Biochem. J. (1981) **197**, 695–706
Printed in Great Britain

Isolation and partial characterization of two antigenic glycoproteins from rye-grass (*Lolium perenne*) pollen

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(*Received 6 April 1981/Accepted 24 April 1981*)

Two glycoproteins have been purified from a buffer extract of rye-grass (*Lolium perenne*) pollen. Both migrated as single bands on sodium dodecyl sulphate/polyacrylamide gels. Glycoprotein 1 (0.8 mg/g of pollen) had an apparent mol.wt. of 33000 and contained 95% protein and 5% carbohydrate. The monosaccharides glucose, galactose, mannose, arabinose and *N*-acetylglucosamine were present in the proportions 3:3:1:2:1. Glycoprotein 2 (0.4 mg/g of pollen) had an apparent mol.wt. of 68000 and contained 88% protein and 12% carbohydrate. The monosaccharides glucose, galactose, mannose, fucose, xylose, arabinose and *N*-acetylglucosamine were present in the proportions 4:7:13:5:8:6:6. This glycoprotein bound concanavalin A and *Lotus tetragonolobus* (asparagus pea) lectin. Radioallergosorbent (RAST) inhibition tests showed that Glycoprotein 1 is an effective allergen, whereas Glycoprotein 2 has less allergenic activity. A method for performing both lectin-binding assays and RAST inhibition tests using microtitre trays is described.

In contrast with the extensive studies of structure and function of animal glycoproteins, there are relatively few reports regarding glycoproteins of plant origin (Sharon & Lis, 1979; Lamport, 1980). In the present study we have isolated two glycoproteins from rye-grass (*Lolium perenne*) pollen. These glycoproteins are of interest from several points of view: firstly, macromolecules of the pollen wall have been implicated in the recognition of pollen by a compatible stigma (Heslop-Harrison, 1975); the glycoproteins studied are present in the pollen wall (Knox *et al.*, 1980) and may be involved in the pollen–stigma recognition. Secondly, both glycoproteins are effective antigens in rabbits; increasingly, immunological techniques are being used to demonstrate markers of identity in plants (Raff *et al.*, 1980) and to monitor molecular changes associated with differentiation (Khavkin *et al.*, 1977, 1979), although the antigens involved have not been chemically defined. The present study contributes to our understanding of the nature of antigenic plant macromolecules. Finally, Glycoprotein 1 is the major allergen of rye-grass pollen and binds specifically to immunoglobulin E of sensitized human subjects.

Abbreviations used: SDS, sodium dodecyl sulphate; IgG, immunoglobulin G; IgE, immunoglobulin E.

Experimental

Materials

Pollen was obtained from Greer Laboratories, Lenoir, NC, U.S.A. Microtitre trays were purchased from Cooke Laboratories, VA, U.S.A. $Na^{125}I$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The lectins concanavalin A, castor-bean (*Ricinus communis*) lectin, peanut (*Arachis hypogaea*) agglutinin, soyabean (*Glycine max*) agglutinin and asparagus pea (*Lotus tetragonolobus*) lectin were purchased from Miles Laboratories, Elkhart, IN, U.S.A. CM-Sepharose 6B, Sephadex G-75, concanavalin A–Sepharose, Protein A, molecular-weight markers, and Phadebas RAST (radioallergosorbent) kit were from Pharmacia Ltd., Uppsala, Sweden. The molecular-weight markers were phosphorylase *b* (mol.wt. 94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100) and α-lactalbumin (14400). Other markers for gel filtration were cytochrome *c* (13500), ovalbumin and bovine serum albumin from Sigma, MO, U.S.A. Urea (spectroscopic grade) and Iodogen were obtained from Pierce Chemicals, Rockford, IL, U.S.A. Acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylenediamine were purchased from East-

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man Kodak, Rochester, NY, U.S.A. Ampholines, pH range 3.5–10 were from LKB, Stockholm, Sweden. Horseradish peroxidase and fungal glucose oxidase were obtained from Calbiochem, Richmond, CA, U.S.A. Monosaccharides (methyl α-mannoside, L-arabinose and D-galactose) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Arabinogalactan-protein from the style of *Gladiolus gandavensis* and its specific antiserum were prepared as previously described (Gleeson & Clarke, 1979, 1980). Bovine serum albumin (Sigma A7638-Globulin-free bovine albumin) was used for all lectin-binding studies on the microtitre trays.

Methods

Isolation of pollen antigens. Pollen (20g) was stirred in 0.01M-Tris/HCl/0.15M-NaCl, pH 8.0 (100ml), for 1h at room temperature. The filtrate was retained and the residue re-extracted with 100ml of the same buffer. The filtrates were combined and centrifuged at 15000*g* ($r_{av.}$ 8cm) at 4°C for 15min and the supernatant brought to 80% saturation with $(NH_4)_2SO_4$ at room temperature during 1.5h. The supernatant retained the yellow pigments of the initial pollen preparation, whereas the precipitate was grey–white in colour. The precipitate was collected by centrifugation at 15000*g* ($r_{av.}$ 8cm) at 4°C for 20min, dissolved in 0.01M-sodium phosphate, pH 6.4 (20ml), and dialysed exhaustively against the same buffer. Two fractions were separated by cation-exchange chromatography; the dissolved precipitate was applied to a column (1.65cm × 29cm) of CM-Sepharose 6B in 0.01M-sodium phosphate buffer, pH 6.4. The material that did not bind was collected, dialysed, concentrated by ultrafiltration (Amicon Diaflo Cell model 202; PM-10 membrane) and applied to a column (2.5cm × 40cm) of Sephadex G-75 in phosphate-buffered saline (0.01M-sodium phosphate/0.15M-NaCl, pH 7.0). Fractions (2ml) were collected, monitored by absorbance at 280nm, examined by SDS/polyacrylamide-gel electrophoresis and by immunoelectrophoresis using antiserum raised to the whole pollen extract. The elution profile is shown in Fig. 1(*a*). The major peak contained a major component of apparent mol.wt. 33000, with several minor components of lower molecular weight. The major component was rechromatographed under the same conditions to give a preparation containing essentially one component, referred to as 'Glycoprotein 1' (Fig. 1*b*).

