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Structural elucidation of hemicelluloses from Vetiver grass

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Abstract

Structure of the hemicelluloses extracted from Vetiver grass (*Vetiveria zizanioides Nash*) leaves was studied. The monosaccharide compositions and the position of the linkages between monosaccharides in the hemicelluloses were defined by TFA hydrolysis and methylation analysis, respectively. ^{13}C NMR and FT-IR spectroscopic methods gave details of the anomeric linkage configuration and confirmed the structure of the hemicelluloses. The proposed structure of the hemicelluloses from the Vetiver grass is an arabinoxylan mainly consisting of (1 → 4)-β-D-xylan backbone substituted in *O*-2 and/or *O*-3 by single α-L-arabinose residue, single α-D-glucuronic acid residue and/or short chains of sugar residues containing arabinose, xylose and galactose. In addition, the (1 → 4)-linked β-D-xylopyranosyl residues in the backbone may contain substituted phenolic acids (e.g. ferulic acid and *p*-coumaric acid). The substituted phenolic acids are esterified via their carboxyl groups to the C-5 hydroxyl of the α-L arabinofuranosyl residues in side chain.

Keywords: Hemicelluloses; Vetiver grass; Molecular structure

1. Introduction

The majority of plant materials are constructed from three major polymeric components: cellulose, hemicelluloses and lignin. The hemicelluloses are estimated to account for one third of all components available in plants. Structures of the hemicelluloses vary significantly in different plants and have been topics of great academic interest. The hemicelluloses from cell wall of the Gramineae family plants such as cereal plants and grasses mainly consist of a chain backbone of (1 → 4)-linked β-D-xylopyranosyl residues to which α-L-arabinofuranose units are linked as side branches (Izydorczyk & Biloaderis, 1995; Sun & Sun, 2002; Xiao, Sun, & Sun, 2001). The manner of attachment of arabinose units to the xylan backbone has been a matter of continuous research. The linkages of Ara_f to C(*O*)3 and to C(*O*)2,3 of xylose residues have been reported (Ebringerova et al., 1990). More recently, the presence of another linkage type, namely Ara_f linked to C(*O*)2 of Xyl_p residues, has been verified for arabinoxylans (Izydorczyk & Biloaderis, 1995). A small proportion of oligomeric side-chains consisting of two or more arabinosyl residues linked via 1 → 2, 1 → 3 and 1 → 5 linkages has been reported although most arabinofuranosyl residues in arabinoxylans are found as monomeric substituents. Terminal galactosyl and glucosyl residues can be present but are usually quantitatively minor and might originate from contaminant polysaccharides (Ebringerova et al., 1990; Ebringerova & Heinze, 2000; Izydorczyk & Biloaderis, 1995; Xiao et al., 2001). Glucuronopyranosyl (and 4-methyl ether) residues were found and attached directly to the C-2 position of xylose from barley straw (Sun & Sun, 2002), rice and rye straws (Izydorczyk & Biloaderis, 1995; Xiao et al., 2001).

Although arabinoxylans from various cereals and/or various plant tissues share the same basic chemical structure, they differ in the manner of substitution of the xylan backbone. The main differences are found in the ratio of arabinose to xylose (Ara/Xyl), in the relative proportions and sequence of the various linkages between these two sugars, and in the presence of other substituents. The ratio of Ara/Xyl in arabinoxylans from wheat endosperm may vary from 0.50 to 0.71 but it is usually lower than that found in bran (1.02–1.07). Similarly, rye bran endosperm arabinoxylans are less substituted (0.48–0.55) than rye bran

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counterparts (0.78) (Izydorczyk & Biloaderis, 1995). In general, arabinoxylans from rice (Shibuya & Iwasaki, 1985) and sorghum (Woolard, Rathbone, & Novellie, 1976) seem to consist of more highly branched xylan backbones than those from wheat, rye, and barley, and they may contain galactose and glucuronic acid substituents, in addition to the pentose sugars.

