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PURIFICATION OF A BASIC GLYCOPROTEIN ALLERGEN FROM POLLEN OF TIMOTHY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

SVEIN HAAVIK*, BERIT SMESTAD PAULSEN and JENS K. WOLD
Department of Pharmacognosy, Institute of Pharmacy, University of Oslo, P.O. Box 1068, Blindern, N-0316 Oslo 3 (Norway)
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SUMMARY

The use of high-performance ion-exchange and size-exclusion chromatography in the purification of the basic timothy pollen allergen antigen 30 (Ag 30) was investigated. The most efficient purification was achieved when an initial purification step on a CM-Sepharose CL-6B column was followed by chromatography on Mono S and TSK G 2000 SW columns. This procedure was highly reproducible and well suited for semi-preparative scale purification of the allergen. The purified allergen gave one band on isoelectric focusing, corresponding to a p*I* of 9.30. On fused rocket immunoelectrophoresis a single precipitate was obtained that coincided with the allergenic activity.

INTRODUCTION

Pollen of timothy (*Phleum pratense*) is one of the most common causes of allergic airway disease in Scandinavia. The allergic reactions are provoked by molecules called allergens, which are extracted from the pollen grains when they are deposited on the mucous membranes of the upper respiratory tract[1]. Weeke *et al.*[2] have demonstrated that eleven different allergens are extracted from timothy *in vitro*. Many investigations have been performed in order to isolate these allergenically active molecules, including ammonium sulphate and ethanol precipitation[3], anion-exchange chromatography on DEAE-cellulose[4] and on QAE-Sephadex[5], extraction with hot phenol and subsequent ethanol precipitation[6], gel filtration on Sephadex G-75[7], Sephadex G-200[7] and Ultrogel AcA 54[5] and affinity chromatography on Con A–Sepharose[7,8] and on a Sepharose–anti-IgE patient IgE immunosorbent[9].

The isolation of pure allergens is a prerequisite for the elucidation of their chemical structures, which may be the key to understanding why some molecules act as allergens[10]. In addition, purified allergens are valuable tools in the diagnosis and therapy of allergic disease[11]. So far, however, only a few timothy pollen allergens have been isolated and none of them has been properly characterized with respect to their chemical structure.

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The timothy pollen allergen antigen 30 (Ag 30) was first isolated by Løwenstein and was reported to be a basic glycoprotein of molecular weight 34,000[5]. Recent investigations in our laboratory have shown that Ag 30 can be purified by Con A-Sepharose chromatography but is easily denatured and thus generally obtained in low yields[8].

The recently developed high-performance liquid chromatographic (HPLC) support Mono S has been used in the purification of stinging insect venoms[12,13] and TSK G 2000 SW and TSK G 3000 SW columns have been used in the purification of allergens from cocksfoot grass (*Dactylis glomerata*) pollen[14].

The aim of this work was to investigate the use of Mono S, Mono Q, TSK G 2000 SW and TSK G 3000 SW columns in the isolation of Ag 30.

EXPERIMENTAL

Materials

Timothy pollen batch 011 379 101 was obtained from Allergon AB (Engelholm, Sweden). CM-Sepharose CL-6B, DEAE-Sephadex, agarose for isoelectric focusing and calibration proteins for molecular weight and p*I* determinations were obtained from Pharmacia (Uppsala, Sweden). Agarose immunoelectrophoresis tablets with tricine buffer (pH 8.60) were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Fuji RX Medical X-ray film was obtained from Fuji Photo Film (Tokyo, Japan). Kodak LX 24 developer and Kodak AL 4 X-ray fixer were obtained from Kodak (Hemel Hempstead, England). Serum from patients allergic to timothy pollen was obtained from Nyegaard (Oslo, Norway). The other chemicals used were of analytical-reagent grade and obtained from Merck (Darmstadt, F.R.G.).

