Antigenic determinants of a plant proteoglycan, the *Gladiolus* style arabinogalactan-protein

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Antiserum has been raised to the arabinogalactan-protein of *Gladiolus* style mucilage. This macromolecule has been characterized and has a structure consistent with a 1→3-linked β -galactan backbone with side branches of 1→6-linked β -galactosyl residues, some of which carry terminal α -L-arabinofuranoside residues [Gleeson & Clarke (1979) *Biochem. J.* **181**, 607–621]. The specificity of the antiserum has been investigated by immunoprecipitation with [3 H]arabinogalactan-protein. The 3 H label was introduced into the arabinogalactan-protein by oxidation of the terminal galactose residues with galactose oxidase, followed by reduction with NaB^3H_4 . The antigenic specificity of the antiserum was shown to be directed towards the carbohydrate component of the arabinogalactan-protein. D-Galactose and L-arabinose were the most effective hapten inhibitors of the antiserum; other monosaccharides, N-acetyl-D-galactosamine, D-galactono-1,4-lactone, D-glucose, D-mannose, L-rhamnose, L-fucose and D-xylose, were all poor inhibitors. The antiserum showed preference for β -galactosides over α -galactosides. Of the haptens examined, the disaccharide 6-O- β -D-galactopyranosyl-D-galactopyranose was the most potent inhibitor. The antigenic features of the arabinogalactan-protein were investigated by examining the interaction of the antiserum with chemically and enzymically modified arabinogalactan-protein. Also, the cross-reactivity of structurally related polysaccharides and glycoproteins with the specific antiserum was assessed by a haemagglutination assay using erythrocytes coupled with specific antiserum. The results indicate that the dominant antigenic determinants of the arabinogalactan-protein are probably the side branches of 1→6-linked β -galactose residues bearing the terminal α -L-arabinose residues.

Many interactions between animal cells are mediated by cell-surface macromolecules (Hughes, 1979; Frazier & Glaser, 1979). There is increasing evidence that surface macromolecules of plant cells are also involved in a variety of cellular recognition reactions (Albersheim & Anderson-Prouty, 1975; Heslop-Harrison, 1978; Clarke & Knox, 1979). Our interest is in the male–female recognition reaction in flowering plants, particularly the role of surface secretions of the female sexual tissues in the capture and recognition of compatible pollen, and in the nurture of the pollen tubes during their growth through the style canal to the ovary. We have shown that the major component of both the receptive stigma surface secretion and the style-canal exudate of *Gladiolus* is an arabinogalactan-protein (Gleeson & Clarke, 1979; Clarke *et al.*, 1979; Gleeson &

Clarke, 1980a). The style material has been well characterized. It is polydisperse in the mol.wt. range 150 000–400 000. It has a low content of associated protein (3%) and the major monosaccharides of the carbohydrate component are galactose and arabinose in the proportions 6:1. The carbohydrate component was shown to be homogenous. Data obtained from methylation analysis, mild acid hydrolysis and enzymic hydrolysis are compatible with a model based on 1→3-linked galactan backbone, branched through C(O)6 to side branches of 1→6-linked galactose residues, some of which carry terminal α -L-arabinofuranoside residues (Fig. 1). Optical-rotation and lectin-binding studies indicated that the galactose residues are in the β -anomeric configuration (Gleeson & Clarke, 1979). Analysis of the stigma arabinogalactan showed it to be structurally similar to the style arabinogalactan-protein (Gleeson & Clarke, 1980a).

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in a single symmetrical peak that eluted with the void volume.

Preparation of a specific antiserum to the *Gladiolus* arabinogalactan-protein. Specific antisera to the isolated *Gladiolus* arabinogalactan-protein were raised in rabbits. Two rabbits (New Zealand White) were inoculated with a relatively high dose of antigen (1 mg) in Freund's complete adjuvant (Difco, Detroit, MI, U.S.A.); the antigen was administered subcutaneously by multiple injections in each of three sites, behind the neck and on each flank, and the rabbits were re-inoculated 28 days later with a further 1 mg of antigen emulsified in Freund's incomplete adjuvant. The rabbits were bled from the ear vein at 7, 10 and 14 days thereafter; the sera from the 7-day bleed gave the highest antibody titre in both rabbits. The sera were frozen in 400 μ l portions at -70°C . Sera when stored at -70°C showed virtually no loss of activity over a 1-year period; however, storage at -20 or 4°C resulted in appreciable loss of activity within months.

Immunodiffusion. Immunodiffusion, using the micro-slide apparatus (Gelman Instrument Co., Ann Arbor, MI, U.S.A.) was performed in 1% (w/v) agarose (Behring Institut, Marburg, Germany) containing 0.15 M-NaCl and 0.02% NaN_3 for 24 h at 37°C in a humidity chamber; the slides were then examined for the presence of immunoprecipitin bands.

