 min after 10 ml of each of the replacing sugar eluents had been applied before elution was continued. The absorbance at 280 nm was recorded and fractions of 2.5 ml were collected and assayed by FRIE. Selected fractions were pooled, dialyzed (MW cut off 3500) against distilled water and lyophilized (Con A-pool).

Con A-pool was redissolved in 600 μ l 0.1 M Na-phosphate buffer, pH 6.8 and subjected to HPLC size exclusion at room temperature on Ultropac TSK G2000SW as described below. Samples of 100 μ l were applied and fractions of 0.175 ml were collected and assayed by FRIE. Fractions containing *Art v* II from six runs were pooled, desalted on a PD-10 column (Pharmacia) and lyophilized. The lyophilized allergen (1.8 mg protein) was designated *Art v* II-B and was stored at -20°C until further characterization.

The recovery of *Art v* II in each step of the isolation procedure was determined by RIE against anti-*Art v* II anti-serum.

HPLC size exclusion

HPLC size exclusion was performed on Ultropac TSK G2000SW (7.5 × 300 mm) (LKB) using the Shimadzu HPLC-system (LC-6A liquid chromato-

graph, SPD-6A UV detector and CR-6A Chromatopac) (Shimadzu corp., Kyoto, Japan). The column was eluted at 0.7 ml/min with 0.1 M Na-phosphate buffer (pH 6.8) and the absorbance was recorded at 280 nm. Samples of 3–5 µg protein in 20 µl were injected in analytical runs. The column was calibrated with ribonuclease A (RIB: MW 13,700), chymotrypsinogen A (CHY: MW 25,000), ovalbumin (OVA: MW 43,000) and bovine serum albumin (BSA: MW 67,000). The void volume was determined with thyroglobulin (MW 669,000).

SDS-PAGE

SDS-gradient PAGE was performed using the Pharmacia PhastSystem with PhastGel Gradient 10–15 (4.5% T, 3% C in the stacking gel and 10–15% T, 2% C in the separation gel). Electrophoresis was performed at 10 mA for 60 Vhr with samples of 120–660 ng protein. The gels were silver-stained with PhastGel Silver Kit. Samples for SDS-PAGE were prepared with the sample buffer of Laemmli (1970) with or without the addition of 5% 2-mercaptoethanol. The Low Molecular Weight Electrophoresis Calibration Kit (Pharmacia) was used for molecular weight determination.

Analytical IEF and crossed immuno-IEF

Analytical IEF was performed in 2 mm thick polyacrylamide gel (5% T, 3% C) containing 5% Ampholine pH 3.5–10 (LKB) using the LKB 2117 Multiphor. Prefocusing of the gel was performed at 30 W for 20 min. Samples containing 1.7–9 µg protein were applied and the electrofocusing was performed at 30 W for 1.5 hr with 1 M NaOH and 1 M H₃PO₄ as the cathodic and anodic electrode solutions, respectively. The Isoelectric Focusing Calibration Kit, pH 3–10 (Pharmacia) was included for pI determination. The gel was fixed with TCA/sulphosalicylic acid and stained with Coomassie brilliant blue R-250 as described by Winter *et al.* (1979).

In crossed immuno-IEF, IEF in the first dimension was performed as described above. Immediately after focusing the gel strip was transferred to a 1% agarose gel by the "laying on"-technique described by Söderholm *et al.* (1975). Second dimension electrophoresis was performed towards the anode at 10 V/cm for 1 hr followed by 2 V/cm for 15 hr into 1% agarose gel containing 50 µl/cm² anti-mugwort antiserum. Other electrophoresis conditions were as described under immunoelectrophoretic methods. The gel was stained with Coomassie brilliant blue R-250.

Immunoelectrophoretic methods

CIE, tandem-CIE, FRIE and RIE were performed essentially as described by Høiby and Axelsen (1983), Krøll (1983), Harboe and Just Svendsen (1983) and Axelsen and Bock (1983), respectively, with modifications as described earlier (Nilsen and Smestad Paulsen, 1986). In some FRIE-experiments, Con A-Sepharose corresponding to 0.2 mg Con A/cm² was

included in the intermediate gel as described by Bøg-Hansen (1983). 25–50 µl/cm² anti-mugwort antiserum or 35 µl/cm² anti-Art v II was included in the antibody-containing gels. The gels were stained with Coomassie brilliant blue R-250.

CRIE, R(R)IE and FR(R)IE were performed as described earlier (Nilsen and Smestad Paulsen, 1986). Briefly, the dried, unstained gel plates were incubated overnight (18–24 hr) with 12.5% serum from mugwort allergic patients (serum pool or individual patient sera as specified in the figure legends), washed and incubated overnight (16–20 hr) in ¹²⁵I-anti-IgE (70,000 cpm/ml), washed, dried and subjected to autoradiography at –80°C.