Preliminary purification procedure I

Timothy pollen (100 g) was defatted by stirring at room temperature with three 500-ml volumes of diethyl ether for 30 min each time. The defatted pollen was dried and extracted with 1000 ml of 0.1 *M* NH_4HCO_3 (pH 8.0) at 4°C for 1 h with magnetic stirring. The pollen was removed by filtration and the filtrate adjusted to 1000 ml with 0.1 *M* NH_4HCO_3 followed by addition of solid $(NH_4)_2SO_4$ (396 g). After dissolution of the $(NH_4)_2SO_4$ the solution was left for 1 h at 4°C and then centrifuged. The precipitate was dissolved in 50 ml of 50 mM NH_4HCO_3 (pH 8.0) and applied at 20 ml/h to a column (100 × 2.5 cm I.D.) of DEAE-Sephadex A-25 that had been equilibrated with 50 m*M* NH_4HCO_3 (pH 8.0). The UV-absorbing material that passed through the column on elution with 50 m*M* NH_4HCO_3 (pH 8.0) (100 ml) was applied to a column (25 × 2.5 cm I.D.) of CM-Sepharose CL-6B. This column was equilibrated and then eluted at 50 ml/h with 50 m*M* NH_4HCO_3 (pH 8.0). The basic allergen was released from the column by elution with a gradient of 0–0.5 *M* NH_4HCO_3 (pH 8.0). The allergen-containing eluate (40 ml) was freeze-dried and stored at −20°C until taken for further purification experiments.

Preliminary purification procedure II

Defatted pollen (200 g) was extracted with 2000 ml of 25 m*M* NH_4HCO_3 (pH 8.0) for 1 h with magnetic stirring. The extract was filtered and the filtrate (2000 ml) was applied at a rate of 100 ml/h to a column (18 × 5 cm I.D.) of CM-Sepharose

CL-6B. After application of the extract, the column was washed with 1000 ml of 25 m*M* NH_4HCO_3 (pH 8.0) and then eluted with (1) 0.1 *M* NH_4HCO_3 (pH 8.0) (500 ml) and (2) 0.2 *M* NH_4HCO_3 (pH 8.0) (500 ml). Fractions of 5.0 ml were collected and assayed for carbohydrate[15] and allergenic activity[16–18]. The fractions containing the basic allergen (Fr. I in Fig. 4) were pooled, desalted and concentrated in an Amicon cell equipped with a Diaflo UM-2 filter (molecular weight cut-off limit 1000). The entire procedure was performed at 4°C.

Mono S and Mono Q chromatography

Chromatography on Mono S HR 5/5 cation exchanger and Mono Q HR 5/5 anion exchanger (Pharmacia) was performed on a Pharmacia fast protein liquid chromatography (FPLC) system equipped with two P-500 pumps controlled by a GP-25 gradient programmer. Chromatograms were recorded by monitoring the absorbance at 280 nm using a Pharmacia UV-1 monitor fitted with a 10 mm path HR cell. Fractions were collected with a Pharmacia FRAC-100 fraction collector.

Amounts of 0.5–10 mg of crude timothy pollen extract or of partly purified basic allergen dissolved in 500 μl of starting buffer were injected into the Mono S HR 5/5 column (50 × 5 mm I.D., particle size 10 μm). The column was eluted at 1 ml/min using the following buffer systems: (1) (A) 10 or 50 m*M* N-2-hydroxyethylpiperazineethanesulphonic acid (HEPES)–NaOH (pH 8.00) (B) 10 or 50 m*M* HEPES–NaOH (pH 8.00) containing 0.5 *M* NaCl; (2) (A) 25 m*M* NH_4HCO_3 (pH 8.0), (B) 0.275 *M* NH_4HCO_3 (pH 8.0); (3) (A) 20 m*M* sodium phosphate (pH 7.00), (B) 20 m*M* sodium phosphate (pH 7.00) containing 0.5 *M* NaCl; (4) (A) 12.5 m*M* malonic acid–12.5 m*M* piperazine (pH 5.50), (B) 0.125 *M* malonic acid–0.125 *M* piperazine (pH 5.50).

Experiments were also performed with 10 and 20% ethylene glycol in the buffers. For each buffer at least five different salt gradients were tried in order to obtain optimal resolution. Fractions of 1 ml were collected and their allergenic activity was assayed by RAST inhibition[18] and/or fused rocket immunoelectrophoresis[16,17].

The Mono S column was run ten times successively using the gradient shown in Fig. 2. The fractions A, B and C were collected, pooled, desalted (Amicon ultrafiltration cell equipped with a Diaflo UM-2 filter) and freeze-dried before chromatography on the TSK G 2000 SW and TSK G 3000 SW columns.

