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ISOLATION AND ANALYSIS OF LIGNIN-CARBOHYDRATE COMPLEXES FROM *LOLIUM MULTIFLORUM*

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Abstract—Lignin-carbohydrate complexes were extracted from grass cell walls by a variety of solvents. The yield of complexes was greatly enhanced if the sample was finely milled in a ball mill; dimethyl sulphoxide and N alkali extractions gave the highest yields. Hydrolysis showed that the carbohydrate fraction of the alkali-extracted complex contained mainly xylose (*ca.* 70%) and arabinose (*ca.* 20%) whereas the dimethyl sulphoxide extracted complex contained glucose (*ca.* 50%), xylose (*ca.* 30%), arabinose (*ca.* 12%) and galactose (*ca.* 5%). The UV spectrum of the dimethyl sulphoxide extracted complex showed lignin absorbance at 280 nm, but, in addition, ester bonding was also observed by the presence of a secondary absorbing region near 325 nm. This secondary absorbing region was absent from the spectrum of the alkali-extracted complexes. Fractionation of the complexes by ethanol precipitation gave a major component which appeared homogeneous by molecular sieve chromatography and had a MW of $\geqslant$ 150,000.

INTRODUCTION

RUMINANT animals have the ability to utilize a large proportion of the structural carbohydrates of the plant cell wall present in their diet as sources of energy. The actual proportion of these polysaccharides which are digested by the animal is dependent on several factors, but one of the most important is the state of maturity of the plant which is closely associated with the lignin content of the plant cell wall. Waite *et al.*[1] reported that as *S*23 ryegrass matured from a young leafy stage through to the seed-setting stage, the apparent digestibility of the organic matter fell from 86 to 62% whilst that of cellulose fell from 92 to 73%, and that of the hemicelluloses from 93 to 56%.

Many workers consider lignin to be undigested, although there are reports to the contrary.[2,3] Waite *et al.*[1] reported that, at the young leafy stage, 23% of the true lignin was digested but, by the seed-setting stage, lignin was undigested. Of the individual plant components that can readily be measured, lignin content shows the highest correlation with digestibility, but the quantitative change in lignin content alone is not sufficient to account for the overall decrease in digestibility and it is believed that lignin interacts with some other plant component(s) to depress the overall digestibility of the plant.[4] As the digestibility of the hemicelluloses in the example given above[1] was reduced to the greatest extent, it would appear logical to suggest that lignin-hemicellulose complexes might be implicated.

[1] WAITE, R., JOHNSTON, M. J. and ARMSTRONG, D. G. (1964) *J. Agr. Sci. Camb.* **62,** 391.
[2] MCANALLY, R. A. (1942) *Biochem. J.* **36,** 392.
[3] SULLIVAN, J. T. (1955) *J. Anim. Sci.* **14,** 710.
[4] MORRISON, I. M. (1972) *J. Sci. Food Agr.* **23,** 455.

The possibility of bonding between lignin and the hemicellulosic polysaccharides is a much debated issue in wood chemistry. Earlier, Merewether[5] came to the conclusion that, although some of the carbohydrate may be free, there exists in wood a lignin-carbohydrate complex. Since then, three laboratories have reported further major evidence to support the lignin-carbohydrate complex theory. Pew,[6] using cellulolytic enzymes, could not remove all the carbohydrate from wood without degrading the lignin. Björkman[7] isolated lignin-carbohydrate complexes from wood in which the carbohydrate composition resembled that found in the hemicellulose fraction of the wood. The lignin-xylan and lignin-carbohydrate complexes isolated by Bolker and Wang[8] could not be separated into individual components by electrophoresis.

In the present study, the extraction of lignin-carbohydrate complexes from perennial grass has been investigated and their composition and homogeneity have been assessed.