Monosaccharide analysis

Monosaccharides in the purified allergen preparation (70 µg protein) were identified and quantified by GLC after methanolysis (1 M HCl in anhydrous methanol, 80°C, 21–22 hr), re-*N*-acetylation, de-*O*-acetylation (Chambers and Clamp, 1971) and TMSi derivatization of the derived methyl glycosides (Sweeley *et al.*, 1963). GLC was performed on a 15 m DB-5 fused silica capillary column with H₂ as carrier gas (2.8 ml/min) and the following temperature program: 140°C when injecting, followed by an increase of 1°C/min to 160°C and 6°C/min from 160°C to 250°C. The chromatograph (Carlo Erba 4200, Milan, Italy) was fitted with split injector (split-flow 15 ml/min) and flame ionization detector.

Amino acid analysis

The purified allergen (12 µg protein) was hydrolyzed under vacuum in 6 M HCl at 110°C for 24 hr. After removal of the HCl under reduced pressure, the amino acid composition was determined using a Biotronik (Munich, F.R.G.) LC 5000 automatic amino acid analyzer.

RESULTS AND DISCUSSION

Isolation procedure

Since Art v II (previously denoted Ag7) was bound to Con A in crossed immunoaffinoelectrophoresis (Nilsen and Smestad Paulsen, 1986), affinity chromatography on Con A-Sepharose was an obvious choice as part of the isolation procedure for Art v II. Initial experiments to fractionate crude mugwort pollen extract directly on the Con A-Sepharose column was, however, not successful. Poor resolution was obtained, possibly because of the presence of large amounts of brown pigments in the extract which was adsorbed to the column and thereby disturbed the separation. Thus, a purification step prior to the Con A-Sepharose step was necessary in order to separate the coloured substances and the (glyco)proteins in the crude extract. Several methods were tested and gel filtration on Ultrogel AcA 54, ion-exchange chromatography on DEAE-Sepharose and preparative IEF in Ultradex granulated gel all