The material bound to the Mono S column was also chromatographed on a Mono Q HR 5/5 column (50 × 5 mm I.D. anion exchanger; Pharmacia) using the following buffer system: (A) 20 m*M* tris(hydroxymethylaminoethane) (Tris)-HCl (pH 7.50), (B) 20 m*M* Tris-HCl (pH 7.50) containing 0.5 *M* NaCl.

Chromatography on Ultropack TSK G 2000 SW and TSK G 3000 SW columns

Chromatography on the Ultropack TSK-G 2000 SW and TSK G 3000 SW columns (300 × 7.5 mm I.D.) (LKB, Bromma, Sweden) was performed with an LKB 2150 HPLC pump and an LKB 2158 Uvicord SD 280 nm UV detector. Fractions were collected with an LKB 2112 Redirac fraction collector. An LKB Ultropack TSK-GSWP column (75 × 7.5 mm I.D.) was used as a pre-column. Samples of 50 μl containing 0.1–1 mg of protein were injected. The columns were eluted at 0.25 ml/min with 50 m*M* sodium phosphate (pH 6.80) containing 0.1 *M* NaCl. Fractions of 0.25 ml were collected and assayed for carbohydrate[15] and for the basic allergen[16,17].

Ovalbumin (MW 43,000), chymotrypsinogen-A (MW 25,000), ribonuclease-A (MW 13,700) and cytochrome *c* (MW 12,500) were used to calibrate the column for molecular weight determinations.

RAST inhibition

RAST inhibition assay of the fractions from the chromatographic columns was performed by a modification[19] of the method of Yman *et al.*[18].

Fused rocket immunoelectrophoresis (FRIE)

FRIE was performed essentially as described by Harboe and Svendsen[16]. Samples of 15 μl of the fractions obtained from the chromatographic columns were applied in each well and allowed to diffuse for 50 min before electrophoresis was started. Electrophoresis was performed at 2 V/cm for 15 h into 1% agarose gel containing 25 m*M* tricine buffer (pH 8.60) (Bio-Rad Labs.) and 5 μl/cm^2 of antibody preparation against whole timothy pollen extract[8]. To detect IgE-binding precipitates, the FRIE plates were incubated with a serum pool from timothy pollen allergics, followed by incubation with [^{125}I]-anti IgE and autoradiography as described by Weeke *et al.*[17].

Isoelectric focusing

Agarose isoelectric focusing at pH 3–10 was performed essentially as described by Peltre *et al.*[20]. Samples of raw extract, Fr. I from the CM-Sepharose column and Fr. C from the Mono S column containing about 10 μg of protein, were applied. A mixture of eleven calibration proteins for p*I* determination (pH 3–10) (Pharmacia) was used to determine the pH gradient.

Carbohydrate assay

The carbohydrate content of the fractions was assayed by the phenol–sulphuric acid method[15].

RESULTS AND DISCUSSION

For the purification of antigen 30 initial experiments were carried out to fractionate the crude timothy pollen extract directly on the Mono S HR 5/5 column. This led to poor resolution and the column rapidly became clogged and discoloured by yellow and brown pigments. These pigments, which are probably polyphenolic, were bound very firmly to the support and disturbed the separation. In addition, phenolic compounds are frequently oxidized to quinones, which may bind to and denature the proteins in the extract[21], *e.g.*, phenolic compounds have been found to denature mitochondrial proteins in apple peel[22]. Because of this we had to perform a preliminary purification in order to concentrate the allergen and remove interfering pigments. In the preliminary purification procedure I this was achieved very efficiently by $(NH_4)_2SO_4$ precipitation and chromatography on columns of DEAE-Sephadex and CM-Sepharose. The single chromatographic step used in preliminary purification procedure II gave a poorer purification, but the interfering pigments were efficiently removed.

The recommended buffer for the Mono S HR 5/5 column at pH 8.00 is 50 m*M* HEPES–NaOH[23]. However, as antigen 30 did not bind to the column at this ionic

strength, the use of buffers with lower ionic strength was investigated. When 10 m*M* HEPES–NaOH was used as the buffer the allergen was bound and could be purified as shown in Fig. 1. The RAST inhibition assay[18] indicated that some allergenic material passed unretarded through the column whereas most of the activity was retained and not released until the elution of the main peak. The RAST inhibition technique as used at present gives a measure of the total allergenic activity of a fraction. However, fused rocket immunoelectrophoresis (FRIE) followed by incubation with a serum pool from timothy allergics, [^{125}I]-anti IgE and autoradiography[16,17] indicated that the basic allergen was present exclusively in the main bound peak. This demonstrates that FRIE is better suited than RAST inhibition for the detection of one specific allergen.