The material bound to CM-Sepharose was eluted with 1M-NaCl/0.01M-sodium phosphate, pH 7.0, dialysed, concentrated and applied to a Sephadex G-75 column under the conditions described for the material that did not bind to CM-Sepharose; the profile is shown in Fig. 2(*a*). The fraction that eluted

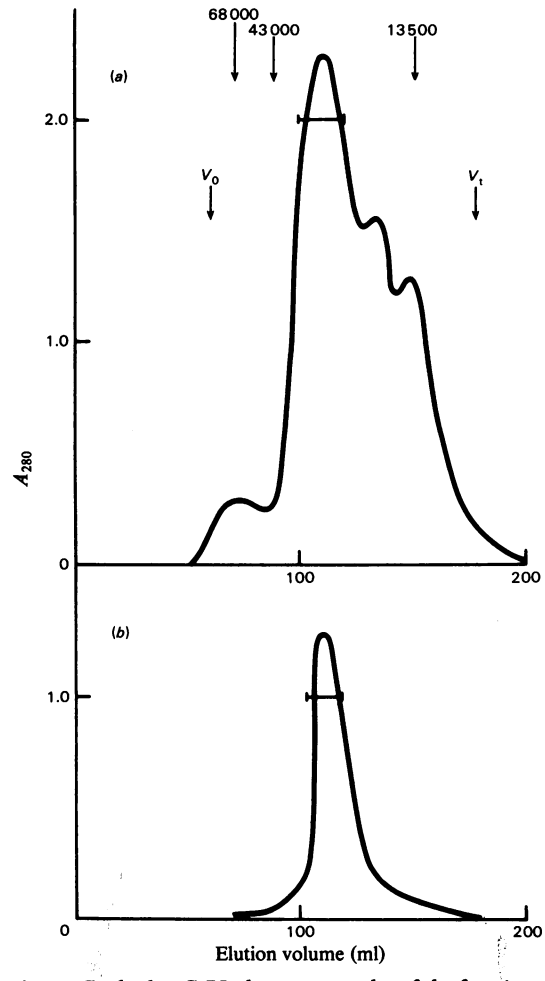

Fig. 1. *Sephadex G-75 chromatography of the fraction of rye-grass pollen extract that did not bind to CM-Sepharose (column dimensions 2.5cm × 40cm) in 0.01M-sodium phosphate /0.15M-NaCl buffer, pH 7.0*

(*a*) Chromatography of pollen extract that did not bind to CM-Sepharose. (*b*) Rechromatography of the major peak from (*a*). ⟷ indicates fractions pooled. The elution volumes of reference molecular-weight markers are shown. V_0, void volume; V_t, total bed volume.

first from the column was rechromatographed under the same conditions, to give a preparation containing essentially one component, referred to as 'Glycoprotein 2' (Fig. 2*b*).

SDS/polyacrylamide-gel electrophoresis. Slab gels containing 12.5% (w/v) polyacrylamide and 0.1% SDS were prepared and electrophoresis was conducted as described by Laemmli (1970).

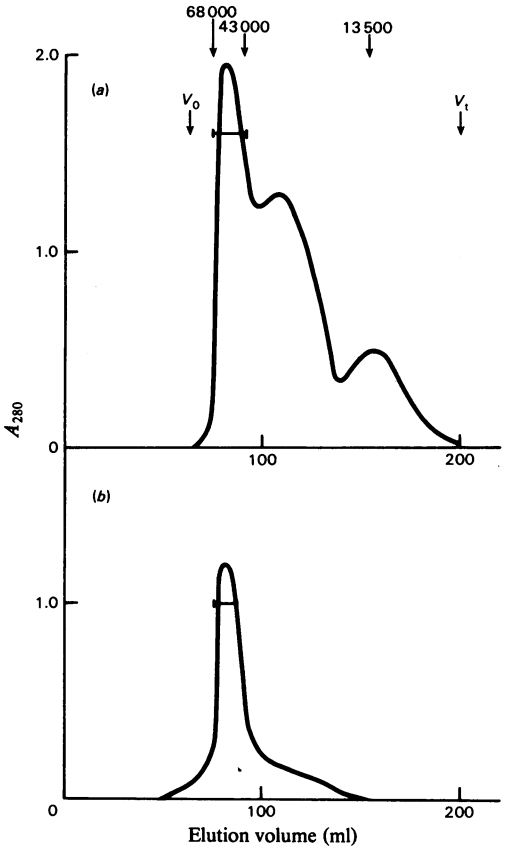

Fig. 2. *Sephadex G-75 chromatography of the fraction of rye-grass pollen extract bound to CM-Sepharose (column dimensions 2.5 cm × 40 cm) in 0.01 M-sodium phosphate/0.15 M-NaCl buffer, pH 7.0*

(*a*) Chromatography of pollen extract that bound to CM-Sepharose. (*b*) Rechromatography of the major peak from (*a*). ⊢——⊣ indicates fractions pooled. The elution volumes of reference molecular-weight markers are shown.

Two-dimensional gel electrophoresis. This was performed as described by O'Farrell (1975).

Ultracentrifugation. The molecular weight of Glycoprotein 1 was determined by the meniscus-depletion sedimentation-equilibrium method of Yphantis (1964) with a Beckman model E ultracentrifuge equipped with interference optics. The sample (0.3 mg/ml in 0.05 M-Tris/HCl/0.1 M-NaCl, pH 7.6) was centrifuged for 20 h at 26000 rev./min at 20°C.

Paper chromatography. This was performed on Whatman no. 3 paper in ethyl acetate/pyridine/water (8:2:1, by vol.). The chromatograms were developed for 18 h and then stained for reducing sugars with silver nitrate (Trevelyan *et al.*, 1950).

Neutral-monosaccharide analysis. Samples [Glycoprotein 1 (1.62 mg) and Glycoprotein 2 (2.00 mg)] were hydrolysed in 2.5 M-trifluoroacetic acid (2 ml) at 100°C for 1 h in a sealed tube under N_2. The samples were reduced with $NaBH_4$ and acetylated (Albersheim *et al.*, 1967). The alditol acetates were separated by g.l.c. on a column (1.85 m × 4 mm diameter) of 3% SP2340 on 100/120 Supelcoport (Supelco Inc., Bellenfonte, PA, U.S.A.), in a model 5710A Hewlett–Packard (Avondale, PA, U.S.A.) gas chromatograph. Chromatography was isothermal at 215°C with a N_2-carrier-gas flow rate of 60 ml/min.

Amino acid analysis. Samples [Glycoprotein 1 (0.72 mg) and Glycoprotein 2 (0.92 mg)] were hydrolysed by boiling under reflux in 6 M-HCl for 24 h under nitrogen. The high-dilution technique of Pusztai & Morgan (1963) was used (protein concentration less than 0.01%).

Determination of specific absorption coefficients. Glycoproteins 1 and 2 were freeze-dried, dried to constant weight at room temperature over P_2O_5, and weighed in a dry atmosphere to prepare solutions (1 mg/ml) in phosphate-buffered saline. The specific absorption co-efficient ($A^{1\%}_{1cm}$) was 12 for Glycoprotein 1. Measurement of the absorption of Glycoprotein 2 was difficult because of its sparing solubility; an estimate ($A^{1\%}_{1cm}$ 10) was obtained by using a more dilute solution (0.2 mg/ml). Concentrations of Glycoproteins 1 and 2 used in subsequent experiments were calculated from these values.

