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Cellulolytic Enzyme-Aided Hemicellulose Extraction and Its Characteristics from Switchgrass

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ABSTRACT

Isolation of intact structure biomass component is crucial to understand the biomass characteristics. Compared to other major biomass component such as cellulose and lignin, hemicellulose remains a challenging component to be isolated from the plant cell wall without significant depolymerization and modification. In this study, a novel cellulolytic enzyme-aided hemicellulose (CEH) isolation method was developed to isolate hemicellulose with near-native branched structure from switchgrass. Structural characteristics of CEH were investigated and compared with hemicelluloses isolated by conventional alkaline extracted hemicellulose (AEH) and DMSO extracted hemicellulose (DMSOH) methods. Gel permeation chromatography (GPC) analysis indicated that the CEH had a weight-average molecular weight of 44 kDa, which was comparable with AEH (43 kDa) but higher than DMSOH (37 kDa). The chemical composition analysis revealed that the CEH retained a higher proportion of glucuronic acid compared to the AEH and DMSOH. The 2D ^{13}C-^{1}H heteronuclear (HSQC) NMR spectra containing β-(1,4)-linked-D-Xylan backbone, non-reducing-end peaks as well as both α and β reducing-end peaks in CEH were comparable with the spectra of the commercial beechwood xylan. The CEH showed a highly branched hemicellulose structure, which retained methoxyl groups, *O*-Acetyl groups, and 4-*O*-methyl-glucuronic acid attached on the xylan backbone.

KEYWORDS

Hemicellulose, Cellulase, Structural properties, Glucuronic acid, Glucuronoarabinoxylan

INTRODUCTION

Lignocellulosic biomass has shown great potential as a feedstock for the production of alternative fuels, chemicals, and materials through various utilization processes. In particular, switchgrass (*Panicum virgatum*), a typical warm-season perennial grass, has been investigated as a promising energy crop for the production of second-generation biofuels.[1] In a typical biorefinery process, cellulose is considered as the primary product stream, while utilization of hemicellulose and lignin is still limited. Recently, the importance of hemicellulose and lignin valorization has been highlighted to improve the total biomass utilization. Xylan, which is a major component of hemicellulose, has several applications including chemicals, biopolymers, and pharmaceutical excipients.[2] However, hemicellulose is still under-utilized primarily due to its complicated chemical compositions and structures.[2]

Structural characteristics of hemicellulose are important information that needs to be collected prior to its utilization, and efficient isolation of hemicellulose from the plant cell wall would be a prerequisite for elucidating its physicochemical properties. The functional groups in the hemicellulose are important for effective biomass utilization. For instance, the acetyl group can affect their properties and interactions with other polymers, thus affecting their solubility and extractability.[3] Subsets or branches of hemicellulose were reported as key recalcitrance-causing factors in switchgrass because they can inhibit saccharification of biomass.[4, 5] Furthermore, it has been reported that the arabinose substitution degree of xylan and uronic acid are two of the key

factors that positively affect enzymatic hydrolysis of biomass, due to the fact that arabinose in xylan or uronic acids-rich polymers may interact with the β-1,4-glucan chains in amorphous regions of cellulose microfibrils via hydrogen bonding which negatively affects cellulose crystallinity.[6-9] The structural properties of hemicelluloses vary depending on the species and other environmental factors.[10] Hemicellulose in switchgrass typically has *β*-(1-4)-linked xylan backbones with an equatorial configuration.[11] Its low degree of polymerization (80-200),[2] complex chemical cross-linking with other biomass components such as lignin, and multi-side-chain structures make it challenging to isolate it with minimal changes to its intact structures.[10] Different isolation strategies have been introduced for understanding the characteristics of hemicellulose.[2] Treatment with aqueous alkali,[12-18] organic solvent,[18-22] hot water and biochemicals method[23-26] have been reported to date. Although hemicellulose can be isolated by applying these methods, there are some limitations in each method such as modification of functional groups of hemicellulose, inefficient isolation yields and other issues.[2, 27] Alkaline treatment, one of the most widely used hemicellulose separation methods, disrupts the cell-wall of biomass by swelling cellulose, hydrolyzing uronic and acetic acid esters linkages, and dissolving hemicellulose, lignin and silica.[28] There have been several studies about the effect of alkaline treatment conditions (e.g., pressure, temperature, concentration, extraction time) on the effectiveness and structures of isolated hemicellulose.[15-18] However, alkaline treatment can cleave ester bonds such *O*-acetyl groups and other branches attached to the xylan backbone that reduces its aqueous solubility and

subsequently affects the valorization of the obtained hemicellulose.[29, 30] Sequential extraction with hot water and alkaline generated low-branched xylans with the weight average molecular weight (M_w) ranged between 3760 and 36,000 g/mol from corn stalks, wheat straw, and bamboo.[14, 15] While the multi-step alkaline extraction techniques showed unavoidable hemicellulose degradation during the process, organic solvent treatment using dimethyl sulfoxide (DMSO),[21, 29] DMSO/water mixture,[19] DMSO/LiCl,[20] and dioxane[31] can extract hemicellulose with minimal cleavage of the acetyl ester bonds and the glycosidic linkages. However, the isolation yields of hemicelluloses by these organic solvents are usually significantly lower (~16%)[20, 21] than the recovery by cold caustic extraction (72-85%).[2] It has been reported that L-arabino-4-*O*-methyl-D-glucurono-D-xylan can be isolated from corn stalk and rice straw using DMSO with minimal degradation.[29] In addition, the DMSO-LiCl extraction proved to be an effective methodology to recover natively acetylated hemicellulose fractions from tomato.[20]

