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Purification of the glycoprotein allergen Ag7 from mugwort pollen by concanavalin A affinity chromatography

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Summary

Two affinity columns comprising immobilized concanavalin A (Con A), Con A-Sepharose and Con A-XP3507, were evaluated for their purifying ability for the glycoprotein allergen Ag7 from a partially purified extract of mugwort pollen. The most pronounced difference between the two columns was the nature of their nonspecific interactions; hydrophobic interactions were dominant with Con A-XP3507, whereas ionic interactions were dominant with Con A-Sepharose. Both Con A-columns were effective for purifying Ag7 with a recovery of 50% after specific elution with displacing sugars. The inclusion of 1.0 M NaCl and 20% ethylene glycol in the elution medium was useful for desorbing nonspecifically bound material, prior to specific elution of adsorbed Ag7 in the presence of the displacing sugars, α -methyl glucoside and α -methyl mannoside. The most efficient purification of Ag7 was achieved with Con A-Sepharose at room temperature rather than at 4°C. Affinity chromatography with Con A-XP3507 resulted in a slightly more contaminated product (purity 54%) than with Con A-Sepharose (purity 64%).

Affinity chromatography; Concanavalin A; Glycoprotein allergen; Mugwort pollen

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Introduction

Pollen of mugwort (*Artemisia vulgaris* L.) is an important cause of allergic airway disease in Northern Europe (Charpin et al., 1974; Spieksma et al., 1980). It has been demonstrated by crossed radioimmuno-electrophoresis (CRIE) that extracts of mugwort pollen contain 8–10 different allergens of variable clinical importance (Ipsen et al., 1985; Smestad Paulsen et al., 1985; Dreborg et al., 1987). One of the important allergens of this pollen is a glycoprotein allergen designated antigen 7 (Ag7). This allergen can easily be identified by immunoaffino-electrophoresis because of its ability to bind the lectin concanavalin A (Con A) (Nilsen and Smestad Paulsen, 1986).

The isolation of pure glycoprotein allergens is a necessary prerequisite for the elucidation of their complete chemical structure which may contribute to our understanding of why some molecules act as allergens. Many different methods have been described for the isolation of pollen allergens. Nevertheless, in most cases the isolation of significant quantities has been a difficult task because of the low amount of allergens present in the pollen and the use of complicated multistep purification procedures (Haavik and Nilsen, 1987). Therefore, it has been proposed that more selective isolation methods such as affinity chromatography should be applied whenever possible (Baldo et al., 1981). Lectin affinity chromatography has been used to purify glycoprotein allergens from many sources including pollen (Haavik et al., 1985; Karlstam and Nilsson, 1982; Corbi and Carreira, 1985), mites (Baldo and Uhlenbruck, 1977) and cow's hair and dander (Bøg-Hansen et al., 1978). Since Ag7 binds to Con A in immunoaffino-electrophoresis (Nilsen and Smestad Paulsen, 1986), we have investigated the possibility of integrating Con A affinity chromatography in the isolation procedure for Ag7 in order to create more efficient and effective purification methods. In accomplishing our task we have also evaluated the separation characteristics of two affinity Con A-columns, (1) the agarose-based Con A-Sepharose and (2) the experimental product Con A-XP3507 where Con A is coupled to the monodisperse acrylic particles XP3507. The material used for the affinity chromatographic studies was a partially purified extract prepared by preparative isoelectric focusing of an extract from mugwort pollen.

Experimental

Materials

Mugwort (*Artemisia vulgaris*) pollen was obtained from Allergon AB (Engelholm, Sweden, batch no. 010182101). Con A-Sepharose (particle size: 45–165 μm , 10–16 mg Con A per ml gel sediment) was obtained from Pharmacia (Uppsala, Sweden). XP3507 (particle size: 20 μm) was kindly provided by Dyno Particles AS, Lillestrøm, Norway. Calibration proteins for molecular weight determination were obtained from Pharmacia (Uppsala, Sweden), agarose immuno-electrophoresis tablets with tricine buffer (pH 8.60) from Bio-Rad Labs (Richmond, CA, U.S.A.), Coomas-

sie brilliant blue R-250 from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and reagents for preparative isoelectric focusing from LKB (Bromma, Sweden). The other chemicals used were analytical-reagent grade and obtained from Merck (Darmstadt, F.R.G.).

