

Glycoprotein Allergens in Pollen of Timothy

II. Isolation and Characterization of a Basic Glycoprotein Allergen

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Abstract. A basic glycoprotein allergen has been isolated from pollen of timothy by a combination of chromatography on columns of CM-Sepharose CL-6B, Bio-Gel P-30, DEAE-Sepharose CL-6B and Ultrogel Aca 44. The allergen which is immunologically identical with the allergen formerly denoted as antigen 30 had an isoelectric point of 9.45. The MW of the glycoprotein was found to be 54,000 and 38,000 by SDS-PAGE and gel filtration, respectively. The allergen contains arabinose (3.1%), fucose (0.7%), xylose (0.7%), mannose (2.1%), galactose (2.8%) and glucose (0.2%). In rocket radioimmuno-electrophoresis the allergen bound IgE from 57 (78%) of 73 patient sera tested. The allergen did not bind Yariv artificial galactosyl- and glucosyl-carbohydrate-binding antigens or a monoclonal antibody against ryegrass pollen glycoprotein allergens.

Introduction

During the past years a great number of investigations has been carried out in order to isolate and characterize allergens [1]. At present, however, the detailed molecular structure is known only for a very limited number of allergens. So far most papers in this field have described partial purification and characterization.

Pollen of timothy (*Phleum pratense*) is one of the most common causes of allergic airway disease in Scandinavia [2]. It has been demonstrated that extracts of timothy pollen contain at least 11 different allergens [3, 4]. One of the allergens that has been studied most extensively is the cathodic allergen usually denoted as antigen 30. Antigen 30 has been reported to be a glycoprotein with MW 34,000 and classified as an intermediate allergen [5]. Recently we have studied the interaction between antigen 30 and lectins and used affinity chromatography on Con A-Sepharose in the purification of the allergen [6]. We also have investigated the use of high-performance gel permeation- and ion-exchange chromatography in the purification of antigen 30 [7]. Although these purification methods were efficient the yield of antigen 30 was low. This was at least partly due to instability and low solubility of the purified allergen [6].

The aim of the present investigation has been to establish an isolation procedure which permits the isolation of antigen 30 in a yield and state of purity sufficient for subsequent meaningful structural investigations.

Materials and Methods

Materials

Pollen of timothy (*Phleum pratense*) batch 011383101 was harvested and delivered by Allergon AB (Engelholm, Sweden). The sheep used for immunization were kindly provided by Mrs. Asta Haavik (Bratland, Norway). Serum from patients allergic to timothy pollen was supplied by Nyegaard & Co A/S (Oslo, Norway). Antiserum to whole timothy pollen extract batch 232581/1 was produced by ALK (Copenhagen, Denmark).

Agarose immuno-electrophoresis tablets with tricine buffer, pH 8.60, acrylamide, N,N-methylene bisacrylamide, and N,N,N',N'-tetramethylethylenediamine were obtained from Bio-Rad Laboratories (Richmond, Calif., USA).

Chromatographic columns, CM-Sepharose CL-6B, DEAE-Sepharose CL-6B, carrier ampholyte for isoelectric focusing (Pharmalyte pH 8-10.5) and Gel-bond* film for agarose were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Fuji RX medical X-ray film was obtained from Fuji Photo Film Co (Japan). X-ray film cassettes and Titan 2HS intensifying screen were obtained from Siemens (FRG). Kodak LX 24 X-ray developer and Kodak AL 4 X-ray fixer were obtained from Kodak (UK). Ultrogel Aca 44 was obtained from LKB (Bromma, Sweden).

Isolation of Timothy Antigen 30

The β -glucosyl and β -galactosyl Yariv compounds [8] were a gift from Dr. M.A. Jermyn (Parkville, Australia). The monoclonal antibody against a ryegrass pollen allergen [9] was a gift from Prof. J. Bradley (Bedford Park, South Australia).

Isolation of the Allergen

Timothy pollen (100 g) was defatted by stirring for 3 × 30 min with diethyl ether (3 × 500 ml). The defatted pollen was extracted with 25 mM NH_4HCO_3 , pH 8.0, (1,000 ml) for 1 h by magnetic stirring. The pollen was then removed by filtration. The filtrate (1,000 ml) was immediately pumped at 100 ml/h into a column (5 × 16 cm) of CM-Sepharose CL-6B which was equilibrated with 25 mM NH_4HCO_3 , pH 8.0. After application of the extract the column was washed with 800 ml 25 mM NH_4HCO_3 , pH 8.0, at 100 ml/h. The flow was then reversed and the column eluted at 75 ml/h with 0.125 M NH_4HCO_3 , pH 8.0.