RESULTS AND DISCUSSION

*S*24 ryegrass tillers, harvested at the seed-setting stage, were rapidly dried at 100° in a current of air. Preliminary extractions were carried out to remove lipids, waxes, low MW phenols and water-soluble carbohydrates. The residue, or crude cell wall preparation, contained cellulose, hemicelluloses and pectic substances, lignin and protein, which presumably arose from intracellular material which had been denatured and hence insolubilized during the preliminary extraction or drying procedure. Although all the available evidence suggests that complexing occurs only between lignin and the hemicellulosic polysaccharides,[4,7] the possibility of lignin-pectin or lignin-protein complexes should not be overlooked and so no attempt was made to remove pectin or protein from the grass samples. Lignin-protein complexes are unlikely to occur as it is only in the primary cell wall that a protein (extensin) has an integral role[9] while lignin is only laid down when secondary thickening of the wall takes place.[10] There is no evidence that protein is an integral component of the secondary cell wall.

A number of different solvents were investigated to determine which were capable of dissolving lignin-carbohydrate complexes from the crude cell wall preparations. The solvents were dimethyl sulphoxide and dimethylformamide, which have high dielectric constants, 4M guanidine hydrochloride (which has been used for the disruption of hydrogen bonding),[11] phenol—acetic acid—water (1:1:1) (which has been used to isolate plant proteins and peptides),[12] 50% acetic acid (which has been used in the extraction of lignin),[13] 15% trichloroacetic acid and N alkali (which is used for the isolation of hemicelluloses from plant holocelluloses).[14] However, the critical factor was the particle size of the sample. When the sample was hammer-milled to pass a sieve of 0·7 mm diameter mesh, very few of the solvents gave significant yields of lignin–carbohydrate complexes. Ball-milling for 24 hr reduced the particle size considerably but still left a fibrous material which provided increased yields of these complexes, but ball-milling for 7 days to reduce the material to a

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[6] PEW, J. C. (1957) *TAPPI* **40,** 553.
[7] BJÖRKMAN, A. (1957) *Svensk Papperstidn.* **7,** 243.
[8] BOLKER, H. I. and WANG, P. Y. (1969) *TAPPI* **52,** 920.
[9] LAMPORT, D. T. A. (1965) *Advan. Botan. Res.* **2,** 151.
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[11] SCHELLMAN, J., SIMPSON, R. B. and KAUZMANN, W. (1953) *J. Am. Chem. Soc.* **75,** 5152.
[12] BAGDASARAN, M., MATHESON, N. A., SYNGE, R. L. M. and YOUNGSON, M. A. (1964) *Biochem. J.* **91,** 91.
[13] KAWAMURA, I. and HIGUCHI, T. (1953) *J. Soc. Textile Cellulose Ind. (Japan)* **9,** 9.
[14] ASPINALL, G. O. (1959) *Adv. Carbohyd. Chem.* **14,** 437.

non-fibrous fine powder was necessary to allow the solvents fully to penetrate the sample and dissolve reasonable amounts of the complexes (see Table 1). The use of toluene in the ball-mill did not have any hydrolytic effect on the cell wall since the toluene, after recovery, contained no extracted material.

TABLE 1. THE EFFECT OF MILLING TREATMENT AND SOLVENT USED ON THE YIELD OF LIGNIN-CARBOHYDRATE COMPLEXES FROM CELL WALLS OF MATURE *S*24 RYEGRASS (% DM)

Solvent	No ball milling ultrasonic treatment	24 hr ball milling ultrasonic treatment	7 days ball milling ultrasonic treatment	7 days ball milling 7 days stirring
Dimethyl sulphoxide	2·4	3·5	7·2	6·9
Dimethylformamide	nil	nd	1·0	1·4
Guanidine hydrochloride (4M)	nil	nd	4·2	4·4
Phenol-acetic acid-water (1:1:1)	0·9	nd	3·3	nd
Acetic acid (50%)	1·1	nd	4·5	3·9
Trichloroacetic acid (15%)	nd	nd	nil	nd
Sodium hydroxide (N)	19·6	nd	24·6	

nd—not determined.