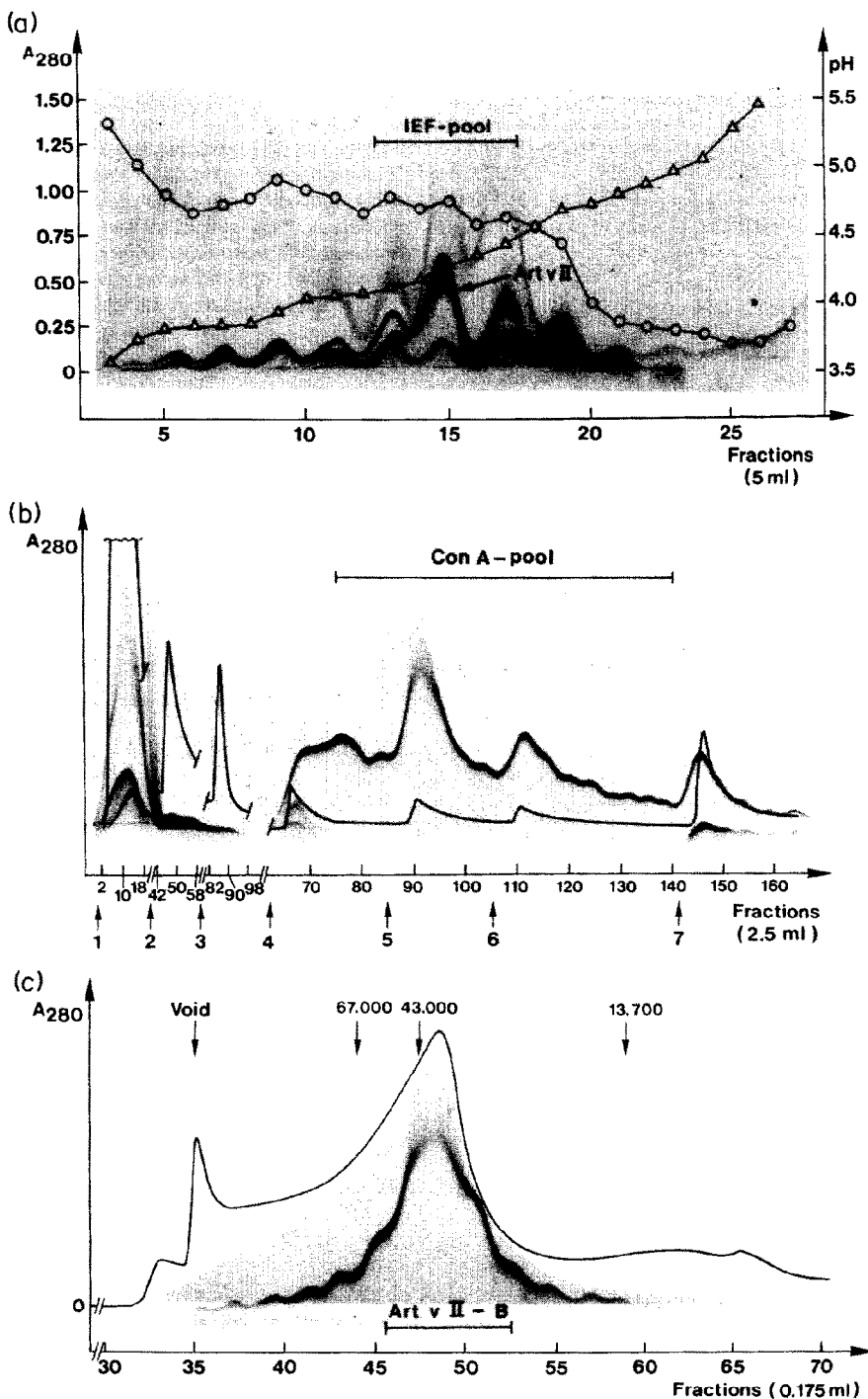


Fig. 1. Isolation of *Art v* II-B. FRIE-patterns with $20 \mu\text{l}/\text{cm}^2$ (a) or $25 \mu\text{l}/\text{cm}^2$ (b, c) anti-mugwort in the antibody-containing gels. (a) FRIE of fractions obtained after preparative IEF (pH gradient 3.5–5) of 1.0 g mugwort pollen extract. $9 \mu\text{l}$ of each fraction (diluted 1:10) was applied. Superimposed: — Δ —: pH-gradient, — $\circ$ —: absorbance at 280 nm. Fractions from seven runs were pooled as indicated (IEF-pool). (b) FRIE of fractions obtained after Con A-Sepharose chromatography of IEF-pool. The column was eluted with (1) 20 mM Tris-buffer and the following eluents in Tris-buffer: (2) 1.0 M NaCl, (3) 20% ethylene glycol, (4) 0.2 M α -MeGlcp, (5) 0.2 M α -MeManp, (6) 0.5 M α -MeManp, (7) 0.5 M α -MeManp, 1.0 M NaCl as indicated by the arrows. $1.5 \mu\text{l}$ of every fourth fraction eluted with eluents 1–3 and every second fraction eluted with eluents 4–7 was applied. Superimposed: —: absorbance at 280 nm. Fractions were pooled as indicated (Con A-pool). (c) FRIE of fractions obtained after HPLC size exclusion of one sixth of Con A-pool. $2 \mu\text{l}$ of each fraction (diluted 1:10) was applied. Superimposed: —: absorbance at 280 nm. Elution volumes of the MW-markers are indicated. Fractions from six runs were pooled as indicated (*Art v* II-B, 1.8 mg protein).

Table 1. Recovery and purification ratio of *Art v* II after each step of the isolation procedure

Fraction	Recovery (%)	Total protein (mg) ^a	Purification ratio ^b
Mugwort pollen extract	100	1300	1.0
IEF-pool	35	130	3.5
Con A-pool	19	4.9	50
<i>Art v</i> II-B	8.2	1.8	58

^aDetermined by amino acid analysis.^bCalculated as:

$$\frac{\text{Total protein in mugwort pollen extract}}{\text{Total protein in fraction recovered in each step}}$$

at equal precipitate height in RIE against anti-*Art v* II.

removed significant amounts of the coloured substances as well as some protein- and carbohydrate-material in the crude extract.

Initially, a purification scheme using ion-exchange chromatography on DEAE-Sephacel Fast Flow as the first purification step, followed by affinity chromatography on Con A-Sephacel at 4°C and ion-exchange chromatography on Mono P, was tried. The *Art v* II-preparation isolated by this method was designated *Art v* II-A. This isolation procedure gave, however, a yield of only 200 µg (protein) *Art v* II from 7.5 g mugwort pollen extract.

To improve the recovery of the isolated allergen, an isolation procedure was developed using preparative IEF as the first step, followed by Con A-Sephacel chromatography at room temperature and HPLC size exclusion on Ultropac TSK G2000SW (Fig. 1). The recovery and purification ratio of *Art v* II after each step in this procedure is given in Table 1. In the preparative IEF-step the Con A-binding allergen *Art v* II was identified by comparing the results from FRIE (Fig. 1a) with the results from a parallel FRIE-experiment with Con A-Sephacel in the intermediate gel as previously described (Nilsen and Smestad Paulsen, 1986). *Art v* II was detected in several fractions in the pH region 4.05–4.65, but fractions with pH 4.10–4.50 contained the major amount of *Art v* II and these fractions were pooled in IEF-pool (Fig. 1a). The subsequent Con A-Sephacel chromatography (Fig. 1b) was performed at room temperature instead of at 4°C since preliminary experiments showed that increasing the temperature led to increased recovery of *Art v* II. Extra washing steps with 1 M NaCl and 20% ethylene glycol in the buffers were included to remove nonspecifically bound material (including significant amounts of coloured substances) before specific elution with α-MeGlcp and α-MeManp was started (Dulaney, 1979). During elution of specifically bound material the flow was stopped for 45 min after applying each of the replacing sugar eluents since preliminary experiments demonstrated that the recovery was increased if the contact time between column and eluent was increased, an effect which may be