According to His Majesty's Initiative, the principal utilisation of the Vetiver grass in Thailand is for preventing soil erosion and conserving soil moisture due to its deep thick root system like on underground curtain. Similar to other undervalued agricultural residues, leaves of the Vetiver grass which has been normally cut every few months are not used as industrial raw materials and are burnt in field or on the side of the road. The main purpose of the work, being presented here just a part, is to assess the possibility of preparing wound dressing materials or other chemicals based on hemicelluloses extracted from the Vetiver grass. Therefore, elucidation of the molecular structure of the Vetiver grass hemicelluloses was of our particular interest.

2. Experimental

2.1. Sample preparation

Vetiver grass (*V. zizanioides*, ecotype Songkhla 3), age of about 9 months, was kindly supplied by Land Development Department (Nakornrachasima, Thailand). The ground leaves were dried in an oven at 60 °C for 16 h before use. The principal composition (%w/w) of the Vetiver grass is 34.46% cellulose, 39.40% hemicelluloses, 7.87% lignin, 3.66% ash and 3.97% protein. The isolation of hemicelluloses was performed in the same manner as described in previous work (Methacanon, Chaikumpollert, Thavorniti, & Suchiva, 2003). Briefly, wax and other extractives were eliminated from the dried grass by extraction with chloroform-methanol in a Soxhlet apparatus. Then, the hemicelluloses were extracted with alkaline solution after delignification with sodium chlorite.

2.2. Characterisation of the extracted hemicelluloses

2.2.1. Monosaccharide identification

To determine the neutral sugar composition of the extracted hemicelluloses, the samples (50 mg) were added with trifluoroacetic acid (100% TFA, 4 ml) in round-bottom flask. The mixtures were left overnight at ambient temperature and subsequently refluxed for 2 h. The solutions were then diluted to 80% TFA using deionised water. After refluxed for 30 min, the solutions were diluted to 30% TFA using deionised water and refluxed for 4 h. The TFA was removed using a rotary vacuum evaporator. Deionised water was then added to the solids to wash them and this was followed by re-evaporation. This procedure was repeated several times until the hydrolysates obtained were neutral. The dry hydrolysate solids were finally dissolved in deionised water (5 ml) and used in the preparation of the alditol acetate detivatives (Blakeney, Harris, Henry, & Stone, 1983) for GC and GC-MS (Chaikumpollert, 2003). The content of total uronic acids was determined by carbazole assay (Chaplin, 1986).

2.2.2. Monosaccharide substituents

The phenolic acids of the samples were determined by alkaline hydrolysis. Samples (200 mg) were saponified with sodium hydroxide (2 M, 10 ml) at room temperature for 20 h under nitrogen. 2,4-Dihydroxybenzoic acid (5 mg/ml in 2 M sodium hydroxide, 100 μl) was added as an internal standard. The samples were acidified with concentrated hydrochloric acid and extracted using three portions of ethyl acetate (2 ml). The ethyl acetate extracts were combined before evaporating off the ethyl acetate. The final residue containing the concentrated alkali-released phenolic acids were redissolved in ethyl acetate (300 μl) and silylated with BSTFA (*N*,*O*-bis(trimethylsilyl) trifluoroacetamide, 100 μl). The mixtures were heated at 60 °C for 30 min with occasional shaking to form the TMS ether derivatives. Solutions of standards (*trans*-*p*-coumaric acid, *trans*-feruric acid and *trans*-sinapic acid, 5 mg/ml in 2 M sodium hydroxide) were treated in the same manner as the samples. TMS ether derivatives of samples and standards were identified by GC (Chaikumpollert, 2003). The extraction of the phenolic acids from the samples and the preparation of their TMS ether derivatives were carried out in the absence of UV and daylight to avoid *cis*–*trans* isomerisation. The derivatives were only stable for a few hours and were therefore analysed immediately after preparation.

In the case of protein, it was determined using HPLC as free amino acids after hydrochloric acid hydrolysis. Samples (100 mg) were added with hydrochloric acid (6 M, 2 ml). The hydrolysis was carried out at 112 °C for 22 h. After that the hydrolysates were derivatised with AccQ® Fluor reagent before analysis by HPLC (Chaikumpollert, 2003).