Two different methods of extraction, namely stirring on a magnetic stirrer and ultrasonic treatment were compared. The yields of complexes, as seen in Table 1, were comparable.

Extraction with N alkali dissolved the highest percentage of the cell wall but the composition of the isolated lignin-carbohydrate complex was very different to that of the other complexes. With the exception of trichloroacetic acid, the other solvents examined were all capable of extracting small but significant amounts of the cell walls (see Table 1) but, of these solvents, dimethyl sulphoxide gave by far the highest yield. The yield from dimethyl sulphoxide extraction was more than seven times that obtained by extraction with dimethylformamide. Repeated extraction with dimethyl sulphoxide by the ultrasonic treatment did not significantly increase the total yield of complex. Of the total complex isolated from the five successive extractions, about 88% was extracted on the first extraction. The monosaccharide composition, as determined by PC, showed that the extracts were quantitatively similar.

TABLE 2. ANALYSIS OF LIGNIN-CARBOHYDRATE COMPLEXES EXTRACTED WITH DIFFERENT SOLVENTS BY ULTRASONIC TREATMENT FROM CELL WALL MATERIAL AFTER BALL MILLING FOR 7 DAYS (% OF LIGNIN-CARBOHYDRATE-COMPLEX)

Solvent	Lignin	Total carbohydrate* As anhydroglucose	As anhydroxylose	Nitrogen
Dimethyl sulphoxide	9·15	88	48	0·23
Dimethylformamide	8·24	87	47	0·77
Guanidine hydrochloride (4M)	5·60	88	50	1·88
Phenol–acetic acid–water (1:1:1)	4·16	86	47	2·35
Acetic acid (50%)	8·75	84	46	1·01
NaOH (N)	13·10	97	59	0·72

* By phenol–sulphuric acid method.[23]

Each extracted lignin-carbohydrate complex has been analysed for lignin, total carbohydrate and nitrogen content (Table 2). The phenol–acetic acid–water and guanidine hydrochloride extracted considerable amounts of nitrogenous materials and small amounts of lignin. All samples contained similar amounts of total carbohydrates. Alkali extracted the greatest percentage of lignin and there was not much difference in the lignin content of the complexes extracted by three remaining solvents although dimethyl sulphoxide extracted the least amount of nitrogenous material (< 1·5% crude protein). The lignin-carbohydrate complex extracted with alkali, as would be expected, had a monosaccharide composition (determined by GLC method) similar to that of grass hemicelluloses, namely *ca.* 70% xylose, 20% arabinose and about 5% each of galactose and glucose (Table 3). The other lignin-carbohydrate complexes gave monosaccharide ratios after hydrolysis different from that of the alkaline extract but were all similar to each other. The approximate composition was 50% glucose, 30% xylose, 12% arabinose and 5% galactose, which is not dissimilar to the overall composition of the cell wall carbohydrates.[1]

TABLE 3. MONOSACCHARIDE COMPOSITION OF LIGNIN-CARBOHYDRATE COMPLEXES EXTRACTED WITH VARIOUS SOLVENTS BY ULTRASONIC TREATMENT (% EACH SUGAR IN HYDROLYSATE AS MEASURED BY GLC METHOD)

Solvent	Arabinose	Xylose	Mannose	Galactose	Glucose
Dimethylsulphoxide	16·5	29·6	*ca.* 1·0	3·3	49·2
Dimethylformamide	17·3	30·4	—	1·8	50·5
Guanidine hydrochloride (4M)	13·3	30·1	1·2	3·5	51·9
Phenol–acetic acid–water (1:1:1)	13·1	28·5	—	3·3	55·1
Acetic acid (50%)	12·2	30·1	—	4·0	53·7
NaOH (N)	17·6	72·4	—	4·8	5·2

