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Structural Studies of Glycoproteins from *Phaseolus vulgaris*

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Sugar analysis, methylation analysis, enzymic hydrolysis and hydrazinolysis were used to study glycoproteins isolated from the primary leaves of *Phaseolus vulgaris* var. Pinto 36 h after sham-inoculation. The glycoproteins contained β-(1→4)-linked D-xylopyranose residues as well as (1→2)-, (1→3)-, and (1→5)-linked L-arabinofuranose residues. Smith degradation of the products of hydrazinolysis indicated that the majority of terminal arabinofuranose residues is linked to O-2 of adjacent residues. Glycoproteins isolated after inoculation with tobacco necrosis virus had a more variable composition and generally contained (1→4)-linked glucose in addition to the above structural elements. Glycoproteins isolated from *P. vulgaris* var. Black Valentine after sham-inoculation were similar to those isolated from the Pinto variety.

Carbohydrate moieties of the glycoproteins appear to be linked *via* hydroxyproline to protein.

The structure of plant glycoproteins has not been widely studied although they are important cell wall constituents,[1] occur in endosperm [2] and cytoplasm,[1] possess lectin activity [3] and may be important for plant virus localization.[4,5] Generally, the carbohydrate moiety is linked to hydroxyproline through α-L-arabinofuranosyl residues [3,6,7] but recently D-galactose has been found to be linked to hydroxyproline [2] and galactose-serine linkages also occur in plant cell wall glycoproteins.[8] L-Arabinose and D-galactose are the most commonly found carbohydrate constituents of plant glycoproteins [2,3,6,7] although, in addition, D-xylose and D-glucose have been found [9,10] as well as D-glucosamine.[11]

Glycoprotein having structural features similar to cell wall glycoproteins but lower hydroxyproline content are produced as a result of mechanical introduction of plant viruses.[4,5] The low hydroxyproline content possibly pointed to an anomalous linkage between carbohydrate and protein moieties and the present study sought to investigate this possibility.

EXPERIMENTAL

Plant and virus. Phaseolus vulgaris L. var. Pinto and Black Valentine received tobacco necrosis virus (Pinto) or were sham-inoculated (Pinto and Black Valentine) as outlined in previous publications.[4,5]

Extraction and purification. Primary leaves harvested 36 h after inoculation were extracted as previously described [4] at 2 °C, unless otherwise stated, yielding non-dialyzable precipitates which were dissolved in 10 % pyridine, insoluble material removed by centrifugation and freeze dried. Lipids were extracted [4] from the dried preparations and the extracted material (in approximately 50 mg batches) dissolved in phosphate buffer (0.02 M, pH 9.0) then chromatographed using a column (2.5 × 50 cm) of Sephadex G-150 developed with the buffer at a flow rate of 3 ml/h. In some cases, the glycoproteins did not dissolve completely in phosphate buffer prior to gel filtration; the insoluble material (up to 30 %) was removed by centrifugation. Fractions (3 ml) were collected; an aliquot of each fraction was diluted and its absorbance at 280 nm measured. Fractions containing the first and second peak of material absorbing at this wavelength were pooled, dialyzed and lyophilized. To measure reproducibility, two preparations from sham-inoculated plants (hereafter referred to as glycoproteins I) and three preparations from TNV-inoculated plants (hereafter referred to as glycoproteins II) were extracted and chromatographed as described.

Hydroxyproline-polysaccharide complexes are extracted from *Lupinus* hypocotyls by alkali at an extraction temperature of 20 °C but not at 2 °C.[9] To test the effect of extraction

temperature on glycoprotein recovery, primary leaves were extracted at 2 °C to yield glycoprotein II (preparation 3) then the leaves were reextracted with 1 M sodium hydroxide at 24 °C. The material extracted at the higher temperature was purified by gel filtration to yield preparation 4, peaks 1 and 2.

To produce enough material for degradation using pronase and hydrazinolysis, batches (approx. 200 mg) of glycoprotein I were purified as described above except the column effluent was monitored at 254 nm with an LKB Uvicord and the first broad peak absorbing at 254 nm was collected, dialyzed and lyophilized.

Acrylamide gel electrophoresis. Electrophoretic separation used a modification of the methods of Ornstein [12] and Davis [13] described previously.[4] The gels were cleaved longitudinally and the halves stained for carbohydrate or protein. Ovalbumin and β-lactoglobulin were used as controls.

