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Fractional and structural characterization of hemicelluloses from perennial ryegrass (*Lolium perenne*) and cocksfoot grass (*Dactylis glomerata*)

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Abstract—Sequential three-stage treatments with 80% EtOH containing 0.2% NaOH, 2.5% H_2O_2–0.2% EDTA containing 1.5% NaOH and 2.5% H_2O_2–0.2% TAED containing 1.0% NaOH at 75 °C for 3 h released 8.0% and 10.4%, 79.1% and 77.0% and 12.9% and 12.5% of the original hemicelluloses from perennial grass and cocksfoot grass, respectively. It was found that the four alkaline peroxide-soluble hemicellulosic fractions contained higher amounts of xylose (33.4–38.2%), uronic acids (9.3–15.3%) and rhamnose (3.0–3.9%), but were lower in glucose (25.1–28.3%), galactose (13.3–15.3%) and mannose (0.4–1.5%) than those of the two alkaline EtOH-soluble hemicellulosic fractions in which glucose (32.9–36.0%), xylose (20.1–22.6%), arabinose (14.1–21.4%), galactose (16.6–19.9%), mannose (4.1–9.9%) and uronic acids (3.4–7.4%) were the major sugar components. ^{13}C NMR spectroscopy confirmed that all the six hemicellulosic fractions were composed of galactoarabinoxylans, 4-*O*-methylglucuronoarabinoxylans and β-glucan. In addition, the studies showed that the four alkaline peroxide-soluble hemicellulosic fractions were more linear and acidic and had larger molecular weights (M_w, 28,400–38,650 g mol^{-1}) than those of the two alkaline EtOH-soluble hemicellulosic fractions (M_w, 16,460–17,420 g mol^{-1}).

Keywords: Perennial ryegrass; Cocksfoot grass; Galactoarabinoxylans; 4-*O*-Methylglucuronoarabinoxylans; β-Glucan

1. Introduction

In the UK and Europe, perennial ryegrass (*Lolium perenne*) and Italian ryegrass (*Lolium multiflorum*) occupy about 70% of the agricultural areas with tall fescue grass (*Festuca arundinacea*), meadow fescue grass (*Festuca pratensis*) and cocksfoot grass (*Dactylis glomerata*) making up the remainder.[1] Similar to other undervalued agricultural residues, these grasses, which are normally cut every few months, are not used as industrial raw materials.

Carbohydrates are the main constituents of the grasses. During maturation, there is an increase of the stem-to-leaf ratio and a secondary thickening and lignification of the cell walls, which results in increased content of structural polysaccharides (mainly cellulose and hemicelluloses) and lignin.[2] Hemicelluloses are classically defined as the alkali-soluble material after removal of the pectic substances.[3] This definition of hemicelluloses is very generic, but is accepted at present. Xylans have been isolated from both root and aerial tissue of perennial ryegrass (*L. perenne*).[3] Methylation analysis

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and studies of partial acid hydrolysis have indicated the similarity between the xylans. It was found that the xylans isolated from tropical grasses were similar in structure to the xylans isolated from temperate grasses.[4] Reid and Wilkie[5] have isolated an acidic arabinoxylan, an acidic galactoarabinoxylan and a β-glucan from both the leaf and stem tissue of *Avena sativa*, and Buchala and Wilkie[6] have isolated similar hemicelluloses from *Ttriticum vulgare*. Blake and Richards[7] prepared both the linear and branched xylans from a legume and showed that each could be further fractionated by ethanol precipitation. Based on an extensive study of extraction and fractionation of the hemicellulosic polysaccharides of S23 ryegrass (*L. perenne*), Morrison[8] demonstrated that there is evidence of the presence of a β-glucan, a highly branched galactoarabinoxylan and a linear xylan. The branched xylan has a similar composition throughout its growth, and gradually increases in concentration during the growth. The linear xylan exhibits considerable polydispersity, in that the xylose:arabinose ratio rises considerably during growth, but its concentration in plant tissue, as a proportion of the total hemicelluloses, slowly declines during growth. Xylans consist of a main chain of β-(1→4)-linked xylopyranose residues to which L-arabinofuranose residues are attached as single-unit side chains, usually to position 3 of the xylose residue. Additionally, single residues of D-glucuronic acid or its 4-*O*-methyl derivative are characteristically attached at position 2.[3] Furthermore, acetic acid, phenolic acids and other cell-wall constituents, for example, lignin linked or associated to the xylans, are likely to influence both the physical state and chemical properties.[9] Grasses are reported to contain 1–3% of bound acetyl groups, and the degree of acetylation increases with maturity.[10] There is a certain amount of ester bonding between phenolic components of lignin and xylose, arabinose and uronic acids of heteroxylans of hemicelluloses.[11] The amount of bonding appears to increase with plant maturity.[12]

Isolation of hemicelluloses actually involves alkaline hydrolysis of ester linkages to liberate them from the lignocellulosic matrix, followed by their extraction into aqueous media. However, the liberation of the xylan component from the cell wall of grasses, cereal straws and woods is restricted by the presence of lignin network as well as ester and ether lignin–hemicellulose linkages. Furthermore, extensive hydrogen bonding between the individual polysaccharide cell-wall components may impede isolation of the hemicellulosic component.[13] Interestingly, owing to a much lower lignin content in the cell walls of grass than that in wood and straw, much of the hemicelluloses can be extracted without delignification.[14] In general, one step of dilute alkali treatment extracts only part of the hemicelluloses from both a holocellulose and a lignified material. Successive treatments with alkali of initially low, and then higher concentration avoid unnecessary exposure of hemicellulosic material to alkali that are more concentrated than that required to extract it.[15] In this case, the hemicellulosic materials from grasses or other plant cell walls are frequently fractionated to give polysaccharides having different structural features. More importantly, studies of such fractionated materials have led to much structural information on molecules in those of the populations of hemicellulosic molecules recovered by the most commonly used procedures. However, the fractionation and characterization of hemicelluloses from various grasses have not yet been studied in detail.[1] We now report the fractional isolation of the hemicelluloses from perennial ryegrass (*L. perenne*) and cocksfoot grass (*D. glomerata*), and describe several structural details and physicochemical properties of the three major hemicellulosic fractions.

