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ARABINOGALACTANS IN A PURIFIED ALLERGEN PREPARATION FROM POLLEN OF TIMOTHY (*PHLEUM PRATENSE*)

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Key Word Index—*Phleum pratense*; Poaceae; timothy; grass pollen; allergen preparation; arabinogalactan; glycoprotein; hydroxyproline.

Abstract—The purified allergen preparation representing a certain fraction of an aqueous timothy pollen extract contained *ca.* 20% carbohydrate, mainly as arabinose (7%) and galactose (13%). The protein content was 63%. Fractionation on DEAE–Sephadex and Sephadex G-100 gave one neutral and two acidic fractions, all containing protein, arabinose and galactose. The structure of the carbohydrate moiety was investigated by methylation analysis, periodate oxidation and enzyme incubation. The acidic fraction contained (1→6)-linked galactose residues, some being substituted on O-3 with arabinose. The neutral fraction consisted of a more extensively branched arabinogalactan with longer side chains of (1→3)- and (1→5)-linked arabinose. The arabinose was present mainly as α-L-arabinofuranosyl residues. Alkaline degradation and subsequent fractionation indicated the presence of a covalent linkage between hydroxyproline and arabinose. Periodate oxidation or incubation with α-L-arabinofuranosidase did not affect the allergenic activity of the extract.

INTRODUCTION

Timothy pollen is a commbn cause of allergic airway disease. Extracts of timothy pollen have been extensively used for hyposensitization and diagnostic purpose ever since immunotherapy with grass pollen extracts was introduced by Noon in 1911[1]. Much work has been carried out to characterize the pollen extracts which have a very complex composition. One of the most brillant investigations was done by Weeke *et al.*[2] who, with the aid of crossed immunoelectrophoresis (CIE) and crossed radioimmunoelectrophoresis (CRIE) detected 28 antigens in an extract from timothy pollen and showed that 11 of these acted as allergens. In this as in most other studies on allergen extracts the researchers concentrated on the protein content in accordance with the general concept that allergens are proteins. A few experiments like those performed by Augustin in 1959[3], indicated this. However, purified allergen preparations frequently contain carbohydrate, which has never been characterized properly. Neither has there been any systematic investigation regarding the possible importance of the carbohydrate on the allergenic activity.

The preparation which is the object of this investigation has been made by removing most of the carbohydrate and protein from a crude aqueous pollen extract.

It has been shown to contain the total allergenic activity of timothy pollen and to be preferable to the crude extract in clinical use[4]. The preparation has been partly characterized previously as to its chemical properties[5], allergenic *in vivo* and *in vitro* properties[6] and immunological properties in the human immunoglobulin E system[7]. However, although the carbohydrate content amounts to *ca* 20% of the dry weight of the material, it has not been further investigated. The purpose of the present study has been to establish the composition and structure of the carbohydrate fraction of the purified timothy pollen allergen preparation, and further, to assess its possible significance in the allergenic activity of the extract.

RESULTS AND DISCUSSION

The purified timothy pollen allergen preparation contained 7.3% arabinose and 13.7% galactose. In addition traces of glucose, xylose and rhamnose were present. The protein content was 63% as determined by the methods of Lowry *et al.*[8] and of Waddell and Hill[9] respectively. Chemical tests for sulphate, phosphate, hexosamine and uronic acid were negative. Furthermore neither hexosamine nor uronic acid could be detected by GC of TMSi-methyl glycosides or by GC/MS after conversion to alditol acetates.

Chromatography of the allergen extract on a DEAE–Sephadex A-25 column gave a neutral fraction (I) and an acidic fraction (II) (Fig. 1). The two fractions were subjected to gel filtration on a calibrated column of Sephadex G-100 (Fig. 2). This furnished one neutral fraction (A) and two acidic ones (B and C) of which the two most carbohydrate-rich, (A and B) were studied further.

The carbohydrate content of the two latter fractions as well as their MW distribution are given in Table 1. The MW-data are of the same order as those previously obtained by MW-determinations of allergens from timothy pollen[10].