The fractions from the CM-Sepharose CL-6B column which contained cathodic allergen were pooled and concentrated to 10 ml in an Amicon ultrafiltration cell equipped with a Diaflo* YM2 membrane (MW cutoff limit 1,000). This solution was applied at 10 ml/h to a column (2.5 × 100 cm) of Bio-Gel P-30 which was eluted with 50 mM NH_4HCO_3 , pH 8.0. The fractions containing cathodic allergen were pooled and applied at 30 ml/h to a column (2.5 × 27 cm) of DEAE-Sepharose CL-6B which had been equilibrated with 25 mM NH_4HCO_3 , pH 8.0. After the pool from the Bio-Gel P-30 column was applied the column was eluted at 30 ml/h with (1) 25 mM NH_4HCO_3 (100 ml) and (2) 0.5 M NH_4HCO_3 (200 ml).

This procedure was repeated 5 times and the fractions from the DEAE-Sepharose CL-6B column which contained cathodic allergen were pooled, concentrated to 30 ml in an Amicon ultrafiltration cell (YM2 membrane). The concentrated solution was divided into 3 equal portions which were chromatographed on a column (2.5 × 90 cm) of Ultrogel Aca 44. This column was eluted at 10 ml/h with 50 mM phosphate buffer, pH 6.80, containing 0.1 M NaCl and 0.02% NaN_3 . The material containing cathodic allergen from three runs on the Aca 44 column was pooled and desalted in the Amicon ultrafiltration cell. The allergen was freeze-dried and stored at -20°C until further characterization.

During the elution of all the columns employed fractions of 5.0 ml were collected. The absorbance of the fractions at 280 nm was read, 100- μ l samples were subjected to phenol sulfuric acid test [10] and 10- μ l samples to fused rocket immuno-electrophoresis (FRIE) [11]. The entire procedure was performed at 4°C.

Production of Antiserum to Timothy Pollen Extract

Three 7-month-old male sheep were immunized intracutaneously with a mixture of 0.5 ml dialyzed timothy pollen extract (Pharmalgen allergenic extract 1:10, Pharmacia Diagnostics AB) and 0.5 ml of Freund's complete adjuvant. 14 and 28 days after the first injection the animals were given booster doses of 0.5 ml allergen extract in 0.5 ml of Freund's incomplete adjuvant. 14 days after the last injection the sheep were slaughtered, the blood (6 liter) collected and the serum pooled. Prior to use in immuno-electrophoresis the globulin fraction was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialyzed against 0.1 M NaCl, 15 mM NaN_3 as described by Harboe and Ingild [12].

Patient Sera

A serumpool was made from 250 patients having RAST-class

2-4 against timothy. 73 individual sera with RAST-class 4 were used to investigate the importance of the isolated glycoprotein as an allergen.

Immuno-electrophoretic Techniques

Rocket immuno-electrophoresis (RIE), FRIE and crossed immuno-electrophoresis (CIE) were performed essentially as described by Axelsen and Bock [13], Harboe and Svendsen [11] and Grubb [14], respectively. The electrophoresis was performed in 25 mM tricine buffer, pH 8.60, with tris-tricine (9.8 and 4.3 g/l), pH 8.60, as electrode buffer. The cathodic gel in RIE and FRIE contained 5.5 μ l/cm² of the antibody preparation against timothy pollen raised in sheep. The second dimension gels in CIE contained 15 μ l/cm² antiserum against timothy pollen (ALK, batch 232581/1).

Detection of IgE-binding precipitates obtained in RIE, FRIE and CIE was performed mainly as described by Weeke et al. [15]. The dried immuno-electrophoretic plates were incubated overnight at room temperature in crossed radioimmuno-electrophoresis (CRIE)-buffer (50 mM phosphate buffer, pH 7.40, with 0.9% NaCl, 0.3% dextran 70 and 0.3% Tween 20) containing 10% serum from nonimmunized sheep and 10% serum from timothy allergics. To investigate if an individual patient serum contained IgE with specificity to the isolated allergen, a RIE-plate of the purified allergen was incubated in a similar way with a solution of this serum. After incubation the plates were rinsed 4 × 30 min in CRIE-washing solution (0.9% NaCl containing 0.3% Tween 20 and 5% CRIE-buffer). The plates were then incubated overnight at room temperature with CRIE-buffer containing ¹²⁵I-labelled sheep anti human IgE corresponding to about 50,000 cpm/ml. After rinsing in CRIE-washing solution for 4 × 30 min the plates were dried and subjected to autoradiography for 3 days at -80°C in X-ray cassettes equipped with Siemens titan 2HS intensifying screens. The X-ray films were developed as described by Kodak.