Rabbit antibodies

Rabbit antibodies against crude mugwort pollen extract (anti-mugwort anti-serum) were a gift from Pharmacia (Uppsala, Sweden, batch no. LJ 327520 840417 UK).

Rabbit antibodies against the glycoprotein allergen Ag7 (anti-Ag7 antiserum) were prepared by immunization with immunoprecipitates of the allergen obtained by crossed immunoelectrophoresis (CIE) essentially as described by Harboe and Closs (1983). Two different batches of Anti-Ag7 (Anti-Ag7 I and Anti-Ag7 II) with different titer were used in this study.

Immunoelectrophoretic methods

Rocket immunoelectrophoresis (RIE) and fused rocket immunoelectrophoresis (FRIE) were performed essentially as described by Axelsen and Bock (1983) and Harboe and Just Svendsen (1983), respectively. In FRIE, samples from the columns (amounts specified in the figure legends) were allowed to diffuse for 30 min before electrophoresis was started. FRIE with Con A-Sepharose corresponding to about 0.2 mg Con A per cm^2 in the intermediate gel was performed as described by Bøg-Hansen (1983). The electrophoresis was performed at 2 V cm^{-1} for 14–17 h into 1% agarose gel containing 25 mM tricine buffer (pH 8.60) and 20–30 $\mu\text{l cm}^{-2}$ of anti-mugwort (FRIE) or 44 $\mu\text{l cm}^{-2}$ of anti-Ag7 antiserum (RIE). After electrophoresis the gel plates were washed and stained with Coomassie brilliant blue.

Preparation of a partially purified extract (IEF-pool) from mugwort pollen

Crude extract of mugwort pollen was prepared essentially as previously described (Nilsen and Smestad Paulsen, 1986). Pollen, (150 g) was extracted with 3 l 75 mM phosphate buffer-NaCl, pH 7.2, containing 2% n-butanol and 5 mM thiourea for 1 h at 4°C . After removal of the pollen by filtration using glass fibre filter paper and suction, the extract was dialyzed (cut off 12 kDa) against $9 \times 7 \text{ l}$ distilled water containing 5 mM thiourea the first 24 h, total dialyzing time was approximately 70 h. The extract was lyophilized (5.3 g).

The crude extract (1.2 g) was purified by preparative isoelectric focusing in a pH gradient 3.5–5 as described elsewhere (Nilsen and Smestad Paulsen, 1990). The glycoprotein allergen Ag7 was detected in fractions with pH in the range from 4.1 to 4.5. These fractions were pooled (120 ml), dialyzed (cut off 12 kDa) against $10 \times 2000 \text{ ml}$ distilled water and lyophilized. The material thus obtained was designated IEF-pool (72 mg).

Preparation of Con A-XP3507

A portion of particles (approximately 2 g sucked-dry weight) was washed on a sintered glass funnel (No. 4) with 200 ml each of water/acetone (1 : 1, v/v), acetone and dry acetone (< 0.01% water content), and sucked dry on the funnel. The dried particles were immediately transferred to a 50 ml flask followed by addition of 16 ml dry acetone and 0.5 g 1,1'-carbonyldiimidazole, and sonicated for 1 min. The reaction was left for 30 min with shaking at room temperature. The activated particles (imidazole carbamate-XP3507) were washed with dry acetone (200 ml), sucked dry and transferred to a 50 ml flask. 8 ml phosphate buffer-NaOH, 50 mM, pH 7.0, containing 100 mg Concanavalin A (Con A, Sigma C-7275) was added to 2 g of the activated particles. The suspension was left for 24 h with gentle shaking at room temperature. The Con A-XP3507 was washed with 200 ml each of cold phosphate buffer and buffer containing 1 M NaCl. The Con A-XP3507 contained 16 mg Con A per g gel, determined by measuring the difference in optical absorption (A_{280}) between the washings recovered after the coupling reaction, and the starting protein solution. The particles were stored at 4°C in 0.02% sodium azide.

