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PROTEIN–POLYSACCHARIDE LINKAGES IN GLYCOPROTEINS FROM *PHASEOLUS VULGARIS*

ROBERT G. BROWN and WARWICK C. KIMMINS
Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada

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Abstract—Serine and hydroxyproline participate in protein–polysaccharide linkages in hydroxyproline–poor glycoproteins from *Phaseolus vulgaris* cv Pinto. Most substituted hydroxyproline residues contain arabinose, galactose and glucose, but some have arabinose only. Serine residues contain arabinose, galactose and glucose.

INTRODUCTION

Following virus inoculation, glycoproteins which contain glucose as the major carbohydrate constituent can be extracted from primary leaves of *Phaseolus vulgaris* [1, 2]. Although the production of glycoproteins is initiated by wounding of leaves during virus inoculation, this response can be suppressed by a virus which causes systemic infection [3]. If virus is omitted and leaves are wounded only, a glycoprotein which contains xylose as the main carbohydrate constituent is produced [4]. Similar glycoproteins have been isolated from the cell wall of *Phaseolus coccineus* and termed 'hydroxyproline–poor glycoprotein' [5]. These glycoproteins have been studied by methylation analysis [2, 4], but information on protein–carbohydrate linkages is lacking and this is the subject of this report.

Table 1. Amino acid composition of glycoproteins extracted at two temperatures in the presence of sodium sulphite (^{35}S)

		Mol (%)	
Amino acid	Temperature	2°	50°
Cysteic acid		0	1
Hydroxyproline		2	1
Aspartic acid		7	5
Threonine		5	5
Serine		7	7
Glutamic acid		10	10
Proline		7	5
Glycine		11	8
Alanine		11	13
Valine		6	8
Methionine		1	1
Isoleucine		5	5
Leucine		7	9
Tyrosine		4	4
Phenylalanine		4	5
Tryptophan		1	1
Lysine		7	5
Histidine		1	2
Arginine		4	7

Table 2. Labelling (^{35}S) and sugar composition of glycoproteins extracted at two temperatures

	Sugars (%)			
Temperature	Arabinose	Galactose	Glucose	Radioactivity (dpm/mg)
2°	69	15	17	23
50°	46	50	4	92

RESULTS

Effect of extraction temperature

Alkali extraction at 2 and 50° in the presence of sodium sulfite resulted in the isolation of glycoproteins which had similar amino acid compositions (Table 1). Cysteic acid was detected only in material extracted at the higher temperature but ^{35}S labelling of glycoprotein extracted at 2° indicated that some carbohydrate was lost even at this temperature (Table 2). Sugar analysis suggested that retention of arabinose and glucose was favoured by the lower extraction temperature.

Purification of glycoproteins

Carbohydrate analysis of 3 glycoprotein preparations revealed that arabinose and glucose were the main components and that the proportion of these two sugars and the carbohydrate content was variable (Table 3). These preparations were pooled and rechromatographed using

Table 3. Carbohydrate composition of glycoproteins from sham-inoculated primary leaves

	Sugars (%)			
Preparation number	Arabinose	Galactose	Glucose	Carbohydrate content (%)
1	66	8	26	13
2	57	tr	43	14
3	18	13	69	7

Table 4 Amino acid composition of glycoproteins and fractions obtained by treatment with alkali and hydrazinolysis

Amino acid (mol. %)	Sephadex G-200		Alkali treated		Hydrazinolysate	
	Peak 1	Peak 2	Fraction 1	Fraction 3	Fraction 1	Fraction 2
Cysteic acid	—	—	2	1	—	—
Hydroxyproline	6	—	5	—	52	44
Aspartic acid	6	6	6	8	—	1
Threonine	5	5	4	5	—	2
Serine	7	5	9	17	—	2
Glutamic acid	9	12	11	9	—	4
Proline	5	6	7	4	—	5
Glycine	8	12	10	14	—	2
Alanine	11	13	10	8	—	3
Valine	7	7	7	4	—	6
Methionine	1	2	1	1	—	2
Isoleucine	4	4	4	3	—	4
Leucine	10	8	7	4	8	3
Tyrosine	2	2	1	1	14	5
Phenylalanine	3	3	2	4	11	3
Lysine	8	8	7	3	—	—
Tryptophan	3	1	4	9	—	2
Histidine	2	2	2	2	14	10
Arginine	4	4	3	4	—	2

