Glycoproteins from the Cell Wall of *Phaseolus coccineus*

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1. The use of a modified sodium chlorite/acetic acid delignification procedure for the solubilization of a hydroxyproline-rich glycoprotein fraction from the depectinated cell walls of *Phaseolus coccineus* is described. 2. The crude glycoprotein was associated with some pectic material; hydroxyproline and serine were the most abundant amino acids, and arabinose, galactose and galacturonic acid the predominant monosaccharides. 3. The bulk of the hydroxyproline is *O*-glycosidically substituted with tetra- and tri-arabinofuranosides. From methylation analysis the linkages in these arabinosides could be inferred. 4. Ion-exchange chromatography of the crude glycoprotein gave one major and two minor hydroxyproline-rich fractions, with similar amino acid but different monosaccharide composition. 5. In the major fraction, serine appears to be *O*-glycosidically substituted with a single galactopyranoside residue that can be removed by the action of α -galactosidase but not β -galactosidase. Removal of arabinofuranoside residues by partial acid hydrolysis greatly enhanced the action of α -galactosidase. 6. Methylation followed by carboxy reduction with LiAl^2H_4 has shown the presence of (1 $\rightarrow$ 4)-linked galacturonic acid in the crude glycoprotein fraction but not in the major fraction from the ion-exchange column. Hence the bulk of the pectic material is not associated with the major glycoprotein component. It is suggested that the glycoprotein is held in the wall by phenolic cross-links. 7. Similarities with the glycopeptide moiety of potato lectin provides further evidence for a class of hydroxyproline-rich glycoproteins with common features.

The presence of a hydroxyproline-rich glycoprotein in the primary cell walls of a wide range of plants is well documented (Lampert, 1965; Brown & Kimmins, 1977). Evidence for its localization has been obtained by using fractionation studies (Lampert & Northcote, 1960; Dougall & Shimbayashi, 1960; King & Bayley, 1965) and radioautography (Roberts & Northcote, 1972). Alkaline degradation of a variety of plant cell walls showed that the bulk of the hydroxyproline is *O*-glycosidically linked to arabinose-containing oligosaccharides (Lampert, 1967). Studies with hydroxyprolinyl arabinosides isolated from suspension-culture tobacco cells have revealed the nature of the *O*-glycosidic linkages and their anomeric configuration (Akiyama & Kato 1977*a,b*). Partial acid hydrolysis of tomato suspension-culture cell walls followed by tryptic digestion of the hydrolysate allowed the isolation of a glycopeptide containing serine substituted with a single galactose residue (Lampert *et al.*, 1973). Similar observations have been made with other cell walls (Cho & Chrispeels, 1976; Kliss, 1976) and a lectin from potato (Allen *et al.*, 1978).

In dicotyledonous-plant cell walls, at least, a glycoprotein containing *O*-glycosidically substituted hydroxyproline and serine is a common feature. It has not been possible to isolate this glycoprotein without recourse to degradative methods. Hence it has proved difficult to assess the relationship of the glycoprotein with other cell-wall polymers and to suggest a role for it (Lampert, 1970; Northcote, 1972; Brown & Kimmins, 1977). Furthermore, all the detailed structural studies have utilized cell walls isolated from plant suspension cultures (Lampert, 1965, 1970; Heath & Northcote, 1971). These differ from whole plant tissues in having higher concentrations of hydroxyproline-containing glycoprotein (Lampert, 1965). Thus there is a need for mild procedures to isolate cell-wall glycoproteins, or glycoprotein-polysaccharide complexes, in a state as near as possible to that in the native walls.

In a previous paper the isolation of non-diffusible hydroxyproline-containing glycoprotein(s) from runner beans by the use of a sodium chlorite/acetic acid delignification procedure was reported (Selvendran, 1975). Although this method suffers from the

disadvantage that certain amino acids undergo modification or are destroyed (Selvendran *et al.*, 1975), it represents a useful technique for isolating cell-wall glycoproteins. In the present paper we report the use of a less-severe delignification with sodium chlorite/acetic acid for isolating hydroxyproline-rich glycoprotein-polysaccharide complexes from the depectinated parenchyma cell-wall material of runner beans. Fractionation studies and methylation analysis have enabled us to elucidate some overall structural features of the glycoproteins.

Experimental

Materials

Plant tissues. Mature runner beans (*Phaseolus coccineus* var. Streamline; average length 30 cm, width 2 cm) used in the present investigation were collected from plants grown in experimental plots near the laboratory. Parenchyma was obtained by scraping the pods (split in half lengthwise) up to the parchment layer.

Chemicals. LiAlH_4 and NaBH_4 were purchased from Fluka (Buchs, Switzerland). Dimethyl sulphoxide, NaH , LiAlH_4 , NaBH_4 and tetrahydrofuran were all obtained from BDH Chemicals (Poole, Dorset, U.K.). Dimethyl sulphoxide (for methylation analysis) was redistilled under vacuum over CaH_2 and stored over molecular sieve 4A. Tetrahydrofuran was redistilled over LiAlH_4 and stored under argon. All other chemicals used were of BDH AnalaR grade or the highest purity available. α -Galactosidase (EC 3.2.1.22) (from green coffee beans) and β -galactosidase (EC 3.2.1.23) (from *Escherichia coli*) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bio-Gel P-2 (100–200 mesh) and Aminex AG50W X2 (200–325 mesh) were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Sephadex G-100 and DEAE-Sephadex (A-50) were from Pharmacia (Uppsala, Sweden).

Dialysis tubing (Visking size 248) was purchased from Medicell International, London, U.K.). Dialysis was performed against distilled water at 2°C in the presence of toluene.

Methods

Amino acid and sugar analyses. Amino acids were released from the preparations by using 6M-HCl hydrolysis at 110°C for 24 h in a sealed tube. Corrections for losses over this period were not made. The liberated amino acids were analysed as their *N*-heptafluorobutyl *n*-propyl ester derivatives by g.l.c. (March, 1975). Total amino acids were determined by the ninhydrin method (Yemm &

Cocking, 1955). Non-peptide-bound hydroxyproline was determined colorimetrically (Kivirikko & Liesmaa, 1959). α -Aminoadipic acid was determined by using a Technicon automatic amino acid analyser.