Affinity chromatography on Con A-Sepharose and Con A-XP3507

Affinity chromatography of IEF-pool was performed with 0.5 ml (6.5×10 mm ID) columns of Con A-Sepharose and Con A-XP3507 at 22°C. The columns were packed by sedimentation. The adsorption capacities of the two different columns for Ag7 from the IEF-pool, were determined as follows: the affinity column was equilibrated in Tris-buffer (Tris-HCl 20 mM, MnSO_4 1 mM, CaCl_2 1 mM, NaN_3 0.02%, pH 7.4) and the test solution consisted of 2 mg ml⁻¹ of the Ag7-containing IEF-pool in Tris-buffer as above. Portions each of 0.5 ml were applied on the column in such a manner that as soon as the first aliquot had just entered into the column, the next portion was applied. Fractions (0.5 ml) were collected at a flow rate of 3 ml min⁻¹ and 15 µl of every third fraction was subjected to RIE against anti-Ag7 antiserum. After having applied a total of 22 mg (11 ml) IEF-pool on each column, the remaining unbound and non-specifically bound material was eluted with: (I) 5 ml Tris-buffer (as above), (II) 5 ml Tris-buffer-NaCl (1 M NaCl in Tris-buffer) and (III) 5 ml Tris-buffer-ethylene glycol (20% ethylene glycol in Tris-buffer) at a flow rate of about 6 ml h⁻¹. After removal of residual ethylene glycol from the columns by washing with 2 ml Tris-buffer, specifically bound material was eluted sequentially at a flow rate of approximately 5 ml h⁻¹ with (a) 3 ml 0.2 M α -methyl glucoside in Tris-buffer, (b) 3 ml 0.2 M α -methyl mannoside in Tris-buffer, (c) 3 ml 0.5 M α -methyl mannoside in Tris-buffer, (d) 3 ml 0.5 M α -methyl mannoside in Tris-buffer-NaCl and (e) 3 ml 0.5 M α -methyl mannoside in Tris-buffer-ethylene glycol. The flow was then stopped for 17 h before elution was continued at the same flow rate with (f) 5 ml 0.5 M α -methyl mannoside in Tris-buffer.

All fractions (1 ml) collected throughout the elution were assayed for UV-absorbing material at 280 nm and for mugwort antigens by FRIE against anti-mugwort antiserum. Con A-binding antigens eluted with Tris-buffer, Tris-buffer-NaCl and Tris-buffer-ethylene glycol were detected by FRIE with Con A-Sepharose in the intermediate gel as previously described (Nilsen and Smestad Paulsen, 1986).

Aliquots of the fractions eluted from the affinity columns were divided into three different pools: (a) pool of fractions eluted without displacing sugar (unbound and non-specifically bound material), (b) pool of fractions eluted with displacing sugar (specifically bound material) and (c) pool of all fractions eluted from the column. The recovery of Ag7 in all pools was determined by RIE against anti-Ag7 antiserum, comparing the pools with reference samples from the solution of IEF-pool applied on the columns.

Fractions eluted from the affinity columns with 0.2 M α -methyl glucoside, 0.2 M α -methyl mannoside and 0.5 M α -methyl mannoside in Tris-buffer were pooled (9 ml), dialyzed (cut off 3.5 kDa) against 4 $\times$ 500 ml distilled water and lyophilized. These fractions were designated as Con A-Sepharose-pool and Con A-XP3507-pool, respectively.

To investigate the effect of temperature on the elution profile and recovery of Ag7 from Con A columns, an identical experiment as that described above was performed at 4°C.

Gel filtration on Ultropac TSK G2000SW

Gel filtration of Con A-Sepharose-pool and Con A-XP3507-pool was performed on Ultropac TSK G2000SW (300 $\times$ 7.5 mm ID) (LKB, Bromma, Sweden) using a Shimadzu LC-6A pump (Shimadzu Corp., Kyoto, Japan) and a Shimadzu SPD-6A UV detector. The elution profile at 280 nm was recorded and integrated with Shimadzu CR6A Chromatopac. Fractions (0.35 ml) were collected with Pharmacia FRAC 100 fraction collector. Samples of 20 μ l containing approximately 6 μ g protein were injected. The column was eluted at 0.7 ml min⁻¹ with 0.1 M sodium phosphate buffer, pH 6.8, and collected fractions were assayed for mugwort antigens by FRIE.

