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Extraction of phenolic-carbohydrate complexes from graminaceous cell walls

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Abstract

Barley straw and a preparation of perennial ryegrass were sequentially extracted with oxalic acid, dimethyl sulphoxide (DMSO) and a "cellulolytic" enzyme preparation Driselase and the fractions studied by methylation analysis, ^{13}C NMR and other methods. Oxalic acid, as expected, solubilised the bulk of the arabinose and ferulic acid in both samples, although appreciable amounts of xylose were also solubilised. DMSO yielded polymeric lignin–carbohydrate complexes (LCC), both of which consisted predominantly of a $\beta(1 \rightarrow 4)$ xylan. From the results of methylation analysis, sensitivity to oxalic acid hydrolysis and size-exclusion chromatography after alkaline hydrolysis, it was evident that lignin polymers were attached to arabinosyl and xylosyl residues by both ester and aryl-ether linkages. After cellulolytic hydrolysis of the residues, thioacidolysis analysis of the lignin component in the DMSO-soluble and Driselase-insoluble fractions from ryegrass revealed differences in every measurable aspect. The DMSO-soluble lignin was found to be more highly condensed, have a higher S/G ratio and have a higher terminal G/internal G ratio.

Keywords: Lignin; Ferulic acid; *p*-Coumaric acid; Hemicellulose

1. Introduction

It is not known, in detail, how lignin is attached to carbohydrate in plant cell walls although the implications are profound. The relationship between the phenolic acids, *p*-coumaric and ferulic acids, and cell wall carbohydrate is better established. Struc-

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turally relevant fragments have been obtained from cell walls of the Gramineae and shown to consist of phenolic acid ester-bound to arabinoxylan through side-chain arabinose residues [1–4]. Ferulic acid has also been found ester-linked to pectic substances and xyloglucan in other plant groups [5,6]. Dimers formed, oxidatively and photochemically, between phenolic acids also occur in the wall in small amounts, where they act as cross-links between polysaccharide chains [7–9]. There is, however, little evidence to suggest that monomeric or dimeric phenolic compounds inhibit hydrolysis of cell walls by rumen microflora to any great extent.

Apparently, of overriding importance in the control of the rate and extent of degradation of the structural polysaccharides by microorganisms is the association of phenolic oligopolymers and polymers (lignin) with cell wall carbohydrate in the form of lignin–carbohydrate complex(es) (LCC) [10]. Although direct evidence for the presence of LCC in non-wood plants is lacking, the weight of indirect evidence coupled with the more extensive knowledge of such structures in woods make their presence difficult to deny. Putative LCC have been isolated from several graminaceous species and aspects of their properties and structure examined [11–13]. The carbohydrate component of graminaceous LCC contains many of the structural features of glucuronoarabinoxylans although suggestions that other wall polysaccharides may complex with lignin cannot be excluded. Rather less attention has been paid to the nature of linkages formed between lignin and carbohydrate and virtually none to the nature of the lignin component itself. LCC isolated from ryegrass by Morrison [14,15] included fractions which could be disrupted by alkali and by reduction with borohydride and others which resisted both treatments. The ability of alkali-treatment at ambient temperature to solubilise a substantial part of the lignin from straw [16] suggests that ester linkages contribute to most phenolic–carbohydrate bridges in this group of plants. Indirect evidence for the presence of alkali-labile linkages formed between lignin and carbohydrate, akin in structure to those formed between phenolic acid and arabinoxylan, has been obtained [17]. Use of alkaline hydrolysis at elevated temperatures has provided further evidence to suggest that esterified ferulic acid units are also etherified to other phenolic compounds adding support to the view that, in Gramineae, this phenolic acid is a major bridge between arabinoxylan and phenolic polymers [18,19]. Ether links between carbohydrate and lignin are suggested by the results of Morrison [15] but have never been characterised.

Microscopic examination of Gramineae cell walls undergoing microbial attack show that some wall types and some parts of individual walls are more susceptible to attack than others [20,21]. This suggests that LCC are not evenly distributed between and within secondary thickened walls and that some heterogeneity of structure exists. Since relatively little difference has been found in the carbohydrate component of LCCs from Gramineae, heterogeneity is more likely to arise because of differences in lignin monomer composition, the types and frequency of intermonomer linkages, the size of the lignin polymer and the nature and degree of lignin–carbohydrate interactions [22,23].