As is apparent from Figs. 1, 2 and 3, the allergen was eluted as broad peaks from the Mono S column. To investigate if this was due to hydrophobic interactions, ethylene glycol was added to the eluent. No significant alteration of the elution profiles could be observed when elution buffer containing 10 or 20% of ethylene glycol was used. This may indicate that the broad peaks are due to microheterogeneity of the allergen. Antigen 30 is a glycoprotein[5,8] and minor structural variations of the carbohydrate part of glycoproteins are frequently encountered[24]. When such molecules are chromatographed in a high-resolution chromatographic system such as that used here, a partial separation of the different molecular species may be achieved, resulting in broad peaks even if the substance is highly purified. This was clearly demonstrated when the purified allergen was rechromatographed on Mono S (Fig. 3). The Mono S column can therefore be used to a certain extent to detect the presence of microheterogeneity of glycoproteins.

The combination of preliminary purification procedure I and chromatography

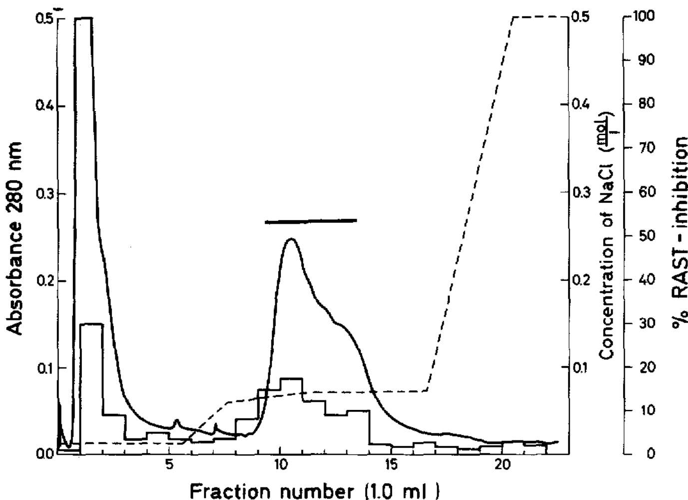

Fig. 1. Chromatography of timothy pollen extract purified by preliminary purification procedure I (7.5 mg) on a Mono S HR 5/5 column (50 × 5 mm I.D.). Buffer A, 10 m*M* HEPES–NaOH (pH 8.00); buffer B, 10 m*M* HEPES–NaOH (pH 8.00) containing 0.5 *M* NaCl. Flow-rate, 1 ml/min; 50-μl samples were subjected to RAST inhibition assay[18]. The horizontal bar indicates the fractions in which antigen 30 was detected by FRIE[16,17].

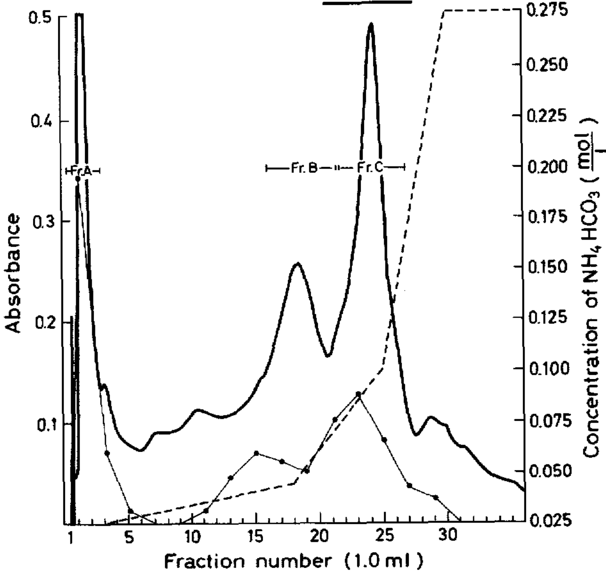

Fig. 2. Chromatography of timothy pollen extract purified by preliminary purification procedure II (4 mg) on a Mono S HR 5/5 column (50 × 5 mm I.D.). Buffer A, 25 m*M* NH_4HCO_3 (pH 8.0); buffer B, 0.275 *M* NH_4HCO_3 (pH 8.0). Flow-rate, 1 ml/min. Thick solid line, absorbance at 280 nm; ●—●, absorbance at 486 nm in the phenol-sulphuric acid carbohydrate assay[15]. Antigen 30 was detected by FRIE[16,17] in the fractions indicated by the horizontal bar.