Chemical analysis. Protein content was measured by nitrogen analysis. Carbohydrate content was estimated with anthrone. [14] Sugars were identified by gas liquid chromatography (GLC) of the alditol acetate derivatives. [15]

The products of pronase treatment and hydrazinolysis, that is amino acids and peptides or amino acid hydrazides, respectively, were measured quantitatively with ninhydrin. [16] Tests for uronic acids and amino sugars used the methods of Dische [17] and Tracey,[18] respectively.

For amino acid analysis, samples were hydrolyzed with 6 M HCl in sealed tubes *in vacuo* at 110 °C for 36 h. The amino acid composition of the hydrolyzate was determined by the method of Moore and Stein [19] using a JEOL Model 5 AH amino acid analyzer.

Methylation analysis. Samples were methylated twice using the Hakomori [20] procedure, hydrolyzed and converted to alditol acetate derivatives which were analyzed by GLC using the following columns: (1) 3 % ECNSS-M on Gas Chrom Q (100–200 mesh) at 165 °C, (2) 3 % OV-225 at 175 °C, (3) SP 1000 capillary column (0.25 mm × 25 m) at 240 °C. For quantitative evaluation of analyses obtained by GLC, a Hewlett Packard 3370 B integrator was used. Mass spectrometry used a Perkin-Elmer 270 combination gas chromatograph-mass spectrometer or, for compounds containing nitrogen, a Varian Mat 311B instrument. A S.C.O.T. OV-225 column was used to separate the compounds by GLC prior to recording mass spectra (MS) in the Perkin-Elmer instrument and an OV-1 column was used in the Varian instrument.

Pronase treatment. As a preliminary experiment, glycoprotein I (peak 1, 35 mg) was treated with pronase (Sigma, protease type VI, 3.4 units/mg) as described by Brown and Kimmins.[4] The products were separated by gel filtration on Sephadex G-150 as described above and the amino acid composition of the two carbohydrate containing fractions having the highest molecular weight determined. Based on the results of these amino acid analyses, the glycoprotein (253 mg) prepared by chromatography of 200 mg batches was treated with appropriate quantities of reagents and the products of enzymic hydrolysis isolated by gel filtration.

Hydrazinolysis. The glycopeptide fraction (30 mg) obtained by pronase digestion was treated with hydrazine (1.5 ml) and hydrazine sulfate (23 mg) as described by Yosizama *et al.*[21] for 10 h at 100 °C. The hydrazinolyzate was dried *in vacuo* over conc. sulfuric acid, then dissolved in water (2 ml) and chromatographed using a column of Sephadex G-25 (1.5 × 50 cm) developed with water (1.2 ml/h). Fractions (1.8 ml) were collected and tested for carbohydrate and amino acid hydrazides.

Two carbohydrate containing fractions were obtained by the above chromatographic procedure. These were dried by lyophilization and weighed. Fraction I (13 mg) was analyzed as follows: (1) part (2 mg) was hydrolyzed using mild conditions (0.01 N HCl, 100 °C, 3 h). The hydrolyzate thus obtained was divided into two parts. One part (2/3) was analyzed by paper chromatography (ethyl acetate, pyridine, water; 8:2:1) using arabinose and hydroxyproline hydrazide as standards and alkaline silver nitrate [22] for development. The other part (1/3) was converted to trimethylsilyl (TMS) derivatives [23] then analyzed by GLC using a 3 % OV-225 column at 110 °C and a SP 1000 column at 130 °C and by GLC-MS using a 3 % OV-1 column at 130 °C. In addition to these GLC procedures, the TMS derivatives were analyzed by GLC-MS as described for cylic (4-*trans*-hydroxy-L-prolyl) -4-*trans*-hydroxy-L-proline. (2) Part (2.0 mg) of fraction I was hydrolyzed using strong conditions (1.0 M HCl, 100 °C, 6 h) and the products analyzed by GLC-MS as TMS derivatives using the same procedures as described above for the products of mild hydrolysis. (3) Part (1.5 mg) was used for molecular weight determination using a column of Sephadex G-50, G-75 calibrated with oligosaccharides obtained from dextran.[24] (4) Part (1.5 mg) was hydrolyzed (0.01 M HCl, 100 °C, 3 h) and the molecular weight distribution of the hydrolyzate determined as described above. (5) Part (2 mg) was methylated and analyzed as described under methylation analysis.

Fraction II (8 mg) obtained by column chromatography of the hydrazinolysate was investigated by methylation analysis which used 2 mg of this material.

