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LIGNIN–CARBOHYDRATE COMPLEXES FROM *LOLIUM PERENNE*

IAN M. MORRISON

Hannah Research Institute, Ayr KA6 5HL, Scotland

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Abstract—Lignin–carbohydrate and lignin–hemicellulose complexes were extracted sequentially from four varieties of *Lolium perenne* which had previously been separated into leaf, leaf sheath and stem tissue. The lignin–carbohydrate complexes from all tissue examined contained high proportions of glucose residues (44–57%). Leaf tissue complexes had the highest glucose content while stem and leaf sheath were very similar. The other neutral sugar residues present in these complexes were mainly arabinose and xylose. The arabinose : xylose ratio was considerably greater in the leaf tissue complex than in the other two tissues in all varieties. The complexes from leaf sheath and stem had higher lignin contents and higher esterified phenolic acid contents than the corresponding leaf tissue. The polysaccharide components of the lignin–hemicellulose complexes contained mainly xylose (63–77%) and arabinose (19–28%). Like the lignin–carbohydrate complexes, the leaf tissue lignin–hemicellulose complexes had lower lignin contents and, in general, greater arabinose : xylose ratios than the corresponding complexes from leaf sheath and stem tissue.

INTRODUCTION

IN A previous publication,[1] methods for the extraction of lignin–carbohydrate complexes from grass cell walls were examined and the compositions of the complexes determined. It was shown that alkali or dimethyl sulphoxide are capable of extracting the largest proportions of the cell walls but the composition of the complexes extracted by the two solvents are very different. The alkali-extracted complex contains about 13% lignin, has a nitrogen content greater than 0·7% and a carbohydrate component having the typical composition of a grass hemicellulose, namely about 70% xylose, 20% arabinose and 5% each of glucose and galactose. The dimethyl sulphoxide extracted complexes have a negligible nitrogen content (<0·2%), a low lignin content (*ca* 9%) and the monosaccharide residues in the carbohydrate portion closely resemble the monosaccharide composition of the intact cell wall, namely about 50% glucose, 30% xylose, 12% arabinose and 5% galactose. Accordingly, alkali-extracted complexes are now referred to as lignin–hemicellulose complexes (LHC) while lignin–carbohydrate complexes (LCC) denote complexes extracted by dimethyl sulphoxide.

The influence of increasing lignification on the decline in digestibility of forage crops has been reviewed.[2] It has been reported[3] that tetraploid varieties of grass have higher digestibilities than the corresponding diploid varieties at similar stages of growth. It is also well established that tissue from different parts of the plant have wide differences

[1] MORRISON, I. M. (1973) *Phytochemistry* **12**, 2979–2984.
[2] MORRISON, I. M. (1972) *J. Sci. Food Agric.* **23**, 455.
[3] DENT, J. W. and ALDRICH, D. T. A. (1968) *J. Br. Grassld. Soc.* **23**, 13.

in digestibility.[4] Presented here, in relation to a detailed analysis of diploid and tetraploid varieties of ryegrass,[5] is an examination of the yield and composition of the LHC and LCC from two diploid and two tetraploid species of ryegrass, harvested at comparable stages of growth, that have been separated into leaf, leaf sheath and stem tissue.

RESULTS AND DISCUSSION

Two early varieties of perennial ryegrass, S24 (diploid) and Barvestra (tetraploid) and two late varieties, S23 (diploid) and Barpastra (tetraploid) were harvested at a similar stage of growth (25–30% headed) and separated into leaf, leaf sheath and stem tissue. From the isolated cell walls of each tissue sample, LCC and LHC were prepared by successive extraction with dimethyl sulphoxide and 0·1N alkali. LCC were extracted first because some of the more labile bonds that occur in these complexes[6] would have been hydrolysed by alkali. The yield of LHC was expected to be lower than that reported previously[1] as successive extraction of LCC and LHC were carried out in the present investigation. It was expected that part of the LCC would be isolated as LHC if no dimethyl sulphoxide extraction had been carried out. Furthermore, the alkali used for extraction was much weaker (0·1N) than that used previously[1] (1N) but still possessed the necessary hydrolytic effect to release most of the LHC from the cell wall.

