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Characteristics of Lignin Fractions from Dilute Acid Pretreated Switchgrass and Their Effect on Cellobiohydrolase from *Trichoderma longibrachiatum*

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To investigate the interactions between acid pretreated switchgrass lignin and cellobiohydrolase (CBH), three different lignin fractions were isolated from dilute acid pretreated switchgrass by (i) ethanol extraction, followed by (ii) dioxane/H_2O extraction, and (iii) cellulase treatment, respectively. Structural properties of each lignin fraction were elucidated by GPC, ^{13}C-NMR, and 2D-HSQC NMR analyses. The adsorptions of CBH to the isolated lignin fractions were also studied by Langmuir adsorption isotherms. Ethanol-extractable lignin fraction, mainly composed of syringyl (S) and guaiacyl (G) units, had the lowest molecular weight, while dioxane/H_2O-extracted lignin fraction had the lowest S/G ratio with higher content of *p*-coumaric acid (*p*CA) unit. The residual lignin fraction after enzymatic treatment had the highest S/G ratio without hydroxyphenyl (H) unit. Strong associations were found between lignin properties such as lignin composition and S/G ratio and its non-productive enzyme adsorption factors including the maximum adsorption capacity and binding strength.

Keywords: characterization, lignin, dilute acid pretreatment, switchgrass, cellobiohydrolase

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INTRODUCTION

Rapid population growth and energy security concerns associated with fossil fuels have led to the development in alternative energy resources like biofuels (Cao et al., 2012). Second-generation biofuel produced from energy crops, agricultural, and forestry residues can avoid competition with food resources, thus it is a promising alternative fuel technology (Yan et al., 2010). Switchgrass has been considered as a potential feedstock for bioethanol production due to its high net energy content, low production costs, low nutrient necessity, high water utilization efficiency, and high pests and diseases tolerance (Samuel et al., 2010).

Pretreatment is an essential step for effective biological conversion of biomass to bioethanol. Diverse pretreatment technologies have been introduced to reduce biomass recalcitrance (Li et al., 2014). Among various pretreatment methods, dilute acid pretreatment has been widely investigated and applied, which typically employing a low concentration of H_2SO_4 (<4%) at moderate

temperatures (140–220°C) (Galbe and Zacchi, 2007; Yao et al., 2010). Compared to other pretreatment methods, by now, this pretreatment method is close to practical application in industrial production due to low chemical cost and utilization with diverse biomass feedstocks including hardwood and agricultural residues (Benjamin et al., 2013; Mesa et al., 2017). Typically, pretreated biomass is then subjected to enzymatic hydrolysis to produce glucose *via* a mixture of enzymes such as endoglucanase (EG), exoglucanase or cellobiohydrolases (CBHs), and β-glucosidase (Abraham et al., 2014). CBHs are a group of cellulases that can hydrolyze glycosidic linkages at a crystalline surface of cellulose (Igarashi et al., 2009). In particular, CBH I (TrCel7A) is one of the most abundant enzymes secreted by *Trichoderma reesei*. It was reported that Cel7A showed higher hydrolytic power than other *T. reesei* enzymes involving cellulose hydrolysis (Yan et al., 2011). In recent years, researches have focused on CBH binding to lignin. Palonen et al. found that CBH I exhibited a higher adsorption affinity to lignin than EG II from *T. reesei* (Palonen et al., 2004). Strong bindings of *Trichoderma reesei* CBH-I and EG-I to both cellulose and lignin were observed using quartz crystal microgravimetry (Martín et al., 2013). Other studies reported that the cellulose binding domain of the effluent enzyme played a significant role in the unspecific binding of cellulases to lignin (Palonen et al., 2004; Rahikainen et al., 2013; Strobel et al., 2015, 2016). *T. longibrachiatum*, which are taxonomically separable from *T. reesei*, could also act as a potential cellulase candidate with high activities (Kubicek et al., 1996).