The aim of this study was to demonstrate the existence of multiple forms of bonding between arabinoxylans and lignin in the cell walls of graminaceous plants. This required a novel approach to retain the lignin–carbohydrate linkage intact during successive solubilisation of fragments from the cell wall and a wide range of spectroscopic

techniques were used to characterise both soluble fragments and residues, together with a novel adaptation of methylation analysis to distinguish between ester- and ether-linked substituents.

2. Experimental

Fractionation of grass and straw samples.—Barley straw (BS) (*Hordeum vulgare* cv. Golden Promise) was harvested in August 1984 at the Rowett Research Institute. The straw was dried, milled to pass a 1 mm sieve and then stored at room temperature.

Whole perennial ryegrass (PR) (*Lolium perenne* cv. Perma) was harvested in June 1988 and stored at −20°C. The frozen grass was repeatedly blended in a Waring blender with iced distilled water and sieved through a 300 μm nylon mesh until all of the green colour had been washed out. The cell wall fraction of ryegrass was then freeze dried and stored at room temperature.

Samples (5g) of barley and ryegrass cell walls were sequentially extracted as detailed below.

Oxalic acid hydrolysis.—Samples were refluxed with 500 mL of 30 mM oxalic acid for 3 h. The insoluble residue was collected by filtration, thoroughly washed with distilled water and freeze dried (oxalate-insoluble fraction). The filtrate (oxalate-soluble fraction), containing oxalic acid, was neutralised with an equimolar amount of calcium acetate and the precipitated calcium oxalate removed by filtration. The freeze dried hydrolysate was then extracted with 5 × 10 mL of methanol to yield methanol-soluble and methanol-insoluble fractions which were then dried.

DMSO extraction.—Samples of oxalate-insoluble fraction (4 g) were milled under liquid nitrogen for 3 min in a SPEX 6700 freezer mill (Spex Industries). The samples were then dried and extracted with 250 mL dimethyl sulphoxide (DMSO) in an ultrasonic bath for 3 h. Filtered DMSO-soluble material was dialysed against distilled water, in the dark, for four days at room temperature and then dried (DMSO-soluble fraction). DMSO-insoluble residues were collected and, after removal of DMSO, freeze dried (DMSO-insoluble fraction).

Driselase hydrolysis.—The mixed polysaccharidase enzyme preparation driselase (5 g, Sigma) was partially purified according to the method of Fry [5]. The final enzyme preparation was dissolved in 50 mL of 100 mM sodium acetate buffer pH 5.0, and stored at −20°C. Samples of DMSO-insoluble fraction (100 mg) were added to 10 mL acetate buffer plus 1 mL of enzyme and incubated at 45°C for 96 h under a layer of toluene. The hydrolysed material was then removed by filtration (Driselase-soluble fraction) and the residue (Driselase-insoluble fraction) washed with distilled water. The soluble and insoluble fractions were freeze dried.

Analytical methods.—Carbohydrate. Total carbohydrates were determined by the phenol-sulphuric acid method [24] and uronic acids by the method of Blumenkrantz and Asboe-Hanson [25]. Monosaccharides were determined as their alditol acetates, prepared according to the method of Blakeney et al. [26] and separated as described by Alexander et al. [27]. Methylation analysis was performed according to Lomax and co-workers

[28,29]. Acetyl groups were determined by the method of Bethge and Lindstrom [30] and analysed as described by Conchie et al. [31].

Phenolics.—Total phenolics were determined by the acetyl bromide method of Morrison [32], using ferulic acid as a standard. Phenolic acids were determined by the HPLC method of Hartley and Buchan [33]. Thioacidolysis followed the method described by Lapierre and co-workers [34,35] with pre-methylation of the samples as described by Lapierre and Rolando [36].

Spectroscopic analysis.—High-resolution ^{13}C NMR spectra of the LCC were recorded in d_6-DMSO at 50 MHz. Chemical shifts are expressed with respect to tetramethylsilane and set from the central resonance of DMSO at 39.5 ppm. Solid-state CP/MAS NMR spectra were obtained at the SERC solid-state NMR service in Durham, at 75.4 MHz for ^{13}C. Assignments were based on published data on lignin and grass cell walls [23,37,38].