Radiolabelling the *Gladiolus* arabinogalactan-protein. (a) ^3H -labelling of terminal galactose residues. The *Gladiolus* arabinogalactan-protein has a high content of terminal galactose residues (29%) (Gleeson & Clarke, 1979) and was labelled by specific oxidation of these residues with galactose oxidase, followed by reduction with NaB^3H_4 (Morell & Ashwell, 1972). The method employed was essentially that of Morell & Ashwell (1972) and Winand & Kohn (1970). The arabinogalactan-protein (1.2 mg) was dissolved in 2 ml of buffer (0.02 M-sodium phosphate/0.045 M-sodium acetate/0.15 M-NaCl/1% toluene, pH 7.0) to which 26 units of horseradish peroxidase (Calbiochem) and 44 units of galactose oxidase (Kabi AB, Stockholm, Sweden) were added. This mixture was incubated at 26°C for 50 h. During the incubation, a precipitate formed that subsequently redissolved. After incubation the reaction mixture was diluted 5-fold with 0.05 M-sodium phosphate buffer, pH 7.0, containing 0.05 M-NaCl. Approx. 10 mCi (1.4 μ mol) of NaB^3H_4 was added and the mixture incubated at room temperature for 30 min. To ensure complete reduction, unlabelled NaBH_4 (3 mg) was added and incubation was continued for a further 15 min. Excess NaBH_4 was then destroyed by lowering the pH to 4.6 with 4 M-acetic acid. The reaction mixture was dialysed for 24 h against distilled water (four changes) and finally equilibrated with 0.15 M-NaCl containing

0.01 M- CaCl_2 . The ^3H -labelled arabinogalactan-protein was then reisolated by affinity chromatography on a column (10 cm $\times$ 1 cm) of tridacnin-Sepharose 4B as previously described (Gleeson *et al.*, 1979). The isolated [^3H]arabinogalactan-protein had a specific activity of approx. 6.7×10^6 d.p.s./mg. A portion of the [^3H]arabinogalactan-protein was hydrolysed with 2.5 M-trifluoroacetic acid at 100°C for 2 h and chromatographed on Whatman no. 3 paper in ethyl acetate/pyridine/water (8:2:1, by vol.) for 18 h. The distribution of the ^3H label on the chromatogram was determined by cutting the chromatogram into strips and counting the radioactivity in each strip; 75–80% of the activity co-chromatographed with galactose and a small percentage of the label (5%) was found close to the origin.

(b) Iodination. Attempts to iodinate the arabinogalactan-protein with ^{125}I by the lactoperoxidase method (Marchalonis, 1969) were unsuccessful; tyrosine is present in the protein moiety of the arabinogalactan-protein (Gleeson & Clarke, 1979) but these residues are presumably inaccessible to iodination by this method.

Quantitative immunoprecipitation studies. Quantitative immunoprecipitation experiments were performed with the ^3H -labelled arabinogalactan-protein. Two different methods of immunoprecipitation were compared. (a) Immunoprecipitation with *Staphylococcus aureus* (Cowan 1; protein A-producing strain) cells. The formalin-fixed and heat-killed *S. aureus* cells were handled as described by Kessler (1975). The cells were stored as supplied as a 10% suspension at -20°C . Before use the cells were centrifuged at 9000 g for 30 s in a Microfuge (Beckman Instrument Co.) and incubated in 0.5% Nonidet P40 in 50 mM-Tris, 5 mM-EDTA, 150 mM-NaCl and 0.02% NaN_3 , pH 7.4 (Tris/EDTA/NaCl buffer) for 15 min at room temperature. The cells were then washed twice in Tris/EDTA/NaCl buffer containing 0.05% Nonidet P40, and finally suspended in this 0.05% Nonidet P40/buffer solution as a 10% (v/v) suspension.

The [^3H]arabinogalactan-protein in phosphate-buffered saline (5 mM-phosphate buffer/0.85% NaCl, pH 7.4) was added to the specific antiserum or to non-immune serum in a total volume of 50 μ l and incubated for 1 h at 37°C in 400 μ l Microfuge tubes. A portion of the 10% (v/v) *S. aureus* suspension was then added, the amount used being sufficient for complete precipitation of the immunoglobulin fraction (100 μ l of cells for undiluted serum; 50 μ l cells for all dilutions of serum). After 15 min at room temperature the cells were spun at 9000 g for 20 s in a Microfuge (Beckman) and washed twice in 200 μ l of Tris/EDTA/NaCl buffer containing 0.05% Nonidet P40. The cells were resuspended using a Super-Mixer (Lab-Line Instruments). The washed

cells were finally resuspended in 200 μ l of 0.5M-NaOH. Portions (50 μ l) were removed, neutralized, liquid scintillant (PCS; Amersham/Searle, Arlington Heights, IL, U.S.A.) (5 ml) added and the radioactivity measured in a liquid-scintillation counter (Isocap 300; Nuclear-Chicago, Des Plaines, IL, U.S.A.).

