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Carbohydrate Polymers 67 (2007) 56–65

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Comparative study of water-soluble and alkali-soluble hemicelluloses from perennial ryegrass leaves (*Lolium peree*)

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Received 6 March 2006; received in revised form 4 April 2006; accepted 10 April 2006
Available online 9 June 2006

Abstract

One water-soluble and four alkali-soluble hemicellulosic preparations from perennial ryegrass leaves were comparatively studied, and their chemical composition, structural features, and physicochemical properties were examined. The relative monosaccharide compositions of the hemicelluloses were determined by high performance anion exchange chromatography after acid hydroloysis. ^{13}C NMR and FT-IR spectroscopic methods gave details of the anomeric linkage configuration and confirmed the structure of the hemicelluloses. The results showed that the water-soluble hemicelluloses probably consist of galactoarabinoxylans, β-glucan, and noticeable amounts of pectic polysaccharides, which had a lower molecular weight (19,360 g mol^{-1}), while the four alkali-soluble hemicellulosic preparations are presumably composed of galactoarabinoxylans, L-arabino (4-*O*-methyl-D-glucurono)xylans, and β-glucan, which had higher molecular weights between 31,060 and 36,140 g mol^{-1}. Galactoarabinoxylans constitute a major fraction of both water- and two alkali-soluble hemicelluloses H_2 and H_3, isolated with a higher concentration of alkali for a shorter period (18% NaOH or 18% KOH at 22 °C for 2 h). They consist of a linear β-(1 → 4) linked xylan backbone to which α-L-arabinofuranose units and/or short chains of sugar residues containing arabinose and galactose are attached as side residues via α-(1 → 3) and/or α-(1 → 2) linkages. On the other hand, the other two alkali-soluble hemicellulosic preparations H_4 and H_5, extracted with a relatively lower concentration of alkali for a longer period (10% NaOH or 10% KOH at 22 °C for 16 h), mainly comprised L-arabino (4-*O*-methyl-D-glucurono)xylans, in which the β-(1 → 4) linked xylan backbone is substituted in O-3 and/or O-2 by signal α-L-arabinose residue and α-D-glucuronic acid or 4-*O*-methyl-glucuronic acid. β-Glucan, both in water- and alkali-soluble hemicelluloses, was characterized as linear homopolymers consisting of both β-(1 → 3) and β-(1 → 4) glucosidic linkages.

Keywords: Perennial ryegrass leaves; Hemicellulosic polymers; Molecular structure; Water- and alkaline-soluble

1. Introduction

The UK and mainland Europe have reached a position where they no longer seek continued growth in food output from their agricultural resources leading to decline in agricultural industry and rural economies. It is reasonable then to look to satisfy other demands by utilisation of those agricultural resources. The grasslands that have supported rural communities for centuries may again sustain those communities when alternative, industrial uses of the resource are considered and implemented. Forage grasses are defined as those grasses that have traditionally been used as fodder or silage for livestock production. In UK and Europe, perennial ryegrass (*Lolium perenne*) and

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doi:10.1016/j.carbpol.2006.04.014

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. Similar to other undervalued agricultural residues, leaves of these grasses, which have been normally cut every few months, are not used as industrial raw materials.

The grass cell wall has been envisioned to be composed of cellulose fibres embedded in an amorphous mixture of hemicelluloses, pectin, lignin, glycoprotein, and minor amounts of starch and silicon. The hemicellulosic fraction is a group of cell wall polysaccharides that account for one third of all components available in temperate grasses (Morrison, 1980). The main hemicelluloses of grasses are glucuronoarabinoxylans, in which the Xyl*p* residues of a β-(1 → 4)-D-xylan backbone are substituted at O-3 with α-L-Ara*f* and at O-2 with α-D-Glc*p*A and/or its 4-*O*-methyl ether (Wilkie, 1979). In addition, *O*-acetyl and *O*-feruloyl esters are present (Wende & Fry, 1997). Hemicellulosic polysaccharides have the ability to bind non-covalently through hydrogen bonding to cellulose and to bind covalently to the lignin (Sun, Sun, & Tomkinson, 2004). The hemicelluloses therefore serve to interconnect the cellulose fibrils and the lignins of the cell wall (Windham, Amos, & Evans, 1987). Our previous studies found that the majority of lignins in cereal straw cell walls are directly linked to arabinose side chains of xylan by ether bonds (Sun & Tomkinson, 2003). Another potential lignin–hemicellulosic linkage is an ester bond between the lignin and carboxyl (C-6) group of uronic acid residues (Sun & Sun, 2002). More importantly, it was reported that ferulic acid ether-linked to lignin formed a cross-link to hemicelluloses through an ester linkage (hemicelluloses–ester–FA–ether–lignin bridges). In these cases, ferulic acid ethers might form cross-links between lignin (at the β-position of the side chains) and hemicelluloses by simultaneous esterification of their carboxyl group to the C-5 position of arabinose substituents of arabinoglucuronoxylans (Ford, 1989; Wende & Fry, 1997). The feruloyl groups are of interest because of their high reactivity in vivo. They can be enzymically dimerized (e.g., by peroxidase + H_2O_2) to form diferuloyl groups, which were identified in the wheat straw cell walls by the cross-linking of arabinoxylans to lignin (Sun, Sun, & Zhang, 2001; Wende & Fry, 1997). It should be noted that *p*-coumaric acid does not have a lignin/hemicelluloses cross-linking function (Sun, Sun, Wang, & Zhu, 2002).

