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PHENOLIC–CARBOHYDRATE COMPLEXES IN THE CELL WALLS OF *LOLIUM PERENNE*

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Key Word Index—*Lolium perenne*; Gramineae; ryegrass; cellulolysis; phenolic–carbohydrate complexes; hemicelluloses.

Abstract—Two classes of phenolic–carbohydrate complexes were purified from the water-soluble products obtained from the digestion of ryegrass cell walls with a cellulase preparation. They contained D-glucose, D-xylose, L-arabinose, D-galactose and D-mannose residues in the ratios 3.6:10:6.3:1.4:2.3 and 5.3:10:3.0:1.1:2.1, respectively, and contained *ca* one ferulic acid residue to every 50–100 neutral sugar residues. The complexes were based on (1 → 4)-β-D-xylan chains to which were attached residues of L-arabinofuranose and D-galactopyranose. Mixed linkage (1 → 3), (1 → 4)-β-D-glucan chains also appeared to be integral components of these complexes. Phenolic–carbohydrate complexes could also be solubilized from the enzyme-resistant residue on hydrolysis under mildly acidic conditions. Two complexes were purified and found to contain the neutral sugar residues listed above, along with small amounts of L-rhamnose, in the ratios 3.5:10:4.9:1.1:3.0 and 3.3:10:3.7:2.8:1.1:2.0, respectively. These latter complexes were also based on galacto-arabinoxylans associated with (1 → 3), (1 → 4)-β-D-glucans and also contained ferulic acid residues which could be released on saponification.

INTRODUCTION

In mature Graminaceous plants, *p*-coumaric and ferulic acid residues constitute 5–10% of the total phenolic component of the cell wall [1]. These phenolic acids were found to be esterified to the lignin core in these grasses and bamboo and could be released from the cell walls by treatment with sodium hydroxide [2]. The isolation of carbohydrate esters of ferulic acid, by digestion of Italian ryegrass cell walls with a cellulase complex secreted by *Aspergillus niger* [3] and the accepted use of sodium hydroxide solutions to extract hemicelluloses from plant cell walls, are consistent with the suggestion that the ester-bound phenolic acids may act as hemicellulose–lignin bridging units.

It was previously observed [4] that, when the cell walls from perennial ryegrass were sequentially treated with a fungal cellulase preparation and a dilute solution of trifluoroacetic acid, each treatment resulted in the release of phenolic acids in carbohydrate-bound forms. In the study reported here, purified preparations of these cellulase-solubilized and mild acid hydrolysed phenolic carbohydrate complexes have been prepared. Furthermore, the structures of the carbohydrate moieties of the complexes have been investigated and compared with those of known components of the cell walls of grasses.

RESULTS

The soluble products from a large scale treatment of cell wall material prepared from mature ryegrass stems with a fungal cellulase preparation were separated by gel chromatography on Sephadex G50 (Fig. 1). Phenolic material was detected throughout the separation range of the column but only fractions eluting as a shoulder before the monosaccharide components were collected. This gave material from which high and low MW contaminants, in the form of the cellulase enzymes and monosaccharides, had been removed. Sufficient quantity was obtained for further analysis. After purification of the fraction by rechromatography on the same column, a single defined peak was obtained which contained both carbohydrate and material absorbing at 280 nm.

The solution of partially purified phenolic–carbohydrate complexes was divided into two equal portions. One portion was made to 1.0 M in sodium hydroxide and the other made to the same volume with water. After stirring for 18 hr under nitrogen at room temperature the solutions were acidified to pH 4.0 and exhaustively extracted with ether. The UV spectra of both the aqueous and ethereal solutions were recorded. The aqueous phase from the water-treated fraction showed absorption maxima at 280 and 320 nm indicative of carbohydrate-bound phenolic acids with no detectable material having been extracted into ether. A large proportion of the phenolic material was found in the ether phase after alkaline hydrolysis with absorption maxima at 283 and 313 nm. The aqueous phase showed a single absorption peak at 275 nm.