Experiments with Yariv Artificial Antigens

(1) RIE of 5 μ g allergen with 0.1 mg/cm² of Yariv β -glucosyl or with β -galactosyl artificial antigens [8] in the intermediate gel was performed. (2) The allergen (1 mg/ml) was incubated with 0.1 mg/ml of each of the Yariv antigens in 1% NaCl for 4 h prior to RIE.

Binding Experiments with Monoclonal Antibody against Ryegrass Pollen Allergen

(1) RIE of 5 μ g of the allergen was performed in 1% agarose gel containing 20 μ l/cm² of the monoclonal antibody preparation FMC-A9 [9]. (2) RIE of 5 μ g of the allergen was performed with 500 μ l/cm² of FMC-A9 in the intermediate gel. (3) The allergen (1 mg/ml) was incubated for 4 h with the antibody preparation FMC-A9 followed by RIE.

Sodium Dodecyl Sulfate Polyacrylamide Gradient Gel (SDS Gradient PAGE) Electrophoresis

This was carried out on slab gels in the discontinuous buffer system as described by Laemmli [16]. The resolving gel contained a linear 7.5-15% polyacrylamide gradient and the stacking gel 4% polyacrylamide. Sample loads of ca. 50 μ g were applied and the electrophoresis allowed to run at 100 V for 16 h. Proteins were stained with Coomassie brilliant blue R-250. For MW determination the following proteins were used as markers: phosphorylase b

(94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α -lactalbumin (14,400).

Isoelectric Focusing

Isoelectric focusing in the pH-range 8–11 was performed in 0–50% sucrose gradient containing Pharmalyte, pH 8.5–10, (Pharmacia Fine Chemicals) diluted 1:16, and 5 mg/ml arginine [17]. The focusing was carried out in an 11-ml electrolysis column for 48 h with a final potential gradient of 1,200 V. After the electrofocusing the column was immediately drained and fractions of 0.25 ml were collected under a slow stream of argon. The pH of each fraction was measured with an Orion research model 601/A digital ion analyzer equipped with an Orion CAT. No. 91-03 glass electrode. 15 μ l of each fraction was subjected to FRIE [11], and the absorbance was read at 280 nm after dilution of the fractions to 1.0 ml with distilled water.

Amino Acid Analysis

The purified glycoprotein was subjected to hydrolysis in 6 M HCl at 110°C for 24 h in vacuo, the amino acid composition was determined by a Bio-Cal automatic amino acid analyzer. Tryptophan was estimated by the method of Edelhoch [18]. Hydroxyproline was assayed by the amino acid analyzer and by the method of Kivirikko and Liesmaa [19].

Monosaccharide Determination

Dried samples (0.1–1 mg) were subjected to methanolysis and subsequent TMSi-derivatization as described previously [20]. TMSi methyl glycosides were separated and quantitated by gas chromatography on a 15-meter DB5-15 W fused silica capillary column. Gas chromatography/mass spectrometry (GC/MS) was performed on a Finnigan 9610 gas chromatograph equipped with a 30-meter SE-54 capillary column coupled to a Finnigan Mat 4021 mass spectrometer.

Carbohydrate Estimation

Carbohydrate estimation was carried out by the phenol sulfuric acid method [10].

Determination of Protein

Protein was determined by the method of Lowry et al. [21] with bovine serum albumin as standard.