TABLE 1. ANALYSIS OF LIGNIN–CARBOHYDRATE COMPLEXES EXTRACTED BY DIMETHYL SULPHOXIDE FROM CELL WALLS OF DIFFERENT VARIETIES OF *Lolium perenne*

Tissue	Yield (% cell wall)	Lignin (% LCC)	Nitrogen (% LCC)	Neutral monosaccharide residues (% neutral sugars) Arabinose	Xylose	Mannose	Galactose	Glucose
S23								
Leaf blade	4·9	6·93	0·23	15·1	22·3	1·3	6·6	54·7
Leaf sheath	7·1	10·07	0·16	12·0	34·3	0·6	3·1	50·0
Stem	7·7	12·83	0·17	12·2	36·7	—	1·6	49·5
Barpastra								
Leaf blade	7·1	6·73	0·18	16·3	21·1	0·7	4·6	57·3
Leaf sheath	8·8	7·60	0·24	16·1	27·3	—	2·8	53·7
Stem	9·1	11·29	0·20	13·8	38·2	—	0·9	47·1

The yields of complexes, as percentages of the cell wall of each tissue, are shown in Tables 1 and 2. A comparison is shown between a diploid variety (S23) and a tetraploid variety (Barpastra); there was little difference between each of the diploid varieties and each of the tetraploid varieties. In all of the ryegrasses examined the yield of LCC was in the order stem tissue > leaf sheath tissue > leaf tissue while for the LHC the order was in general leaf tissue > leaf sheath tissue > stem tissue. The two diploids gave similar yields of each complex from similar tissue as did the tetraploids but the tetraploids gave higher yields of each complex than the corresponding diploids except for the LHC from stem tissue.

The analysis of the complexes for lignin and nitrogen is also shown in Tables 1 and 2. In virtually all of the samples, the LHC had higher lignin and nitrogen contents than the corresponding LCC. The order of lignin content for both types of complex was stem tissue > leaf sheath tissue > leaf tissue which is the same order as was found in the

[4] JOHNSTON, M. J. and WAITE, R. (1965) *J. Agric. Sci. Camb.* **64**, 211.
[5] WAITE, R. (1968–71) *Hannah Dairy Res. Inst. Rep.* 63.
[6] MORRISON, I. M. unpublished results.

intact cell walls. The nitrogen contents of the complexes are not thought to have any structural significance. The likely source is from residual protein in the cell wall co-extracted with the LCC and LHC. As proteins are generally more soluble in alkali than dimethyl sulphoxide, this lends support to this view. However, it has been reported that lignin does contain some nitrogenous constituents.[7,8]

TABLE 2. ANALYSIS OF LIGNIN–HEMICELLULOSE COMPLEXES EXTRACTED BY 0·1 N NaOH AFTER PRELIMINARY EXTRACTION WITH DIMETHYL SULPHOXIDE FROM CELL WALLS OF DIFFERENT VARIETIES OF *Lolium perenne*

Tissue	Yield (% cell wall)	Lignin (% LHC)	Nitrogen (% LHC)	Neutral monosaccharide residues (% neutral sugars) Arabinose	Xylose	Mannose	Galactose	Glucose
S23								
Leaf blade	9·1	7·20	1·20	27·3	63·0	0·3	4·9	4·5
Leaf sheath	8·7	11·60	0·88	28·1	63·0	—	4·7	4·2
Stem	6·1	14·31	0·61	19·0	76·6	—	2·5	1·9
Barpastra								
Leaf blade	13·0	7·31	0·97	25·2	64·6	0·2	4·7	5·2
Leaf sheath	11·4	10·23	0·70	21·2	70·7	—	3·3	4·9
Stem	4·2	13·48	0·48	21·3	70·1	—	3·4	5·2