Hydrolytic enzymes such as cellulase and hemicellulase can selectively degrade the target polysaccharides.[25] Enzyme applications have been used with alkaline as a separation process for hemicelluloses and cellulose isolation from cellulosic fibers.[23-26, 32] Employing the sequential two-stage treatments enhanced the selectivity of hemicellulose and purity of cellulose.[26] High purity xylan fractions with relatively high yield (60% of original pulp xylan) or high molecular weight (up to 40 kDa) fractions were obtained from hardwood Kraft pulp by a combination of specific xylanase/endoglucanase treatments and alkaline extraction.[25] Arabinoxylan in corn fiber was

solubilized in the form of xylo-oligosaccharides and only polymeric hemicellulose fraction remained in the residual solids after soaking in aqueous ammonia pretreatment followed by cellulase hydrolysis.[23]

Despite these efforts, structural modification of hemicellulose during the alkali isolation process and/or the relatively low recovery yield and poor selectivity remain challenges in hemicellulose isolation.[2] In this study, peracetic acid delignification followed by cellulase-aided hemicellulose isolation was developed to overcome these limitations. Peracetic acid delignification instead of alkaline extraction reduced the chemical modification of hemicellulose structure. With cellulase treatment, the morphological changes of the lignocellulosic fiber were localized to cellulose degradation, which facilitated the diffusion of hemicelluloses from the fiber matrix, leading to improved hemicellulose selectivity.[26] The selective effects of cellulase on cellulose aids on retaining the integrity of hemicellulose during the enzymatic hydrolysis process. The structural characteristics of the isolated hemicellulose, termed as CEH, from switchgrass were analyzed in this study. Specifically, the molecular weights, chemical compositions, structural properties including functional groups of CEH were all investigated. In addition, alkaline extracted hemicellulose (AEH) and DMSO extracted hemicellulose (DMSOH) were also carried out with the same switchgrass and the characterization results were compared with CEH.

MATERIALS AND METHODS

Substrates and reagents. Switchgrass (*Panicum virgatum*) was obtained from Samuel Roberts Noble Foundation in Ardmore, OK, grown in 2011 and harvested in 2012. The raw materials were pulverized and screened to 40-mesh using a Wiley mill prior to the hemicellulose isolation. All the chemicals used in this study were of analytical grade and were purchased from Sigma-Aldrich (Sigma-Aldrich, Inc. St. Louis, MO).

Extractives Removal and Delignification. To remove extractives, milled switchgrass was Soxhlet-extracted with toluene/ethanol mixture (2:1, *v*:*v*) for 8 h followed by acetone extraction for 4 h. Extractives-free materials were dried in a fume hood at 25 °C overnight. Solids were delignified at 25 °C by a peracetic acid (PAA) treatment for 24 h. Specifically, thirty grams of biomass (dry basis) was added into a solution mixture composed of 105.00 g of peracetic acid (32% diluted in acetic acid) and 24.00 g of deionized water. After the delignification, the mixture was transferred into a Buchner funnel, vacuum-filtered, and washed with deionized water until a neutral pH was obtained. The moisture contents of the samples were measured by a Halogen moisture analyzer. The holocellulose samples used for hemicellulose extraction were never dried to avoid fiber hornification which negatively affects the following hydrolysis process.

Hemicellulose Isolation. The overall isolation procedure for each method is summarized in **Figure 1**.

Alkaline Hemicellulose Extraction. Delignified switchgrass samples (~2.50 g) were slurred with 37.5 ml aqueous sodium hydroxide solution (17.5%) in 50 ml centrifuge tubes. The tubes

were screwed-capped and shaken (180 rpm) for 16 h in an incubator shaker at room temperature. The slurry was then filtered microfiber glass filter by vacuum filtration. The filtrates were adjusted to pH 5.5 by acetic acid, followed by precipitation of hemicellulose in 95% ethanol (four times volume of neutralized filtrate). After overnight standing, the suspension was centrifuged at 8,000 rpm for 10 min and the supernatant was decanted. The precipitate was washed and centrifuged with 50 ml of 95% ethanol three times, followed by freeze-drying. This hemicellulose fraction isolated from switchgrass with the alkaline solution was named as AEH.

DMSO Hemicellulose Extraction. Isolation of natively acetylated hemicellulose by DMSO extraction from plant cell wall has been reported in several previous studies.[19-21] In this study, a modified DMSO extraction was applied to prepare and explore the structures of hemicellulose in switchgrass. In brief, hemicelluloses were extracted from the PAA delignified switchgrass with DMSO (1.00 g dry weight of biomass for 40 ml solution) at 70 °C in an N_2 atmosphere with continuous agitation (100 rpm) for 5 h. The suspension was filtered through a polystyrene membrane with additional ~20 ml distilled water. The DMSO extracts and washing liquor were adjusted with acetic acid to pH 3.5 followed by being added to ethanol (160 ml) and finally left in a refrigerator at 4 °C for 12 h. The precipitated hemicellulose was then isolated by centrifugation at 8,000 rpm and 4 °C for 15 min followed by purification with methanol washing 3 times. The precipitated hemicellulose fraction was then vacuum-dried at 60 °C. For further purification, the dry extract was dissolved in 50 ml of deionized water and precipitated with 200 ml of cold 95%

ethanol at 4 °C overnight. The precipitate was recovered by centrifugation (15 min, 8,000 rpm, 4 °C), washed with ethanol 95%, freeze-dried, and named as DMSOH.

