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Studies on the Starch and Hemicelluloses Fractionated by Graded Ethanol Precipitation from Bamboo *Phyllostachys bambusoides* f. shouzhu Yi

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Supporting Information

ABSTRACT: Starch from bamboo *Phyllostachys bambusoides* f. shouzhu Yi evaluated by means of solid-state ^{13}C CP/MAS NMR and X-ray diffraction showed a typical B-type pattern with a very low degree of crystallinity (10.9%). In addition to starch, alkali-soluble hemicelluloses were further fractionated by graded precipitation at ethanol concentrations of 0 (HA), 15, 30, 45, 60, and 75% (v/v). Chemical composition and structural features of the six hemicellulosic subfractions were investigated by a combination of sugar analysis, GPC, FT-IR, GC-MS, 1D (^1H and ^{13}C) and 2D (HSQC) NMR spectra, and thermal analysis. The results showed that the bamboo hemicelluloses were *O*-acetylated 4-*O*-methyl-glucuronoarabinoxylans (GAX) consisting of a linear (1→4)-*β*-D-xylopyranosyl backbone decorated with branches at *O*-3 of α-L-arabinofuranosyl (5−12 mol %) or at *O*-2 of 4-*O*-methylglucuronic acid units and acetyl groups (0.8−11 mol %). The molecular weights of these polysaccharides ranged between 13400 and 67500 g/mol, and the molar ratios of A/X and G/X increased with ascending ethanol concentrations. Moreover, *xylo*-oligosaccharides (XOS) with DP 1−6 were produced by enzymatic hydrolysis of hemicelluloses and the total yields of XOS were range of 21.5 to 40.6%. The structure−property relationships were also established in order to improve enzyme accessibility.

KEYWORDS: bamboo, starch, hemicelluloses, chemical composition, structural features, enzymatic hydrolysis, *xylo*-oligosaccharides, glucuronoarabinoxylans

INTRODUCTION

The major challenges in the increasing use of fossil feedstocks for the production of energy, chemicals, materials, and food ingredients are from environment and climate. As a sustainable alternative to petroleum derivatives, biomass is recently attracting much attention.[1−3] Starch and hemicelluloses are two components of most important polysaccharides in plant cell walls. It is necessary to study their structural features before converting them into value-added products through economically viable processes. Moreover, the utilization of starch from food plants is limited in developing countries, especially in China, due to the competition between food and bioethanol.[4] Therefore, it is a promising way to find a new starch source from nonfood plants for the growing demand.

Starch, semicrystalline granules, is the main source of carbohydrate in food and nonfood industry. Starch is composed of two polysaccharides: the almost linear α-(1→4) glucan amylose and α-(1→4) glucan with α-(1→6)-branched amylopectin.[5] Typically, natural starch granules range in degrees of crystallinity from about 15 to 45%.[6] They are formed and stored in higher plants such as seed embryo, tuber, and cereal endosperm. Widely accepted models of starch involve three main forms, A, B and C, according to different botanical origins. The A polymorph occurs frequently in cereal starch, and the B polymorph is characteristic of tuber and amylose-rich starches, while the C type starch is a mixture of both A and B types. Currently, much attention has been devoted to the study of the starch arising from food plants. However, little research has been carried out on the starch present in bamboo, which is usually wasted during the isolation of hemicelluloses and lignin.

Hemicelluloses are considered to be the second most abundant polysaccharides in nature after cellulose. They are not single homogeneous polymers but contain different heterogeneous polymers mainly consisting of xylose, arabinose, galactose, mannose, 4-*O*-methyl-D-glucuronic acid and so on.[7] The most common hemicellulosic biopolymers are glucuronoarabinoxylans in herbaceous crops.[8] Besides, naturally occurring xylan contains native acetyl groups.[9] In recent years, the xylan originated from hemicelluloses has been used extensively as a food ingredient, for medical use, in materials, and for energy. As it is well-known, *xylo*-oligosaccharides (XOS) are sugar oligomers produced via the hydrolysis of xylan. They have been referred to as prebiotics that are important ingredients of functional food improving health and stimulating the growth of intestinal bifidobacteria.[10] On the other hand, from a nutritional point of view, XOS are usually considered to be nondigestible oligosaccharides. Moreover, hemicelluloses can be used as a source of xylose for the production of xylitol or for the preparation of ethers and esters which

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can be used as thermoplastic materials for water-soluble food-packaging films, coatings, and capsules.[11] In addition, significant attention has been paid to simultaneous bioconversion of hemicelluloses and cellulose into ethanol by fermentation as reported by Krishna and Chowdary.[12] Therefore, understanding the chemical composition, structural features, and location of these polysaccharides is beneficial to pave the way for the research of hemicelluloses toward those value-added products. However, due to the structural complexity and high heterogeneity of hemicelluloses, their utilization is relatively limited on an industrial scale. In view of this fact, some fractionation techniques involving stepwise addition of ammonium sulfate, ethanol and potassium iodine−iodine as well as ion-exchange chromatography with DEAE column have been used in an attempt to obtain more homogeneous hemicellulosic fractions.[13−16] The graded ethanol precipitation technique is the way that polysaccharides could be selectively precipitated to fractionate by stepwise addition of ethanol.[8] Generally, it yields arabinoxylan families varying in their branching patterns with increasing concentration of the ethanol.[17]

As one of the most important agricultural resources, bamboo belonging to Gramineae Bambusoideae is widely distributed in China (about 300 species), Japan and South-East Asian countries and has the characteristics of high productivity and rapid growth. Bamboo hemicelluloses (*Phyllostachys reticulata* C. Koch) were characterized as 4-*O*-methyl-D-glucuronic acid, L-arabinose and D-xylose in a molar ratio of 1.0:1.3:25, respectively, by Meakawa.[18] Similar sugar composition was also found in other bamboo species.[19−22] However, the hemicelluloses obtained in the above research were relatively more heterogeneous rather than homogeneous, and fractionation techniques and structural characterization were not extensively performed. In addition, until now, the sugar composition and structure of the hemicelluloses from bamboo species *Phyllostachys bambusoides* f. shouzhu Yi, which is the most widely planted in Sichuan and Zhejiang provinces in China, is less clear. More importantly, this is the first study on the subfractionation of hemicelluloses from *Phyllostachys bambusoides* f. shouzhu Yi by graded ethanol precipitation.