The neutral monosaccharide residues present in the carbohydrate portion of the complexes are given in Tables 1 and 2. The LCC from leaf tissue had significantly higher glucose and galactose contents than the LCC from leaf sheath and stem tissues. Consequently, the combined arabinose and xylose content of leaf tissue LCC was lower. The arabinose to xylose ratio for both types of complex was in the order leaf tissue > leaf sheath tissue > stem tissue. In the case of the LHCs, the carbohydrate fraction consisted of at least 89% arabinose and xylose and, therefore, these complexes are likely to contain arabinoxylan polymer chains. The arabinose to xylose ratios from the LHCs may be lower than those occurring *in situ* since alkaline treatment may cause the loss of arabinose residues from hemicelluloses. In addition to the sugars shown in Tables 1 and 2, small amounts (>0·5%) of fucose and rhamnose were found which are probably derived from pectin. Small amounts of acidic sugars were also found in the hydrolysates but they were not characterized.

Further evidence for the similarity between leaf sheath and stem tissue was found in the UV spectra of their LCC and LHC. Typical spectra of the LCC are shown in Fig. 1 in the range 250–400 nm; the spectra of the LHCs have been discussed previously.[1] For both types of complex, the leaf sheath spectra only differ from the stem spectra in intensity of absorption. The major absorbing area of the LCC is around 325 nm, which indicates ester bonding, but a shoulder is clearly visible at 280 nm, confirming the presence of lignin. The spectra of LHC from leaf sheath and stem tissue show only one major absorbing area at 280 nm with a slight shoulder at 310 nm. The ratio of the absorbance of the LCC of stem tissue to that of leaf sheath tissue at both 280 and 325 nm is the same as the ratio of the absorbance of the LHC of stem tissue to that of leaf sheath tissue at 280 nm, namely 1·1 : 1. This is the same ratio as the lignin content of intact stem cell walls to leaf sheath cell walls. The LCC and LHC are thus believed to be representative fractions of the cell wall and that ester bonding in the

[7] BONDI, A. and MEYER, H. (1948) *Biochem. J.* **43**, 248.
[8] MEYER, H. and BONDI, A. (1952) *Biochem. J.* **52**, 95.

LCC and cell wall is associated with the lignin portion. The spectra of the LCC from leaf tissue gave a shoulder at 280 nm and a small absorbance at 325 nm while the LHCs show only a shoulder at 280 nm.

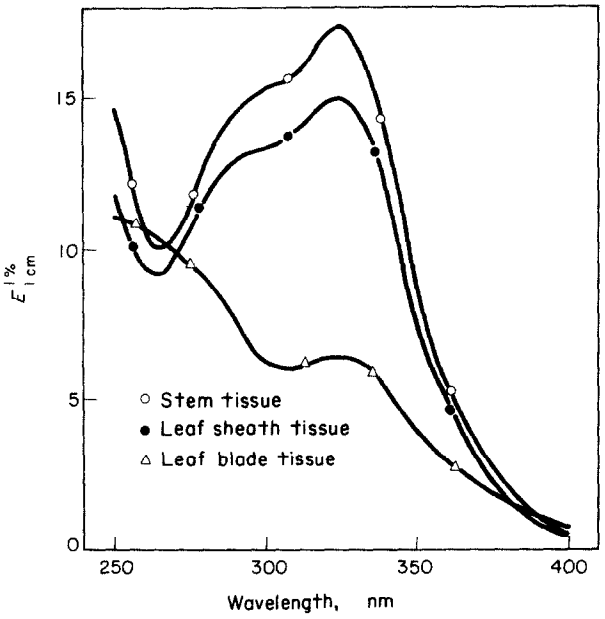

FIG. 1. UV SPECTRA OF LIGNIN–CARBOHYDRATE COMPLEXES FROM S23 RYEGRASS CELL WALLS. SOLVENT 10% DIMETHYL SULPHOXIDE IN WATER.

The UV spectra of the LCCs are very different from those previously reported for a mature grass;[1] the major absorbing area was around 280 nm with a shoulder around 325 nm. The amounts of esterified material present in the cell wall which absorb around 325 nm are dependent on the stage of maturity of the plant.[6,9] The esterified elements, which have been reported as *p*-coumaric and ferulic acids, rise in concentration during the early stages of growth but then decline until maturity and senescence when they are barely detectable.