Infrared spectra of the LCCs were recorded from 0.8 mg of the fraction, in 13 mm KBr discs on a Perkin–Elmer 580B spectrophotometer. UV spectra were recorded from 50% aqueous DMSO solution, with and without sodium acetate on a Perkin–Elmer 124 double beam spectrometer.

3. Results and discussion

Oxalic acid hydrolysis.—Oxalic acid hydrolysis was used in an attempt to disrupt phenolic–carbohydrate complexes which involved bonding to arabinose side-chains of arabinoxylan, by selectively hydrolysing labile arabinofuranosyl linkages [39,40]. It was anticipated that structures involving phenolic acid units not further linked to other (phenolic) structures would be solubilised while those forming part of more complex structures would remain with the oxalate-insoluble residues [19,41]. Under the conditions used, cleavage of arabinofuranosyl links by oxalic acid hydrolysis was essentially complete [42] although the recovery of feruloyl-arabinose [43] showed that ester bonds were not cleaved. Mild acid hydrolysis removed 24.3 and 39.6% of dry matter from barley straw and ryegrass respectively. Most of the arabinose (barley straw, 90.4; ryegrass, 96.2%) and ferulic acid (barley straw, 51.2; ryegrass, 79.1%) initially present was solubilised whereas lignin and *p*-coumaric acid were less extensively solubilised (Tables 1 and 2). However there were also xylosyl residues in the methanol-soluble fraction (Tables 1 and 2), presumably oligomeric, and this indicated that xylopyranosyl linkages were also attacked. Although a proportion of methanol-soluble xylose units could have been released from xyloglucan [40], amounts of this polymer are small in Gramineae cell walls and most must have come from arabinoxylan. Comparison of results from methylation analysis of the starting materials and oxalate-insoluble fractions (Tables 3 and 4) showed that the residual xylose was predominantly the 2,3 *O*-methyl derivative demonstrating that the oxalate-insoluble arabinoxylans were debranched but not hydrolysed. These xylans may have been stabilised by non-covalent association with cellulose, from which some of them were released by DMSO extraction. Much of the original ryegrass pectic material (uronic acids and galactose) was found in the methanol-insoluble fraction.

Table 1
Composition of barley straw cell wall and fractions

	Percent of dry weight of fraction						
	Cell wall	Methanol-soluble	Methanol-insoluble	Oxalate-insoluble	DMSO-soluble	DMSO-insoluble	Driselase-insoluble
Rhamnose	0.07	0.08	0.19	0.00	0.00	0.00	0.00
Fucose	0.00	0.23	0.00	0.00	0.00	0.00	0.00
Arabinose	2.81	12.51	1.77	0.35	0.63	0.45	0.55
Xylose	19.89	27.32	19.26	12.55	23.42	9.93	9.29
Mannose	0.47	0.57	1.08	0.02	0.20	0.17	0.13
Galactose	1.05	3.15	2.39	0.14	0.31	0.16	0.11
Glucose	43.08	5.03	15.99	43.89	3.33	55.01	51.63
Uronide	2.39	1.06	5.48	1.78	1.74	1.02	1.19
Acetyl	3.03	n.d	n.d	1.98	2.31	1.73	2.08
Ferulic acid	0.45	1.74	0.52	0.29	0.48	0.27	0.29
p-Coumaric acid	1.33	1.78	0.40	1.66	1.81	1.85	2.70
Lignin [a]	10.05	7.18	11.13	10.82	16.95	6.45	9.26

[a] Lignin calculated as total phenolics – total phenolic acids. n.d Not determined.

From the oxalate extraction it was possible to deduce the extent to which arabinosyl residues bridged between xylan and lignin. If arabinofuranosyl linkages were completely cleaved by oxalate then the small proportion (~ 20%) of the arabinosyl residues not solubilised at that stage (Tables 3 and 4) must have remained coupled to polymeric lignin by non-glycosidic linkages. The recovery of 2,3,5-tri-*O*-methyl arabinose following methylation suggests that the non-glycosidic bonds were ester or ester-like; ester links being cleaved by the dimethyl sulphinyl anion. All barley straw and ryegrass