Cellulolytic Enzyme-Aided Hemicellulose Extraction. A commercial cellulase (*Trichoderma reesei ATCC 26921*, endoglucanases-rich) with an enzymatic activity of 1 U/mg was supplied by Sigma-Aldrich, Inc. *T. reesei* endoglucanases were proved to have only trace of the xylanase activities that practically no effect on the hemicellulose.[33] Cellulase can cleave cellulose chains, which further weakens the fiber structure and loosens the interaction between hemicelluloses and cellulose. With appropriate cellulase treatment, hemicellulose with relatively high purity and yield can be expected.

PAA delignified switchgrass (1.00g) was ball-milled in a zirconium oxide jar (Retsch PM 100, Newton, PA) at 600 rpm for 3h. Enzyme treatment was conducted with cellulase (30 mg enzyme/g holocellulose) in 50 mM sodium citrate buffer at 37 °C and pH 5.0 with 200 rpm shaking in an incubator for 72 h. The insoluble residues were filtered through glass Buchner funnel and washed with deionized water for subsequent hemicellulose isolation. After the enzyme treatment, hemicellulose polysaccharides supernatant was precipitated in 65 % (*v*/*v*) ethanol (four-volume times) and recovered by centrifugation and freeze-drying. The dry cellulase extract named cellulolytic enzyme-aided hemicellulose (CEH) was purified through the same procedure as described in the DMSO extraction section.

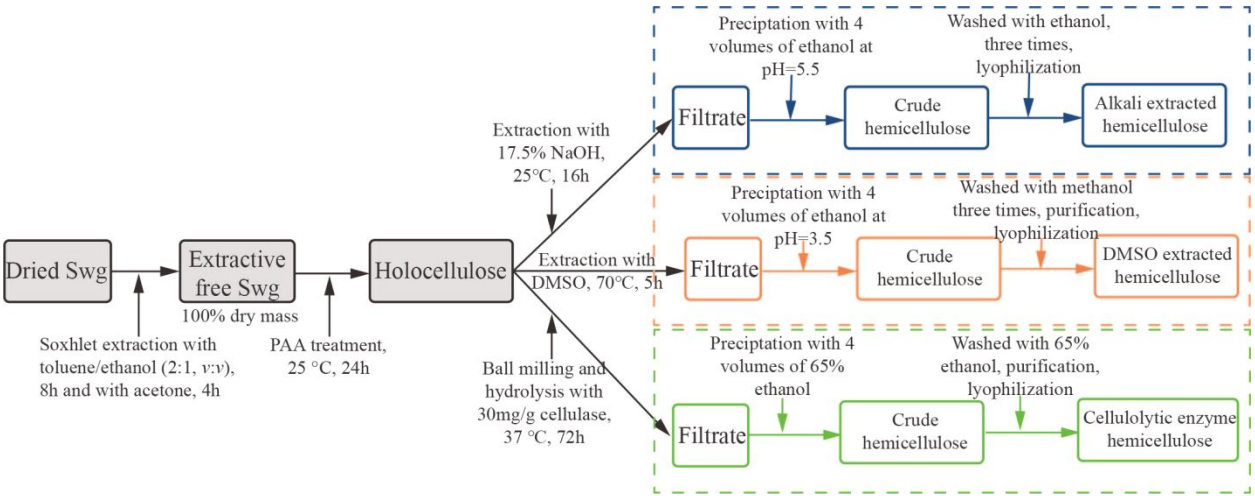

Figure 1. Scheme of hemicellulose isolation with alkaline, DMSO and cellulolytic enzyme methods

Molecular Weight Analysis for Hemicellulose. The molecular weight analysis was performed as described in a previous study.[11] In brief, the hemicellulose samples (~2 mg) were dissolved in 2.0 ml of 0.1M sodium nitrate/0.02% sodium azide mixture (pH~11) at room temperature. Molecular weight analysis was performed using a Wyatt gel permeation chromatography/size exclusion chromatography (GPC) system equipped with light scattering and viscometer detectors as well as a refractive index (RI) detector with the following conditions: Waters Ultrahydrogel 120, 250, and 500 connected in series along with Ultrahydrogel guard column, 0.1 M sodium nitrate with 0.02% sodium azide eluent, 0.5 ml/min flow rate at 25 °C column oven temperature, and 280 nm sample detection. The data acquisition and processing were controlled by ASTRA software with the dn/dc value taken to be 0.145, according to the literature.[34]

Chemical compositional analysis. The carbohydrates and Klason lignin analysis of switchgrass and solid residues after hemicellulose extraction were measured according to the NREL Laboratory Analytical Procedures (LAP) "Determination of Structural Carbohydrates and Lignin in Biomass Procedures"[35]. For quantifying the carbohydrates and Klason lignin in the isolated hemicellulose, the analysis procedure was modified as described in the literature.[11, 36] In brief, ~5 mg samples were hydrolyzed in 4% aqueous sulfuric acid (1.5 ml) at 121 °C for 1h along with sugar recovery standards. The monosaccharide content of the hydrolyzed sample was determined using a HPAEC-PAD Dionex 3000 Ion Chromatograph (IC) equipped with a CarboPac PA-20 column. The monosaccharides were eluted by 2mM NaOH, while the uronic acids were eluted with 400mM NaAc and 100mM NaOH by a modification of the method described in the literature.[36]

