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METHYLATION ANALYSIS OF CELL WALL GLYCOPROTEINS AND GLYCOPEPTIDES FROM *CHLAMYDOMONAS REINHARDII*

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Abstract—Cell wall glycoproteins from *Chlamydomonas reinhardii* and the glycopeptides produced by the action of thermolysin were subjected to standard methylation analysis. GC–MS of the methylated alditol acetates revealed short oligosaccharides some of which show branching. *O*-glycosidically linked galactofuranosyl residues are present. The asymmetric distribution of the major *O*-glycosidic linkages is also reported.

INTRODUCTION

Hydroxyproline containing glycoproteins in the cell walls of higher plants is well documented [1, 2]. However, structural information on these polymers is limited due to problems of isolating them in an unmodified form [3, 4]. In order to overcome these difficulties Lamport [5] has proposed using the lower algae as a source of intact cell wall glycoproteins. *Chlamydomonas reinhardii* possesses a cell wall with a hydroxyproline-rich glycoprotein as its main component [6, 7]. It resembles the cell wall glycoprotein of higher plants [8] but is readily solubilized in its native state [7, 9]. The major cell wall glycoprotein (2BII) is characterized by large amounts of hydroxyproline and the sugars arabinose, galactose and mannose [7]. It is also capable of reversible self-assembly into a crystalline lattice [8]. Enzymatic degradation of 2BII with the endopeptidase thermolysin has revealed the asymmetric distribution of hydroxyproline and structurally different domains of the glycoprotein [10]. Studies using circular dichroism have shown a thermolysin fragment (T2) to possess 85% polyproline II conformation conferring on it a rigid rod-like structure [11]. In this paper we report the use of standard methylation analysis to determine the major *O*-glycosidic linkages in *C. reinhardii* cell wall glycoproteins and glycopeptides.

RESULTS AND DISCUSSION

Cell wall glycoproteins

Extraction of *C. reinhardii* cell wall material (CWM) with 2M Na perchlorate solubilises *ca* 94% w/w of the material [7]. Fractionation of the soluble products on Sepharose 2B yields two major glycoproteins, 2BI and 2BII, present in the ratio 1:2.8. All three products (CWM, 2BI and 2BII) were subjected to methylation analysis. The IR spectrum of the methylated polymers showed no broad –OH absorption at *ca* 3400 cm^{-1} suggesting methylation to be essentially complete. Separation and qualitative analysis of the partially methylated alditol acetates (PMAA) was performed by GC–MS and the results are shown in Table 1. Quantitative analysis showed the overall recoveries to be in broad agreement with those obtained by direct analysis.

The predominant derivative found in CWM and 2BII was 3,5-Me_2 arabinitol. The presence of this and 2,3,5-Me_3 arabinitol suggests the occurrence of arabinofuranosyl residues. Virtually all of the arabinose can be accounted for by terminal end (1 → 2)-linked ara*f* units. The identification of a small amount of unmethylated arabinose indicates the occurrence of (1 → 5)-linked ara*f* with branch points through $C(O)_2$ and $C(O)_3$. This type of linkage is commonly found in arabinans of higher plants [12–14].

The bulk of the galactose occurred in CWM and 2BII; methylation analysis revealed the presence of several partially methylated galactitol acetates. In both preparations the occurrence of 2,3,5,6-Me_4- and 2,3,4,6,-Me_4 galactitol indicates the presence of both terminal galactofuranosyl and galactopyranosyl residues. The presence of terminal gal*f* residues was confirmed by reference to an authentic spectrum of 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methylhexitol [15]. The occurrence of gal*f* units is of interest as they have not been previously reported in plant glycoproteins or polysaccharides. However, they have been detected in a fungal glycopeptide [16]. In CWM the remaining galactose is accounted for by (1,3,6)-linked gal*f*, (1 → 3)- and (1 → 4)-linked gal*p*, (1 → 6)-linked gal*f* and (1,3,6)-linked gal*p*. Both fractions contained small amounts of (1 → 4)-linked glc*p*.