Table 2
Composition of ryegrass cell wall and fractions

	Percent of dry weight of fraction						
	Cell wall	Methanol-soluble	Methanol-insoluble	Oxalate-insoluble	DMSO-soluble	DMSO-insoluble	Driselase-insoluble
Rhamnose	0.20	0.12	0.58	0.00	0.00	0.00	0.00
Fucose	0.07	0.17	0.00	0.00	0.00	0.00	0.00
Arabinose	5.71	20.78	1.66	0.36	1.01	0.56	0.46
Xylose	20.89	27.64	20.21	13.19	30.37	9.13	6.55
Mannose	0.13	0.05	0.00	0.07	0.06	0.13	0.12
Galactose	1.61	3.61	2.34	0.12	0.45	0.17	0.13
Glucose	45.49	1.89	9.96	52.58	5.85	55.80	38.91
Uronide	7.24	1.36	14.41	2.23	5.94	1.86	0.69
Acetyl	2.75	n.d	n.d	1.65	3.43	1.20	1.18
Ferulic acid	1.07	2.56	1.16	0.37	0.91	0.24	0.17
p-Coumaric acid	0.69	0.91	0.37	0.72	1.47	0.61	1.02
Lignin [a]	6.18	4.77	8.11	5.16	7.36	4.26	7.29

[a] Lignin calculated as total phenolics – total phenolic acids. n.d Not determined.

Table 3
Methylation analysis of barley straw cell wall and fractions

Sugar	*O*-Methyl derivative					
	Percent of dry weight of fraction					
		Cell wall	Oxalate-insoluble	DMSO-insoluble	DMSO-soluble	Driselase-insoluble
Arabinose	2,3,5	1.56	0.73	1.07	0.44	0.82
	2,3	0.35	—	—	—	—
	2,5	0.43	—	—	—	—
	3,5	0.22	—	0.18	—	—
Xylose	2,3,4	0.37	0.33	0.62	0.16	0.22
	2,3	14.48	20.26	38.79	13.66	18.68
	2	2.76	1.21	1.41	0.83	1.08
	3	1.23	0.97	1.01	0.79	0.84
	Un-Me	2.68	—	—	0.24	—
Rhamnose	2,3,4	0.04	—	—	—	—
Glucose	2,3,4,6	1.04	0.44	0.94	0.41	0.02
	2,3,4	0.14	—	—	—	—
	2,3,6	33.25	45.22	2.15	47.74	39.25
	2,4,6	1.01	—	2.64	0.87	—
	2,3	1.69	1.25	—	1.09	0.69
	3,6	0.85 [a]	—	—	0.42	—
	2	0.33	—	—	—	—
	6	0.35	—	—	—	—
	Un-Me	0.68	—	—	—	—
Galactose	2,3,4,6	0.53	0.21	—	0.34	0.13
	2,6	0.43	0.84	—	0.80	—
Total carbohydrate		64.42	71.46	49.15	67.58	62.03

[a] Includes some 4,6-di-*O*-Me-Glc.

residues yielded 2,3,5-*O*-methyl arabinose consistent with the presence of bridging units of the lignin–ester–arabinofuranosyl–xylan type; more being recovered from the lignified barley straw. In the ryegrass, the oxalate-insoluble fraction additionally yielded 2,3-di-*O*-methyl arabinose suggesting the presence of an ether linkage between lignin and O-5 of arabinose. Ether linkages, like glycosidic linkages would be stable under the conditions of the methylation step.

Thus both ester and ether linkages were present between arabinose and lignin in ryegrass but only ester linkages were detected in barley straw.

DMSO extraction.—DMSO solubilised 7.8 and 10.9% of the oxalate-insoluble barley straw and ryegrass fractions, which represented 5.9 and 6.6% of the original sample weight, respectively. The carbohydrate in the DMSO-soluble samples consisted, predominantly, of $\beta(1 \rightarrow 4)$ xylan. Recovery of 2,3,6- and 2,4,6-*O*-methylglucose, from methylation analysis, also suggested the presence of some mixed-linked glucan (Tables 3 and 4). These structures were confirmed by high-resolution ^{13}C NMR spectroscopy (Fig. 1). Both NMR and infrared spectroscopy (IR) of the DMSO-soluble samples indicated a substantial acetyl content, shown by the characteristic IR absorptions at 1250 and 1733 cm^{-1}, although the residue was also acetylated (Tables 1 and 2). Absorption