The pH of each of the aqueous phases was adjusted to 7.0 and the samples were refractionated on the Sephadex G50 column. The untreated material was indistinguishable from the original material in its elution profile but the material hydrolysed by alkali was separated into three fractions. Two fractions contained carbohydrate and two contained material absorbing at 280 nm (Fig. 2). The elution volume of the slower moving carbohydrate fraction coincided with that of the untreated material; therefore, the more mobile carbohydrate fraction had apparently increased in molecular size.

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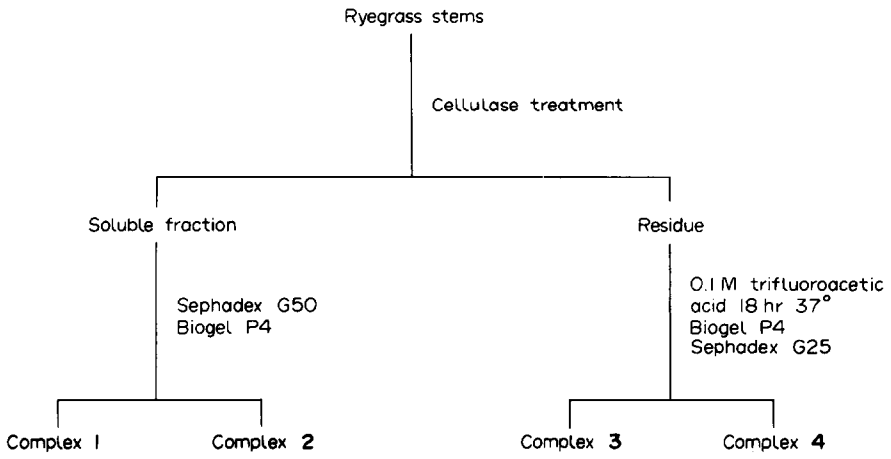

Fig. 1. Scheme for the isolation of phenolic–carbohydrate complexes 1–4 from ryegrass stems.

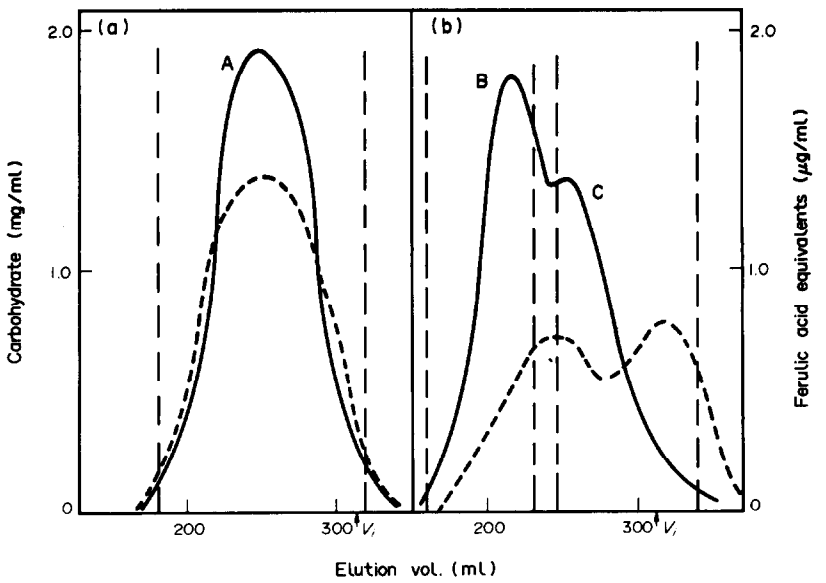

Fig. 2. Chromatography of the partially purified phenolic–carbohydrate complexes on Sephadex G50 after ether extraction. (a) Before treatment with 1.0 M sodium hydroxide; (b) after treatment with 1.0 M sodium hydroxide. Fractions A, B and C are indicated in the figure. (——) Total carbohydrate; (– – –) phenolic material.

Representative aliquots of each carbohydrate-containing fraction were hydrolysed and the neutral monosaccharides determined by GC of the aldononitrile peracetates (Table 1). The material with the highest apparent molecular size contained a large proportion of D-xylose residues but also contained significant proportions of L-arabinose and D-glucose residues. The increase in the mobility of this fraction on Sephadex chromatography may have been due either to a real increase in molecular size by, for example aggregation of the carbohydrate chains, or to a reduction in the affinity between the fraction and the column matrix [5]. The original material and the two carbohydrate-containing sub-fractions obtained on saponification were each re-fractionated by chromatography on Bio-gel P4 (Fig. 3), a polyacrylamide gel which is not reported to have the affinity for aromatic compounds which is shown by Sephadex gels. The neutral sugar composition of the carbohydrate-containing fractions is shown in Table 2.