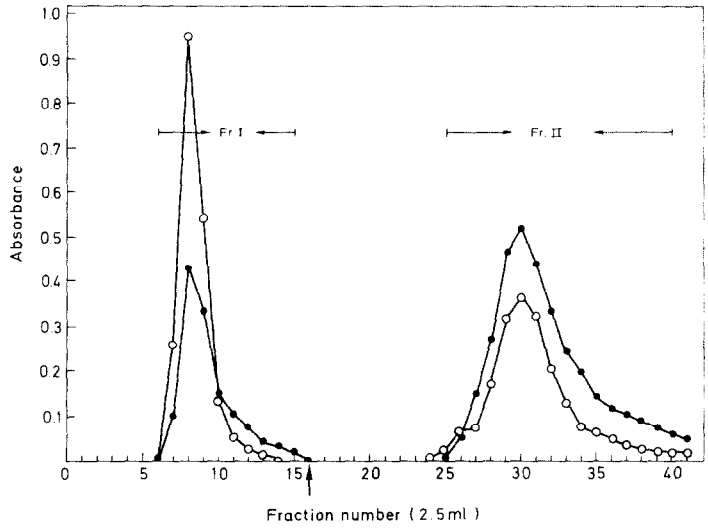

Fig. 1. Elution of allergen extract from timothy pollen on DEAE-Sephadex A-25. The column (30 × 2.5 cm) was eluted with 0.05 M Tris-HCl buffer pH 7.2. At fraction no. 16 the buffer was changed to buffer containing 0.25 M NaCl as indicated by the arrow. No carbohydrate or protein was eluted with buffer containing 0.5 M and 1.0 M NaCl. Fractions I and II were collected as indicated on the figure. (○) Carbohydrate; (●) protein.

The fractionation procedure did not lead to the isolation of pure carbohydrate or particular carbohydrate-rich fractions. A more successful separation into such fractions may be achieved by lectin affinity chromatography on tridacnin–Sepharose 4B as described by Gleeson *et al.*[11]. The restricted amount of material available in this study is the main reason why other fractionation techniques were not attempted.

Periodate oxidation was carried out on the total preparation. The periodate consumption was 0.74 mol/mol anhydro sugar. The product after oxidation was reduced with sodium borohydride, subjected to methanolysis[12], silylated and analysed by GC. This revealed a destruction of 52% of the arabinose and 67% of the galactose during the periodate treatment (Table 2). The only low MW alcohol detected was glycerol, which would be formed, e.g. from (1→5)-linked pentofuranose and from (1→6)-linked hexopyranose units. Due to a lack of material a complete Smith degradation could not be carried out. Mild acid hydrolysis[13] removed 70% of the arabinose; this indicates that most of the arabinose exists as arabinofuranose. Incubation of the allergen preparation with α-L-arabinofuranosidase[14] led to removal of 55% of the arabinose, demonstrating that most of the arabinose occurs as α-linked L-arabinofuranosyl units. During the enzyme incubation the digest became turbid suggesting that removal of terminal arabinose residues reduced the solubility of the arabinogalactan protein. Gleeson *et al.*[13] made similar observations in the study of an arabinogalactan protein from *Gladiolus* style.

The results of methylation analysis of fractions (A) and (B) are given in Table 3. Peak areas were used as an indication of their relative proportions. The results are consistent with those obtained by weak acid hydrolysis and enzymolysis, thus further supporting the evidence that most of the arabinose exists as arabinofuranosyl units.

From the data obtained the following conclusions may be drawn regarding the molecular structure. Fraction (A) contains (1→6)-linked galactopyranosyl units with branching at O-3 of approximately every second residue with (1→3)- and (1→5)-linked α-L-arabinofuranosyl side chains. The average side chain length is *ca.* three residues. In addition the fraction contained some (1→4)-linked glucose. This could originate from starch, known to be present in pollen. However, on incubation with α-amylase, maltose could not be conclusively identified in the digest. Even so, the existence of a small proportion of starch in the pollen preparation must be regarded as a possibility. Fraction (B) also contained (1→6)-linked galactopyranosyl units with branch points at O-3 but the degree of branching was less than in (A). In this fraction the arabinose appeared to exist almost solely as single terminal α-L-arabinofuranosyl residues linked to the galactan backbone. Possible structures of the arabinogalactan regions of the two fractions are given in Fig. 3.

Incubation with α- and β-D-galactosidase did not lead to detectable (GC) liberation of galactose. This may be due to the restricted amount of galactose end groups and/or steric hindrance. It thus remains to establish the anomeric configuration of the galactosidic linkages.

Arabinogalactans and arabinogalactan proteins have been isolated from a wide range of plant species and have been found in most plant tissues[15, 16]. The structures of the arabinogalactans from timothy pollen have some similarities with the structure of an arabinogalactan protein isolated from the female reproductive tissues of flowering plants[17]. However, a major difference is the apparent absence