2.2.3. Linkage analysis

The positions of linkages between monosaccharides in polysaccharides are defined by methylation analysis The procedure used was slightly modified from the method of Ciucanu and Kerek (1984). The hemicelluloses samples (100 mg) in dimethylsulphoxide (DMSO, 10 ml) were added finely powdered sodium hydroxide (400 mg). The mixtures were then stirred under nitrogen at 60 °C for 1 h. After that methyl iodide (CH_3I, 2 ml) was added and stirred under nitrogen at 60 °C for 3 h. To remove dimethylsulphoxide and other impurities, the methylated products were dialysed against deionised water using membrane (MWCO: 1000) until the dialysates were neutral. Then, retentates were freeze-dried using lyophiliser. To ensure complete methylation of the polysaccharide, the methylation was repeated five times. After that, the methylated hemicelluloses (5 mg) were hydrolysed using formic acid (90% w/w, 3 ml) for 2 h at 100 °C. After removal of formic acid using rotary evaporation at 40 °C, the hydrolysis was continued by treatment with sulphuric acid (0.25 M, 1 ml) and heated at 100 °C for 12 h. The cooled solution was neutralised with barium carbonate, centrifuged, and concentrated under the reduced pressure at 40 °C before derivatised to alditol acetate (Blakeney et al., 1983) and subsequent analysed by GC and GC-MS (Chaikumpollert, 2003). The methylated alditol acetates were identified by comparison with mass spectra by Jansson, Kenne, Liedgren, Lindberg, and Lonngren (1976) and relative retention time of methylated alditol acetates was compared with Carpita and Shea (1989). The effective carbon response factors (Sweet, Shapiro, & Albersheim, 1975) were used for calculation of the molar quantities of permethylated alditols.

2.2.4. Spectroscopic analysis

^{13}C-NMR spectra (75 MHz) were recorded on a DPX-300 Nuclear Magnetic Resonance Spectrometer. Dry samples (150 mg) were dissolved in sodium deuteroxide (0.5 M NaOD, 2 ml). The NMR tube diameter was 5 mm and the number of scans was approximately 30,000. Tetramethylsilane (TMS) was used as an internal reference.

FT-IR spectra of samples were recorded on a Perkin Elmer system 2000 FTIR spectrometer with 30 numbers of scan in the spectral range of 4000–370 cm^{-1}, using a KBr disc containing 5–10% finely ground samples.

3. Results and discussion

3.1. Monosaccharide identification

The extracted hemicelluloses consisted of two fractions: water-soluble and water-insoluble fractions. Their monosaccharide compositions were identified and quantified as shown in Table 1. The major monosaccharide in both soluble and insoluble fractions is xylose (ca. 46 and 63%, respectively) followed by arabinose (ca. 16 and 10%, respectively). This result suggested that the hemicelluloses from the Vetiver grass are mainly composed of arabinoxylan. The Ara/Xyl ratio of water-soluble and water-insoluble hemicelluloses was approximately 0.4 and 0.2, respectively, which indicated that water-soluble hemicelluloses were more highly branch than water-insoluble one. This observation was corresponded well to the previous reports: the lower the arabinose content indicating a lower degree of branching of the xylan chains, the lower the solubility of the polymer (Ebringerova et al., 1990; Ebringerova & Heinze, 2000). In addition, the presence of lower branching of the xylan in water-insoluble fraction resulted in occurrence of crystallinity as evidenced by X-ray diffraction, which consequently affected to higher thermal stability of the sample.

In addition to xylose and arabinose, glucose appeared as a noticeable quantity, which is presumably as glucan (Sun, Sun, Fowler, & Tomkinson, 2002; Xiao et al., 2001). Galactose may be end-group residues in side chain of the samples (Sun et al., 2002). Uronic acid was also found as 8% in the water-soluble fraction whereas that in the water-insoluble one could not be estimated because of the difficult solubility of the sample in water.

