ISOLATION AND CHARACTERIZATION OF SOLUBLE POLYSACCHARIDES FROM *CALAMAGROSTIS ANGUSTIFOLIA* KOM

Xue-Fei Cao,[a] Tongqi Yuan,[b] Shaoni Sun,[b] and Run-Cang Sun [a,b,]*

Sequential treatments of dewaxed *Calamagrostis angustifolia* Kom with water (60 ºC and 90 ºC), 70% ethanol, and 70% ethanol containing 0.2%, 1.0%, 2.0%, 4.0%, and 8.0% NaOH at a solid to liquid ratio of 1:25 (g/mL) at 80 ºC for 3 h yielded 36.2% soluble polysaccharides of the dry dewaxed material. The eight polysaccharide fractions obtained were comparatively studied by sugar analysis, GPC, FT-IR, ^{1}H and ^{13}C-NMR, and 2D-NMR (HSQC) spectroscopy. The results showed that the water-soluble polysaccharides might contain noticeable amounts of β-D-glucan, as well as some pectic substances and galactoarabinoxylan. 70% ethanol-soluble polysaccharide was mainly arabinogalactan. The five alkali-soluble hemicelluloses were mainly galactoarabinoxylans. The Ara/Xyl and Ara/Gal values of H_5-H_8 fractions decreased with the increment of NaOH concentration from 1.0% to 8.0%. Meanwhile, the molecular weights had a declining trend from ~60,000 to ~40,000 g/mol. The smaller sized and more branched polysaccharides tended to be extracted in the early stages under milder conditions, and the larger molecular sized and more linear hemicelluloses tended to be isolated under more highly alkaline conditions.

Keywords: Sugars; Soluble polysaccharides; Calamagrostis angustifolia Kom*; NMR; Sequential extractions*

*Contact information: a: State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, 510640, Guangzhou, China; b: Institute of Biomass Chemistry and Technology, Beijing Forestry University, 100083, Beijing, China; *Corresponding author: rcsun3@bjfu.edu.cn (R.C. Sun)*

INTRODUCTION

Synthetic fiber, plastic, and rubber, as the most commonly used macromolecular materials, have been applied in people's daily life and industry for many years. With the opening up of applications for these synthetic materials, their consumption has increased each year. However, the resources of the most synthetic materials come almost entirely from petroleum and natural gas. As we all known, fossil fuels are limited and non-renewable, and they are consumed increasingly to meet the huge demands of energy and petroleum-based products. It is a hard and undeniable fact that the fossil fuels will be exhausted some day. The energy issue and the shortage of products based on the crude oil will become a severe problem that we have to face up to. It will be very likely to become one of the key factors impeding economic growth of the world and a source of potential conflicts. When that day is eventually reached, the only alternative resource for any substance produced today by the petrochemicals-based industries will be renewable resources.

Due to their relatively low content of lignin, grasses are easily separated into

readily utilizable components, and therefore considered as one of the potential resources for the biological production of fuel and materials. *Calamagrostis angustifolia* Kom is one of the main pasture crops and has a wide distribution in northeastern and northern China and the Inner Mongolia wetland plains of China. It has a powerful capacity of regeneration and a short growth cycle, so that it can complete its growth within two months. It can also be reaped two times every year. The harvest time has a great influence on the nutrient component of this pasture. Livestock is willing to eat the pasture reaped in the first seasonal crop, which has a higher content of crude proteins. However, the second harvest of this pasture is usually used to build grass huts or even is discarded as an underutilized excess material. In consideration of its important role in the pastures in the north of China, it is necessary to study the utilization of this waste to be something more valuable, and then promote local development in these areas.

In wood and grass, the soluble polysaccharides that are present are mainly comprised of hemicelluloses, which are classically defined as the alkali-soluble material after removal the pectic substances (Lamport 1970). Hemicelluloses are the second most abundant plant renewable materials after cellulose (Sun et al. 2001), and they comprise roughly one-fourth to one-third of most plant materials of all land, fresh water plants, and some seaweed. They are branched polymers of low molecular weight with a degree of polymerization of 80 to 200. Their general formulates are $(C_5H_8O_4)_n$ and $(C_6H_{10}O_5)_n$, called pentosans and hexosans, respectively (Kacuráková et al. 2000). Hemicelluloses consist of various different sugar units such as D-xylose, D-mannose, D-glucose, L-arabinose, D-galactose, D-glucuronic acid, and D-galacturonic acid, which are arranged in different proportions and substituents (Sun et al. 2003). Other sugars such as L-rhamnose and L-fucose may also be present in small amounts, and the hydroxyl groups of sugars can be partially substituted with acetyl groups (Girio et al. 2010). According to Doblin et al. (2001), hemicelluloses include xyloglucans, mannans, glucomannans, mixed-linkage glucans, and xylans, and the most important biological role of hemicelluloses is their contribution to strengthening the cell wall by interaction with cellulose and lignin. Xyloglucans are the most abundant hemicelluloses in primary walls of spermatophytes except for grasses. Mannans and glucomannans are often acetylated, and they can be separated from softwood in large amounts. β-(1→4)-linked glucans with interspersed single β-(1→3)-linkages are well known in grasses, and these can be quite easily extracted without alkali. Xylans contain a backbone of D-xylopyranosyl residues, linked together by β-(1→4)-glycosidic bonds. These xylans usually contain many arabinose residues attached to the backbone and are known as arabinoxylans and glucuronoarabinoxylans. There are a number of L-arabinofuranose and D-glucuronic acid (or its 4-*O*-methyl derivative) residues (through position C-2 or C-3 or both) attached to the xylans backbone as single-unit side chains (MacGregor and Fincher 1993). In addition, phenolic acids such as ferulic and *p*-coumaric acids, which have intimate connection with the linkage between hemicelluloses and lignin, have been found to be esterified to *O*-5 of some L-arabinofuranose residues in arabinoxylans (Prates et al. 2001). Hemicelluloses can be neutral or acidic depending on whether they contain 4-*O*-methyl-D-glucuronopyranosyl or D-glucuronopyranosyl substituents.

Grass hemicelluloses form covalent bonds with lignin and ester linkages with acetyl units and hydroxycinamic acids, restricting the liberation of hemicelluloses from the cell wall matrix. Hydrogen bonding between the individual polysaccharide cell wall

components may also impede the isolation of hemicelluloses component (Kačuráková et al. 2000). Although many methods have been used to separate hemicelluloses from plant cell walls, alkaline extraction is widely used due to its easy operation and high efficiency. By this process, cellulose, hemicelluloses, and lignin can be roughly fractionated, and then they can be widely utilized in different fields. Although forage grasses are considered to be abundant biomass suitable for bio-fuels and bio-chemicals, there have been no reports on the structures and physiochemical properties of the polysaccharides from *Calamagrostis angustifolia* Kom. Since the structure and functions are intimately related, the structure information of this material is essential for its potential utilization. Therefore, the main purposes of this paper are to study the isolation of polysaccharides and determine their structural properties.

EXPERIMENTAL

Materials

Calamagrostis angustifolia Kom was harvested in October from a farm in Northeast China. It was dried in sunlight, cut into small pieces and then dried at 60 ºC in an oven for 16 h before use. The material, which passed through a 60-mesh screen and was retained on an 80-mesh screen, was collected for subsequent experimentation. In addition, the main composition of this material was analyzed (Nykter et al. 2008). After the wax and water-soluble polysaccharides were removed, the material consisted of approximately 43.2 wt% cellulose, 36.5 wt% hemicelluloses, and 20.3 wt% lignin.

