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SERINE-*O*-GALACTOSYL LINKAGES IN GLYCOPEPTIDES FROM CARROT CELL WALLS*†

YONG-PILL CHO and MAARTEN J. CHRISPEELS‡
Department of Biology, John Muir College, University of California, San Diego, La Jolla, CA 92093, U.S.A.

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Key Word Index—*Daucus carota*; Umbelliferae; carrot; cell wall protein; extensin; glycoprotein; serine-*O*-galactose.

Abstract—Cell walls obtained from carrot disks aged for 6 days were treated with mild acid to remove the arabinosyl sidechains from the hydroxyproline residues of extensin, and subsequently digested with trypsin. The peptides in the tryptic digest were fractionated according to MW by gel filtration and further purified with Dowex 50X2. The peptides were rich in hydroxyproline and contained small amounts of carbohydrate, especially galactose. Treatment of the glycopeptides with NaOH in the presence of Na_2SO_3 resulted in a considerable loss of serine residues (up to half in some fractions) and the formation of cysteic acid. Free carbohydrate, consisting mostly of galactose, was released by this treatment. Treatment with NaOH in the presence of $NaBH_4$ resulted in the release of carbohydrate sidechains which primarily contained galactitol and galactose. The data indicate that the serine-*O*-galactosyl linkage occurs in glycopeptides of different sizes and is most abundant in the hydroxyproline-rich glycopeptide fractions.

INTRODUCTION

The primary walls of plant cells contain the hydroxyproline-rich glycoprotein extensin. Work in our laboratory has shown that this glycoprotein is synthesized in the cytoplasm and subsequently secreted into the cell wall where it becomes bound to the hemicellulose cell wall matrix [1, 2]. Lamport [3, 4] has advanced the hypothesis that extensin is involved in the control of cell wall extension through its involvement in a protein–glycan network. We have obtained evidence which supports this hypothesis and which suggests that there is a causal relationship between excessive extensin deposition in the wall of pea stems and the cessation of elongation [5]. It is not known however how extensin crosslinks the cell wall macromolecules to regulate cell extension. The glycoprotein contains numerous hydroxyproline-*O*-arabinoside linkages which give rise to short oligo-arabinoside sidechains [6]. These sidechains are known to be formed in the cytoplasm before the protein is secreted [7,8] and they are thought not to be involved in cross-linking cell wall macromolecules [4, 9, 10, 11]. Recently Lamport *et al.* [12] reported the existence of a serine-*O*-galactosyl linkage in a glycopeptide isolated from tomato cell wall. It has been postulated [4, 17] that this linkage may be involved in cross-linking other cell wall macromolecules but there is as yet no evidence for this hypothesis. As a preliminary to investigating the biosynthesis of this linkage we wanted to determine whether the serine-*O*-galactosyl linkage also occurs in other plant species, and whether it occurs abundantly in the extensin molecule. For this study we used aged carrot disks, which synthesize large amounts of extensin [13] and are a convenient system to study the biosynthesis and the secretion of this glycoprotein. The evidence presented here is the first confirmation of the discovery by Lamport *et al.* [12] of this new glycopeptide linkage. Furthermore, the serine-*O*-galactosyl linkage appears to be of widespread occurrence in the glycopeptides obtained by enzymatic digestion of the cell walls.

RESULTS AND DISCUSSION

Isolation and characterization of cell wall glycopeptides

Purified cell walls were first given a mild acid treatment, and then digested with trypsin in order to obtain peptides from the cell wall. The effect of these treatments on the solubilization of bound sugars and amino acids was followed by measuring the release of ^{14}C-arabinose and ^{14}C-proline and ^{14}C-hydroxyproline from the walls of carrot disks which had been allowed to incorporate ^{14}C-arabinose and ^{14}C-proline. The results showed that the mild acid hydrolysis (pH 1·4, 90° for 90 min) released more than 80% of the bound ^{14}C-arabinose and about 30% of the ^{14}C-proline plus hydroxyproline. The subsequent trypsin digestion released 10% of the ^{14}C-arabinose and 40% of the ^{14}C-proline plus hydroxyproline, while the remaining radioactivity remained in the residue. Similar results were obtained when the hydroxyproline content of the three fractions was determined chemically. Thus, the trypsin-solubilized peptides represent about 40% of the total hydroxyproline-rich cell wall protein. The peptides were then fractionated according to

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‡ To whom requests for reprints should be addressed.