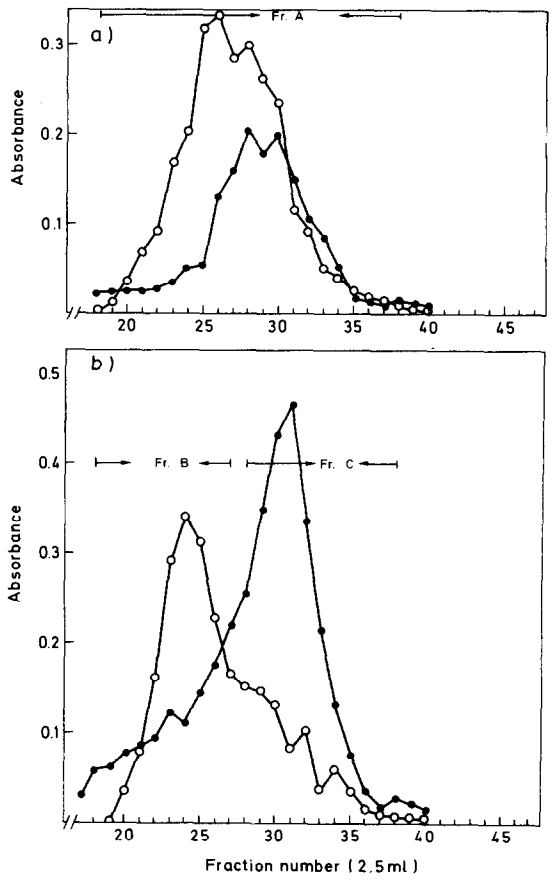

Fig. 2. Gel filtration on Sephadex G-100 (80 × 1.5 cm) with 0.05 M Tris–HCl buffer pH 7.2. The column was calibrated for MW-determination by reference dextrans and globular proteins. Blue dextran MW 2×10^6 was eluted at fraction 15, dextran MW 19 000 at fraction 22, ovalbumin MW 45 000 at fraction 23, dextran MW 7 500 at fraction 28, cytochrome *c* MW 13 000 at fraction 31, dextran MW 2000 at fraction 37 and $K_2Cr_2O_7$ at fraction 42. (○) Carbohydrate; (●) protein. (a) Fraction I; (b) fraction II. Fractions A, B and C were collected as indicated in the figure.

of (1→3)-linked galactose in the galactan backbone of timothy pollen arabinogalactan. Gleeson *et al.*[17] stated that this open-branched structure is particularly suited for interaction with other molecules and that the arabinogalactan proteins of the stigma may serve a function in the recognition of compatible pollen. In view of these speculations it was somewhat interesting to find that pollen contained extractable components of similar structure. Amino acid analysis of the unfractionated preparation revealed an amino acid composition common to plant glycoproteins (Table 4). The hydroxyproline content was shown to be 0.75% by the method of ref. [18].

Table 2. Arabinose and galactose content after periodate oxidation of the total preparation. Determined by GC as TMSi-methylglycosides. Given as % (w/w)

Oxidation time	Arabinose	Galactose
0	7.3	13.7
15 min	5.7	7.4
1 hr	5.4	6.3
6 hr	3.4	4.1

Since we failed in separating carbohydrate from protein, the existence of covalent carbohydrate–protein linkages was suspected. One type of such linkage commonly found in plant glycoproteins is the serine-*O*-galactosidic linkage. Hillestad and Wold [19] have demonstrated this mode of covalent carbohydrate–protein connection in a glycoprotein from *Cannabis sativa*. The linkage is alkali labile and treatment with alkaline tritiated borohydride will lead to a cleavage of the glycopeptide linkage by the β-elimination reaction resulting in the release of oligosaccharides containing terminal tritiated galactitol[20]. After having subjected the extract to such treatment the product was methanolysed, silylated and analysed by GC–RC. No radioactive alditol could be detected, but the activity of the reducing agent may have been too small. Hounsell *et al.*[21] recently showed that only 1% of the activity was found in the oligosaccharide fraction.

Another mode of covalent connection between carbohydrate and protein is the hydroxyproline–arabinose linkage. This linkage was first demonstrated by Lamport and Miller and has been shown to have a wide distribution in the Plant Kingdom[22]. To reveal the possible presence of such linkages the preparation was subjected to alkaline degradation[23] followed by chromatography on a Bio-Gel P-6 column. The eluate from the Bio-Gel P-6 column contained carbohydrate of lower MW than the native extract. As glycosidic linkages are resistant to the alkaline conditions used, the finding indicates degradation of protein bound to carbohydrate. This proves the presence of glycoprotein in the extract.