Isolation of Polysaccharides

The scheme for sequentially extracting polysaccharides with hot water, aqueous ethanol and ethanol solution with sodium hydroxide is shown in Fig. 1. Specifically, the dried sample was first treated by refluxing in a Soxhlet apparatus with toluene-ethanol (2:1, v/v) for 6 h to remove fats and waxes. The dewaxed material (15 g) was then successively treated with distilled water at 60 ºC and 90 ºC, and then 70% ethanol at 80 ºC with a constant solid to liquor ratio 1:25 (g/mL) for 3 h under stirring. After filtration in a Büchner funnel, the residue was washed thoroughly with the distilled water and then dried in an oven at 60 ºC for 16 h. The filtrate was concentrated with a rotary evaporator under reduced pressure, and then precipitated in 2 volumes of 95% ethanol at room temperature under stirring. The precipitate was recovered by centrifugation, washing with 70% ethanol, and freeze-dried. These polysaccharides were named as H_1, H_2, and H_3, respectively.

Then the residue was successively extracted with 70% ethanol containing 0.2%, 1.0%, 2.0%, 4.0%, and 8.0% NaOH with a 1:25 solid to liquor ratio (g/mL) at 80 ºC for 3 h under stirring. The insoluble residue was filtrated as crude cellulose. The filtrates were neutralized to pH 5.5-6.0 with 6 M HCl and then concentrated at reduced pressure. These polysaccharides were precipitated and separated as the method mentioned above, and designated as H_4, H_5, H_6, H_7, and H_8, respectively.

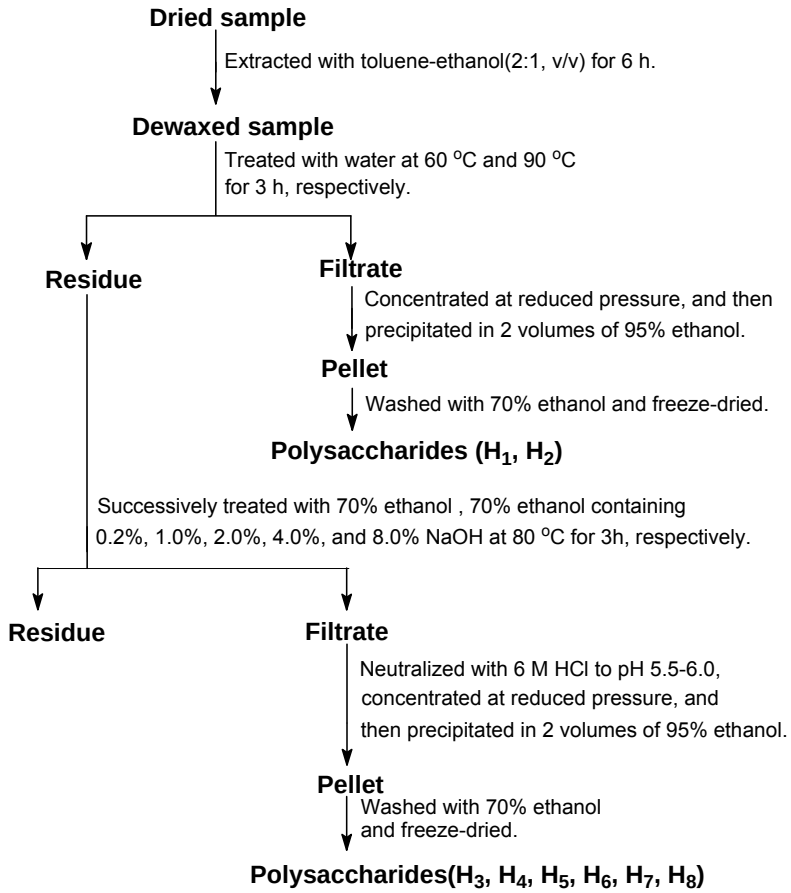

Fig. 1. Scheme for isolation of polysaccharides from *Calamagrostis angustifolia* Kom.

Analytical Methods

The neutral/acid sugar composition of the isolated polysaccharides was detected by a high-performance anion-exchange chromatography (HPAEC). About 5 mg sample of polysaccharides was hydrolyzed with 1.475 mL of 6% H_2SO_4 for 2.5 h at 105 ºC. After hydrolysis, the mixture was filtered and diluted 50-fold, and injected into the HPAEC (Dionex ICS3000, U.S.) with pulsed amperometric detector, AS50 autosampler, the CarbopacTM PA-20 column (4×250 mm, Dionex), and the guard PA-20 column (3×30 mm, Dionex). Samples injected into the system were eluted with 0.018 M NaOH (carbonate free and purged with nitrogen) with post-column addition of 0.3 M NaOH at a rate of 0.5 mL/min. Run time was 45 min, followed by 10 min elution with 0.2 M NaOH to wash the column and then 15 min elution with 0.018 M NaOH to re-equilibrate the column. The uronic acids were eluted with 0.4 M NaOH for 20 min at a rate of 1 mL/min with post-column addition of 0.3 M NaOH at a rate of 0.5 mL/min. Neutral sugars and uronic acids were separated in a 5 mM NaOH isocratic (carbonate free and purged with nitrogen) condition for 20 min, followed by a 0-75 mM NaAc gradient in 5 mM NaOH for 15 min. Then the columns were washed with 200 mM NaOH to remove carbonate for 10 min. This was followed by a 5 min elution with 5 mM NaOH to re-equilibrate the column before the next injection. The total analysis time was 50 min, and the flow rate was 0.4 mL/min. Calibration was performed with a standard solution of L-rhamnose, L-arabinose, D-glucose, D-xylose, D-mannose, D-galactose, glucuronic acid, and

galacturonic acid. The analysis of sugar composition in present study was run in duplicate, and average values were calculated for all eight polysaccharide fractions.

The weight-average (M_w) and number-average (M_n) molecular weights of these polysaccharide fractions were determined by gel permeation chromatography (GPC) on a PL aquagel-OH 50 column (300×7.7 mm, Polymer Laboratories Ltd.), calibrated with PL pullulan polysaccharide standards (peak average molecular weights 738, 12200, 100000, 1600000, Polymer Laboratories Ltd.). A flow rate of 0.5 mL/min was maintained. The eluent was 0.02 N NaCl in 0.005 M sodium phosphate buffer (pH 7.5). Detection was achieved with a differential refractive index detector (RID). The column oven was kept at 30 ºC. All fractions were dissolved in the eluent, pH 7.5 at a concentration of 0.1% before measurement.

The FT-IR spectra of the eight polysaccharides samples were obtained on a FT-IR spectrophotometer (Tensor 27) using a KBr disc containing 1% finely ground samples. All the spectra were measured in the frequency range from about 4000 to 400 cm^{-1}, and 32 scans were taken per sample.