This paper, on one hand, it provides some structural information on bamboo (*Phyllostachys bambusoides* f. shouzhu Yi) starch by solid-state ^{13}C CP/MAS NMR and X-ray diffraction (XRD). On the other hand, selective precipitation with graded ethanol technique was employed to obtain the higher molecular weight and more homogeneous bamboo hemicellulosic fractions. Moreover, the aim is to investigate their detailed chemical composition and the structural characteristics by a comprehensive set of analytical methods, including HPAEC, FT-IR, GPC, GC-MS, 1D (^1H and ^{13}C) and 2D (HSQC) NMR spectra. Furthermore, enzymatic hydrolysis and thermogravimetric analysis of hemicelluloses were also carried out to obtain XOS and later prepare biomass-based materials according to the structure−function relationships, respectively.

MATERIALS AND METHODS

Materials. Bamboo culms (*Phyllostachys bambusoides* f. shouzhu Yi, 3 years old) were harvested from Bamboo Garden of North-Western Sciences and Technology of Agricultural and Forest University (Yangling, China). The raw material was first dried in an oven at 60 °C for 16 h and then ground in a mill to a particle size of less than 1 mm. Chemical analysis showed its composition to be 73.0% holocellulose, 24.7% pentosans, 24.0% Klason lignin, 3.1% extractives, and 2.2% ash on a dry weight basis according to the method of Leopold[23] and Tappi standards T 223 cm-01, T 222 om-02, T 204 cm-97, T 211 om-02 (Tappi 2002), respectively. Chemicals used were analytical grade or best available.

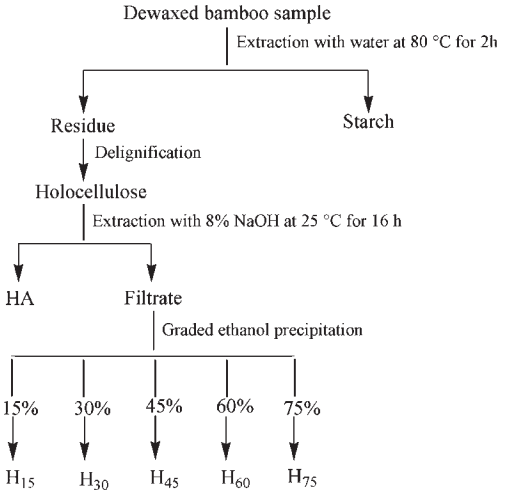

Figure 1. Scheme for fractionation of starch and hemicelluloses from bamboo species *Phyllostachys bambusoides* f. shouzhu Yi.

Fractionation of Starch and Hemicelluloses. Wax and other extractives were removed from bamboo powder by refluxing in a Soxhlet apparatus for 6 h with toluene−ethanol (2:1, v/v).[24] The defatted bamboo (15 g) was first treated with water with a solid to liquid ratio of 1:20 (g/mL) at 80 °C for 2 h. After filtration, the filtrates (300 mL) were concentrated to about 60 mL at reduced pressure, and then mixed with three volumes of 95% ethanol for isolating the water-soluble polysaccharides (starch fraction, 2.1% of defatted bamboo). The water-insoluble residue was then delignified with 6% sodium chlorite at pH 3.6−3.8, adjusted with acetic acid, at 75 °C for 2 h. Further, the holocellulose was extracted with 8% NaOH solution at 25 °C for 16 h. The extract (190 mL) was neutralized with 6 M HCl to pH 5.5 and concentrated to about 160 mL. The precipitated hemicellulose A was recovered by filtration and labeled as HA. Moreover, the supernatant was further subfractionated by graded precipitations at ethanol concentrations of 15, 30, 45, 60, and 75% (v/v) sequentially, corresponding to five subfractions, which were designated H_{15}, H_{30}, H_{45}, H_{60} and H_{75}, respectively. The scheme for fractionation of starch and hemicelluloses from bamboo species *Phyllostachys bambusoides* f. shouzhu Yi is illustrated in Figure 1. All the fractions were obtained by freeze-dried.

Monosaccharide Analysis and Gel Permeation Chromatography. High performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detector was used to analyze the monomeric sugars that were liberated by hydrolysis of starch and hemcellulose fractions (5 mg) with 1 M H_2SO_4 at 105 °C for 2.5 h. After hydrolysis, the sample was diluted 50-fold, filtered and then injected into the HPAEC system (Dionex, ICS-3000, USA) with an AS50 autosampler and a Carbopac PA-20 column (4 × 250 mm, Dionex). Neutral sugars and uronic acids were separated in a 5 mM NaOH (carbonate free and purged with nitrogen) for 20 min, followed by a 0−75 mM NaAc gradient in 5 mM NaOH for 15 min. Then the column was washed with 200 mM NaOH to remove carbonate for 10 min, and followed a 5 min elution with 5 mM NaOH to re-equilibrate the column before the next injection. The total analysis process was performed at 30 °C.

The molecular weights of the water-soluble arabinoxylans (WSAX) in starch fraction and hemicellulosic subfractions were determined by gel permeation chromatography on a PL aquagel-OH 50 column (300 × 7.7 mm, Polymer Laboratories Ltd.) with a differential refractive index

detector (RID), which was eluted with 5 mM sodium phosphate buffer (pH 7.5) containing 0.02 N NaCl at a flow rate of 0.5 mL/min and kept at 30 °C. The concentration of polysaccharides was 0.1%, and the molecular weight calibration cure was obtained using PL pullulan polysaccharide standards (M_w of 783, 12200, 100000, and 1600000, Polymer Laboratories Ltd.).

FT-IR and NMR Spectroscopy. The FT-IR spectra of the starch and hemicellulosic samples were obtained on a spectrophotometer (Tensor 27) in the range 4000−400 cm^{-1} using a KBr disk containing 1% of finely ground samples. The solution-state ^1H and ^{13}C NMR spectra were obtained on a Bruker AVIII 400 MHz spectrometer. The hemicellulosic sample (20 mg for ^1H, 80 mg for ^{13}C) was dissolved in 1 mL of D_2O. The chemical shifts were calibrated relative to the signals from H_2O, used as an internal standard, at 4.7 ppm for the ^1H NMR spectra. ^{13}C NMR spectra were obtained at 25 °C after 30000 scans. A 30° pulse flipping angle, a 9.2 μs pulse width, and a 2 s delay time between scans were used. The heteronuclear single quantum coherence (HSQC) spectra were acquired over a t_1 spectral width of 20000 Hz and a t_2 width of 3600 Hz, and the acquired time per scan (AQ) is 0.1409 s. The numbers of scan (NS) was 64. The delay between transients was 1.5 s, and the delay for polarization transfer was set to correspond to an estimated average $^1H-^{13}C$ coupling constant of 145 Hz.

The developed techniques of solid-state ^{13}C cross-polarization/magic angle spinning nuclear magnetic resonance (CP/MAS NMR) spectroscopy provide a powerful tool for the structural characterization of the starch polymorphs. The spectra were obtained at 100.6 MHz for ^{13}C on a Bruker AV-III 400 MHz spectrometer at room temperature (25 °C). The sample (ca. 100 mg) was packed in 4 mm zirconia (ZrO_2) rotor and spun at 5 kHz. Contact time and repetition time were 1 ms and 2 s, respectively.