Neutral sugars were released from the various preparations by using either 2M-trifluoroacetic acid hydrolysis at 120°C for 2 h or 1M- H_2SO_4 hydrolysis at 100°C for 2.5 h, and the liberated sugars were analysed as their alditol acetates by g.l.c.; a correction was applied for incomplete release of rhamnose (Selvendran *et al.*, 1979). Total sugar was determined by the phenol/ H_2SO_4 method (Dubois *et al.*, 1956).

Spectrophotometry. I.r. spectrophotometric analysis was performed with a Pye Unicam AP-200G i.r. spectrophotometer. Samples were prepared in CCl_4 or as KBr discs.

Cell-wall preparation. Cell-wall material of parenchymatous tissue was prepared by sequential extraction of fresh wet ball-milled tissue with aq. 1% (w/v) sodium deoxycholate, phenol/acetic acid/water (2:1:1, w/v/v) and aq. 90% (v/v) dimethyl sulphoxide (O'Neill & Selvendran, 1980; Ring & Selvendran, 1978).

Fractionation of cell-wall material. Cell-wall material (1g/100ml) was depectinated by sequential extraction twice with water, pH 4.0, at 80°C for 2 h and once with ammonium oxalate, pH 5.5, at 80°C for 2 h. The insoluble residue was suspended in distilled water (1g/100ml) in a loosely stoppered flask and heated to 70°C. Acetic acid (0.12%, v/v) and sodium chlorite (0.3%, w/v) were added and the contents were stirred for 15 min. The treatment was repeated and the mixture was stirred for a further 15 min. To prevent a build-up of chlorine dioxide gas, the flask was continuously flushed with argon. After cooling, the contents of the flask were filtered through sintered glass and the residue was washed with 5 bed volumes of distilled water. The filtrate was purged with argon to remove chlorine dioxide, dialysed and freeze-dried to yield the crude chlorite-soluble glycoprotein fraction. The residue on the sinter was further washed with 500 ml of distilled water and freeze-dried.

Effect of delignification treatment on lysozyme. As a control, the effect of sodium chlorite/acetic acid on lysozyme was studied. Lysozyme (250 mg) was dissolved in 25 ml of distilled water and subjected to the delignification treatment described above. Treated lysozyme (35 mg) was dissolved in 0.5M-acetic acid, and placed on a column (56 cm $\times$ 1.1 cm) of Sephadex G-100 and eluted with the same solution at a flow rate of 25 ml/h. Fractions (10 ml) were collected and portions (1 ml) of each were hydrolysed with an equal volume of 12M-HCl. After neutralization, the total amino acid was determined by the ninhydrin method (Yemm &

Cocking, 1955). The elution profile of untreated lysozyme (50mg) was recorded with the same column under identical conditions. The presence of protein in the fractions was determined by the absorption at 280nm.

Isolation of hydroxyproline arabinosides. The chlorite-soluble glycoprotein fraction (20mg) was suspended in 2.50ml of saturated Ba(OH)₂ solution and heated at 90°C for 8h. The suspension was cooled, and adjusted to pH 7.0 with 0.25M-H₂SO₄ and filtered. The filtrate was concentrated to a small volume (about 1.5ml) by rotary evaporation and placed on a column (90cm × 1.6cm) of Bio-Gel P-2. The column was eluted with 0.1M-acetic acid at a flow rate of 30ml/h. Fractions (5ml) were collected and analysed for non-peptide bound hydroxyproline and total sugar. Appropriate fractions were pooled and freeze-dried. The material was dissolved in a small volume (1ml) of 0.02M-HCl and placed on a column (36cm × 1.6cm) of Aminex ion-exchange resin (H⁺ form). Elution was carried out with a linear decreasing pH gradient (0.02–0.2M-HCl). Fractions (2.5ml) were collected and portions (250μl) of each were analysed for non-peptide-bound hydroxyproline and total sugar. Appropriate fractions were pooled and freeze-dried. The relative amounts of hydroxyproline and arabinose were determined by g.l.c. after appropriate hydrolysis.

Ion-exchange chromatography. The chlorite-soluble glycoprotein fraction (30mg) was dissolved in 0.05M-sodium acetate buffer, pH 5.2, and filtered. The clear supernatant was placed on a column (15cm × 1.5cm) of DEAE-Sephadex (Cl⁻ form). The column was eluted at a flow rate of 20ml/h with (a) 100ml of sodium acetate buffer, (b) a linear gradient of 0–0.6M-NaCl in sodium acetate buffer (total volume 200ml) and (c) 100ml of 1M-NaCl in sodium acetate buffer. Fractions (4ml) were collected and portions (250μl) of each were assayed for total sugar. Hydroxyproline was determined colorimetrically after acid hydrolysis with an equal volume of 12M-HCl for 24h at 110°C. Appropriate fractions were pooled, dialysed and freeze-dried.

Partial acid hydrolysis. Fraction D-2 (major peak from DEAE-Sephadex chromatography) was subjected to partial acid hydrolysis with 12mm-oxalic acid for 5h at 100°C to remove arabinose residues (Fincher *et al.*, 1974). The product was dialysed and freeze-dried.

α- and β-Galactosidase treatments. (a) α-Galactosidase. About 300μg of fraction D-2 (either complete or acid-treated) was dissolved in 2ml of 0.05M-sodium citrate/phosphate buffer, pH 6.0, and incubated with 500μl (1 unit) of coffee-bean α-galactosidase at 37°C for 7 days in the presence of toluene (1 unit of enzyme will hydrolyse 1.0μmol of *p*-nitrophenyl α-D-galactose/min at 25°C). The incubation mixture was dialysed and freeze-dried

(Allen *et al.*, 1978). After hydrolysis with 1M-H₂SO₄ the neutral sugars were determined by g.l.c.