The column was calibrated with bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa). The void volume was determined with thyroglobulin (669 kDa).

Results and Discussion

Adsorption capacity of the affinity columns for the allergen Ag7

The adsorption capacity of Con A-Sepharose and Con A-XP3507 for Ag7 from the IEF-pool was studied by frontal analysis and eluted fractions were assayed for Ag7 by rocket immunoelectrophoresis (RIE) against anti-Ag7 antiserum. A plateau was reached for both adsorbents when approx. 13 mg IEF-pool (26 mg IEF-pool per

ml adsorbent, 3.6 mg total protein per ml adsorbent) had passed through the columns. The concentration of Ag7 in the eluate at the plateau was approx. 6% and 3% of the concentration of Ag7 in the solution of IEF-pool applied on Con A-Sepharose and Con A-XP3507, respectively. These results may indicate that the Ag7 eluted at the plateau is a low-affinity form which constitutes only a minor fraction of the total Ag7 present in IEF-pool, and that only the capacity for this low-affinity form of Ag7 was exceeded in this study. The existence of at least two forms of Ag7 with varying capability to interact with Con A has previously been demonstrated (Nilsen and Smestad Paulsen, 1986). Since the plateau was reached when the same amount of IEF-pool had been applied on both columns, these results indicate that the two affinity adsorbents have the same capacity for the low-affinity form of Ag7.

The capacity of the adsorbents for the high-affinity form(s) of Ag7 was not exceeded even when 22 mg IEF-pool had been applied on each column (6 mg total protein per ml gel). Thus no comparison can be made between the capacity of the two adsorbents for the high-affinity forms of Ag7.

Nonspecific adsorption effects of the affinity columns

Nonspecific interactions between Con A and different proteins and glycoproteins in Con A-affinity chromatography have been demonstrated previously (Haavik et al., 1985; Dulaney, 1979).

Fig. 1 shows the A_{280} elution profile of IEF-pool from the two affinity columns, and fused rocket immunoelectrophoresis (FRIE) of the fractions is shown in Fig. 2. The results obtained demonstrated that NaCl eluted only small amounts of UV-absorbing and antigenic material from the Con A-XP3507 column, while a significant amount of nonspecifically adsorbed material was eluted from the Con A-Sepharose column under these conditions. Successive elution with ethylene glycol eluted a sharp peak from the Con A-XP3507 column and a smaller peak from the Con A-Sepharose column. Therefore, the nonspecific adsorption on Con A-XP3507 seems to be primarily due to hydrophobic interactions, while ionic interactions are most important for Con A-Sepharose. As judged from Figs. 1 and 2 the total nonspecific adsorption seems to be of the same order of magnitude for both columns.

Elution and purification of bound Ag7

Components bound to the Con A-columns were eluted with different concentrations of the displacing sugars α -methyl glucoside and α -methyl mannoside in Tris-buffer (Figs. 1a-c and 2a-c). FRIE of the collected fractions (Fig. 2) demonstrated that Ag7 behaved similarly on the two Con A columns. The major fraction of Ag7 was eluted with 0.2 M α -methyl glucoside and 0.2 M α -methyl mannoside from both columns. Further elution with 0.5 M α -methyl mannoside did not result in elution of further peaks. However, Ag7 is not the only antigen eluted under these conditions, at least two contaminating antigens are present and these contaminating