Methylation Analysis. The methylation analysis was conducted by the method of Laine et al.[25] with minor modification. Briefly, the dried sample (10 mg) was dissolved with 2 mL of DMSO under nitrogen and then methylated with 0.2 mL of CH_3I and 40 mg of NaOH powder. Partially methylated alditol acetate was prepared from the fully methylated sample by acid hydrolysis with 1 M H_2SO_4 at 105 °C for 2.5 h and the reduction of the hydrolysate using $NaBH_4$, followed by acetylation with acetic anhydride. The alditol acetate was analyzed by GC−MS, using a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm). Helium was used as a carrier gas at a constant flow rate of 1 mL/min. The oven conditions included an initial temperature of 80 °C for 2 min, then to 200 at 25 °C/min, further to 270 at 10 °C/min, and finally to 280 at 10 °C/min for 5 min. The inlet temperature was kept constant at 260 °C. The mass range used in this study was 29−500 m/z. Peak assignments were made based on the retention time and mass spectra. Identification of methylated sugars was performed by gas chromatography−mass spectrometry (GC−MS) and relative GC retention time. Molar ratios were estimated from peak area and response factor analysis.[26]

Enzymatic Hydrolysis. The hemicellulosic samples (4 mg) in 50 mM sodium acetate buffer pH 5 (1 mL) was incubated with *endo*-(1,4)-β-D-xylanase (10 U/mL) at 50 °C for 24 h. After inactivation of the enzyme (100 °C, 10 min) the *xylo*-oligosaccharides (XOS) were analyzed by HPAEC-PAD with an AS50 autosampler and a Carbopac PA-100 column (4 × 250 mm, Dionex). XOS was separated in 0−80 mM NaAc gradient in a 100 mM NaOH isocratic (carbonate free and purged with nitrogen) for 15 min, followed by a 80−300 mM NaAc gradient in 100 mM NaOH for 10 min, then a 10 min elution with 100 mM NaOH to re-equilibrate the column before the next injection. The total analysis process was performed at 30 °C with a flow rate of 0.4 mL/min. Xylose, xylobiose, xylotriose, xylotetrose, xylopentose and xylohexose were used as standards.

Thermogravimetric Analysis. Thermal analysis of the hemicellulosic samples was performed using thermogravimetric analysis (TGA) on a simultaneous thermal analyzer (SDT-60, Shimadzu). The apparatus was continually flushed with a nitrogen flow of 25 mL/min. The sample was weighed between 8 and 12 mg and heated from room temperature to 600 °C at a rate of 10 °C/min.

X-ray Diffraction. The crystallinity of starch was measured using a XRD-6000 instrument (Shimadzun) with Cu Kα radiation source (λ = 0.154 nm) at 40 kV and 30 mA. The sample was scanned at a speed of 1°/min, in the 2θ range 5−35°, and a step size of 0.04° at room temperature. The degree of crystallinity of starch was quantitatively estimated, following the method of Nara and Komiy.[27]

Table 1. Monosaccharide Compositions of Starch and Hemicellulosic Subfractions from Bamboo

		molar composition[c] (mol %)					molar ratio	
fraction[a]	yield[b] (%)	Ara	Gal	Glc	Xyl	GlcA	Ara/Xyl	GlcA/Xyl
starch	2.1	2.69	0.87	95.17	1.27	0	2.118	0
HA	3.7	5.75	0.04	8.36	84.30	1.55	0.068	0.018
H_{15}	1.8	5.84	0.04	9.05	83.26	1.81	0.070	0.022
H_{30}	3.2	6.44	0.13	10.29	81.10	2.04	0.079	0.025
H_{45}	5.1	5.86	0.17	17.01	74.58	2.38	0.078	0.032
H_{60}	4.8	7.40	0.65	35.18	53.33	3.44	0.139	0.064
H_{75}	1.5	9.65	1.05	24.27	61.60	3.43	0.157	0.057

[a] HA, H_{15}, H_{30}, H_{45}, H_{60}, and H_{75} represent the subfractions precipitated at ethanol concentrations of 0, 15, 30, 45, 60, and 75% (v/v), respectively. [b] Based on defatted bamboo (wt %). [c] Expressed in relative molar percentages.

RESULTS AND DISCUSSION

Monosaccharide Composition. The monosaccharide composition of the starch and six hemicellulosic fractions is presented in Table 1. As can be seen, the fraction of starch contained a significant amount of glucose (95.2%) together with traces of arabinose (2.7%) and xylose (1.3%), indicating that water-soluble arabinoxylan fragments were also present in the bamboo starch fraction in a very small amount. The dominating monomeric sugar components of alkali-soluble hemicelluloses afforded by graded precipitation with ethanol were identified as xylose (53.3−84.3%), followed by glucose, arabinose and glucuronic acid, while galactose was a trace constituent. This sugar composition suggested that the bamboo hemicelluloses were potential glucuronoarabinoxylan (GAX)-type polysaccharides, which is in agreement with those found in other bamboo species.[18,20] The existence of glucose (8.4−35.2%) probably arose from (1→3)(1→4)-β-D-glucan. In addition, small amounts of glucuronic acid (1.6−3.4%) were also released. Those glucuronic acids were originated from 4-*O*-methylglucuronic acids as side chains, which can be confirmed by NMR. 4-*O*-Methyl-D-glucuronic acid was also found in polysaccharides of both bamboo species *Sasa senanensis* Rehd. and *Phyllostachys reticulate* C. Koch by Yoshida et al.[20] and Maekawa,[18] who gave a similar molar ratio of G/X (0.04), respectively. No glucuronic acids were detected in the water-soluble arabinoxylans, implying evidence of difficult isolation of acidic hemicelluloses from the water process.

In general, arabinose (A) to xylose (X) ratio is indicative of the degree of linearity or branching of hemicelluloses. From Table 1, the A/X ratio in the fractions continuously increased from 0.068 to 0.157 as increasing concentrations of the ethanol from 0 to 75%, and a similar trend of G/X ratio was also observed. These results indicated that the more branched hemicellulosic fractions were precipitated at higher ethanol concentrations, which were in

close agreement with those found for hemicelluloses of wheat bran, malt, and barley by several investigators[13,14,28] using ethanol or ammonium sulfate graded precipitation techniques. However, in comparison, the xylan from bamboo seems to be more linear than those of the hemicelluloses. On the other hand, it also suggested that the higher the degree of substitution, the more hemicelluloses appeared to be soluble in aqueous solution, which required a very much higher concentration of ethanol to precipitate from the solution. Therefore, a remarkably low solubility of the fraction HA appeared to be a plausible reason for the experimental fact that it could be collected as precipitation without any ethanol as soon as the extract was neutralized with 6 M HCl to pH 5.5.