Solution-state ^{1}H-NMR and ^{13}C-NMR spectra were obtained with a Bruker AVIII 400 MHz spectrometer (operating frequency of 100.6 MHz). The sample (20 mg for ^{1}H, 80 mg for ^{13}C) was dissolved in 1 mL D_2O. The ^{13}C-NMR spectrum was recorded at 25 ºC after 30000 scans. A 30° pulse flipping angle, a 9.2 μs pulse width, a 1.36 s acquisition time, and 2 s relaxation delay time between scans were used. Heteronuclear Single Quantum Coherence (HSQC) data were also obtained on a Bruker AVIII 400 MHz spectrometer after 128 scans with 20 mg sample dissolved in 1 mL D_2O. The spectral width was 2200 Hz for the ^{1}H-dimensions and 15400 Hz for the ^{13}C-dimensions. The number of collected complex points was 1024 for ^{1}H-dimension with a relaxation delay of 1.5 s. The number of scans was 128, and 256 time increments were recorded in the ^{13}C-dimension. The $^1J_{CH}$ used was 146 Hz. Prior to Fourier transformation, the data matrixes were zero filled up to 1024 points in the ^{13}C-dimension.

RESULTS AND DISCUSSION

Yield of Polysaccharides

The polysaccharides, which are extensively distributed in the plant cell wall, can be extracted with diverse solvents, and their yield and composition can vary depending on the method of isolation. In order to extract the polysaccharides from *Calamagrostis angustifolia* Kom as entirely as possible, and then further study their structural characteristics, the dewaxed sample was sequentially treated with distilled water at 60 ºC and 90 ºC, 70% ethanol, and 70% ethanol containing sodium hydroxide at different concentrations. The yields of polysaccharides (% dried dewaxed material) are illustrated in Table 1. As can be seen, a total amount of 36.24% hemicelluloses was isolated during the eight steps. The amounts 9.72%, 0.70%, 1.27%, 0.86%, 10.10%, 5.72%, 7.57%, and 0.30% of the soluble polysaccharides (% dry matter) were released from each successive extraction progress, respectively. It should be noted that the water-soluble polysaccharides accounted for 28.8% of the totally extracted fractions, and almost all of them were released at 60 ºC by water treatment. These results are probably due to the relatively high content of glucose-rich substances in grass. As we know, ethanol has been used

routinely as precipitant in the isolation of polysaccharides. However, it is interesting to note that in this research when the residue was treated with 70% ethanol, some polysaccharides could dissolve in the aqueous alcohol and then they were precipitated as usual. The reason for this phenomenon was probably that some polysaccharides were soluble in aqueous ethanol at a relatively higher temperature. However, as the solvent cooled down, they would precipitate out of the solution. Then, as the sodium hydroxide was added into the 70% ethanol solution, the release of hemicelluloses increased significantly. Taken together, the five-step sequence of alkali treatments took up 67.7% of the total yield of polysaccharides. The first step of alkali extraction did not release a large amount of hemicelluloses. As the concentration of sodium hydroxide was increased from 0.2% to 1.0%, the yield of hemicelluloses rose dramatically from 0.86% to 10.10%. This observation suggested that only when the alkali concentration exceeds certain content can the linkages between lignin and hemicelluloses be cleaved effectively. When the residue was finally extracted with 70% etnanol containing 8.0% NaOH, the yield of hemicelluloses was very low, which indicated that almost all of the polysaccharides had already been released from the material.

Table 1. Yield of Polysaccharides (% Dried Dewaxed Material) Obtained by Successive Treatments of the Grass under Different Conditions.

	Polysaccharide fraction[a]								
	H_1	H_2	H_3	H_4	H_5	H_6	H_7	H_8	Total
Yield	9.72	0.70	1.27	0.86	10.10	5.72	7.57	0.30	36.24

[a] H_1, H_2, H_3, H_4, H_5, H_6, H_7, and H_8 represent the polysaccharides obtained by sequential treatments of dewaxed material with water at 60 ºC and 90 ºC, 70% ethanol, 70% ethanol containing 0.2%, 1.0%, 2.0%, 4.0%, and 8.0% NaOH with a 1:25 solid to liquor ratio (g/mL) at 80 ºC for 3 h, respectively.

Content of Neutral Sugars and Uronic Acids

It is well known that the polysaccharide components differ with respect to their location in the plant cell walls and extraction processes. The main sugars are xylose, arabinose, glucose, galactose, mannose, rhamnose, and uronic acids. To analyze the difference among these polysaccharides, the content of neutral sugars and uronic acids of the eight polysaccharides were detected, and the data are illustrated in Table 2. According to the different extraction processes, the polysaccharides were fractionated into water-soluble (H_1, H_2), 70% ethanol-soluble (H_3), and alkali-soluble (H_4-H_8) hemicelluloses.

The H_1 and H_2 fractions extracted by hot water had a similar content of sugars. Glucose, galactose, arabinose, xylose, and galacturonic acid were the predominant sugar components; in particular glucose took up over 50% of the total sugars. This high content of glucose might originate from the β-D-glucan of the plant. No starch was present in water-soluble polysaccharides, and that was confirmed by a positive test with KI/I_2. However, comparing H_1 with H_2, they had some differences in the content of galacturonic acid (12.51% in H_1, 4.44% in H_2) and xylose (6.17% in H_1, 14.69% in H_2). Low xylose content meant less hemicellulosic fraction in H_1 (Wedig et al. 1987), while a high galacturonic acid content in H_1 correspondingly indicated more pectic polysaccharides (Gross and Wallner 1979). This indicated that the hot water treatment probably released

noticeable amounts of β-D-glucan and some amounts of pectic polysaccharides and galactoarabinoxylans (Xu et al. 2007). The presence of pectic polysaccharides in the water-soluble fraction from agriculture residues had been demonstrated in our previous studies on wheat straw and some shrubs (Sun et al. 1996, Sun et al. 1998). The fraction of the 70% ethanol-soluble polysaccharide (H_3) was mainly composed of galactose (45.77%), arabinose (23.86%), glucose (11.74%), and xylose (9.10%), indicating the presence of a kind of arabinogalactan. Arabinogalactan could be dissolved in a low content of ethanol, and then precipitated with a high concentration of that. This was in accordance with the results of a recent report (Suarez et al. 2005).

Table 2. The Content of Neutral Sugars and Uronic Acids (Relative % Polysaccharides, w/w) in Isolated Polysaccharide Fractions

Neutral sugars/ uronic acids	Polysaccharide fraction[a]							
	H_1	H_2	H_3	H_4	H_5	H_6	H_7	H_8
Rhamnose	ND[b]	2.72	ND	ND	ND	ND	ND	ND
Arabinose	8.49	7.36	23.86	11.69	14.07	11.96	9.74	6.77
Galactose	19.61	14.97	45.77	4.24	5.16	2.60	1.58	0.90
Glucose	50.19	52.31	11.74	3.68	4.12	5.69	12.83	13.16
Xylose	6.17	14.69	9.10	79.31	75.68	78.76	75.05	78.25
Mannose	2.63	2.48	6.35	ND	ND	ND	ND	ND
Glucuronic A	0.39	1.04	0.47	0.49	0.16	0.12	0.08	0.04
Galacturonic A	12.51	4.44	2.72	0.59	0.81	0.86	0.71	0.88
Ara/Gal	2.31	2.03	1.92	0.36	0.37	0.22	0.16	0.13
Ara/Xyl	1.38	0.50	2.62	0.15	0.19	0.15	0.13	0.09

[a] Corresponding to the polysaccharide fractions in Fig. 1.
[b] ND = not detectable.