Sephadex G-200 (Fig. 1). Amino acid and carbohydrate analysis of the two peaks obtained indicated that the lower MW peak contained less hydroxyproline and more glucose than the higher MW peak (Tables 4 and 5). Uronic acids could not be detected in either fraction and both fractions were homogeneous by disc gel electrophoresis in agreement with previous results [1, 4].

Treatment with alkali and hydrazinolysis

Treatment of the higher MW material (Peak 1) with alkali containing sodium sulphite at 50° released carbohydrate by a β-elimination reaction and produced two protein fractions, 1 and 3 (Fig. 2). Following β-elimination of carbohydrate side chains, sodium sulphite converts the olefinic derivative of serine to cysteic acid [6]. Amino acid analysis of these fractions indicated the presence of cysteic acid in both fractions, whereas, hydroxyproline was only found in the higher MW fraction (Table 4).

Sugar analysis indicated that fractions 1 and 2 had similar sugar compositions (Table 5). The lower MW fraction contained mainly glucose with smaller quantities of galactose and arabinose indicating that all three sugars had been attached to the glycoprotein through serine.

Hydrazinolysis followed by gel filtration produced two carbohydrate containing fractions (Fig. 3). Sugar analysis of the higher MW fraction indicated the presence of mainly glucose with small quantities of arabinose and galactose. The lower MW fraction, on the other hand, contained only arabinose (Table 5). Amino acid analysis indicated that both fractions were substantially enriched in hydroxyproline (Table 4).

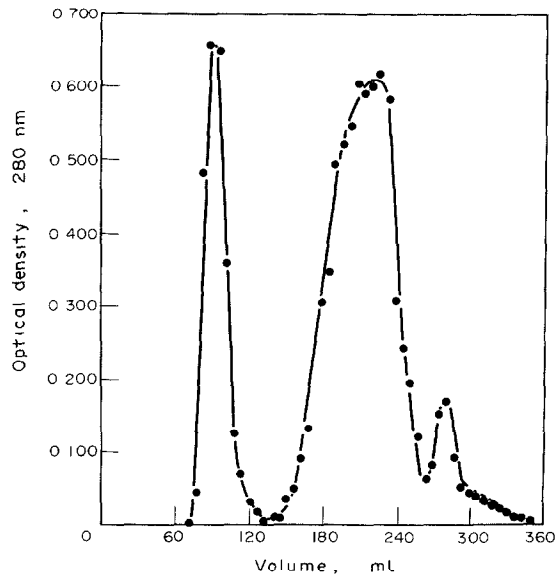

Fig. 1. Absorption of fractions obtained by column chromatography using Sephadex G-200. Peak 1, 78–120 ml; peak 2, 126–264 ml. Yields: 292 mg placed on column; peak 1, 80 mg; peak 2, 193 mg.

Table 5. Carbohydrate composition of glycoproteins and fractions obtained by treatment with alkali and hydrazinolysis

Sample	Sugars (%)			
	Arabinose	Mannose	Galactose	Glucose
Sephadex G-200				
Peak 1	52	3	14	31
Peak 2	10	3	11	76
Alkali-treated				
Fraction 1	8	—	12	80
Fraction 2	8	—	25	67
Hydrazinolysate				
Fraction 1	12	—	8	80
Fraction 2	100	—	—	—