From the ratio of terminal to branch point residues (CWM 2.7:1, 2BII 2.1:1) it is clear that terminal residues are in excess. A possible explanation for the underestimation of branch points is the glycoprotein nature of the polymers. In these some of the 'branch points' will be represented by amino acids and are not included in the analysis. However, from these data it is possible to infer the presence of short oligosaccharides (DPN *ca* 4) with some branching. By direct analysis and assuming that all the sugar residues are attached to hydroxyproline, an average DPn of 6 is obtained. This

Table 1. Partially methylated alditol acetates from *C. reinhardii* cell wall material

PMAA	RR_t*		Proportions (mol%) of PMAA		
	a	b	CWM	2BI	2BII
2,3,5-Me_3Ara†	0.44	0.46	8.0 (38.5)‡	2.5 (16.9)	7.9 (45.9)
3,5-Me_2Ara	0.80	0.91	30.6	5.9	29.0
Arabinitol	2.66	3.73	2.2	2.5	1.1
2,3,4-Me_3Xyl	0.56	0.65	5.4 (2.9)	1.7 (2.4)	6.4 (4.6)
2,3,4,6-Me_4Man	0.98	1.00	9.8 (23.1)	11.7 (62.7)	7.4 (9.2)
3,4,6-Me_3Man	1.78	1.88	tr	—	1.6
2,4,6-Me_3Man	1.88	2.11	8.7	33.3	7.3
4,6-Me_2Man	3.35	3.32	5.7	24.7	—
2,3,5,6-Me_4Gal	1.08	1.14	9.8 (29.8)	3.1 (16.9)	11.1 (34.9)
2,3,4,6-Me_4Gal	1.17	1.23	4.0	1.4	4.3
2,4,6-Me_3Gal	2.04	2.24	4.0	8.1	—
2,3,6-Me_3Gal	2.20	2.37	2.2	1.6	2.4
2,3,5-Me_3Gal	2.80	3.34	1.7	—	1.0
4,6-Me_2Gal	4.35	3.57	tr	—	11.0
2,5-Me_2Gal	4.81	3.87	5.3	—	5.3
2,4-Me_2Gal	5.10	6.45	0.3	2.3	—
2,3,6-Me_3Glc	2.28	2.47	2.3 (6.6)	1.2 (0.9)	4.2 (5.4)

* R_t relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on (a) OV-225 at 180° and (b) ECNSS-M at 170°.

† 2,3,5-Me_3Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol etc.

‡ Values in parentheses obtained by direct sugar analysis using GC.

may indicate the presence of glycosidic linkages to another amino acid(s). *N*-Glycosidic linkages (involving asparagine and *N*-acetyl glucosamine) and *O*-glycosidic linkages (involving serine and galactose) are common in plant glycoproteins [2]. We have been unable to detect amino sugars in either preparation, therefore serinyl-*O*-glycosides may be present. Furthermore, Miller *et al.* [17] did not detect mannose in hydroxyprolylglycosides isolated from *C. reinhardii*. Unlike the cell wall glycoproteins of higher plants, *Chlamydomonas* glycoproteins contain arabinose, galactose and glucose [17]. Also methylation analysis has shown the absence of (1 → 3)-linked ara*f* residues in *C. reinhardii* which are present in higher plant hydroxyprolylarabinosides [4, 18].

The bulk of the mannose derivatives are found in fraction 2BI. This is a minor component of the cell wall and may be responsible for the promotion of assembly of 2BII [8]. It is clear from the data of Table 1 that 2BI is substantially different from 2BII. All the partially methylated mannitol acetates are accounted for by terminal, (1 → 3)- and (1,2,3,)-linked man*p* units. Based on the relative amounts of terminal to linear and branched mannosyl units an average DPn of 5 is obtained. It is interesting to note that mannose residues have been implicated in accurate reassembly of *C. reinhardii* cell wall glycoproteins [8]. The possibility therefore exists that mannose containing oligosaccharides adopt a specific conformation and may form recognition sites for crystallization.

Thermolysin glycopeptides

Enzymatic hydrolysis of the major glycoptotein 2BII with the endopeptidase thermolysin, followed by fractionation on Sepharose 6B, produced three glycopeptide fractions, T1, T2 and T3 present in the ratio 1.9:4.3:1.0 [10]. Insufficient amounts of T3, the least glycosiated fraction, were available for methylation analysis. Both T1 and T2 were methylated and the distribution of the major *O*-glycosidic linkages along the polypeptide chain determined. The results are shown in Table 2.

The predominant methylated derivative of T2 is 3,5-Me_2 arabinitol; 2,3,5-Me_3 arabinitol is again present confirming the occurrence of arabinofuranosyl residues. As with 2BII, T2 shows an absence of (1 → 3)-linked ara*f* making it unlikely that it contains hydroxyprolyl-arabinosides encountered in higher plant cell walls [4, 18]. Appreciable amounts of terminal gal*f* and (1 → 3)-linked gal*p* were detected. Also present in lesser concentrations were (1,3,6)-linked gal*f*, terminal xyl*p*, gal*p* and man*p*, (1 → 4)-linked gal*p*, (1 → 6)-linked gal*f*, (1 → 4)-linked glc*p* and (1 → 2)-linked man*p*. A small amount of unmethylated glucose was found. Whether this represents highly branched glucosyl units or is due to undermethylation is unclear. Again the average DPn by methylation (*ca* 2) is considerably lower than that obtained from the hydroxyproline:total sugar ratio (*ca* 5.7).