The unsaponified material (fraction A) did not elute as a single peak on Bio-gel P4. A large proportion of the carbohydrate was eluted at the included volume of the gel and this peak, which also appeared in the elution profiles for fractions B and C, did not contain any material absorbing at 280 nm. From the analysis, this fraction contained predominantly D-glucose residues and appeared to represent the carbohydrate of a true molecular size corresponding to a K_D of 0·6 on the Sephadex G50 column.

Two unresolved carbohydrate-containing peaks in the elution profile from fraction A corresponded closely to two peaks in the elution profile of fraction B. The two

Table 1. Neutral sugar compositions of fractions from chromatography of the partially purified phenolic carbohydrate complexes on Sephadex G50

Elution volume (ml)	Monosaccharide composition (molar %)			Total (μmol/ml)
	Glc	Xyl	Ara	
(a) Before treatment with 1.0 M NaOH				
220	78	13	6	3.5
250	75	18	5	7.9
280	70	23	4	1.0
(b) After treatment with 1.0 M NaOH				
220	51	39	7	8.7
250	72	22	3	2.8
280	73	21	4	3.6

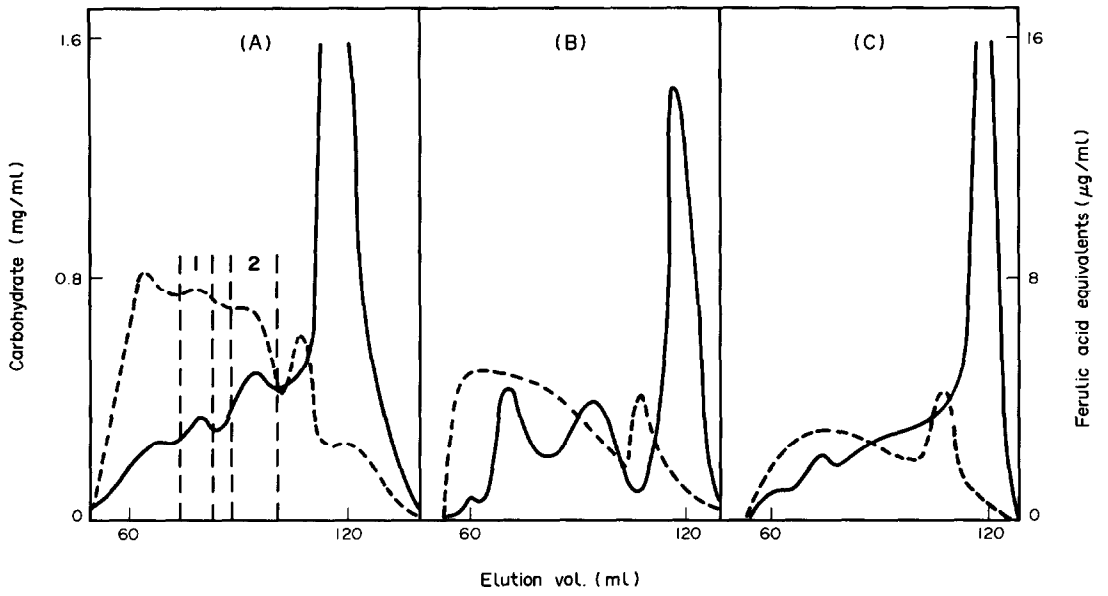

Fig. 3. Chromatography of fractions A–C on Bio-gel P4. The subfractions designated as complexes 1 and 2 are indicated in the figure. (——) Total carbohydrate; (– – –) phenolic material.