Results and Discussion

Isolation

A wide variety of different extraction buffers, pollen:buffer ratios, methods and duration of extraction etc. has been used in the preparation of timothy pollen extracts. Nielsen et al. [22] used nondefatted pollen and performed the extraction in a Braun cell homogenizer for 5 $\times$ 60 s using 0.1 M sodium bicarbonate (1:10) as extraction buffer. Malley et al. [23] extracted ether-defatted pollen in 0.127 M phosphate buffer, pH 10.4, (1:3) containing 10% glucose by

magnetic stirring for 24 h, while Puttonen and Maasch [24] used pollen that was defatted with light petroleum ether and performed the extraction in 0.125 M NH_4HCO_3 for 16 h. In our hands extracts of diethyl ether-defatted pollen seemed to be better suited for fractionation experiments than extracts of nondefatted pollen. The yield of antigen 30 was not improved when the extraction lasted for more than 1 h. This is in agreement with previous investigators who have demonstrated that timothy pollen allergens and especially the cathodic allergens are extracted rapidly [6, 25, 26].

By the initial purification step on the CM-Sephacrose CL-6B column the allergen was concentrated and most enzymes and pigments were removed (fig. 1a, 2a). It turned out to be essential that such substances which otherwise could have denatured the allergen [6] were removed at an early stage in the purification. On the Bio-Gel P-30 column low MW pink colored protein and some high MW carbohydrates and proteins were removed (fig. 1b, 2b). The low-MW material resembles the basic, pink colored allergen C which has been isolated from Kentucky blue grass pollen [27].

The allergen was further purified by chromatography on the columns of DEAE-Sephacrose CL-6B (fig. 1c, 2c) and Ultrogel AcA 44 (fig. 1d, 2d). When the pooled material from the Ultrogel AcA 44 column was chromatographed on an Ultrogel AcA 54 column no further purification was achieved.

Characterization

In CIE of the isolated allergen one broad cathodic precipitate was obtained (fig. 3). The strongest radio-staining in CRIE (fig. 3) as well as in all the other IgE-binding experiments (fig. 2, 5, 6) was located in the area enclosed by the precipitate. This phenomenon which is frequently encountered in CRIE [15] indicates that allergen is precipitated in this area and that this allergen has greater capability of binding IgE than the allergen in the precipitation arc.

The purified allergen gave one band corresponding to MW 54,000 in SDS gradient PAGE (fig. 4). The band was somewhat broader than the bands of the calibration proteins. This phenomenon is frequently encountered in PAGE of glycoproteins.

The MW determined by gel filtration on all the columns used (table I) was 38,000. This is somewhat higher than the values previously obtained by us [6, 7] (32,000) and by Löwenstein [5] (34,000). The discrepancy between the MW determined by SDS-PAGE