Table 1
Monosaccharide content of the extracted hemicelluloses

Hemicellulose fraction	Monosaccharide content (%w/w)				
	Ara	Xyl	Gal	Glc	Uronic acid[a]
Water-soluble	16.11	46.34	9.04	13.28	8.00
Water-insoluble	10.08	63.49	2.05	8.65	n.d.

n.d., not determined.
[a] Determined by carbozole assay.

3.2. Monosaccharide substituents

As previously been reported, phenolic acid compounds and protein link to arabinoxylan backbone via arabinose residues Although the phenolic acid compounds in the samples were not detected from the GC result due to probably their presence in small amount (<0.05%), they were found in the ^{13}C-NMR result (Section 3.4).

Protein present as total amino acid in soluble and insoluble fractions was found to be 0.59 and 0.16%, respectively. A board distribution of amino acids identified was shown in Fig. 1. The individual amino acids found in the water-soluble and water-insoluble fractions were similar with glutamic and aspartic acids being the two major amino acids. It is noticeable that hydroxyproline which has

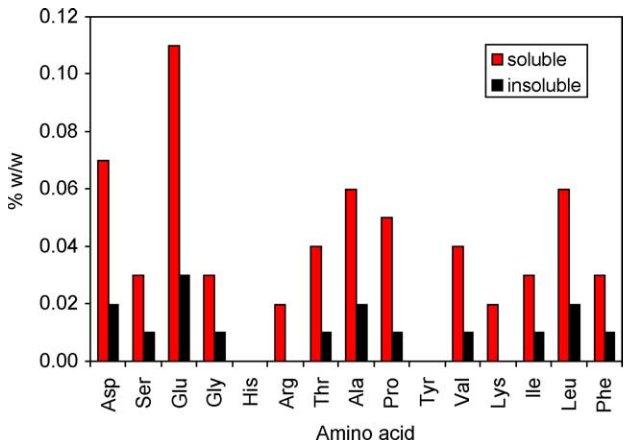

Fig. 1. Individual amino acid content present in the water-soluble and water-insoluble hemicelluloses; Asp:aspartic acid, Ser:serine, Glu:glutamic acid, Gly:glucine, His:histidine, Arg:arginine, Thr:threonine, Ala:alanine, Pro:proline, Tyr:tyrosine, Val:valine, Lys:lysine, Ile:isoleusine, Leu:leucine, Phe:phenylalanine.

Table 2
Mole percentages of the partially methylated alditol acetates obtained from methylation analysis

Deduced glycosidic linkage	Mole (%)	
	Soluble	Insoluble
1,4-Xyl$_p$	83.02	85.60
1,2,4-Xyl$_p$	2.86	0.40
1,3,4-Xyl$_p$	9.54	10.00
Terminal-Ara$_f$	0.95	1.20
1,2-Ara$_f$	0.76	0.80
1,3,5-Ara$_f$	0.95	1.20
Unmethylated Xyl$_p$	1.91	0.80
Unmethylated Glc$_p$	Trace	Trace
Unmethylated Gal$_p$	Trace	Trace

previously been reported in plant glycoproteins was hardly found in these samples.

3.3. Glycosidic linkage position

Methylation analysis (Table 2) revealed that the water-soluble hemicelluloses from Vetiver grass consist of a (1 → 4)-linked D-xylan backbone due to the presence of the non-terminal unsubstituted xylose residues (83.02 mol%), single substituted residues at *O*-2 (2.86 mol%) and ones at *O*-3 (9.54 mol%).

Generally, the arabinose residues are in furanose form and attached to the xylan backbone as single units (terminal residues), 2-, 3- and/or 5-linked residues (Ebringerova et al., 1990). The terminal Ara$_f$ (0.95 mol%), 1,2-Ara$_f$ (0.76 mol%) and 1,3,5-Ara$_f$ (0.95%) were also found in the hemicellulose samples. It is notable that the numbers of arabinose side chains detected are less than the number of branch points probably due to losses in an experimental work up. Since it has been reported that the 2,3,5-tri-*O*-methyl-arabinitol derivative is very volatile as indicated by its low retention time on GC analysis and is so easily lost during the analysis (Dupont & Selvendran, 1987).