Immunodiffusion and immunoelectrophoresis. Antisera were raised in rabbits to the whole pollen preparation and to the purified components by using the immunization schedule described by Livingston (1974). The following amounts of eliciting antigen were used in each injection: pollen extract, 2 mg; Glycoprotein 1, 0.5 mg; Glycoprotein 2, 1.0 mg. Antisera were stored at −20°C in 200 μl portions. Double diffusion was conducted as described by Ouchterlony (1958). Immunoelectrophoresis was conducted by the method of Schiedigger (1955) in 1% agarose gels in 0.2 M-sodium barbital, pH 8.8 (current, 3 mA/cm of gel width; antigens, 100 μg in 10 μl; antisera were undiluted).

Lectin affinity chromatography. (*a*) Concanavalin A–Sepharose chromatography: pollen extract (18 mg in 3 ml) was dialysed against 0.1 M-sodium acetate / 1 mM-$MgCl_2$ / 1 mM-$MnCl_2$ / 1 mM-$CaCl_2$ / 0.5 M-NaCl, pH 6.0, and applied to a column (0.8 cm × 18 cm) of concanavalin A–Sepharose in the same buffer at 4°C. The unbound fraction and the fraction eluted with 0.1 M-methyl α-mannoside in the same buffer were dialysed against water and freeze-dried. (Yield: unbound fraction 9 mg; bound fraction 5 mg.) These fractions were examined by

SDS/polyacrylamide-gel electrophoresis and by immunoelectrophoresis by using antisera to pollen extract, Glycoprotein 1 and Glycoprotein 2.

(*b*) Tridacnin–Sepharose chromatography: tridacnin–Sepharose (1.5ml) was prepared and used as described by Gleeson *et al.* (1978). Pollen extract (5mg in 1ml) was dialysed against 0.15M-NaCl/0.01M-$CaCl_2$ and applied to a column (0.5cm × 4cm) of Tridacnin–Sepharose in the same buffer. The unbound fraction and the fraction eluted with 0.1M-lactose were dialysed against water and freeze-dried. (Yield: unbound fraction 3.6mg; bound fraction 0.1mg.)

Lectin binding to Glycoproteins 1 and 2. Lectins (concanavalin A, soya-bean agglutinin, castor-bean lectin, peanut agglutinin and *Lotus tetragonolobus* lectin were iodinated by using the Iodogen technique of Fraker & Speck (1978). The specific radioactivities were 1.08×10^8c.p.m./μg, 3.93×10^7c.p.m./μg, 2.53×10^8c.p.m./μg, 1.5×10^7c.p.m./μg and 1.16×10^8c.p.m./μg respectively. The binding assays were performed in flexible polyvinylchloride microtitre trays, which adsorb many proteins and glycoproteins (Marier *et al.*, 1979; Howlett & Clarke, 1981).

The glycoproteins being examined (40μl of a 50μg/ml solution in phosphate-buffered saline) were pipetted into individual wells of the tray. After 3h incubation at room temperature, unbound glycoproteins were removed from individual wells with a Pasteur pipette fitted with a flexible plastic tube tip and stored for use in subsequent experiments. In this way, excess glycoprotein was removed without disturbing the adsorbed layer. Approx. 2% of the total glycoprotein applied was adsorbed. Remaining potential binding sites in the wells were blocked by adding 200μl of 1% bovine serum albumin in phosphate-buffered saline to individual wells. After 5min, this solution was removed by suction. The wells were then washed twice with portions (200μl) of 1% bovine serum albumin in phosphate-buffered saline; after the final wash the plate was drained by inverting it on paper tissues, and it was allowed to air-dry at room temperature. The plate could either be used immediately or stored at −20°C for periods of at least 1 month with no subsequent loss of lectin-binding or antibody-binding capacity. Experiments with concanavalin A were performed in 0.1M-sodium acetate/1mM-$MgCl_2$/1mM-$CaCl_2$/1mM-$MnCl_2$/0.5M-NaCl/1% bovine serum albumin, pH6.0. The other lectins were diluted with 1% bovine serum albumin in phosphate-buffered saline. Solutions of various concentrations of unlabelled lectin were prepared in 1% bovine serum albumin in phosphate-buffered saline and a constant amount of ^{125}I-labelled lectin was added to each solution. Portions (50μl) were then delivered to each well. For sugar-inhibition experiments, the monosaccharides specific for each lectin were preincubated with the lectin solution for 30min at room temperature before addition to the wells. After 6h incubation at room temperature, the lectin solutions were removed by suction and the wells washed twice with 1% bovine serum albumin in phosphate-buffered saline and five times with water as described above. Individual wells were cut from each plate and counted for radioactivity in a gamma counter (Packard 5110 Auto-Gamma scintillation spectrometer).

Solid-phase radioimmunoassay. This assay is a modification of the technique of Marier *et al.* (1979) and was used to examine the interactions of rye-grass pollen glycoproteins with antiserum specific for terminal arabinose and galactose residues, which had been prepared previously (Gleeson & Clarke, 1980), to a defined arabinogalactan-protein isolated from *Gladiolus* style mucilage (Gleeson & Clarke, 1979). A preliminary experiment was performed to determine whether the arabinogalactan-protein, which contains 90% carbohydrate and 3% protein, would bind to the microtitre plate. [^{3}H]Arabinogalactan-protein was prepared by galactose oxidase treatment followed by reduction with NaB^3H_4 (Gleeson & Clarke, 1980). [^{3}H]Arabinogalactan-protein (40μl of a 40μg/ml solution in phosphate-buffered saline; 40000c.p.m.) was added to each well and incubated at 4°C for 16h; the solution was then removed by suction and the plate washed (thrice with 1% bovine serum albumin in phosphate-buffered saline; eight times with water). The wells were cut and washed individually with 3 × 0.1ml portions of 0.2% Nonidet P40 in phosphate-buffered saline. The washings were combined and counted for radioactivity. A total of 4% of the radioactivity added was recovered in the detergent washings, so that at least 64ng of arabinogalactan-protein bound per well. This amount of binding of a macromolecule containing 90% carbohydrate is comparable with that of a glycoprotein such as IgG, containing 3% carbohydrate (2ng bound per well) (Marier *et al.*, 1979).

Glycoprotein 1 or 2 or arabinogalactan-protein (40μl of a 50μg/ml solution in phosphate-buffered saline) were added to each well of the microtitre tray. After 3h at room temperature, non-adsorbed antigens were removed with a Pasteur pipette fitted with a flexible plastic tip and stored. These solutions could be re-used in subsequent experiments, since only nanogram amounts of the added antigen bind to the well. Any remaining potential binding sites on the plate were saturated by washing the wells three times with 200μl of 1% bovine serum albumin in phosphate-buffered saline. The plate was drained on a tissue, and dilutions of antiserum (50μl in 1% bovine serum albumin in phosphate-buffered saline) were added to each well and incubated for 3h. The wells were washed (thrice with 200μl of 1% bovine

serum albumin in phosphate-buffered saline; eight times with water), and the plate drained on a tissue. Protein A (50μl), iodinated by the technique of Fraker & Speck (1978), was then added to detect IgG binding to the wells. The specific activity of ^{125}I-labelled Protein A ranged from 1×10^6 to 6×10^6 c.p.m.$\cdot \mu g^{-1}$. After 5h, the plates were extensively washed and the wells cut and counted for radioactivity in the Packard gamma counter.