(b) β-Galactosidase. About 300μg of fraction D-2 (either complete or acid-treated) was dissolved in 2ml of phosphate-buffered saline (10mM-NaCl in 50mM-sodium phosphate buffer), pH 7.2, and incubated with 25μl (37 units) of *Escherichia coli* β-galactosidase at 37°C for 24h in the presence of toluene (1 unit will hydrolyse 1.0μmol of lactose/min at 37°C). The incubation mixture was dialysed, freeze-dried and analysed as above.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Sodium dodecyl sulphate/polyacrylamide-gel (5.2% total acrylamide and 2.63% cross-linking) electrophoresis of the unfractionated glycoprotein was performed by Dr. R. Begbie of the Protein Biochemistry Department, Rowett Research Institute, Aberdeen, Scotland, U.K. The gels were stained with Coomassie Blue or dansylhydrazine (Eckhardt *et al.*, 1976).

Methylation analysis. Methylation analysis of the crude chlorite-soluble glycoprotein and fraction D-2 was carried out as previously described (O'Neill & Selvendran, 1980). Carboxy reduction of partially methylated uronic acid-containing material was carried out with LiAlH₄ (Ring & Selvendran, 1978; Lindberg, 1972). Partially methylated alditol acetates were separated by g.l.c. (Ring & Selvendran, 1978). Combined g.l.c.–mass spectrometry was performed on a Pye 104 gas chromatograph interfaced to an AEI MS30 mass spectrometer in the electron-impact mode. The information was acquired with continuous scanning (10s/decade) on an AEI DS 50 SM computer system. Qualitative identification of each partially methylated alditol acetate was made from (a) methoxy substitution pattern from g.l.c.–mass spectrometry, (b) relative retention time (*R_r*) on two columns (3% OV-225 at 180°C and 3% ECNSS-M at 170°C) relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, and (c) monosaccharide composition of the unmethylated material. Quantitative results were obtained by using peak area analysis from temperature-programmed g.l.c.: 5min at 150°C, then 0.5°C/min to 210°C.

Results and Discussion

Isolation of cell-wall material

The sequential extraction procedures previously described (O'Neill & Selvendran, 1980) were used for the preparation of cell-wall material of runner-bean parenchyma. The cell walls are essentially free of cytoplasmic contaminants, and the constituent polymers have undergone minimal degradation (Ring & Selvendran, 1978). Fresh tissue (100g) gave about 1g (dry wt.) of cell-wall material. The

purity of the preparation is reflected in the relatively high content (0.45%, w/w) of hydroxyproline.

Fractionation of the cell-wall material

To isolate the cell-wall glycoprotein with minimal contamination with pectic substances, prior depectination of the cell-wall material is essential. Extraction of the cell-wall material (3.0g) with hot water and hot ammonium oxalate solubilized about 32% (w/w) of the total wall material. Subsequent analysis of the depectinated cell-wall material showed it to contain 5.6% (w/w) protein, of which 0.75% (w/w) was hydroxyproline and 10% (w/w) uronic acid-containing polymers. In a previous paper (Selvendran *et al.*, 1975) the use of a 4 h sodium chlorite/acetic acid treatment for the solubilization of cell-wall glycoprotein was described. We have subsequently found that two 15 min treatments with 0.12% (v/v) acetic acid and 0.3% (w/v) sodium chlorite at 70°C released about 80% (w/w) of the hydroxyproline in a non-diffusible form. The chlorite-soluble material accounted for about 7% (w/w) of the cell-wall material. The amino acid and

monosaccharide compositions of the depectinated cell-wall material and the chlorite-soluble and -insoluble fractions are given in Table 1. For the chlorite-soluble fraction, the protein content was calculated as 14.5% (w/w) from the recovery of amino acids; carbohydrate accounted for 72% (w/w). The ratio of hydroxyproline to arabinose was 1:4.4 and that of serine to galactose was 1:4.3, implying the presence of oligosaccharides attached to these amino acids. Interestingly, the overall amino acid compositions of material solubilized by either a 30 min or a 4 h treatment (Selvendran, 1975) are essentially similar except for the lysine content. However, the monosaccharide compositions are not comparable. Hence the glycoprotein is probably solubilized first, prolonged treatment resulting in the solubilization of other wall polysaccharides (hemicelluloses?). Morrison (1974), working with grass cell walls, has also observed an increased release of carbohydrate on extended periods of delignification.

During sodium chlorite/acetic acid treatment free ϵ -amino groups of lysine undergo oxidation, yielding α -amino adipic acid. In the g.l.c. system employed

Table 1. *Amino acid and monosaccharide compositions of the fractionated cell-wall material from runner beans*
For experimental details see the text. Results are expressed as (i) mol/100 mol of amino acids and (ii) mol/100 mol of monosaccharides.

	Depectinated cell-wall material	Chlorite-soluble glycoprotein	Insoluble residue after chlorite extraction
(i) Amino acid			
Ala	6.0	3.2	8.8
Gly	7.5	4.5	8.3
Val	5.2	4.4	6.0
Thr	4.8	3.7	4.7
Ser	10.2	13.9	8.5
Leu	7.5	2.5	9.5
Ile	3.6	1.5	4.5
Pro	6.1	7.0	5.3
Hyp	12.1	35.3	4.3
Asp	8.1	5.0	9.5
Phe	4.0	1.4	4.5
Glu	9.9	7.8	13.1
Lys	8.1	7.5*	8.8*
Tyr	2.5	0.9	0.2
Arg	4.0	1.4	4.0
His	0.4	0.0	0.0
Recovery (% w/w)	5.6	14.5	4.2
(ii) Monosaccharide			
Rha	2.1	3.4	1.7
Ara	5.9	46.1	2.5
Xyl	3.9	1.3	3.3
Man	2.8	0.0	2.6
Gal	7.5	16.6	6.5
Glc	67.4	2.5	67.6
GalUA	10.4	30.1	15.8
Recovery (% w/w)	66.4	71.0	84.2

* Sum of lysine and α -amino adipic acid; see the text.

for the analysis of amino acids, these two components co-chromatographed. Analysis by ion-exchange chromatography, which facilitates a good separation between these components, indicated that about 30% (w/w) of the lysine had been oxidized. By contrast, the 4 h treatment modified about 82% (w/w) of the lysine (Selvendran *et al.*, 1975). Clearly, a shorter sodium chlorite/acetic acid treatment allows isolation of similar amounts of hydroxyproline-rich glycoprotein but with less modification of the constituent polymers and less contamination.