2. Results and discussion

2.1. Fractional yield of hemicelluloses

Organosolv pulping processes involve the treatment of lignocellulosic substances with organic solvent–water media in the presence or absence of a catalyst (acid or alkali); hence, they have lower environmental impact and lower energy consumption. This process allows the fractionation of lignocellulosic materials into three major components by separation of the residue from black liquor: cellulosic fibre, hemicelluloses and lignin.[16,17] In this case, the solvent primarily acts on the promotion of vegetal tissue impregnation.[18] Interestingly, a considerable extraction of hemicelluloses could be achieved using aqueous organic solvents under alkaline conditions. From the degraded or solubilized hemicelluloses, sweetening materials such as xylitol, food additives and novel polymers can be produced.[19] In addition, during organosolv treatment, lignin is also dissolved or degraded by cleavage of such bonds as α-aryl ether and arylglycerol-β-aryl ether in the lignin macromolecule,[20] which results in an increase in release of the hemicellulosic polymers. In this study, the first treatment of the dried perennial grass and cocksfoot grass with 80% ethanol containing 0.2% NaOH at 75 °C for 3 h resulted in a release of 8.0% and 10.4% of the original hemicelluloses, respectively. As the data shown in Table 1, the organosolv treatment also released substantial amounts of chlorophyll (90% of the original chlorophyll). This high solubility of chlorophyll was probably due to the reason that the chlorophyll is present mainly on the surface of the grass, from where it dissolves easily in the organosolv treatment. It should be noted that the treatment with 80% ethanol also dissolved small amounts of other compounds such as lignin, wax, free sugars and oligosaccharides.[9]

Table 1. The yield of hemicelluloses (% dry matter) solubilized in the sequential treatment of perennial grass (*Lolium perenne*) and cocksfoot grass (*Dactylis glomerata*) with 80% EtOH containing 0.2% NaOH, 2.5% H_2O_2–0.2% EDTA containing 1.5% NaOH and 2.5% H_2O_2–0.2% TAED containing 1.0% NaOH at 75 °C for 3 h

Yield (%)	Perennial grass	Cocksfoot grass
Solubilized hemicelluloses in 80% EtOH containing 0.2% NaOH	3.3	3.5
Solubilized hemicelluloses in 2.5% H_2O_2–0.2% EDTA containing 1.5% NaOH	32.5	25.8
Solubilized hemicelluloses in 2.5% H_2O_2–0.2% TAED containing 1.0% NaOH	5.3	4.2
Total solubilized hemicelluloses	41.1	33.5
Residue (crude cellulose)	28.4	30.9

It is well known that hydrogen peroxide reacts with lignin under alkaline conditions and has been widely used for many years to bleach high-lignin wood pulps. The bleach effect of hydrogen peroxide has been attributed to its ability to react with various coloured carbonyl-containing structures in lignin. This reaction has been explained through the reactions of the hydroperoxide anion (HOO^-), formed in alkaline media.[21] On the other hand, hydrogen peroxide is unstable under alkaline conditions and readily decomposes into hydroxyl radicals ($HO^{\cdot}$) and superoxide anion radicals ($O_2^{-\cdot}$). There are many pathways to generate $HO^{\cdot}$, such as by reaction of H_2O_2 with the reduced transition metal ions, Ti^{3+}, Fe^{3+} and Cu^{2+}.[22] In addition, ascorbate can also reduce H_2O_2 to the $HO^{\cdot}$ radical in the presence of trace metal ions in polysaccharides.[23] These radicals are thought to cause the oxidation of lignin structures, which leads to the introduction of hydrophilic (carboxyl) groups, cleavage of some interunit bonds and eventually, the dissolution or degradation of lignin and hemicelluloses.[24] In the present study, to remove interference from metal ions or inactivate metal ions, 0.2% EDTA, a metal chelating agent, was added during the alkaline peroxide treatment. As expected, treatment of the 80% ethanol-extracted perennial grass and cocksfoot grass residues with 2.5% H_2O_2–0.2% EDTA containing 1.5% NaOH at 75 °C for 3 h released 79.1% and 77.0% of the original hemicelluloses, in addition to degradation of 29.5% and 55.8% of the original lignin, respectively. These end products of the solubilized hemicelluloses were white, whereas the extracts by alkali without hydrogen peroxide were brownish in colour. Obviously, the yield of hemicelluloses was higher than that of lignin, indicating that the alkaline peroxide extraction of the 80% ethanol-treated grasses under the conditions given favoured the release of hemicelluloses. Similar results were observed in previous studies on maize stems, wheat straw and other agricultural residues using alkaline peroxide in which the delignification reaction and the dissolution of hemicelluloses were strongly dependent on pH, and as the reaction pH became more alkaline, increasing amounts of hemicelluloses were noted.[21,24–26]

More recently, one of the most common approaches to pulp bleaching in the industry is the modification of the peroxide bleaching chemistry and technology as well as to improve the bleach stability at lowest cost. It was found that TAED is a well-established low-temperature bleach activator. It reacts with peroxide to provide effective low-temperature bleaching via the production of the peracetic acid anion, at temperatures and residence times for which peroxide alone would be ineffective.[27] TAED can be used at low levels, under alkaline conditions, to boost the brightening performance of peroxide and perform rapid bleaching.[28] To increase whiteness of the fibres (residue of the peroxide treatment) and reduce the loss of fibre strength, minor quantities (0.2%) of TAED were used in the third-stage treatment of the alkaline peroxide-treated grass residues. The results showed that the third treatment with 2.5% H_2O_2–0.2% TAED containing 1.0% NaOH at 75 °C for 3 h led to further release of 12.9% and 12.5% of the original hemicelluloses and 11.5% and 9.6% of the original lignin from the alkaline peroxide-treated residues of perennial grass and cocksfoot grass, respectively. The yield of white crude cellulose was found to be 28.4% from perennial grass and 30.9% from cocksfoot grass, which are relatively free of the bound lignin. These results revealed that TAED-activated peroxide, except for bleaching cellulose, is a strong agent both for removing the residual lignin and for releasing the residual hemicelluloses from the grasses.