The chemical composition of cell walls of grasses have been reported by a number of authors (Brecker et al., 2005; Buchala, 1974; Chaikumpollert, Methacanon, & Suchiva, 2004; Jones, 1970; Sullivan, Phillips, & Routley, 1960; Waite & Corrod, 1959; Yoshida, Kuno, Saito, Aoyama, & Kusakabe, 1998) and a detailed examination of the changes in the hemicelluloses of ten varieties of temperate grasses with increasing maturity has been published (Morrison, 1974, 1980). These reports considered in detail the changes of the different types of hemicelluloses, particularly with regard to the linear and the branched xylans. It was found that a strong correction existed between the lignin and hemicellulose concentrations, and the varieties with higher lignin concentrations showed a higher linear:branched ratio and the ‘linear’ components also had higher xylose:arabinose ratios. Although forage grasses are considered abundant, renewable sources for the production of xylitol from the hydrolysates of xylan, the reports on the structures and the physiochemical properties of the hemicelluloses from perennial ryegrass are limited. The main purpose of the work, being presented here just a part, is to assess the possibility of producing xylitol and other chemicals based on hemicelluloses extracted from the perennial ryegrass. Therefore, elucidation of the molecular structure and the physiochemical properties of the perennial ryegrass hemicelluloses was of our particular interest.

2. Experimental

2.1. Materials

Perennial ryegrass leaves (*Lolium perenne*) used in this experiment, age of two months, cut in mid-August 2005, was kindly supplied by Biochem Wales Ltd, and was approximately 10 cm in height. The leaves were dried in an oven at 60 °C for 16 h and then ground by hand before use.

2.2. Extraction of water-soluble and alkali-soluble hemicelluloses

Prior to hot water treatment, the ground grass leaves were first extracted with chloroform–ethanol (2:1, v/v) in a Soxhlet extractor for 5 h. Hot water treatment was carried out in a 1000 ml glass reactor at atmospheric pressure. The extractive free powder (20.0 g) was treated with 500 ml distilled water for 4 h at 80 °C. The green slurry was filtered through a 45-μm nylon cloth, resulting in a green suspension and the insoluble residue fraction left in the cloth. The residue was subsequently washed with distilled water and ethanol, and then oven dried at 60 °C for 16 h. The supernatant from the hot water treatment was concentrated to about 60 ml on a rotary evaporator under reduced pressure at bath temperatures not exceeding 50 °C. The water-soluble hemicellulosic fraction was then recovered by precipitation of the concentrated water extracts in 3 volumes of 95% ethanol. The precipitates that formed were recovered by filtration, washed with acidified 70% ethanol and air-dried, and labelled as water-soluble hemicellulosic fraction H_1. The residue was then delignified with 1.2% sodium chlorite at pH 3.6–3.8, adjusted with 10% acetic acid, at 75 °C for 2 h (Lawther, Sun, & Banks, 1995). The alkali-soluble hemicelluloses were extracted from the holocellulose with 18% NaOH at 22 °C for 2 h, 18% KOH at 22 °C for 2 h, 10% NaOH for 16 h at 22 °C, and 10% KOH for 16 h at 22 °C with a holocellulose to extractant ratio of 1:20 (g/ml), respectively. After the solid material was extensively washed with water, and the filtrate and washings were combined. The extract was neutralized with aqueous 6 M hydrochloric acid or 6 M acetic acid solution to pH 5.5. The released hemicelluloses were precipitated by pouring the neutralized supernatant fluid into 3 volumes of 95% ethanol (22 °C, 5 h). After filtration, the four pellets of the hemicelluloses were thoroughly washed with acidified 70% ethanol and air-dried, and considered to be alkali-soluble hemicellulosic fractions H_2, H_3, H_4, and H_5, respectively. The sequential treatments of perennial ryegrass leaves and isolation of water- and alkali-soluble hemicelluloses were illustrated in Fig. 1. All experiments were performed at least in duplicate. Yield of hemicelluloses is given on a dry weight basis related to the perennial ryegrass leaves (Table 1).

2.3. Structural and physiochemical characterization of hemicelluloses

The chemical composition of the five hemicellulosic polymers (10 mg) were determined by hydrolysis with 1.48 ml of 6% H_2SO_4 for 2.5 h at 100 °C. The analysis of the individual sugars in the hydrolysate was carried out by high performance anion exchange chromatography using pulsed amperoteric detection 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. Uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl assay (Blumenkrantz & Asboe-Hansen, 1973). Method for measurement of the hemicellulosic molecular weights has been described in previous papers (Lawther et al., 1995; Sun, Lawther, & Banks, 1996). The hydrolysis and analyses were conducted in duplicate, and the values of individual monosaccharide residues were within ±5%.

FT-IR spectra of the hemicellulosic samples were obtained on an FT-IR spectrophotometer (Nicolet 750) in the range 4000–400 cm^{-1} using a KBr disc containing