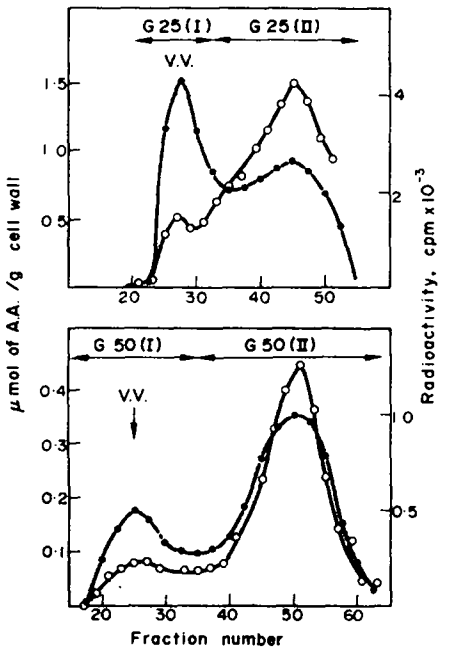

Fig. 1. Chromatography of cell wall peptides on Sephadex G-25 (top) and G-50 (bottom). Profiles of radioactivity in ^{14}C-proline and hydroxyproline (●—●) and ninhydrin-reactive material (○···○).

MW by gel filtration, first on Sephadex G-25 and then on Sephadex G-50. The results of such a fractionation are shown in Fig. 1 which contains both radioactivity profiles (^{14}C-proline plus hydroxyproline) as well as ninhydrin profiles. It is clear from the fractionation on Sephadex G-25 that the tryptic digest contained peptides of different sizes: some larger ones which were excluded by G-25 and will be referred to as G-25(I), and a majority of smaller peptides which will be referred to as G-25(II). Since more than 70% of the combined ^{14}C-proline plus hydroxyproline is in hydroxyproline it appears that the larger peptides contained more hydroxyproline. The G-25(I) fraction was further fractionated on Sephadex G-50 into two fractions: one containing the largest peptides, called G-50(I), and one containing the smaller ones, called G-50(II). Assays for carbohydrate showed that a considerable amount of carbohydrate material eluted with the void volume in both the G-25 and the G-50 columns (data not shown). All the fractions were therefore passed through a Dowex 50X2 column to remove the free carbohydrates. More than 90% of the peptide material was retained by the resin, and after the column had been thoroughly washed with 1 mM pyridine formate pH 3·3 the peptides were eluted with 0·75 M of the same buffer. The 4 peptide fractions differed considerably in their hydroxyproline content as shown in Table 1. Invariably, the larger glycopeptides contained more hydroxyproline. The glycopeptide nature of the eluted glycopeptides was indicated by the presence of bound carbohydrate ranging from 2% (mol of sugar per mol of amino acid) in G-25(II) to 40% in G-50(I). The larger hydroxyproline-rich glycopeptides were also richest in carbohydrate. An analysis of the sugar composition of the glycopeptides showed that arabinose was the major neutral sugar (up to 70% of the total neutral sugars) and that minor amounts of galactose, glucose and mannose were present. Subsequently, slightly more stringent conditions for the acid hydrolysis step prior to trypsin digestion (pH 1 for 60 min at 100°) were used. This eliminated most of the arabinose from the various glycopeptide fractions. The slightly more acidic conditions had only a small effect on the Sephadex G-25 and G-50 profiles, generally in the direction of a reduction in the size of the glycopeptides. In all the subsequent experiments described here the more acidic conditions of hydrolysis were used. Typical carbohydrate to protein ratios and neutral sugars compositions of the glycopeptide fractions obtained in this way are also shown in Table 1. Galactose is by far the most abundant sugar in all the fractions, although the larger glycopeptides still contained significant quantities of arabinose. Minor and variable amounts of mannose and glucose were also present.

Characterization of glycopeptide linkages

To find out whether serine and/or threonine residues were involved in the attachment of the carbohydrate the glycopeptide fractions were treated with dilute alkali in the presence of sodium sulfite. Glycosylated serine and threonine residues are destroyed in this procedure and converted respectively to cysteic acid and 2-amino-3-sulfonylbutyric acid [14, 15]. The serine, threonine and cysteic acid* content of the glycopeptides determined before and after NaOH–$NaSO_3$ treatment of the glycopeptides is shown in Table 2. The results show that there is considerable destruction of serine residues (up to 50%) and less disappearance of threonine residues (up to 20%). Serine destruction is greatest in those glycopeptides which are richest in serine and hydroxyproline. Further treatment of the G-50(II) fraction with pronase and fractionation of the digest on Sephadex G-15 resulted in a glycopeptide fraction which contained 45% hydroxyproline and 18% serine, with 60% of the serine residues being destroyed by the alkaline treatment. Treatment with NaOH–Na_2SO_3 did not result in a stochiometric conversion of serine and threonine to cysteic acid plus 2-amino-3-sulfonyl butyric acid, as measured with the amino acid analyzer; this lack of stochiometry has also been observed by other workers [12, 16]. Control experiments with proteins without sugar residues (e.g. bovine serum albumin and glucagon) showed that 8–12% of the threonine residues and 15–20% of the serine residues were destroyed if the proteins were treated with NaOH–Na_2SO_3 under the conditions used here. These results suggest that there is non-specific destruction of these residues unrelated to the alkaline hydrolysis of the glycopeptide linkages. The observed destruction of threonine is well within the range of the non-specific destruction caused by the alkaline treatment of proteins. It can therefore not be concluded that threonine is involved in glycopeptide linkages. The observed destruction of serine, especially in the G-50(I) and G-50(II) fractions is considerably greater than the non-specific destruction of serine suggesting that serine may be involved in a glycoprotein linkage.