Inhibition studies. Preliminary experiments were performed in which wells were coated with test glycoproteins or arabinogalactan-protein; specific antisera were serially diluted and added to each well; IgG binding was detected by addition of ^{125}I-labelled Protein A (see Fig. 7*a* below). From these saturation curves, a concentration of antiserum that gave 70% maximal binding in the arabinogalactan-protein–anti-(arabinogalactan-protein) interaction was selected, and mixtures with inhibitors were prepared at these antisera concentrations. For inhibition studies, threefold serial dilutions of inhibitors were preincubated for at least 1h with the antiserum at the concentration selected. Solutions of the inhibitor–antiserum mixture were then added to the antigen-coated wells; the trays were incubated, drained, washed and incubated with ^{125}I-labelled Protein A as described above.

Radioallergosorbent inhibition tests. The radioallergosorbent inhibition test (RAST inhibition; Gleich *et al.*, 1975) was used to determine the allergenicity of Glycoproteins 1 and 2. The test was performed both with commercial discs coated with *Lolium perenne* pollen extract, and in wells of microtitre trays coated with *Lolium perenne* pollen extract.

Threefold serial dilutions of *Lolium perenne* pollen extract (300μg/ml) and both Glycoproteins 1 and 2 (120μg/ml) were made with serum (1:3 dilution with 1% bovine serum albumin in phosphate-buffered saline) of a subject showing severe clinical symptoms of allergy to rye-grass pollen. After 2h incubation at room temperature, portions of the mixtures (50μl) were added to tubes containing discs coated with *Lolium perenne* pollen extract or to wells of microtitre trays that had been coated with *Lolium perenne* pollen extract (300μg/ml) as described for the lectin-binding studies. The discs or wells coated with antiserum–inhibitor mixtures were incubated for a further 4h at room temperature, and washed [thrice with 1% bovine serum albumin in phosphate-buffered saline (200μl); eight times with water]. ^{125}I-labelled anti-(human IgE) was diluted in 1% bovine serum albumin in phosphate-buffered saline (5000c.p.m./ng) and 50μl (16000c.p.m.) was added to each well and to the tubes containing the discs. After 5h incubation, the discs and the wells were washed [thrice with 1% bovine serum albumin in phosphate-buffered saline (200μl); eight times with water] and drained on tissue paper. The wells were cut from the plates and placed in tubes, as were the commercial discs, and counted in a gamma counter.

Results

Purification of rye-grass pollen glycoproteins

Two glycoproteins of the pollen extract were purified by ion-exchange chromatography and gel filtration. Coomassie Blue staining of SDS/polyacrylamide gels indicated that these glycoproteins are major components of the pollen extract, and are present in approximately equal amounts. The yields of purified glycoproteins were low (Glycoprotein 1, 0.8mg/g of pollen, 26.6mg/g of pollen extract; Glycoprotein 2, 0.4mg/g of pollen, 10.3mg/g of pollen extract).

Properties of Glycoprotein 1

The purified material had an apparent mol.wt. of 29000 as determined by ultracentrifugation, 31000 by Sephadex G-75 gel filtration (Fig. 1*b*) and 33000 by SDS/polyacrylamide-gel electrophoresis. It behaved as a single component on SDS/polyacrylamide-gel electrophoresis (Fig. 3*a*), although two-dimensional gel electrophoresis resolved the purified material into three components with pI values 5.1, 5.3 and 5.5 (Fig. 3*b*). Antisera were raised to both unfractionated rye-grass pollen extract and to the purified material. Both antisera gave two bands on immunodiffusion (Figs. 4*a* and 4*b*) and at least two bands on immunoelectrophoresis against the purified material (Fig. 5*c*). This purified glycoprotein was an effective allergen as measured by the radioallergosorbent (RAST) inhibition assay performed in microtitre trays and on commercial disc kits (Fig. 8).

Properties of Glycoprotein 2

The purified material had an apparent mol.wt. of 67000 as determined by SDS/polyacrylamide-gel electrophoresis (Fig. 3*a*) and 58000 by Sephadex G-75 gel filtration (Fig. 2*b*). A single band that stained for both protein and carbohydrate was obtained by SDS/polyacrylamide-gel electrophoresis (Fig. 3*a*). This glycoprotein has a high isoelectric point (>9.0) that could not be determined precisely under the experimental conditions used, as the range of the isoelectric-focusing gels did not exceed pH9.0. Purified Glycoprotein 2 was extremely difficult to handle, as it became quite insoluble during the last stage of purification, and a low yield was obtained. Attempts to maintain it in solution by addition of 0.1% Nonidet P40 or Triton X-100 were unsuccessful. The insoluble material was an effective antigen and gave intense sharply defined bands in both immunodiffusion (Fig. 4*c*) and

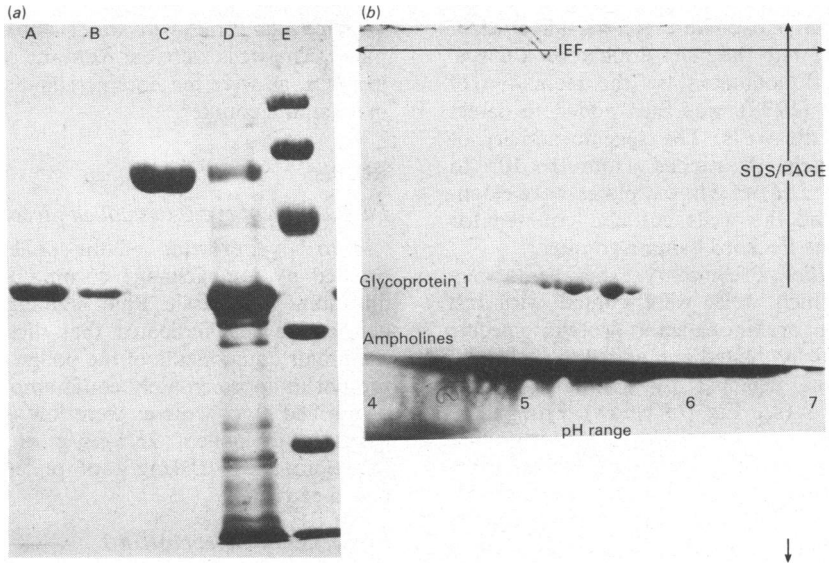

Fig. 3. (*a*) *SDS/polyacrylamide-gel electrophoresis of Glycoprotein 1 (A, B), Glycoprotein 2 (C), pollen extract (D), molecular-weight markers 94000, 67000, 43000, 30000, 20100 and 14400 (E) and (b) two-dimensional gel electrophoresis of Glycoprotein 1*

(*a*) Gels containing 12.5% acrylamide and 0.1% SDS were stained with Coomassie Blue. (*b*) First dimension: isoelectric focusing (IEF) in 8 M-urea, pH gradient 4–7; second dimension: SDS/polyacrylamide-gel electrophoresis (SDS/PAGE).