Nuclear Magnetic Resonance (NMR) Characterization for Hemicellulose. For two dimensional (2D) ^{13}C-^{1}H heteronuclear (HSQC) NMR analysis of the switchgrass whole cell wall (WCW) and holocellulose, ball-milled sample was prepared and dissolved in DMSO-d_6/HMPA-d_{18} (4:1) according to the method described in previous literature.[37] The swelling of ball-milled cell wall materials in the bi-solvent system provides a gel that permits spectra with reasonable dispersion and resolution to be acquired.[37] The isolated hemicellulose samples were also prepared for NMR analysis as follow: 20 mg of each sample was added into 0.6 ml D_2O. The dissolved samples were directly transferred into 5 mm NMR tubes. Solution-state 2D HSQC NMR spectra

of WCW, holocellulose and hemicellulose samples were obtained using a Bruker Avance III 400 MHz spectrometer.

The NMR experiment was conducted at 298 K equipped with a 5 mm Broadband Observe probe (5 mm BBO 400 MHz W1 with Z-gradient probe, Bruker) and a Bruker standard pulse sequence ('hsqcetgpsi2') with the following parameters: spectral width of 11 ppm in F2 (^{1}H) with 2048 data points and 190 ppm in F1 (^{13}C) with 256 data points; 128 scans (NS) and 1 s interscan delay (D1).

All the data was processed using the TopSpin 3.5 software (Bruker BioSpin). Assignments for correlations were from the extensive HSQC NMR data of the purchased beechwood xylan and monosaccharide standards (Sigma, Knoxville, USA, purity>90%), along with data from a long history of NMR of both WCW and isolated hemicellulose in previous studies.[38-40]

FTIR analysis. The functional group present in the hemicellulose were identified by FTIR. The infrared spectrum was recorded on a PerkinElmer Spectrum 100 FTIR. FTIR spectra were obtained by averaging 32 scans from 4000 to 600 cm^{-1}.

RESULTS AND DISCUSSION

Hemicellulose Yield and Molecular weight. In this study, PAA delignification method was used to produce holocellulose prior to hemicellulose extraction.[41] After delignification, the loss of substrate (including lignin, ash and a small quantity of carbohydrates) from the extractives-free switchgrass was 16.4%. The hemicellulose isolation yields, based on the hemicellulose content (27.66%) of the raw switchgrass, are presented in **Table 1**. The conventional aqueous alkaline

extraction resulted in the highest yield of hemicellulose recovery (89.5%) in switchgrass. The hemicellulose isolation yield was 47.7% in the cellulolytic enzyme-aided hemicellulose (CEH) isolation method and only 8.0% in the form of DMSO extracted hemicellulose (DMSOH).

Table 1. Yield and the weight-average (M_w), number-average (M_n) molecular weights in g/mol, and the polydispersity index (PDI=M_w/M_n) of the hemicellulose samples.

Samples	Time(h)	Temp (℃)	Yield (%) *	M_w (kDa)	M_n (kDa)	PDI
AEH	16	25	89.5±1.8	42.9	22.0	1.95
DMSOH	5	70	8.0±0.2	37.4	19.3	1.94
CEH	72	37	47.7±1.8	44.2	27.6	1.60

* Yields were based on hemicellulose content of the raw switchgrass.

As shown in **Table 1**, the molar mass of the alkaline-extracted, DMSO-extracted and cellulolytic enzyme-aided hemicelluloses was determined by an aqueous phase Wyatt GPC/SEC system. An additional low molecular weight (M_w ~7000 Da) fraction was observed in the CEH (data not shown), this is consistent to the results reported by Hakala et al.[25] The observed low molecular weight of CEH could be due to the small molecules in the isolated hemicellulose generated by cellulase during the extraction procedure. However, the cellulolytic enzyme-aided hemicellulose still showed comparable molecular weight with AEH while DMSOH had the lowest M_w and M_n. The values are also in the comparable ranges with the molecular weights of other species such as corn stover (52k – 56k of M_w/ 46k- 47k of M_n), rice straw (31k – 53k of M_w/ 10k- 17k of M_n), and wheat straw (42k – 45k of M_w) reported in the previous studies. [42-44]

Chemical Compositions of Hemicelluloses. The sugar profiles analysis of raw-switchgrass, solid residues after each extraction and recovered hemicelluloses were shown in **Figure 2** (see **Table S1**).