explained by a slow rate of dissociation of the Con A-*Art v* II-complex (Dulaney, 1979). The specifically bound *Art v* II was recovered both in fractions eluted with α-MeGlcp and in fractions eluted with α-MeManp (Fig. 1b) in accordance with previous results from CIE with Con A-Sephacel in the intermediate gel (Nilsen and Smestad Paulsen, 1986) where multiple forms of *Art v* II with varying capability to interact with Con A was demonstrated. *Art v* II-containing fractions were pooled as indicated in Fig. 1b (Con A-pool). In addition to the *Art v* II recovered in Con A-pool (Table 1), less than 5% *Art v* II was eluted in the unbound fraction, approximately 9% was eluted in the early α-MeGlcp fractions not included in the pool and approximately 6% was recovered in the fractions eluted with α-MeManp and NaCl. In the final purification step on Ultropac TSK G2000SW (Fig. 1c) *Art v* II was eluted in the MW range 27,000–50,000 with a peak value of 39,000. The *Art v* II-containing fractions were pooled as indicated in the figure and the allergen preparation obtained by this method was denoted *Art v* II-B. The purification ratio for *Art v* II-B (Table 1) indicate that *Art v* II constitutes only 1.7% of the total protein in mugwort pollen extract.

The recovery of *Art v* II in this isolation procedure was still relatively low (8.2%, Table 1) which may be due to repeated dialysis of dilute solutions and problems with low solubility of some of the fractions after lyophilization.

Purity of the isolated fractions

CIE (Fig. 2a) and CRIE of *Art v* II-A gave only one precipitate corresponding to *Art v* II, whereas scanning of a Coomassie-stained SDS-PAGE gel (results not shown) indicated that *Art v* II-A contained about 10% contaminating protein. Results from HPLC size exclusion (Fig. 4a) also indicated that *Art v* II-A contained some minor contaminants. However, the peaks (a) and (b) in the HPLC chromatogram (Fig. 4a) may be due to aggregate formation of *Art v* II, since other experiments in our laboratory (unpublished results) indicate that purified *Art v* II has a tendency to aggregate under the conditions used for HPLC. The MW of the material in peak (b) (72,000) is consistent with an aggregate of two *Art v* II-molecules (four *Art v* II-subunits; see discussion below). The material in peak (a) is eluted in the void volume of the column and may thus contain higher MW aggregates of *Art v* II.

CIE/CRIE of *Art v* II-B (Figs 2c, f) revealed the presence of a minor contaminating antigen. The apparent IgE-binding ability of this antigen may be caused by coprecipitation of *Art v* II in its precipitation arc. SDS-PAGE of *Art v* II-B revealed only one broad band (Fig. 3) and HPLC size exclusion (Fig. 4b) gave one peak constituting 92% of the UV-absorbing material.

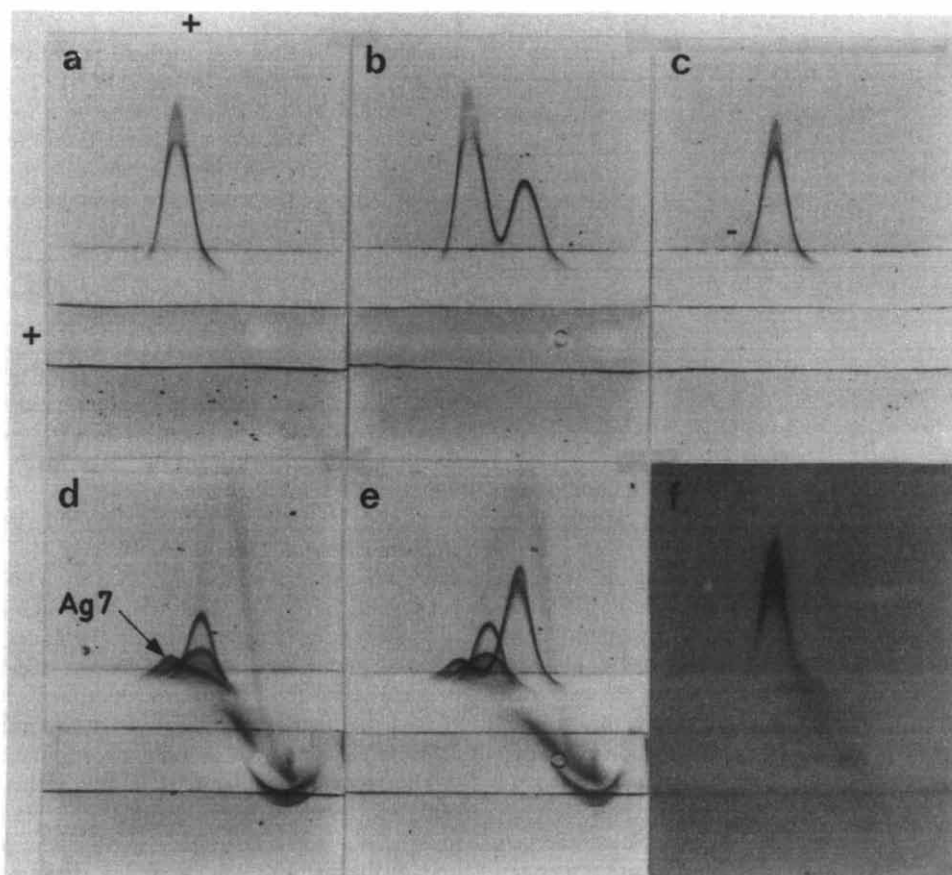


Fig. 2. CIE, CRIE and tandem-CIE of the purified allergen preparations. (a) CIE of *Art v* II-A. (b) Tandem-CIE of *Art v* II-A (left) and *Art v* II-B (right), (c) CIE of *Art v* II-B, (d) CIE of mugwort pollen extract, (e) Tandem-CIE of mugwort pollen extract (left) and *Art v* II-B (right) and (f) CRIE of *Art v* II-B (5 days autoradiography). 0.35 μ g (protein) *Art v* II-A/B and 52 μ g (8.5 μ g protein) mugwort pollen extract were applied and 30 μ l/cm² anti-mugwort antiserum was included in the antibody-containing gels. The anode was positioned as indicated in (a).