D-Galactose and D-glucose were found in small amounts from monosaccharide analysis. Unfortunately, their linkages cannot be determined due to the presence of unmethylated galactose and glucose in methylation analysis. The previous work, Izydorczyk and Biloaderis (1995), has suggested that D-galactose was present as end-group pyranose residues, possibly terminating short side-chains. D-glucose may be present as the β-glucan which is widely distributed in cell walls of various monocotyledons (Sun et al., 2002). D-Glucuronic acid might be another branch residue substituted onto the main chain of the samples which would not be also detected in the analyses used in this study. Furthermore, uronic acids are generally quite resistant to acid hydrolysis. Hence the uronic acids and neutral monosaccharides to which they are attached are lost (Carpita & Shea, 1989).

In addition to sugars, substituted phenolic acids and proteins can also link to backbone as branched chains (Ebringerova & Heinze, 2000; Sun et al., 2002). However, the linkages between them cannot be detected by methylation analysis. For the hemicellulose samples, though the substituted phenolic acids were not found (Section 3.2), the ^{13}C-NMR results showed the resonances at 177 ppm which may be attributed to carbonyl resonances from cinnamic acids (or phenolic acid) and esters. This signal was very weak, which verified very small amount of phenolic acids present in the samples. From literature data, the substituted phenolic acids are linked via their carboxyl groups to the C(*O*)5 hydroxyl of the single α-L-arabinofuranosyl residues located on the C(*O*)2 and/or C(*O*)3 of the (1 → 4)-linked β-D-xylopyranosyl residues in the backbone (Ebringerova & Heinze, 2000; Izydorczyk & Biloaderis, 1995).

In the case of water-insoluble hemicelluloses, the linkage position between monosaccharide and their quantities were similar to those present in the water-soluble hemicelluloses

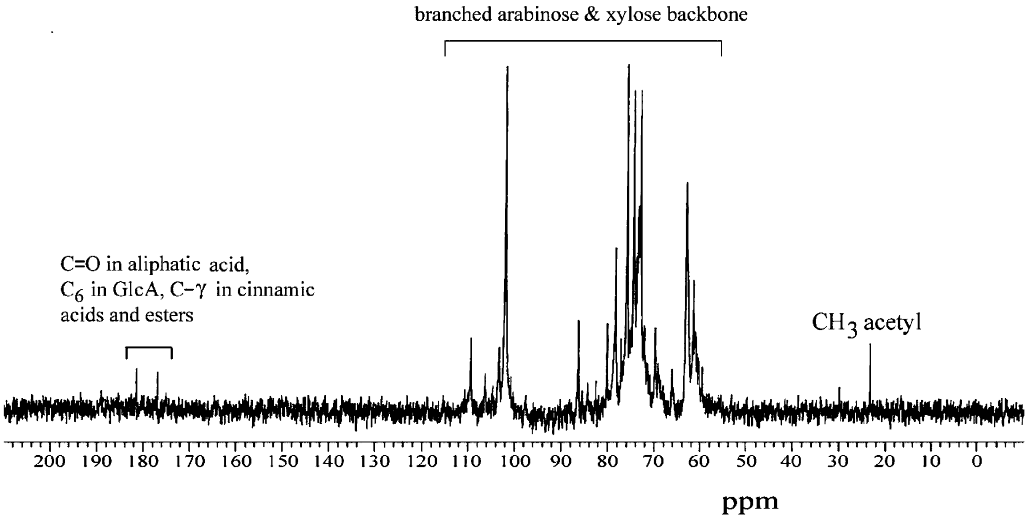

Fig. 2. ^{13}C-NMR spectrum of the water-soluble hemicelluloses.