The titration of specific antiserum with [3 H]-arabinogalactan-protein (2500 d.p.s., approx. 0.3 μ g) is shown in Fig. 2. Equivalence was obtained by using a dilution of antiserum of 1 in 4, and 45% of the total radioactivity was precipitated. The extent of non-specific precipitation, determined by using non-immune serum in the same assay, was consistently below 0.5% of the total radioactivity.

(b) Indirect antibody precipitation. The [3 H]-arabinogalactan-protein in phosphate-buffered saline was added to specific antiserum or non-immune serum in a total volume of 75 μ l and incubated for 1 h at 37°C. Goat anti-(rabbit globulin) (25 μ l) was then added at a concentration pre-determined to give optimal precipitation. The mixture was incubated for a further 1 h at 37°C, centrifuged at 9000g for 1 min, and reincubated at 4°C overnight. The immunoprecipitate was collected by centrifugation at 9000g for 1 min, and washed three times with 200 μ l portions of phosphate-buffered saline. The precipitate was then redissolved and the radioactivity measured as described above. Titration of specific antiserum by this method showed a maximum of 25% of the total radioactivity precipitated at equivalence. Non-specific precipitation ranged

Fig. 2. Titration of the specific antiserum with [3 H]-arabinogalactan-protein

A portion (25 μ l) of [3 H]-arabinogalactan-protein (2500 d.p.s. or about 0.3 μ g) in phosphate-buffered saline, pH 7.0, was added to a range of dilutions of the specific antiserum in a total volume of 50 μ l and incubated for 1 h at 37°C. *Staphylococcus aureus* cell suspension (10%, v/v) was added and the mixture incubated at room temperature for 15 min. The cells were washed and the radioactivity determined as described under 'Methods'. Each determination was carried out in duplicate.

between 1 and 5% of the total radioactivity. Thus immunoprecipitation by this method gave lower specific precipitation and higher non-specific precipitation than immunoprecipitation using *S. aureus* cells. Method (a) was used for subsequent experiments in the present study. The relative efficiency of immunoprecipitation with *S. aureus* cells has been noted previously (Kessler, 1975).

Inhibition studies were performed by preincubation of the antiserum with inhibitor in phosphate-buffered saline, pH 7.0, at 37°C for 30 min. The final dilution of the antiserum in this incubation mixture was 1 in 4. Portions of the antiserum-inhibitor mixture (25 μ l) were placed in 400 μ l Microfuge tubes together with 25 μ l of [3 H]-arabinogalactan-protein (2500 d.p.s. or approx. 0.3 μ g) and incubated at 37°C for 1 h. *S. aureus* cells (50 μ l of a 10% suspension) were added and the incubation continued at room temperature for 15 min. The cells were washed and the radioactivity measured as described above. Control assays in the absence of inhibitors were performed at the same time. All inhibition assays were performed in duplicate, and control assays in triplicate.

Enzymic and chemical modifications of the *Gladiolus arabinogalactan-protein*. (a) Enzymic hydrolysis. The arabinogalactan-protein was treated with α -L-arabinofuranosidase as previously described (Gleeson & Clarke, 1979). Analysis of the treated material showed that all arabinose residues were removed (Gleeson & Clarke, 1979); this arabinose-depleted material is referred to as the 'galactan-protein'.

(b) Mild acid hydrolysis. The arabinogalactan-protein was hydrolysed in 12.5 mM-oxalic acid at 100°C for 5 h and the hydrolysate fractionated by precipitation with 80% (v/v) ethanol (Gleeson & Clarke, 1979). Analysis of the fractions showed that all the arabinose had been removed, as well as 25% of the galactose residues, which were released both as the free monosaccharide and as galactosyl oligosaccharides (Gleeson & Clarke, 1979). Methylation analysis of the galactan remaining after hydrolysis (ethanol-insoluble fraction) indicated that the molecules had been degraded both at the side chains and at the backbone (Gleeson & Clarke, 1979).

(c) Periodate oxidation. The arabinogalactan-protein (5 mg) was dissolved in sodium periodate (0.02M; 5 ml) and incubated at 4°C in the dark. Samples (100 μ l) were removed at intervals (1, 4, 7, 24, 48, 72 h), diluted to 25 ml with distilled water and the A_{223} measured. Oxidation was complete after 7 h. During oxidation of the arabinogalactan-protein, 0.92 mol of periodate/mol of saccharide residues were consumed. The theoretical periodate uptake, based on methylation data (Gleeson & Clarke, 1979) is 0.83 mol of periodate/mol of saccharide

residues. To monitor the efficiency of the periodate-oxidation procedure, a sample of maltose was oxidized at the same time under the same conditions; the observed periodate uptake equalled the theoretical value. The oxidized arabinogalactan-protein was dialysed exhaustively against phosphate-buffered saline, pH 7.0, and stored at 4°C. Under these conditions the oxidized arabinogalactan-protein remained in solution, but after freeze-drying or freezing the solution at -20°C, it became totally insoluble.