2.2. Sugar composition of hemicelluloses

The hemicelluloses are a mixture of a number of different polysaccharides, and their composition can vary depending on the method of isolation. The sugar composition of the six hemicellulosic fractions from perennial grass and cocksfoot grass is given in Table 2. Evidently, there were no significant differences in the sugar composition of the corresponding hemicellulosic fractions between fractions H_1 and H_2, H_3 and H_4, and between fractions H_5 and H_6, indicating a similar structure of the corresponding hemicelluloses isolated from both perennial grass and cocksfoot grass. As can be seen, the H_1 and H_2 samples that were isolated with 80% ethanol from perennial grass and cocksfoot grass had the higher content of D-glucose (32.9–36.0%), probably arising from the presence of a mixed linkage β-(1→3)- and (1→4)-D-glucan in these tissues. Henry and Stone[29] and Gordon et al.[30] have independently reached similar conclusions by quite different methods. Based on the examination of the chain length and link-

Table 2. The content of neutral sugars (relative % dry weight, w/w) and uronic acids (% dry weight, w/w) in isolated hemicellulosic fractions

Sugars (%)	Hemicellulosic fraction					
	H_1[a]	H_2[a]	H_3[b]	H_4[b]	H_5[c]	H_6[c]
Arabinose	21.4	14.1	18.5	20.4	19.0	19.4
Rhamnose	1.6	0.8	3.0	3.9	3.4	3.1
Galactose	19.9	16.6	15.3	14.3	13.5	13.3
Glucose	32.9	36.0	28.3	25.4	25.1	25.6
Xylose	20.1	22.6	33.4	35.6	38.2	37.4
Mannose	4.1	9.9	1.5	0.4	0.7	1.2
Uronic acids	3.4	7.4	9.3	12.7	15.3	14.3

[a] H_1 and H_2 represent for the hemicellulosic fractions solubilized during the treatment of perennial grass and cocksfoot grass with 80% EtOH containing 0.2% NaOH at 75 °C for 3 h.

[b] H_3 and H_4 represent for the hemicellulosic fractions solubilized during the sequential extraction of 80% ethanol-treated perennial grass and cocksfoot grass with 2.5% H_2O_2–0.2% EDTA containing 1.5% NaOH at 75 °C for 3 h.

[c] H_5 and H_6 represent for the hemicellulosic fractions solubilized during the sequential extraction of alkaline peroxide-treated perennial grass and cocksfoot grass with 2.5% H_2O_2–0.2% TAED containing 1.0% NaOH at 75 °C for 3 h.

age composition of water-soluble β-glucans produced in vitro from [^{14}C]-D-glucose by ryegrass membranes, Henry and Stone[29] reported that 64% of the radiolabelled β-D-glucosyl residues were 3-substituted, 33% were 4-substituted and 3% were non-reducing terminal residues, indicating that the average degree of polymerization of the radiolabelled sequences was 33. On the basis of a study of polysaccharides from white clover (*Trioflium pratense* L. cv. Aberystwyth s100) and perennial grass (*L. perenne* L. cv. Perma), Gordon et al.[30] indicated that there was evidence of the presence of small amounts of (1→3)-linked units exception for (1→4)- and terminally linked glucose. They also implied that these glycosidic linkages were present in greatest amount in early-cut ryegrass (2.8% of total identified sugars) and in amounts that decreased with increasing maturity in the other Gramineae examined. Similarly, a high content of glucose in all the alkaline peroxide-soluble hemicellulosic fractions of H_3, H_4, H_5 and H_6, obtained from the 80% ethanol-treated perennial grass and cocksfoot grass, suggested that β-glucan may also be a constituent of these hemicellulosic preparations. Such glucans were also known to be present in non-endospermic tissues of barley, wheat, rye, maize and bamboo, and the ratio of β-(1→3)- to β-(1→4)-linkages in the β-glucans decreased concomitantly with tissue maturity.[31] In addition, as expected, xylose (20.1–22.6%), arabinose (14.1–21.4%) and galactose (16.6–19.9%) were the major hemicellulosic constituents, but small amounts of other sugars in fractions H_1 and H_2 demonstrated that the 80% ethanol-soluble hemicelluloses consist mainly of a highly branched galactoarabinoxylan. Small amounts of uronic acids (3.4–7.4%) presumably arose from the 4-*O*-methyl-glucuronoarabinoxylans or pectic substances, even though grasses have a low content of pectin.

As the data show in Table 2, six sugars were found in four alkaline peroxide-soluble samples. Xylose (33.4–38.2%) was always the most abundant, and in decreasing order were glucose (25.1–28.3%), arabinose (18.5–20.4%), galactose (13.3–15.3%), rhamnose (3.0–3.9%) and mannose (0.4–1.5%), the last always being present in minor amount. Obviously, the proportions of xylose residues and uronic acids (9.3–15.3%) in the four samples extracted with alkaline peroxide were higher than those of the two hemicellulosic fractions isolated with 80% ethanol, while the concentrations of glucose and galactose residues were lower than those of the first two fractions. The proportion of arabinose residues remained relatively constant. Glucose residues, mainly from β-glucan and xylose-, arabinose and galactose residues from galactoarabinoxylans were the dominating hemicellulosic constituents, but noticeable amounts of uronic acids (D-glucuronic acid or its 4-*O*-methyl ether) indicated a tentative presence of 4-*O*-methyl-glucuronoarabinoxylans. Such a higher content of D-xylose residues indicates that the hemicelluloses, mainly galactoarabinoxylans, isolated with alkaline peroxide, were less branched in structure than the corresponding two hemicellulosic fractions extracted with 80% ethanol. In addition, there are some changes taking place in the composition of the galactoarabinoxylans between the four alkaline peroxide-soluble hemicellulosic fractions of H_3, H_4, H_5 and H_6. The ratio of D-xylose to L-arabinose residues in H_5 and H_6 was higher than that in H_3 and H_4, indicating that a more linear galactoarabinoxylan could be isolated with 2.5% H_2O_2–0.2% TAED containing 1.0% NaOH than from the 2.5% H_2O_2–0.2% EDTA-treated residues of perennial grass and cocksfoot grass, and it is more strongly associated with cellulose microfibrils. In other words, the more highly branched galactoarabinoxylans are more readily extracted by aqueous ethanol and alkali than the more linear polymers.