Table 4
Methylation analysis of ryegrass cell wall fractions

Sugar	*O*-Methyl derivative					
	Percent of dry weight of fraction					
		Cell wall	Oxalate-insoluble	DMSO-soluble	DMSO-insoluble	Driselase-insoluble
Arabinose	2,3,5	2.37	0.63	1.03	0.51	0.49
	2,3	0.39	—	—	0.64	—
	2,5	0.51	—	—	—	—
	3,5	0.36	0.24	0.56	0.18	—
Xylose	2,3,4	0.23	0.52	0.84	0.32	0.18
	2,3	7.85	15.52	42.93	12.25	5.66
	2	3.78	0.91	1.72	0.84	0.94
	3	1.89	1.08	4.29	0.92	0.58
	Un-Me	2.39	0.47	—	0.37	—
Rhamnose	3	0.38	—	—	—	—
Glucose	2,3,4,6	1.07 [a]	0.23	0.23	0.24	1.51
	2,3,4	—	—	—	—	—
	2,3,6	34.39	54.02	6.43	59.33	43.16
	2,4,6	1.38	—	1.43	—	—
	2,3	0.96	1.10	—	1.37	0.49
	3,6	0.38	—	—	—	—
	2	0.36	—	—	—	—
	6	0.27	—	—	—	—
	Un-Me	0.84	—	—	—	—
Galactose	2,3,4,6	0.71	—	0.37	0.18	0.32
	2,6	—	0.77	—	0.90	—
Total carbohydrate		60.51	75.49	59.83	78.05	53.33

Results expressed as % dry weight.
[a] Includes some 2,3,4,6-tetra-*O*-Me Gal.

bands at 1120 and 630 cm^{-1} indicated the presence of residual DMSO in the fraction. The major carbohydrate bands (990, 1030–1040, 1070–1090 and 1150–1180 cm^{-1}) were typical of xylan and/or glucan. Little or no nitrogen was found by elemental analysis of the LCCs.

The main components remaining after DMSO extraction were acetylated xylans, cellulose and lignin (Tables 1 and 2). Most of the original *p*-coumaric acid was still present in this fraction. The remaining xylans after DMSO extraction had a higher proportion of branch points than those extracted (Tables 3 and 4). Some of the branches may have been glucuronyl residues but in the DMSO-insoluble fractions the total amount of uronic acid present which probably included pectic residues, is less than the number of branches so the remainder were probably non-glycosidic, i.e. aryl-ethers.

Approximately 10% of the original, acetyl bromide lignin was present in the DMSO-soluble fractions (Tables 1 and 2). The presence of lignin was demonstrated by UV and by IR absorptions in the aromatic region, 1400–1650 cm^{-1} for syringyl/guaiacyl groups. UV absorbing and carbohydrate components could not be separated by gel filtration chromatography, showing that a lignin–carbohydrate complex was present. On treatment with 1 M NaOH, to cleave ester linkages, the DMSO soluble

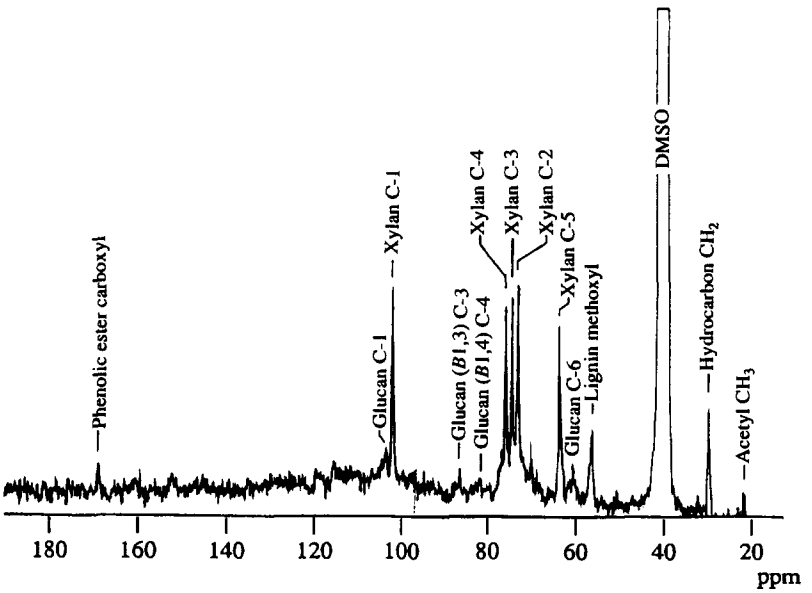

Fig. 1. ^{13}C NMR spectrum of DMSO-soluble fraction of barley straw.

fraction from ryegrass was substantially disrupted and could be resolved, by gel chromatography, into carbohydrate and phenolic fractions, as previously demonstrated by Morrison [14,15], while the corresponding extract of barley straw was incompletely resolved (data not shown). Lack of disruption by NaOH, under these conditions, would suggest ether linkages between carbohydrate and lignin.