By use of a modified carbazole assay (Selvendran *et al.*, 1979) it was shown that an appreciable quantity of uronic acid-containing material was also solubilized during chlorite treatment (Table 1, column 2; also Table 2, column 3). The uronic acid does not arise by oxidation at C-6 of the constituent sugars of polysaccharides during delignification (Morrison, 1974). It is not clear whether the contaminating pectic material is present as (a) a co-solubilized component, (b) an associated component of the glycoprotein *in vivo*, or (c) an aggregate formed after extraction (Knee, 1973).

Effect of sodium chlorite/acetic acid treatment on lysozyme

Treatment of lysozyme with sodium chlorite/acetic acid at 70°C for 30 min does not appear to cleave the molecule into small fragments, since 95% of the material can be recovered after 24 h dialysis. The results of gel filtration of untreated and treated lysozyme on Sephadex G-100 are shown in Fig. 1. The altered elution profile of the treated lysozyme suggests only a slight decrease in molecular weight. The peak is broader, and this could be due to an increase in the number of acidic groups (e.g. conversion of lysine residues into α -amino adipic acid residues) and a change in the conformation of the molecule, possibly by modifications of some functional groups, which permit greater hydrophilic interaction with Sephadex. From the limited data available it is difficult to assess all the affects of delignification on a standard protein.

Isolation of hydroxyproline arabinosides

The chlorite-soluble glycoprotein fraction on alkaline degradation followed by chromatography

Table 2. *Amino acid and monosaccharide compositions of cell-wall-material fractions isolated by DEAE-Sephadex chromatography*

For experimental details see the text. Results are expressed as (i) mol/100 mol of amino acids and (ii) mol/100 mol of monosaccharides.

	Fraction ...	D-1	D-2	D-3
(i) Amino acid				
Ala		4.1	3.4	2.4
Gly		5.0	4.6	2.9
Val		2.6	4.4	2.5
Thr		4.1	3.9	4.5
Ser		9.9	15.0	17.9
Leu		4.1	2.1	2.1
Ile		6.1	1.5	2.0
Pro		8.5	6.5	5.3
Hyp		27.4	37.4	34.2
Asp		6.7	4.9	6.2
Phe		1.7	0.9	1.1
Glu		15.4	7.3	11.6
Lys*		1.7	6.7	5.2
Tyr		2.7	1.1	2.1
Arg		0.0	0.3	0.7
Recovery (% w/w)		13.3	24.6	22.9
(ii) Monosaccharide				
Rha		1.2	1.4	4.6
Ara		27.9	73.5	30.9
Xyl		7.7	2.1	12.6
Gal		49.9	11.0	9.3
Glc		8.7	1.4	2.0
GalUA		4.5	10.6	40.6
Recovery (% w/w)		53.4	56.9	49.3
Recovery from column (% w/w)		7.8	75.4	15.8

* Sum of lysine and α -amino adipic acid; see the text.

on Bio-Gel P-2 yielded one major peak (B-1) containing non-peptide bound hydroxyproline and neutral sugar (Fig. 2a). Analysis, after appropriate hydrolysis, showed fraction B-1 to be a glycopeptide containing mainly hydroxyproline (85 mol/100 mol of amino acid) and arabinose as the sole monosaccharide. The ratio of hydroxyproline to arabinose varied across the peak from 1:3.9 to 1:2.8, with a ratio of 1:3.5 at peak maxima. A small peak eluted after fraction B-1 was found to correspond to free hydroxyproline and accounted for 10% (w/w) of the total hydroxyproline. This showed that the bulk of the hydroxyproline is glycosylated with arabinose-containing oligosaccharides.

A component containing neutral sugar but no non-peptide bound hydroxyproline was detected at the column void volume (V_0) (Fig. 2). On analysis it was found to contain protein (14%, w/w) and carbohydrate (86%, w/w). Serine, glycine and glutamic acid were the predominant amino acids, with only small amounts of hydroxyproline. Arabinose, galactose and glucose were the major neutral sugars. Hence little (if any) hydroxyproline is covalently associated with high-molecular-weight material in the alkaline hydrolysate.

Fractionation of fraction B-1 by ion-exchange chromatography revealed two major peaks (BA-1 and BA-2) containing non-peptide-bound hydroxyproline and neutral sugar (Fig. 2b). They were present in the ratio 1:0.6 with respect to hydroxyproline content. After hydrolysis, fractions BA-1 and

BA-2 were shown to contain hydroxyproline and arabinose in the ratios 1:3.9 and 1:3.1 respectively. So the bulk of the hydroxyproline is *O*-glycosidically substituted with oligosaccharides containing 3 or 4 arabinose units. Similar elution profiles were obtained with partial-alkaline-degradation products of depectinated cell-wall material (M. A. O'Neill & R. R. Selvendran, unpublished work). These results show that treatment with sodium chlorite/acetic acid causes little (if any) hydrolysis of arabinofuranose residues associated with the glycoprotein.

DEAE-Sephadex chromatography

The conditions for fractionation of the chlorite-soluble fraction were chosen so that uronic acid-containing material would be retarded by the column. The fractionation yielded one major and two minor fractions containing hydroxyproline and

Fig. 1. Gel filtration of untreated and chlorite-treated lysozyme on Sephadex G-100

The column of Sephadex G-100 (56 cm $\times$ 1.1 cm) was equilibrated and eluted with 0.5 M-acetic acid, and the effluent was analysed as described in the text. $\blacktriangle$, Untreated lysozyme; $\triangle$, treated lysozyme. V_0 , Column void volume, determined with Blue Dextran; V_e , elution volume of tyrosine.