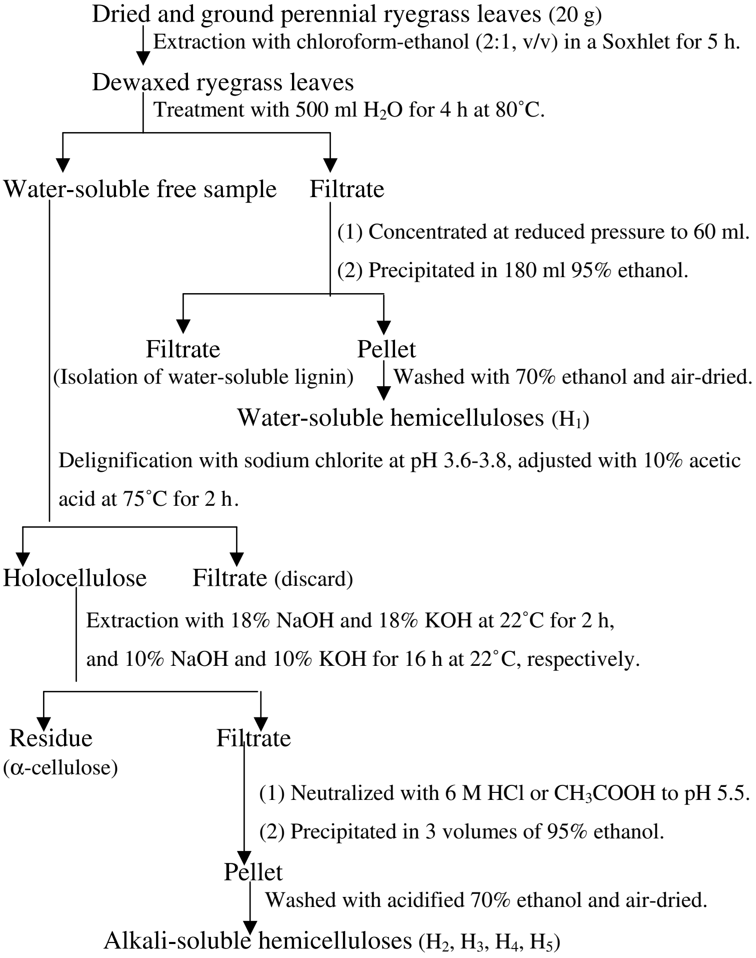

Fig. 1. Scheme for isolation of hemicelluloses from perennial ryegrass leaves.

Table 1
Yield and extraction conditions of the hemicelluloses obtained from perennial ryegrass leaves

Fraction no.	Extractant	Temperature (°C)/times (h)	Dry material (g)/extractant (ml)	Yield (% dry grass)
H_1[a]	Distilled water	80/4	20/500	8.4
H_2[b]	18% NaOH	22/2	3.0/60	17.6
H_3[c]	18% KOH	22/2	3.0/60	17.1
H_4[d]	10% NaOH	22/16	3.0/60	22.6
H_5[e]	10% KOH	22/16	3.0/60	21.9

[a] H_1 represents for the water-soluble hemicellulosic preparation solubilized during the distilled water treatment of dewaxed perennial ryegrass leaves for 4 h at 80 °C.

[b] H_2 represents for the alkali-soluble hemicellulosic preparation isolated with 18% NaOH for 2 h at 22 °C from the delignified grass leaves.

[c] H_3 represents for the alkali-soluble hemicellulosic preparation isolated with 18% KOH for 2 h at 22 °C from the delignified grass leaves.

[d] H_4 represents for the alkali-soluble hemicellulosic preparation isolated with 10% NaOH for 16 h at 22 °C from the delignified grass leaves.

[e] H_5 represents for the alkali-soluble hemicellulosic preparation isolated with 10% KOH for 16 h at 22 °C from the delignified grass leaves.

1% finely ground samples. The solution-state ^{13}C NMR spectrum was obtained on a Bruker MSL300 spectrometer at 74.5 MHz. The sample (80 mg) was dissolved in 1 ml D_2O (99.8% D) by adding three drops of NaOD 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.

Thermal analysis of the hemicellulosic samples was performed using thermogravimetric analysis (TGA) and differential thermal analysis (DTA) on a simultaneous thermal analyzer (SDT Q600, TA Instrument). The apparatus was continually flushed with nitrogen. The sample weighed between 9 and 11 mg and heated from room temperature to 600 °C at a rate of 10 °C per minute.

3. Results and discussion

3.1. Yield of water- and alkali-soluble hemicelluloses

The hemicellulosic polymer is a mixture of a number of different polysaccharides, and the yield and composition of the polymer can vary depending on the method of isolation (Morrison, 1974). To isolate the pure hemicelluloses, a prior treatment by extraction with organic solvents is required to remove the non-cell wall components such as wax and chlorophyll. It was found that the pre-treatment with chloroform–ethanol (2:1, v/v) under the condition used removed almost of the chlorophyll, wax, and other extractives with a combined yield of 5.3% dry matter. Following treatment with hot water for 4 h at 80 °C released 24.0% dry weight of the grass leaves, which contained 35.0% water-soluble hemicelluloses and 10.4% lignin together with some amounts of protein, ash, and starch. Similar results have been found in our previous studies on low molecular weight of hemicelluloses obtained by extraction with hot water from wheat straw (Lawther et al., 1995).

The delignification with acid chlorite was next examined at 75 °C for 2 h. In this case, the loss of the dry matter was 26.1% including lignin, residual protein, ash, and starch as well as minor quantities of polysaccharides. It was accepted that there were dangers of oxidation and depolymerization and that these could not be avoided but they were believed to be slight. Based on the investigation of hemicelluloses obtained during the process of delignification from oat tissues, Buchala, Fraser, and Wilkie (1972) indicated that 1% of the plant tissues was normally not accounted for, due to solubility losses during the delignification procedure, and arabinose, fructose, galactose, and xylose were the major sugars in the hydrolysates. The proportion of glucose in each of the hydrolysates was high (average ca. 35%); less than 10% of the hemicellulosic β-glucan was solubilized during the delignification procedure. It also reported that up to 8% of the total hemicelluloses from different oat tissues was lost on delignification (Buchala et al., 1972). On the other hand, it was assumed that if the polysaccharides in plant samples were identical then they would be similarly, or identically, affected; there seemed little danger that delignification would upset comparative studies unless residual lignin impeded dissolution or there were solubility losses similar to those above. There was no evidence that the duration of the delignification had any significant effect on the proportions of the sugars in hydrolysates (Reid & Wilkie, 1969).