To confirm that cysteic acid was indeed formed during the NaOH–Na_2SO_3 treatment, ^{35}S-labelled Na_2SO_3 was used. The resulting labelled peptide was desalted by gel filtration on Sephadex G-25, hydrolyzed with 6N HCl and the products of hydrolysis were separated by PC.

* Cysteic acid, 2-amino-3-sulfonylbutyric acid were not separated on the amino acid analyzer.

Table 1. Hydroxyproline, carbohydrate content and sugar composition of the different glycopeptide fractions

	G-25(I)	G-25(II)	G-50(I)	G-50(II)
Hydroxyproline*	26·1	6·6	29·4	24·6
mol of amino acids	1	1	1	1
mol of carbohydrate	0·156	0·016	0·588	0·133
Araginose†	2·0	3·4	29·4	6·0
Mannose	7·4	6·0	11·1	10·7
Galactose	84·6	82·9	49·2	75·8
Glucose	6·0	7·7	10·3	7·6

* Hydroxyproline values are mol% of the neutral and acidic amino acids. † Sugar composition is in mol% of the neutral sugars.

Only one major radioactive spot was present and it co-chromatographed with cysteic acid, but not with homocysteic acid, a close relative of 2-amino-3-sulfonyl butyric acid [15]. Similar results were obtained with a second chromatographic system. In control experiments an equivalent amount of glucagon (2 mg) was treated in the same way. Again a radioactive compound which cochromatographed with cysteic acid was formed, but it had only 12% as much radioactivity as was present in an equivalent amount of a G-50(II)-treated sample. This also suggests that some non-specific destruction of serine on addition of sulfite can occur under these conditions.

The carbohydrate released during the alkaline hydrolysis was separated from the peptides and glycopeptides by another passage through a Dowex 50X2 column. The released carbohydrate constituted 55% of the total carbohydrate of the G-25(I) glycopeptide fraction. The neutral sugar composition of the total glycopeptide fraction was 4·6% arabinose, 11·7% mannose, 77·7% galactose and 6·0% glucose, while the carbohydrate released by the alkaline treatment had the following composition: 5·6% mannose, 82·0% galactose and 12·4% glucose. Galactose was the most abundant sugar in the carbohydrate released by alkaline treatment of the glycopeptides.

The identity of the sugars involved in the carbohydrate protein was examined by treating the glycopeptides from the G-25(I) and G-25(II) fractions with NaOH in the presence of $NaBH_4$. This has been shown to result in the reduction of the reaction products after the base catalyzed hydrolysis has occurred. The serine residues are converted to alanine residues and the sugars involved in the linkage are converted to their corresponding alditols [14]. After the glycopeptides had been treated with $NaOH–NaBH_4$, the released carbohydrates were separated from the remaining peptides and glycopeptides by passage over a Dowex 50X2 column; the released carbohydrates were then hydrolyzed and the resulting mixture of sugars and alditols directly acetylated. An analysis of the reaction products by gel chromatography showed that only one neutral sugaralditol, galactitol, had been formed during the reductive base-catalyzed hydrolysis of the glycopeptides. This suggests that galactose was the only neutral sugar involved in linking the released carbohydrates to the polypeptide chains. This was true for both the G-25(I) and the G-25(II) fractions. The total amount of galactose in the released carbohydrates was also determined in these same samples and the data showed that 65% of the total galactose was present at the reducing ends of the sidechains and was reduced to galactitol during the $NaOH–NaBH_4$ treatment. Since galactose was the main sugar these results suggest that the carbohydrate sidechains were quite short, mostly only one or two residues long. This was confirmed by fractionating the released carbohydrates on a Sephadex G-15 column which had been standardized with glucose, sucrose and raffinose. More than 80% of the released carbohydrate material eluted in the mono and disaccharide region of the column.