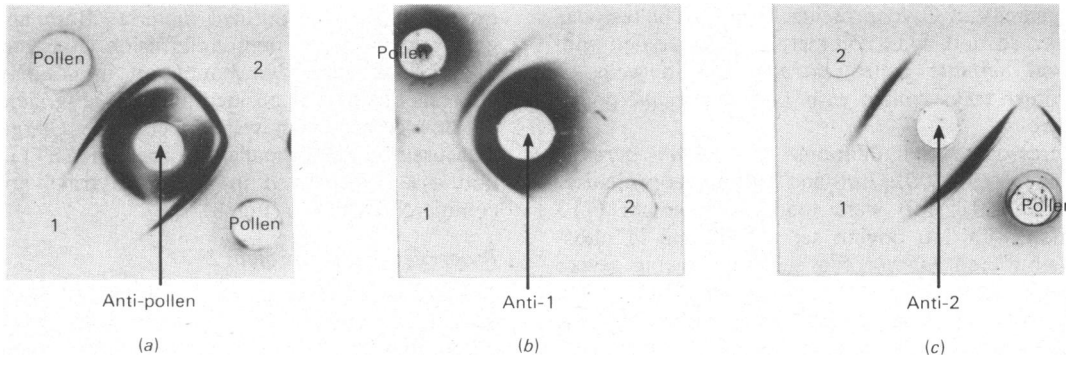

Fig. 4. *Immunological properties of purified Glycoproteins 1 and 2*

The Figures depict immunodiffusion of pollen extract ('Pollen'), Glycoproteins 1 and 2 ('1' and '2') against (*a*) anti-(pollen extract) serum ('Anti-pollen'), (*b*) anti-(Glycoprotein 1) serum ('Anti-1') and (*c*) anti-(Glycoprotein 2) serum ('Anti-2').

immunoelectrophoresis (Fig. 5*d*), when antisera raised to the purified material were used. In contrast with Glycoprotein 1, Glycoprotein 2 was a much less effective allergen when tested in the radio-allergosorbent (RAST) inhibition assay using either commercial discs or microtitre trays (Fig. 8).

Analysis of purified rye-grass pollen glycoproteins

Both glycoproteins stained for carbohydrate and protein in SDS/polyacrylamide gels. The amino acid and monosaccharide composition of the glycoproteins is given in Table 1. The amino acid analyses

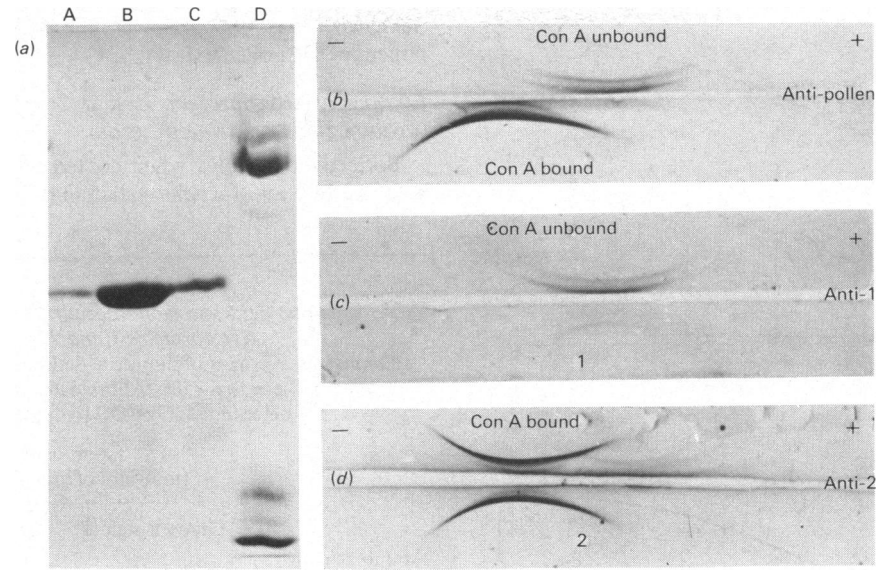

Fig. 5. *Identification of pollen components in concanavalin A–Sepharose bound and unbound fractions*
(*a*) SDS/polyacrylamide gel of Glycoprotein 1 (A), concanavalin A, unbound fractions (B, C) and concanavalin A bound (D). (*b–d*) Immunoelectrophoresis of Glycoproteins 1 and 2 ('1' and '2') and the concanavalin A ('Con A') bound and unbound fractions against antisera to pollen extract ('Anti-pollen'), Glycoprotein 1 ('Anti-1') or Glycoprotein 2 ('Anti-2').

were unremarkable, except for the presence of 2 and 4 mol of hydroxyproline per mol of Glycoproteins 1 and 2 respectively.

Monosaccharide analysis showed glucose, galactose, mannose, arabinose and *N*-acetylglucosamine in both glycoproteins. In addition, Glycoprotein 2 contained xylose and fucose (Table 1). The same range of monosaccharides in the hydrolysates detected by g.l.c. was also detected by paper chromatography. From the monosaccharide analyses, Glycoprotein 1 contained 5% and Glycoprotein 2 contained 12% carbohydrate.

In the present study we have adapted the microtitre-tray technique used for radioimmunoassay (Marier *et al.*, 1979), for lectin-binding studies. By using this method, nanogram quantities of bound lectin can be detected rapidly, with less than 5% variation between replicates. Commercially obtained samples of horseradish peroxidase and glucose oxidase, both bound concanavalin A, consistent with their high content of α1,2-linked and terminal mannose residues (Fig. 6*a*). The specificity of binding was shown by inhibition with 0.2M-methyl α-mannoside at a particular concanavalin A concentration (50μg/ml). The binding of five lectins to Glycoproteins 1 and 2 was examined by the same technique. None of the lectins tested bound to Glycoprotein 1, but concanavalin A and *Lotus* lectin bound to Glycoprotein 2 (Fig. 6*b*). The binding of concanavalin A was inhibited 95% by 0.2M-methyl α-mannoside and of *Lotus* lectin 92% by 0.2M-L-fucose, whereas monosaccharides such as galactose had no effect on the binding. Background amounts of lectin binding to bovine serum albumin were detected. A detailed discussion of the use of this assay for examining lectin–glycoprotein interactions is presented elsewhere (Howlett & Clarke, 1981).