The paper chromatography and GLC described above for fraction I required hydroxyproline hydrazide and cyclic (4-*trans*-hydroxy-L-prolyl)-4-*trans*-hydroxy-L-proline as standards. The first compound was synthesized by hydrazinolysis of L-hydroxyproline methyl ester; the structure of the product was verified

by nuclear magnetic resonance and MS. The cyclic compound was synthesized from L-hydroxyproline methyl ester as described by D'Alagni and Pispisa [25] and the product had a melting point of 242–247 °C (decomp.). A 3 % OV-1 column at a starting temperature of 160 °C increasing to 240 °C at 10 °C/min was used for GLC-MS of the silyl derivative of this compound.

Smith degradation. The remaining parts of fractions I and II (4 and 6 mg, respectively) were degraded using the Smith procedure as described by Lindberg *et al.*[26] with the following modifications. After periodate oxidation, the product was purified by gel filtration using Sephadex G-25 (1.5 × 50 cm) and copper reduction [27] for monitoring fractions. The oxidized product was reduced with sodium borohydride and excess reagent removed by ion exchanging sodium with Dowex 50 and codistilling the boric acid thus generated with methanol. Before addition of Dowex 50, the solution was cooled to 2 °C and maintained at this temperature until the methanol codistillation step which was done at 30 °C under reduced pressure. After methylation, part of fractions I and II (1.5 and 2 mg, respectively) was hydrolyzed, converted to alditol acetate derivatives and analyzed by GLC while the remainder of each fraction was treated with 50 % aqueous acetic acid (100 °C, 1 h), reduced ($NaBH_4$ in dioxane-ethanol, 3:1), remethylated (CD_3I) then analyzed by GLC.

Xylanase treatment. Glycoprotein I extracted at 2 °C from *P. vulgaris* var. Black Valentine (24 mg) was suspended in 0.1 M acetate buffer (pH 5.5, 2 ml) and xylanase (14 μg in 0.1 ml water) was added. The mixture was incubated at 37 °C while the production of reducing groups was measured every 30 min for 4 h. Incubation was continued for 12 h to insure complete hydrolysis; then the hydrolyzate was converted to alditol acetate derivatives and analyzed by GLC. The xylanase was produced by *Trichoderma viride* (All Japan Biochemicals Co., Nishinomiya, type Onozuka SS) and isolated as described by Sinner. [28] The preparation had the following specific activities: xylanase 101.5; CM cellulase 0.22; mannanase 0.00; β-xylosidase < 0.02; β-glucosidase; < 0.03 and β-galactosidase; 0.00.

RESULTS

Extraction and purification. Separation of glycoproteins by gel filtration produced two

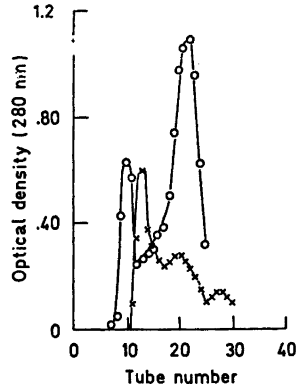

Fig. 1. Absorption (280 nm) of diluted fractions obtained by column chromatography using Sephadex G-150 of glycoproteins I (preparations 1) (○) or glycoproteins II (×). Glycoproteins I: peak 1 (tubes 8–12 incl.); peak 2 (tubes 18–25 incl.). Glycoprotein II: peak 1 (tubes 11–16 incl.); peak 2 (tubes 18–23 incl.). Yields: glycoprotein I; 66 mg placed on column; peak 1, 26 mg; peak 2, 21 mg. Glycoprotein II; 46 mg placed on column; peak 1, 22 mg; peak 2, 16 mg.

Table 1. Carbohydrate and protein content of glycoproteins.

Sample	Preparation No.	Glycoproteins I, % Carbohydrate	Protein[a]	Glycoproteins II, % Carbohydrate	Protein[a]
Peak 1	1	12	–	14	–
	2	17	74	16	77
	3	–	–	12	74
	4[b]	–	–	4	–
Peak 2	1	6	–	11	–
	2	7	76	13	77
	3[b]	–	–	4	–

[a] Protein was measured by nitrogen analysis and assuming a nitrogen content of 13.65 % based on the amino acid analysis. [b] Extracted at 24 °C.

Table 2. Sugar composition of glycoproteins.