It is known that alkali is very efficient to extract hemicelluloses from plant cell walls. In this study, all the five alkali-soluble hemicelluloses were precipitated in 2 volumes of 95% ethanol. As can be seen in Table 2, the percentage of xylose was relatively stable, ranging from 75.05% to 79.31%, suggesting the high proportion of xylan in alkali-soluble hemicelluloses. Arabinose, galactose, and glucose, ranging from 6.77% to 14.07%, 0.9% to 5.69%, and 3.68% to 13.16%, respectively, were the relatively higher sugar components. Small amounts of uronic acids (<1%) were identified, and no rhamnose and mannose were detected. As the concentration of NaOH increased, the content of glucose increased, whereas the proportion of arabinose, glucuronic acid, as well as the ratios of arabinose to galactose (Ara/Gal) and xylose (Ara/Xyl) decreased accordingly. The decline of the ratio of Ara/Gal might be explained by the alkaline degradation of the oligomeric side chains of arabinose and galactose residues. Moreover, it should be noted that the ratio of arabinose to xylose could imply the degree of linearity or branching of hemicellulosic fraction (Wedig et al. 1987). A decreasing content of arabinose and glucuronic acid indicated that the hemicelluloses became more linear. In contrast to this decreasing tendency, the increasing glucose content might result from the degradation of cellulose, such as would occur due to the peeling reaction, which would

take place even in mild alkaline solution. Therefore, we could make a conclusion that galactoarabinoxylans might be the main component of the five alkali-soluble hemicelluloses, and the higher branched hemicelluloses were more easily soluble in alkaline solution than the linear hemicelluloses.

Molecular Weight Distribution

The molecular weight and its distribution are important parameters of macromolecules, especially in the fine chemical industry. The distribution has a huge effluence on the physical characteristics of the products and the final application of polymers. Therefore, to further investigate the difference of the eight polysaccharides, they were analyzed by the gel permeation chromatography (GPC), and their weight-average (M_w), number-average (M_n), and distribution of molecular weights are shown in the Table 3. Obviously, the hot water-soluble fractions H_1 and H_2, of which M_w were 11,670 g/mol and 19,470 g/mol respectively, showed a relatively lower degree of polymerization than others. The 70% ethanol-soluble polysaccharides H_3 with a M_w value of 33,430 g/mol, while the five alkali-soluble hemicelluloses (H_4, H_5, H_6, H_7, and H_8) displayed a higher M_w value from 38,630 to 60,320 g/mol.

Relating to the sugar analysis, we could presume that the shorter, smaller, and more branching polysaccharides could be solubilized more easily by the hot water treatment, while the alkali-soluble hemicelluloses consisted of longer, larger, and less branching polymers. It is worthy to note that, as the NaOH concentration was increased, the M_w value of these fractions increased from ~40,000 (H_4) to ~60,000 (H_5 and H_6) then back to ~40,000 g/mol (H_7 and H_8). This indicated that the relatively smaller hemicelluloses were extracted first by mild alkaline solution.

With the increment of NaOH concentration, the relatively larger hemicelluloses were released. Meanwhile the peeling reaction of hemicelluloses became severe under high NaOH concentration conditions, and the larger polymers were degraded into smaller ones. Corresponding with the M_w decrease, the content of glucose of H_5-H_8 rose remarkably from 4.12 to 13.16 %. It can be concluded that the cellulose experienced a noticeable degradation and that the same was true for the hemicelluloses. In addition, it should be noted that molecular weights of polysaccharide polymers varied depending on the extraction methods. Besides solvent quality, chain aggregation may be partially responsible for such a wide variation in the estimates of molecular weight of these polymers (Izydorczyk and Biliaderis 1995).

Table 3. Weight-Average (M_w) and Number-Average (M_n) Molecular Weights and Polydispersity (M_w/M_n) of the Isolated Polysaccharide Fractions

	Polysaccharide fraction[a]							
	H_1	H_2	H_3	H_4	H_5	H_6	H_7	H_8
M_w	11670	19470	33430	40590	60000	60320	38630	40190
M_n	8170	10860	11770	18880	25740	24190	17900	16170
M_w/M_n	1.43	1.79	2.84	2.15	2.33	2.49	2.16	2.49

[a] Corresponding to the polysaccharide fractions in Fig. 1

Generally, it is important to get polymers with narrow polydispersity because of their better physicochemical stability. Similarly, the isolation of various narrow polydispersity polysaccharides from a plant is equally fundamental. As shown by the data in Table 3, the two hot water-soluble polysaccharides had a narrow distribution (1.43 in H_1, 1.79 in H_2), while the mass distribution of the five alkali-soluble hemicelluloses ranged from 2.15 to 2.49. The ratio of M_w/M_n of the 70% ethanol-soluble polysaccharide reached 2.84, so it was the highest of all the fractions.

FT-IR Spectra

In order to identify the structural characteristics of each polysaccharide obtained under different conditions, FT-IR spectroscopy was used to analyze the functional groups and structural information. The FT-IR spectra of H_1 to H_4 are shown in Fig. 2 (a). The strong and wide absorption centered at 3410 cm^{-1} is attributed to the hydroxyl stretching vibration, and the bands at 2923 and 2853 cm^{-1} arise from the C-H asymmetric and symmetrical vibrations in methyl and methylene groups, respectively. The small peak at 1461 cm^{-1} is related to the scissoring bending of methylene. The strong absorption band at ~1635 cm^{-1} is characteristic of the bending mode of the absorbed water for polysaccharides having a strong affinity for water (Nacos et al. 2006; Le Troedec et al. 2008). The peak at ~1371 cm^{-1} represents the C-H bending vibration of methyl group. The presence of a shoulder at 1731 cm^{-1} in the spectrum of H_3 extracted with 70% ethanol suggests that H_3 contained carbonyl groups, such as acetyl, uronic, and ester groups, etc. (Tserki et al. 2005). However, this signal disappeared in the spectra of water-soluble polysaccharides fractions, indicating that carbonyl group has a chance of survival during 70% ethanol treatment at 80 ºC. The medium band at 1543 cm^{-1} in the spectrum of H_3 and small band at 1517 cm^{-1} in the spectrum of H_4 are related to the aromatic skeletal vibration of the associated lignin in polysaccharides (Xu et al. 2006). The maximum absorption bands in the mid-infrared region at 1200-800 cm^{-1} have been shown to be useful for the identification of polysaccharides with different structures and compositions (Israilides et al. 2007). The bands in 1200-1000 cm^{-1} are dominated by ring vibrations overlapped with stretching vibration of C-OH side groups and the C-O-C glycosidic bond vibration (Kačuráková et al. 2000). In the spectrum of H_4, the bands at 991 and 1166 cm^{-1} are related to the presence of arabinoxylans, which indicated the presence of the arabinosyl side chain (Kačuráková et al. 2000).

The FT-IR spectra of H_5, H_6, H_7, and H_8 are shown in Fig. 2 (b). Most of the bands of these four fractions were rather similar, especially among H_6, H_7, and H_8. The bands at 1445, 1326, 1249, 1157, 1121, 1094, 1043, 991, 898, and 878 cm^{-1} have a close contact with hemicelluloses. The very weak absorption at 1763 cm^{-1} belongs to a γ-lactone corresponding to lignans (Pavliková et al 2004; Tan et al. 2005). The band at 1445 cm^{-1} relates to the CH_2 symmetric bending vibration. The peak observed at 1326 cm^{-1} is attributed to the bending vibration of C-H and C-O groups of the aromatic ring in polysaccharides (Nacos et al. 2006; Le Troedec et al. 2008). The small peak at 1249 cm^{-1} is assigned to the C-O stretching of the aryl group in lignin (Le Troedec et al. 2008). The band at 1157 cm^{-1} relates to the C-O, C-O-C stretching or the contribution of C-OH bending. The sharp peak at 1121 cm^{-1} is typical of C-O, C-C stretching. The bands at 1094 cm^{-1} and 1043 cm^{-1} are due to the ring vibration and COH bending modes, which are strongly influenced by the degree of branching and hydration.