Molecular Weight. The weight-average (M_w) and number-average (M_n) molecular weights of the seven fractions were determined by gel permeation chromatography (GPC). As shown in Table 2, all of the fractions exhibited high M_w ranging between 13400 and 67500 g/mol. The M_w of water-soluble arabinoxylans in the starch fraction was 13400 g/mol. For alkali-soluble hemicelluloses, an increase in precipitated ethanol concentrations from 0 to 60% resulted in an increasing trend of M_w value from 24400 to 67500 g/mol, although the fraction H_{75} had a relatively low M_w (26900). In this case, it indicated that a much higher molecular weight obtained in a range of ethanol concentration (0−60%) corresponded with a more branched fraction. However, it should be noted that the molecular weights of polysaccharides vary significantly depending on the method of their estimation. Schooneveld-Bergmans et al.[14] showed that the little branched wheat bran GAX had a high molecular weight as estimated by light scattering. In addition, Maekawa[18] reported that M_w determined from sedimentation equilibrium of DMSO extraction and then water-soluble bamboo xylan (*Phyllostachys reticulata* C. Koch) by a short column method was 27200, while M_n estimated from osmotic pressure measurement of 5% NaOH-extracted one was 25700. In comparison, a broad range of molecular sizes (estimated by GPC) could be obtained using our approach. Furthermore, the polydispersity index is defined by the ratio M_w/M_n.[29] As expected, the relatively low polydispersity indices (1.3−2.0) proved a narrow distribution of all the hemicellulosic fractions, indicating the chemical and structural homogeneity for each hemicellulosic fraction employed the selective fractionation with graded ethanol precipitation technology in the present study.

Table 2. Weight-Average (M_w) and Number-Average (M_n) Molecular Weights (g/mol) and Polydispersity (M_w/M_n) of Starch and Hemicellulosic Subfractions

	WSAX in starch[a]	HA	H_{15}	H_{30}	H_{45}	H_{60}	H_{75}
M_w	13400	24400	21300	33000	55000	67500	26900
M_n	7900	17500	16300	20800	34300	32800	19000
M_w/M_n	1.7	1.4	1.3	1.6	1.6	2.0	1.4

[a] Water-soluble arabinoxylans (WSAX) in starch fraction.

FT-IR Spectra. The FT-IR spectra of fractions (starch, HA, H_{15}, and H_{30}) are shown in Figure 2. Evidently, all fractions give rather similar characteristic absorption of typical polysaccharides in the region 800−1500 cm^{-1}. As can been seen from Figure 2, the absorptions at 1463 and 1405 cm^{-1} are attributed to the CH_2 and CH or OH bending, respectively. A very small band at 1162 cm^{-1} arises from C−O and C−O−C stretching with some contribution of OH bending in arabinoxylans (AX). The C−C stretching gives the absorption at 1116 cm^{-1}. The ring vibrations at 1088 and 990 cm^{-1} are influenced by the degree of branching and hydration. Moreover, AX shows the main band maximum at about 1045 cm^{-1} due to COH bending mode. Another important band appears at about 899 cm^{-1}, corresponding to the glycosidic C_1−H deformation mode with ring vibration contribution for the β-anomer form of the pyranoid ring, which suggests β-glycosidic linkages between the sugar units. Furthermore, the broad band at 1640 cm^{-1} is assigned to the H−O−H angle vibration. A weak absorption at 1249 cm^{-1} relates to the C−O linkage in the acetyl group, indicating the structural feature

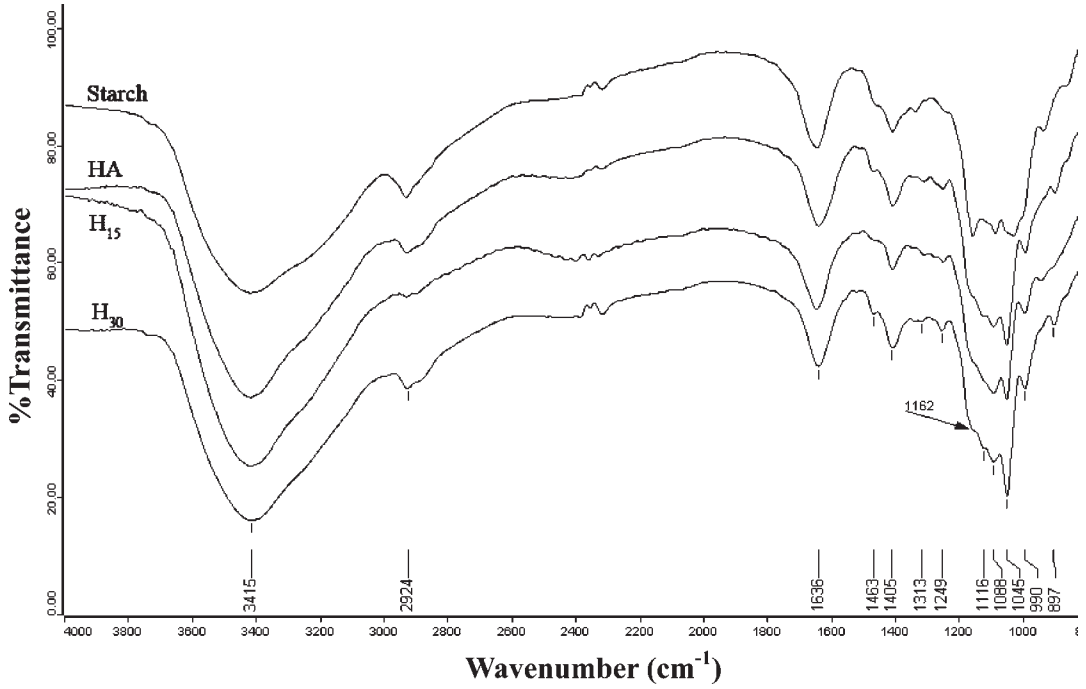

Figure 2. FT-IR spectra of starch fraction and hemicellulosic subfractions of HA, H_{15}, and H_{30}.