Affinity chromatography of pollen extract

The ability of the pollen extract to bind to two lectins, the mannose-binding lectin concanavalin A and the galactose-binding lectin tridacnin, was tested. A bound (5mg) and unbound (9mg) fraction was obtained after affinity chromatography of the extract (18mg) on concanavalin A–Sepharose (Fig. 5). The bound fraction contained a component that gave a reaction of identity with Glycoprotein 2 in immunodiffusion and immunoelectrophoresis (Fig. 5*d*), as well as several minor components. The unbound fraction contained Glycoprotein 1 and several minor components (Fig. 5*c*). Tridacnin–

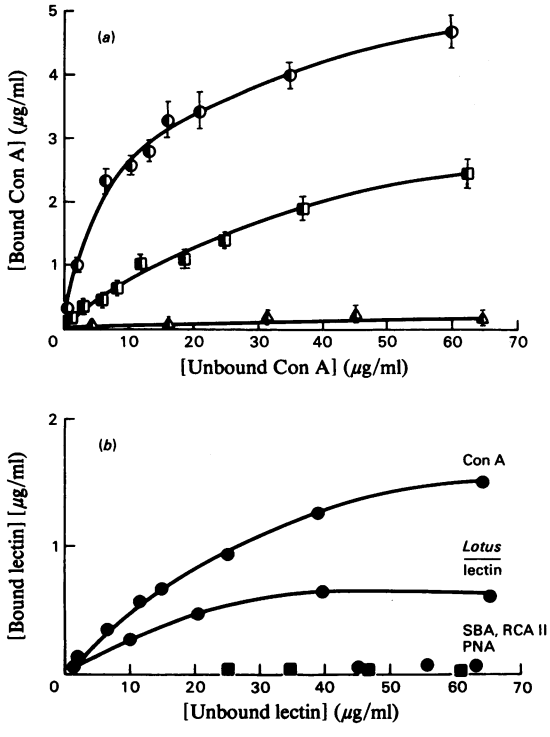

Fig. 6. (*a*) *Concanavalin A (Con A) binding to glycoproteins of known composition and* (*b*) *lectin binding to Glycoproteins 1 and 2*

(*a*) Wells of a microtitre tray were coated with glucose oxidase (◐), horseradish peroxidase (◧) or bovine serum albumin (▲) (40μl of a 50μg/ml solution in 1mM-$MgCl_2$/1mM-$CaCl_2$/1mM-$MnCl_2$/0.1M-sodium acetate/0.5M-NaCl, pH 6.0). Solutions containing various concentrations of unlabelled concanavalin A were prepared in 1% bovine serum albumin in the same buffer and a constant amount of ^{125}I-labelled concanavalin A (Con A) was added Portions (50μl, 20000c.p.m.) were then delivered to the glycoprotein-coated wells. After incubation for 6h at 20°C, wells were washed and counted for radioactivity. Each point represents the mean of three determinations. (*b*) Glycoproteins 1 (■) and 2 (●) (40μl of 50μg/ml in phosphate-buffered saline) were added to wells of a microtitre tray. Solutions of unlabelled lectins, *Lotus* lectin, castor-bean lectin (RCA II), peanut agglutinin (PNA) and soya-bean agglutinin (SBA) were prepared at various concentrations in bovine serum albumin in phosphate-buffered saline. Solutions of concanavalin A were prepared in the same acetate buffer as described in (*a*). Constant amounts of ^{125}I-labelled lectins were added to the homologous unlabelled solutions. Portions (50μl) of these lectin solutions (concanavalin A, 10000c.p.m.; *Lotus* lectin, 10000c.p.m.; castor-bean lectin, 15000c.p.m.; peanut agglutinin, 4000c.p.m.; soya-bean agglutinin, 10000c.p.m.) were then added to the glycoprotein-coated wells. After 6h at 20°C, solutions were removed and wells were washed and counted for radioactivity. Each point represents the mean of two determinations. Glycoprotein 2 bound concanavalin A and *Lotus* lectin, but not soya-bean agglutinin, castor-bean lectin or peanut agglutinin. Glycoprotein 1 bound none of the lectins tested.

Sepharose was not effective in binding any component of the pollen extract.

Binding of Glycoproteins 1 and 2 to specific anti-(arabinogalactan-protein) serum

Both glycoproteins, when coated on to the plate, bound the anti-(arabinogalactan-protein) serum.

Table 1. *Amino acid and monosaccharide composition of Glycoproteins 1 and 2*

Results are averages of duplicate determinations that differed by less than 5%. Abbreviations used: ND, not determined; Cys(O_3H), cysteic acid

	Composition (mol/mol of glycoprotein)	
	Glycoprotein 1	Glycoprotein 2
Amino acid		
Lys	25	33
His	3	8
Arg	6	24
Trp	ND	ND
Cys(O_3H)	2	0
Asp	26	58
Thr	19	29
Ser	14	43
Glu	22	43
Pro	14	41
Gly	28	54
Ala	24	46
½-Cys	2	7
Val	15	39
Met	2	11
Ile	11	31
Leu	11	43
Tyr	8	26
Phe	9	25
Hyp	2	4
Monosaccharide		
Ara	2	6
Xyl	0	8
Fuc	0	5
Man	1	13
Gal	3	7
Glc	3	4
GlcNAc*	1	6
GalNAc*	0	0
Carbohydrate (% in sample)	5	12

* From amino acid analysis.

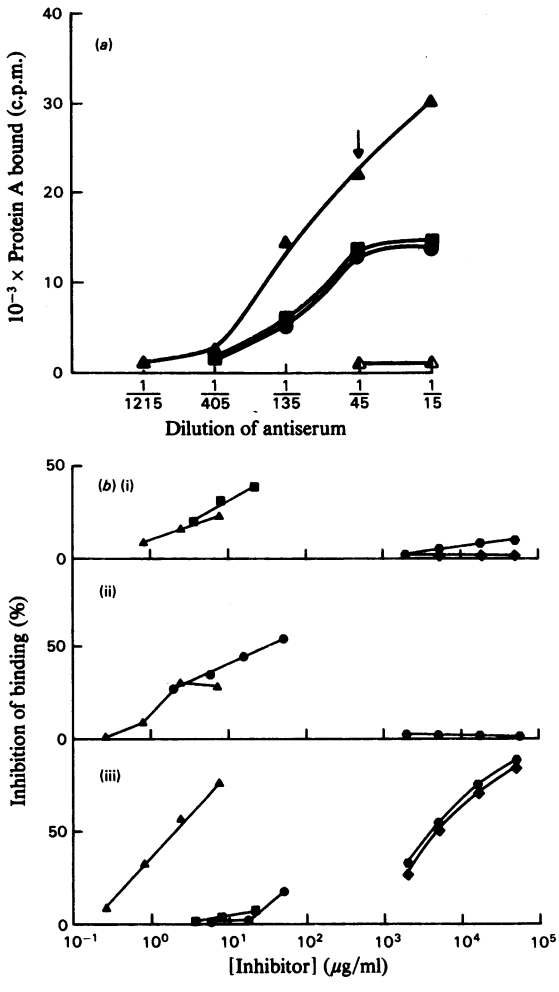

Fig. 7. (*a*) *Binding of Glycoproteins 1 and 2 and arabinogalactan-protein to anti-(arabinogalactan-protein) serum and* (*b*) *inhibition of binding of Glycoproteins 1 and 2 and arabinogalactan-protein to anti-(arabinogalactan-protein) serum*