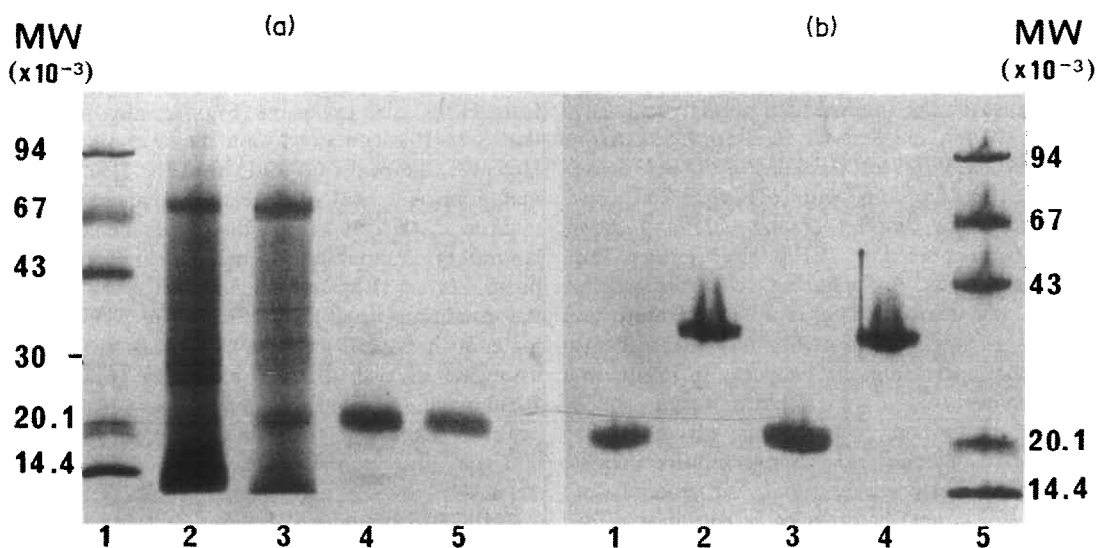


Fig. 3. (a) SDS-PAGE (PhastGel Gradient 10–15) of fractions recovered after each purification step. Lane 1: Molecular weight references, lane 2: mugwort pollen extract, 650 ng protein; lane 3: IEF-pool, 350 ng protein; lane 4: Con A-pool, 220 ng protein; lane 5: *Art v* II-B, 120 ng protein. The gel was stained with PhastGel Silver Kit. (b) SDS-PAGE (PhastGel Gradient 10–15) of the purified allergen prepared with or without mercaptoethanol (ME) in the sample buffer. Lane 1: *Art v* II-B (120 ng protein) with ME; lane 2: *Art v* II-B without ME; lane 3: *Art v* II-A (120 ng protein) with ME; lane 4: *Art v* II-A without ME; lane 5: molecular weight references. The gel was stained with PhastGel Silver Kit.

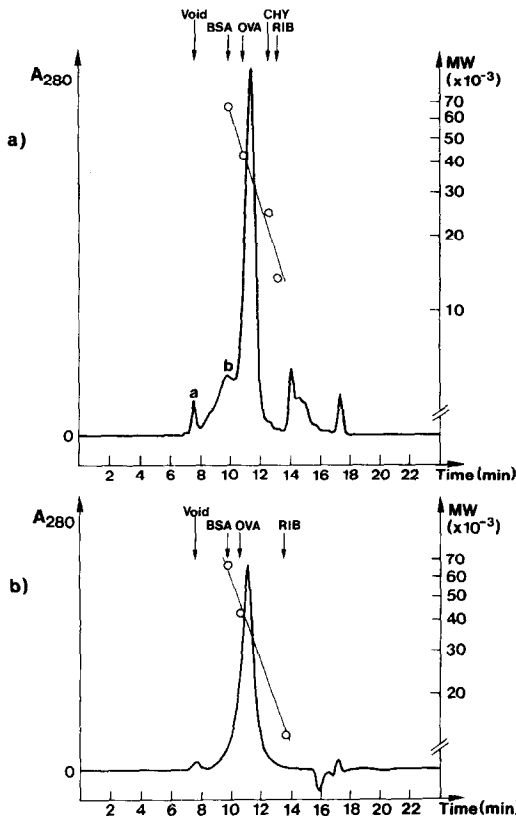


Fig. 4. HPLC size exclusion of the purified allergen preparations. (a) *Art v* II-A (5 µg protein) and (b) *Art v* II-B (3 µg protein). The elution volumes of the molecular weight references (BSA: 67,000, OVA: 43,000, CHY: 25,000 and RIB: 13,700) are indicated.