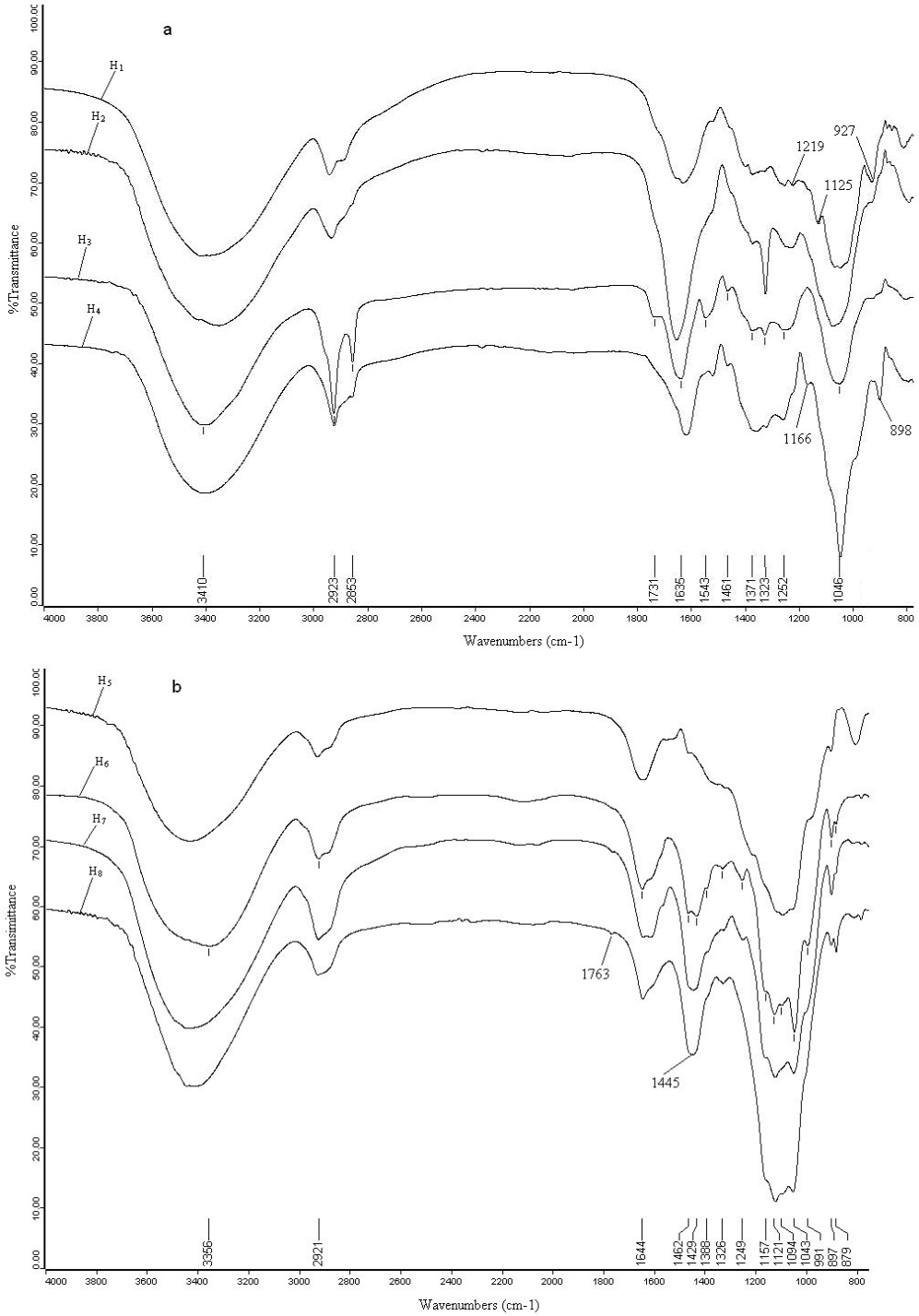

Fig. 2. FTIR spectra of the hemicellulosic fractions (a, H_1, H_2, H_3, and H_4), (b, H_5, H_6, H_7, and H_8)

The intensity changes of the bands at 1157 and 991 cm^{-1} can be suggested to reflect the arabinosyl substituent contribution and, therefore, used for the identification of arabinoxylan structures (Kačuráková et al. 2000). As can be seen, with the increasing of the concentration of NaOH, the intensity decrease at 1157 and 991 cm^{-1} indicates that the content of arabnosyl substituent decreased accordingly. This result was in line with the

data in sugar analysis. The small sharp band at 897 cm^{-1} is attributed to the glycosidic C_1-H deformation mode with ring vibration contribution and OH bending modes. The presence of bands at 897 and 879 cm^{-1} is characteristic of β-glycosidic linkages between the sugar units and pyranoid ring conformation of xylan units (Komiyama et al. 2008; Kačuráková et al. 2000).

^{1}H and ^{13}C and 2D HSQC NMR Spectra

With developments in appropriate analytical methods, advances in the elucidation of the structure of various plant polysaccharides have been possible. Nuclear magnetic resonance (NMR) has proven invaluable in identifying the polymer backbone and the type of side-chain. NMR spectroscopy, a non-destructive probe of molecular structure, has been widely used for structural elucidation of carbohydrates. Because of the complex composition and structure of the sample, the signals in ^{1}H and ^{13}C NMR spectra always overlap badly, and the assignments become difficult to make. However, by virtue of the method of HSQC we were able to assign various polysaccharides easily. Therefore, the ^{1}H, ^{13}C, and HSQC spectra of the fraction H_5 are shown in Figs. 3 and 4, respectively. The main hemicelluloses cross-signals assigned in the HSQC spectrum are listed in Table 4.

As can be seen from Fig. 3, the anomeric signals in the ^{1}H NMR spectrum of H_5 were assigned according to sugar analysis and literature data (Chiarini et al. 2004), and they were found in the region of 4.30 to 5.60 ppm. The relevant signals appeared in two regions, namely, the anomeric region (δ 5.60-4.90 for α-anomers and δ 4.90-4.30 for β-anomers), and the ring proton region (δ 4.50-3.00). Therefore, the signals at 5.31 and 4.48 ppm are assigned to the terminal arabinose residues and xylose residues, respectively (Bengtsson and Åman 1990). This confirmed that xylan is linked by β-glycosidic bonds, which is consistent with the presence of the small peak at 897 cm^{-1} in the FT-IR spectrum of H_5. Terminal arabinofuranosyl residues are linked at O-2 and/or O-3 xylose residues (Saha 2003). The signal at 5.46 ppm arises from terminal arabinose residues linked to position O-3 in xylose residues. The two signals at 5.31 and 5.22 ppm are assigned as the anomeric protons of terminal α-L-arabinfuranosyl residues substituted at O-2 and O-3, respectively (Westerlund et al. 1993).