Table 2. Neutral sugar compositions of sub-fractions from chromatography of fractions A–C on Bio-gel P4

Elution volume (ml)	Monosaccharide composition (molar %)				
	Glc	Xyl	Ara	Gal	Man
(a) Fraction A					
76	5	54	26	5	10
96	20	47	17	6	10
120	86	11	2	0	1
(b) Fraction B					
70	14	43	27	6	10
94	25	47	14	5	10
120	78	15	5	1	2
(c) Fraction C					
94	38	49	7	3	3
120	56	35	6	0	3

peaks in the latter were made more distinct by the removal, as fraction C, of contaminating carbohydrate which does not appear to have been bound to phenolic material prior to alkaline hydrolysis.

A peak at K_D 0.85, which absorbed strongly at 280 nm, was common to all three fractions but was not associated with carbohydrate. Apart from this peak, material absorbing at 280 nm in fractions B and C occurred primarily in the high MW region and tailed off markedly towards the included volume for the column. There was no evidence to confirm whether this *A* was associated with carbohydrate or not. The two unresolved carbohydrate-containing peaks for fraction A did appear to be associated with material absorbing at 280 nm and their UV spectra showed peaks at both 280 and 320 nm, indicating the presence of carbohydrate-bound phenolic esters.

These two phenolic–carbohydrate complexes (complexes **1** and **2**) contained 50–100 neutral sugar residues for each phenolic acid moiety and consisted of D-glucose,

D-xylose, L-arabinose, D-galactose and D-mannose in the ratios 3.6:10.0:6.3:1.4:2.3 and 5.3:10.0:3.0:1.1:2.1, respectively. The quantitative values for phenolic components are based on the UV spectra of the material released by alkaline hydrolysis and extractable into ether with ferulic acid used as standard. Ferulic acid was identified by GC as the major phenolic component in an alkaline hydrolysate of mixed complexes **1** and **2** but other unidentified components were also present. Since the residue after alkaline hydrolysis still absorbed at 280 nm, it is likely that both complexes contained more highly polymerized phenolic components which were bound to the complexes by bonds other than ester bonds.

The complexes from fraction A having K_D 0.17–0.67 and from fraction B having K_D 0.00–0.75 were permethylated and the resultant partially methylated sugars obtained on hydrolysis were analysed as their acetylated aldononitriles. The molar proportions of the partially methylated sugars are shown in Table 3; in this table, since no standards were available, the mannose components have not been accounted for. The two fractions were very similar in their structural details, confirming that they were probably derived from the same phenolic–carbohydrate complexes. The complexes were based on (1 → 4)-β-D-xylan chains. Since 2,4,6-tri-*O*-methyl-D-gluconitrile acetate was poorly resolved from the tetra-*O*-methyl derivatives of glucose and galactose on column A, its presence and amount were determined using column B.

The only major difference between the carbohydrate composition of the methylated, intact complexes and that of the complexes hydrolysed by alkali was in the relative proportions of 2,3-di-*O*-methyl-D-xylose and 2-*O*-methyl-D-xylose. The lower proportion of the latter sugar, in the product hydrolysed by alkali, indicates that C-3 of some of the D-xylose residues were derivatized in the intact material by groups which were removed on alkaline hydrolysis. Such groups could be the phenolic acids.

The insoluble residue, which remained after the cellulase treatment was treated with dilute trifluoracetic acid solution (Fig. 1) under conditions chosen to favour the hydrolysis of furanosidic linkages but having little effect on other glycosidic bonds, and the soluble products were fractionated on Bio-gel P4 (Fig. 4). Most of the carbohydrate present was eluted close to the included volume of the column and was partially separated into a hexose-rich fraction (G) and a pentose-rich fraction (H). At least three other carbohydrate-containing fractions were identified. Fractions D and E were either partially or completely excluded from the gel whereas fraction F was present as a leading shoulder on the hexose-rich fraction G. Fraction F appeared to be associated with phenolic material but it was not found possible to completely separate it from free monosaccharides.

Fractions D and E (K_D 0·00 and 0·12) appeared to have high MWs and were rich in xylose residues as well as containing glucose and arabinose residues (Table 4). They were, therefore, designated as phenolic–carbohydrate complexes **3** and **4**. Both fractions were associated with phenolic material which gave absorption maxima at 280 and 320 nm and their UV spectra were similar to those previously reported for carbohydrate esters of ferulic acid. Alkaline hydrolysis of these complexes gave small amounts of ferulic acid which were identified by GC. Other minor phenolic components were present but were not positively identified. The complexes, on rechromatography on Bio-gel P4 and Sephadex G25, appeared to be homogeneous. Although G25 is reported to exclude molecules of a greater size than Bio-gel P4, the fractions were both retarded in their elution volume and remained associated with the phenolic material.