Removal of sialic acid from glycoproteins. Sialic acid was removed from glycoproteins by hydrolysis in 0.05 M-H₂SO₄ at 80°C for 1 h (Kieda *et al.*, 1978). The desialylated glycoproteins were dialysed exhaustively against distilled water and freeze-dried.

Haemagglutination assay using specific antiserum coupled to erythrocytes. The globulin fraction of the anti-(arabinogalactan-protein) serum was precipitated with 40%-satd. (NH₄)₂SO₄, washed once with 40% (NH₄)₂SO₄, dissolved in distilled water and dialysed exhaustively against 0.9% NaCl. The globulin fraction (1 mg) was coupled to human erythrocytes (125 µl of packed cells in 2 ml of 0.9% NaCl) by using 0.1% (w/v) chromic chloride [diluted from a 'matured' 1% (w/v) stock solution] as described by Parish & McKenzie (1978). Various amounts of the 0.1% (w/v) chromic chloride were tested, and the amount that gave optimal coupling of the globulin fraction to the erythrocytes was used. The washed coupled erythrocytes were diluted to a 2% (v/v) suspension and stored at 4°C, in the presence of 0.5% bovine serum albumin as a stabilizer, for periods up to one week. Before use they were washed once and resuspended to 2% (v/v) in phosphate-buffered saline, pH 7.4.

The cross-reactivity of a number of polysaccharides and glycoproteins with the anti-(arabinogalactan-protein) serum was assessed by a haemagglutination assay using the specific-antiserum-coupled erythrocytes. Doubling dilutions of the samples (25 µl) were made in a microtitre tray (Disposable Products, Adelaide, S. Australia, Australia) and an equal volume of the coupled erythrocytes was added. The trays were incubated at room temperature for 1 h and the lowest dilution at which agglutination was detected was taken as the end point. As controls to the specificity of the haemagglutination, assays were performed in a similar manner by using untreated erythrocytes as well as erythrocytes coupled with non-immune serum.

Results

The immune sera of both rabbits gave two bands with the *Gladiolus* arabinogalactan-protein in immunodiffusion tests; however, in both cases the

sera had to be concentrated 2-fold for these precipitin bands to be observed. The following studies on the specificity of the antiserum to *Gladiolus* arabinogalactan-protein were carried out with serum obtained from a single rabbit.

Inhibition of the [³H]antigen-antibody binding with saccharide inhibitors

The specificity of the anti-(arabinogalactan-protein) serum was determined initially by studying the ability of various mono- and di-saccharides to inhibit binding of specific antiserum to [³H]arabinogalactan-protein. The hapten-inhibition curves of the [³H]antigen-antibody binding by mono- and di-saccharides are shown in Fig. 3. The hapten concentrations required for 50% inhibition of the binding between [³H]arabinogalactan-protein and antiserum are shown in Table 1. Of the haptens examined, the disaccharide 6-*O*-β-D-galactopyranosyl-D-galactopyranose was the most potent inhibitor. It was approx. 80 times more effective on a molar basis than the next most powerful inhibitor, methyl β-D-galactopyranoside, and 280 times more effective than D-galactose. The antiserum showed preference for the galactosides in the β-configuration, as the β-galactosides examined, methyl β-D-galactopyranoside, 3-*O*-β-D-galactopyranosyl-D-arabinose and 4-*O*-β-D-galactopyranosyl-D-glucopyranose (lactose) were all considerably better inhibitors (about 10 times) than the only galactoside in the α-configuration tested, methyl α-D-galactopyranoside. The monosaccharide L-arabinose was equally as effective an inhibitor as D-galactose. However, D-arabinose in the disaccharide 3-*O*-β-D-galactopyranosyl-D-arabinose did not enhance the inhibitory capacity of the galactoside.

The inhibition curves of the effective inhibitors all showed a maximum of 70–80% inhibition at the highest concentrations tested. At a concentration of

Table 1. Comparison of saccharides as hapten inhibitors of the [³H]arabinogalactan-protein-specific antiserum binding

Data obtained from Fig. 3.