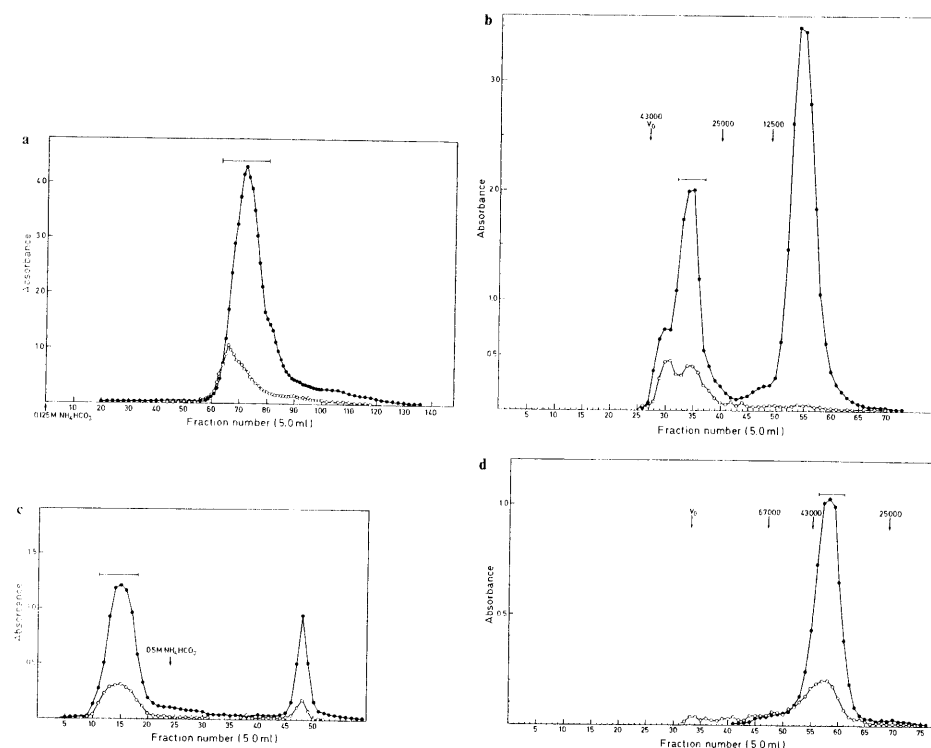


Fig. 1. Chromatographic steps in the isolation of the glycoprotein allergen. ● = Absorbance at 280 nm, ○ = absorbance at 486 nm in the phenol sulfuric acid carbohydrate test [10]. The fractions indicated were pooled and subjected to the next chromatographic step. The elution volumes of cytochrome c (MW 12,500), chymotrypsinogen A (MW 25,000), ovalbumin (MW 43,000), bovine serum albumin (MW 67,000) and blue dextran (V_0) which were used as calibration substances for MW determination on the gel filtration columns are indicated in b and d. a Elution of the CM-Sephacrose CL-6B (5 $\times$ 16 cm) column at 75 ml/h with 0.125 M NH_4HCO_3 , pH 8.0. 1,000 ml timothy pollen extract was applied at 100 ml/h to the column and it was washed at 100 ml/h with 800 ml 25 mM NH_4HCO_3 , pH 8.0, before elution with 0.125 M NH_4HCO_3 was started. b Chromatography of the pooled fractions from the

CM-Sephacrose CL-6B column (concentrated to 10 ml) on the column (2.5 $\times$ 100 cm) of Bio-Gel P-30. The column was eluted at 10 ml/h with 50 mM NH_4HCO_3 . c Chromatography of the pooled fractions from the Bio-Gel P-30 column (30 ml) on the column (2.5 $\times$ 27 cm) of DEAE-Sephacrose CL-6B. The column was equilibrated with 25 mM NH_4HCO_3 , pH 8.0, before the material was applied and it was eluted at 30 ml/h with (1) 25 mM NH_4HCO_3 , pH 8.0, (100 ml), and (2) 0.5 M NH_4HCO_3 , pH 8.0. d Chromatography of one third of the pooled material from 5 runs of the DEAE-Sephacrose CL-6B column on the column (2.5 $\times$ 90 cm) of Ultrogel AcA 44. 10 ml concentrated solution was applied to the column which was eluted at 10 ml/h with 50 mM phosphate buffer, pH 6.80, containing 0.1 M NaCl and 0.02% NaN_3 .

(54,000) and gel filtration (38,000) was considerably higher (+42%). However, abnormally high MW estimates of glycoproteins are frequently encountered in SDS-PAGE [28]. Consequently we regard 38,000 as the most correct MW estimate for antigen 30.

In isoelectric focusing one peak with a maximum corresponding to a pI of 9.45 was obtained (fig. 5). The FRIE pattern revealed accordance between UV-absorbing and IgE-binding material (fig. 5). The somewhat irregular shape of the peak may indicate