Table 2. Destruction of serine and threonine and formation of cysteic acid as a result of treating the glycopeptides with $NaOH–Na_2SO_3$

	G-50(I)	G-50(II)	G-25(II)
Hydroxyproline	33·7*	20·3	6·6
Serine	18·3	14·1	11·2
Threonine	6·8	8·2	7·8
ΔSerine	−9·2	−4·8	−0·4
ΔThreonine	−1·4	−1·4	−0·3
ΔCysteic acid†	+5·0	+2·7	+0·4

* All numbers indicate mol% of the neutral and acidic amino acids. † Cysteic acid (derived from serine) and 2-amino-3-sulfonyl butyric acid (derived from threonine) were not separated on the amino acid analyzer.

Calculations show that there is about a one to one relationship between the amount of serine destroyed due to hydrolysis of the glycopeptide linkage and the amount of galactitol formed. For example, G-25(I) contained 15·6 mol of carbohydrate per 100 mol of amino acid. Treatment of the glycopeptide with $NaOH–Na_2SO_3$ released 55% of the carbohydrate and resulted in the destruction of 8·2 mol% of serine out of a total of 18%. If we assume that 20% of the serine residues are destroyed non-specifically 8·6 mol of carbohydrate were released per 6·6 mol of glycosidically linked serine destroyed. Since 75% of the carbohydrate was galactose and 2/3 of the galactose could be reduced by the $NaOH–NaBH_4$ treatment it appears that 4·3 residues of galactitol were formed per 6·6 mol of serine destroyed.

Taken together our findings confirm and extend the original observation by Lamport [12] reporting the existence of a serine-*O*-galactosyl linkage in a glycopeptide isolated from tomato cell walls. The results reported here suggest that this linkage occurs in glycopeptides of various sizes, but is most abundant in the largest hydroxyproline-rich fractions. The data also indicate that no other neutral sugars are involved in glycopeptide linkages with serine (or threonine) residues. The carbohydrate side chains in the glycopeptides contain only one or two sugar residues. The simplest interpretation of these data is that extensin contains a variety of short oligosaccharide sidechains, some attached to hydroxyproline resi-

dues and some to serine residues. It is of course possible that these sidechains were longer prior to the mild acid treatment of the cell walls, and that extensin may be involved in crosslinking various matrix macromolecules as has been suggested by Lamport [3, 4] and by Albersheim *et al.* [11, 17] but our data do not lend support to such a hypothesis.

EXPERIMENTAL

Tissue and incubation. Small disks (1 mm, thick diam. 8 mm) of carrot root phloem parenchyma tissue were incubated at 30° with continuous agitation in H_2O containing 50 μg/ml of chloramphenicol. H_2O was changed once a day for 6 days. The disks were thoroughly washed with H_2O immediately after cutting and again just before homogenization. Normally lots of 500–800 g of tissue were incubated and processed at one time.

Preparation of cell walls. Usually 20 g batches of disks were homogenized in 200 ml of cold H_2O. The resulting homogenate was centrifuged at 1500 *g* for 3 min to sediment the walls. The walls were resuspended in H_2O and resedimented $\times 3$ to remove cytoplasmic contaminants. The walls were then washed 2 $\times$ with 0·2 M $CaCl_2$ to remove ionically bound proteins and again resuspended in H_2O. The washed walls were boiled for 30 min to solubilize pectic substances, sedimented and washed once more with H_2O. The walls were then treated with dil. HCl to hydrolyze pentosans and especially the arabinose sidechains of extensin. Removal of the sidechains renders the protein susceptible to proteolytic attack [1, 6]. Two different conditions were used for the mild acid hydrolysis: pH 1·4 for 90 min at 90° and pH 1 for 60 min at 100°. The acid treatment extracted not only pentoses but also a considerable proportion of the cell wall protein. The acid treated walls were washed again to remove the hydrolyzed material and resuspended in 1·5 l. of 100 mM tris buffer pH 8 containing 15 mM $CaCl_2$.

Extraction and fractionation of cell wall peptides. The resuspended acid-treated walls were incubated with trypsin (5 mg of trypsin per g of cell wall) in 100 mM tris pH 8 with 15 mM $CaCl_2$ for 24 hr at 37°. The residue was removed by centrifugation at 3000 *g* for 10 min and the solubilized material was boiled for 10 min and then lyophilized. The residue was dissolved in 10 mM pyridine–formate pH 3·3. The trypsin extract was fractionated by gel filtration on Sephadex G-25 and G-50. To separate the neutral carbohydrates from the peptides and glycopeptides the pooled fractions from the Sephadex columns were diluted $\times$ 10, absorbed on Dowex 50X2 (pyridine form) and the ion exchange columns thoroughly washed with 1 mM pyridine-formate pH 3·3. The peptides and glycopeptides were then eluted with 0·75 M pyridine–formate pH 3·3. The eluted peptide fraction was concentrated on a rotary evaporator at 40° and desalted by passage over a Sephadex G-25 column. The desalted material was used for further analyses.