UV spectra of the DMSO-soluble fractions showed major absorptions around 280 and 320 nm. On the addition of sodium acetate, a noticeable hypsochromic shift occured in the DMSO-soluble fraction from barley straw and a shift of λ_{max} to 280 nm in the corresponding ryegrass fraction. This indicated that a high proportion of the carboxyl groups (e.g. in ferulate and *p*-coumarate in the barley LCC were present in their free form, rather than ester linkages to carbohydrate, since the addition of acetate would have had no effect or would have produced a bathochromic shift if in the ester form [44]. The DMSO extraction afforded the opportunity to study lignin components not involved in large insoluble polymeric networks separately from those that were. The structures of these two lignin moieties are compared in the next section.

Driselase hydrolysis.—Residues from DMSO extractions were treated with the "cellulolytic" enzyme preparation Driselase to selectively hydrolyse the carbohydrate component. Driselase was selected because of its wide spectrum of enzyme activities and its lack of non-specific esterase activity [40]. Soluble fractions from the Driselase extraction were not analysed due to impurities in the Driselase preparation, but the residues were characterised. The DMSO-insoluble residue from ryegrass was more extensively hydrolysed than the corresponding fraction of barley straw, leaving only 12.1% of the original material, compared to 31.8% in barley. There was no evidence of selective hydrolysis and methylation analysis (Tables 3 and 4) confirmed that all

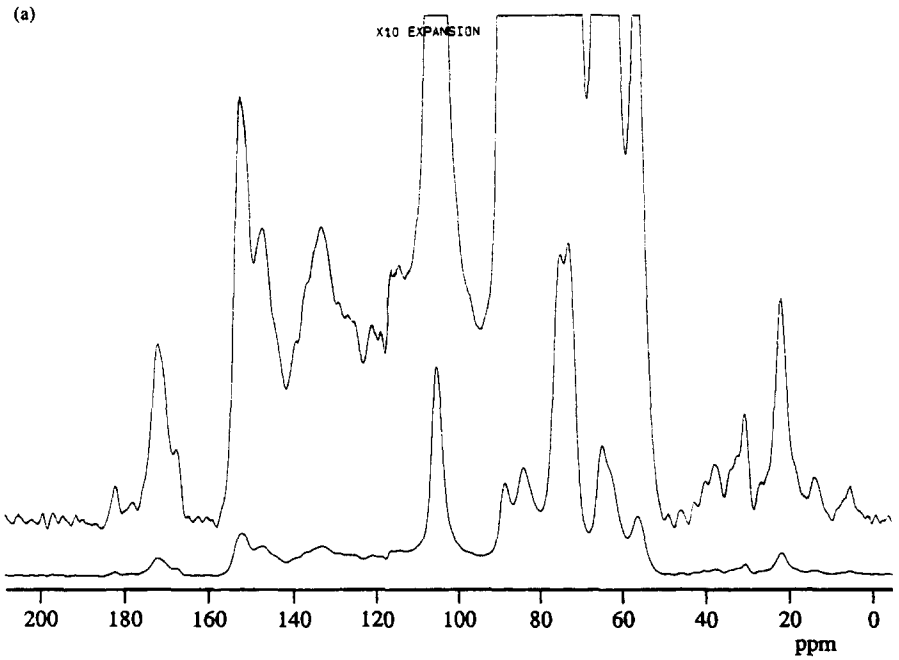

Fig. 2. ^{13}C CP/MAS NMR spectra of Driselase-insoluble fractions: (A) barley straw; (B) ryegrass.

glycosidic linkage types were hydrolysed to some extent. CP/MAS ^{13}C NMR Spectra of the driselase residues are shown in Fig. 2 and the assignments in Table 5. The proportion of crystalline cellulose remaining (C-4, 89 ppm), particularly in the ryegrass residue, was considerably greater than is normally found in intact walls. Branched xylose units, as previously attributed to ether linkages to lignin, were also present (Tables 3 and 4). Loss of phenolic material and acetyl accompanied hydrolysis of polysaccharide with the result that there was little net accumulation of either in the driselase-treated residues (Tables 1 and 2). In this and most other respects the driselase insoluble residues closely resembled the residues of barley and ryegrass recovered after extended rumen incubation [45]. Such results indicate that in this fraction of the wall at least, lignin must occur in numerous discrete units attached to polysaccharide, which can be separated as the polysaccharide is depolymerised, and not as an extended graft polymer.