Fig. 2. Fractionation of hydroxyprolinyl arabinosides obtained from chlorite-soluble glycoproteins of cell-wall material

(a) A sample (about 6 mg) of hydroxyproline arabinosides was applied to a column (96 cm $\times$ 1.1 cm) of Bio-Gel P-2 equilibrated and eluted with 0.1 M-acetic acid. Fractions (5 ml) were collected and assayed for neutral sugar ($\circ$) and non-peptide bound hydroxyproline ($\bullet$). (b) Fractions included under B-1 were rechromatographed on a column (36 cm $\times$ 1.6 cm) of Aminex AG50W X2 with a linear gradient of 0.02–0.2 M-HCl (----). Fractions (2.5 ml) were assayed as indicated above.

neutral sugars (Fig. 3). The major component (D-2) accounted for 75% (w/w) of the material recovered from the column and was eluted as a single peak by 0.11 M-NaCl. Each component was isolated and the amino acid and monosaccharide compositions were determined (Table 2). With respect to amino acid composition, each fraction shows similarity in being rich in hydroxyproline and serine. Fraction D-1 is fairly rich in glutamic acid, but is eluted with buffer at pH 5.2 only, implying the presence of glutamine. In fractions D-2 and D-3 the presence of mainly glutamic acid is assumed.

With respect to carbohydrate composition (Table 2) a considerable variation within the fractions was observed. Fraction D-1 had a very low uronic acid content and it was eluted with sodium acetate buffer only. On the other hand, fraction D-3 was eluted with 1 M-NaCl and contained a high proportion of uronic acid. This fraction accounted for the bulk of the pectic-type material solubilized during delignification. The major fraction D-2 was eluted with the NaCl gradient (at 0.11 M-NaCl) and was found to contain a small proportion of uronic acid. As previously stated, it is difficult to ascertain whether the uronic acid-containing material is associated with the glycoprotein *in vivo* or is complexed with it during the extraction procedure.

Several differences are observed with respect to

the neutral monosaccharide content of each fraction (Table 2). The predominant sugars in fraction D-1 are galactose and arabinose. The ratio of hydroxyproline to arabinose is 1:3.7, implying the presence of hydroxyproline tetra- and tri-arabinofuranosides. However, the ratio of serine to galactose is 1:18.1, which is not consistent with serine being substituted with short-chain galactose-containing oligosaccharides. The large amount of galactose may be attributed to the presence of neutral galactan-type polysaccharides solubilized by chlorite treatment. The presence of an appreciable amount of glucose could be attributed to the presence of some hemicellulosic polysaccharides. Fraction D-2 contained predominantly arabinose, with smaller amounts of galactose. The ratio of hydroxyproline to arabinose is 1:3.5 and that of serine to galactose is 1:1.2. The latter ratio is consistent with the serine being *O*-glycosidically substituted with a single galactose residue (Lamport *et al.*, 1973). Fraction D-3 also had arabinose as the predominant neutral sugar. Surprisingly, xylose is the next most abundant, the significance of which is not known. The ratio of hydroxyproline to arabinose is 1:1.2 and that of serine to galactose is 1:0.6.

Partial acid hydrolysis

Hydrolysis of fraction D-2 with 12.5 mM-oxalic

Fig. 3. Elution profile of chlorite-soluble glycoprotein(s) obtained from cell-wall material from DEAE-Sephadex A-50. The chlorite-soluble glycoprotein mixture (about 30 mg) was applied to a column (15 cm $\times$ 1.6 cm) of DEAE-Sephadex (Cl⁻ form) equilibrated with 50 mM-sodium acetate buffer, pH 5.2. Elution was carried out with 100 ml of 50 mM-sodium acetate buffer, pH 5.2, followed by a linear gradient of 0–0.6 M-NaCl in sodium acetate buffer. Firmly bound material was eluted with 1.0 M-NaCl (—). Fractions (4 ml) were collected and assayed for neutral sugar (O), uronic acid ($\square$) and, after acid hydrolysis, for hydroxyproline ($\bullet$). The numbered peaks are discussed in the text.

acid for 5 h at 100°C removed the arabinose residues almost completely (approx. 5% of original remaining). The hydrolysed material contained virtually all the galactose and amino acids of the original product. From the acid-lability, the presence of arabinofuranose residues can be inferred. Because the galactose is acid-stable, the presence of galactopyranose residues may be assumed. Galactose is thus linked to the protein by an acid-stable linkage. Other workers have shown the presence of a serinyl *O*-galactoside unit in wall glycoproteins (Cho & Chrispeels, 1976; Kliss, 1976). Fraction D-2 is rich in serine and therefore we assume the presence of this linkage. We have been unable to detect *N*-acetylglucosamine, and therefore a glycosylamine linkage to asparagine seems unlikely (Spiro, 1973).

Galactosidase treatment

On the assumption of the presence of serinyl *O*-galactoside, it was decided to determine the anomeric configuration of the linkage (Allen *et al.*, 1978). Samples of fraction D-2 (either complete or acid-treated) were incubated with either α - or β -galactosidase, and the treated material was isolated and analysed for remaining carbohydrate (Table 3). In both preparations only α -galactosidase had any detectable effect. It has been shown that the removal of arabinofuranose residues by partial acid hydrolysis greatly facilitated the enzymic cleavage of galactopyranose units by α -galactosidase but not by β -galactosidase (Allen *et al.*, 1978). So the bulk of the serine in fraction D-2 is probably α -*O*-glycosidically substituted with a single galactopyranose residue.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Attempts were made to fractionate the chlorite-soluble glycoprotein by using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. No bands were detected with Coomassie Blue. However, when the gel from a comparable experiment without any added fixative was sliced and analysed for hydroxyproline after 6 M-HCl hydrolysis, the bulk of

the glycoprotein was shown to remain at the point of application. The failure of Coomassie Blue to stain satisfactorily may be because it is a hydrophobic molecule and is prevented from reaching reactive sites by the hydrophilic sugar residues. Further chlorite treatment modifies the lysine residues and destroys tyrosine. As these residues are involved in binding Coomassie Blue, it is not surprising that the glycoprotein fails to stain satisfactorily. Dansyl-hydrazine also failed to stain the glycoproteins, but the reasons for this are not clear.