Sample	Preparation No.	Glycoproteins I, % Ara[a]	Xyl	Man	Gal	Glc	Glycoproteins II, % Ara	Xyl	Man	Gal	Glc
Peak 1	1	39	47	2	6	6	18	2	2	4	73
	2	14	61	18	4	3	15	59	12	6	8
	3	–	–	–	–	–	31	–	13	16	40
	4[b]	–	–	–	–	–	33	7	7	17	36
Peak 2	1	14	43	2	14	24	6	11	3	2	78
	2	24	47	5	6	17	5	21	8	5	61
	3[b]	–	–	–	–	–	52	7	9	15	17

[a] Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose and Glc, glucose. [b] Extracted at 24 °C.

fractions (Fig. 1). Each was homogeneous by disc gel electrophoresis and showed coincident protein and carbohydrate bands. Although there was variation in the degree of separation and relative amounts of peak 1 and 2 in subsequent preparations, all showed a similar distribution of components during gel filtration.

Chemical analysis. Carbohydrate and protein accounted for most of the weight of the glycoprotein preparations (Table 1). The sugar composition of the glycoproteins indicated that more variation occurred in glycoproteins II than glycoproteins I (Table 2). The most abundant sugars were arabinose and xylose although in some cases glucose accounted for a considerable portion of the carbohydrate content, particularly in glycoproteins II. Colourimetric tests for uronic acids using native glycoproteins I and amino sugars after acid hydrolysis indicated that the content of these sugars was less than 0.5 % for uronic acids and 0.1 % for amino sugars. In addition, the

Table 3. Amino acid composition of glycoproteins and glycopeptides (Preparation 1).

Amino acid	Molar ratio Glycoprotein I Peak 1	Glycoprotein II Peak 1	Glycoprotein II Peak 2	Glycopeptides[a] Peak 1	Glycopeptides[a] Peak 2
Lysine	8	10	7	9	9
Histidine	2	1	1	7	6
Ammonia	13	90	84	15	20
Arginine	4	5	4	3	3
Unknown[b]	trace	trace	trace	10	8
Hydroxyproline	trace	trace	trace	18	9
Aspartic acid	8	11	11	7	12
Threonine	4	5	7	3	6
Serine	5	10	7	9	9
Glutamic acid	9	14	13	4	8
Proline	4	6	6	4	4
Glycine	7	14	20	9	13
Alanine	8	6	6	1	11
Valine	6	9	8	5	7
Methionine	1	1	1	–	–
Isoleucine	5	5	4	2	3
Leucine	10	9	8	3	5
Tyrosine	3	3	3	3	2
Phenylalanine	4	4	4	2	2

[a] Glycopeptides produced by pronase treatment of glycoprotein I (peak 1) and separation of the carbohydrate containing fractions by gel filtration. [b] The molecular weight and colour coefficient for ninhydrin were assumed to be the same as hydroxyproline for calculating molar ratios.

Table 4. Methylation analysis of glycoproteins (preparation 2).

Sugar[a]	Tg[b] SP 1000	ECNSS-M	OV-225	Molar proportions (%) Glycoprotein I Peak 1	Glycoprotein I Peak 2	Glycoprotein II Peak 1	Glycoprotein II Peak 2
2,3,5-Tri-*O*-Me-Ara	0.55	0.48	0.45	2	—	—	1
2,3,4-Tri-*O*-Me-Xyl	0.74	0.66	0.55	2	2	3	5
3,5-Di-*O*-Me-Ara	0.92	0.91	0.80	—	8	2	3
2,5-Di-*O*-Me-Ara	0.96	1.06	0.89	—	16	1	1
2,3,4,6-Tetra-*O*-Me-Glc or -Man	1.00	1.00	1.00	2	—	1	7
2,3-Di-*O*-Me-Xyl	1.20	1.54	1.19	38	33	57	26
2,3,6-Tri-*O*-Me-Glc	1.84	2.48	2.29	3	1	3	43
Ara	—	—	—	3	4	5	—
Xyl	—	—	—	9	25	—	—
Man	—	—	—	17	—	22	—
Gal	—	—	—	3	—	2	—
Glu	—	—	—	19	—	5	—

[a] See Table 2 for abbreviations. [b] Tg is the retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

sugar composition of glycoprotein I (peak 1) remained unchanged following reduction,[29] further indicating the absence of uronic acids.

The glycoproteins had similar amino acid compositions (Table 3). The ammonium content was high in glycoproteins II following hydrolysis, otherwise the analyses showed only minor variation. All analyses indicated the presence of only traces of hydroxyproline and a more acidic unknown component which reacted with ninhydrin to produce a product absorbing more strongly at 440 nm than at 520 nm.