Immunochemical characterization of *Art v* II-A and *Art v* II-B

Tandem-CIE of *Art v* II-A and *Art v* II-B demonstrated that the two allergen preparations were antigenically identical (Fig. 2b). Tandem-CIE of mugwort pollen extract and *Art v* II-B (Fig. 2e) verified that the isolated allergen was identical with the glycoprotein allergen we have previously denoted Ag7 (Fig. 2d). This allergen is probably identical with Ag 3 in the study of Ipsen *et al.* (1985 and Ipsen, personal communication). CRIE of *Art v* II-B (Fig. 2f) demonstrated that the isolated allergen had retained its IgE-binding ability.

For classification of *Art v* II as an allergen, R(R)IE of *Art v* II-A was performed with a panel of 40 patient sera. The patient sera were selected as described under materials and methods and results with a selection of sera are shown in Fig. 5. Figures 5a-c, e and f show examples of sera giving specific radiostaining (i.e. radiostaining with an intensity greater than the negative controls) and Figs 5d, g show examples of sera giving only nonspecific radiostaining of the precipitate of *Art v* II-A. The results showed that 5 (33%) of the 15 sera from patients with clinical mugwort allergy and 13 (52%) of the 25 RAST-class 4 patient sera gave specific radiostaining

of the precipitate of *Art v* II-A. This indicates that in total about 45% of the mugwort allergic patients have specific IgE against *Art v* II and that *Art v* II can be classified as an intermediate allergen. These results are significantly lower than the results obtained in our earlier study where 92% of the patient sera gave visible radiostaining of the precipitate of Ag7 (*Art v* II) in CRIE of mugwort pollen extract (Nilsen and Smestad Paulsen, 1986). This discrepancy may have several explanations, one being differences in the selection of patient sera in the two studies. With the relatively low number of patients used in these studies, the patient selection becomes a critical parameter as can be seen from the different results obtained with the two groups of patient sera used in the present study. Another explanation may be partial loss of some allergenic epitopes during the isolation of *Art v* II, e.g. because of selective removal of some of the molecular forms of *Art v* II with different pI (see discussion below) or with different carbohydrate structures (Nilsen and Smestad Paulsen, 1986). Finally, the results in Figs 5h, i which demonstrate that nonspecific radiostaining of *Art v* II may occur, point to the possibility that the number of patient sera containing specific IgE against *Art v* II was overestimated in our earlier study, since such negative controls were not included then.

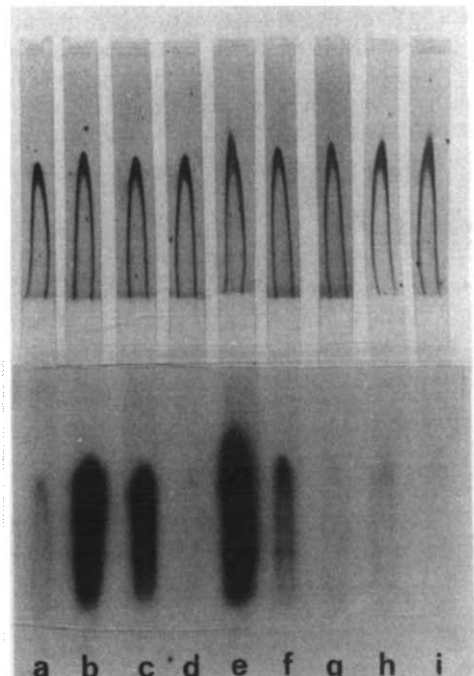


Fig. 5. R(R)IE of the purified allergen (*Art v* II-A) with a selection of patient sera. 0.2 µg samples of *Art v* II-A was applied in RIE with 35 µl/cm² anti-mugwort in the antibody-containing gel. The individual rockets were incubated with: (a)–(d) clinical patient sera, (e)–(g) RAST class 4 patient sera, (h) 0.9% NaCl and (i) serum from a non-allergic individual, followed by incubation with ¹²⁵I-anti IgE and autoradiography (4 days). On the top are shown the Coomassie brilliant blue-stained rockets, on the bottom the corresponding autoradiographs.

Table 2. Physicochemical parameters of the purified allergen			
pI	4.10, 4.20, 4.35		
	4.45, 4.55, 4.65, (4.80)		
MW, HPLC size exclusion: <i>Art v</i> II-A	34,000		
	<i>Art v</i> II-B		
MW, SDS-PAGE: with reducing agent	20,000		
	without reducing agent		
Carbohydrate/Protein ^a	35,000		
	1/10		

^aCarbohydrate and protein contents were determined by monosaccharide composition analysis and amino acid composition analysis, respectively.

Physicochemical characterization of Art v II-A and Art v II-B

The MW of the purified allergen (Table 2) was estimated both by SDS-PAGE (Fig. 3) and by HPLC size exclusion (Fig. 4). HPLC gave slightly different results for the two allergen preparations with estimated MWs of about 34,000 for *Art v* II-A and about 38,000 for *Art v* II-B.