The homogeneity of ryegrass LCC were assessed by molecular sieve chromatography on both Sephadex gels and controlled-pore glass granules. As many of the complexes were water insoluble, the eluant used throughout was 10% dimethyl sulphoxide in water. As well as dissolving the complexes the incorporation of dimethyl sulphoxide should reduce the possibility of aggregation of polysaccharides caused by inter-chain hydrogen bonding. The strong absorbance of dimethyl sulphoxide (76% transmittance at 280 nm) does not allow this solvent to be used alone since it interferes with the lignin determination at 280 nm. The LCC were eluted at the void volume from all Sephadex gels up to G150 and from CPG-10-370Å glass granules in a single symmetrical peak which contained both lignin and carbohydrate. In molecular sieve materials of greater porosity, namely G200 and CPG-10-1250Å the complexes were retained to a slight extent and gave skew peaks but as before the carbohydrate and lignin co-chromatographed indicating that they were covalent complexes.

[9] HARTLEY, R. D. (1973) *Phytochemistry* **12**, 661.

The origin of the large quantities of glucose residues found in ryegrass LCC is at present being investigated. Xyloglucans, similar in composition to the ryegrass LCC, have been reported from many plant sources[10–12] but may occur only in primary cell walls.[13] Rees *et al.*[14] extracted a water-soluble component from mustard seeds which appeared to be cellulose held in solution by an encrustation of hemicellulosic and pectic polysaccharides. The LHCs were not homogeneous on molecular sieve chromatography but in all samples, carbohydrate and lignin were associated implying that these complexes were typical hemicellulosic polysaccharides covalently bound to lignin by alkali-stable bonds.

EXPERIMENTAL

Two perennial ryegrass varieties (*Lolium perenne* L.) with normal diploid chromosome systems, S23 and S24, were examined, together with two corresponding tetraploid varieties, Barpastra and Barvestra. The tillers were harvested at a similar stage of growth when the heads were 25–30% emerged. Immediately on harvesting, the tillers were separated into leaf, leaf sheath and stem subsamples. The general procedures for drying, hammer-milling, preparation of cell walls, ball-milling and extraction of LCC and LHC have been previously described.[1] The LCC were isolated by a method involving extraction at room temp. with dimethyl sulphoxide for 7 days. LHC were extracted with 0·1N NaOH. Lignin, nitrogen and total carbohydrate were determined as before.[1]

Analysis of neutral monosaccharides. Samples (5 mg: in duplicate) were hydrolysed by heating at 100° for 16 hr with N H_2SO_4 (1 ml) containing myoinositol (2 mg/ml) as internal standard. After neutralization with $BaCO_3$, and filtering, excess Ba^{2+} ions were removed from the filtrate with Amberlite IR 120 (H^+) resin. After refiltering, the filtrate was evaporated to dryness before being reduced with a solution of $NaBH_4$ (10 mg/ml) at room temp. for 1 hr. HOAc was added to destroy excess $NaBH_4$, the sample evaporated to dryness and then re-evaporated 5× with 5% HOAc in MeOH (2 ml) to remove borate ions as the volatile methyl borate. The dry sample was acetylated with Ac_2O (1 ml) at 100° for 1 hr. On cooling to 5° $CHCl_3$ (2 ml) was added and the soln of alditol acetates was extracted with 2 × 5 ml 2N NaOH and 2 × 5 ml H_2O to remove Ac_2O. The $CHCl_3$ soln was filtered through Whatman 1PS-filter paper and evaporated to dryness. The alditol acetates were analysed by the method of Sawardeker *et al.*[15] using a 1·5 m column and isothermal conditions at 180°.

Molecular sieve chromatography. Sephadex gels or Corning controlled-pore glass granules were suspended in 10% dimethyl sulphoxide in H_2O washed 2× with the solvent and allowed to equilibrate. After packing the columns, and washing with 10 bed vol. of solvent, they were ready for use. Column effluents were monitored for carbohydrate by the PhOH–H_2SO_4 method and for lignin by UV absorption at 280 nm.

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