Since UV-absorbing material yielding thioacidolysis products typical of polymeric lignin (Table 6) remained insoluble during oxalate hydrolysis and DMSO extraction, but was released by subsequent hydrolysis of the remaining polysaccharides with Driselase, it seems likely that this fraction of the lignin was attached covalently to xylose residues and not through arabinose sidechains. Direct lignin–cellulose links are not ruled out by these results but are generally considered unlikely.

The CP/MAS NMR spectra (Fig. 1) allowed features of the lignin structure to be deduced, although the signal intensities in the aromatic region (110–160 ppm) were less

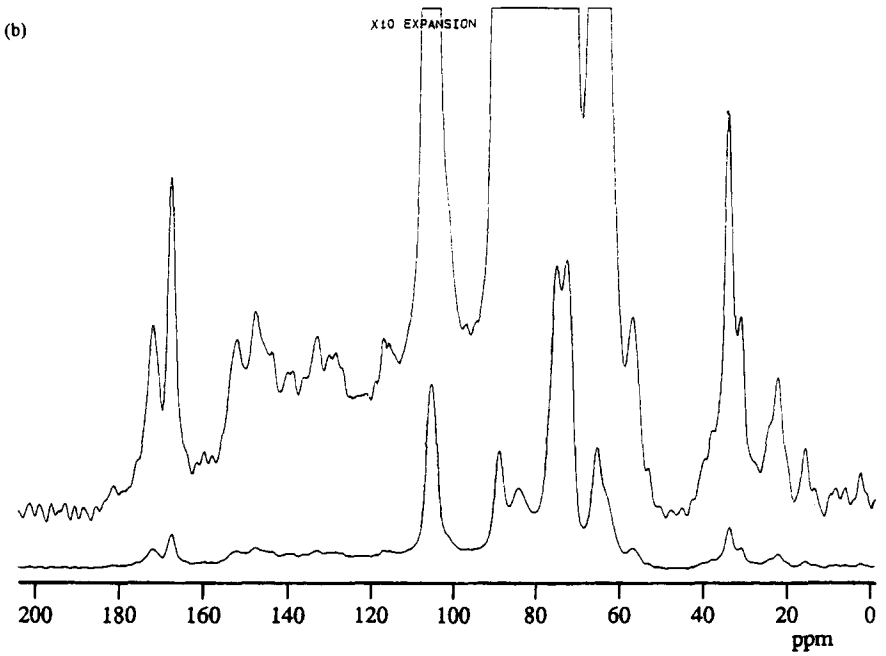

Fig. 2 (continued).

Table 5
Assignments of signals from CP/MAS ^{13}C NMR

Chemical shift (ppm)	Principal assignment(s)
181	Phenolic acid carboxyl, non-esterified
173	Acetyl carboxyl
168	Phenolic ester carboxyl, cutin carboxyl
152	Syringyl, ether-linked, C-3/5
149	Guaiacyl, ether-linked, C-3
147	G and S, not ether-linked, C-3/5
141	Phenolic acid C-a
136	Syringyl C-1
133	Guaiacyl C-1, syringyl C-4
127	Phenolic acid C-1/2
116	Guaiacyl C-5
105	Cellulose/xylan C-1
89	Crystalline cellulose C-4
84	Amorphous cellulose/xylan C-4
72–76	Cellulose C-2/3/5; xylan C-2/3
65	Crystalline cellulose C-6
62	Amorphous cellulose C-6/xylan C-5
56	Lignin methoxyl
33	Cuticular wax methylene
30	Cutin methylene
21	Acetyl methyl

Table 6
Thioacidolysis analysis of ryegrass DMSO-soluble and Driselase-insoluble fractions