2.3. Weight-average molecular weight (M_w)

The molecular weights of the six hemicellulosic fractions were further determined by gel-permeation chromatography (GPC), and their weight-average (M_w) and number-average (M_n) molecular weights and polydispersity

(M_w/M_n) are given in Table 3. Obviously, the first two hemicellulosic fractions (H_1 and H_2), solubilized during the alkaline 80% ethanol, showed a much lower degree of polymerization with M_w values between 16,460 and 17,420 g mol^{-1} than those of the last four hemicellulosic fractions (H_3, H_4, H_5 and H_6) with M_w values from 28,400 to 38,650 g mol^{-1}, released during the sequential alkaline peroxide treatments. Additionally, the relatively higher M_w (36,140, 38,650 g mol^{-1}) of the last two hemicelluloses isolated with alkaline peroxide and 0.2% TAED than those of the hemicellulosic fractions of H_3 and H_4 (M_w, 28,400, 33,810 g mol^{-1}), extracted with alkaline peroxide and 0.2% EDTA, implied that the alkaline peroxide–0.2% TAED treatment under the conditions used did not degrade the macromolecular structure of hemicelluloses to any noticeable extent, and the first treatment with alkaline 80% ethanol under the conditions given favoured release of the small molecular size of hemicelluloses. Furthermore, the analysis showed that the first two polymeric hemicelluloses solubilized during the alkaline 80% ethanol treatment gave a more narrow molar mass distribution corresponding to polydispersity indexes of 2.36 for H_1 and 2.51 for H_2 as compared to those of the four alkaline peroxide-soluble hemicellulosic products of H_3, H_4, H_5 and H_6, which have polydispersity indexes between 3.62 and 4.68.

Table 3. Weight-average (M_w) and number-average (M_n) molecular weights and polydispersity (M_w/M_n) of the hemicellulosic fractions isolated from perennial grass and cocksfoot grass

	Hemicellulosic fractions[a]					
	H_1	H_2	H_3	H_4	H_5	H_6
M_w	17420	16460	28400	33810	36140	38650
M_n	7390	6570	7840	8090	7830	8250
M_w/M_n	2.36	2.51	3.62	4.18	4.62	4.68

[a] Corresponding to the hemicellulosic fractions in Table 2.

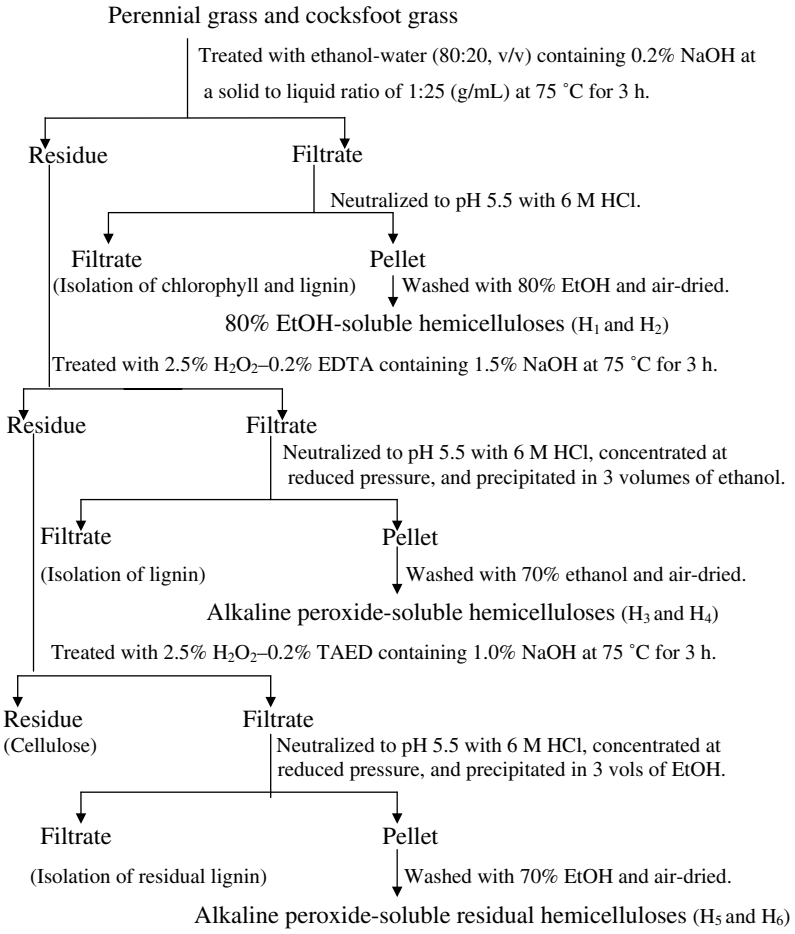

Figure 1. Scheme for fractional isolation of hemicelluloses from perennial grass and cocksfoot grass.