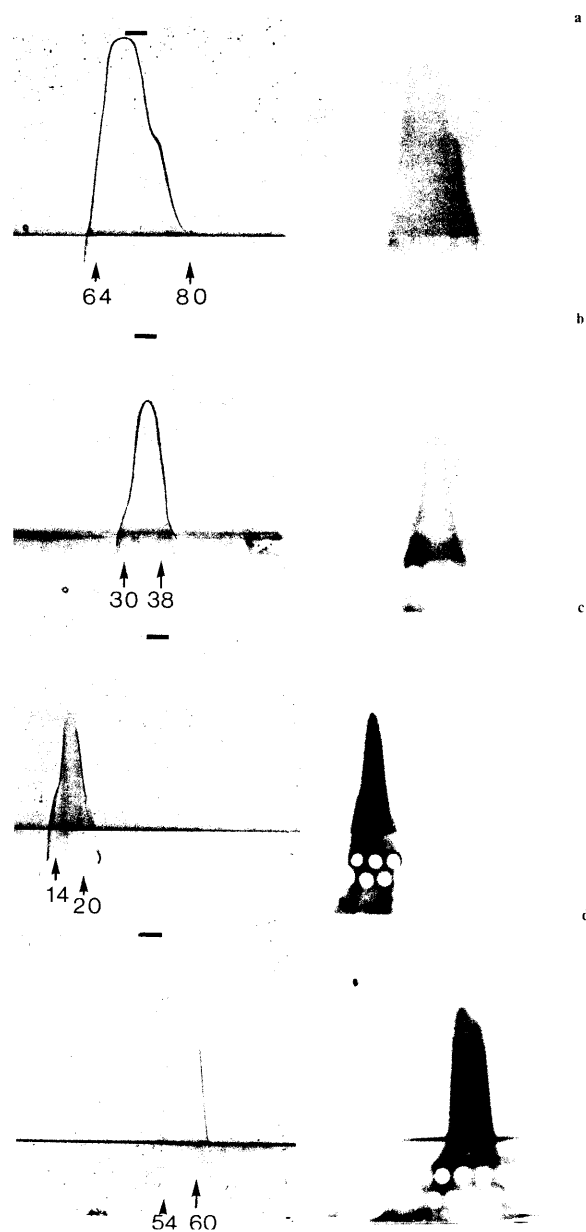


Fig. 2. FRIE [11] of the fractions from the columns of CM-Sepharose CL-6B (a) Bio-Gel P-30 (b) DEAE-Sepharose CL-6B (c) and Ultrogel AcA 44 (d) 10- μ l samples of the fractions were placed in the wells and allowed to diffuse for 50 min. Electrophoresis was then performed towards the cathode at 2 V/cm for 15 h into 1% agarose gel, pH 8.60, containing 5.5 μ l/cm² antibody preparation against timothy pollen extract. To the left are shown the FRIE-plates after staining with Coomassie brilliant blue and to the right are shown autoradiographs of the corresponding plates after incubation with the serum pool from timothy pollen allergies and ¹²⁵I-labelled anti IgE [15]. Fraction numbers are indicated on the Coomassie brilliant blue-stained plate.

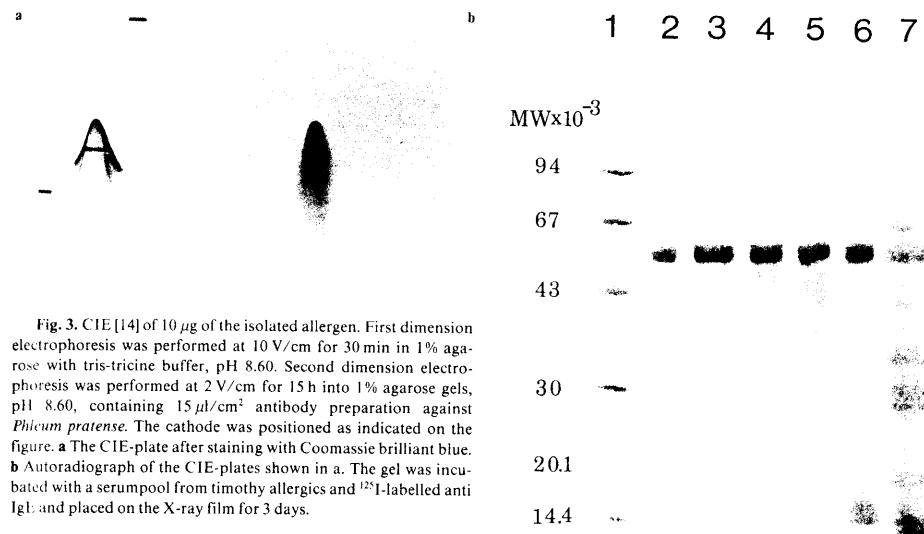


Fig. 3. CIE [14] of 10 μ g of the isolated allergen. First dimension electrophoresis was performed at 10 V/cm for 30 min in 1% agarose with tris-tricine buffer, pH 8.60. Second dimension electrophoresis was performed at 2 V/cm for 15 h into 1% agarose gels, pH 8.60, containing 15 μ l/cm² antibody preparation against *Phleum pratense*. The cathode was positioned as indicated on the figure. a The CIE-plate after staining with Coomassie brilliant blue. b Autoradiograph of the CIE-plates shown in a. The gel was incubated with a serum pool from timothy allergies and ¹²⁵I-labelled anti IgE and placed on the X-ray film for 3 days.

Table I. Properties of the isolated glycoprotein allergen

pI 0-50% sucrose gradient	9.45
MW Bio-Gel P-30	38,000
MW Ultrogel AcA 44	38,000
MW Ultrogel AcA 54	38,000
MW SDS gradient PAGE	54,000
Carbohydrate content	9.6%

that the allergen exists in multiple forms with slightly different isoelectric points. The presence of a non-ionic solute as sucrose in the pI-gradient decreases the dielectric constant of water. As a consequence of this the pI determined in sucrose gradients is dependent on the sucrose concentration. For instance Flatmark et al. [17] demonstrated that the pI of cytochrome c was reduced about 0.3 pH units when the sucrose concentration was increased from 0 to 50%. This may indicate that the true pI of the allergen may be higher than 9.45 and thus explain its previously observed cathodic migration at pH 10.0 [6].