	S/G	Terminal G/ internal G	Terminal S/ internal S	Condensed lignin [a] (% dry weight)
Fraction				
DMSO-soluble	1.5	1.6	0.1	84.9
Driselase-insoluble	0.8	0.8	0.1	39.8

[a] Condensed lignin = total lignin (acetyl bromide) − total monomeric thioacidolysis products.

than expected from the acetyl bromide results as is normally observed [46]. The spectrum of the barley residue was typical of angiosperm lignins with a relatively high proportion of β-aryl ether linked syringyl units (153 ppm). The ryegrass residue, however, had relatively few syringyl units. A prominent ester peak at 168 ppm and considerable intensity around 130 ppm were consistent with the presence of bound phenolic acids, but these features are also present in the spectrum of cutin and signals at 29 and 32 ppm characteristic of the wax and ester methylene carbons of cutin were clearly visible. This indicates that the phenolic moiety of cutin, whose structure remains uncharacterised, contributed to the aromatic region of the ryegrass spectrum, although comparison of the relative sizes of the aromatic and hydrocarbon signals [47] suggests that its contribution was not dominant. Cutin methylene groups were also visible in the straw spectrum but the signal intensities were low. Otherwise, the CP/MAS spectra were in good agreement with the thioacidolysis results which showed that the lignin was typical in structure [22,38] with linear chains of syringyl units and a cross-linked guaiacyl network (Table 6).

Both the cross-linked network and the esterified phenolic acids would appear in the condensed fraction after thioacidolysis. Thioacidolysis showed that lignin present in the DMSO-soluble fraction differed substantially from that of the driselase-insoluble fraction (Table 6). The greatest differences were seen in the ryegrass fractions. Differences between the corresponding fractions from barley straw were similar but less distinct. The DMSO-soluble lignin fraction had a higher "condensed" lignin content, to which esterified phenolic acids may have contributed, but the uncondensed fraction had more syringyl and less internal (linear) guaiacyl residues than the driselase residues. The ferulic acid content of the DMSO-soluble fraction was much higher than that of the residue. The higher terminal G/internal G ratio in the former fraction suggested that the lignin existed as smaller, more discrete polymers and/or as a more branched structure. Smaller polymers are a more likely explanation for the solubility of this lignin fraction.

Histological studies have previously suggested differences in syringyl/guaiacyl contents between secondary and primary cell walls [48,49]. Secondary cell wall layers are thought more easily degraded by microorganisms than the primary cell wall layer of lignified cell walls [50,51]. It is possible that the small, highly condensed, syringyl rich DMSO-soluble lignin molecules originate in secondary cell walls. Secondary walls, unlike the primary wall layer, respond to alkali-treatment losing their ability to stain positive with the lignin stain acid phloroglucinol. This would be consistent with the response of DMSO-soluble LCC to alkali. The higher molecular weight, less condensed

guaiacyl rich LCC may originate in the more resistant primary cell wall layers or be an additional component of secondary wall layers. Cutinised epidermal cell walls also appear, from the CP/MAS data, to be resistant to enzymic degradation.

It seems likely that Graminaceous lignin does not form a single macromolecular complex throughout the wall but exists as small units attached to carbohydrate, some of which can be solubilised as the carbohydrate is enzymically degraded while the remainder, different in structure, is able to protect the carbohydrate from degradation and hence remains in an insoluble LCC.

4. Conclusions

A great variety of lignin–arabinoxylan linkages were evident in both species. Evidence was obtained for ester linkages between lignin and arabinose side chains for which 5-*O*-feruloyl-arabinose [1,3] is a convenient model. There was also evidence for ether linkages through the 5-position of arabinose in ryegrass. Alkali treatment of the DMSO-soluble fraction of ryegrass cleaved what may be assumed to be ester linkages between lignin and xylose, and the methylation data provided evidence for ether linkages through the 2-*O* and 3-*O* positions of xylose in the DMSO and driselase residues.

Comparison of the DMSO-soluble and driselase-insoluble fractions provided clear evidence of heterogeneity in the lignin components, with cutin making an additional contribution to the least soluble fraction. Both fractions appeared to be attached to both arabinose and xylose.

The presence of multiple forms of LCC has important implications for the agro-industrial use of plant cell walls. It would also implicate different control mechanisms in lignin biogenesis at different stages of cell wall development, which may provide practical tools for plant geneticists.

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