2.4. FTIR spectra

The FTIR spectra of the alkaline ethanol-soluble hemicellulosic fraction H_1 (spectrum a), alkaline peroxide–0.2% EDTA-soluble hemicellulosic fraction H_3 (spectrum b) and alkaline peroxide–0.2% TAED-soluble hemicellulosic fraction H_5 (spectrum c) obtained from perennial ryegrass are illustrated in Figure 2. The absorption at 3436 cm^{-1} is attributed to the stretching of –OH groups and that at 2932 and 2850 cm^{-1} is attributed to C–H stretching. The band at 1634 cm^{-1} is due to the bending mode of the absorbed water. Obviously, all the three hemicellulosic fractions showed the typical signal pattern for the hemicellulosic moiety, and had a specific band maximum in the 1200–1000 cm^{-1} region, which is dominated by ring vibrations overlapped with stretching vibrations of side groups (C–OH) and the glycosidic bond vibration (C–O–C).[32] Interestingly, in the carbonyl stretching region, a shoulder at 1739 cm^{-1} in the spectrum of the alkaline ethanol-soluble hemicelluloses is assigned to the acetyl and uronic ester groups of the hemicelluloses or to the ester linkage of the carboxyl group of ferulic and/or *p*-coumaric acids. The disappearance of this signal in the spectra (b and c) of alkaline peroxide-soluble hemicellulosic fractions verified that the treatment with alkaline peroxide–0.2% EDTA or alkaline peroxide–0.2% TAED under the conditions given completely cleaved this ester bond from the hemicelluloses. As expected, the absence of a signal at 1720 cm^{-1} for carbonyl stretching in all the three spectra implied that both alkaline ethanol and alkaline peroxide treatments under the conditions used did not significantly attack or oxidize the glycosidic linkages and hydroxyl groups of hemicelluloses. A small band at 928 (spectra a and b) or 908 cm^{-1} (spectrum c), which is due to the C-1 group frequency or ring frequency, is indicative of β-glycosidic linkages between the xylose units in the hemicelluloses.[33] The small bands at 1471, 1421, 1378, 1324 and 1262 cm^{-1} represent C–H stretching and C–O or OH bending vibration in hemicelluloses. Evidently, the absence in all three spectra of a band at 1520 cm^{-1}, which is due to aromatic skeletal vibrations in bound lignin, indicates that the hemicellulosic fractions are free of the associated lignin.

Figure 3 illustrates the FTIR spectra of the alkaline ethanol-soluble hemicellulosic fraction H_2 (spectrum a), alkaline peroxide–0.2% EDTA-soluble hemicellulosic fraction H_4 (spectrum b) and alkaline peroxide–0.2% TAED-soluble hemicellulosic fraction H_6 (spectrum c) obtained from cocksfoot grass. Obviously, the spectra b and c appear to be rather similar, indicating a similar structure of two hemicellulosic fractions, which corresponds to their sugar composition. In spectra b and c, the absorbances at 1638, 1417, 1320, 1258, 1157, 1040 and 900 cm^{-1} are associated with hemicelluloses, in which the two bands at 1157 and 1040 cm^{-1} are typical of arabinoxylans. In spectrum a, a small absorbance at 1739 cm^{-1} is indicative of the carbonyl of acetyl, feruloyl, *p*-coumaroyl, etc. groups in hemicelluloses and lignin, and the absorbance at 1712 cm^{-1} arises from the

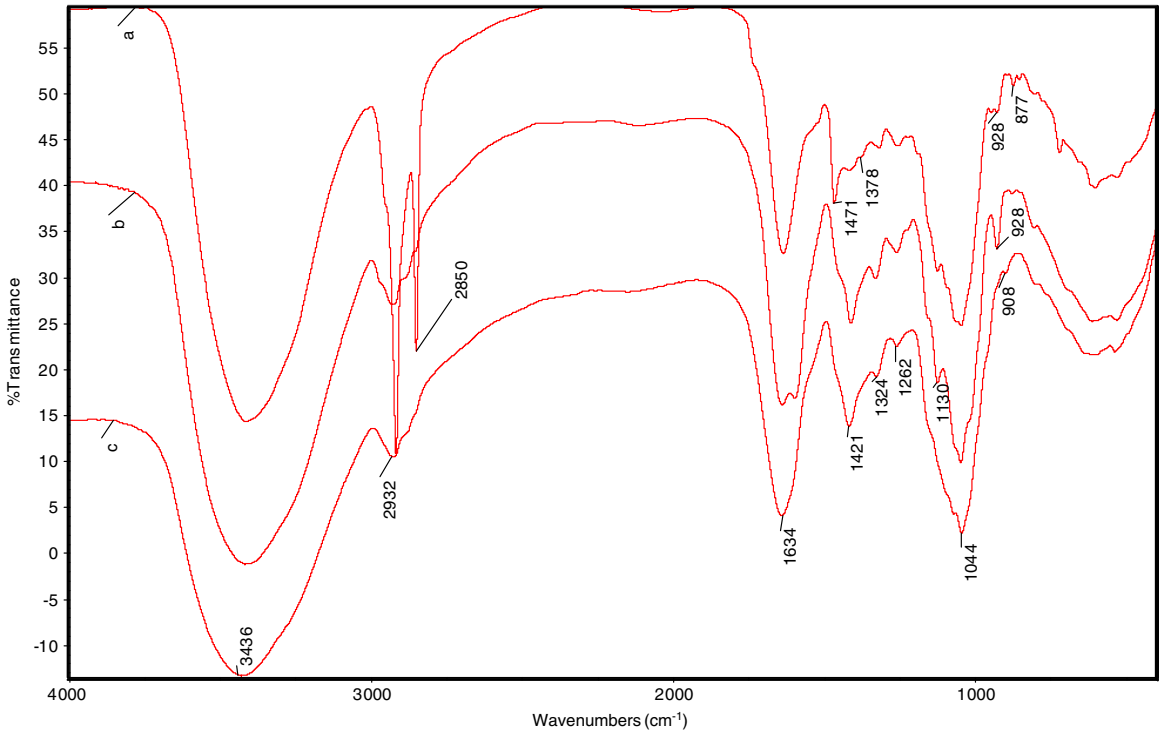

Figure 2. FTIR spectra of hemicellulosic fractions H_1 (spectrum a), H_3 (spectrum b) and H_5 (spectrum c).