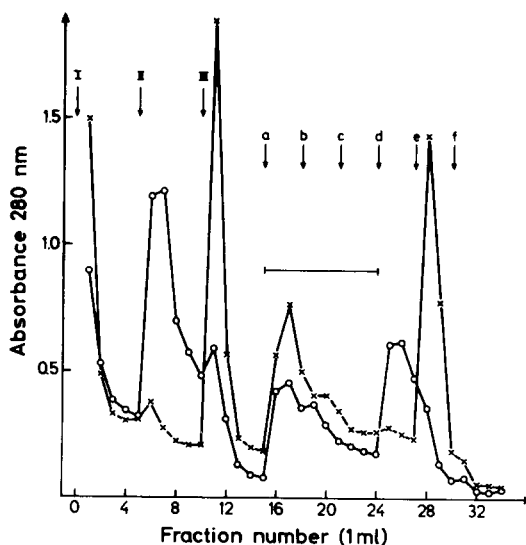


Fig. 1. Affinity chromatography at room temperature of IEF-pool on Con A-XP3507 ($\times$ — $\times$) and Con A-Sepharose ($\circ$ — $\circ$). The columns were eluted with: (I) Tris-buffer, (II) Tris-buffer-NaCl, (III) Tris-buffer-ethylene glycol, followed by: (a) 0.2 M α -methyl glucoside, (b) 0.2 M α -methyl mannoside, (c) 0.5 M α -methyl mannoside (all in Tris-buffer), (d) 0.5 M α -methyl mannoside in Tris-buffer-NaCl, (e) 0.5 M α -methyl mannoside in Tris-buffer-ethylene glycol and (f) 0.5 M α -methyl mannoside in Tris-buffer after the flow had been stopped for 17 h. The horizontal bar indicates fractions pooled in Con A-XP3507-pool and Con A-Sepharose-pool, respectively.

antigens seem to be present in higher amounts in the fractions obtained from Con A-XP3507 than in the fractions from Con A-Sepharose. The higher amount of contaminating material is also reflected in the higher absorbance at 280 nm of the fractions from Con A-XP3507 (Fig. 1a-c).

Addition of 1.0 M NaCl to the α -methyl mannoside eluent resulted in elution of a further minor peak from Con A-XP3507 and a major peak from Con A-Sepharose (Fig. 1d), while inclusion of 20% ethylene glycol in the α -methyl mannoside eluent gave rise to a major peak from Con A-XP3507 and only a minor peak from Con A-Sepharose (Fig. 1e). This elution pattern is consistent with the results discussed above for nonspecific adsorption to the columns. However, examination of the FRIE-pattern (Fig. 2d, e) reveals that these peaks seen in the A_{280} profile (Fig. 1d, e) are not due to elution of increased amounts of Ag7, but rather to elution of increased amounts of one of the contaminating antigens. These results indicate that contrary to the results obtained for some other glycoproteins (Dulaney, 1979) inclusion of NaCl or ethylene glycol in the buffer when purifying Ag7 on Con A-columns would be disadvantageous. This is because elution of Ag7 is not significantly influenced by addition of these reagents, while contaminating material is eluted to a greater extent.