SDS-PAGE of *Art v* II-A/B gave one band with an apparent MW of about 35,000 under nonreducing conditions and one band with an apparent MW of about 20,000 when the sample was reduced with 2-mercaptoethanol (Fig. 3b). These results indicate that *Art v* II is composed of two identical or nearly identical subunits covalently linked by one or more disulfide bond(s). The discrepancy between the MW of 35,000 for the unreduced allergen which would give a MW of 17,500 for two identical subunits and the observed MW of 20,000 for the reduced *Art v* II may be explained by the fact that the MW of the unreduced allergen was estimated on the basis of a

standard curve made from reduced standard proteins. This may lead to an underestimation of the MW of the unreduced allergen (Lambin, 1978). On the other hand, the MW of glycoproteins is often overestimated in SDS-PAGE (Leach *et al.*, 1980). It is therefore likely that the MW of *Art v* II is about 35,000, which is in agreement with the results from gel filtration, and that the MW of the subunit is less than 20,000.

Staining of the SDS-PAGE gel with periodic acid-Schiff reagent (Zacharius *et al.*, 1969) verified the glycoprotein nature of *Art v* II.

Analytical IEF of *Art v* II-B (Fig. 6b) gave six clearly visible bands and one very faint band in the pI region 4.10–4.80. Crossed immuno-IEF (Fig. 6a) demonstrated antigenic identity between all these bands indicating that *Art v* II consists of 6 (7) isoforms with pI 4.10, 4.20 (major component), 4.35, 4.45, 4.55, 4.65, (4.80). These results are in agreement with earlier results from crossed immuno-IEF of a partly purified extract of mugwort pollen where Ag7 (*Art v* II) gave a non-symmetric precipitate in the pI-region 4.10–4.65 with a peak value at 4.3 (Nilsen and Smestad Paulsen, 1986). pI-heterogeneity has earlier been demonstrated in a number of allergens including the pollen allergens AgE from ragweed (King, 1972; King *et al.*, 1974), Group I allergen from rye-grass (Marsh, 1975; Howlett and Clarke, 1981) and Ags 19, 23 and 25 from birch (Ipsen and Løwenstein, 1983). The observed pI-heterogeneity of *Art v* II may be caused by deamidation of the amino acid residues asparagine and glutamine as first

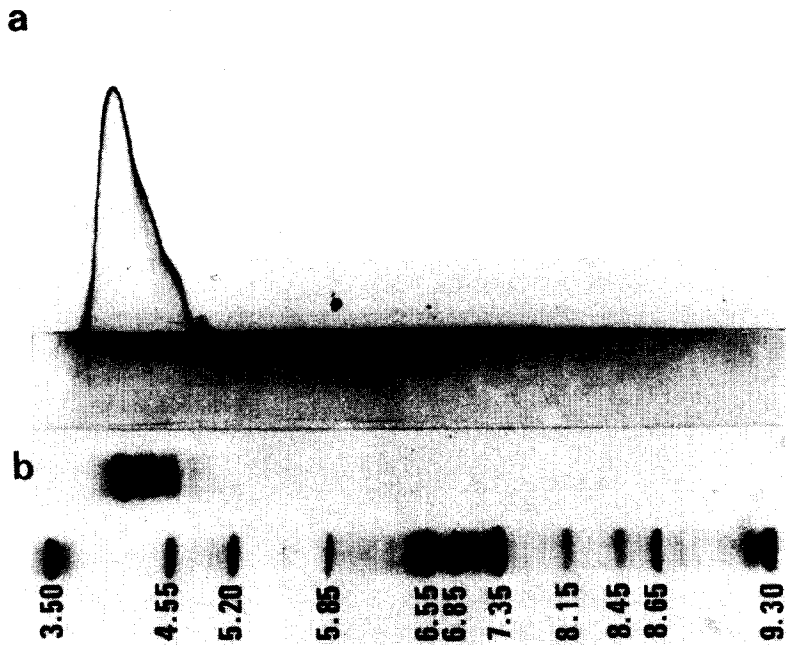


Fig. 6. Analytical IEF and crossed immuno-IEF of the purified allergen (*Art v* II-A). (a) Crossed immuno-IEF of 1.7 μ g *Art v* II-A with 50 μ l/cm² anti-mugwort in the antibody-containing gel. (b) IEF in pH-gradient 3.5–10 of 9.6 μ g *Art v* II-A (top) and calibration proteins for pI determination (bottom). The pI of the calibration proteins are indicated.

demonstrated for cytochrome c (Flatmark, 1966; Flatmark and Sletten, 1968). It is less likely that the pI-heterogeneity is caused by heterogeneity in the carbohydrate moiety since monosaccharide composition analysis revealed only neutral monosaccharides, but further structural analysis of the allergen is necessary to determine the cause of this phenomenon. The presence of several isoforms with different pI may explain why *Art v* II gives an asymmetric precipitate in CIE. However, the purified allergen preparations (Figs 2a, c) gave more symmetric CIE-precipitates of *Art v* II than the crude extract (Fig. 2d). This may reflect differences in the relative amount of the different isoforms of *Art v* II in crude extract and in the purified allergen preparations indicating that some of the isoforms have been selectively removed during the purification process.