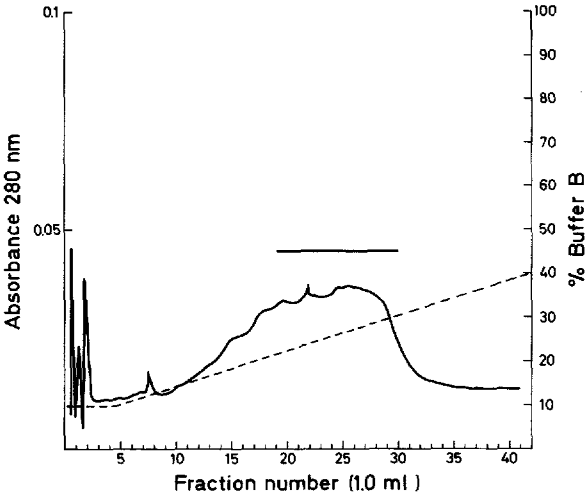

Fig. 3. Chromatography of Fr. b form the TSK G 2000 SW column (Fig. 7) on a Mono S HR 5/5 column (50 × 5 mm I.D.). Buffer A, 12.5 mM malonate–12.5 m*M* piperazine (pH 5.50); buffer B, 0.125 *M* malonate–0.125 *M* piperazine (pH 5.50). Flow-rate, 1 ml/min. Antigen 30 was detected by FRIE[16,17] in the fractions indicated by the horizontal bar.

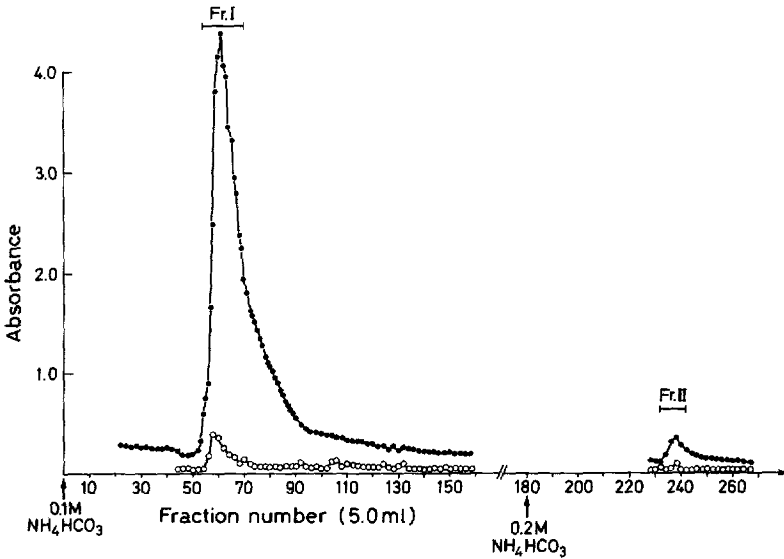

Fig. 4. Chromatography of timothy pollen extract (2000 ml) on a column (18 × 5 cm I.D.) of CM-Sepharose CL-6B. After application of the extract the column was washed with 1000 ml of 25 m*M* NH_4HCO_3 (pH 8.0) before elution with 0.1 and 0.2 *M* NH_4HCO_3 was started as indicated. Throughout the experiment the column was eluted at 100 ml/h. ●, Absorbance at 280 nm; ○, absorbance at 486 nm in the phenol-sulphuric acid test[15].

on Mono S resulted in a very efficient purification of antigen 30. However, as we also wanted to investigate the use of the HPLC techniques at an earlier stage in the purification, the preliminary purification procedure II was developed. The CM-Sepharose CL-6B column was found to be well suited for this purpose as it was not clogged by the raw pollen extract and could be eluted at high flow-rates. The main part of the basic allergen was bound to this column and could be eluted with 0.1 *M* NH_4HCO_3 (Figs. 4 and 5). Fr. I from the CM-Sepharose CL-6B column (Fig. 4) was