Glycopeptide T1 differs from T2 both in amino acid and sugar composition [10]. Methylation analysis also shows

Table 2. Partially methylated alditol acetates from glycopeptides T1 and T2

PMAA	RR_t*		Proportion (mol%) of PMAA	
†	a	b	T1	T2
2,3,5-Me_3Ara	0.44	0.46	5.3 (36.5)‡	8.3 (48.6)
3,5-Me_2Ara	0.80	0.91	25.7	30.6
Arabinitol	2.57	3.73	1.6	0.8
2,3,4-Me_3Xyl	0.58	0.64	3.6 (4.4)	4.4 (3.7)
2,3,4,6-Me_4Man	0.97	0.99	8.0 (21.3)	3.8 (2.0)
3,4,6-Me_3Man	1.75	1.85	0.7	2.0
2,4,6-Me_3Man	1.89	2.07	21.2	—
2,3,5,6-Me_4Gal	1.07	1.13	10.5 (29.1)	13.0 (38.7)
2,3,4,6-Me_4Gal	1.16	1.22	3.1	4.2
2,4,6-Me_3Gal	1.95	2.27	—	17.0
2,3,6-Me_3Gal	2.20	2.46	3.7	2.9
2,3,5-Me_3Gal	2.74	3.34	—	2.8
4,6-Me_2Gal	4.38	3.87	7.1	—
2,5-Me_2Gal	4.10	3.70	1.1	5.2
2,3,6-Me_3Glc	2.30	2.51	2.1 (8.7)	2.2 (7.0)
Glucitol	6.70	—	6.3	2.8

* R_t as Table 1.

† Abbreviations as Table 1.

‡ Figures in parentheses obtained by direct sugar analysis using GC.

it to differ in certain *O*-glycosidic linkages. Arabinose again is present as terminal and (1 → 2)-linked ara*f* residues in similar amounts to those found in T2. A significant difference is the absence of (1 → 3)-linked gal*p* but with a concomitant appearance of (1,2,3)-linked gal*p*. The major distinction from T2 is the presence of appreciable quantities of terminal and (1 → 3)-linked man*p* with lesser amounts of (1 → 2)-linked man*p*. From the relative amounts of mannose derivatives an average DPn of 2.7 is obtained. The ratio of terminal to remaining sugar derivatives yields a DPn of 2.3 which again is lower than that obtained by assuming that all the sugars are *O*-glycosidically linked to hydroxyproline.

Although the data from standard methylation analysis must be interpreted with caution, they allow several general features to be established. The cell wall glycoproteins of *C. reinhardii* solubilized by Na perchlorate are of high MW and contain a high proportion of sugar [7]. Our data suggest a model in which the protein backbone is glycosylated with short oligosaccharides, some of which are branched. The methylation data for glycopeptides T1 and T2 are also consistent with this model.

Unlike higher plant cell wall glycoproteins [3, 4, 19] *C. reinhardii* glycoproteins are glycosylated with mannose containing oligosaccharides. A further difference is the absence of (1 → 3)-ara*f* residues implying that *C. reinhardii* differs in its hydroxyprolyl glycoside composition from all other plants so far analysed. The unusual occurrence of galactofuranosyl residues in *C. reinhardii* glycoproteins and glycopeptides is another distinguishing feature but the significance of these residues is at present unclear. Thus, caution must be exercised when comparing cell wall glycoproteins of the lower algae with those of higher plants. Methylation of the glycopeptides produced by the action of thermolysin has shown the asymmetric distribution of certain *O*-glycosidic linkages. This suggests that each domain within the glycoprotein may have a specific function, which is in part dependent on the type of oligosaccharide present.

EXPERIMENTAL

Cell wall preparation and fractionation. Cells of *C. reinhardii* were grown and their cell walls harvested as previously described [9]. Wall components solubilized by 2M Na perchlorate were prepared and purified on a Sepharose 2B column [7].

Thermolysin digest. The purified glycoprotein, 2BII, was digested with thermolysin (EC 3.4.24.4, Calbiochem) and the glycopeptides fractionated on Sepharose 6B [10].

Sugar analysis. Neutral sugars were released using M H_2SO_4 for 2.5 hr at 100° and analysed by GC [20].

Methylation analysis of the various preparations (3–5 mg) was carried out as previously described [21]. Partially methylated alditol acetates were separated by GC [22]. GC–MS was performed in the electron impact mode [22]. Peak areas were converted into mole values using the appropriate correction factors [23].

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