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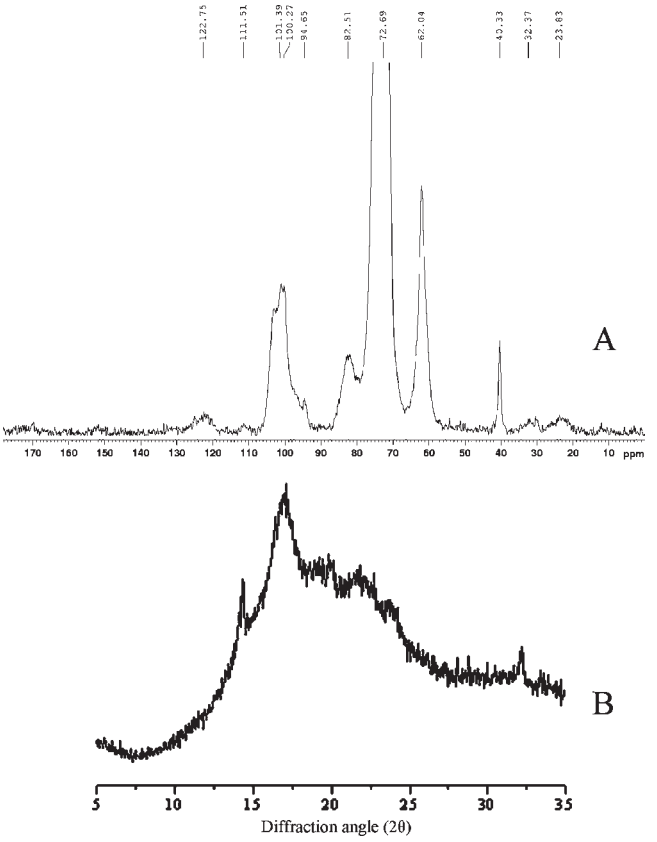

Figure 3. ^{13}C CP/MAS NMR (A) and XRD (B) spectra of starch.

of acetylation for bamboo hemicelluloses. In addition, the strong band at 3415 cm^{-1} is originated from O−H stretching, whereas the C−H stretching vibration of CH_2 group appears at 2924 cm^{-1}. Similarly, the absorbances at 1458, 1406, 1153, 1111, 1083, 1046, and 899 cm^{-1} are associated with the fractions H_{45}, H_{60}, and H_{75} for the typical hemicelluloses (Figure S1 in the Supporting Information).

Structural Analysis of Starch by ^{13}C CP/MAS NMR and XRD. Figure 3A shows a typical ^{13}C CP/MAS NMR spectrum of bamboo starch. Carbon chemical shifts for starch have been identified in accordance with the previous papers:[30,31] 94.5−103.0 ppm for C_1, 72.7 ppm for C_2, C_3, and C_5, 82.5 ppm for C_4, and 62.0 ppm for C_6. In addition, the two shoulders appearing at δ 103.0 and 94.5 ppm arise from noncrystalline material for C_1, and the resonance at δ 82.5 ppm is originated from noncrystalline material for C_4. Generally, the C_1 position exhibits characteristic chemical shift patterns that can reveal the nature of crystallinity in starch. For an A-type crystal, the C_1 gives a triplet pattern at ~102, 101, and 100 ppm, while for a B-type crystal, the C_1 becomes a doublet each at ~101 and 100 ppm.[30] From this point of view, the results for bamboo starch showed two obvious peaks at 101.4 and 100.2 ppm for C_1 indicating a B-type crystal. Moreover, the X-ray diffraction (XRD) pattern of the bamboo starch (Figure 3B) also displayed a typical B-type diffraction pattern with strong reflection at 2θ of about 5°, 14.7°, 17°, 20°, 22° and 23°, corresponding to the structural evolution in terms of ^{13}C CP/MAS NMR, and the degree of crystallinity of bamboo starch calculated from Figure 3B was only 10.9%. The interpretation of the crystallization of starch may be complicated. At present, the most probable mode is accepted to be a double helix and the uniquely shaped amylopectin molecule.[31] Therefore, such a low degree of crystallinity indicated a high content of amylose in bamboo starch, which can be viewed as a good potential source for production of ethanol.

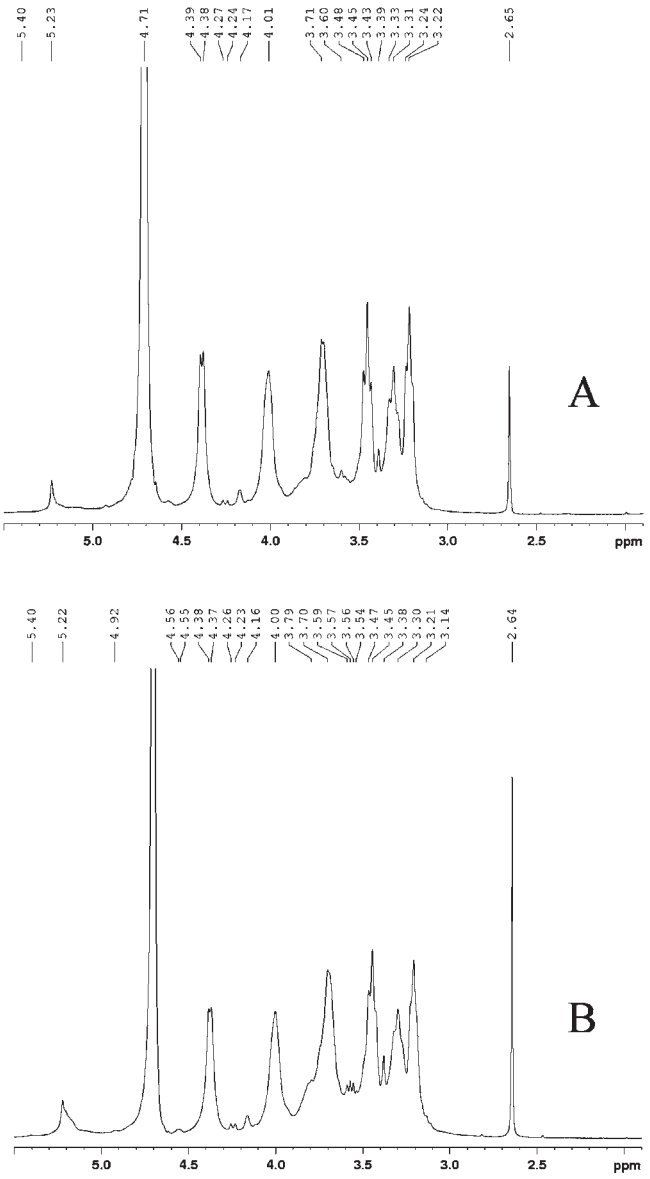

Figure 4. ^{1}H NMR spectra of hemicellulosic subfractions HA (A) and H_{45} (B).