The effects of alkali and extraction duration on the yield of hemicelluloses are shown in Table 1. Treatment of the holocellulose with 18% NaOH at 22 °C for 2 h, 18% KOH at 22 °C for 2 h, 10% NaOH for 16 h at 22 °C, and 10% KOH for 16 h at 22 °C yielded 17.6%, 17.1%, 22.6%, and 21.9% of the hemicelluloses (percent dry matter), respectively. The total yield of hemicellulosic preparations, including the hot water-soluble part (8.4%), was over 25% of the initial dry weight. As would be expected, the differences in the yield of two alkali-extractable hemicellulosic preparations were not distinct, 17.6% with 18% NaOH and 17.1% with 18% KOH, or 22.6% with 10% NaOH and 21.9% with 10% KOH. However, in a comparison of extracting power of aqueous alkali, sodium hydroxide is slightly more powerful than potassium hydroxide. This is in agreement with studies of the extraction of hemicellulosic polymers from wheat straw (Lawther, Sun, & Banks, 1996). At equal concentrations, sodium hydroxide is slightly more effective than potassium hydroxide for the removal of hemicelluloses, corresponding to the increasing alkali strength from KOH to NaOH. In addition, as the data shown in Table 1, the yield of hemicelluloses increased with increase in extracting time even though the extraction was performed under a relatively lower alkaline concentration. The yield of the hemicelluloses obtained by extraction with 18% NaOH at 22 °C for 2 h accounted for 17.6%, whereas it increased to 22.6% as 10% NaOH was used for 16 h at 22 °C. Similar observations were found between 18% and

10% KOH extractions. This increment was probably due to the direct consequence of favourable effect of reaction time on the diffusion of the hemicelluloses dissolved by alkali. Taken together, the total yield of hemicellulosic preparations, including the hot water-soluble part ranged between 25.5% and 31.0% of the initial dry weight.

3.2. Content of neutral sugars and uronic acids

Neutral sugars, expressed as a relative percentage of dry weight, and uronic acids, represented for a percentage of dry weight of hemicelluloses, in one water-soluble and four alkali-soluble hemicellulosic preparations are given in Table 2. Xylose (26.0%), glucose (24.2%), arabinose (19.8%), and galactose (19.1%) are the major sugar components of the water-soluble hemicellulosic fraction compared to predominance of xylose (37.6–44.5%) in the four alkali-soluble hemicellulosic preparations. The amounts of rhamnose and uronic acids were greater in water-soluble hemicelluloses (H_1, 6.8%, 9.1%) than in the alkali-soluble hemicelluloses (H_{2-5}, 0.1–2.0%, 5.8–6.3%), and no significant differences were found between the four alkaline hemicellulosic preparations. This indicated that the hot water treatment probably released both low molecular weight of galactoarabinoxylans and some amounts of pectic polysaccharides exception for noticeable amounts of β-glucan, and the four alkali-soluble hemicellulosic preparations were less branched in structure than the water-soluble hemicellulosic fraction. The presence of pectic polysaccharides in hot water-soluble fraction from agricultural residues has been widely demonstrated in our previous studies on wheat straw polysaccharides (Sun, Lawther, & Banks, 1998). Interestingly, the two alkali-soluble hemicellulosic samples H_2 and H_3, isolated with 18% NaOH and KOH, have a markedly consistent composition, indicating a similar structure of the hemicelluloses. On the other hand, there are major changes taking place in the composition of the two other alkali-soluble hemicellulosic samples H_4 and H_5. As the alkaline concentration decreased from 18% to 10% and the extraction duration extended to 16 h at 22 °C, the content of galactose decreased substantially from 14.5 % in H_2 and 12.7% in H_3 to 3.2% in H_4 and 7.3% in H_5. In contrast to this decreasing trend, the content of glucose increased significantly from 25.1% in $H_2$to 39.4% in H_4 as the NaOH concentration decreased from 18% to 10%. This phenomenon suggested that treatment of the delignified grass leaves with 10% NaOH for a longer period may result in a remarkable degradation of cellulose, since the glucose liberated was partially attributed to the cellulose fraction. This was confirmed by a lower yield of cellulose (22.7% dry weight of the grass leaves) isolated with 10% NaOH for 16 h at 22 °C, than that of the cellulose (28.2% dry weight of the grass leaves) extracted with 18% NaOH at 22 °C for 2 h.

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

Sugars (%)	Hemicellulosic preparations[a]				
	H_1	H_2	H_3	H_4	H_5
Arabinose	19.8	17.1	17.0	11.0	15.5
Rhamnose	6.8	2.0	1.9	0.6	0.1
Galactose	19.1	14.5	12.7	3.2	7.3
Glucose	24.2	25.1	28.7	39.4	34.7
Xylose	26.0	38.5	37.6	44.5	42.1
Mannose	3.8	2.9	2.2	1.4	0.1
Uronic acids	9.1	5.8	6.1	5.9	6.3

[a] Corresponding to the hemicellulosic preparations in Table 1.