Amino acid composition of glycopeptides. Peptides were hydrolyzed in 6N HCl for 16 hr at 110° in sealed vials; HCl was removed by rotary evaporation and the residue washed 2 $\times$ with H_2O. Residue was dissolved in Na citrate buffer and the amino acid composition (neutral and acidic amino acids only) of the sample determined with an automatic amino acid analyzer which was temp programmed to separate hydroxyproline from aspartic acid. Total amino acid content of the samples was determined with ninhydrin [18].

Carbohydrate content and sugar composition of glycopeptides. The glycopeptides were hydrolyzed with 2N TFA for 1 hr at 121° in sealed vials. The TFA was removed by rotary evaporation, and the residue was washed 2 $\times$ with H_2O and then dissolved in H_2O. Total carbohydrate content was measured with the phOH–H_2SO_4 method [19], using *glu* as a standard. To determine sugar composition of the samples the sugars were converted to their corresponding alditol acetates and then separated by GLC using 3% ECMSS-M on Gas-Chrom Q and a temp. program of 170°–210° (2° per min) after 8 min at 170° [17, 20, 21].

Cleavage of alkali-labile glycopeptide bonds (β-elimination). Glycopeptide samples were lyophilized and redissolved in 2 ml of 0·25 N NaOH, and incubated for 5 hr at 50°. The serine–galactose linkage is known to be hydrolyzed by these conditions [12]. In some expts M Na_2SO_3 was included to convert the glycosidically-linked serine and threonine to cysteic acid and 2-amino-3-sulfonyl butyric acid. In other expts 100 mM Na_2SO_3 and 400 μCi of Na_2SO_3 labelled with ^{35}S (sp. act. 0·5 mC/mg) were used to obtain the labelled derivatives. In further expts M $NaBH_4$ was included with the NaOH to reduce the sugar residues involved in the glycopeptide linkage and convert them to the corresponding alditols.

Determination of the amino acids involved in the glycopeptide linkage. The amino acid composition of the NaOH–$NaSO_3$ treated samples was compared with controls and the mol% decrease in serine and threonine and the increase in cysteic acid was calculated. Cysteic acid and 2-amino-3-sulfonyl butyric acid are not separated on the automatic amino acid analyzer. The NaOH–Na_2SO_3–$^{35}SO_3$ treated samples were first desalted on Sephadex G-25 to remove the contaminating $^{35}SO_3^{2-}$, then hydrolyzed with 6N HCl and the radioactive products separated by descending PC (phOH–H_2O 8:2) after running for 4½ days, or MeOH–H_2O–C_5H_5N, 80:20:3 run overnight. Chromatograms were sprayed with ninhydrin to locate the standards. Radioactivity was located by cutting the paper into 2·5 cm strips which were counted in a liquid scintillation counter.

Determination of the sugar involved in the glycopeptide linkage. Glycopeptides were treated with NaOH in the presence of $NaBH_4$ as described above, and the unreacted $NaBH_4$ decomposed by the dropwise addition of HOAc. Borate was removed by rotary evaporation as Me borate after resuspending the sample in MeOH. The material was then resuspended in 1 mM pyridine–formate pH 3·3 and applied to a Dowex 50X2 column. Carbohydrates which had been cleaved from the glycopeptides were not absorbed on the resin and were collected in the eluate. This material was lyophilized, hydrolyzed with TFA, and alditols converted to their corresponding alditol acetates. The step involving the reduction of the sugars *after* TFA hydrolysis was omitted from the usual procedure for the preparation of alditol acetates.

Labelling with ^{14}C-proline. Carrot disks (10 g) aged for 6 days were incubated with 20 μCi of ^{14}C-proline (New England Nuclear sp. act. 180 mCi/mmol) for 2 hr or with 20 μCi of ^{14}C-L-arabinose (New England Nuclear 9·8 mCi/mmol). The cell walls were isolated and fractionated as described above. There is extensive conversion of ^{14}C-proline to ^{14}C-hydroxyproline, and in the cell wall about 75% of the radioactivity is in ^{14}C-hydroxyproline. Thus the radioactivity profiles represent primarily ^{14}C-hydroxyproline.

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