Methylation analysis

Both chlorite-soluble glycoprotein material and fraction D-2 were subjected to methylation analysis. I.r. spectrometry of the methylated products revealed no broad -OH absorption at 3400 cm⁻¹, suggesting the methylation to be essentially complete. Separation and quantitative analysis of the partially methylated alditol acetates was performed by g.l.c.-mass spectrometry. Both products gave 11 identifiable methylated neutral sugar derivatives (Table 4). Quantitative analysis showed the overall recoveries of methylated derivatives, except those of galactose, to be in broad agreement with those obtained by direct analysis. The discrepancy in galactose values may be attributed to β -elimination of alkali-labile serinyl *O*-galactoside bonds during polyalkoxide formation (Jansson *et al.*, 1976). Complete β -elimination may have been prevented by ionization of hydroxy groups of arabinose residues attached to hydroxyproline. In several glycoproteins (and some lectins) the glycosylated hydroxyproline and serine residues are close together, so that the ionized arabinose residues could have an inhibitory effect on β -elimination of serinyl *O*-galactoside residues. Allen *et al.* (1978) have reported incomplete β -elimination of serinyl *O*-galactoside residues in potato lectin, which have comparable arabinose residues, at pH 13.7.

The presence of 2,3,5-tri-, 3,5-di- and 2,5-di-*O*-methylarabinitol confirms the evidence from partial acid hydrolysis of the occurrence of arabinofuranosyl residues. Similarly, the finding of 2,3,4,6-tetra-*O*-methylgalactitol indicates the presence of

Table 3. *Galactosidase treatment of glycoproteins obtained from cell-wall material*

The effects of treatment with α - and β -galactosidase on the carbohydrate compositions of (a) DEAE-Sephader fraction D-2 and (b) the same glycoprotein after treatment with 12.5 mM-oxalic acid at 100°C for 5 h are shown. For experimental details see the text. Results are expressed as molar ratios relative to hydroxyproline.

	(a)			(b)		
	Untreated	α -Galactosidase-treated	β -Galactosidase-treated	Untreated	α -Galactosidase-treated	β -Galactosidase-treated
Ara	3.4	3.4	3.4	0.2	0.2	0.2
Gal	0.5	0.3	0.5	0.6	0.02	0.6

Table 4. *Partially methylated alditol acetates obtained from fractions of cell-wall material*

Results of methylation analysis on (i) chlorite-soluble glycoprotein and (ii) DEAE-Sephadex fraction D-2 are shown. For experimental details see the text. Results are expressed as percentages of total peak area of identifiable sugars from temperature-programmed g.l.c.

Partially methylated sugar	R_t^*	(i) Chlorite-soluble glycoprotein	(ii) DEAE-Sephadex fraction D-2
3,4-Di- <i>O</i> -methylrhamnitof†	0.90	1.0	0.5
2,3,5-Tri- <i>O</i> -methylarabinitol	0.46	18.5	22.9
3,5-Di- <i>O</i> -methylarabinitol	0.83	37.7	35.3
2,5-Di- <i>O</i> -methylarabinitol	0.90	19.4	18.7
2,3-Di- <i>O</i> -methylarabinitol	1.09	3.4	3.4
2-Mono- <i>O</i> -methylarabinitol	1.90	0.2	1.2
2,3,4-Tri- <i>O</i> -methylxylitol	0.61	0.9	0.7
2,3-Di- <i>O</i> -methylxylitol	1.19	2.1	1.9
2,3,4,6-Tetra- <i>O</i> -methylgalactitol	1.19	10.1	9.4
2,4,6-Tri- <i>O</i> -methylgalactitol	2.03	0.1	0.7
2,3,6-Tri- <i>O</i> -methylglucitol	2.30	7.0	5.3
2,3-Di- <i>O</i> -methylgalactitol	4.28	0.0 (19.7‡)	0.0 (0.0‡)

* Relative retention time, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on 3% OV-225 at 180°C.

† 3,4-Di-*O*-methylrhamnitof corresponds to (1→2)-linked rhamnose etc.

‡ Values in parentheses obtained after carboxy reduction of partially methylated material.

galactopyranosyl residues. In both products the bulk of the methylated neutral sugar derivatives can be accounted for by terminal (1→2)-linked and (1→3)-linked arabinofuranosyl residues. These are present in the proportions 1.2:1.9:1 in fraction D-2. Akiyama & Kato (1977a,b) have determined the structure of hydroxyproline arabinosides from tobacco cell-wall material and have proposed a sequence for the arabinose residues. Their work showed that the tetra-arabinoside has terminal (1→2)-linked and (1→3)-linked L-arabinose residues in the proportions 1:2:1. By analogy we propose the tetra-arabinoside from runner-bean cell wall to have the sequence:

The tri-arabinoside probably has the sequence:

Of the remaining derivatives, the most abundant is 2,3,4,6-tetra-*O*-methylgalactitol, which corresponds to terminal galactose. We propose that this is α -*O*-glycosidically linked to serine.

Other methylated sugar derivatives were found, and these correspond to (1→4)-linked glucose, (1→5)-linked arabinose, (1→4)-linked xylose, (1→2)-linked rhamnose and terminal xylose. These residues are probably derived from pectic-type and hemicellulosic-type material. It is, however, not known whether any of these residues are covalently linked with the wall glycoprotein or are artifacts of the extraction procedure. Derivatives corresponding to (1,3,5)-linked arabinose and (1→3)-linked galactose were found only in fraction D-2.

We have been unable to detect (1→6)- and (1,3,6)-linked galactose residues. Thus it would seem unlikely that the runner-bean cell-wall glycoprotein has a 3,6-linked arabinogalactan type of polysaccharide covalently associated with it (Keegstra *et al.*, 1973; Mort, 1978). However, the possibility that the arabinogalactan was *O*-glycosidically linked to serine and was lost by β -elimination cannot be discounted. Evidence for the presence of arabinogalactan-peptide/protein complexes in plant extracts has been put forward by a number of workers (Fincher *et al.*, 1974; Hillestad *et al.*, 1977; Clarke *et al.*, 1979).