Non-peptide bound hydroxyproline was eluted from the Bio-Gel P-6 column with a volume corresponding to a considerably higher MW than that of free hydroxyproline. PC of appropriate fractions revealed spots which contained arabinose and hydroxyproline and whose mobilities were consider-

Table 1. Carbohydrate composition of fraction A and B. Determined by GC as TMSi-methylglycosides

Fraction	Arabinose	Galactose	Glucose	MW (dextran)
A	12.0%	7.0%	2.0%	$5–20 \times 10^3$
B	1.0%	4.0%	—	$5–25 \times 10^3$

Table 3. Alditol acetates obtained from methylated fractions A and B. Reduced with $NaBD_4$

R_{TMG}	Alditol acetate		Area of total peak area (%) A	B
0.48	2,3,5-Tri-*O*-methylarabinitol	45, 118, 161	7	1
0.59	2,3,4-Tri-*O*-methylarabinitol	117, 118, 161, 162	tr	tr
0.82	3,5-Di-*O*-methylarabinitol	45, 161, 190	tr	—
0.89	2,5-Di-*O*-methylarabinitol	45, 118, 233	2	—
1.06	2,3-Di-*O*-methylarabinitol	118, 189	5	—
1.08	2,3,5,6-Tetra-*O*-methylgalactitol	89, 117, 118, 161	—	tr
1.14	2,3,4,6-Tetra-*O*-methylgalactitol	45, 118, 161, 162, 205	1	tr
1.69	2-Mono-*O*-methylarabinitol	118	1	—
2.37	2,3,6-Tri-*O*-methylglucitol	45, 118, 162, 189, 233	4	—
2.55	2,3,4-Tri-*O*-methylgalactitol	118, 162, 189, 233	4	3
4.24	2,4-Di-*O*-methylgalactitol	118, 189	5	1

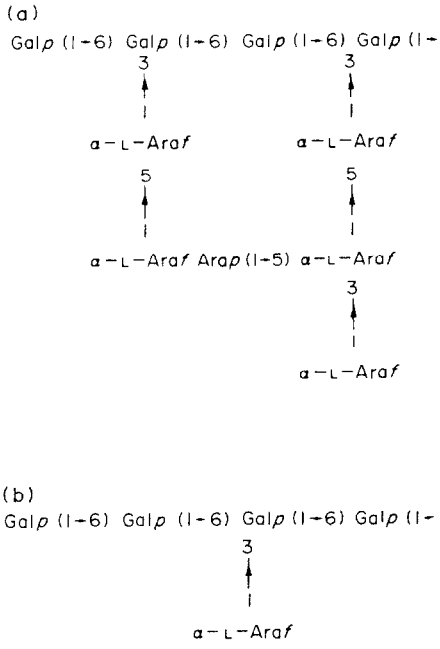

Fig. 3. Possible structures for the arabinogalactans isolated from the allergenic preparation from timothy pollen. In addition to the linkages indicated in the figure some of the arabinose is probably linked to hydroxyproline thus forming an integral part of glycoproteins with arabinose and/or arabinogalactan side chains. (a) Fraction A: this fraction also contains some (1→4)-linked glucopyranosyl units; (b) fraction B.

Table 4. Amino acid composition of the extract. The results are given in mol% of total amino acids

Aspartic acid	10.5
Threonine	5.9
Serine	5.5
Glutamic acid	12.9
Proline	5.6
Glycine	7.2
Alanine	16.7
Valine	6.7
Methionine	1.0
Isoleucine	3.6
Leucine	5.9
Tyrosine	1.9
Phenylalanine	4.1
Histidine	1.5
Lysine	8.4
Arginine	2.5
Hydroxyproline	0.6*

*Obtained by the colorimetric method [18].

ably lower than that of free hydroxyproline or free arabinose. This indicates the presence of alkali stable hydroxyproline arabinosides which in turn may indicate the presence of a glycoprotein with *O*-glycosidic linkages between arabinose and the hydroxyl group of hydroxyproline. Parallel to this investigation it was shown in our laboratory that major allergens from birch and alder are glycoproteins [16, 24]. However, this paper presents for the first time the nature of the covalent linkage between carbohydrate and protein in an allergenic preparation, except for the thio-glucoside residue found in cod allergen [25]. The finding indicates that at least some of the allergens in timothy pollen may be glycoproteins. This is in agreement with results obtained by Löwenstein [10]. He found that one of the purified allergens from timothy pollen (Ag 30) contained only 50% protein.

After periodate oxidation (0.01 M $NaIO_4$, pH 4, 4°) at intervals ranging from 15 min to 6 hr the allergenic activity of the extract was tested by the RAST-inhibition test [26]. In this test the allergenic activity is measured as the ability of the tested substance to compete with allergens bound to a cyanogen bromide-

activated paper disc in binding specific IgE. However, no alteration of the allergenic activity of the preparation could be detected after periodate oxidation (Fig. 4). Incubation with α-L-arabinofuranosidase (6 hr and 24 hr) followed by testing in the RAST-inhibition test system gave similar results (Fig. 5). According to these experiments it seems that the carbohydrate which was destroyed or removed was of no importance for the allergenic activity of the extract. This means that the actual carbohydrate does not form an integral part of the allergen determinants. Neither does it contribute to the conformation of the allergen, necessary for binding of IgE. The experiment may indicate that the carbohydrate is without significance for the activity of the allergens in timothy pollen. To prove this, however, studies in which all of the carbohydrate is destroyed/removed have to be performed. Treatment with specific glycosidases will probably be a preferable way to remove carbohydrate as the use of chemical agents may result in simultaneous alteration of protein structure. Generally there are many ways to explain an observed decrease in allergenic activity.