Determination of Structure of Hemicelluloses by ^{1}H, ^{13}C and 2D HSQC NMR. The ^{1}H NMR spectrum of fraction HA is shown in Figure 4A. General speaking, the signals of anomeric protons of the arabinofuranosyl units appear in the δ 5.2−5.4 ppm region while those from the xylopyranosyl residues are at δ 4.4−4.7 ppm. As can be seen, the relative complexity of the structures was exhibited by (i) major signals at δ 4.39 (H-1), 4.01 (H-5eq), 3.71 (H-4), 3.45 (H-3), 3.31 (H-5ax) and 3.22 ppm (H-2), corresponding to nonsubstituted (1→4)-β-D-Xyl*p* residues; (ii) minor signals at δ 4.27 (H-5), 3.86 (H-3), 3.58 (H-2), 3.24 (H-4) and 3.39 ppm (OCH_3-4), originating from 4-*O*-methyl-α-D-Glc*p*A acid (1→2) residues; and (iii) a very small signal at δ 5.40 ppm, indicating the Ara*f* units monosubstituted at *O*-3 of Xyl*p* residues.[32,33] Figure 4B illustrates the ^{1}H NMR spectrum of fraction H_{45}; quite similar signals were also observed.

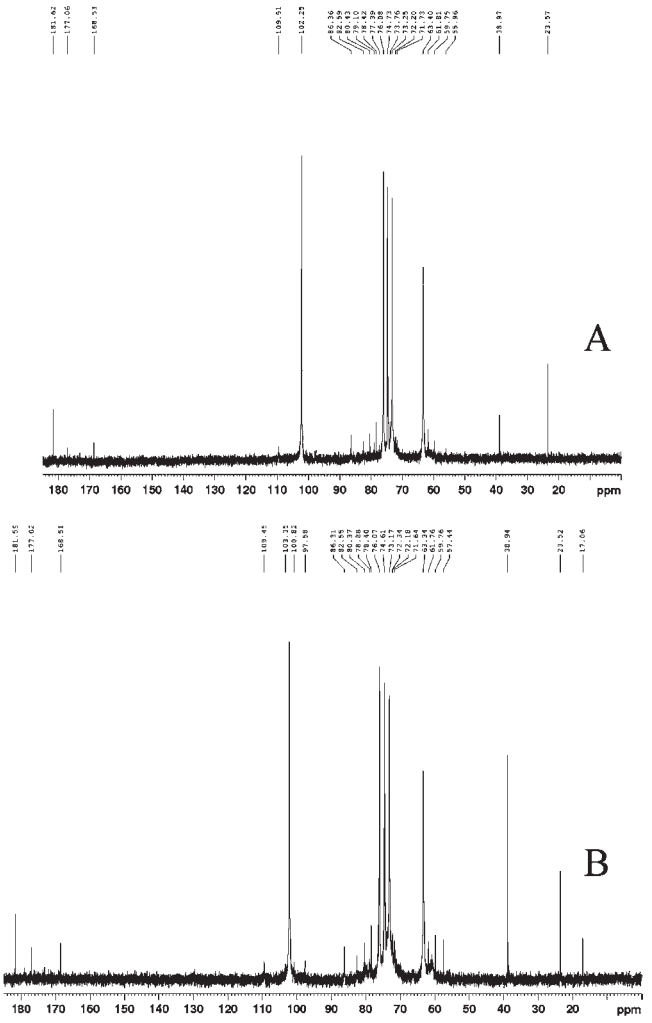

Figure 5. ^{13}C NMR spectra of hemicellulosic subfractions HA (A) and H_{45} (B).

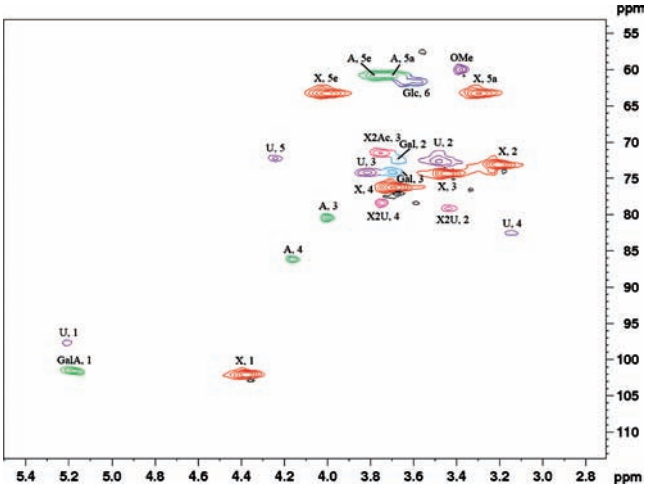

Figure 6. HSQC (D_2O, 25 °C) spectra of hemicellulosic subfraction HA. Designations are as follows: X, Xyl*p* unit; A, Ara*f* unit; U, 4-*O*-Me-α-D-Glc*p*A unit; Gal, →4)-*β*-D-Gal*p*-(1→ unit; Glc, (1→4)-linked α-D-Glc*p* unit; GalA, (1→4)-α-D-GalA.

The ^{13}C NMR spectrum of HA (Figure 5A) shows five main signals at *δ* 102.25 (C-1), 76.08 (C-4), 74.73 (C-3), 73.25 (C-2), and 63.40 ppm (C-5), corresponding to (1→4) linked *β*-D-Xyl*p* residues. Other less intense signals at *δ* 109.51(C-1), 82.59 (C-2), 80.43 (C-3), 86.36 (C-4) and 61.81 ppm (C-5) are attributed to Ara*f* units. Small signals at *δ* 177.06, 72.20, 73.76, 73.25, and 59.75 ppm, are characteristic, respectively, of COOH, C-2, C-3, C-5 and OCH_3-4 of 4-*O*-methy-α-D-glucuronic acid residues.[33] Moreover, the ^{13}C NMR spectrum of H_{45} (Figure 5B) presented the same general features as already observed in the case of HA, which indicated that the fraction H_{45} had a similar backbone of polysaccharide with that of HA.

More specific information about fraction H_{45} was obtained by 2D HSQC NMR spectrum (Figure 6). Obviously, the marked ^{1}H/^{13}C cross-peaks in the HSQC spectrum at *δ* 4.37/102, 5.21/97.7, and 4.16/86.1 ppm confirmed the structural element of (1→4)-*β*-D-xylan backbone, 4-*O*-Me-α-D-Glc*p*A-(1→2) units at position *O*-2, and Ara*f* residues at *O*-3, respectively. In addition, very low amounts of (1→4)-α-D-Glc*p* (3.69/74.1 ppm) and (1→4)-α-GalA (5.17/101.5 ppm) units were also observed.[34,35] Furthermore, the HSQC also provided additional evidence for the presence of Xyl*p* units attached with acetyl groups (*δ* 3.75/71.53 ppm) at position 2. This result implied that the polysaccharide from bamboo is monoacetylated at the C-2 position of Xyl*p* in spite of the absence of a signal at *δ* 2.11 ppm in the ^{1}H NMR spectrum, which might be explained by removal of acetyl groups under the alkaline conditions used. The xylan acetylated at *O*-2 was also isolated from cell walls of bamboo shoot,[36] and Meakawa[18] first prepared a xylan containing 6.5% of native acetyl groups by extraction with DMSO from bamboo holocellulose (*Phyllostachys reticulata* C. Koch).