The resonances at 4.46, 3.21, 3.48, 3.68, 4.02, and 3.30 ppm in the ^{1}H-NMR spectrum are attributed to H-1, H-2, H-3, H-4, H-5eq, and H-5ax of the (1→4)-β-D-xylopyranosyl (Broberg et al 2000), and the main 1,4-linked β-D-xylopyranosyl units were obviously observed by five strong signals at 101.7, 72.7, 73.7, 76.4, and 63.0 ppm in the ^{13}C-NMR spectrum, which are assigned to C-1, C-2, C-3, C-4, and C-5 positions of xylan backbone, respectively (Izydorczyk and Biliaderis 1995; Roubroeks et al 2000). Relatively weaker signals at 107.7, 80.8, 77.3, 84.8, and 61.3 ppm in the ^{13}C NMR spectrum were obtained for the samples C-1, C-2, C-3, C-4, and C-5, indicating the presence of α-L-arabinfuranosyl linked to β-D-xylans, respectively. The signals could also be found in the ^{1}H NMR spectrum at 5.31, 4.08, 3.83, 4.19, 3.83, and 3.68 ppm, representing H-1, H-2, H-3, H-4, H-5eq, and H-5ax of α-L-arabinfuranosyl, respectively (Bengtsson and Åman 1990; Izydorczyk and Biliaderis 1995; Roubroeks et al 2000). The signals at 102.7, 73.3, 75.6, 69.2, 76.1, and 60.9 ppm originated from the C-1, C-2, C-3, C-4, C-5, and C-6 of (1→4)-β-D-glucopyranosyl oligosaccharides (Igartuburu et al. 2001).

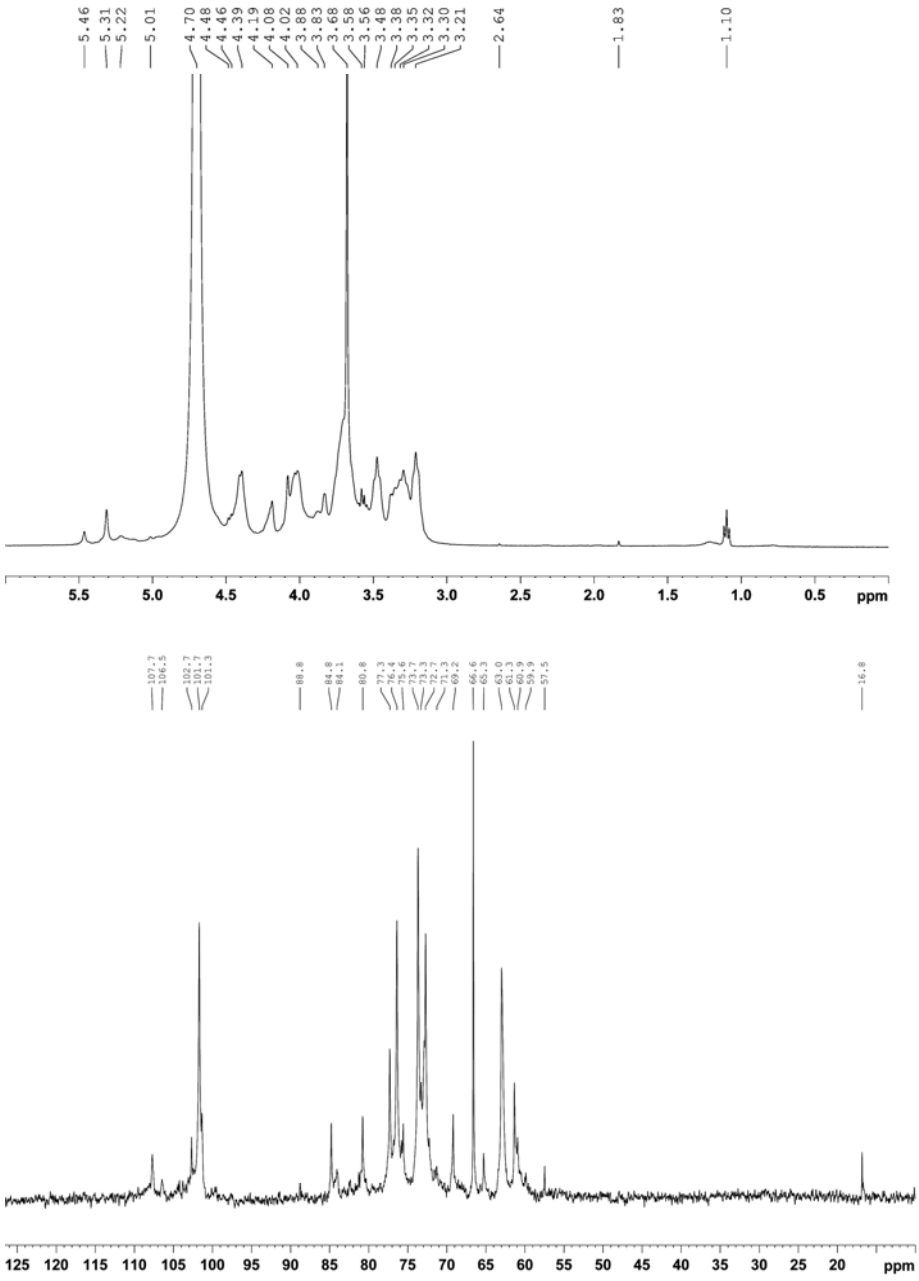

Fig. 3. ^{1}H and ^{13}C NMR spectra of hemicellulosic fraction H_5 extracted with 70% ethanol containing 1.0% NaOH for 3 h at 80 ºC

In addition, the small peaks at 3.38 ppm in the ^{1}H NMR spectrum and 59.9 ppm in the ^{13}C NMR spectrum came from methoxyl group of the 4-*O*-methoxyl group of glucuronic acid residue in xylan (Sun and Sun 2002), and this is in line with the low uronic acid content of sugar analysis well. The signals of a triplet around 1.10 ppm and a quartet around 3.56 and 3.58 ppm in the ^{1}H NMR spectrum and resonances at 16.8 (not shown) and 57.5 ppm in the ^{13}C NMR spectrum are attributed to the trace of ethanol residue in the freeze-dried fraction. A strong peak at 4.70 ppm belongs to the residual

solvent (HDO). The assignments of (1→4)-β-D-xylopyranosyl, α-L-arabinfuranosyl, and (1→4)-β-D-glucopyranosyl oligosaccharides in the HSQC spectrum are similar to that of our previous studies (Peng et al. 2010; Yuan et al. 2010).

As may be gathered from the results of sugar analysis, FT-IR, and ^{1}H, ^{13}C, and HSQC NMR spectra, the structure of H_5 can be defined as a linear backbone of (1→4)-*β*-D-xylopyranosyl units, which is substituted on O-2 and/or O-3 by single residues or short chains, like single arabinose residue or a short chain of sugar residues containing arabinose and galactose residues (Saha. 2003; Morrison. 1974). In addition, small amounts of (1→4)-β-D-glucopyranosyl oligosaccharides in H_5 might came from the degradation of cellulose.