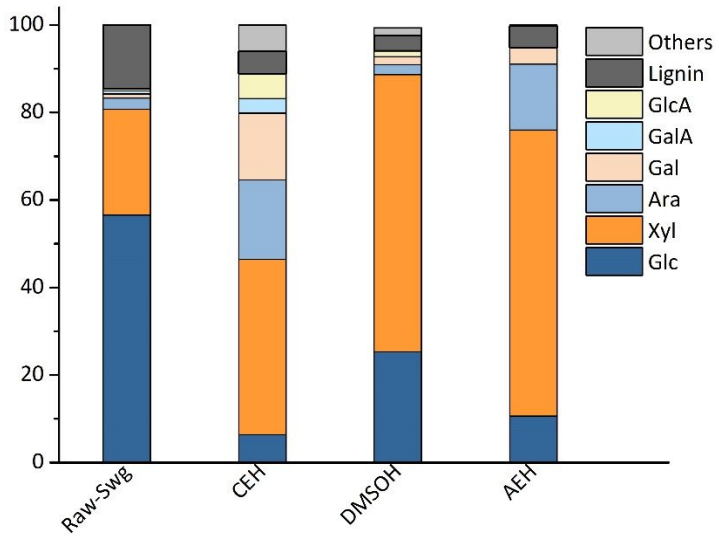

Figure 2. Chemical compositions of raw switchgrass and isolated hemicellulose samples. (Raw-Swg: Extractives-free switchgrass, CEH: cellulolytic enzyme-aided hemicellulose, DMSOH: DMSO extracted hemicellulose, AEH: alkaline extracted hemicellulose, Glc: glucose, Xyl: xylose, Ara: arabinose, Gal: galactose, GalA: galacturonic acid, GlcA: glucuronic acid, Others: ash and other unidentified components)"

The sugar composition, acid insoluble lignin (AIL) and acid soluble lignin (ASL) contents of the raw switchgrass and three hemicelluloses are shown in **Figure 2** and **Table S1**. Raw switchgrass had a significantly higher proportion of lignin (14.91%) and glucose (56.54%) compared with each

extracted hemicellulose. These hemicelluloses differed in their sugar content. Fractions AEH and DMSOH contained 84.20% (*w*/*w*) and 68.77% (*w*/*w*) of hemicellulose, whereas fraction CEH contained 82.45% (*w*/*w*) of hemicellulose, of which half (40.07%) was xylose. Xylose was the main constituent sugar in the extracted hemicelluloses from switchgrass, and arabinose was the second major sugar in CEH (18.16%) and AEH (15.04%). However, in DMSOH, the glucose content was higher than the other hemicelluloses as the second major sugar. This was because DMSO was also capable of partially dissolving low degree of polymerization cellulose besides hemicellulose.[45] Galactose content was the lowest neutral sugar in DMSOH (1.83%) and AEH (3.76%), but was much higher in CEH (15.24%). This was possibly due to the cellulase selectively hydrolyzed cellulose and remained galactose linked to hemicellulose xylan backbone. It has been shown that the cellulase is deficient in a galactosidase.[33] Thus, the CEH contains high galactose content and is more soluble with a much higher galactose substitution. As shown in Figure2, CEH was also enriched in glucuronic acid (GlcA) and galacturonic acid (GalA) compared to the other two types of hemicellulose. This is consistent with the FTIR results that the cellulolytic enzyme-aided hemicellulose contained uronic acid groups (Supplemental Figure S3). In addition, the DMSOH have smaller GlcA (1.33%) proportions compared with the CEH, while no uronic acid was detected in AEH. Li et al. reported that the arabinose in hemicellulose was partially linked with the cellulose in the amorphous regions, leading to an efficient cellulase access for initial enzymatic hydrolysis of cellulose.[6] It was also evidenced that uronic acids-rich polymers also had

similar interactions with β-1,4-glucan chains that reduce cellulose crystallinity.[8] In addition, as the hemicellulose has relatively short chains, it would pack rigidly into the oriented cellulose microfibrils by some cross-bridging or looping.[46] When cellulose microfibrils in the amorphous region were hydrolyzed using cellulolytic enzyme, those bridge occurred between cellulose and hemicellulose could disappear, leading to the release of hemicellulose with higher uronic acid than traditional chemical extraction. As a result, much higher content of arabinose and uronic remained in the CEH after the enzymatic hydrolysis of cellulose-rich holocellulose compared to DMSOH and AEH.

Under the experimental conditions used in this study, 65% aqueous ethanol wash was efficient for the removal of glucose degraded from cellulose and dissolved in the enzymatic hydrolysate. In conclusion, the cellulolytic enzyme-aided method was efficient to selectively hydrolyze cellulose from switchgrass leaving hemicellulose in the supernatant, indicating that this method was more selective and effective for the extraction of uronic acid-rich hemicellulose from switchgrass.

Structural Properties of Hemicelluloses.

Structural properties of WCW and holocellulose from switchgrass in different chemical shift regions including alkyl region (δ_C/δ_H: 10-50/0.5-3.0 ppm); aliphatic (lignin sidechain and polysaccharide) region (δ_C/δ_H: 50-90/2.5-5.5 ppm); polysaccharide anomeric (δ_C/δ_H: 90-110/4.0-5.5 ppm) and lignin aromatic region (δ_C/δ_H: 105-150/6.0-8.5 ppm) are illustrated in the 2D HSQC NMR spectra (**Figure 3**).[47] Even though the HSQC NMR analysis is still semiquantitative method

and challenging to directly compare with bulk compositional analysis, this analysis can provide important structural information of biomass component.