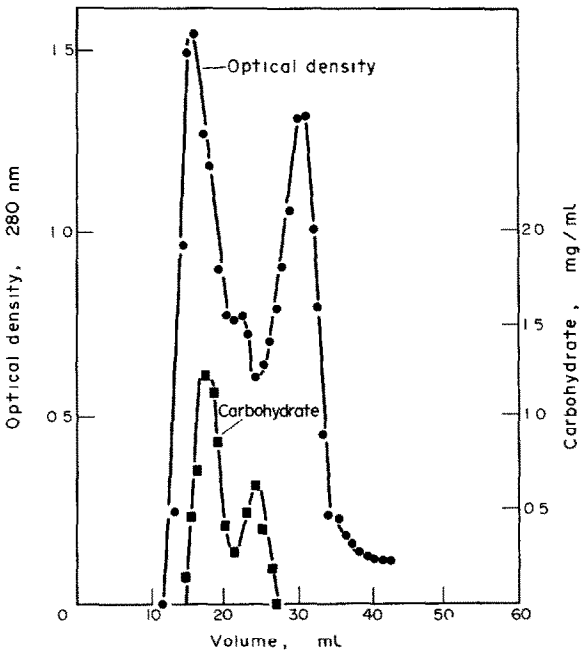

Fig. 2. Absorption and carbohydrate content of fractions obtained by gel filtration using Sephadex G-200 of alkali–sodium sulphite treated glycoprotein. Fraction 1, 11–21 ml; fraction 2, 22–27 ml; fraction 3, 28–34 ml.

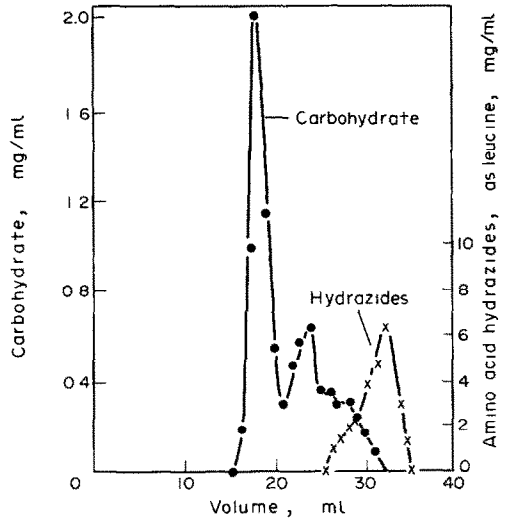

Fig. 3. Carbohydrate and amino acid hydrazide content of fractions obtained by gel filtration of the hydrazinolysate of peak 1 using Sephadex G-25. Fraction 1, 16–21 ml; fraction 2, 22–25 ml.

Purification of glycoproteins using Sepharose-6BCL

Use of Sepharose in place of Sephadex G-200 to purify glycoprotein preparations resulted in the isolation of two soluble peaks as obtained with Sephadex G-200 but, in addition, a Sepharose-insoluble fraction was isolated. On contact with the Sepharose gel, most of the glycoprotein lost its solubility and formed a band on the top of the column. The top few mm of the column gel were collected at the end of the elution period and as much of the Sepharose as possible removed by differential centrifugation. The carbohydrate and amino acid analysis of these 3 fractions were similar to each other (Tables 6 and 7). Treatment with alkali–sodium sulphite (^{35}S) followed by gel filtration using Sepharose-6BCL produced two protein peaks from each Sepharose-soluble glycoprotein and solubilised one protein fraction from the Sepharose-insoluble material. The lower MW fractions had more cysteic acid than the high MW material but the fraction solubilised from Sepharose-insoluble material had the most cysteic acid (Table 7). This was confirmed by ^{35}S labelling (Table 8). Treatment of peak 2 with alkali–sodium borohydride produced 3 carbohydrate fractions. Analysis of the two fractions which had a lower MW than the starting material indicated similar sugar compositions and confirmed that arabinose, galactose and glucose were linked to serine (Table 6).

DISCUSSION

Use of alkali for extraction of glycoproteins has the potential disadvantage that carbohydrate consituents attached to serine or threonine may be lost by β-elimination reactions. In the present study, labelling with ^{35}S indicates that about 25% of carbohydrate side chains attached to serine are lost during alkali extraction. Amino acid analysis i.e. the cysteic acid content, suggests that the loss may be less than 25%.