Inhibitor	Concentration required for 50% inhibition (mM)
6- <i>O</i> -β-D-Galactopyranosyl-D-galactopyranose	0.08
Methyl β-D-galactopyranoside	6.6
3- <i>O</i> -β-D-Galactopyranosyl-D-arabinofuranose	7.6
4- <i>O</i> -β-D-Galactopyranosyl-D-glucopyranose (lactose)	12.5
D-Galactose	23
L-Arabinose	30
Methyl α-D-galactopyranoside	100

Fig. 3. Saccharide inhibition of the $[^3\text{H}]$ arabinogalactan-protein-specific antiserum binding. Inhibition of binding of $[^3\text{H}]$ arabinogalactan-protein (2500 d.p.s. or about $0.3\text{ }\mu\text{g}$) to specific antiserum (dilution 1 in 4). The immunoprecipitation assays were performed as described under 'Methods'. The inhibitor concentration is that in the incubation mixture before addition of *Staphylococcus aureus* cells. All inhibition assays were performed in duplicate and analyses varying by more than $\pm 10\%$ were rejected and the assay repeated. Triplicate assays in the absence of inhibitors were performed at the same time and the average of these assays was used in calculating the percentage inhibition. Symbols: $\blacklozenge$, 6-*O*- β -D-galactopyranosyl-D-galactopyranose; $\bullet$, methyl β -D-galactopyranoside; $\square$, 3-*O*- β -D-galactopyranosyl-D-arabinose; $\blacktriangle$, 4-*O*- β -D-galactopyranosyl-D-glucopyranose (lactose); $\circ$, D-galactose; $\blacksquare$, L-arabinose; $\blacklozenge$, methyl α -D-galactopyranoside; $\blacksquare$, L-rhamnose; $\ominus$, *N*-acetyl-D-galactosamine; $\diamond$, D-xylose; $\bullet$, D-glucose; $\star$, L-fucose; $\otimes$, D-mannose; $\triangle$, D-galactono-1,4-lactone.

100 mM, both D-galactose and L-arabinose showed 70% inhibition; there was only a marginal increase in inhibition when both monosaccharides, each at 100 mM, were tested together.

A range of other related monosaccharides, i.e. *N*-acetyl-D-galactosamine, D-galactono-1,4-lactone, D-glucose, D-mannose, L-rhamnose, L-fucose and D-xylose, were all poor inhibitors.

Inhibition of the $[^3\text{H}]$ antigen-antibody binding with enzymically and chemically modified arabinogalactan-protein and arabinoxylan

The antigenic features of the arabinogalactan-protein were investigated by examining the consequence of modifying the arabinogalactan-protein both enzymically and chemically. This was assessed by assaying the ability of the modified arabinogalactan-protein to inhibit binding between $[^3\text{H}]$ -arabinogalactan-protein and specific antiserum. The results are shown in Fig. 4. On a weight basis, the galactan-protein gave an inhibition curve similar to that given by the native arabinogalactan-protein, and both gave 100% inhibition at $250\text{ }\mu\text{g}/\text{ml}$. The oxalic acid-treated arabinogalactan-protein also gave an

inhibition curve similar to that produced by the native material; however, at low concentrations, the oxalic acid-treated material was a slightly more effective inhibitor than either the native arabinogalactan-protein or the galactan-protein, and at high concentrations it was a slightly less effective inhibitor, giving a maximum inhibition of 90% at $250\text{ }\mu\text{g}/\text{ml}$. Periodate oxidation destroyed virtually all the antigenic determinants, as the oxidized material was only a weak inhibitor (Fig. 4). The polysaccharide arabinoxylan was not an effective inhibitor of the antigen-antibody binding.

Antigenic cross-reactivity of polysaccharides and glycoproteins with anti-(arabinogalactan-protein) serum

The cross-reactivity of a number of polysaccharides and glycoproteins with the anti-(arabinogalactan-protein) serum was assessed by using a haemagglutination assay with the specific antiserum coupled to erythrocytes. The relevant structural features of the polysaccharides and glycoproteins examined are given in Table 2, together with the results, which are expressed as the lowest con-

Fig. 4. Inhibition of the [^3H]arabinogalactan-protein-specific antiserum binding with enzymically and chemically modified arabinogalactan-protein and arabinoxylan

Inhibition of binding of [^3H]arabinogalactan-protein (2500 d.p.s. or about $0.3\text{ }\mu\text{g}$) to specific antiserum (dilution 1 to 4). The immunoprecipitation assays were performed as described under 'Methods'. The inhibitor concentration is that in the incubation before addition of *Staphylococcus aureus* cells. All assays were performed in duplicate and those varying by more than $\pm 10\%$ were rejected and the assay repeated. Triplicate assays in the absence of inhibitors were performed at the same time and the average of these assays were used in calculating the percentage inhibition. Symbols: ●, unlabelled arabinogalactan-protein; □, galactan-protein; ○, oxalic acid-treated arabinogalactan-protein; ▲, periodate-oxidized arabinogalactan-protein; ■, arabinoxylan.