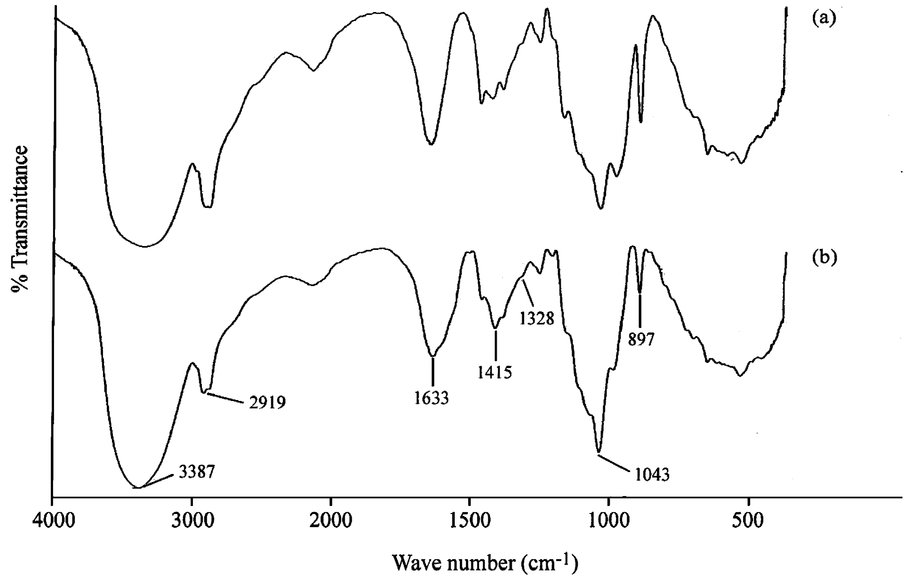

Fig. 3. FT-IR spectra of the hemicelluloses: (a) water-soluble and (b) water-insoluble.

except the amount of 3-*O*-methyl-xylose. The number of 3-*O*-methyl-xylose in the water-soluble hemicelluloses (2.86%) was significantly higher than that in the water-insoluble ones (0.40%). It indicated that the arabinoxylan in water-soluble fraction had more branch points than that in water-insoluble fraction. The results corresponded well to the Ara/Xyl ratio obtained from the monosaccharide analysis (Section 3.1).

3.4. Spectroscopic analysis

To obtain further information about the anomeric linkage configuration of hemicelluloses, the ^{13}C-NMR spectroscopic analysis was performed. The ^{13}C-NMR spectrum of the water-soluble hemicelluloses is illustrated in Fig. 2. The main 1,4-linked β-D-Xyl$_p$ units were obviously characterised by five strong signals at 102.2, 76.0, 74.6, 73.5 and 63.3 ppm which respectively were assigned to C-1, C-4, C-3, C-2 and C-5 of the β-D-Xyl$_p$ units. The signals at 109.5, 86.5, 80.2, 78.5 and 61.7 ppm correspond to C-1, C-4, C-2, C-3 and C-5 of α-L-Ara$_f$ residues, respectively (Bradbury & Jenkins, 1984). The resonance at 181.6 ppm may be attributed to carbonyl resonance from acetyl groups in aliphatic acid and C-6 of glucuronic acid residue in the xylan. The signal at 59.7 ppm was assigned to 4-*O*-methoxyl group of glucuronic acid residue. The signal at 23.4 ppm was most likely due to CH_3 in acetyl in hemicelluloses. All data from NMR stated that the anomeric