(*a*) Wells of microtitre tray were coated with 40μl of a 50μg/ml solution of Glycoprotein 1 (■), Glycoprotein 2 (●), arabinogalactan-protein (▲) or bovine serum albumin (Δ). Dilutions of antiserum to arabinogalactan-protein were prepared in 1% bovine serum albumin in phosphate-buffered saline and 50μl portions were added to the coated wells. After 16h at 4°C, wells were washed and ^{125}I-labelled Protein A (40000c.p.m. in 50μl) added to each well. The arrow indicates dilutions of antiserum used in inhibition experiments (*b*). Each point represents the mean of two determinations. (*b*) Wells of a microtitre tray were coated as described for (*a*) and solutions of inhibitors Glycoprotein 1 (■), Glycoprotein 2 (●), arabinogalactan-protein (▲), D-galactose (♦) or L-arabinose (⬟) prepared in a dilution of anti-(arabinogalactan-protein) serum (1:45 in 1% bovine serum albumin in phosphate-buffered saline). ^{125}I-labelled Protein A (40000c.p.m. in 50μl) was added to each well. (i) Inhibition of the Glycoprotein 1–anti-(arabinogalactan-protein) serum interaction (no inhibitors, 12000c.p.m. bound); (ii) inhibition of Glycoprotein 2–anti-(arabinogalactan-protein) serum interaction (no inhibitors, 17000c.p.m. bound); (iii) inhibition of arabinogalactan-protein–anti-(arabinogalactan-protein) interaction (no inhibitors, 26000c.p.m. bound). Each point represents the mean of two determinations.

Glycoprotein 1 effectively inhibited (38% at 20μg/ml) the Glycoprotein 1–anti-(arabinogalactan-protein) interaction. This interaction was less effectively inhibited by arabinogalactan-protein (22% at 8μg/ml). The monosaccharides galactose and arabinose were ineffective inhibitors, even at concentrations of 0.26M and 0.32M respectively. A similar pattern was obtained for the Glycoprotein 2–anti-(arabinogalactan-protein) interaction (Fig. 7*b*). Both glycoproteins were tested for their capacity to inhibit

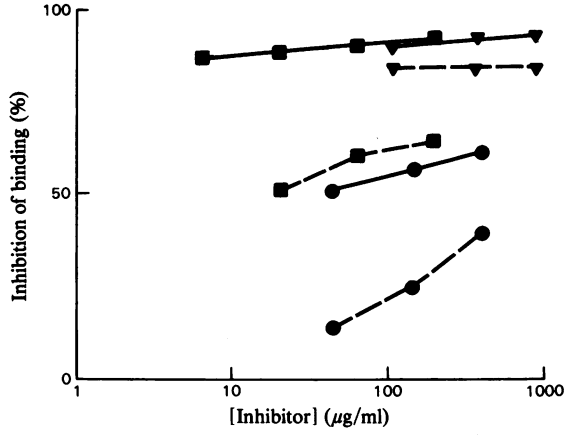

Fig. 8. *Radioallergosorbent inhibition test* (*RAST inhibition test*) *performed on commercial L. perenne discs* (----) *and microtitre tray wells coated with L. perenne pollen extract* (——)

Coated wells and discs were incubated with serial dilutions of inhibitors, Glycoprotein 1 (■), Glycoprotein 2 (●), and *L. perenne* extract (▼) in a 1:3 dilution of serum from a subject allergic to rye-grass pollen. After washing, ^{125}I-labelled anti-(human IgE) (16000c.p.m., 50μl containing 3ng) was added to both discs and wells, which were then washed and counted for radioactivity in a gamma counter. The allergic serum in the absence of inhibitors bound 8600c.p.m. in the disc assay and 6000c.p.m. in the microtitre-tray assay. Serum from a non-allergic subject in the absence of inhibitors bound 460c.p.m. in the disc assay and 360c.p.m. in the microtitre-tray assay.

the arabinogalactan-protein–anti-(arabinogalactan-protein) interaction (Fig. 7). Glycoprotein 1 was a poor inhibitor of this interaction, whereas Glycoprotein 2 gave a 20% inhibition at 50μg/ml; higher concentrations of Glycoproteins 1 and 2 were not tested. The inhibition of this reaction by the monosaccharides D-galactose and L-arabinose with this technique was essentially the same as that established previously by immunoprecipitation (Gleeson & Clarke, 1980).

Allergenic properties of Glycoproteins 1 and 2

The glycoproteins had different capacities for inhibiting binding of anti-IgE to serum of a rye-grass-sensitive human subject. This test is a standard assay *in vitro* for allergenicity (Gleich *et al.*, 1975). By using this microtitre tray technique, Glycoprotein 1 effectively inhibited 90% of the binding at 10μg/ml; however, Glycoprotein 2 was less effective, giving 50% inhibition at 20μg/ml (Fig. 8).

Discussion

In the present study we have isolated and partially characterized two soluble glycoprotein components from rye-grass (*Lolium perenne*) pollen. These glycoproteins are chemically and immunologically distinct; both apparently occur as monomers with no subunit structure, as similar values for molecular weight were obtained by SDS/polyacrylamide-gel electrophoresis and gel filtration. The molecular-weight values for Glycoprotein 1 were confirmed by ultracentrifugation; Glycoprotein 2 could not be examined by this technique because it precipitated from solution during the final stage of purification. Once precipitated, the material could not be re-dissolved by dilution, high salt concentration or the presence of detergents. This is unusual behaviour for a glycoprotein; as it was quite soluble in the original pollen extract, it may have been held in solution by close association with other pollen components.

The proportions of individual amino acids in the two glycoproteins were quite different. The amino acid analysis of Glycoprotein 1 is almost identical with that published for an allergen preparation (Group 1 allergen) from rye-grass pollen, by Johnson & Marsh (1965, 1966*a*,*b*). The electrophoretic properties and monosaccharide compositions of Glycoprotein 1 were also extremely similar to those of Group 1 allergen. On the basis of all these similarities, it is likely that Glycoprotein 1 described in the present paper and the Group 1 allergen are, in fact, the same components of rye-grass pollen.