concentration giving detectable haemagglutination. Haemagglutination with the native *Gladiolus* style arabinogalactan-protein was detectable at $31\text{ }\mu\text{g/ml}$. The galactan-protein and the oxalic acid-treated arabinogalactan-protein showed no detectable haemagglutination at $500\text{ }\mu\text{g/ml}$ and $1000\text{ }\mu\text{g/ml}$ respectively. The antiserum cross-reacted strongly with the closely related arabinogalactan from *Gladiolus* stigma surface exudate and to a lesser extent with arabinogalactans from *Triticum* and from *Lolium multiflorum* (rye-grass) endosperm cell-culture medium. The stigma surface exudate of *Lilium longiflorum* also contains arabinogalactans (Aspinall & Rosell, 1978). Two groups of arabinogalactans have been separated from this exudate by affinity chromatography on tridacnin-Sepharose 4B. Both the bound and unbound fractions contain arabinogalactans (Gleeson & Clarke, 1980b) that cross-reacted strongly with the specific antiserum. The wheat arabinoxylan cross-reacted strongly, haemagglutination being detected at a concentration of $15\text{ }\mu\text{g/ml}$. A pectin preparation containing a mixture of cell-wall components cross-reacted strongly, as did the galactomannan from *Ceratonia siliqua* seeds. Mannan, dextran, araban and gum arabic showed essentially no cross-reactivity. A number of glycoproteins were also examined; all except for ovalbumin were desialylated to expose terminal galactose

Table 2. Cross reactivity of polysaccharides and glycoproteins with antiserum to *Gladiolus* arabinogalactan-protein

The antiserum to *Gladiolus* arabinogalactan-protein was coupled to human erythrocytes as described under 'Methods'. Cross-reactivity of the polysaccharides and glycoproteins was assessed by a haemagglutination assay using erythrocytes coupled with specific antiserum. Control assays were performed by using untreated erythrocytes as well as erythrocytes coupled with non-immune serum.

	Source	Main structural features	Lowest concentration giving detectable haemagglutination ($\mu\text{g/ml}$)	Reference
Polysaccharides				
(a) Arabinogalactan-protein	<i>Gladiolus</i> style canal	1 $\rightarrow$ 3-linked β -galactan backbone with side branches of 1 $\rightarrow$ 6 β -galactose residues, some of which carry the terminal α -L-arabinose (<i>f</i>) residues	31	Gleeson & Clarke (1979)
(b) Arabinogalactan-protein treated with α -L-arabinofuranosidase	<i>Gladiolus</i> style canal	As above, with all α -L-arabinose residues removed	>500	Gleeson & Clarke (1979)
(c) Arabinogalactan-protein treated with oxalic acid	<i>Gladiolus</i> style canal	Molecule fragmented at both side chains and backbone; free of arabinose residues	>1000	Gleeson & Clarke (1979)
(d) Arabinogalactan	<i>Gladiolus</i> stigma surface secretion	1 $\rightarrow$ 3-linked β -galactan backbone with side branches of 1 $\rightarrow$ 6-linked β -galactose residues, some of which carry the terminal α -L-arabinose (<i>f</i>) residues	62	Gleeson & Clarke (1980a)
(e) Arabinogalactan-protein	<i>Lolium multiflorum</i> (rye-grass)	As for (d) above	250	Anderson <i>et al.</i> (1977)