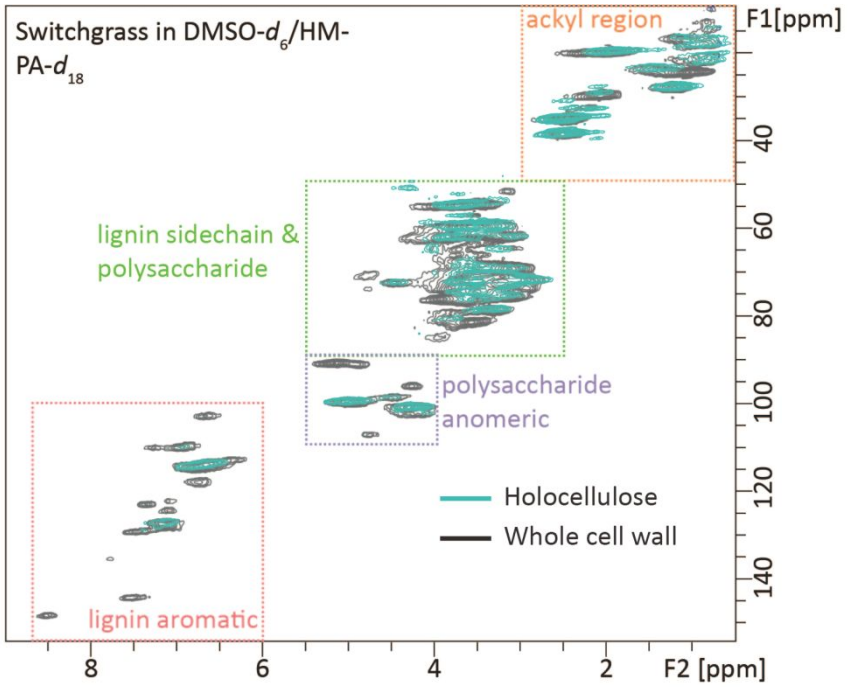

Figure 3. The overlay 2D ^{1}H-^{13}C HSQC spectra of the whole cell wall and isolated holocellulose from switchgrass

The WCW and holocellulose NMR spectra present contours from polysaccharides and lignin in switchgrass. The contours of the lignin sidechain and aromatics were significantly removed during the PAA delignification process, whereas there was no discernible difference between the correlation peaks of polysaccharides from holocellulose and those peaks from WCW except the absence of α-D-galactose and cellulose residues signals in holocellulose.[47, 48] This is consistent with the previous studies that crystalline cellulose is not swelled significantly in these gel-solvents with limited mobility, therefore, it is practically "invisible" in the HSQC spectra.[4, 49] The chemical

shifts ($^{13}C/^{1}H$ ppm) of WCW and holocellulose from switchgrass in 2D NMR regions were presented in **Figure S1**. The main C1/H1 correlation peaks in this research, listed in **Table S2**, were assigned according to previous studies.[4, 38, 39, 49, 50]

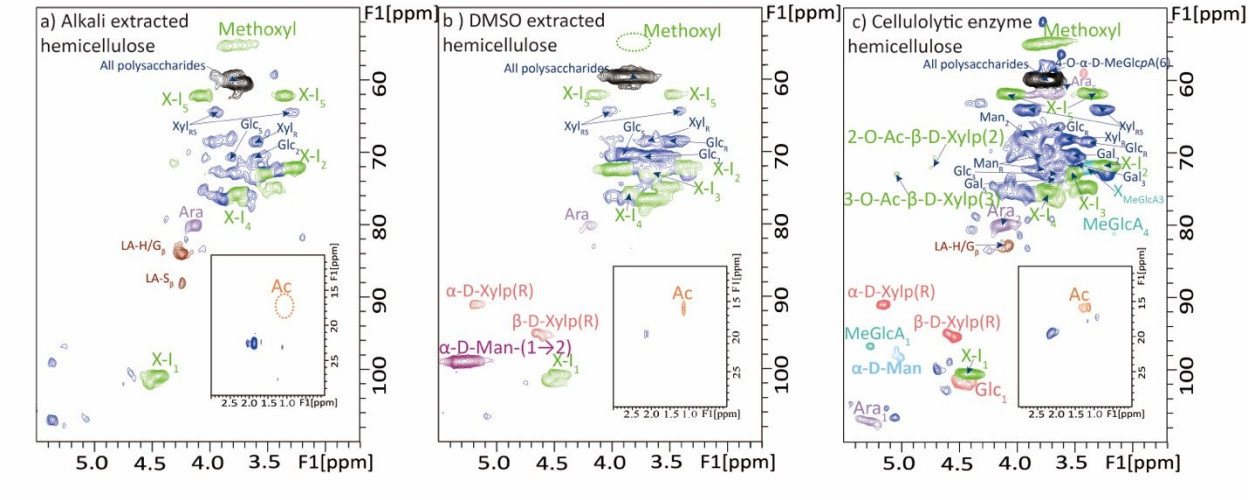

Figure 4. 2D ^{1}H-^{13}C HSQC NMR spectra of hemicelluloses: a) AEH, b) DMSOH, c) CEH in D_2O. Part of the alkyl region (δ_C/δ_H: 10-30/0.5-3.0 ppm) was inserted at the non-signaled area of each spectrum. The assignments are based on NMR data from model compounds in the same solvent, and from other references.[4, 21, 50, 51] X-I, xylan internal unit; LA-H/G_β, lignin *β*- aryl ether (*β*-*O*-4-H/G); LA-S_β, lignin *β*-aryl ether (*β*-*O*-4-S); Ac, Acetyl group; MeGlcA, 4-*O*-methylglucuronic acid.