Glycosidic Linkage Composition. To further study the nature of glycosidic linkages and substitution pattern, seven fractions of polysaccharides were investigated by methylation analysis, and the results are given in Table 3. Starch fraction contained a high amount of 2,3,6-Me_3-Glc*p* (90%) and a low amount of 2,3,4-Me_3-Glc*p* (3.5%), which suggested the presence of amylose with a long 1→4 linked glucose backbone and amylopectin with side chains by 1→6 linkage, corresponding to the result of XRD. Additionally, highly substituted water-soluble arabinoxylans in starch fraction consisted of only 14% unsubstituted xylose, 55% monosubstituted and 31% terminal xylose. While arabinose was chiefly terminally linked, however, 1→5 (14%) and 1→2 (27%) linked arabinose residues were also detected, indicating the attachment of a few *p*-coumaric acids or ferulic acids at the *O*-5 position of α-arabinosyl units and the presence of α-L-Ara-(1→2)-α-L-Ara-(1→3) as side chains in the water-soluble arabinoxylans. These nonterminal arabinose units were also reported in pericarp tissues of grasses.[37−40]

In the six fractions of little substituted alkali-soluble hemicelluloses, varying between 51% and 80% of the xylose residues were unsubstituted. Approximately 0.8−11% of the xylose units were monosubstituted at the *O*-2 position, which was from 4-*O*-methylglucuronic acid unit and acetyl group contribution. Another 5−12% of the xylose residues were monosubstituted at the *O*-3 position by arabinose, whereas the remaining residues were terminal. As expected, the increase in ethanol concentration levels produced arabinoxylan fractions with a decreasing content of unsubstituted xylose residues but an increasing amount of monosubstituted units. This corresponds well to the results of sugar analysis. For arabinose, it was present almost exclusively as a terminal residue and progressively increased in branched arabinoxylan. In addition to arabinoxylan, the majority of glucose in those subfractions appeared to be derived from coextracted

Table 3. GC−MS Results of Methylation Analysis of Starch and Hemicellulosic Subfractions

methylated sugar	mass fragments (m/z)	type of linkage	molar ratio						
			starch	HA	H_{15}	H_{30}	H_{45}	H_{60}	H_{75}
		Galactose							
2,3,4,6-Me_4-Gal[a]	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	β-D-Gal*p*-(1→	1.2	0.1	0.1	0	0	2.4	2.7
2,3,6-Me_3-Gal	43, 45, 71, 87, 99, 101, 113, 117, 129, 233	→4)-β-D-Gal*p*-(1→	1.8	0.2	0.1	0.8	0.2	0.2	0.2
		Arabinose							
2,3,5-Me_3-Ara	43, 45, 71, 87, 101, 117, 129, 161, 233	α-D-Ara*f*-(1→	43.7	24.8	22.5	33.8	45.7	55.0	59.1
2,3-Me_2-Ara	43, 87, 101, 117, 129, 189	→5)-α-D-Ara*f*-(1→	10.7	0	0	0	0	0.2	0.5
3,5-Me_2-Ara	43, 45, 71, 87, 99, 101, 117, 129, 161, 233	→2)-α-D-Ara*f*-(1→	20.0	0.2	0	0	0.7	0.3	0.7
		Xylose							
2,3,4-Me_3-Xyl	43, 45, 87, 101, 117, 129, 161	β-D-Xyl*p*-(1→	2.2	3.5	2.1	1.2	0.8	3.2	0.3
2,3-Me_2-Xyl	43, 87, 101, 117, 129, 189	→4)-β-D-Xyl*p*-(1→	1.0	21.1	9.7	9.1	5.5	6.7	2.1
3-Me-Xyl	43, 45, 87, 99, 101, 129	→4)-β-D-Xyl*p*-(1→ 2 ↑	2.0	0.3	0.1	0.3	0.3	1.5	0.1
2-Me-Xyl	43, 85, 101, 117, 127, 159, 261	→4)-β-D-Xyl*p*-(1→ 3 ↑	1.9	1.3	0.7	1.4	0.8	1.6	0.2
		Glucose							
2,3,4,6-Me_4-Glc	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	β-D-Glc*p*-(1→	2.4	0.1	0.1	0.1	0.2	0.7	0.2
2,4,6-Me_3-Glc	43, 45, 71, 87, 101, 113, 117, 129, 161, 233	→3)-β-D-Glc*p*-(1→	0	0.8	0.8	0.9	1.5	1.3	1.1
2,3,6-Me_3-Glc	43, 45, 71, 87, 99, 101, 113, 117, 129, 233	→4)-β-D-Glc*p*-(1→	67.1	3.8	3.1	3.4	5.3	4.1	2.0
2,3-Me_2-Glc	43, 58, 85, 87, 101, 117, 127, 142, 159, 161, 201, 261	→4)-β-D-Glc*p*-(1→ 6 ↑	2.1	0.4	0	0	0.3	2.0	0.4
2,3,4-Me_3-Glc	43, 58, 87, 99, 101, 117, 129, 161, 189, 233	→6)-β-D-Glc*p*-(1→	2.6	0	3.2	3.5	3.7	1.1	0.2

[a] 2,3,4,6-Me_4-Gal = 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl-D-glucitol, etc.

Table 4. Composition and Total Yield of *xylo*-Oligosaccharides Released by Enzyme from Bamboo Hemicellulosic Subfracions

fraction[a]	molar composition[b] (mol %)						total yield[c]
	xylose	xylobiose	xylotriose	xylotetraose	xylopentose	xylohexose	
HA	2.3	49.4	28.9	17.4	1.7	0.3	23.1
H_{15}	2.4	49.8	28.7	17.3	1.5	0.3	21.5
H_{30}	2.1	50.6	27.7	17.6	1.7	0.3	32.5
H_{45}	2.1	51.0	27.6	17.2	1.8	0.3	26.3
H_{60}	2.5	55.3	27.4	14.1	0.7	nd[d]	38.9
H_{75}	4.5	74.5	15.9	5.1	nd	nd	40.6

[a] Corresponding to the hemicellulosic subfraction in Table 1. [b] Expressed in relative molar percentages. [c] Based on the original xylose (w/w%). [d] Not detectable.