The determination of the mode of linkage of uronic acid-containing polymers is difficult owing to selective losses and degradation during methylation (Lindberg *et al.*, 1975; O'Neill & Selvendran, 1980). However, by using carboxy reduction with LiAlD_4 , it is possible to get an estimate of uronic acid linkages (O'Neill & Selvendran, 1980). This introduces two deuterium atoms at C-6, allowing unambiguous identification of the appropriate derivative(s) by g.l.c.-mass spectrometry. The methylated products from both the chlorite-soluble glycoprotein fraction and fraction D-2 were subjected to this procedure. On analysis, only the unfractionated material showed incorporation of deuterium (Table 4, values within parentheses). The methylated sugar derivative corresponded to (1→4)-linked galacturonic acid. As this could not be detected in fraction D-2, it is proposed that the bulk of the pectic material solubilized by chlorite treatment is not (covalently) associated with the major wall glycoprotein fraction. The detection of a small amount of

uronic acid-containing material in fraction D-2 by the modified carbazole method (Selvendran *et al.*, 1979) may be of doubtful significance, because it involves the use of suitable correction factors for interference from neutral sugars; fraction D-2 is rich in neutral sugars.

General Discussion

A major problem in studying the structural aspects of cell-wall glycoproteins from higher plants is the isolation of these proteins (Brown & Kimmins, 1977). In certain tissues, alkaline extraction of the walls releases a hydroxyproline-containing component (Bailey & Kauss, 1974; Monro *et al.*, 1974). In others, including runner bean, the hydroxyproline-rich glycoprotein is tenaciously bound to the cell-wall complex (Heath & Northcote, 1971; Selvendran, 1975; Selvendran *et al.*, 1975). Therefore most information so far obtained on structural features comes from studies on degraded and modified glycoproteins and glycopeptides (Brown & Kimmins, 1977). We have found that it is possible to isolate a hydroxyproline-rich polymer in non-diffusible form with minimal modification by using a short chlorite/acetic acid treatment. Mort (1978) has used a similar procedure with suspension-culture tomato cell walls.

The present and previous studies (Selvendran, 1975; Selvendran *et al.*, 1975) do not allow us to propose a definite mechanism whereby the wall glycoprotein is solubilized. Under the conditions used, neither *O*-glycosidic nor peptide bonds are likely to be hydrolysed appreciably. Ferulic acid ester type of linkages is unlikely to occur, since the glycoprotein is not solubilized by alkali (Hartley, 1971; Morrison, 1977). Further, we failed to detect any ferulic acid or *p*-coumaric acid in an alkaline extract of the cell-wall material (M. A. O'Neill & R. R. Selvendran, unpublished work). From the known action of sodium chlorite/acetic acid on phenolic or polyphenolic material, we propose that the glycoprotein is held in the cell wall by phenolic

cross-links. Since parenchymatous tissues are devoid of phloroglucinol/HCl-staining lignin elements, the phenolic cross-links could be glycoprotein-protein or glycoprotein-polysaccharide.

A noteworthy feature of the chlorite-soluble glycoprotein from runner-bean (order Leguminosae) cell wall is its similarity with material solubilized by chlorite treatment of suspension-culture tomato (order Personatae) cell walls (Mort & Lamport, 1977). Table 5 summarizes the similarities between the two preparations. The chlorite treatment may act in a specific way to solubilize the hydroxyproline-rich glycoprotein(s).

Analysis of cell-wall material from a variety of sources shows that hydroxyprolinyl arabinosides and serinyl *O*-galactosides are of universal occurrence (Brown & Kimmins, 1977). This conservation of structure strongly points to the wall protein having a unique fundamental role in dicotyledonous-plant cell walls.

Determination of the function(s) of the cell-wall glycoprotein(s) depends, in part, on their association with other cell-wall polymers. Keegstra *et al.* (1973) suggested that the polysaccharide and protein polymers of the primary cell walls of suspension-culture sycamore cells are covalently linked to form a macromolecular complex. However, the evidence for a covalent linkage of the glycoprotein to a (1,3,6)-linked arabinogalactan was obtained with material secreted into the growth medium by the suspension cultures. These complexes have subsequently been shown to be distinct from cell-wall glycoprotein (Pope, 1977). From our work we have also been unable to demonstrate a direct covalent linkage of glycoprotein to polysaccharide. However, the glycoprotein could be linked to polysaccharides via phenolic cross-links. Such cross-links would be disrupted by sodium chlorite/acetic acid treatment, rendering the glycoprotein in a soluble form.

Fractionation of the chlorite-soluble glycoprotein by ion-exchange chromatography enabled us to remove the bulk of the uronic acid-containing material from the major hydroxyproline-rich poly-

Table 5. Comparison of hydroxyproline-rich glycoproteins obtained from cell-wall material after sodium chlorite/acetic acid treatment

Both preparations were obtained by mild sodium chlorite oxidation. For experimental details see the text. (i) Chlorite-soluble glycoprotein from runner bean; (ii) chlorite-soluble glycoprotein from tomato suspension-culture cells (Mort & Lamport, 1977). Results are expressed as relative molar ratios.

	(i) Runner bean	(ii) Tomato
Arabinose/hydroxyproline ratio	4.6	5.04
Galactose/serine ratio	4.2	4.2
Galacturonic acid/serine ratio	7.6	7.0
Rhamnose/serine ratio	0.6	1.7
Glucose/serine ratio	0.6	0.84
Hydroxyproline (mol/100 mol)	35.3	37.5
Serine (mol/100 mol)	13.9	12.6

mer. Three fractions were obtained, all rich in hydroxyproline but differing in monosaccharide composition. It may be that these represent three distinct groups of hydroxyproline-rich glycoproteins within the cell wall. Similar inferences from the incorporation of radioactive amino acids into wall glycoproteins and kinetic studies on the rate of labelling have been made (Pope, 1977). However, the glycoproteins may represent a family with different extents of glycosylation, heterogeneity being a common feature of glycoproteins (Beeley, 1974).