Amino acid analysis revealed the presence of amino acids normally found in plant glycoproteins (table II). The amino acid composition was in reason-

Fig. 4. SDS-slab gel-electrophoresis in 7.5-20% PAGE [16]. Samples of ca. 50 μ g were applied and electrophoresis allowed to run at 100 V for 16 h. Proteins were stained with Coomassie brilliant blue R-250. 1 = Phosphorylase b (MW 94,000), bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), carbonic anhydrase (MW 30,000), trypsin inhibitor (MW 20,100) and α -lactalbumin (MW 14,400). The MW of the bands is indicated on the figure; 2 and 3 = pool Ultrogel AcA 44; 4 = pool DEAE-Sepharose CL-6B; 5 = pool Bio-Gel P-30; 6 = pool CM-Sepharose CL-6B; 7 = raw extract of timothy pollen.

able agreement with the composition reported by Löwenstein [5], the main difference being the presence of half-cysteine found by us, and that we also assayed for hydroxyproline. The hydroxyproline-arabinose linkage is a widely distributed covalent connection between carbohydrate and protein in plant glycoproteins [29]. Previously we have demonstrated the presence of this type of linkage in a purified allergen preparation from pollen of timothy [20]. This finding may indicate that the hydroxyproline-arabinose bond constitutes at least a part of the protein-carbohydrate linkages in the glycoprotein.

The allergen contained arabinose, fucose, xylose, mannose, galactose and traces of glucose (table III). The same monosaccharides were detected in the al-

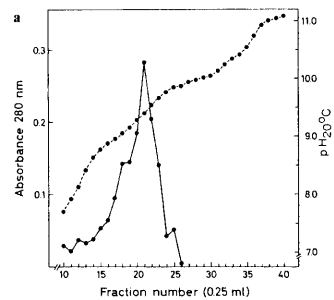


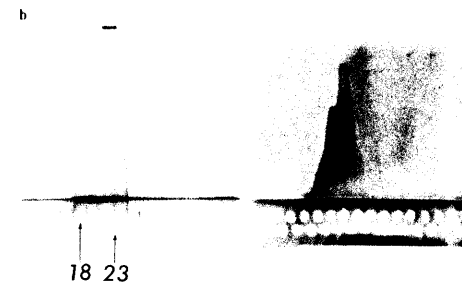
Fig. 5. Isoelectric focusing of 1 mg of the isolated allergen in 0–50% sucrose gradient containing Pharmalyte, pH 8.5–10, diluted 1:16 and 5 mg/ml arginine [17]. The focusing was performed in an 11-ml electrofocusing column for 48 h with a final potential gradient of 1,200 V. **a** —●— = the pH-gradient; —○— = absorbance at 280 nm of the fractions collected from the electrofocusing column.

Table II. Amino acid composition of the allergen (vacuum hydrolysis for 24 h in 6 M HCl)

Amino acid	% w/w	Number of residues/molecule
Asp or Asn	8.8	29.1
Thr	3.9	14.7
Ser	5.5	24.0
Glu or Gln	8.9	26.2
Pro	6.1	23.9
Gly	3.9	26.0
Ala	4.6	24.6
1/2-Cys	0.5	1.8
Val	5.5	21.1
Met	1.3	3.8
Ile	4.3	14.5
Leu	6.3	21.2
Tyr	5.9	13.8
Phe	5.4	14.0
His	2.5	6.9
Lys	6.1	18.1
Arg	5.7	13.9
Trp ^a	4.6	9.4
Hypr. ^b	0.6	2.0
Total	90.4	309.0

^a Determined by the method of *Edelhoeck* [18].

^b Average of the values determined by the amino acid analyzer and the method of *Kivirikko and Liesmaa* [19].



b FRIE [11] of the fractions obtained after isoelectric focusing. FRIE was performed as described in the legend to figure 2. To the left is shown the FRIE-plate after staining with Coomassie brilliant blue and to the right is shown the autoradiograph of the FRIE-plate after incubation with the serum pool from timothy pollen allergies and ¹²⁵I-labelled anti IgE [15].