β-(1→3)(1→4) glucans because both 3- and 4-linked glucose residues were identified. The ratios of β-(1→4)/β-(1→3) linkages ranged from 2:1 to 5:1. From this, it can be calculated that the subfraction obtained at a higher ethanol concentration had a lower β-(1→4)/β-(1→3) ratio, similar to the stepwise precipitation with $(NH_4)_2SO_4$.[13] Some minor populations of 1→4 linked galactose and 1→4,6 linked glucose residues were also present, as evidenced by the occurrence of 2,3,6-Me_3-Gal and 2,3-Me_2-Glc, respectively. Uronic acid residues were suggested by the increase in 2,3,4,6-Me_4-Glc because of carboxyl-reduction prior to methylation. The uronic acid is originated from 4-*O*-methyl-derivative (diagnostic MS fragments, 129, 189 and 201).

Production of *xylo*-Oligosaccharides by Enzymatic Hydrolysis. A series of *xylo*-oligosaccharides (DP 1−6) formed by endoxylanase treatment of the six hemicellulosic fractions were monitored using HPAEC, and the results are presented in Table 4. The total yield of degradability was shown to range from 21.5 to 40.6% based on the original xylose, the lowest of which was observed for H_{15} and the highest was found for H_{75}. The very low degradability of HA and H_{15} may be explained by the little substituted backbone. Theoretically, less substituted arabinoxylans should be more prone to enzymatic hydrolysis by xylanases. However, because of possible intermolecular associations with other arabinoxylans or β-glucans and interchain aggregation, these

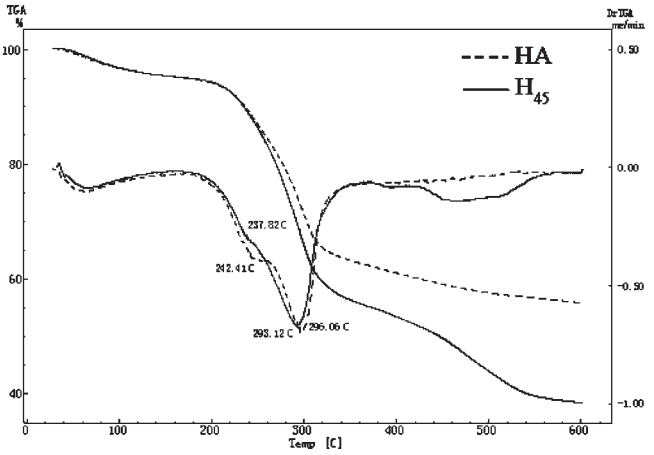

Figure 7. TGA and DTG curves of hemicellulosic subfractions HA and H_{45}.

polymers might in fact be protected from enzymatic attack due to reduced accessibility of xylanases to their backbone. It can therefore be concluded that the effect of enzyme-resistance on hydrolysis of XOS was mainly caused by aggregation among molecules rather than substitution of the backbone in case of more linear xylans. As mentioned in Monosaccharide Composition, bamboo hemicelluloses were more linear than others. HPAEC analysis of the hydrolysis mixtures showed that the XOS consisted predominantly of xylobiose, xylotriose and xylotetraose, followed by xylose and xylopentose, while xylohexose was a trace constituent. From Table 4, it is clear that the increase in ethanol concentration, namely, branched GAX, produced XOS with an increasing amount of xylobiose but decreasing contents of xylotriose and xylotetraose. This indicated that the more branched GAX contained relatively few regions of three or more contiguous unsubstituted xylose residues. By combination of the above results, a difficult hydrolysis of the little substituted bamboo glucuronoarabinoxylans by an endoxylanase was observed, probably due to their sensitive aggregation, however, the presence of substitutions (degree of branches) on the xylan backbone played an important role in limiting the composition of XOS production. Thereby, understanding the structural characterization of hemicelluloses will be helpful in improving the enzymatic hydrolysis treatment in release of XOS.

Thermogravimetric Analysis. Thermogravimetric analysis (TGA) is one of the most common techniques used to rapidly investigate the mass loss and compare thermal behavior during pyrolysis of materials. Figure 7 shows the measured TGA curves and their first derivatives of the thermogravimetric (DTG) curves of fractions HA and H_{45} (heating rate β = 10 °C/min). From the TGA curves, it can be clearly seen that weight loss of both fractions took place mainly in the temperature range of 200−320 °C and HA is thermally more stable than H_{45}. It indicated that linear hemicelluloses showed the characterization of more thermal stability than the branched ones during pyrolysis. In addition, the DTG curve for HA exhibited the peak maximum at 296 °C with a shoulder at 242 °C, and for H45 at 293 and 237 °C, respectively. Two distinct peaks revealed that the thermal degradation of polysaccharide occurred in two steps. According to Daniel et al.'s view,[41] the first peak, a shoulder on the main degradation peak, is attributed to the low-temperature polymerization process, which results in the formation of char, carbon monoxide, carbon dioxide and water. The second one, at somewhat higher temperatures, is attributed to generate volatile anhydrosugars and related monomeric compounds. However, the overall decomposition mechanism of hemicellulose pyrolysis also involves competing reactions. Concerning DTG curves, obviously, in comparison to HA, the shoulder of H_{45} is less pronounced, and the maximum and shoulder peaks shifted 3 and 5 °C to lower temperature, respectively. This phenomenon can also partially support the above structure−thermal stability relationships.

Conclusion. Bamboo (*Phyllostachys bambusoides* f. shouzhu Yi) starch, displaying the characteristic of typical B-type pattern, may be considered as a new starch source for food and nonfood industry due to its very low degree of crystallinity. Meanwhile, highly substituted water-soluble arabinoxylans constituted a rather minor portion of starch fraction. Besides terminally linked arabinose, the side chains of α-L-Ara-(1→2)-α-L-Ara-(1→3) were also confirmed in water-soluble arabinoxylans. In comparison, the alkali-soluble hemicelluloses, fractionated into six subfractions by graded ethanol precipitation, were *O*-acetylated glucuronoarabinoxylan-type polysaccharides with the structural element of →4)[2-*O*-Ac][4-*O*-Me-α-D-Glc*p*A-(1→2)][α-L-Ara-*f*-(1→3)]-*β*-D-Xyl*p*-(1→. Moreover, the branched alkali-soluble hemicelluloses with high molecular weights but lower thermal stability tended to be precipitated at somewhat higher ethanol concentrations. Furthermore, a lack of hydrolysis of the little substituted bamboo glucuronoarabinoxylans by an endoxylanase was also observed, while the composition of XOS production depended on the presence of substitutions on the xylan backbone. Additionally, the graded ethanol selective precipitation technology was useful in fractionation of the high molecular weight and more homogeneous bamboo hemicelluloses, which can be utilized in production of XOS by enzyme.

■ ASSOCIATED CONTENT

Supporting Information. FT-IR spectra of hemicellulosic subfractions of H_{45}, H_{60}, and H_{75}. This material is available free of charge via the Internet at http://pubs.acs.org.

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