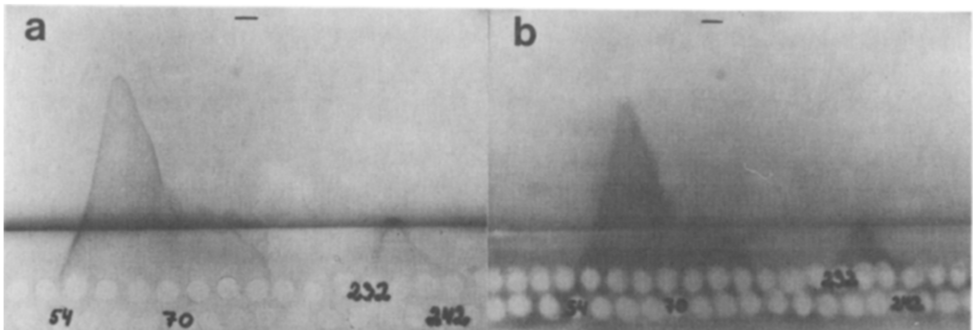

Fig. 5. FRIE of the fractions collected from the CM-Sepharose CL-6B column (Fig. 4). Samples of 15 μl were placed in the wells. The fraction numbers are indicated. After 50 min, electrophoresis was performed at 2 V/cm for 15 h into 1% agarose gel (pH 8.60) containing 5 μl/cm^2 of antibody preparation against whole timothy pollen extract. The cathode was positioned as indicated. (a) The gel stained by Coomassie Brilliant Blue; (b) autoradiograph of the FRIE plate after incubation with serum pool from timothy pollen allergics and [^{125}I]-anti IgE[17].

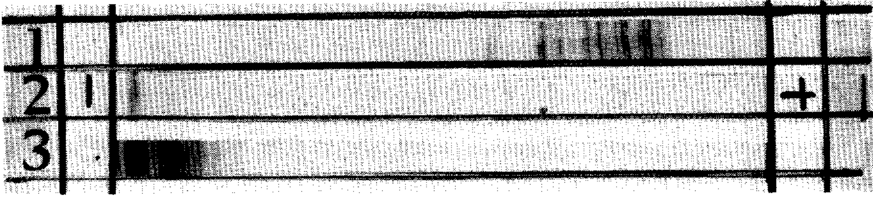

Fig. 6. Flat bed isoelectric focusing in 1% agarose gel (0.5 mm × 10 × 24 cm) (pH 3–10). Focusing was performed at 15 W for 1 h. The gel was fixed in a solution of trichloroacetic acid and sulphosalicylic acid and stained with Coomassie Brilliant Blue. (1) Raw timothy pollen extract; (2) Fr. C from the Mono S column (Fig. 2); (3) Fr. I from the CM-Sepharose CL-6B column (Fig. 4).

fractionated into three main peaks (Fr. A, B and C) on Mono S HR 5/5 (Fig. 2). Of the eluents tested, NH_4HCO_3 buffer was found to give the best resolution; NH_4HCO_3 also has the advantage that it can be removed by freeze-drying.

Fr. C, which contained most of the allergen, gave one band on isoelectric focusing at pH 3–10 corresponding to a p*I* of about 9.30 (Fig. 6). This is in reasonable agreement with Løwenstein[5], who reported that antigen 30 had an isoelectric point

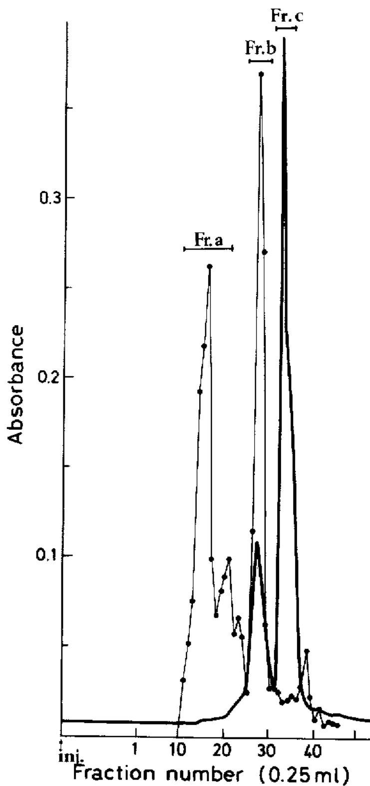

Fig. 7. Chromatography of Fr. C Mono S (Fig. 2) on a TSK G 2000 SW column (300 × 7.5 mm I.D.). The column was eluted at 0.25 ml/min with 50 m*M* sodium phosphate (pH 6.80) containing 0.1 *M* NaCl. Thick solid line, absorbance at 280 nm; ●—●, absorbance at 486 nm in the phenol–sulphuric acid carbohydrate assay[15].