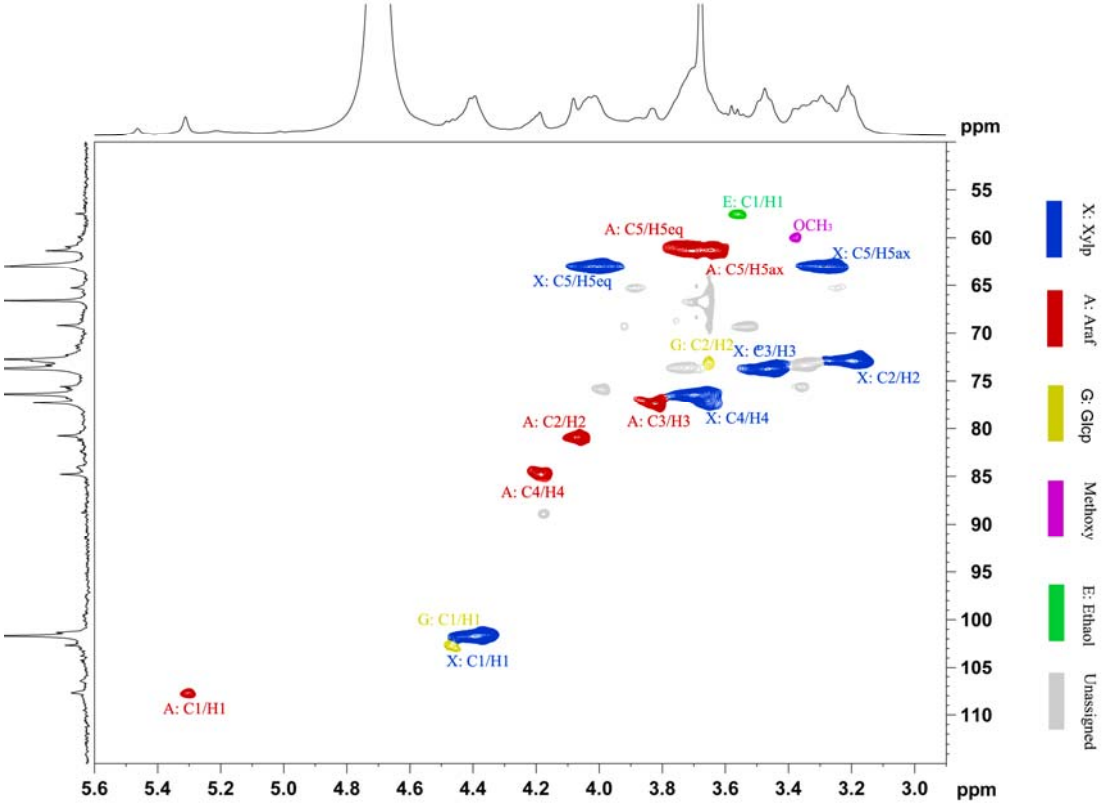

Fig. 4. HSQC spectrum (in D_2O) of hemicellulosic fraction H_5

Table 4. Assignment of ^{13}C-^{1}H Cross-Signals in the HSQC Spectrum of the Hemicellulosic Fraction H_5

Saccharide(unit)	*NMR*	**Assignment**							
		1	2	3	4	5eq[a]	5ax[b]	6eq	6ax
(1→4)-β-D-Xylp	^{13}C	101.7	72.7	73.7	73.4	63.0			
	^{1}H	4.46	3.21	3.48	3.68	4.02	3.30		
α-Araf	^{13}C	107.7	80.8	77.3	84.8	61.3			
	^{1}H	5.31	4.08	3.83	4.19	3.83	3.68		
β-D-Glcp-(1-4)-	^{13}C	102.7	73.3	75.6	69.2	76.1		60.9	
	^{1}H	4.46	3.66	-	-	-		-	

[a] eq: equatorial, [b] ax: axial

CONCLUSIONS

The sequential treatments of dewaxed *Calamagrostis angustifolia* Kom with hot water, 70% ethanol, and 70% ethanol containing 0.2%, 1.0%, 2.0%, 4.0%, and 8.0% NaOH yielded 36.2% polysaccharides from the dry dewaxed material. It is concluded that water-soluble hemicelluloses presumably consist of noticeable amounts of β-D-glucan, as well as small amounts of pectic substances and galactoarabinoxylans, while the five alkali-soluble hemicelluloses are probably mainly composed of galactoarabinoxylans. The smaller sized and more branched polysaccharides tended to be extracted in the early stages of relatively mild conditions, and the larger molecular sized and more linear hemicelluloses tended to be isolated under alkaline conditions of treatment. A higher concentration of alkali, however, would lead to degradation of the polysaccharides, and then the weight of molecular declined. With the increasing concentration of NaOH from 1.0% to 8.0%, the content of glucose increased, but the content of arabinose and the values of Ara/Gal and Ara/Xyl decreased, accordingly. Based on the results of sugar analysis, FT-IR, and NMR spectra analyses, H_5 was mainly formed by a linear backbone of (1→4)-*β*-D-xylopyranosyl units, which is substituted on O-2 and/or O-3 by single residues or short chains. These branches may be single arabinose residues or a short chain of sugar residues containing arabinose and galactose residues.

ACKNOWLEDGEMENTS

The authors are grateful for the financial support from State Forestry Administration (200804015), Major State Basic Research Projects of China (973-2010CB732204, National Natural Science Foundation of China (30930073, 30710103906), and China Ministry of Education (111).

REFERENCES CITED

Bengtsson, S., and Åman, P. (1990). "Isolation and chemical characterization of water-soluble arabinoxylans in rye grain," *Carbohydr. Polym*. 12(3), 267-277.

Broberg, A., Thomsen, K. K., and Duus, J. Ø. (2000). "Application of nano-probe NMR for structure determination of low nanomole amounts of arabinoxylan oligosaccharides fractionated by analytical HPAEC-PAD," *Carbohydr. Res.* 328(3), 375-382.

Chiarini, L., Cescutti, P., Drigo, L., Impallomeni, G., Herasimenka, Y., Bevivino, A., Dalmastri, C., Tabacchioni, S., Manno, G., Zanetti, F., and Rizzo, R. (2004). "Exopolysaccharides produced by *Burkholderia cenocepacia* recA lineages IIIA and IIIB," *J. Cyst. Fibros.* 3(3), 165-172.

Doblin, M. S., De Melis, L., Newbigin, E., Bacic, A., and Read, S. M. (2001). "Pollen tubes of *Nicotiana alata* express two genes from different beta-glucan synthase families," *Plant Physiol*. 125(4), 2040-2052.

Girio, F. M., Fonseca, C., Carvalheiro, F., Duarte, L. C., Marques, S., and Bogel-Lukasik, R. (2010). "Hemicelluloses for fuel ethanol: A review," *Bioresour. Technol*. 101(13), 4775-4800.

Gross, K. C., and Wallner, S. J. (1979). "Degradation of cell wall polysaccharides during Tomato fruit ripending," *Plant Physicology.* 1(63), 117-120.

Igartuburu, J. M., Pando, E., Luis, F. R., and Gil-Serrano, A. (2001). "A hemicellulose B fraction from grape skin (*Vitis vinifera*, palomino variety)," *J. Nat. Prod.* 64(9), 1174-1178.

Israilides, C., Kletsas, D., Arapoglou, D., Philippoussis, A., Pratsinis, H., Ebringerová, A., Hromádková, Z., and Harding, S. E. (2007). "*In vitro* cytostatic and immunomodulatory properties of the medicinal mushroom *Lentinula edodes*," *Phytomedicine*. 15(6-7), 512-519.

Izydorczyk, M. S., and Biliaderis, C. G. (1995). "Cereal arabinoxylans: Advances in structure and physicochemical properties," *Carbohydr. Polym*. 28(1), 33-48.