Methylation analysis. All preparations of glycoproteins were subjected to methylation analysis.The results presented for glycoproteins I had the best agreement between the sugar composition before methylation and recovery of *O*-methyl derivatives; however, methylation of preparation 1 yielded the same methylated sugars. Unmethylated sugars were present in glycoproteins I after two treatments using the Hakomori procedure (Table 4). Although unmethylated sugars were detected, no peaks were found during GLC–MS which would indicate mono-*O*-methylated pentoses or di-*O*-methylhexose; that is, the residues appeared to be methylated completely or not at all. Attempts to improve efficiency by pronase digestion and mild acid hydrolysis (0.01 N HCl, 100 °C, 3 h) before methylation were unsuccessful; that is, the methylation analyses after these treatments were very similar to the analyses obtained using native glycoproteins. Glycoprotein II (preparation 2, peak 1) had about the same composition of methylated sugars as glycoprotein I. Glycoproteins II having glucose as the most abundant sugar contained more 2,3,4,6-tetra-*O*-methylhexose and 2,3,6-tri-*O*-methylglucose; the ratio of tetra to tri was in the range 1 to 3 – 7.

No unmethylated sugars were detected following methylation of the glucose-rich preparation (glycoprotein II, peak 2) indicating that methylation was complete.

2,3,5-Tri-*O*-methylarabinose indicated terminal arabinofuranose units whereas 3,5- and 2,5-di-*O*-methylarabinose demonstrated (1→2)- and (1→3)-linked arabinofuranose residues, respectively. 2,3,4-Tri-*O*-methylxylose indicated terminal xylopyranose units and 2,3-di-*O*-methylxylose could have arisen from (1→5)- or (1→4)-linked residues. 2,3,4,6-Tetra-*O*-methylhexose demonstrated terminal glucose or mannose residues and 2,3,6-tri-*O*-methylglucose revealed the presence of (1→4)- or (1→5)-linked glucose residues. Since mild acid hydrolysis of glycoproteins I and II released only arabinose, xylofuranose and glucofuranose residues are not present.

Pronase treatment. Glycoprotein I (peak 1) was hydrolyzed with pronase and two glycopeptide fractions isolated. Amino acid analysis (Table 3) indicated that the glycopeptide fractions were enriched in hydroxyproline and

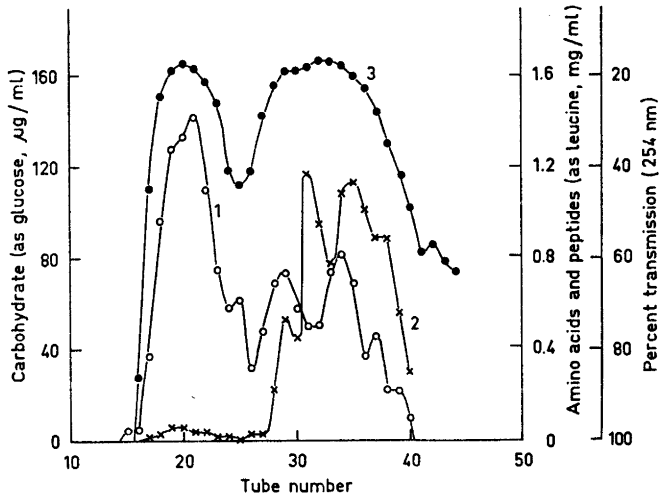

Fig. 2. Gel filtration (Sephadex G-150) of pronase digest of glycoprotein I. Yield: 32 mg (tubes 17 to 25 incl.) of 253 mg placed on column. Curve 1: carbohydrate. Curve 2: peptides and amino acids. Curve 3: absorbance at 254 nm.

an unknown compound. This suggested that hydroxyproline was important for linkage of carbohydrate and protein moieties. This procedure removed 80 – 90 % of the protein, therefore, a larger quantity of glycoprotein I was hydrolyzed with pronase and the products of hydrolysis fractionated by gel filtration (Fig. 2). The glycopeptide fraction was treated with hydrazine without analysis.

Hydrazinolysis. Gel filtration of the products of hydrazinolysis resulted in the isolation of two fractions rich in carbohydrate (Fig. 3). Paper chromatography of fraction I, $[\alpha]_{578}$ + 8 °, after mild acid hydrolysis resulted in the detection of arabinose (R_F 0.36) but no hydroxyproline hydrazide (R_F 0.54). In addition, arabinose was the only sugar detected by GLC after mild hydrolysis (Table 5). Acid hydrolysis with 1 M HCl released xylose and glucose in addition to arabinose. GLC indicated that cyclic (4-*trans*-hydroxy-L-prolyl)-4-*trans*-hydroxy-L-proline was not present in either hydrolyzate although more volatile unidentified components were detected. The unidentified compounds gave mass spectra which were similar to each other and indicated that they were not carbohydrate derivatives.