Knowledge that both hydroxyproline and serine are involved in protein–polysaccharide linkage poses the question, of which carbohydrates moieties are attached to serine and which are attached to hydroxyproline? Alkali treatment to remove carbohydrate attached to serine demonstrated that arabinose, galactose and glucose are attached to this amino acid. Addition of sodium borohydride to prevent base degradation following base elimination yielded the same result. Hydroxyproline residues also contain these 3 sugars but some hydroxyproline residues contain only arabinose. The sugar composition of the high MW fractions following alkali treatment and hydrazinolysis is similar. Both fractions comprise sugars attached to hydroxyproline.

Previous studies [1, 4] have indicated that arabinose occurs in the furanose form and may be (1 → 2), (1 → 3), or (1 → 5)-linked; whereas, glucose occurs as β-(1 → 4)-

Table 6. Carbohydrate composition of glycoprotein fractions purified by gel filtration using Sepharose-6BCL and products obtained by treating peak 2 with alkali–sodium borohydride

	Sugars (%)		
Sample	Arabinose	Galactose	Glucose
Peak 1	16	6	78
Peak 2	27	10	63
Sepharose-insoluble	9 (5)	* (50)	91 (45)
Peak 2			
Fraction 2	19	25	56
Fraction 3	9	48	48

*Galactose present was assumed to have been derived from Sepharose and therefore omitted. Figures in parantheses give actual sugar analysis.

Table 7. Amino acid composition of glycoproteins obtained by gel filtration using Sepharose-6BCL and fractions produced by alkali treatment of these glycoproteins

Amino acid (Mol %)	Peak 1			Peak 2			Sepharose-insoluble	
	Native	*Alkali-treated*		Native	*Alkali-treated*		Native	*Alkali-treated*
		1*	2		1	2		
Cysteic acid	—	—	3	—	—	2	—	15
Hydroxyproline	2	2	2	2	—	3	2	5
Aspartic acid	6	9	8	7	8	7	8	8
Threonine	5	5	4	5	5	7	5	3
Serine	7	7	5	7	5	6	6	6
Glutamic acid	8	8	8	9	12	10	9	8
Proline	6	6	6	6	5	5	7	7
Glycine	10	11	12	11	12	12	11	8
Alanine	11	8	12	8	12	10	11	7
Valine	7	6	8	5	6	6	7	5
Methionine	1	2	2	1	1	—	2	1
Isoleucine	6	5	4	5	4	5	5	4
Leucine	12	7	6	10	10	11	9	6
Tyrosine	4	3	3	4	2	2	2	3
Phenylalanine	4	4	3	4	3	4	5	3
Lysine	8	8	4	7	5	7	5	5
Tryptophan	1	—	6	2	2	2	2	2
Histidine	1	2	2	2	3	2	1	1
Arginine	4	5	5	6	6	4	4	3

*1 and 2 refer to the high and low MW fractions respectively.

linked residues forming chains of low MW. This study indicates that galactose is attached to the glycoprotein mainly through serine. Although this sugar is a minor component, it appears to be a legitimate part of the glyco-moiety. No information about its linkage is available. The low arabinose content of the two fractions obtained following alkali treatment, in contrast to the high arabinose content of the starting material, indicates that many serine residues have arabinose side chains of low MW. These arabinose units were probably not eluted during gel filtration after the alkali treatment.

The production of two protein peaks by alkali treatment suggests the presence of at least two glycoproteins. One, having most of its carbohydrate linked through serine, so that loss of carbohydrate effectively reduces the MW of the protein moiety. The other, having most carbohydrate linked through hydroxyproline, is unaffected by alkali treatment. The glycoproteins have only 10–15% carbohydrate, therefore, this explanation is only feasible if the carbohydrate side chains cause aggregation of the glycoproteins. This receives some support from the study of Sepharose-insoluble glycoproteins. Sepharose appears to selectively precipitate those glycoproteins which contain most of their carbohydrate moieties linked through serine. This is supported by ^{35}S labelling and the high cysteic acid content of the solubilized protein following alkali–sodium sulphite (^{35}S) treatment of Sepharose-insoluble glycoproteins. Presumably Sepharose would selectively precipitate the high MW aggregates.