An aliquot of complex **3** was treated sequentially with 0.3 M sodium borohydride and 1.0 M sodium hydroxide solutions. Reduction with borohydride caused an overall decrease in the intensity of *A* in the UV spectrum of the complex but no detectable material was extracted into ether from a solution at pH 4 and there was no change in the monosaccharide residues present in the complex. After alkaline hydrolysis, most of the material absorbing at 280 nm was extractable into ether at pH 4 but the carbohydrate portion was again unaltered.

Complex **4** was permethylated and the partially methylated sugars released on acid hydrolysis were determined as their aldononitrile acetates by GC (Table 5). The identification of 2,3-di-*O*-methylxylononitrile acetate as a major component of the mixture again suggested that these complexes were based on (1 → 4)-linked chains of D-

Table 3. Composition of partially methylated sugars obtained from hydrolysates of methylated phenolic–carbohydrate complexes

	Source of complex		
Methylated sugar	Fraction A (original) Composition (molar %)	Fraction B (OH^--treated)	Mode of linkage
2,3,4-Me_3-Xyl	3	2	Xyl *p* (1 →
2,3-Me_2-Xyl	26	30	→ 4) Xyl *p* (1 →
2-Me-Xyl	20	14	→ 3,4) Xyl *p* (1 →
3-Me-Xyl	1	1	→ 2,4) Xyl *p* (1 →
2,3,5-Me_3-Ara	18	19	Ara *f* (1 →
2,3,4,6-Me_4-Glc	12	13	Glc *p* (1 →
2,4,6-Me_3-Glc	4	5	→ 3) Glc *p* (1 →
2,3,6-Me_3-Glc	10	9	→ 4) Glc *p* (1 →
2,3,4,6-Me_4-Gal	6	7	Gal *p* (1 →

The material used was a mixture of complexes **1** and **2** and similar material after alkaline hydrolysis.

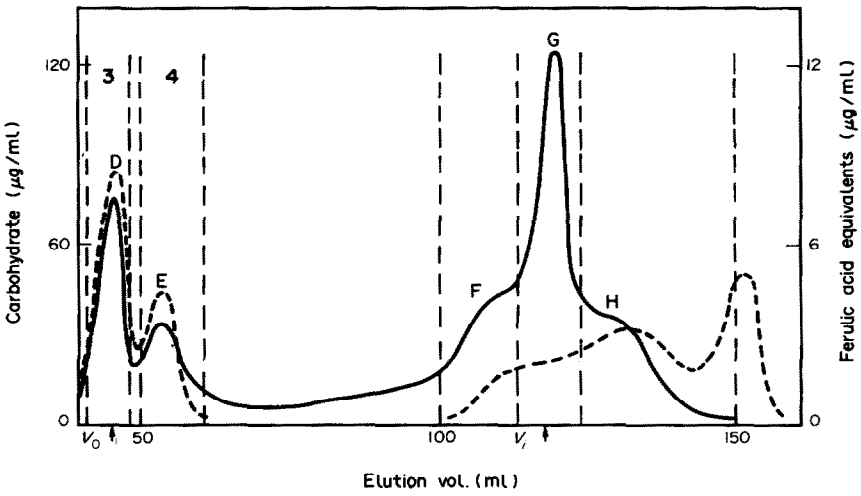

Fig. 4. Chromatography of the soluble products from the 0.1 M trifluoroacetic acid hydrolysis of the cellulase-treated cell walls on Bio-gel P4. The material taken as fractions D–H are indicated in the figure. Complexes **3** and **4** are fractions D and E, respectively. (——) Total carbohydrate; (– – –) phenolic material.

