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DELIGNIFICATION AND HEMICELLULOSE EXTRACTION OF CELL WALLS OF *LOLIUM PERENNE* AND *TRIFOLIUM PRATENSE*

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Key Word Index—*Lolium perenne*; Gramineae; ryegrass; *Trifolium pratense*; Leguminosae; red clover; cell walls; delignification; hemicelluloses; gel filtration.

Abstract—The rates of delignification of samples of *Lolium perenne* at four different stages of maturity and a sample of *Trifolium pratense* by the action of sodium chlorite–acetic acid have been determined. For samples with lignin contents of $<8\%$, delignification was essentially complete within 30 min. The yield and composition of hemicelluloses obtained by alkaline extraction of cell walls was dependent on the duration of the delignification reaction.

INTRODUCTION

The plant cell wall comprises the polysaccharides cellulose, hemicelluloses and pectin together with the aromatic polymer lignin. Since lignin acts as a cementing agent in the wall[1] it has been considered necessary to delignify the cell walls by chemical means in order that the hemicellulosic polysaccharides can be completely extracted. One of the most frequently used methods for delignification employs chlorine dioxide [2] which is prepared *in situ* from sodium chlorite and acetic acid [3, 4]. Since these techniques were used for delignifying woods, the reaction times were lengthy. Whistler *et al.* [5] showed that a much shorter period of reaction (1 hr) was required to delignify *Zea mays* cobs and that the resultant holocellulose contained only 10% of the original lignin. Extended delignification (10-hr period) did not completely remove the remainder of the lignin but caused a very significant loss of carbohydrate from the holocellulose. However, in *Avena sativa* [6] the loss of cell wall carbohydrate was usually small but could amount to 10% of the total hemicellulose and, from the carbohydrate composition it appeared that a glucan was preferentially lost.

As our interests are concerned with the nature of the carbohydrates in the plant cell wall and the digestion of these components in the rumen, and since most forage crops when harvested at stages of growth which are of agricultural importance have low lignin contents (3–8%), it was necessary to determine the rate of delignification of these plant materials during the early part of the delignification reaction and to ascertain how much and what type of carbohydrate would be lost during the delignification reaction. Since it has been reported [7] that there are differences in the composition of the hemicelluloses extracted with alkali before and after delignification of grasses, the extraction of the hemicellulosic carbohydrates with different concentrations of alkali has also been investigated in relation to the rate of delignification.

RESULTS AND DISCUSSION

The rates of delignification of the four ryegrass samples and the clover sample are shown in Fig. 1. The rate of removal of lignin from the grass samples in the first 30 min of reaction was dependent on the amount of lignin originally present in the sample. The least mature samples were delignified very rapidly while the more mature ones were delignified more slowly. During the 2 hr period of delignification that was investigated the lignin content of all samples had decreased to a constant value of between 1 and 2% of the original plant dry

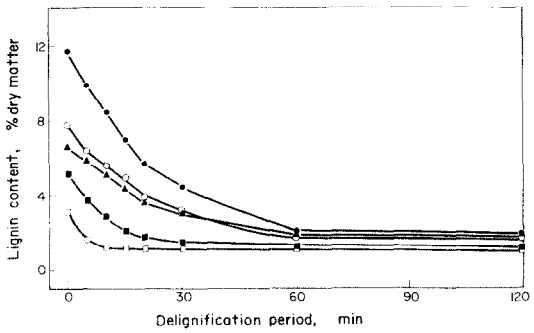

Fig. 1. Rate of delignification of four grass (*Lolium perenne*) samples and one clover (*Trifolium pratense*) sample. □—□ Grass sample 1; ■—■ grass sample 2; ○—○ grass sample 3; ●—● grass sample 4; △—△ clover sample.

matter after about 1 hr. When one sample was delignified for an extended period (7 hr) the lignin content only decreased further by a very small amount. However it was observed, in agreement with the results of Whistler *et al.* [5], that the hemicellulosic polysaccharides were removed at a continuously increasing rate the longer the delignification reaction was allowed to proceed.