Table 8. Radioactive (^{35}S) labelling of cysteic acid after alkali treatment

Fraction	Radioactivity present in cysteic acid (dpm/mg glycoprotein)
Peak 1:	
Fraction 1	4470
Fraction 2	57500
Peak 2:	
Fraction 1	10100
Fraction 2	86000
Sepharose-insoluble (solubilized by alkali treatment)	292000

Previous studies have demonstrated that following virus inoculation glycoproteins enriched in glucose [1, 4] were produced; whereas, wounding without virus resulted in the production of glycoproteins enriched in xylose [4]. In the present study, primary leaves were wounded in the absence of virus, yet glycoproteins enriched in glucose were obtained. Investigations have demonstrated that many variables affect the type of glycoprotein obtained and this will be the subject of another publication.

EXPERIMENTAL

Production and purification of glycoproteins. *P. vulgaris* cv Pinto was sham-inoculated as outlined in refs [1, 2]. The plants were grown for 20 days in sand on a 12–12 hr light regime in a growth chamber. Primary leaves were harvested 36 hr after inoculation and glycoproteins extracted and purified as previously described. The number of plants which were convenient to work with resulted in yields of 55–100 mg after purification by gel filtration using Sephadex G-150. Three batches were pooled to give *ca* 200 mg material which was rechromatographed on either Sephadex G-200 or Sepharose-6BCL using a column (2.5 × 50 cm) developed with Pi buffer (0.02 M, pH 9) at a flow rate of 6 ml/hr. Fractions (6 ml) were collected and A at 280 nm measured.

Effect of extraction temperature. To measure the loss of carbohydrate attached to serine during extraction, primary leaves were harvested and each pair divided. One lot was extracted by alkali containing M Na_2SO_3 (^{35}S; 1000 dpm/mg) at 2° while the other lot was extracted at 50°. The extraction time was *ca* 30 min. Once extracted the glycoproteins were purified as described above; after chromatography using Sephadex G-150, the prepns were analysed.

Alkali treatment. Samples were dissolved in NaOH (0.2 M, 20 mg/ml) and incubated at 50° for 5 hr. Na_2SO_3 (^{35}S) was added (5×10^5 dpm/mg) at zero time. After 1 hr, Na_2SO_3 in 4 equal aliquots was added at 1 hr intervals to give a final concn of 1 M. Following alkali treatment, samples were neutralized and chromatographed on Sephadex G-200 or Sepharose-6BCL (1×50 cm) developed with Pi buffer (0.02 M, pH 9) at a flow rate of 1 ml/hr. The gel used was the same as that used to purify the material being treated. Untreated glycoproteins were used to measure the elution vol. of the material before alkali treatment. Where indicated, $NaBH_4$ was added in place of Na_2SO_3.

Hydrazinolysis. The procedure used was as in ref. [4]. The hydrazinolysate was chromatographed on Sephadex G-25 (1×30 cm) developed with H_2O at a flow rate of 1 ml/hr.

Radioactivity determination. was measured by scintillation counting. After hydrolysis samples were separated by PC (EtOH–HOAc–H_2O, 65:1:34) and the radioactivity of material with the same R_f as cysteic acid determined. With most samples, all radioactivity corresponded to cysteic acid; however, some samples had some radioactivity at the origin or with a higher R_f than cysteic acid. This did not exceed 25% of the total radioactivity.

Chemical analysis. Sugars were analysed by GLC [7] and amino acids were determined with an amino acid analyser by the method of ref. [8]. Total carbohydrate was measured with anthrone [9].

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