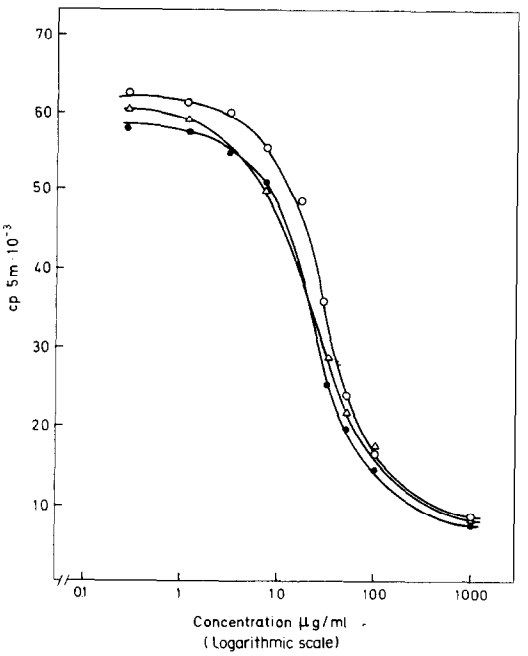

Fig. 5. RAST-inhibition of allergenic preparation from timothy pollen after incubation with α - L - arabinofuranosidase. (○) Control of native extract; (●) 6 hr incubation; (△) 24 hr incubation. After 24 hr incubation the arabinose content of the extract was reduced to 45%.

EXPERIMENTAL

Materials. The allergen preparation (batch no. 526/16-1) was manufactured by Nyegaard & Co. A/S (Oslo) and supplied as freeze-dried material. This purified extract was prepared from pollen of *Phleum pratense* L. delivered by Allergon, Engelholm (Sweden) who had cultivated the plant and collected the pollen which was stated to be 95% pure (5% plant debris and <0.1% foreign pollen). α-D-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) from *Coffea arabica* seeds was obtained from Boehringer. β-D-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) from *Escherichia coli* grade IV was obtained from Sigma.

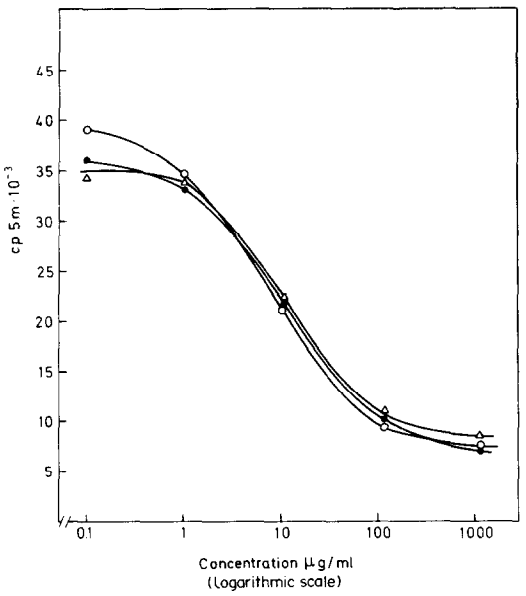

Fig. 4. RAST-inhibition of allergenic preparation from timothy pollen after periodate oxidation (0.01 M $NaIO_4$, pH 4, 4°). (○) Extract not subjected to periodate oxidation; (●) 1 hr oxidation; (△) 6 hr oxidation. The curve after 15 min oxidation is omitted as it was identical to the curve of the control. The content of arabinose and galactose after periodate oxidation is given in Table 3.

General methods. Carbohydrate estimation was carried out by the PhOH–H_2SO_4 method [27] with D-galactose and L-arabinose (2:1) as standard. Protein was determined by the method of ref. [8] and by the direct spectrophotometric method described in ref. [9], both methods used bovine serum albumin as standard. SO_4^{2-} was estimated according to the method of ref. [28]. Test for Pi [29] was performed after hydrolysis of the extract by 5 M H_2SO_4 for 3 hr at 100°. Hexosamine was tested for by the Elson–Morgan reaction, as modified in ref. [30]. Amino acids were analysed on a Bio Cal BC 200 automatic amino acid analyser after hydrolysis of the sample (10 mg) with 6 M HCl (2 ml) for 18 hr at 110° under N_2. The same hydrolysate was assayed for hydroxyproline by the method of ref. [18]. All the GC analyses were performed on a FID instrument using N_2 as the carrier gas. For GC/MS the column was coupled with a Micromass 7070F mass spectrometer.