Molecular weight determination of fraction I before and after mild acid hydrolysis indicated that before hydrolysis, $\overline{M}_w$ was 27 800 and $\overline{M}_n$ was 15 900. The ratio $\overline{M}_w/\overline{M}_n$ was 1.75 indicating a heterogeneous distribution of chain lengths. The highest fragment had a molecular weight of 48 800, whereas the smallest fragment had a molecular weight of 3 360.

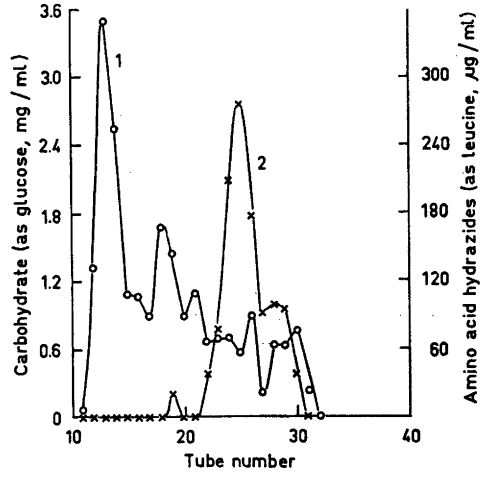

Fig. 3. Fraction of hydrazinolysate by gel filtration (Sephadex G-25). Yields: Fraction I; 13 mg (tubes 11 – 16 incl.) and fraction II; 8 mg (tubes 17 – 20 incl.). Curve 1: carbohydrate. Curve 2: amino acid hydrazides.

Table 5. Analysis of trimethylsilylated acid hydrolyzates of fraction I obtained by hydrazinolysis.

Compound	Ta[c]		Ta (standards)		% [d]	
	OV-225	SP 1000	OV-225	SP 1000	Mild acid	Strong acid
Arabinose[a]	1.00	1.00 (1.08)	1.00	1.00 (1.10)	69	31
Arabinose[a]	1.40	1.50 (1.73)	1.40	1.54 (1.72)		27
Xylose	1.54	1.80	1.53	1.75	–	14
Xylose	1.96	2.82	1.95	2.79	–	4
Mannose	–	–	1.53	–	–	–
Mannose	–	–	2.76	–	–	–
Glucose[b]	2.48	–	2.57	–	–	–
Glucose[b]	4.50	–	4.61	–	–	–
Unknown 1	1.17	0.96	–	–	12	12
Unknown 2	–	1.29	–	–	6	6
Unknown 3	–	2.15	–	–	13	–

[a] The figures in brackets indicate additional peaks found after mild hydrolysis of fraction I and, in the case of the standards, phenyl-α-L-arabinofuranoside. [b] Glucose was identified separately using a column of OV-225 at 130 °C and the quantity of this sugar is not included in the calculation of percent. [c] Retention time relative to silylated β-arabinose. [d] Percent obtained by integration and uncorrected for detector response.

After mild hydrolysis $\overline{M}_w$ was 8 990, $\overline{M}_n$ was 1 600 and the ratio $\overline{M}_w/\overline{M}_n$ was 5.6 indicating very large differences in chain length. The highest molecular weight was 34 490 whereas the lowest was 170.

Methylation analysis of fraction I (Table 6, column a) indicated the presence of terminal arabinofuranose and glucopyranose units. In addition (1→2)- and (1→3)-linked arabinofuranose units were demonstrated. 2,3-Di-*O*-methylarabinose could have arisen from either (1→4)- or (1→5)-linked arabinose; however, since arabinose occurs in the furanose form when (1→2)- and (1→3)-linked, the latter possibility is considered to be more likely. The presence of 2,3-di-*O*-methylxylose also indicated (1→4)- or (1→5)-linkages; however, the result of mild acid hydrolysis eliminates the latter possibility.

Methylation of fraction II (Table 6, column d) demonstrated terminal arabinofuranose in addition to (1→2)- and (1→3)-linked arabinofuranose units.

Smith degradation. Analysis of the products of the modified Smith degradation prior to treatment with 50 % acetic acid indicated that all terminal glucose, (1→4)-linked xylose, and (1→5)-linked arabinose residues of fraction I had been oxidized (Table 6, column b). The presence of 2,3,5-tri-*O*-methylarabinose in both

Table 6. Methylation analysis of fractions I and II obtained by hydrazinolysis.