Immunologically the Glycoproteins 1 and 2 were also distinct and showed no cross-reactivity on immunodiffusion. Glycoprotein 1 behaved as a single component on SDS/polyacrylamide-gel electrophoresis, but was resolved into two components on immunodiffusion and immunoelectrophoresis. A similar observation was reported for Group 1 allergen by D. G. Marsh [unpublished work cited by Lowenstein (1978)]. This characteristic of the Glycoprotein 1 was confirmed by two-dimensional gel electrophoresis, which revealed three distinct components (pI 5.1, 5.3, 5.5). Similar pI values for the Group 1 allergen components separated by starch-gel electrophoresis have been recorded (Johnson & Marsh, 1966*a*), and interpreted in terms of differing glutamine/glutamate ratios in the individual components. By contrast, Glycoprotein 2 behaved as a single component on SDS/polyacrylamide-gel electrophoresis, immunodiffusion and immunoelectrophoresis.

The two glycoproteins also differed with respect to their monosaccharide composition: both are complex in that five different monosaccharides were present in hydrolysates of Glycoprotein 1, and seven in hydrolysates of Glycoprotein 2. Insufficient material was available to examine the nature of the presumed carbohydrate–protein linkage or to establish the arrangement of monosaccharides within the carbohydrate component by methylation analysis; however, some deductions can be drawn from a consideration of the monosaccharide analysis and the binding interactions of the glycoproteins with lectins and defined antisera.

This approach was must useful for Glycoprotein 2, which contained 12% carbohydrate, with monosaccharides arabinose, xylose, fucose, mannose, galactose, glucose and *N*-acetylglucosamine present (respectively) as 6, 8, 5, 13, 7, 4 and 6 residues per molecule of glycoprotein. This glycoprotein bound specifically to concanavalin A, both in affinity-chromatographic procedures and in the microtitre-tray binding assay, indicating the presence of mannose or glucose residues either in terminal positions or in α1,2 linkage. It also bound specifically to the *Lotus* lectin, indicating the presence of terminal α-fucose residues. These data, and the high proportion of *N*-acetylglucosamine in the glycoprotein, are consistent with at least part of the carbohydrate of Glycoprotein 2 being present as a $(Man)_x$–$(GlcNAc)_2$ oligosaccharide linked to asparagine, the core structure of many animal glycoproteins, as well as several glycoproteins of plant origin such as soya-bean agglutinin (Lis & Sharon, 1978) and bromelain, a proteinase from pineapple (*Ananas comosus*) (Ishihara *et al.*, 1979). The structure of bromelain is particularly relevant, since a fucose residue is present in α-linkage to C-3 of the asparagine-linked *N*-acetylglucosamine. Furthermore, a terminal xylose residue is present in β1,2 linkage to a core mannose residue. The proportions of mannose, xylose, fucose and *N*-acetylglucos-

amine found in Glycoprotein 2 could be accounted for in a similar structure. The other monosaccharides present (galactose, glucose and arabinose) may be organized in a separate carbohydrate chain, possibly through Hyp–Ara or Hyp–Gal linkages. The finding that Glycoprotein 2 does not bind to any of the galactose-specific lectins tested indicates that terminal galactose residues are not likely to be a feature of the molecule. This, in conjunction with the effective binding of Glycoprotein 2 to an antiserum raised to purified arabinogalactan-protein, suggests the presence of terminal arabinose residues. The specificity of this antiserum is for side branches of the arabinogalactan protein that contain β1,6-linked galactose residues, some of which are substituted through C(O)3 to terminal L-arabinofuranose residues (Gleeson & Clarke, 1980). It is also possible that part of the arabinose and xylose could be accounted for as a fragment of arabinoxylan, the major hemicellulose of monocotyledonous plants (Burke *et al.*, 1974). It is not likely, however, that an arabinoxylan–glycoprotein association would be maintained throughout the isolation procedure. In summary, the data indicates that the monosaccharides are arranged so that at least some of the arabinose and fucose residues are terminal, the mannose and/or glucose residues are either terminal or α1,2-linked, and the galactose residues are not in terminal positions.

The indirect study of monosaccharide arrangement was not so useful for Glycoprotein 1, which had a lower carbohydrate content (5%) and did not bind any of the lectins tested. The absence of concanavalin A binding both in affinity chromatography and in the microtitre-tray assay, was surprising in view of the proportion of mannose and glucose residues present; presumably they are in non-terminal positions or in linkages other than α1,2. The absence of binding to galactose-binding lectins also indicates the non-terminal situation of the galactose residues. The presence of a low extent of binding to the anti-(arabinogalactan-protein) serum, suggests a terminal position for the arabinose residues.

These lectin- and antiserum-binding studies give only limited information regarding the arrangement of monosaccharide residues within the glycoprotein. However, where, as in the present case, only microgram quantities of material are available for analysis, they do give useful information. The method devised for examining glycoprotein–lectin interactions is rapid, highly reproducible and extremely sensitive. A fuller description of this method is published elsewhere (Howlett & Clarke, 1981).

With regard to the allergenic properties of the glycoproteins, it is clear from RAST inhibition studies that Glycoprotein 1 is a more effective allergen than Glycoprotein 2 on a weight basis. In previous publications these pollen glycoproteins have been referred to by various names; Glycoprotein 1 corresponds to 'Group 1 allergen' (Johnson & Marsh, 1965; Knox *et al.*, 1980) and Glycoprotein 2 probably corresponds to both 'Antigen A' (Augustin *et al.*, 1971; Smart & Knox, 1980; Knox *et al.*, 1980) and 'Group IV allergen' (Marsh, 1975). A major difference between these glycoproteins was believed to be their allergenicity; in the present study we show that both glycoproteins inhibit binding of serum from a rye-grass-pollen-allergic patient to rye-grass pollen extracts, although Glycoprotein 1 is much more effective than Glycoprotein 2. Because both glycoproteins are in fact allergenic (as measured by RAST inhibition), and both are antigenic, we have designated them 'Glycoproteins 1 and 2'.

Similar information on the allergenicity of the glycoproteins was obtained by using a commercial test kit and a method using the same sequence of binding on the microtitre tray. The advantage of the microtitre-tray method is that solid-state extracts are prepared by adsorption to plastic, obviating the covalent coupling to paper discs required for the commercial kits. The method, like that for lectin binding, is rapid, cheap and reproducible.

Questions regarding the role of these glycoproteins as major pollen-wall components remain unanswered, as does the question of the mechanism of their allergenicity. The data presented here are a basis on which experimental approaches to understanding the biological functions of these molecules could be founded.

We are grateful to Dr. C. Roxburgh and Dr. E. F. Woods, Division of Protein Chemistry, C.S.I.R.O., Parkville, Vic., Australia, for performing the amino acid analyses and ultracentrifugation, and to Professor R. B. Knox of this School and Professor B. A. Stone, Department of Biochemistry, La Trobe University, Bundoora, Vic., Australia, for critical review of the manuscript. This work was supported by a grant from the Asthma Foundation of Victoria and, in part, by a grant from the Australian Research Grants Committee.

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