The protein to carbohydrate ratio of the purified allergen was found to be 10:1 as estimated by amino acid analysis and monosaccharide composition analysis of *Art v* II-A.

Amino acid analysis of *Art v* II-A (Table 3) revealed a relatively low content of the basic amino acids lysine and arginine, consistent with the observed pI of the allergen in the region 4.10–4.80. The molecule contains only 0.6% phenylalanine and at least 4.6% half-cystine which calculated on the molecular basis will give at least 14 residues half-cystine per allergen molecule. This indicates a possibility for at least seven intra- or interchain disulfide bridges in the *Art v* II-molecule.

Monosaccharide composition analysis demonstrated that the allergen contained mannose, *N*-acetyl-glucosamine, galactose and glucose (Table 4). The estimated number of mannose and *N*-acetylglucosamine residues per *Art v* II-molecule combined with the observed high affinity of this allergen for Con A, a lectin with a high affinity for high-mannose oligosaccharides

Table 4. Monosaccharide composition of *Art v* II-A determined by GLC of TMSi-methylglycosides^a

Monosaccharide	Residues/100 residues	Number of residues per <i>Art v</i> II-molecule ^b
Mannose	70.7	13.4 (13)
<i>N</i> -acetyl-glucosamine	17.0	3.2 (4)
Glucose	7.0	1.3 (1)
Galactose	5.3	1.0 (1)
Total	100.0	18.9 (19)

^aData from one analysis.

^bAssuming a MW of 35,000.

(Ohyama *et al.*, 1985) suggests that *Art v* II may contain *N*-linked high-mannose oligosaccharide chains. High-mannose oligosaccharide chains have been found in numerous animal glycoproteins and during the last decade this type of oligosaccharide chains have also been demonstrated in an increasing number of plant glycoproteins (Kornfeld and Kornfeld, 1985; Faye *et al.*, 1989). On the basis of monosaccharide composition and lectin-binding, it has been proposed that the carbohydrate part of the allergen Glycoprotein 2 from rye-grass pollen contain a related structure (Howlett and Clarke, 1981). Further structural analysis of the carbohydrate part of *Art v* II will be published elsewhere (Nilsen *et al.*, manuscript in preparation).

Comparison of *Art v* II and other purified allergens from *Artemisia vulgaris* and *Artemisia scoparia*

De la Hoz *et al.* (1989) have recently reported the purification and characterization of an allergen, *Art v* I, from mugwort pollen. The information given about the physicochemical parameters of this allergen is unfortunately limited and it is not reported whether *Art v* I is a glycoprotein. The pI of *Art v* I (4.4) is within the pI region we find for the allergen purified in this study but no pI-heterogeneity was reported for *Art v* I. Since many of the allergens in mugwort pollen extract seem to have pIs between 4 and 5 (unpublished observations) this is not a reliable parameter for distinguishing between the allergens. However, the MW of *Art v* I (47,000 by HPLC size exclusion and 60,000 by SDS-PAGE) is significantly different from the MW of the allergen purified in the present study. The MW of *Art v* I determined by SDS-PAGE was also independent of treatment of the sample with reducing agents. It is therefore most likely that the allergen described in our study is different from *Art v* I and we propose to designate this allergen *Art v* II according to the allergen nomenclature system recommended by the International Union of Immunological Societies (IUIS) (Marsh *et al.*, 1986).

Jaggi and Gangal (1987) have reported the isolation of a glycoprotein allergen, denoted Art VI b, from pollen of *Artemisia scoparia* and the authors suggest that this allergen is similar to *Art v* II (Ag7) from pollen of *Artemisia vulgaris*. The results in the present study do not confirm such a similarity since most of the physicochemical parameters of Art VI b (protein/carbohydrate ratio: 5.80/1, MW determined by gelfiltration: 14,300, pI: 3.80) differ from the physicochemical parameters we find for *Art v* II.

Table 3. Amino acid composition of *Art v* II-A^a (vacuum hydrolysis in 6 M HCl at 110°C for 24 h)^b

Amino acid	Residues/100 residues	Calculated residues per <i>Art v</i> II-molecule ^c
Asx	16.3	47.9 (48)
Thr	4.7	13.8 (14)
Ser	5.7	16.8 (17)
Glx	12.0	35.2 (35)
Pro	6.1	17.9 (18)
Gly	10.8	31.8 (32)
Ala	8.1	23.8 (24)
1/2 Cys	4.6	13.5 (14)
Val	5.1	15.0 (15)
Met	1.8	5.3 (5)
Ile	2.6	7.6 (8)
Leu	5.5	16.2 (16)
Tyr	5.0	14.7 (15)
Phe	0.6	1.8 (2)
His	3.2	9.4 (9)
Lys	3.6	10.6 (11)
Arg	4.4	12.9 (13)
Total	100.1	294.1 (296)

^aThe content of tryptophan could not be determined under these conditions.

^bData from one analysis.

^cAssuming a MW of 35,000 and a carbohydrate content of 9.2% (carbohydrate/protein: 1/10).

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