Both lignin and proteins have the ability to absorb light in the region of 280 nm. The various lignin-carbohydrate complexes all showed a maximum in this region. The $E^{1\%}_{1\,cm}$ values are consistent with a summation of the protein and lignin contributions if it is assumed that lignin is a stronger absorber than protein. There was, however, a second absorbing area, centred around 325 nm, in the spectrum of all except the alkaline extracted complexes. This is the spectral region where esters absorb and as esters are cleaved by alkali, the alkaline-extracted complexes would not be expected to contain any of these bonds. Ester bonds involving ferulic and *p*-coumaric acids have been reported in milled-wood lignins from grasses[15] and in carbohydrate esters released from grass cell walls by the action of cellulase.[16]

The solutions that remained after precipitation of the lignin-carbohydrate complexes in 80% ethanol still contained small amounts of lignin and carbohydrate. As far as could be ascertained by colorimetric methods, the material not precipitated accounted for about 10% of the total extracted solid. Fractional precipitation was attempted in order to gain some information on the distribution of carbohydrate and lignin in the complexes. In a typical experiment, a dimethyl sulphoxide extract was fractionally precipitated with ethanol. Up to 50% ethanol concentration, no material was precipitated. Between 50 and 75% ethanol concentration, 84% of the carbohydrate (estimated by the phenol-sulphuric acid method)

[15] HIGUCHI, T., ITO, Y., SHIMADO, M. and KAWAMURA, I. (1967) *Phytochemistry* **6**, 1551.
[16] HARTLEY, R. D. (1973) *Phytochemistry* **12**, 661.

was precipitated (sample A). Concentration of the residual solution to 0·1 of its original volume and addition of 10 volumes of ethanol–ether (1:1) precipitated a further 11% of the carbohydrate (sample B) while the remainder was left in solution (sample C). Each of the three subsamples was analysed by molecular sieve chromatography on Sephadex G150. All three samples contained a component with MW $\geqslant$ 150 000 which was eluted in the void volume and which contained both lignin and carbohydrate. The ratio of lignin to carbohydrate (as determined by the ratio of the absorbance at 280 nm to the phenol–sulphuric acid values) varied quite considerably, carbohydrate being the dominant component of A while there was very little carbohydrate in C. It is suggested that a series of lignin-carbohydrate complexes with a range of lignin to carbohydrate ratios are extracted with dimethyl sulphoxide and that the procedure above has arbitrarily produced fractionation. No lower molecular weight components were found in A but such components, containing both lignin and carbohydrate, were found in B and C. It is not known if these components were cleaved from higher molecular weight components during the fractionation procedure.

The full spectra of the fractions from the Sephadex G150 column separations of samples A, B and C which were eluted at the void volume ($K_{av} = 0$) were examined in the range 250–430 nm. Each showed maximum absorbance at about 280 nm with secondary absorbance around 325 nm. This indicates that esterified components are strongly associated with the lignin component of the complexes. The individual monosaccharide composition of each of these fractions eluted at the void volume was quantitatively identical to the composition of the original sample, showing that no fractionation of different carbohydrate polymers had occurred.

Molecular sieve chromatography of the dimethyl sulphoxide extracted lignin-carbohydrate complex on Sephadex G200 gave a single component eluted at the void volume. However the shape of the distribution was distinctly skew on the low molecular weight side suggesting that two components could be present but this could not be confirmed.

The alkaline extract, on neutralization to pH 5 with acetic acid, remained soluble. It is thus not classified as an A-type hemicellulose.[17] Since it was precipitated by the addition of ethanol it must be a B-type hemicellulose. The structural features which classify a hemicellulose as one or other of these types are not well defined but it is believed that A-types are virtually linear molecules. The presence of side chains of L-arabinose or 4-*O*-methyl-D-glucuronic acid may be other determining factors.