The effect of short exposures to the delignifying reagents on the extraction of the total hemicellulose from grass tissue was investigated as follows. All samples were given a total delignification period of 1 hr with the reagents being added every 15 min. However, the 1 hr period was interrupted after n min and hemicellulose Fraction 1 was extracted from the residue with alkali. The alkali-free residue was delignified for a further $(60-n)$ min before being re-extracted with alkali of the same molarity to yield hemicellulose Fraction 2. The yield and lignin content of hemicellulose Fractions 1 and 2 (calculated on a lignin-free basis and shown as a percentage of the cell wall) changed depending on the length of the initial delignification period. Hemicellulose Fraction 1 increased in yield as the initial period of delignification increased up to about 20–30 min but then noticeably declined. The yield of hemicellulose Fraction 2 from the sample that had no initial period of delignification ($n = 0$) was quite high but gradually declined to a negligible amount the longer the initial period of delignification had been. The sum of Fractions 1 and 2, or total hemicellulose, was not constant, gradually rising with the length of the initial period of delignification up to 15–20 min and then declining to a value very similar to that observed initially. The results given in Fig. 2 are for the extraction (with M KOH) of the grass sample used previously with a lignin content of 12% (5 g grass per 100 ml M KOH). Extraction of clover with M KOH and extraction of grass and clover samples with 0·1 and 4·5 M KOH gave essentially similar results. The only significant difference, as was expected, was that the greater the molarity of the alkali used for extraction, the greater was the yield of total hemicellulose.

The monosaccharide compositions of the hemicellulose Fractions 1 and 2 obtained after the different lengths of the initial delignification period as shown in Fig. 2 were determined and the results are shown in Table 1. The sample used was the S23 rye grass with lignin content of 12%, and the hemicellulose fractions were extracted with M KOH/(5 g sample/100 ml alkali). From previous results [7] it has been shown that the major polysaccharides in the hemicellulosic fraction of grasses are an arabinoxylan, a galactoarabinoxylan and a glucan. This is in agreement with results from other members of the Gramineae [8–11]. Therefore, when considering the monosaccharide residues present in the various hemicellulose fractions, the arabinose, xylose and galactose residues ought to be considered separately from the glucose residues. The glucose content of the hemicellulose fraction obtained from lignified tissue was relatively low but the proportion in the hemicellulose rose steadily during the first 10 min of delignification and then remained fairly constant. When the

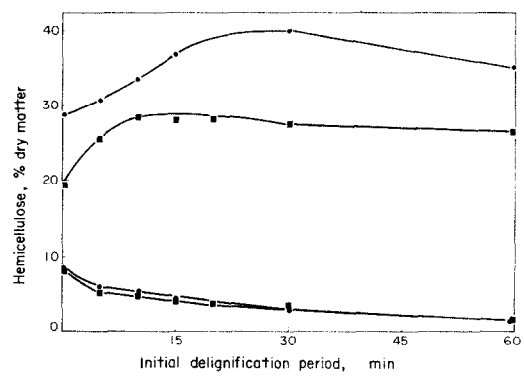

Fig. 2. Yields of hemicellulose fractions from two grass samples obtained after different periods of delignification. Upper graphs are Fractions 1 and lower graphs are Fractions 2. The total time of delignification for all samples was 60 min.

Table 1. Neutral monosaccharide composition of hemicellulose fractions from ryegrass obtained after different delignification times (% each sugar in hydrolysate as measured by GLC method)

Hemicellulose fraction	Delignification reaction time (min)*	Neutral monosaccharide residues				Radio of xylose:arabinose
		Arabinose	Xylose	Galactose	Glucose	
1A	0	20·7	64·7	6·6	8·0	3·1
1B	5	17·7	65·8	6·5	9·9	3·7
1C	10	16·7	66·3	5·4	11·7	4·0
1D	15	14·2	69·6	4·9	11·4	4·9
1E	20	16·1	67·6	5·7	10·7	4·2
1F	30	15·6	67·4	5·8	11·2	4·3
1G	60	13·6	68·0	5·6	12·8	5·0
2G	0	12·7	68·4	5·6	13·3	5·4
2F	30	13·8	64·4	6·2	15·6	4·7
2E	40	15·5	61·6	6·8	16·1	4·0
2D	45	14·1	63·2	6·8	15·9	4·5
2C	50	18·6	56·4	8·1	16·9	3·0
2B	55	17·9	56·6	8·3	17·2	3·2
2A	60	18·6	56·2	8·9	16·2	3·0

* The delignification reaction time for hemicellulose Fraction 2 was calculated as $60 - T(1)$ min.

partially delignified residues were further delignified so that the total reaction time was 1 hr and then re-extracted to give the second hemicellulose fraction, the glucose content again increased as the length of the second period of delignification increased. Taken overall, the total amount of glucose residues extracted in hemicellulose Fractions 1 and 2 was constant, irrespective of the time of the initial period of delignification.