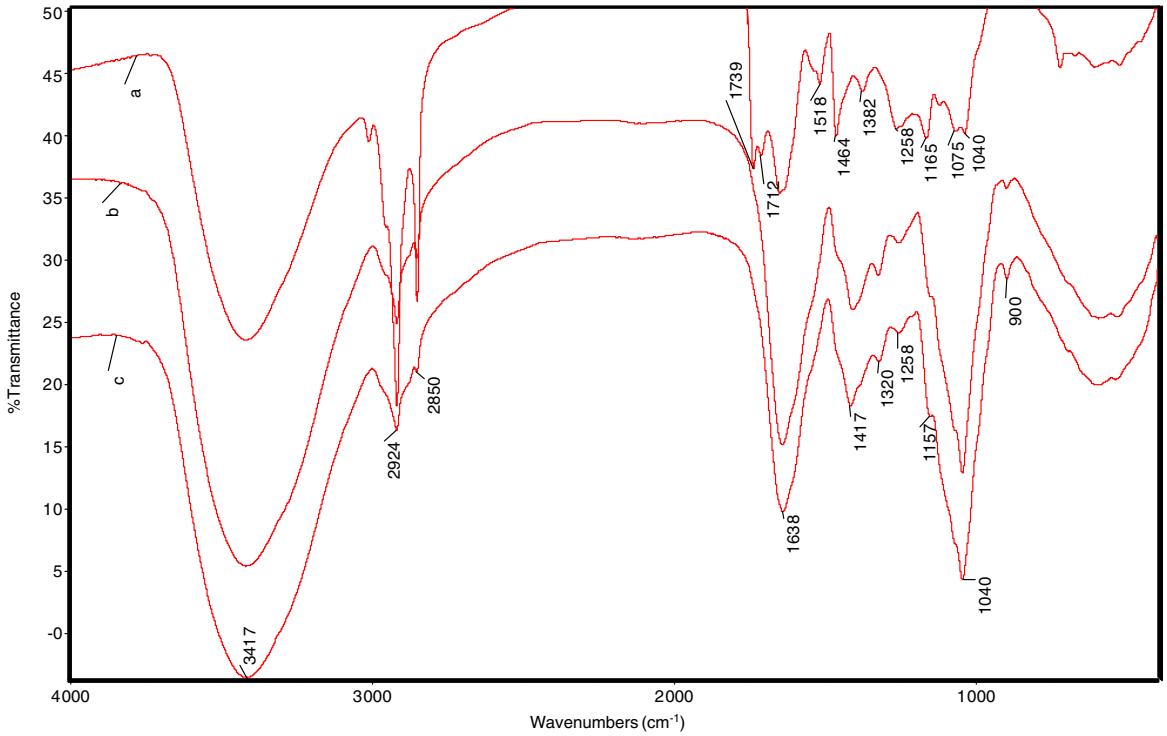

Figure 3. FTIR spectra of hemicellulosic fractions H_2 (spectrum a), H_4 (spectrum b) and H_6 (spectrum c).

C═O in unconjugated ketone (β-carbonyl) and carboxylic acid in associated lignin and/or hydroxycinnamic acids. An intensive absorption at 1518 cm^{-1} is characterized by aromatic skeleton vibrations in bound lignin or ferulic and *p*-coumaric acids. This phenomenon indicates that the hemicellulosic fraction H_2, extracted with 80% ethanol under alkaline conditions from cocksfoot grass, contained noticeable amounts of ferulic and *p*-coumaric acids and/or lignin.

2.5. ^{13}C NMR spectra

Nuclear magnetic resonance (NMR) spectroscopy has proven invaluable in studying the molecular structures of hemicellulosic polymers. In particular, ^{13}C NMR spectroscopy, a non-destructive probe of molecular structure, has become a method of choice for structural elucidation of native hemicelluloses that allows for rapid determination of the nature, configuration and relative content of monosaccharide residues constituting the hemicelluloses as well as the type and number of specific linkages.[34] To obtain further information about the anomeric linkage configuration of the hemicelluloses, the main fraction H_3, isolated with alkaline peroxide–0.2% EDTA from the alkaline 80% ethanol-treated perennial grass, was analyzed by ^{13}C NMR spectroscopy in D_2O, and its spectrum is shown in Figure 4. The main (1→4)-linked β-D-Xyl*p* units were obviously characterized by five strong signals at 107.1, 84.4, 81.4, 78.8 and 66.6 ppm, which originate from C-1, C-4, C-3, C-2 and C-5 of the β-D-Xyl*p* units, respectively. The signals at 88.7, 85.2 (data not shown) and 63.4 ppm are assigned to C-4, C-3 and C-5 of α-L-Ara$_f$ residues, respectively, in which the signal at 63.4 ppm was overlapped with the signal for C-6 in β-glucan. Two signals at 105.6 and 77.1 ppm (data not shown) are attributed to C-1 and C-4 in α-Gal*p* residues. Small amounts of glucuronic acid and 4-*O*-methyl-glucuronic acid were also present as identified from the spectrum with signals at 104.2 (C-1 in Glc*p*A), 74.8 (C-5 in Glc*p*A) (data not shown) and 59.8 ppm (4-*O*-methoxyl group of glucuronic acid). Two signals at 82.3 (data not shown) and 63.4 ppm (overlapped with C-5 of α-L-Ara$_f$ residues) are due to C-5 in the β-Glc*p*-(1→4)- and C-6 in β-Glc*p*-(1→3)-linkages of β-glucans.[35] The carbonyl resonance at 182.7 ppm may originate from acetyl groups in an aliphatic acid and C-6 of glucuronic acid and/or 4-*O*-methylglucuronic acid in hemicelluloses.[36,37] The signal at 19.8 ppm relates to CH_3 in the acetyl group in hemicelluloses. These observations suggested that the hemicellulosic fraction H_3 isolated with alkaline peroxide–0.2% EDTA consists of galactoarabinoxylans, 4-*O*-methylglucuronoarabinoxylans and β-glucans. The current results were consistent with the studies of glycosidic linkages of legume, grass and cereal straw cell-wall polysaccharides by Gordon and co-workers.[30] They demonstrated that arabinose was found to occur predominantly as the terminally linked sugar, but some in-chain units were linked, and (1→3)-linkages were also detected. Galactose residues were found as (1→4)- and