Methanolysis [12] *and gas chromatography.* The dried sample (*ca* 1 mg) was heated with 1 M HCl in dry MeOH in a sealed tube for 24 hr at 80°; mannitol was used as int. standard. TMSi derivatives of the methyl glycosides were prepared with pyridine–HMDS–TMCS (5:2:1) according to ref. [31]. The TMSi methyl glycosides were chromatographed on a column (390 × 0.2 cm) of 3% SE-52 on Varaport using a temp. programme starting at 155° with an increase of 1°/min for 10 min, followed by an increase of 2°/min to 185° and finally 4°/min to 250°. N_2 at 30 ml/min was used as carrier gas.

Hydrolysis of the polysaccharide moiety. The sample (1 mg) was hydrolysed in 90% HCO_2H (1 ml) for 6 hr at 100° under N_2. 4 ml H_2O was then added and the soln heated for 2 hr at 100°. The hydrolysate was evaporated to dryness under red. pres. below 40°.

Conversion of monosaccharides to alditol acetates. Ca 5 mg extract was subjected to HCO_2H-hydrolysis and the evaporated hydrolysate boiled with 10 ml MeOH and 1 ml dry Dowex 50 W × 8[H^+] for 24 hr. Any uronic acid methyl esters were then selectively reduced with $NaBD_4$. Excess $NaBD_4$ neutralized with Dowex 50 W × 8[H^+] and boric acid removed as methyl borate by additions of MeOH and subsequent evaporations. The methyl glycosides were hydrolysed with HCO_2H and free monosaccharides reduced with $NaBH_4$. Excess $NaBH_4$ was destroyed by addition of HOAc and H_3BO_4 removed as before. Acetylation was carried out by heating with Ac_2O at 100° for 30 min. The alditol acetates were analysed by GC/MS on a column (200 × 0.2 cm) of 3% OV-225 at 210°.

Fractionation. The allergen preparation (25 mg) dissolved in 0.05 M Tris–HCl buffer pH 7.2 (1 ml) was applied to a column (30 × 2.5 cm) of DEAE–Sephadex A-25 and eluted with the same buffer; 2.5 ml fractions were collected. The carbohydrate and protein contents were detected as described above. When the eluate contained no more carbohydrate, the column was eluted with the Tris–HCl buffer containing 0.25 M NaCl. The neutral fraction (I) and acidic fraction (II) isolated (Fig. 1) were desalted (Amicon Ultrafiltration Cell with Diaflo UM-2 filter) and freeze-dried. The lyophilized material was chromatographed on a column of Sephadex G-100 (80 × 1.5 cm) eluted with 0.05 M Tris-HCl pH 7.2 (2.5 ml fractions). The column had previously been calibrated for MW estimation with reference dextrans (gift from Dr. K. Granath, Pharmacia, Uppsala), and with globular proteins.

Periodate oxidation. The sample (10 mg) was subjected to oxidation with 0.01 M $NaIO_4$ in 0.1 M NaOAc buffer pH 4 (5 ml) at 4° in the dark. The consumption of periodate was followed spectrophotometrically at 223 nm[32], and the reaction terminated after 24 hr by addition of ethylene glycol. Free aldehyde groups were reduced by $NaBH_4$, the soln desalted, freeze-dried and subjected to methanolysis and analysis of monosaccharides by GC as described earlier. For testing of allergenic activity after $NaIO_4$ oxidation samples (0.5 ml) were taken from the oxidation mixture after 15 min, 1 hr and 6 hr. The reaction was then immediately stopped by the addition of ethylene glycol, $NaBH_4$ (1 mg) and one drop of HOAc, and an aliquot of the soln tested by the RAST-inhibition method[26]. The rest was desalted on a Bio-Gel P-2 column, freeze-dried, subjected to methanolysis and GC of TMSi-methylglycosides.

Methylation analysis[33]. The sample (5 mg) was dissolved in dry DMSO (1.5 ml), the vial flushed with N_2 and 2 M methylsulfinyl anion[34] in DMSO (2 ml) was added. The mixture was kept on an ultra-sonic bath for 30 min and then left without stirring overnight at room temp. The vial was then placed on ice and MeI (1.5 ml) was added. After ultra-sonic agitation at room temp. for 1 hr the vial was opened and excess MeI removed by flushing with a stream of N_2. The residue was dialysed and evaporated to dryness. The product was hydrolysed by HCO_2H and the monosaccharides reduced with $NaBD_4$. Excess $NaBD_4$ was destroyed by addition of HOAc and H_3BO_4 removed as methyl borate by additions of MeOH and subsequent evaporations. Acetylation was carried out by heating in Ac_2O at 100° for 30 min and the partially methylated alditol acetates were analysed by GC/MS on a column (400 × 0.2 cm) of 3% OV-225 on Varaport isothermally at 200°.