Xylose to arabinose ratios are indicative of the degree of linearity or branching of hemicelluloses (Wedig, Jaster, & Moore, 1987). A high xylose to arabinose ratio would indicate a high degree of polymerisation with little bonding with other monosaccharide constituents. A low xylose to arabinose ratio suggest a short-chain polymer with a large amount of branching with other monosaccharides. As the data given in Table 2, the sugar compositions of H_2 and H_3 isolated with 18% alkali, were very similar, indicating again a similar structure of the hemicelluloses. A high content of xylose (37.6–38.5%) and noticeable amounts of arabinose (17.0–17.1%) and galactose (12.7–14.5%) suggested that they could contain high branched galactoarabinoxylans similar to those reported by Morrison (1974) from S23 ryegrass (*Lolium perenne*). It is very likely that a greater amount of arabinose was found in grass leaves than in wheat straw, and the ratio of arabinose to xylose was fourfold higher in ryegrass leaves (approximately 1:2) than the wheat straw (approximately 1:8). The compositions of the H_4 and H_5 preparations were also very similar, in which the content of arabinose (11.0–15.5%) and galactose (3.2–7.3%) was lower than in H_2 and H_3, however, the xylose–arabinose and xylose–galactose ratios were higher. These two preparations are therefore less branched galactoarabinoxylans or arabinoxylans of very similar structure than those of the hemicellulosic samples of H_2 and H_3. In addition, acidic sugars (probably glucuronic acid and/or 4-*O*-methyl-glucuronic acid) occurred in small amounts in all the hydrolysates of the four alkali-soluble hemicellulosic preparations, implying that these hemicelluloses are also composed of L-arabino (4-*O*-methyl-D-glucurono)xylans. Furthermore, a large amount of glucose found in the hydrolysate of water-soluble hemicelluloses probably arose from the presence of a mixed linkage β(1 → 3) (1 → 4)-D-glucan in perennial ryegrass leaves. Non-cellulose β-D-glucans have been identified as cell wall components of other five grasses with a yield range between 4.1% and 9.7% (*Zea mays*, *Hordeum vulgare*, *Triticum vulgare*, *Secale cereal*, and *Sorghum bicolour*). These polysaccharides are dissociated from the cell wall matrix of non-endospermic tissues by hot water and alkali and have been characterized as linear homopolymers consisting of both β-(1 → 3) and β-(1 → 4) glucosidic linkages. The mean ratio of (1 → 3) to (1 → 4) linkages has been estimated at 3:7 (Nevins, Yamamoto, & Huber, 1978). This apparent similarity in the β-D-glucans from various species indicated a highly conserved glucosyl sequence in the plant family Gramineae, such as barley, rye, oat, maize, and bamboo (Buchala & Wilkie, 1973). It is of interest to note that the ratio of β-(1 → 3) to β-(1 → 4) linkages decreased with increasing plant maturity and the degree of polymerization (DP) of the various β-D-glucans fell as each tissue matured. Gel-filtration experiments indicated that the β-D-glucans did not have molecular weight significantly different from those of the xylan-type hemicelluloses (Buchala & Wilkie, 1973).

3.3. Weight-average molecular weight

In this study, the molecular weights of the hemicellulosic polymers were analysed by gel permeation chromatography (GPC). The GPC revealed that the weight-average (M_w) molecular weight of 19,360 g mol^{-1} for water-soluble hemicelluloses and 31,060–36,140 g mol^{-1} for four alkali-soluble hemicellulosic preparations (Table 3), indicating that the hemicelluloses released during the hot water treatment had a much lower molecular weights than those the hemicelluloses isolated with aqueous alkali from delignified holocellulose of perennial ryegrass leaves. This phenomenon suggested that the hot water treatment released only low molecular polysaccharides such as galactoarabinoxylans and pectic substances as well as β-glucan, while the alkaline extraction of delignified grass leaves dissolved the hemicelluloses having a high molecular weight and the hemicellulosic polymers were not substantially degraded under the alkaline extraction conditions used. It should be noted that molecular weights of polysaccharide polymers vary depending on the method for their estimation. In addition, depending on solvent quality, chain aggregation may be partially responsible for such a wide variation in the estimates of molecular weight of these polymers (Izydorczyk & Biliaderis, 1995).

Table 3
Weight-average (M_w) and number-average (M_n) molecular weights and polydispersity (M_w/M_n) of the hemicellulosic preparations isolated from perennial ryegrass leaves

	Hemicellulosic preparations[a]				
	H_1	H_2	H_3	H_4	H_5
M_w	19,360	34,160	36,140	31,060	32,790
M_n	7000	7710	7940	7780	7400
M_w/M_n	2.77	4.43	4.55	3.99	4.43

[a] Corresponding to the hemicellulosic preparations in Table 1.

The elution profiles of hot water-soluble hemicelluloses declared a very narrow distribution of molecular size as indicated by its polydispersity (M_w/M_n) value of 2.8, whereas the four alkali-soluble hemicellulosic samples showed a very broad distribution of molecular weight. The high ratio of weight-average molecular weight to number-average molecular weight (M_w/M_n) ranged between 4.0 and 4.6 for alkali-extractable grass hemicelluloses, which strongly pointed to their inherent polydispersity.

3.4. FT-IR spectra

Fig. 2 shows the FT-IR spectra of the water-soluble hemicellulosic fraction (spectrum a) and the holocellulose (spectrum b) obtained from perennial ryegrass leaves. The hydroxyl group and ether signals (C–O–C) in sugar units are absorbed as strong intensities at 3425 and 1064 cm^{-1} or a shoulder at 1069 cm^{-1}, respectively. Bands between 1170 and 1000 cm^{-1} are typical of arabinoxylans, since the presence of the arabinosyl side-chains is shown by a high-intensity peak at 1130 cm^{-1} in the spectrum of water-soluble hemicellulosic fraction and a relatively low-intensity peak at 1161 cm^{-1} in the spectrum of holocellulose. The bands at 1646, 1413, 1242 cm^{-1} in the spectrum of water-soluble hemicellulose (spectrum a) are originated from carboxylate anion, methyl C–H wagging vibrations,