Table 2—continued

	Source	Main structural features	Lowest concentration giving detectable haemagglutination ($\mu\text{g/ml}$)	Reference
(f) Arabinogalactan-peptide	<i>Triticum</i> (wheat)	As for (d) above	500	Fincher <i>et al.</i> (1974); Neukom & Markwalder (1975)
(g) Arabinogalactan	(i) <i>Lilium longiflorum</i> stigma exudate	Galactan containing 1→3 and 1→6 linkages substituted with Araf, Galp, Rhap, and GlcpA		Aspinall & Rosell (1978)
	(ii) Tridacnin-bound fraction		78	Gleeson & Clarke (1980b)
	(iii) Tridacnin-unbound fraction		155	Gleeson & Clarke (1980b)
(h) Gum arabic	<i>Acacia senegal</i> (Sigma)	1→3-linked β -galactan backbone with side chains of 1→6-linked β -galactose residues substituted with Rhaf, Araf, Galp, GlcpA, Arap	8000	Aspinall (1969)
(i) Arabinoxylan	<i>Triticum</i> (wheat)	1→4-linked β -xylan backbone with single α -L-arabinose (f) residues as side branches	15	Perlin (1951); Neukom <i>et al.</i> (1967)
(j) Araban	Koch-Light	Contains a branched polymer of L-arabinose	8000	Hough & Powell (1960)
(k) Galactomannan	<i>Ceratonia siliqua</i> gum locust bean seeds (Sigma)	1→4-linked β -mannan with single 1→6-linked galactose residues as side branches	250	Smith & Montgomery (1959)
(l) Pectin	Citrus (Sigma)	Mixture of soluble cell-wall components	125	
(m) Mannan	<i>Saccharomyces cerevisiae</i> (baker's yeast)	1→6-linked α -mannan with side branches of 1→2- and 1→3-linked mannose residues	8000	Ballou (1974)
(n) Dextran	<i>Leuconostoc mesenteroides</i> (Pharmacia)	1→6-linked α -glucan with side branches of 1→3-linked α -glucose residues	8000	Walker (1978)
Glycoproteins				
(1) Ovalbumin	Chicken	Terminal saccharide sequence (Man) _n -GlcNAc . . .	>4000	Spiro (1973)
(2) Asialofetuin	Foetal-calf serum serum	β -Gal-(1→3)- α -GalNAc . . .	>8000	Spiro (1973)
		β -Gal-(1→4)- β -GlcNAc . . .		Spiro & Bhoyroo (1974)
(3) Asialotransferrin	Human serum (Miles)	β -Gal-GlcNAc	>8000	Jamieson <i>et al.</i> (1971)
(4) Asialothyroglobulin	Porcine (Sigma)	(Man) _n -GlcNAc . . .	>4000	Fukuda & Egami (1971a,b)
		β -Gal-(1→4)- β -GlcNAc . . .		Gottschalk & Graham (1959)
(5) Asialomucin	Bovine sub-maxillary glands (Sigma)	GalNAc . . . (major)	>8000	Bertolini & Pigman (1970)
		Gal . . . (minor)		

residues. However, none of the glycoproteins showed any haemagglutination at the highest concentrations used.

Discussion

Many cell-surface components of animal cells, yeast cells and other micro-organisms are antigenic. In some cases the antigenic components are the carbohydrate moieties of surface glycoconjugates, e.g. human blood-group-specific antigens (Watkins, 1978) and microbial surface antigens (Jann & Westphal, 1975).

Increasingly, immunological approaches are being used to investigate the nature and function of plant surface components (Knox, 1980), but, in most cases, little chemical characterization of the antigenic components has been undertaken. We have raised a specific antiserum to the *Gladiolus* style arabinogalactan-protein and have carried out immunochemical studies to describe the nature of the antigenic determinants. The antiserum is directed towards the carbohydrate component of the arabinogalactan-protein. This is indicated by the destruction of antigenic determinants by periodate oxidation (Fig. 4). Further, hapten-inhibition studies showed that the specificity of the antiserum was directed to the monosaccharide components D-galactose and L-arabinose. This was confirmed by the ability of the antiserum to bind the polysaccharides arabinoxylan and galactomannan, which have a high proportion of accessible terminal arabinose and galactose residues respectively. Although hapten-inhibition studies indicated a preference for galactosides in the β -configuration, α -galactosides were also effectively bound in both the hapten inhibition and the haemagglutination assays. This is in keeping with the β -configuration of galactose residues in the arabinogalactan-protein (Gleeson & Clarke, 1979).

None of the monosaccharides tested gave 100% inhibition of the antigen-antibody binding. This probably reflects the relatively low affinity of monosaccharides for the antibody-binding sites compared with that of the antigenic determinants of the arabinogalactan-protein, suggesting that the determinants include a number of saccharide residues. The binding sites of other anti-glycosyl antibodies are known to extend over a number of saccharide residues, for example a hexasaccharide in the dextran-anti-dextran interaction (Kabat, 1966) and a tetrasaccharide in the antigenic determinants of human blood-group antigens A and B (Lloyd *et al.*, 1966). Other carbohydrate-binding proteins, e.g. lectins and glycosidases, are also known, in a number of cases, to accommodate an extended region of the saccharide chain in their binding sites (Goldstein & Hayes, 1978; Blake *et al.*, 1967).

Inhibition studies of the anti-(arabinogalactan-protein) serum with disaccharides showed that 6-*O*- β -D-galactopyranosyl-D-galactopyranose was a far more potent inhibitor than was the monosaccharide D-galactose, also suggesting that the binding site can accommodate at least two galactose residues.

Further characterization of the antigenic determinants by hapten inhibition is limited by the availability of model oligosaccharides. Therefore an alternative approach was adopted; the arabinogalactan-protein was chemically and enzymically modified and the effect of these modifications on the antigenic determinants was assessed.