Sugar[a]	Tg[b]			Molar proportions (%)					
	SP 1000	ECNSS-M	OV-225	Fraction I			Fraction II		
				a	b	c	d	e	f
2,3,5-Tri-*O*-Me-Ara	0.55	0.48	0.45	21	22	55	20	16	55
3,5-Di-*O*-Me-Ara	0.92	0.91	0.80	13	28	–	30	34	7
2,5-Di-*O*-Me-Ara	0.96	1.05	0.89	24	50	45	50	50	37
2,3,4,6-Tetra-*O*-Me-Glc	1.00	1.00	1.00	8	–	–	–	–	–
2,3-Di-*O*-Me-Ara	1.12	1.35	1.07	12	–	–	–	–	–
2,3-Di-*O*-Me-Xyl	1.20	1.54	1.19	22	–	–	–	–	–

[a+b] See Table 4.

fractions (columns b and e) indicated that some of the modified sugar residues had been hydrolyzed probably by acid generated when Dowex 50 was used to remove excess sodium borohydride. Following hydrolysis with 50 % acetic acid and remethylation, an increased content of 2,3,5-tri-*O*-methylarabinose was observed with a corresponding decrease of 3,5-di-*O*-methylarabinose (columns c and f). These results indicate that most terminal arabinose units were linked to O-2 of adjacent arabinose residues.

Effect of variety variation. Phaseolus vulgaris var. Black Valentine was sham-inoculated, extracted at 2 °C then subsequently at 24 °C and glycoproteins I (peak 1) isolated by gel filtration. Glycoprotein I extracted at 2 °C contained 5 % carbohydrate and 70 % protein, whereas the material extracted at 24 °C contained 7 % carbohydrate and 51 % protein. The sugar composition (Table 7) was similar to other preparations of glycoprotein I. Methylation analysis (Table VIII) indicated the same glycosidic linkages as found in glycoproteins isolated from *P. vulgaris* var. Pinto.

Treatment of glycoprotein I extracted at 2 °C with xylanase produced reducing groups equivalent to 416 μg xylose (theoretical yield, 400 μg). GLC indicated that the hydrolyzate contained xylose and a smaller quantity of arabinose.

Table 7. Sugar composition (%) of glycoproteins I from *P. vulgaris* var. Black Valentine.

Sugars	2 °C[a]	24 °C[a]
Arabinose	41	26
Xylose	31	17
Mannose	13	22
Galactose	6	7
Glucose	9	28

[a] Temperature of extraction.

Table 8. Methylation analysis[a] of glycoproteins I from *P. vulgaris* var. Black Valentine.

Sugars	Molar proportions (%) 2 °C	24 °C
2,3,5-Tri-*O*-Me-Ara	11	7
2,3,4-Tri-*O*-Me-Xyl	5	14
3,5-Di-*O*-Me-Ara	6	–
2,5-Di-*O*-Me-Ara	11	13
2,3-Di-*O*-Me-Ara	8	13
2,3-Di-*O*-Me-Xyl	53	16
2,3,6-Tri-*O*-Me-Glc	–	13

[a] See Tables 4 and 6 for abbreviations and Tg values.

DISCUSSION

Following mechanical introduction of plant viruses, glycoproteins have been isolated and characterized [4] and their production studied.[5] The aim of the present work was to characterize glycoproteins produced when virus was omitted but leaves were brushed with Celite in the normal manner (sham-inoculation). During this investigation it became apparent that differences in glycoprotein composition occurred. To determine the cause of this variability, the effect of extraction temperature was investigated as well as duplicate samples from sham-inoculated plants and triplicate samples from TNV-inoculated plants. In addition, two varieties, Pinto and Black Valentine, were used for the sham-inoculation experiments.