Oxalic acid hydrolysis[13]. The sample (10 mg) was heated with 12.5 mM oxalic acid (2 ml) for 5 hr at 100°. The hydrolysate was desalted on a Bio-Gel P-2 column, freeze-dried, subjected to methanolysis and monosaccharides analysed by GC as TMSi-methylglycosides.

Incubation with α-L-arabinofuranosidase. The enzyme α-L-arabinofuranosidase, kindly provided by Professor M. Neukom, Zürich, was isolated from Pectinol R-10[14]. The extract (3 mg) with enzyme suspension (0.1 ml) was incubated for 24 hr at 37° in citrate–phosphate buffer 0.1 M pH 4 (0.1 ml). After incubation for 6 and 24 hr, samples (50 μl) were taken out for testing of allergenic activity[26]. The rest was separated into low and high MW fractions on a Bio-Gel P-2 column. The low MW fraction was silylated[31] and analysed by GC. The high MW fraction was subjected to methanolysis, silylation and analysis by GC.

Incubation with α-D-galactosidase. The preparation (4 mg) in 0.1 M citrate–phosphate buffer pH 6 (0.1 ml) was incubated for 24 hr at 37° under toluene with 0.1 ml α - D - galactosidase suspension (5 mg/ml). The mixture was then deionized with Zeolite DM-F(CO_3^{2-}) resin, evaporated to dryness, silylated[31] and analysed by GC.

Incubation with β-D-galactosidase. The preparation (5 mg) in 0.1 M Pi buffer pH 7 (1 ml) was incubated for 15 hr at 37° under toluene with 0.05 ml β - D - galactosidase suspended in 2.2 M $(NH_4)_2SO_4$. The rest of the procedure was as described for incubation with α - D - galactosidase.

Alkaline degradation in the presence of tritiated borohydride. To the extract (15 mg) dissolved in 0.5 ml H_2O was added 10 mg $NaBH_4$ to reduce any free aldehyde groups. After 2 hr at room temp. 60 mg $NaBH_4/NaBT_4$ with sp. act. 4 μCi/mmol and 1.5 ml 0.1 M NaOH was added. The mixture was heated in a stoppered vessel at 50° for 8 hr [35]. It was then diluted with H_2O, neutralized to pH 5 with HOAc and freeze-dried. The residue was dissolved in 1 ml H_2O and applied to a column (26 × 1.5 cm) of Bio-Gel P-6 which was eluted with H_2O. Fractions of 1 ml were collected and assayed for carbohydrate and protein according to the methods described previously[8, 27]. 10 μl of each fraction was added to a filter paper (diam. 1.5 cm) which was transferred to a scintillation vial containing 5 ml scintillation liquid (PPO 5 g, dimethyl POPOP 0.05 g/l. in toluene). ^3H was measured in a Mark II liquid scintillation system. Appropriate fractions were evaporated to dryness and subjected to methanolysis and silylation[31]. The TMSi-derivatives were analysed by GC-RC isothermally at 160° on a column (400 × 0.2 cm) of 3% SE-52 on Varaport 30 coupled to a Nuclear® radioactivity detector for GC. The carrier gas was Ar at 60 ml/min.

Alkaline degradation. The extract (15 mg) was treated with saturated, aq. $Ba(OH)_2$ on a reflux for 8 hr[23]. After neutralization with 0.5 M H_2SO_4 and filtration the soln was evaporated to dryness. The residue was dissolved in 0.5 ml H_2O, applied to a column (26 × 1.5 cm) of Bio-Gel P-6 and eluted with H_2O. The fractions (1 ml) were assayed for non-peptide-bound hydroxyproline[18] and for pentose as described in ref. [23]. Appropriate fractions were combined and subjected to PC on Whatman No. 1 paper with EtOAc–HOAc–HCO_2H–H_2O (18:3:1:4). Carbohydrate was detected by periodate–benzidine[36] and hydroxyproline by isatin[37].

RAST-inhibition test. 30 μl of diluted allergen soln (0.01–1000 μg/ml) was added to 20 μl test serum (serum pool from 30 non-hyposensitized individuals allergic to timothy pollen,

diluted 1+2 with 0.9% NaCl). After 1.5 hr incubation at room temp. a RAST-disc with covalently bound allergens from the timothy extract was added (Paper discs CNBr-activated according to the method of ref. [38] and allergens coupled as described in ref. [26]). This was incubated overnight at room temp., the discs then rinsed 3 × 10 min (2.5 ml RAST rinsing soln, Pharmacia). After the third rinse the supernatant was completely removed by suction and 50 μl anti-IgE ^{125}I added [Phadebas (Pharmacia) RAST anti-IgE ^{125}I]. After further incubation overnight at room temp. and rinsing 4 × 10 min the radioactivity of the dry disc was counted in a γ-counter.