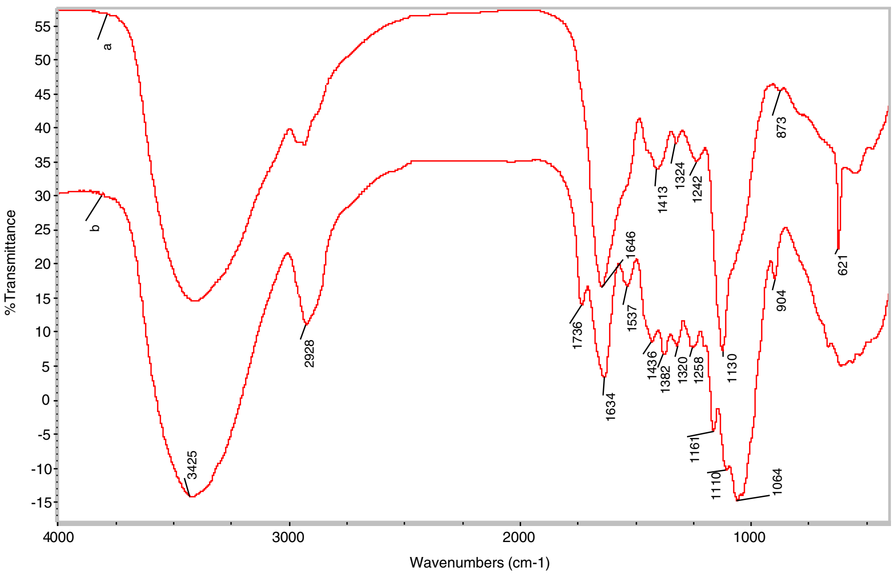

Fig. 2. FT-IR spectra of water-soluble hemicellulosic fraction (spectrum a) and holocellulose (spectrum b) obtained from perennial ryegrass leaves.

and carbonyl absorbance in pectic substances, confirming that the water-soluble hemicellulosic fraction contained noticeable amounts of pectic polysaccharides, which corresponded to the results obtained by sugar analysis.

In spectrum b, the absorption at 1634 cm^{-1} in the spectrum of holocellulose was principally associated with absorbed water, since holocellulose usually have a strong affinity for water, and in the solid state these macromolecules may have disordered structures which can easily be hydrated (Chaikumpollert et al., 2004). It is noticeable that a small band at 1736 cm^{-1} in holocellulose is due to the acetyl, uronic, and ferulic ester groups of the polysaccharides. The small bands at 1436, 1382, 1320, and 1258 cm^{-1} represent C–H and C–O stretching and CH or OH bending in polysaccharides. The C–H stretching vibrations give a signal at 2928 cm^{-1}. A small but sharp band at 904 cm^{-1} in spectrum b (holocellulose), which is due to the C-1 group frequency or ring frequency, is characteristic of β-glycosidic linkages between the sugar units (Sun et al., 1996).

The FT-IR spectra of the four alkali-soluble hemicellulosic preparations are illustrated in Fig. 3. As expected, the four spectral profiles and relative intensities of the most bands were rather similar, indicating a similar structure of the four hemicellulosic samples. The absorbances at 1421, 1378, 1328, 1258, 1204, 1165, 1044, and 904 cm^{-1} are associated with hemicelluloses. Two bands at 1165 and 1044 cm^{-1} are typical of arabinoxylans. In comparison with the spectra of water-soluble hemicellulosic fraction, a sharp band at 904 cm^{-1} in Fig. 3 demonstrated that the alkaline treatment under the conditions given did not cleave the β-glycosidic linkages between the sugar units from the backbone of hemicelluloses. Obviously, the disappearance of a band at 1510 cm^{-1} relating to the bound lignin stated that the four hemicellulosic preparations are free of associated lignin.

3.5. ^{13}C NMR spectra

The advance in the elucidation of the fine structure of plant polysaccharides has been possible via developments in appropriate analytical methods. In particular, NMR has proven invaluable in studying the molecular structures of hemicellulosic polymers. ^{13}C NMR spectroscopy, a non-destructive probe of molecular structure, has become a method of choice for structure elucidation of native hemicelluloses, e.g., arabinoxylans, and its spectroscopy allows for fast determination of the nature, configuration, and relative content of monosaccharide residues constituting the hemicelluloses as well as the type and amount of specific linkages (Izydorczyk & Biliaderis, 1995). In order to get a deeper insight into the branched structure of the hemicelluloses, a ^{13}C NMR spectrum of the hemicellulosic preparation H_2 isolated with 18% NaOH at 22 °C for 2 h was recorded as shown in Fig. 4. The main 1,4-linked β-D-Xyl*p* units were obviously observed by five strong signals at 104.4, 78.4, 76.8, 75.4, and 65.6 ppm which were, respectively, assigned to C-1, C-4, C-3, C-2, and C-5 of the β-D-Xyl*p* units. The signals at 111.5, 89.0, 82.5, and 64.0 ppm (data not shown) are attributed to C-1, C-4, C-2, and C-5 of α-L-Ara_f residues, respectively. These data revealed that the anomeric configuration of the D-xylopyranose residues is β while the L-arabinofuranose residue is α. Small amounts of glucuronic acid and 4-*O*-methyl-glucuronic acid were also present as identified from the spectrum with signals at 86.5 (C-4 in Glc*p*A), 72.0 (C-5 in Glc*p*A), and 61.7 ppm (4-*O*-methoxyl group of glucuronic acid). Two weak signals at 74.6 and 68.4 ppm arise from C-4 and C-2 in α-galactose residues. In the light of this, it is possible that the alkali-soluble hemicellulosic preparation H_2 is composed of galactoarabinoxylans and L-arabino (4-*O*-methyl-D-glucurono)xylans. Such a conclusion has been also drawn from legume, grass, and cereal straw cell