Table III. Monosaccharide composition of the allergen determined by GLC as TMSi-methylglycosides

Monosaccharide	% w/w	Number of residues/molecule
Arabinose	3.1	8.9
Fucose	0.7	1.8
Xylose	0.7	2.0
Mannose	2.1	4.9
Galactose	2.8	6.6
Glucose	0.2	0.5
Total	9.6	24.7

lergen when it was purified by affinity chromatography on Con A-Sepharose [6]. That material, however, contained considerably more glucose. The allergen 'glycoprotein 2' which was isolated from ryegrass pollen by *Howlett and Clarke* [30] contained the same neutral monosaccharides and, in addition, glucosamine was detected by amino acid analysis. In the amino acid analysis of the allergen isolated by us a peak with a retention time corresponding to glucosamine was obtained, too. But no amino sugar could be detected by GC/MS of the amino acid hydrolysate or the methanolysate (1 M HCl) of the allergen.

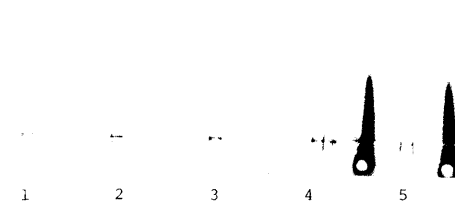


Fig. 6. RIE [13] of 2.5-μg samples of the isolated glycoprotein. The electrophoresis was performed at 2 V/cm for 15 h into 1% agarose gel, pH 8.60, containing 3.5 μg/cm² antibody preparation against timothy pollen extract. The gel was rinsed, pressed and dried before the individual rockets were incubated with solutions of different sera followed by incubation with ¹²⁵I-labelled anti IgE and autoradiography. To the left is shown the Coomassie brilliant blue-stained rocket and to the right is shown the corresponding autoradiograph. The rockets are incubated with 300 μl of (1) 0.9% NaCl, (2) a serum pool from persons having RAST-class 0 to timothy pollen, (3) the serum pool from 250 timothy allergies, (4) and (5) serum from persons with RAST-class 4 to timothy pollen. The sera were diluted 1:10 in CRIE-buffer containing 10% serum from non-immunized sheep.

The Yariv β-galactosyl and β-glucosyl artificial antigens [8] did not precipitate the allergen or affect its migration in RIE. These artificial antigens have been shown to precipitate arabinogalactan proteins. The β-glucosyl Yariv compound has been used in the purification of an arabinogalactan protein from gladiolus-style mucilage [31].

The monoclonal antibody against ryegrass pollen allergen FMC-A9 [9] did not bind to the isolated allergen. This antibody has been shown to react with a variety of grass pollen components including a component very similar to the allergenic glycoprotein 2 isolated by *Howlett and Clarke* [30]. It has been speculated if this monoclonal antibody possibly binds to a cross-reactive carbohydrate determinant which has been detected in several plant extracts including a timothy pollen extract [32]. Our experiments demonstrate that antigen 30 does not possess this cross-reactive carbohydrate determinant.

Allergenic in vitro Properties

By incubation of the precipitate in RIE with 0.9% NaCl and serum from nonallergics prior to incubation with ¹²⁵I-labelled anti IgE the specificity of the radiostaining was clearly demonstrated (fig. 6). It may be difficult to reveal the importance of an allergen in CRIE as the antigens are only partially separated and

coprecipitation frequently occurs. Previously the precipitate of antigen 30 has been reported to be associated with unspecific radiostaining and it has been speculated if coprecipitation is responsible for the radiostaining observed with many of the patient sera [4, 33]. Our investigation demonstrated that the serum from 57 (78%) of the 73 patients with clinical timothy pollen allergy contained IgE with specificity to the purified antigen 30. This indicates that antigen 30 can be classified as an intermediate allergen.

Acknowledgements

This investigation was supported by a grant from the Norwegian Medical Depot and by Nyegaard & Co. A/S (Oslo, Norway) who provided the pollen and ¹²⁵I-labelled anti IgE. The authors are indebted to Dr. *Knut Sletten*, Institute of Biochemistry, University of Oslo for performing the amino acid analysis and to *Per Helland*, Rikshospitalet (Oslo, Norway) for performing the GC/MS. The skilful assistance of Dr. *Jan Haavik*, Institute of Biochemistry, University of Bergen in the immunization experiments is gratefully acknowledged.

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Received: January 21, 1985

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