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REFERENCES

1. Noon, L. (1911) *Lancet* **1**, 1572.
2. Weeke, B., Löwenstein, H. and Nielsen, L. (1974) *Acta Allergol.* **29**, 402.
3. Augustin, R. (1959) *Immunology* **2**, 230.
4. Frostad, A. B., Grimmar, Ø., Sandvik, L. and Aas, K. (1980) *Allergy* **35**, 81.
5. Grimmer, Ö., Andersen, I., Dale, S., Oseid, S., Thorsdalen, N., Skålhegg, B. and Aas, K. (1977) *Int. Arch Allergy Appl. Immun.* **55**, 29.
6. Frostad, A. B., Bolle, R., Grimmer, Ö. and Aas, K. (1977) *Int. Arch. Allergy Appl. Immunol.* **55**, 35.
7. Grimmer, Ö., Bolle, R., Frostad, A. and Aas, K. (1977) *Int. Arch Allergy Appl. Immunol.* **55**, 41.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol Chem.* **193**, 265.
9. Wadell, W. J. and Hill, C. (1956) *J. Lab. Clin. Med.* **48**, 311.
10. Löwenstein, H. (1978) *Allergy* **33**, 30.
11. Gleeson, P. A., Jermyn, M. A. and Clarke, A. E. (1979) *Analyt. Biochem.* **92**, 41.
12. Chambers, R. E. and Clamp, I. R. (1971) *Biochem. J.* **125**, 1009.
13. Gleeson, P. A. and Clarke, A. E. (1979) *Biochem. J.* **181**, 607.
14. Neukom, H., Providoli, L., Gremli, H. and Hui, P. A. (1967) *Cereal Chem.* **44**, 213.
15. Clarke, A. E., Anderson, R. L. and Stone, B. A. (1979) *Phytochemistry* **18**, 521.
16. Vik, H., Elsayed, S., Paulsen, B. S. and Apold, J. (1982) *Int. Arch. Allergy Appl. Immunol.* (in press).
17. Gleeson, P. A. and Clarke, A. E. (1979) in *Arabinogalactan Protein News* Vol. 2, p. 9. University of Melbourne, Australia.
18. Kivirikko, K. I. and Lesmaa, M. (1959) *Scand. J. Clin. Lab. Invest.* **11**, 128.
19. Hillestad, A. and Wold, J. K. (1977) *Phytochemistry* **16**, 1947.
20. Aminoff, D., Gathman, W. D., McLean, C. M. and Yadomae, T. (1980) *Analyt. Biochem*, **101**, 44.
21. Hounsell, E. F., Wood, E. and Feizi, T. (1981) *Carbohydr. Res.* **90**, 283.
22. Lamport, D. T. A. and Miller, D. H. (1971) *Plant Physiol.* **48**, 454.
23. Lamport, D. T. A. (1967) *Nature* **216**, 1322.
24. Florvaag, E., Elsayed, S. and Paulsen, B. S. (1982) *Int. Arch. Allergy Appl. Immunol.* (in press).
25. Elsayed, S. and Bennich, H. (1975) *J. Immunol.* **4**, 203.
26. Yman, L., Ponterius, G. and Brandt, R. (1975) *Dev. Biol. Standard* **29**, 151.
27. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Analyt. Chem.* **28**, 350.
28. Antonopoulos, C. A. (1962) *Acta Chem. Scand.* **16**, 1521.
29. Fiske, C. H. and Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375.
30. Gatt, R. and Berman, E. R. (1966) *Analyt. Biochem.* **15**, 167.
31. Sweeley, C. C., Bentley, R., Makita, M. and Wells, W. W. (1963) *J. Am. Chem. Soc.* **85**, 2497.
32. Dixon, J. S. and Lipkin, D. (1954) *Analyt. Chem.* **26**, 1092.
33. Hakomori, S. (1964) *J. Biochem.* **55**, 205.
34. Sandford, P. A. and Conrad, H. E. (1966) *Biochemistry* **5**, 1508.
35. Lamport, D. T. A., Katona, L. and Roerig, S. (1973) *Biochem. J.* **133**, 125.
36. Gordon, H. T., Thornborg, W. and Werum, L. N. (1956) *Analyt. Chem.* **28**, 849.
37. Acher, R., Fromageot, C. and Iutisz, M. (1950) *Biochim. Biophys. Acta* **5**, 81.
38. Ceska, M., Eriksson, R. and Varga, I. M. (1972) *J. Allergy Clin. Immunol.* **49**, 1.