As all of the arabinose residues in grass hemicelluloses are associated with xylose residues in the form of arabinoxylans, it is important to consider the molar ratios of these sugar residues. In these experiments, for hemicellulose Fraction 1, the xylose:arabinose ratio tended to rise, the longer the delignification period. The xylose: arabinose ratio for hemicellulose Fraction 2 were similar to Fraction 1.

Buchala *et al.* [6] have shown that the delignification liquors from *A. sativa* tissue contained carbohydrate that was polymeric in structure. Qualitatively, the sugar residues present were similar to those found in the total hemicellulose but quantitatively, a glucan predominated. When the delignification liquors from grass samples were fractionated on a column of Sephadex G50, *ca* 60% of the total carbohydrate eluted was present as a single sharp peak in the void volume indicating that the MW was >10000 (i.e. DP $>$ 75 pentose units). For the sample extensively examined above (Table 1) and delignified for a period of 1 hr this sharp peak from the Sephadex G50 column contained arabinose (16%), xylose (24%), galactose (24%) and glucose (35%) residues. The UV spectrum of the above material, showed a shoulder around 280 nm indicating that a small amount of lignin was still covalently bonded to the polysaccharide; the sample did not contain protein. The longer the delignification reaction was allowed to continue, the greater was the amount of carbohydrate in the delignification liquors and the ratio of the individual monosaccharide residue in the material excluded from the Sephadex G50 column also changed. With a delignification period of only 5 min and using the S23 ryegrass sample with lignin content of *ca* 3%, the composition was arabinose (21%), xylose (13%), galactose (6%) and glucose (60%). The values altered progressively with longer delignification periods and after 1 hr the composition was arabinose (26%), xylose (30%), galactose (11%) and glucose (32%).

It was suspected that sodium chlorite might cause oxidation of the cell wall polysaccharides, so samples of cellulose and a grass hemicellulose were individually submitted to the delignification procedure for 1 hr. The soluble products were chromatographed on Sephadex G50 and examined for total carbohydrate and uronic acids by the methods of Dubois *et al.* [12] and Blumenkrantz and Asboe-Hansen [13]. The treatment of cellulose gave no soluble carbohydrate product and the products from the grass hemicellulose gave an

identical ratio of uronic acid to total carbohydrate as was present in the original sample. This shows that no oxidation to uronic acid takes place and that no oxidation takes place at other carbon atoms in the sugar ring.

EXPERIMENTAL

General methods. Hemicellulose samples (5 mg; in duplicate) were hydrolysed by heating with 2 M CF_3COOH as previously described[14]. The derived glycitols were acetylated and estimated quantitatively by GLC on a column (1·5 m) of 3% EC'NSS-M on Gas-Chrom 2 as described previously[15]. Lignin was determined by the acetyl bromide method[16].

Plant material. The samples of S23 ryegrass (*Lolium perenne*) have been described previously [17]. The sample of red clover (*Trifolium pratense* cv. Hungaropoly) was obtained in the same way. Crude cell wall preparations were isolated as described previously [17]. Whatman cellulose powder was used as the cellulose sample and the grass hemicellulose was isolated from S23 rye grasses (lignin content 12%) by extraction with M KOH following delignification. After neutralization with HOAc, the hemicellulose was precipitated with 3 vol. of EtOH.

Delignification of the plant tissue. The procedure used was essentially that of Wise *et al.* [4] where HOAc and $NaClO_2$ were added to the reaction mixture every 15 min. The reaction was stopped at the appropriate times by immediately cooling the sample and filtering through ice. The partially delignified residues were washed copiously with H_2O and dried by solvent exchange through EtOH, Me_2CO and Et_2O.

Extraction of the hemicelluloses. Delignified plant tissues were extracted with KOH of varying molarity by rolling in sealed polypropylene bottles from which the air had been displaced by N_2. After filtering and washing the residue with H_2O, the pH of the filtrate was adjusted to 5 with HOAc and the hemicellulose precipitated by adding 3 vol. of 95% EtOH. The extracts were dried by solvent exchange as above.

Purification of delignification liquors. N_2 was bubbled through the liquors plus H_2O washings to remove any traces of dissolved ClO_2. The sol was concentrated to a small vol and applied to a column of Sephadex G50 (90 × 4 cm) which was eluted with H_2O. The column effluent was collected on a fraction collector and the tubes were analysed for total carbohydrate by the PhOH H_2SO_4 [12] method. The appropriate tubes were pooled, evaporated to dryness and the individual monosaccharide residues in the polysaccharide determined as described under General Methods.

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