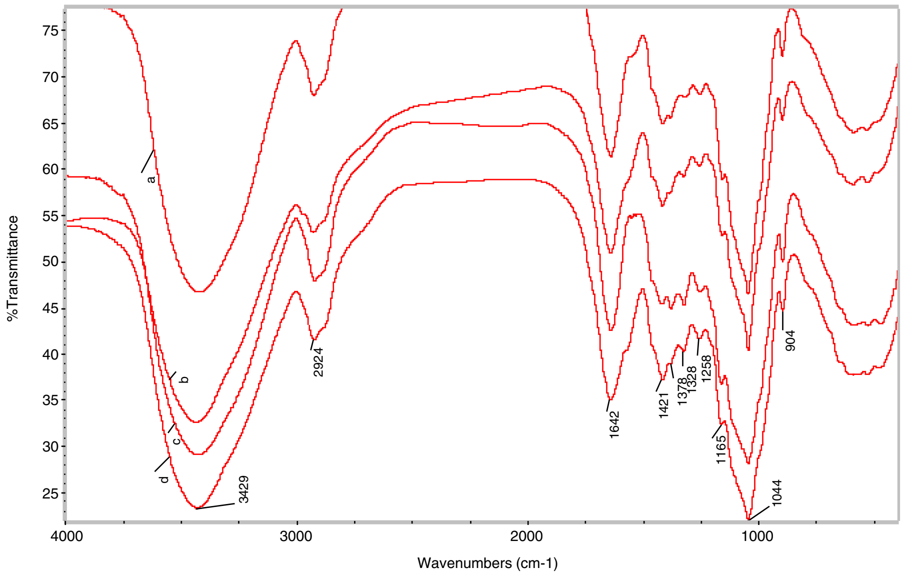

Fig. 3. FT-IR spectra of alkali-soluble hemicellulosic preparations H_2 (spectrum a), H_3 (spectrum b), H_4 (spectrum c), and H_5 (spectrum d).

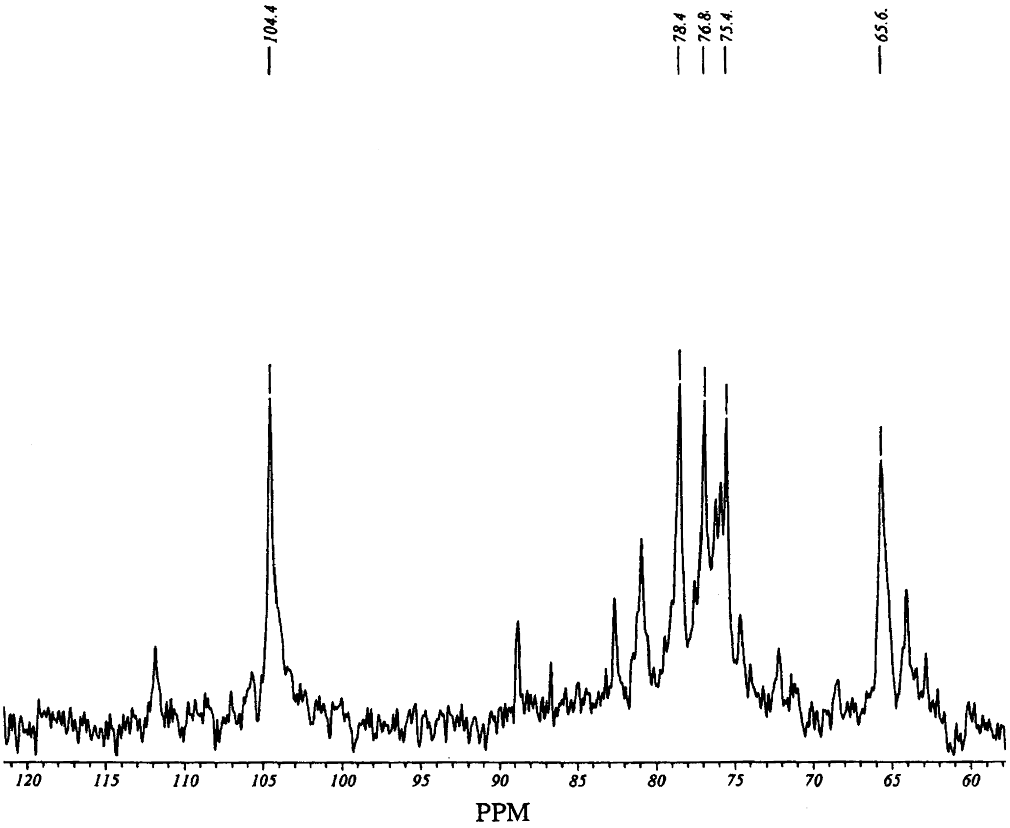

Fig. 4. ^{13}C NMR spectrum of alkali-soluble hemicellulosic preparation H_2 extracted with 18% NaOH for 2 h at 22 °C from the delignified perennial ryegrass leaves.

walls before and after extensive degradation by rumen microorganisms (Gordon, Lomax, & Chesson, 1983). They reported that arabinose was found to occur predominantly as the terminally linked sugar, but some in-chain units linked and (1 → 3) were also detected. Galactose residues were found as (1 → 4)- and (1 → 6)-linked units but mostly terminally linked. Rhamnose was linked primarily by (1 → 2)-bonds with some indication of branched units. The other sugar found, mannose, was largely terminally linked.