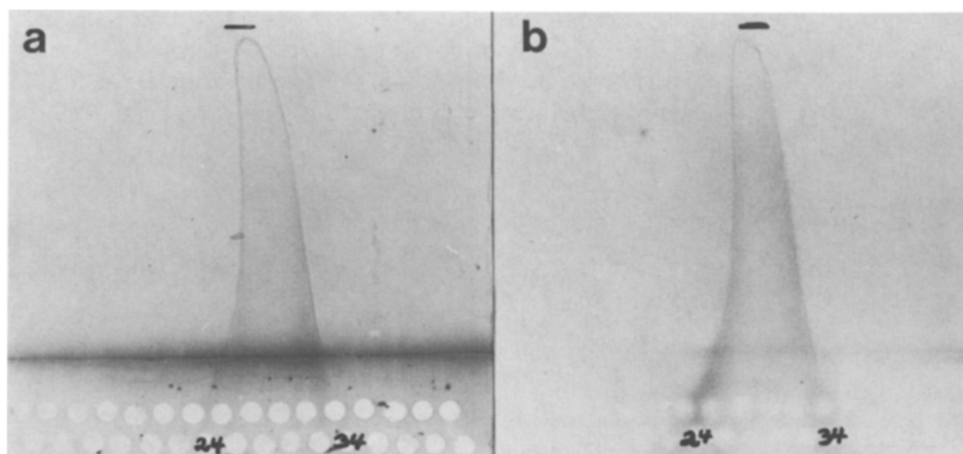

Fig. 8. FRIE[16] of the fractions from the TSK G 2000 SW column (Fig. 7). The fraction numbers are indicated. FRIE was performed as described in the legend to Fig. 5. (a) The gel stained by Coomassie Brilliant Blue; (b) autoradiograph of the FRIE plate after incubation with serum pool from timothy pollen allergics and [^{125}I]-anti IgE[17].

of 9.4. Attempts to fractionate Fr. C (Fig. 2) further on a Mono Q HR 5/5 anion exchanger were not successful.

Previously Calam *et al.*[14] used TSK G 2000 SW and TSK G 3000 SW columns to fractionate allergen extracts from cocksfoot grass pollen. They recommended the use of size exclusion chromatography as the initial fractionation method. However, the size exclusion columns that we used (300 × 7.5 mm I.D.) have a much lower capacity and resolving power than the ion exchangers. Moreover, chromatography on TSK-G SW columns involves the use of buffers with a high salt concentration and desalting has to be performed before ion-exchange chromatography can be carried out. Hence we used chromatography on TSK G 2000 SW as the final purification step (Fig. 7). On this column Fr. C from the Mono S column (Fig. 2) was fractionated into three main peaks (Fig. 7). By FRIE it was demonstrated that the elution profile of antigen 30 (Fig. 8) corresponded to the elution of carbohydrate and protein in the second peak (Fr. b) in Fig. 7. The molecular weight of the allergen was estimated to be 32 000, which is in good agreement with Løwenstein[5], who found that antigen 30 had a molecular weight of 34 000. In order to investigate if Fr. c in Fig. 7 was due to dissociation of the allergen during chromatography. Fr. b was rechromatographed on the TSK G 2000 SW column. In this experiment, however, only one peak corresponding to MW 32 000 was eluted, thus demonstrating that Fr. c in Fig. 7 is not formed by degradation of antigen 30.

CONCLUSION

The use of HPLC techniques in the isolation of antigen 30 from timothy pollen should be preceded by removal of pigments and other interfering substances from the crude extract. Then highly purified antigen 30 can be obtained rapidly by a combination of chromatography on Mono S and TSK G 2000 SW columns. Thus our

results are in agreement with those of Calam *et al.*[14], who suggested that such techniques will probably play an increasingly important role in the micro-scale identification and isolation of allergens. To our knowledge, this investigation is the first to employ HPLC techniques in the purification of a previously characterized, well defined allergen.

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