Structural differences among the AEH, DMSOH, and CEH were also compared in the aliphatic

regions (**Figure. 4**). Aliphatic regions of 2D ^{13}C-^{1}H HSQC NMR spectra show the internal xylan signals from C1/H1 to C5/H5. The methoxyl group (-OCH_3) identified at δ_C/δ_H 55.6/3.72-3.76 ppm in the NMR spectra of AEH and CEH[4, 52] could be aroused from the C-6 in 4-*O*-methyl group of uronic acid residues,[50, 53] but it might be also possibly detected from the residual lignin in the extracted hemicellulose (Figure 2). Disappearance of this methoxyl peak in hemicellulose was also reported after DMSO extraction in a previous study.[54] A peak at δ_C/δ_H 60.1/3.42 ppm representing C6/H6 correlations of 4-*O*-α-D-glucuronic acid (MeGlcA) was observed in CEH and DMSOH, while not in AEH (**Figure 4**). In CEH, another peak representing uronic acid as branch chain of the hemicellulose like C1/H1 of 4-*O*-α-D-(MeGlcA) to (1→4)-β-D-Xyl*p* was detected at δ_C/δ_H 101.4/ 4.61 ppm. Furthermore, the presence of uronic acid groups was also confirmed with observed peaks in the aliphatic and polysaccharide anomeric regions of CEH. The isolated hemicellulose using cellulase showed the glucuronoarabinoxylan (GAXs) with relatively large proportion of α-(1,2)-linked-D-glucuronic acid (GlcA) and MeGlcA (Figure 4). The GlcA and MeGlcA substitutions on β-(1,4)-D-xylopyranose backbone was reported in the previous study.[55]

The correlation peaks of acetyl group in CEH and DMSOH were observed at δ_C/δ_H 20.2/1.7-2.1

ppm, while it was absent in AEH. In addition, the correlation peak from acetyl group (C1/H1) of DMSOH was less intense than the CEH, indicating that the hemicellulose extraction with DMSO under given condition removed some acetyl groups. The acetyl group content could affect properties of hemicelluloses and interactions with other polymers. For instance, it can affect the water solubility and extractability of hemicellulose.[3] Furthermore, CEH showed low intensity of 2-*O*-Ac-β-D-Xyl*p* C2/H2 at δ_C/δ_H 73.5/4.64 ppm and 3-*O*-Ac-β-D-Xyl*p* C3/H3 correlation peaks at δ_C/δ_H 75.0/4.94 ppm, whereas both of the contours were absent in DMSOH. Correlation peaks from *O*-acetyl-Xyl*p* group were not detected in the 2D NMR spectra of AEH, which is consistent with the previous observation.[2]

In summary, cellulolytic enzyme method, which minimizes the loss of intact structure of hemicellulose, including GlcA, MeGlcA, and *O*-acetyl groups, becomes a novel method to assess the initial hemicellulose structure in order to ascertain their industrial application. According to the NMR results, the CEH from switchgrass retains much native structure of xylans, which consists of a linear backbone of (1→4)-linked D-xylopyranosyl units (Xyl*p*), partially *O*-3 substituted with

L-arabinofuranosyl (Ara*f*) units, and *O*-2 substituted essentially with 4-*O*-methyl-D-glucuronosyl units (MeGlc*p*A).[56]

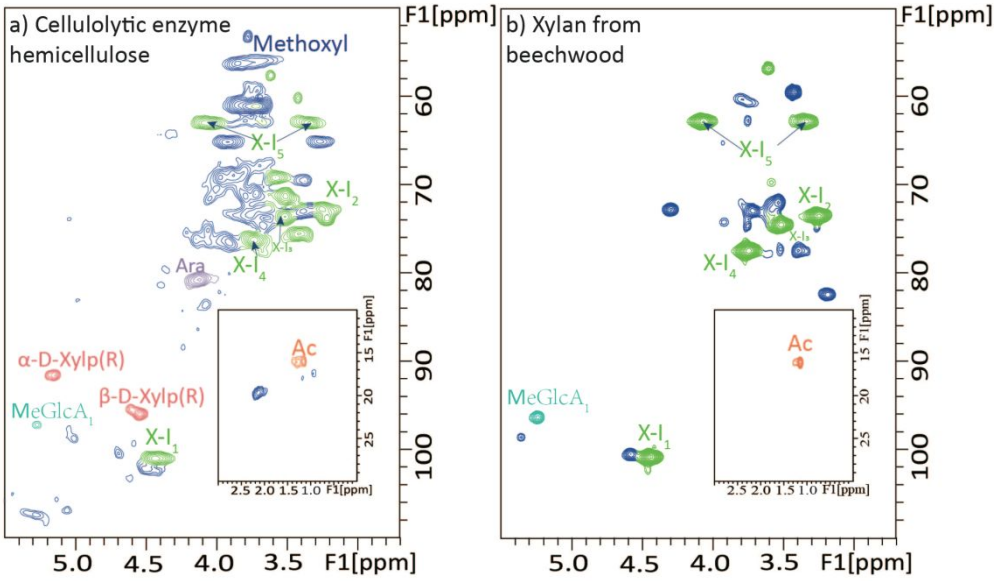

Figure 5. 2D ^{1}H-^{13}C HSQC spectra from CEH and beechwood xylan in D_2O: a) CEH, b) xylan from beechwood. Part of the alkyl region (δ_C/δ_H: 10-30/0.5-3.0 ppm) was inserted at the non-signaled area of each spectrum. X-I, xylan internal unit; Ac, Acetyl group; MeGlcA, 4-*O*-methylglucuronic acid.