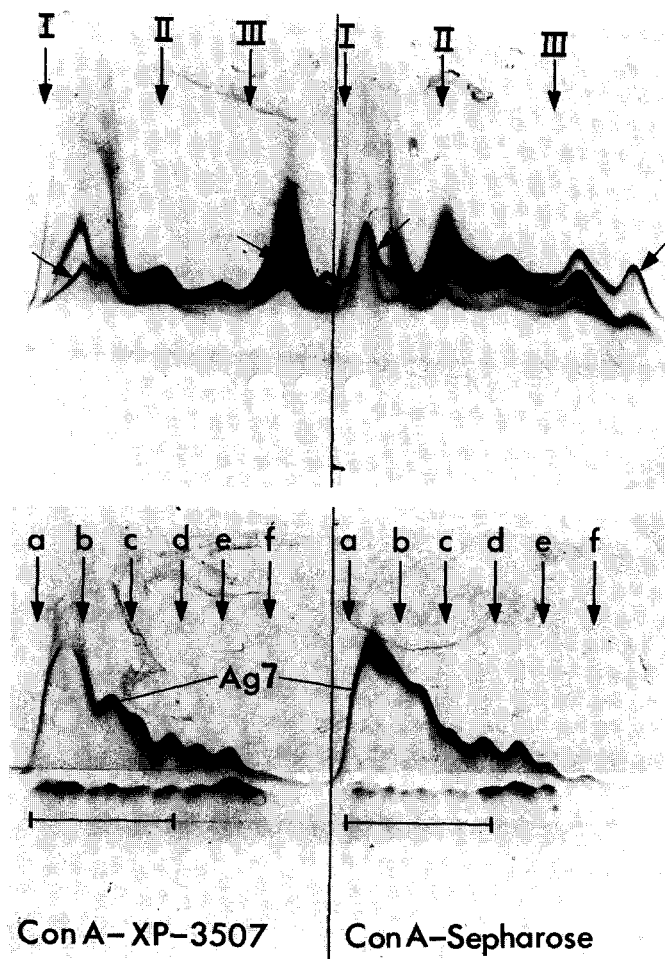


Fig. 2. FRIE of the fractions collected from Con A-XP3507 and Con A-Sepharose during elution of unbound and non-specifically bound material (I-III), and after elution of bound Ag7 (a-f) (see Fig. 1). Samples each of 10 μ l (I-III) or 1 μ l (a-f) were applied in the wells and allowed to diffuse for 30 min before electrophoresis was performed at 2 V cm^{-1} for 15 h into 1% agarose gel (pH 8.60) containing 20 μ l cm^{-2} of anti-mugwort antiserum. Arrows indicate the precipitate of the Con A-binding antigen, Ag7. The horizontal bars indicate fractions pooled in Con A-XP-3507-pool and Con A-Sepharose-pool, respectively.

The elution profile of Ag7 from the Con A-Sepharose column is influenced by the temperature used. When affinity chromatography of IEF-pool on Con A-Sepharose was performed at 4°C, Ag7 was eluted in a greater volume (1.5 to 1.7-fold) than when elution was performed at room temperature. This is probably due to a slower rate of dissociation of the glycoprotein-Con A complex at lower temperatures. It has been reported for several glycoproteins earlier (Dulaney, 1979). The results suggest that elution of Ag7 from Con A columns should be performed at

room temperature rather than at 4°C.

Recovery of Ag7 from the Con A columns

The recovery of Ag7 from the two Con A columns was determined by RIE with monospecific antiserum against Ag7. Less than 5% of the Ag7 applied on the columns was recovered in the fractions eluted without displacing sugar in the eluent (unbound and nonspecifically bound material). The recovery of specifically bound Ag7 (which was eluted with eluents containing displacing sugar) was 50% of the applied Ag7 for both Con A columns.

Increasing the contact-time did not influence the recovery of Ag7 from the column at room temperature. Thus the columns after elution with 0.5 M α -methyl mannoside in Tris-ethylene glycol buffer were left overnight (17 h) before elution was effected with 0.5 M α -methyl mannoside in Tris-buffer. It was found that at room temperature, no additional Ag7 was eluted from the columns under these conditions (Figs. 1f and 2f).

Evaluation of purity of Ag7 by gel filtration

The fractions eluted with 0.2 M α -methyl glucoside, 0.2 M α -methyl mannoside and 0.5 M α -methyl mannoside from the two Con A-columns, were pooled and marked as Con A-XP3507-pool and Con A-Sepharose-pool. In order to evaluate the