Kacuráková, M., Capek, P., Sasinková, V., Wellner, N., and Ebringerová, A. (2000). "FT-IR study of plant cell wall model compounds: Pectic polysaccharides and hemicelluloses," *Carbohydr. Polym*. 43(2), 195-203.

Kačuráková, M., Ebringerová, A., Hirsch, J., and Hromádková, Z. (1994). "Infrared study of arabinoxylans," *J. Sci. Food. Agr*. 66(3), 423-427.

Komiyama, H., Kato, A., Aimi, H., Ogihara, J., and Shimizu, K. (2008). "Chemical structure of kenaf xylan," *Carbohydr. Polym*. 72(4), 638-645.

Lamport D. (1970). "Cell wall metabolism," *Annu. Rev. Plant Physiol*. 21(1), 235-270.

Le Troedec, M., Sedan, D., Peyratout, C., Bonnet, J. P., Smith, A., Guinebretiere, R., Gloaguen, V., and Krausz, P. (2008). "Influence of various chemical treatments on the composition and structure of hemp fibres," *Composites Part A*. 39(3), 514-522.

MacGregor, A. W., and Fincher, G. B. (1993). "Carbohydrates of the barley grain," In: *Barley: Chemistry and Technology*, MacGregorm, A. W., and Bhatty, R. S. (eds.), American Association of Cereal Chemists, St. Paul, MN.

Morrison, I. M. (1974). "Changes in the hemicellulosic polysaccharides of rye-grass with increasing maturity," *Carbohydr. Res*. 36(1), 45-51.

Nacos, M. K., Katapodis, P., Pappas, C., Daferera, D., Tarantilis, P. A., Christakopoulos, P., and Polissiou, M. (2006). "Kenaf xylan-A source of biologically active acidic oligosaccharides," *Carbohydr. Polym*. 66(1), 126-134.

Nykter, M., Kymalainen, H. R., Thomsen, A. B., Lilholt, H., Koponen, H., Sjoberg, A. M., and Thygesen, A. (2008). "Effects of thermal and enzymatic treatments and harvesting time on the microbial quality and chemical composition of fibre hemp (Cannabis sativa L.)," *Biomass Bioenerg*. 32(5), 392-399.

Pavliková, D., Pavlík, M., Vašičková, S., Száková, J., Tlustoš, P., Vokáč, K., and Balík, J. (2004). "Separation of organic compounds binding trace elements in seeds of Leuzea carthamoides (Willd.) DC," *Applied Organometallic Chemistry*. 18(12), 619-625.

Peng, F., Ren, J. L. Xu, F., Bian, J., Peng, P., and Sun, R. C. (2010). "Fractionation of alkali-solubilized hemicelluloses from delignified *Populus gansuensis*: Structure and properties," *J. Agric. Food. Chem.* 58(9), 5743-5750.

Prates, J., Tarbouriech, N., Charnock, S., Fontes, C., Ferreira, L., and Davies, G. (2001). "The structure of the feruloyl esterase module of xylanase 10B from *Clostridium thermocellum* provides insights into substrate recognition," *Struc.* 9(12), 1183-1190.

Roubroeks, J. P., Andersson, R., and Aman, P. (2000). "Structural features of (1 -> 3),(1 -> 4)-beta-D-glucan and arabinoxylan fractions isolated from rye bran," *Carbohydr. Polym*. 42(1), 3-11.

Saha, B. C. (2003). "Hemicellulose bioconversion," *J. Ind. Microbiol. Biotechnol.* 30(5), 279-291.

Suarez, E. R., Kralovec, J. A., Noseda, M. D., Ewart, H. S., Barrow, C. J., Lumsden, M. D., and Grindley, T. B. (2005). "Isolation, characterization and structural determination of a unique type of arabinogalactan from an immunostimulatory extract of *Chlorella pyrenoidosa*," *Carbohydr. Res.* 340(8), 1489-1498.

Sun, R. C., Fang, J. M., Goodwin, A., Lawther, J. M., and Bolton, A. J. (1998). "Fractionation and characterization of polysaccharides from abaca fibre," *Carbohydr. Polym*. 37(4), 351-359.

Sun, R. C., Fang, J. M., Tomkinson, J., Geng, Z. C., and Liu, J. C. (2001). "Fractional isolation, physico-chemical characterization and homogeneous esterification of hemicelluloses from fast-growing poplar wood," *Carbohydr. Polym*. 44(1), 29-39.

Sun, R. C., Lawther, J. M., and Banks, W. B. (1996). "Isolation and physicochemical characterisation of xylose-rich pectic polysaccharide from wheat straw," in: *Pectins and Pectinases*, Visser, J., and Voragen, A. G. J. (eds.), Elsevier, Amsterdam.

Sun, R. C., and Sun, X. F. (2002). "Fractional and structural characterization of hemicelluloses isolated by alkali and alkaline peroxide from barley straw," *Carbohydr. Polym*. 49(4), 415-423.

Sun, R. C., Sun, X. F., and Tomkinson, J. (2003). "Hemicelluloses and their derivatives," in: *Hemicelluloses: Science and Technology*, Gatenhole P., and Tenkanen M. (eds), American Chemical Society, Washington, D.C.

Tan, X. Q., Chen, H. S., Liu, R. H., Tan, C. H., Xu, C. L., Xuan, W. D., and Zhang, W. D. (2005). "Lignans from *Trachelospermum jasminoides*," *Planta Med*. 71(1), 93-95.

Tserki, V., Zafeiropoulos, N. E., Simon, F., and Panayiotou, C. (2005). "A study of the effect of acetylation and propionylation surface treatments on natural fibres," *Composites Part A*. 36(8), 1110-1118.

Wedig, C. L., Jaster, E. H., and Moore, K. J. (1987). "Hemicellulose monosaccharide composition and in vitro disappearance of orchard grass and alfalfa hay," *J. Agric. Food. Chem*. 35(2), 214-218.

Westerlund, E., Andersson, R., and Åman, P. (1993). "Isolation and chemical characterization of water-soluble mixed-linked [beta]-glucans and arabinoxylans in oat milling fractions," *Carbohyd. Polym*. 20(2), 115-123.

Xu, F., Geng, Z. C., Sun, J. X., Liu, C. F., Ren, J. L., Sun, R. C., Fowler, P., and Baird, M. S. (2006). "Fractional and structural characterization of hemicelluloses from perennial ryegrass (*Lolium perenne*) and cocksfoot grass (*Dactylis glomerata*)," *Carbohydr. Res*. 341(12), 2073-2082.

Xu, F., Sun, J. X., Geng, Z. C., Liu, C. F., Ren, J. L., Sun, R. C., Fowler, P., and Baird, M. S. (2007). "Comparative study of water-soluble and alkali-soluble hemicelluloses from perennial ryegrass leaves (*Lolium peree*)," *Carbohyd. Polym*. 67(1), 56-65.

Yuan, T. Q., Xu, F., He, J., and Sun, R. C. (2010). "Structural and physico-chemical characterization of hemicelluloses from ultrasound-assisted extractions of partially delignified fast-growing poplar wood through organic solvent and alkaline solutions," *Biotechnol. Adv.* 28(5), 583-593.

Article submitted: April 29, 2011; Peer review completed: May 26, 2011; Revised version received and accepted: June 15, 2011; Published: June 16, 2011.