Glycoproteins extracted at 2 °C from *P. vulgaris* var. Pinto after sham- or TNV-inoculation had about 14 % carbohydrate whereas material extracted at 24 °C contained less carbohydrate and consequently was not investigated further. Extraction at 2 °C and 24 °C of *P. vulgaris* var. Black Valentine after sham-inoculation, produced glycoprotein preparations having lower carbohydrate content than similar preparations from the Pinto variety. The low protein content of material extracted from Black Valentine at 24 °C indicated that more impurities were extracted at the higher temperature. The sugar composition of glycoproteins extracted after sham-inoculation was approximately the same regardless of which variety was used. Glycoproteins extracted after TNV inoculation had a more variable sugar composition which may be caused by the disruption of cellular processes by the virus following mechanical inoculation. Generally, the presence of virus caused a decreased content of xylose with a corresponding increase in

glucose. The amino acid composition of glycoproteins from *P. vulgaris* var. Pinto were similar to each other, to other analyses,[4] to glycoproteins from *P. vulgaris* var. Black Valentine,[30] and except for the low content of hydroxyproline to plant cell wall glycoproteins.[11] Methylation analysis of glycoproteins from Pinto after TNV-inoculation and both varieties after sham-inoculation indicated that the same glycosidic linkages were present in all preparations. Investigation of glycoproteins rich in glucose indicated that this sugar occurred as either terminal or (1→4)-linked residues. The ratio of tetra to tri-*O*-methylglucose indicated that glucose probably occurred as low molecular weight oligosaccharides. Perhaps this low degree of polymerization accounts for the relative ease with which these glycoproteins are fully methylated in contrast to preparations rich in xylose. Xylose was invariably (1→4)-linked whereas arabinose occurred as (1→2)- and (1→3)-linked arabinofuranose units.

Methylation analysis of glycoproteins extracted at 2 °C from *P. vulgaris* var. Black Valentine indicated the same glycosidic linkages as found in Pinto except for the absence of terminal glucose units and the presence of terminal xylose units. Extraction at 24 °C provided material having similar linkages except for the absence of (1→2)-linked arabinofuranose units and the presence of (1→4)-linked glucose. No evidence of incomplete methylation was found in either preparation. Xylose was produced when material extracted at 2 °C was treated with xylanase indicating that β-(1→4)-D-xylopyranose linkages were present.

Treatment of the glycoproteins with pronase hydrolyzed sufficient peptide bonds so that subsequent gel filtration removed about 90 % of the amino acid residues. Amino acid analysis revealed substantial enrichment of hydroxyproline in the carbohydrate containing fractions and implicated this amino acid in carbohydrate-protein linkage. Enrichment of an unidentified acidic component also occurred and the structural significance of this compound remains to be determined.

Hydrazinolysis allowed removal by gel filtration of more of the amino acid residues not attached to carbohydrate and there is considerable evidence[7] which indicates that few, if any, glycosidic linkages are cleaved by this procedure. Nevertheless, some low molecular weight carbohydrate components were lost during gel filtration. Although only results of hydrazinolysis of glycoprotein rich in xylose are presented, the same procedure was applied to glucose-rich glycoproteins; however, the low recovery caused by incomplete separation of carbohydrate from amino acid hydrazides during gel chromatography did not permit further structural studies.

Methylation analysis of the products of hydrazinolysis indicated the presence of the same linkages as found in the original glycoprotein, but in addition, (1→5)-linked arabinofuranose units were found. During methylation analysis of native glycoproteins, GLC revealed many peaks which mass spectrometry demonstrated were not sugars. GLC of the methylated product of hydrazinolysis detected only sugars and all peaks were identified. In addition, there was no evidence of incomplete methylation as occurred using native glycoproteins from Pinto.

The two fractions isolated after hydrazinolysis of Pinto glycoproteins had an average degree of polymerization based on the results of methylation analysis of about 2 for the fraction eluted first and 4 for the fraction eluted second. Furthermore, molecular weight determination by gel filtration indicated a very much higher degree of polymerization than did the methylation analysis. This result suggests that the apparent degree of polymerization is an artifact of hydrazinolysis. Indeed, Heath and Northcote[7] have shown that adjacent hydroxyproline residues in protein will dimerize during hydrazinolysis. However, the cyclic dimerization product was not detected in the hydrolyzates of the products of hydrazinolysis nor was hydroxyproline hydrazide. This discrepancy must involve arabinofuranose linkages since mild acid hydrolysis caused a ten-fold decrease in degree of polymerization as indicated by $\overline{M}_n$.

Smith degradation of the products of hydrazinolysis indicated that terminal arabinose units are linked to O-2 of adjacent residues whereas arabinose units closer to the reducing end are (1→3)-linked. Although the structural significance of the (1→5)-linked arabinose

units is difficult to evaluate, it is probably that (1→4)-linked xylose units are on the perifery and that these chains can be quite long. The low optical rotation of the products before Smith degradation is consistent with β-(1→4)-linked D-xylose units and α-linked L arabinofuranose units. Other studies have demonstrated conclusively the presence of L-arabinofuranose [2,8] in plant derived material which is α-linked. [2,8,31]

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