The MW range of hemicelluloses is not easy to determine by molecular sieve chromatography since it is reported that aggregation can occur in aqueous solutions.[18] The lignin-hemicellulose complex extracted with alkali was analysed on Sephadex G100. At least five components were observed ranging in MW from > 100 000 to about 10 000. The most important observation was that lignin and carbohydrate were associated in each of the five components, indicating that a true complex and not a mixture was present. It is probable that many of the components were associated forms of the lowest MW component. Its MW (*ca.* 10 000) is consistent with the reported DP of ryegrass xylans at around 50.[19]

EXPERIMENTAL

All reagents used for extraction were of the highest purity available and, where possible, were redistilled before use.

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[18] BLAKE, J. D. and RICHARDS, G. N. (1971) *Carbohyd. Res.* **18,** 11.
[19] ALAM, M. and MCILROY, R. J. (1967) *J. Chem. Soc.* **C,** 1577.

Plant material. A mature sample of *S*24 ryegrass was used. After drying in a forced-draught oven at 100–110° for 45 min the sample was milled to pass a 0·7 mm dia. screen.

Preparation of crude cell walls. The grass dry matter was extracted with azeotropic EtOH–C_6H_6 (1:2) in a Soxhlet followed by H_2O at 60–65° until the filtrate contained no carbohydrate. The residue, or crude cell wall material, was dried by solvent exchange through EtOH, Me_2CO and Et_2O (yield 78%).

Ball milling of cell walls. Crude cell walls in toluene were milled in a porcelain pot with a steatite charge (Pascall Engineering Co. Ltd.) for periods up to 7 days. Toluene was removed by filtration and the sample air-dried to a buff powder (yield = 100%).

Extraction of lignin-carbohydrate complexes. Crude cell walls were extracted with the appropriate solvents (except NaOH) by stirring for 7 days in a stoppered flask on a magnetic stirrer or by ultrasonic treatment (MSE 100W instrument operated at frequency of 20 000 Hz) for 30 min taking care to keep the temp. below 40°. In both methods the sample-solvent ratio was 1:20. The lignin-carbohydrate complexes were isolated by centrifuging off the residue at 12 000 *g* and washing the residue once with extractant. The combined centrifugates were added to 4 vols. EtOH and the precipitated complexes collected by centrifuging, before drying by solvent exchange as above. The extraction with N NaOH was done by rolling the samples overnight in alkali in polypropylene bottles in which the air had been replaced by N_2 before sealing. The residue was removed by filtration and the extract neutralized with HOAc; the complex was then precipitated by the addition of EtOH to give a concentration of 80% EtOH.

Molecular sieve chromatography was done on columns of Sephadex G150 and G200 (90 × 1·5 cm) using either H_2O or 10% dimethyl sulphoxide as eluant.

Hydrolysis of the complexes was achieved by either N H_2SO_4 or 2N trifluoroacetic acid at 100° for 18 hr in sealed tubes. The H_2SO_4 was neutralized with $BaCO_3$, the solution filtered through a glass fibre filter paper (GF/A), treated with Amberlite 1R120 (H^+) resin to remove Ba^{2+} ions, filtered and evaporated to dryness. Trifluoroacetic acid was removed on a rotary evaporator below 40°.

PC estimation of monosaccharides. Monosaccharides were separated in EtOAc–HOAc–HCOOH–H_2O (18:3:1:4) and EtOAc–Py–H_2O (10:4:3) and estimated by the colorimetric method of Wilson.[20]

GLC estimation of reducing sugar. Monosaccharides were reduced with $NaBH_4$ and then acetylated by the method of Albersheim *et al.*[21] except that the alditol acetates were dissolved in $CHCl_3$ and excess Ac_2O was removed by extraction with NaOH. The alditol acetates were separated essentially as described by Sawardeker *et al.*[22] except that a 1·5 m column was used. *Total carbohydrate* was determined by the PhOH–H_2SO_4 method.[23] *Lignin* was determined by the acetyl bromide procedure.[4,24] *Nitrogen* was determined by the semi-micro Kjeldahl method using a colorimetric technique to determine the NH_3 released.[25]

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