The glycoprotein isolated from the cell wall of runner-bean parenchyma shows many similarities with the glycopeptide portion of a lectin isolated from potato (Allen *et al.*, 1978; Murray & Northcote, 1978). Both have a similar composition, and in both the cell-wall glycoprotein and the lectin the arabinose is linked to the hydroxyproline residues of the polypeptide chain. The arabinose is present as the furanoside and probably as the β -anomer. In both products one galactose residue is α -O-glycosidically linked to serine. The occurrence of glycoproteins having comparable structures in the water-soluble and -insoluble residues of plant tissues is noteworthy. Such conservation of structure suggests an underlying role for these groups of compounds. In the insoluble residue (cell-wall material) of runner bean the glycoprotein is associated with α -cellulose (Selvendran, 1975) and cannot be released by hydrogen-bond-breaking reagents. Phenolic or similar cross-links (Whitmore, 1978a,b) may therefore play a major role in determining the properties of the glycoprotein.

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References

- Akiyama, Y. & Kato, K. (1977a) *Agric. Biol. Chem.* **40**, 2343–2348
- Akiyama, Y. & Kato, K. (1977b) *Agric. Biol. Chem.* **41**, 79–81
- Allen, A. K., Desai, N. N., Neuberger, A. & Creeth, J. M. (1978) *Biochem. J.* **171**, 665–674
- Bailey, R. W. & Kaus, H. (1974) *Planta* **119**, 233–245
- Beeley, J. G. (1974) *Biochem. Soc. Symp.* **40**, 27–36
- Brown, R. G. & Kimmins, W. C. (1977) in *International Review of Biochemistry: Plant Biochemistry II* (Northcote, D. H., ed.), vol. 13, pp. 183–209, University Park Press, Baltimore
- Cho, Y.-P. & Chrispeels, M. J. (1976) *Phytochemistry* **15**, 165–169
- Clarke, A. E., Anderson, R. L. & Stone, B. A. (1979) *Phytochemistry* **18**, 521–540
- Dougall, D. K. & Shimbayashi, K. (1960) *Plant Physiol.* **35**, 396–404
- Dubois, M., Gilles, K. A., Hamilton, J. D., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356
- Eckhardt, A. E., Hayes, C. E. & Goldstein, I. J. (1976) *Anal. Biochem.* **73**, 192–197
- Fincher, G. B., Sawyer, W. H. & Stone, B. A. (1974) *Biochem. J.* **137**, 535–545
- Hartley, R. D. (1971) *J. Chromatogr.* **54**, 335–344
- Heath, M. F. & Northcote, D. H. (1971) *Biochem. J.* **125**, 953–961
- Hillestad, A., Wold, J. K. & Paulsen, B. S. (1977) *Carbohydr. Res.* **57**, 135–144
- Jansson, P.-E., Kenne, L., Liedgren, H., Lindberg, B. & Lonngrén, J. (1976) *Chem. Commun. Univ. Stockholm* **8**, 1–75
- Keegstra, K., Talmadge, K. W., Bauer, W. D. & Albersheim, P. (1973) *Plant Physiol.* **51**, 188–196
- King, N. J. & Bayley, S. T. (1965) *J. Exp. Bot.* **16**, 294–303
- Kivirikko, K. I. & Liesmaa, M. (1959) *Scand. J. Clin. Lab. Invest.* **11**, 128–133
- Kliss, F. M. (1976) *Plant Physiol.* **57**, 224–226
- Knee, M. (1973) *Phytochemistry* **12**, 637–653
- Lamport, D. T. A. (1965) *Adv. Bot. Res.* **2**, 151–212
- Lamport, D. T. A. (1967) *Nature (London)* **216**, 1322–1324
- Lamport, D. T. A. (1970) *Annu. Rev. Plant Physiol.* **21**, 235–270
- Lamport, D. T. A. & Northcote, D. H. (1960) *Nature (London)* **188**, 665–666
- Lamport, D. T. A., Katona, L. & Roerig, S. (1973) *Biochem. J.* **133**, 125–131
- Lindberg, B. (1972) *Methods Enzymol.* **28**, 179–195
- Lindberg, B., Lonngrén, J. & Svensson, S. (1975) *Adv. Carbohydr. Chem. Biochem.* **31**, 185–240
- March, J. F. (1975) *Anal. Biochem.* **69**, 420–442
- Monro, J. A., Bailey, R. W. & Penny, D. (1974) *Phytochemistry* **13**, 375–382
- Morrison, I. (1974) *Phytochemistry* **14**, 505–508
- Morrison, I. (1977) *Carbohydr. Res.* **57**, C4–C6
- Mort, A. J. (1978) Ph.D. Thesis, University of Michigan
- Mort, A. J. & Lamport, D. T. A. (1977) *Anal. Biochem.* **82**, 289–309
- Murray, R. H. A. & Northcote, D. H. (1978) *Phytochemistry* **27**, 623–629
- Northcote, D. H. (1972) *Annu. Rev. Plant Physiol.* **23**, 113–132
- O'Neill, M. A. & Selvendran, R. R. (1980) *Carbohydr. Res.* in the press
- Pope, D. G. (1977) *Plant Physiol.* **59**, 894–900
- Ring, S. G. & Selvendran, R. R. (1978) *Phytochemistry* **17**, 745–752
- Roberts, K. & Northcote, D. H. (1972) *Planta* **107**, 43–51
- Selvendran, R. R. (1975) *Phytochemistry* **14**, 2175–2180
- Selvendran, R. R., Davies, A. M. C. & Tidder, E. (1975) *Phytochemistry* **14**, 2169–2174
- Selvendran, R. R., March, J. F. & Ring, S. G. (1979) *Anal. Biochem.* **96**, 282–292
- Spiro, R. G. (1973) *Adv. Protein Chem.* **27**, 349–467
- Whitmore, F. (1978a) *Phytochemistry* **17**, 421–425
- Whitmore, F. (1978b) *Plant Sci. Lett.* **13**, 241–245
- Yemm, E. W. & Cocking, E. C. (1955) *Analyst (London)* **80**, 209–212