Beechwood xylan, a high purity *O*-acetyl-(4-*O*-methylglucurono) xylan polysaccharide composed of 1,4-linked, *β*-D-xylopyranose in the backbone with abundant acetyl groups side-chain substitutions, was analyzed to support distinction of ^{13}C-^{1}H correlation peaks from xylan backbone.[10, 57] Similar contours of internal (1→4)-*β*-D-Xyl*p* of CEH and commercial xylan indicated that the cellulolytic enzyme method did not significantly modify the xylan structures from switchgrass. From the internal xylan units of the beechwood xylan, correlation peaks of the

internal xylan appeared at δ_C/δ_H 101.07/4.43 ppm (X-I_1), δ_C/δ_H 62.38/4.08 ppm (X-I_{5eq}) and at δ_C/δ_H 62.38/3.35ppm (X-I_{5ax}).[39]

For further analysis, xylose and glucose structures were also analyzed by the same NMR analysis method as CEH (**Figure S1**). The sugar NMR data were compared with the correlation peaks signals of CEH. Two reducing-end peaks (both α and β isomers) of CEH and xylose shared the same chemical shifts at δ_C/δ_H 91.53/5.20 ppm ($R_{\alpha1}$) and δ_C/δ_H 96.16/4.59 ppm ($R_{\beta1}$), respectively[39]. A few weak signals from monosaccharide residues in CEH appeared in the NMR spectra. They are possibly from residues of hemicellulose branches, supporting the possibility that the CEH has remained more branches than AEH and DMSOH.

CONCLUSIONS

Understanding of native hemicellulose characteristics is important in biomass utilization. In this study, a novel hemicellulose extraction method using cellulolytic enzyme and peracetic acid was developed to retain the intact structures of hemicellulose, in particular, the information of branch structures with uronic acids. The cellulolytic enzyme-aided hemicellulose isolation led to 47.7% hemicellulose yield (based on the hemicellulose content of raw switchgrass) and retained all essential functional groups and the substituted components on xylan backbone from switchgrass compared to other chemical methods using alkaline or DMSO. Chemical composition and 2D HSQC NMR results showed that there was a high proportion of GlcA and MeGlcA in the CEH. NMR analysis also revealed that alkaline isolation method resulted in breaking of the *O*-acetyl groups and DMSO extraction led to cleavage of methoxyl groups of hemicellulose, while

cellulolytic enzyme-aided method retained both methoxyl group and *O*-acetyl groups in the CEH. The cellulolytic enzyme-aided hemicellulose isolation method resulted in comparable molecular weights and polydispersity index with other isolated hemicelluloses. This proposed method enables isolation of hemicellulose with its essential and intact structures of hemicellulose, thus help to provide structural insights on the recalcitrance of hemicellulose and wall polymers interaction related to cell wall network construction. This study can also be applied in understanding the effects of pretreatments on biomass with hemicellulose as well as in the development of plant cell wall engineering for reducing recalcitrance.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Table S1. Composition analysis of raw switchgrass, hemicellulose samples, and residues.

Table S2. Assignment of ^{13}C-^{1}H HSQC NMR spectra of hemicelluloses from switchgrass biomass.

Table S3. The main functional groups assignment of hemicellulose from switchgrass in FTIR spectra.

Figure S1. Assignments for the HSQC spectra of WCW and isolated holocellulose from switchgrass.

Figure S2. 2D ^{1}H-^{13}C HSQC spectra of CEH and other saccharide models in D2O. a) CEH, b) xylose, c) glucose

Figure S3. FTIR spectra of alkaline extracted CEH, DMSOH, and AEH.

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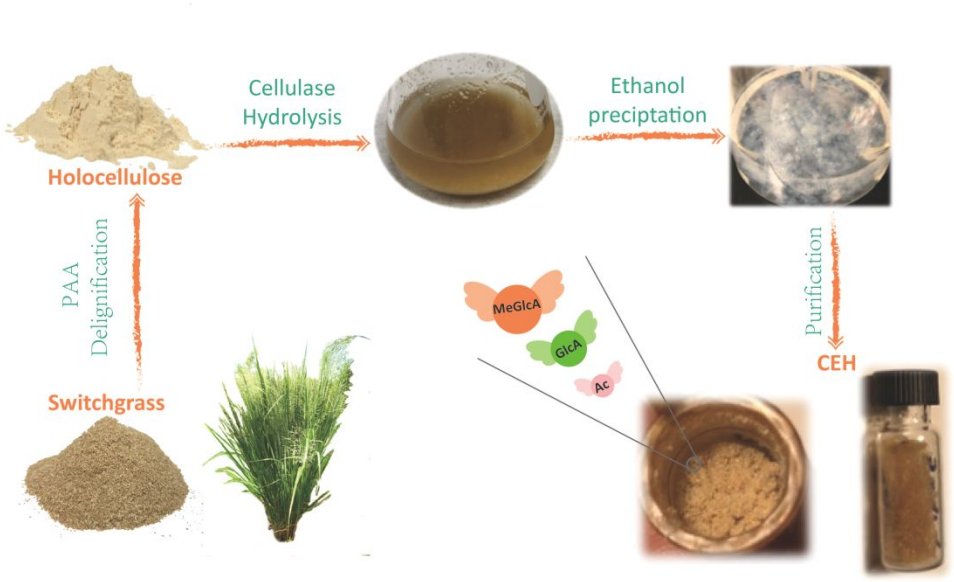
GRAPHICAL ABSTRACT



To further increase the potential applications of hemicellulose, an eco-friendly promising protocol was introduced to isolate hemicellulose from switchgrass.