Periodate oxidation of the arabinogalactan-protein causes extensive degradation of side branches containing 1 $\rightarrow$ 6-linked galactose residues, terminal galactose and arabinose residues, but no oxidation of the 1 $\rightarrow$ 3-linked galactan backbone would be expected. The loss of antigenic determinants after periodate oxidation indicates that the antigenic determinants are located on the side branches. This location of determinants at the side branches is a common feature of polysaccharide antigens (Jann & Westphal, 1975); e.g. the antigenic determinants of a number of yeast mannans are side branches containing 1 $\rightarrow$ 3- and 1 $\rightarrow$ 2-linked mannose residues (Ballou, 1970). Apparently, in the arabinogalactan-protein, both arabinose and galactose are included in the antigenic determinants as both monosaccharides inhibited the antigen-antibody binding. The question of the number of sets of specific antibodies has not been resolved. However, it is likely that a single set of specific antibodies capable of binding to determinants containing both D-galactose and L-arabinose is involved, as a mixture of these monosaccharides inhibited the antigen-antibody binding to a similar extent as did the individual monosaccharides. There is some indirect evidence that the galactose residues may be more important than the arabinose residues in determining the extent of binding by the specific antiserum; thus the antiserum can bind to at least two galactosyl residues; it also has a high affinity for both the arabinose-free galactan-protein and oxalic acid-treated arabinogalactan-protein. In contrast, arabinoxylan was a poor inhibitor of the binding (Fig. 4). Oxalic acid hydrolysis of the arabinogalactan-protein not only removes all the arabinose but also fragments the molecule at both the side branches and the backbone, to give an increased proportion of 1 $\rightarrow$ 6-linked galactose residues (Gleeson & Clarke, 1979). This high proportion of 1 $\rightarrow$ 6-linked galactose residues could account for its efficiency as an inhibitor.

The differences in effectiveness of various polysaccharides in the two assays reflects the fundamental differences in the assays; the inhibition

assays measure relative affinities of the determinants for the antiserum; on the other hand the haemagglutination assay, using antibody-coated erythrocytes, measures the extent of cross-linking between the cells and may be dependent on the number and distribution of the determinants, as well as their affinity. The cross-reactivity of related arabinogalactans confirms the specificity of the antiserum. The arabinogalactan from *Gladiolus stigma* is structurally most similar to the native antigen and this is reflected in its strong cross-reactivity with the antiserum. The arabinogalactan-protein from *Lolium multiflorum* endosperm cells cross-reacts to a lower extent, possibly owing to its more highly branched nature, and the low molecular weight of the wheat arabinogalactan-peptide (22000) is consistent with its poorer cross-reactivity. Although gum arabic shares some structural features with the *Gladiolus* style arabinogalactan-protein, there are major differences in the terminal sequences, consistent with its poor cross-reactivity. The araban did not cross-react; this polysaccharide preparation contains a number of monosaccharide components, but the proportion and arrangement of the arabinose residues is not known. Arabinoxylan, which had a low affinity for the antiserum, cross-reacted strongly in the haemagglutination assay, presumably because the xylan chains are extensively substituted with terminal arabinose residues. Neither the oxalic acid-treated *Gladiolus* arabinogalactan-protein nor the galactan-protein showed any detectable haemagglutination. The fragments of the arabinogalactan-protein remaining after oxalic acid hydrolysis may have been too small to allow effective cross-linking of the erythrocytes. The non-reactivity of the galactan-protein was, however, unexpected, but may be related to its altered solubility properties (Gleeson & Clarke, 1979).

None of the glycoproteins examined showed any detectable haemagglutination; all except ovalbumin had terminal non-reducing galactose residues, and the non-reactivity probably reflects a low content of these residues in the glycoproteins. In contrast, galactomannan, which has high content of terminal α -galactose residues, cross-reacted strongly.

A number of mouse myeloma immunoglobulin A proteins have been described that also bind specifically to polysaccharides containing 1 $\rightarrow$ 6-linked β -D-galactosides (Glaudemans, 1975). Oligosaccharides containing these linkages were more potent inhibitors of the myeloma proteins than was the monosaccharide, with the 1 $\rightarrow$ 6-linked β -D-galactotetraose being the most effective inhibitor tested (Jolley *et al.*, 1974; Glaudemans, 1975). The immunoglobulin A protein of one of these, the J539 myeloma, has been shown to bind to the *Gladiolus* arabinogalactan-protein (Gleeson & Clarke, 1979) as well as to a number of other arabino-3,6-

galactans (Baldo *et al.*, 1978b). Thus the specificity of this myeloma protein is similar to the anti-(arabinogalactan-protein) serum described here, except that the anti-(arabinogalactan-protein) serum also binds terminal α -L-arabinofuranose, residues, whereas the myeloma proteins tested were not inhibited by α -L-arabinofuranoside (Potter *et al.*, 1972).

In summary, the antiserum to *Gladiolus* arabinogalactan-protein is directed to side chains of the carbohydrate component; both monosaccharides, D-galactose and L-arabinose, contribute to the antigenic determinants.

Carbohydrate-specific antibodies, together with lectins, are extremely useful tools in investigations of both the form and function of glycoconjugates. The antiserum described here provides an additional specific reagent for such investigations.

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