Table 4. Monosaccharide composition of fractions from chromatography of the soluble products from mild acid hydrolysis of cellulase treated cell walls on Bio-gel P4

	Neutral sugar composition (molar %)					
Fraction	Glc	Xyl	Ara	Gal	Man	Rha
D	13	37	18	11	4	18
E	15	46	17	4	5	13
F	22	49	23	< 1	< 1	5
G	60	19	18	0	< 1	2
H	21	17	17	0	0	45

Table 5. Composition of the hydrolysate of complex **4** after permethylation

Methylated sugar	Composition (molar %)	Mode of linkage
2,3,4-Me_3-Xyl	9	Xyl *p* (1 →
2,3-Me_2-Xyl	46	→ 4) Xyl *p* (1 →
2-Me-Xyl	4	→ 3,4) Xyl *p* (1 →
3-Me-Xyl	3	→ 2,4) Xyl *p* (1 →
2,3,4-Me_3-Ara	17	Ara *p* (1 →
2,3-Me_2-Ara	2	→ 5) Ara *f* (1 →
		→ 4) *Ara p* (1 →
2,4,6-Me_3-Glc	3	→ 3) Glc *p* (1 →
2,3,6-Me_3-Glc	2	→ 4) Glc *p* (1 →
2,3,4,6-Me_4-Gal	6	Gal *p* (1 →
2,3,6-Me_3-Gal	7	→ 4) Gal *p* (1 →

xylopyranose residues. A peak having the R_t of 2,3,4-tri-*O*-methylarabinonitrile acetate was attributed to the possible presence of L-arabinopyranose residues. This peak may have contained a component derived from L-rhamnose residues, which were present in these complexes, but no standard methylated sugars were available for comparison. Some of the residues in the main chain of the xylan were substituted at O-2 or O-3 and the polysaccharide contained non-reducing terminal residues of D(L)-galactopyranose, D-xylopyranose and L-arabinopyranose. A peak corresponding to 2,3,6-tri-*O*-methylgalactonitrile acetate may be indicative of (1 → 4)-linked D-galactopyranose residues further substituted by some of the terminal residues above.

DISCUSSION

Complexes **1** and **2** may be regarded as former components of the cell walls of ryegrass stems which were released into solution by hydrolysis of some of the glycosidic bonds within the polysaccharides from where they had been derived. The ratio D-xylose–L-arabinose in the hydrolysates of these complexes (10:6.3 and 10:3.0) is comparable with that from the phenolic acid–carbohydrate complexes from Italian ryegrass which contained D-glucose, D-xylose and L-arabinose residues in the ratio 6.7:10:4.8 [3]. The relatively high proportion of L-arabinose residues found in these complexes may be due to the inability of the (1 → 4)-β-D-xylanase in the 'cellulase' preparation to cleave the (1 → 4) bonds adjacent to D-xylose residues which are substituted by other components.

Lignin–carbohydrate complexes have been isolated from perennial ryegrass [6, 7] and a comparison of their composition with the phenolic–carbohydrate complexes isolated here suggests that some similarity of origin does exist. A lignin–carbohydrate complex containing D-glucose, D-xylose, L-arabinose and D-galactose residues in the ratio 16.7:10.0:4.0:1.7 was also found to contain ferulic acid and *p*-coumaric acid residues linked by ester bonds. On alkaline hydrolysis, one phenolic acid residue was released from this complex for *ca* every 80 neutral sugar residues.

Methylation analysis showed that (1 → 4)-linked D-xylopyranose units were the principal features of the complex while non-reducing terminal arabinofuranose

and galactopyranose residues appeared to be important substituents to what were assumed to be (1 → 4)-β-D-xylan chains. Such xylans have been isolated from the cell walls of many Graminaceous plants [8] and the L-arabinose and D(L)-galactopyranose substituents to these polysaccharides account for almost all of the arabinose and galactose residues found in secondary plant cell walls [9].

The presence in the phenolic–carbohydrate complexes of residues of 3- and 4-linked D-glucopyranose residues, in the ratio 2:1, indicated that they were derived from a β-D-glucan containing both (1 → 3) and (1 → 4) linkages and not from cellulose. These non-cellulosic β-glucans have been isolated from mature tissue of other species of grass and from bamboo where they are associated with an acidic xylan [10].

The number of non-reducing terminal D-glucose residues may have been overestimated. Residues of D-mannose constituted a significant proportion of the complexes and the 2,3,4,6-tetra-*O*-methyl derivatives of D-glucose and D-mannose have similar GC R_ts. Although D-mannose residues have been reported to be minor constituents of the cell walls from the aerial organs of several grasses [6, 11–13], their structural relationship with other cell wall constituents is not known.