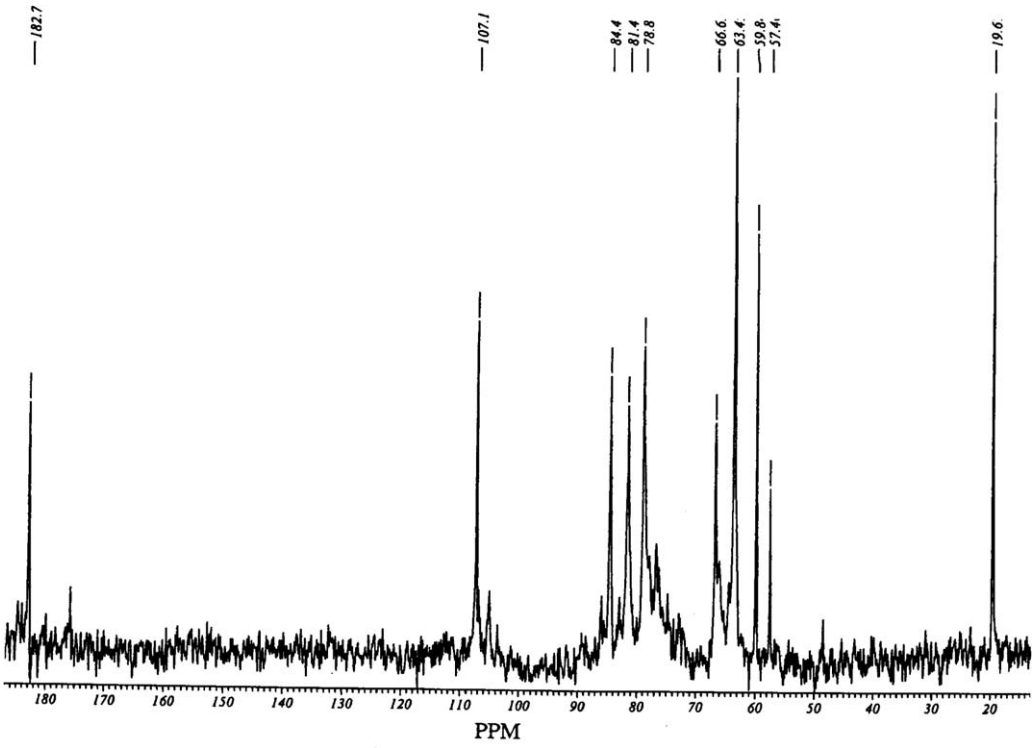

Figure 4. ^{13}C NMR spectrum of hemicellulosic fraction H_3.

(1→6)-linked units but mostly as terminally linked units. The other sugars found, rhamnose and mannose, were largely terminally linked.

2.6. Thermal analysis

Thermogravimetric analysis (TGA) and differential thermal analysis (DTA) were used to study the thermal stability of the hemicelluloses isolated, and their curves for alkaline ethanol-, alkaline peroxide–0.2% EDTA- and alkaline peroxide–0.2% TAED-soluble hemicellulosic fractions of H_1, H_3 and H_5 from perennial grass are shown in Figure 5. As can be seen from the figure, the three hemicellulosic fractions of H_1 (a), H_3 (b) and H_5 (c) began to decompose at 220, 198 and 180 °C, respectively. At 50% weight loss, the decomposing temperature of the alkaline ethanol-soluble hemicellulosic fraction H_1, alkaline peroxide–0.2% EDTA-soluble fraction H_3 and the alkaline peroxide–0.2% TAED-soluble hemicellulosic fraction H_5 occurred at 350, 400 and 330 °C, respectively. The initial weight loss was probably due to generation of non-combustible gases such as CO, CO_2, formic acid and acetic acid, whereas the significant (maximum) weight loss at 310 °C for H_1, 315 °C for H_3 and 290 °C for H_5 indicated the onset of pyrolysis and generation of combustible gases.[28] By comparing the values of the initial and maximum weight loss, it was found that both alkaline peroxide–0.2% EDTA-soluble hemicellulosic fraction H_3 and alkaline ethanol-soluble

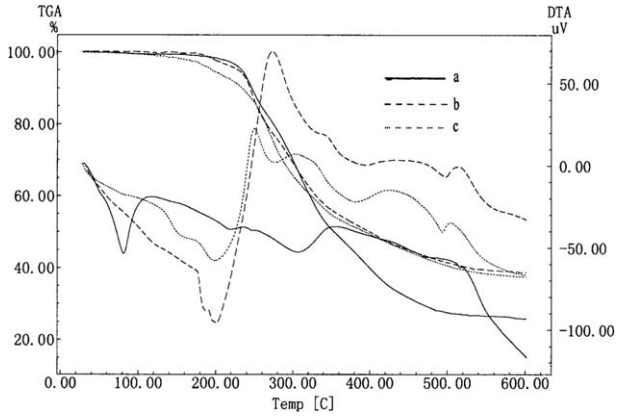

Figure 5. Thermograms of hemicellulosic fractions of H_1 (a), H_3 (b) and H_5 (c).

fraction H_1 had a higher thermal stability than that of the alkaline peroxide–0.2% TAED-soluble hemicellulosic preparation H_5.

3. Conclusions

The above results showed that the three sequential treatments with 80% ethanol containing 0.2% NaOH, 2.5% H_2O_2–0.2% EDTA containing 1.5% NaOH and 2.5% H_2O_2–0.2% TAED containing 1.0% NaOH at 75 °C for 3 h together yielded 41.1% hemicelluloses from