In addition, the occurrence of β-glucans in the alkali-soluble hemicellulosic preparation H_2 is characterized by two signals at 80.7 (C-4 in β-Glc*p*-(1 → 4)) and 63.0 ppm (C-6 in β-Glc*p*-(1 → 3)) (Roubroeks, Andersson, & Aman, 2000). Still, it is very difficult to compare β-glucans from other hemicelluloses because the spectra are probably overlapped or very similar. It has been reported that in addition to (1 → 4) and terminally linked glucose, there was an evidence of the presence of small amounts of (1 → 3)-linked units. These were present in greatest amount in early cut ryegrass (2.80% of total identified sugars) and amounts which decreased with increasing maturity (Gordon et al., 1983). Similar trend was observed for cereal β-glucans. Based on an extensive study of structural features of (1 → 3), (1 → 4)-β-D-glucan, and arabinoxylan fractions isolated from rye bran, Roubroeks et al. (2000) demonstrated that the proportion of (1 → 4)-linkages in cereal β-glucan exceeds that of (1 → 3)-linkages by at least a factor of two.

3.6. Thermal analysis

Fig. 5 illustrates the TGA/DTA curves of the water-soluble hemicelluloses H_1 (a), and the two alkali-soluble hemicellulosic preparations H_3 (b) isolated with 18% KOH at 22 °C for 2 h, and H_5 (c) extracted with 10% KOH for 16 h at 22 °C from the delignified ryegrass leaves. As shown in the figure, the TGA curves of the water-soluble hemicellulosic fraction H_1 and the two alkali-soluble hemicellulosic preparations H_3 and H_5 began to decompose at 223, 220, and 205 °C, respectively, and the maximum rate of weight loss was observed between 280 and 360 °C for H_1 and between 260 and 320 °C for H_3 and H_5 with a similar maximum of the exothermic peak at 308 °C in DTA thermograms. 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 indicated the onset of pyrolysis and generation of combustible gases (Sun & Tomkinson, 2003). At 10% weight loss the degradation temperature of the water-soluble hemicellulosic fraction H_1 and the two alkali-soluble hemicellulosic preparations H_3 and H_5 occurred at 292, 280, and 245 °C, respectively. This phenomenon suggested that the alkali-soluble hemicellulosic

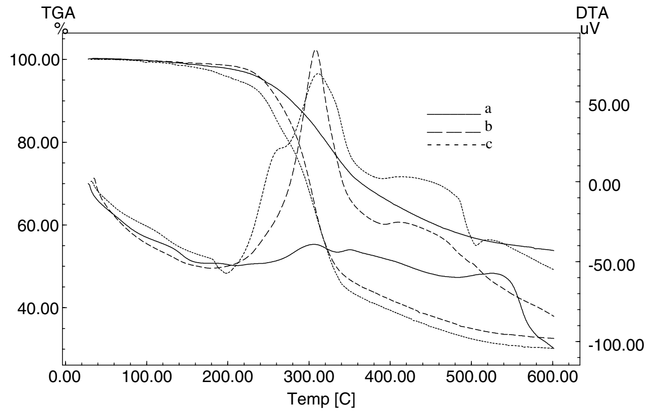

Fig. 5. Thermogram of hemicellulosic preparations H_1 (a), H_3 (b), and H_5 (c).

fraction H_3 extracted with a higher concentration of alkali for a shorter period (18% KOH at 22 °C for 2 h) had a higher thermal stability than that of the alkali-soluble hemicellulosic preparation H_5 isolated with a relatively lower concentration of alkali for a longer period (10% KOH for 16 h at 22 °C), corresponding to their molecular weights in Table 3. That is, the thermal stability of the alkali-soluble hemicelluloses increased with an increment in their molecular weight. However, this increasing trend was not parallel to that of the water-soluble hemicelluloses H_1.This is largely due to the different chemical composition between the water-soluble and the alkali-soluble hemicelluloses.

4. Conclusions

It is concluded that water-soluble hemicelluloses, accounted for 8.4% of the dry perennial ryegrass leaves, presumably consist of galactoarabinoxylans, β-glucan, and noticeable amounts of pectic polysaccharides, which had a lower molecular weight (19,360 g mol^{-1}) but a relatively higher thermal stability, while the four alkali-soluble hemicellulosic preparations, amounted to 17.1–22.6% of the dry matter, are probably composed of galactoarabinoxylans, L-arabino (4-*O*-methyl-D-glucurono)xylans, and β-glucan, which had a higher molecular weight between 31,060 and 36,140 g mol^{-1} but a lower thermal stability. The (1 → 4)-xylopyranose backbone is substituted on O-2 and/or O-3 by single residues or short chains. These branches may be single arabinose residue, single glucuronic acid or 4-*O*-methyl-glucuronic acid residues or a short chain of sugar residues containing arabinose and galactose. The results from the sugar analysis also revealed that the galactoarabinoxylans released during the hot water treatment and extracted with a higher concentration of alkali for a shorter period (18% NaOH or 18% KOH at 22 °C for 2 h) were more branched than the corresponding hemicelluloses isolated with a lower concentration of alkali for a longer period (10% NaOH or 10% KOH at 22 °C for 16 h) from the delignified ryegrass leaves. β-Glucan was characterized as linear homopolymers consisting of both β-(1 → 3) and β-(1 → 4) glucosidic linkages.

Acknowledgements

The authors wish to express their gratitude for the financial supports from Wales Development Agency and National Natural Science Foundation of China (No. 30430550), Guangdong Natural Science Foundation (No. 05103548), and Ministry of Education, China.

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