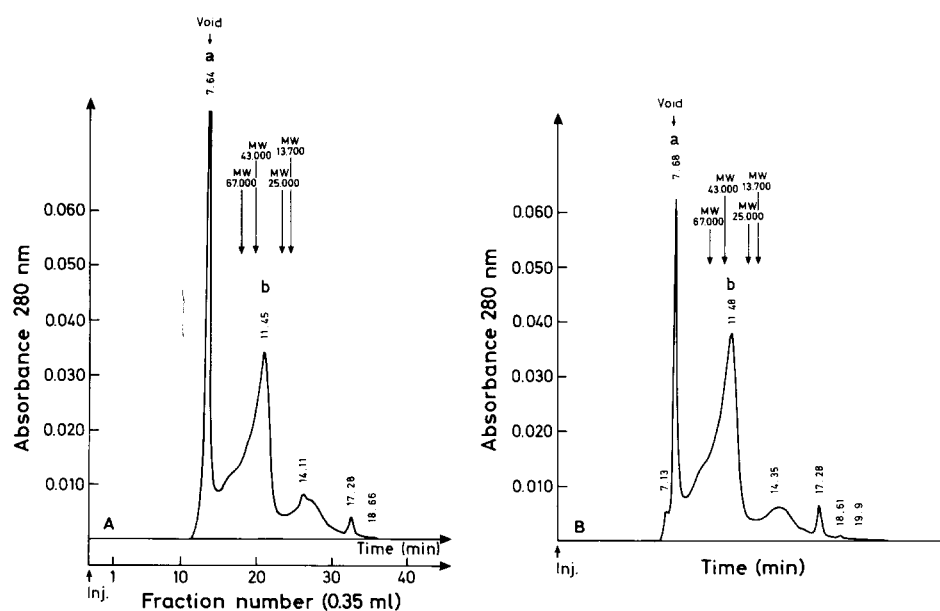


Fig. 3. Gel filtration of Con A-XP3507-pool (A) and Con A-Sepharose-pool (B) on Ultropac TSK G2000SW. The position of the MW-markers is indicated and elution times are given above each peak.

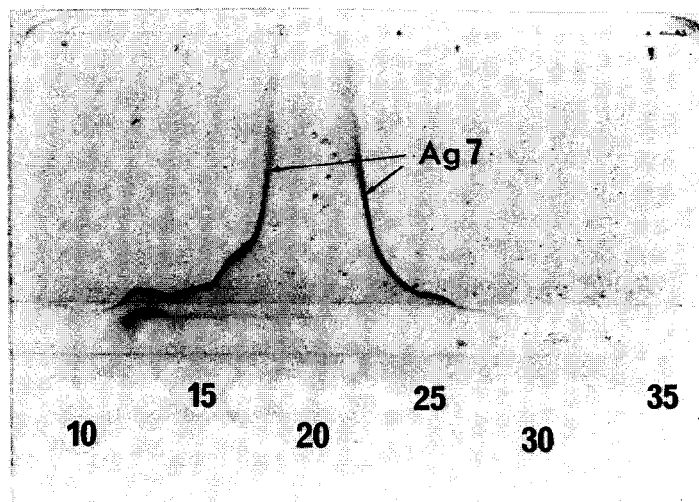


Fig. 4. FRIE of fractions from gel filtration of Con A-XP3507-pool on Ultropac TSK G2000SW (Fig. 3, left panel). The fraction numbers and the precipitate of Ag7 are indicated. Samples each of 15 μ l were applied in the wells and electrophoresis was performed as described in Fig. 2 with 15 μ l cm^{-2} anti-mugwort antiserum in the antibody-containing gel.

purity of the two pools, the fractions were analyzed by gelfiltration on Ultropac TSK G2000SW. Fig. 3 shows that although the two A_{280} elution profiles were similar, the peaks did have some quantitative differences with respect to the amount of material eluted in the different peaks. Peak b, which contained the major part of Ag7 as revealed by FRIE (Fig. 4), constituted about 54% of the total UV-absorbing material in Con A-XP3507-pool and approx. 64% of the total UV-absorbing material in Con A-Sepharose-pool. Peak a, which contained the major part of a contaminating antigen, constituted about 30% of the UV-absorbing material in Con A-XP3507-pool and 18% of the UV-absorbing material in Con A-Sepharose-pool. These results indicate that Con A-XP3507-pool contains slightly larger amounts of contaminating material than Con A-Sepharose-pool, which is consistent with the results in Figs. 1 and 2.

The major fraction of Ag7 eluted from the Ultropac TSK G2000SW column was eluted in peak b with elution time 11.45 min corresponding to a molecular weight of 33 kDa (Fig. 4). Small amounts of Ag7 were also eluted in a shoulder of peak b, corresponding to a molecular weight of 80–90 kDa, and in the void volume of the column. This elution pattern may be due to the formation of aggregates of higher molecular weights from Ag7-molecules, a phenomenon often encountered with glycoproteins (Pharmacia, 1985).

In conclusion, the results in this study show that both Con A-columns are effective for purifying Ag7, but the use of Con A-Sepharose at room temperature gave the best result for purification of Ag7 from pollen of mugwort.

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