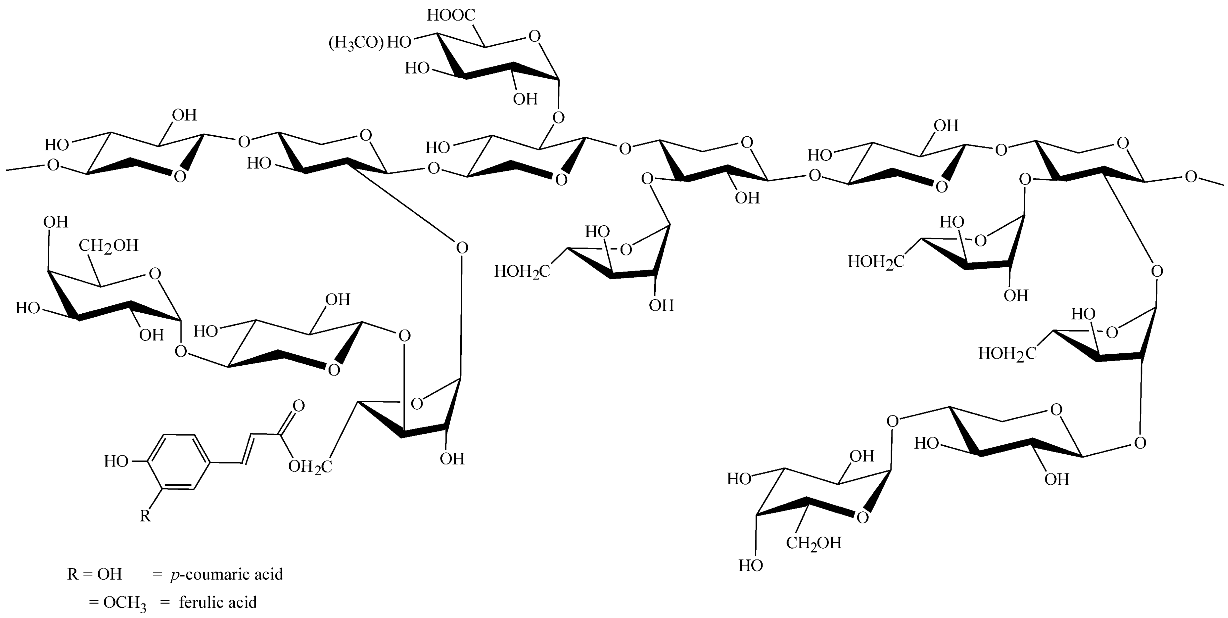

Fig. 4. Proposed structure of hemicelluloses from the Vetiver grass.

configuration of the D-xylopyranose residues is β while the L-arabinofuranose residue is α.

The FT-IR spectra of the hemicelluloses, water-soluble and water-insoluble, are illustrated in Fig. 3. As expected, the two spectral profiles and relative intensities of most bands were rather similar, indicating a similar structure for both hemicellulose samples. The absorption at 1633 cm^{-1} was principally associated with absorbed water, since the hemicelluloses usually have a strong affinity for water, and in the solid state these macromolecules may have disordered structures which can easily be hydrated (Kacurakova, Belton, Wilson, Hirsch, & Ebringerova, 1998). The small bands at 1480, 1328 and 1255 cm^{-1} represent C–H stretch and CH or OH bending in hemicelluloses (Sun, Lawther, & Banks, 1998). The band at 1388 and 1169 cm^{-1} attribute to C–H deformation and C–O–C vibration in hemicelluloses, respectively. Bands between 1125 and 1000 cm^{-1} were typical of xylans. The prominent band at 1043 cm^{-1} was attributed to the C–O, C–C stretching or C–OH bending in hemicelluloses. The sharp band at 897 cm^{-1} corresponding to the C-1 group frequency or ring frequency, was characteristic of β-glycosidic linkages between the sugar units (Gupta, Madan, & Bansal, 1987).

4. Conclusions

It is concluded that the hemicellulose samples from the Vetiver grass is an arabinoxylan mainly consisting of a backbone of 1,4-linked xylopyranosyl units (Fig. 4). The (1 → 4)-xylopyranose backbone is substituted on *O*-2 and/or *O*-3 by single residue or short chains. These branches may be single arabinose residue, single glucuronic acid residue or a short chain of sugar residues containing arabinose, xylose and galactose. In addition, the (1 → 4)-linked β-D-xylopyranosyl residues in the backbone may contain substituted phenolic acids (ferulic acid and *p*-coumaric acid). The substituted phenolic acids are esterified via their carboxyl groups to the C(*O*)5 hydroxyl of the single α-L-arabinofuranosyl residues located on the C(*O*)2 and/or C(*O*)3 of the (1 → 4)-linked β-D-xylopyranosyl residues in the backbone.

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