perennial grass and 33.5% from cocksfoot grass, in which the treatment with 2.5% H_2O_2–0.2% EDTA containing 1.5% NaOH released 79.1% and 77.0% of the original hemicelluloses from the alkaline ethanol-treated perennial grass and cocksfoot grass, respectively. It was found that there were no significant differences in chemical composition and structural features between the corresponding hemicellulosic fractions obtained from the two different grasses. However, noticeable differences were observed among the different hemicellulosic fractions. Sugar analysis and ^{13}C NMR spectroscopy confirmed that the hemicellulosic fractions obtained from both perennial grass and cocksfoot grass were composed of galactoarabinoxylans, 4-*O*-methylglucuronoarabinoxylans and β-glucan. Galactoarabinoxylans in the hemicellulosic fractions of H_1 and H_2 extracted with 80% ethanol containing 0.2% NaOH from both perennial grass and cocksfoot grass were more branched than those in the hemicellulosic fractions of H_3 and H_4 extracted with 2.5% H_2O_2–0.2% EDTA containing 1.5% NaOH, and they were the most linear in the hemicellulosic fractions of H_5 and H_6 extracted with 2.5% H_2O_2–0.2% TAED containing 1.0% NaOH. This indicated that the hemicelluloses of galactoarabinoxylans containing a high degree of side-chain substitution are more ethanol soluble and bind less tightly to cellulose, whereas molecules with infrequent side chains are less extractable and bind more tightly to cellulose. On the other hand, a reverse trend was observed for 4-*O*-methylglucuronoarabinoxylans, which are more acidic for the hemicellulosic fractions of H_3, H_4, H_5 and H_6 extracted with alkaline peroxide than for the preparations of H_1 and H_2 isolated with alkaline ethanol, and 4-*O*-methylglucuronoarabinoxylans in the last two fractions of H_5 and H_6 are the most acidic hemicelluloses. The content of β-glucan was higher in the two alkaline ethanol-soluble hemicellulosic fractions than in the four alkaline peroxide-soluble fractions, implying that β-glucan is more organosolv-soluble and binds less tightly to cellulose in the cell walls of grasses. In addition, the results from FTIR showed that the alkaline ethanol-soluble hemicellulosic fraction obtained from cocksfoot grass contained noticeable amounts of lignin, whereas the corresponding fraction isolated from perennial grass under the same extraction condition used are free of the associated lignin, indicating that the hemicelluloses and lignin are more tightly linked in the cell walls of cocksfoot grass than in perennial grass.

4. Experimental

4.1. Materials

Perennial ryegrass (*L. perenne*) and cocksfoot grass (*D. glomerata*), age of 10 weeks, were harvested from the farm of Biochem Wales Ltd in mid-September 2005, and were approximately 15 cm in height. They are freed from any weeds by hand and transported to the laboratory as quickly as possible. The samples were dried in a forced-draught oven at 60 °C for 16 h and then ground by hand before use.

4.2. Fractional extraction of the hemicelluloses

In order to remove chlorophyll, wax, low-molecular-weight hemicelluloses and other soluble components, ground perennial ryegrass and cocksfoot grass (30.0 g) were first treated with 600 mL of 80% aq EtOH containing 0.2% NaOH for 3 h at 75 °C, respectively. The green slurry was filtered through a 45-μm nylon cloth, resulting in a green suspension along with an insoluble residue fraction left on the cloth. The residue was thoroughly washed with 80% EtOH, and then oven dried at 60 °C for 16 h. The supernatant was neutralized with 6 M HCl to pH 5.5 and then kept for 5 h at 22 °C to precipitate the low-molecular-weight hemicelluloses released. The precipitates that formed were recovered by filtration, washed with acidified 70% EtOH and air-dried, and labelled as 80% EtOH-soluble hemicellulosic fractions H_1 from perennial grass and H_2 from cocksfoot grass. The residue was then sequentially extracted with 2.5% H_2O_2–0.2% EDTA (ethylenediaminetetraacetic acid) containing 1.5% NaOH at 75 °C for 3 h, and 2.5% H_2O_2–0.2% TAED (tetraacetylethylenediamine) containing 1.0% NaOH at 75 °C for 3 h with a solid to liquid ratio of 1:20 (g/mL). The solubilized hemicelluloses were separated from the insoluble residue by filtration with a nylon cloth. The residue was subsequently washed with distilled water and 95% EtOH, and then oven dried at 60 °C for 16 h. EtOH in the combined supernatant was removed with a rotary vacuum evaporator at 40 °C. Then the supernatant was neutralized to pH 5.5 with 6 M HCl, concentrated under reduced pressure and then mixed with 3 vols of 95% EtOH for isolation of the hemicelluloses liberated. The isolated hemicelluloses were purified by washing with acidified 70% EtOH at room temperature and drying in air. Note that the hemicelluloses solubilized during the treatment of the 80% EtOH-extracted perennial grass residue with alkaline peroxide containing 0.2% EDTA and alkaline peroxide containing 0.2% TAED were labelled as hemicellulosic fractions H_3 and H_5, and the hemicelluloses released during the corresponding alkaline peroxide treatment of the 80% EtOH-extracted cocksfoot grass residue were named as the hemicellulosic fractions H_4 and H_6, respectively. A scheme for the sequential treatments of perennial ryegrass and cocksfoot grass, and the isolation of hemicelluloses solubilized is shown in Figure 1. All experiments were performed at least in duplicate. Yields of hemicelluloses are given on a dry-weight basis related to the starting grass (Table 1).

4.3. Characterization of hemicellulosic fractions

The neutral sugars in the six hemicellulosic fractions were liberated by hydrolysis of the polymers with 6% H_2SO_4 for 2.5 h at 100 °C. The analysis of the individual sugars in the hydrolyzate was carried out by high-performance-anion exchange chromatography using a Dionex GP50 gradient pump, ED50 electrochemical detector, AS50 autosampler and a Carbopac™ PA1 column. Samples injected into the system were eluted with 0.004 M NaOH (carbonate free and purged with helium) with post-column addition of 0.3 M NaOH at a rate of 1 mL/min. Run time was 45 min, followed by 8 min elution with 0.5 M NaOH to wash the column and then 15 min elution with 0.004 M NaOH to re-equilibrate the column. The analysis was quantified against two separate standard solutions using Chromeleon™ computer software. Total uronic acid content was determined colorimetrically by the method of Blumenkrantz and Asboe-Hansen.[38] Methods for measurement of the hemicellulosic molecular weights and thermal analysis have been described in the previous papers.[39,40] The hydrolyses and analyses were conducted in duplicate, and the values of individual sugars were within ±5%.

The FTIR spectra of the hemicelluloses were recorded from a KBr disc containing 1% finely ground samples on a Nicolet 750 FTIR spectrophotometer in the range 4000–400 cm^{-1}. The solution-state ^{13}C NMR spectrum was obtained on a Bruker MSL300 spectrometer operating in the FT mode at 74.5 MHz. The sample (80 mg) was dissolved in 1 mL D_2O (99.8% D) with overnight stirring at room temperature. The spectrum was recorded at 25 °C after 30,000 scans. A 60° pulse flipping angle, a 3.9 μs pulse width and a 0.85 s acquisition time were used.

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