The striking feature of complexes **3** and **4** is their similarity to complexes **1** and **2**. They also appear to be based on (1 → 4)-β-D-xylan chains which are substituted with D(L)-galactose and L-arabinose residues. The presence of L-arabinose residues was not expected in view of the acid conditions used in the preparation of the complexes. However, on methylation analysis a component from complex **3** was identified as 2,3,4-tri-*O*-methylarabinonitrile acetate. This indicates that the arabinose residues in this complex are not in the acid-labile furanose form but the more acid-stable pyranose form.

Further investigation of the mild acid-solubilized phenolic carbohydrate complexes will be necessary before their structures and relationships with the other components of the cell wall are completely understood. However, their dissolution under mild conditions only after treatment of the cell walls with a fungal 'cellulase' complex, either before or after much of the carbohydrate had subsequently been removed by borohydride reduction [4], implies that they are closely associated with lignin and may be covalently bound to it via L-arabinofuranosyl bridging groups. Alternatively, the 'cellulase'-solubilized complexes may be distributed throughout those regions of the cell walls which are available for enzymatic hydrolysis and are removed from it at the same rate as dry matter is removed from the walls [14, 15].

EXPERIMENTAL

Source of enzymes. The source of the crude cellulase (ex *Trichoderma viride*) and the method used to remove low MW contaminants have been described previously [4].

Plant material. Preparation of cell wall material from mature stem tissue of perennial ryegrass cv S24 (*Lolium perenne*) and its digestion by the fungal 'cellulase' have already been described [4]. The cell wall material which remained insoluble after cellulase treatment was removed by centrifugation (2000 *g* for 20 min) and thoroughly washed with H_2O. The combined aq. extracts were then lyophilized. Part of the residue (200 mg) was stirred with 0.1 M trifluoroacetic acid (TFA) (10 ml) for 18 hr at 37°. The residue was centrifuged-off as above and the combined aq. soln was evaporated to dryness.

Molecular sieve chromatography. Carried out on Sephadex G25 and G50 (Pharmacia) (90 × 3 cm i.d.) and Bio-gel P4 (Biorad Labs.) (90 × 1.5 cm i.d.) columns eluted with 0.02 M NaCl (pH 5.8) containing 0.02% (w/v) sodium azide. The excluded and included vols. were determined with Blue Dextran and D-glucose, respectively.

Total carbohydrate in the eluates was determined by the Ph–OH–H_2SO_4 method [16]. Phenolic components were detected by UV *A* at 280 nm and quantified relative to a standard of ferulic acid.

Gas–liquid chromatography. Carried out on a dual FID instrument using glass columns (2 m × 4 mm i.d.) packed with: (A) 3% (w/w) SP 2340 on 100–200 mesh Supelcoport; or (B) 5% (w/w) OV 225 on 100–200 mesh Chromosorb W AW DMCS. N_2 was the carrier gas at 45 ml/min.

Carbohydrate analysis. Water-soluble fractions were hydrolysed with 2.0 M TFA for 1 hr at 121° [17]. The mixture was evaporated to dryness and the neutral sugars were analysed by GC as their aldononitrile peracetates [18].

Methylation analysis of the phenolic carbohydrate complexes. Carried out by the method of Hakomori [19] as described elsewhere [20], followed by 2.0 M TFA acid hydrolysis. The partially methylated sugars were analysed as the aldononitrile peracetates by GC. The columns used were as above, using the following temp. programmes: (A) 150–225° at 2°/min; and (B) 160–210° at 1°/min.

Phenolic acid analysis. Carried out by GC [21].

Reduction of the purified complex. Carried out in 0.3 M $NaBH_4$ soln at pH 7 for 18 hr at room temp. Excess $NaBH_4$ was removed with HOAc. The soln (pH 4) was extracted with Et_2O (× 3) to remove any phenolic material released from the complex and borate ions were removed from the soln by repeated evaporation to dryness with 5% HOAc in MeOH.

Alkaline hydrolysis of the reduced complex. Carried out in 1.0 M NaOH as described previously [4].

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