Lignin is the most abundant non-carbohydrate component in plant cell walls and is considered a leading contributor involved in biomass recalcitrance. Lignin is typically derived from three hydroxycinnamyl alcohols (monolignols) that include *p*-coumaryl, coniferyl, and sinapyl alcohols which are connected *via* various types of linkages including aryl ether and carbon–carbon bonds (Ralph et al., 2004; Ragauskas et al., 2014). It is widely accepted that lignin inhibits enzymatic hydrolysis of lignocellulosic biomass (Zeng et al., 2014; Lu et al., 2016; Saini et al., 2016; Yang et al., 2016). The adsorption of enzymes onto isolated lignins from dilute acid or hot water pretreated biomass has been studied (Zheng et al., 2013; Yu et al., 2014; Li et al., 2016; Lu et al., 2016; Sun et al., 2016). They reported key lignin-enzyme binding effects including the effects of condensed phenolic OH groups, methoxy groups, and the degree of lignin condensation on cellulase binding.

The difficulty in elucidating the mechanism of cellulase adsorption to lignin is partly due to the structural complexity of lignin. Thus, a method to fractionate lignin and get lignin fractions with distinctive structure is needed. Lignin fractionation using different hydrogen-bonding capacity organic solvents was introduced to elucidate structural properties of lignin (Mörck et al., 1986, 1988; Yuan et al., 2009). In order to fractionate lignin with various properties that might affect its adsorption capabilities to enzymes, in this study, three different lignin fractions were separated from the dilute acid pretreated switchgrass using ethanol extraction (Fraction NO.1 lignin), followed by 96% dioxane extraction (Fraction NO.2 lignin), and then overloading with cellulase to recover lignin residue (Fraction NO.3 lignin). Characteristics of each lignin fraction were analyzed by gel permeation chromatography (GPC), ^{13}C-nuclear magnetic resonance (NMR) and 2D-heteronuclear single quantum coherence (HSQC) NMR. Non-productive enzyme adsorption factors of lignin were evaluated by measuring the adsorption of CBHs to lignin samples. The correlations between physicochemical characteristics of lignin and lignin-enzyme adsorption parameters were also discussed.

EXPERIMENTAL

Materials

Samples of the lowland cultivar Alamo switchgrass (*Panicum virgatum*), which were grown in 2011 and harvested in 2012 were provided by the Samuel Roberts Noble Foundation in Ardmore, OK. The chemical reagents were purchased from Fisher Scientific (USA) and used as received without further purification unless otherwise specified. Protease (Cat. No. 10165921001) was purchased from Sigma Chemical Company (USA), and CBH (Cat. No. 37329-65-0) was purchased from Megazyme (Ireland).

Dilute Acid Pretreatment of Switchgrass

The biomass sample was Wiley-milled through a 2 mm screen and then extracted by toluene/ethanol (2:1, *v/v*) for 8 h, followed by water and acetone for 3 h per each extraction to remove extractives (Hao et al., 2017). The extractive-free material was loaded to a 1 L 4560 Parr reactor (Parr Instrument Company, Moline, IL, USA) with 1% sulfuric acid (*v/v*) solution at a 10% solid loading (*w/w*). The pretreatment temperature was set at 150 ± 2°C and kept for 10 min (±0.5 min), which was the optimum dilute-acid pretreatment condition for switchgrass in the previous study (Zhou et al., 2012). The stirring speed was 2.5 Hz and the heating rate was 3°C/min. To stop the pretreatment process, the Parr reactor was quenched in an ice water bath. The pretreated switchgrass was seperated and washed with deionized water until the effluent pH was neutral, then the biomass was air-dried overnight. The yield for pretreatment was 43% by mass of the initial substrate.

Fractionation of Lignin from Pretreated Switchgrass

Lignin fractions were separated from dilute acid pretreated switchgrass as shown in **Figure 1**. The detailed method was the same as described in a previous study (Yao et al., 2017). In brief, the pretreated switchgrass was first extracted with anhydrous ethanol (liquid:solid = 10:1) for 24 h (×2) and ethanol-solved lignin fraction was purified as NO.1 lignin. The air-dried solid residues after ethanol extraction was treated by 96% dioxane (*v/v*) to get the dioxane-water soluble lignin (NO.2 lignin). The last lignin fraction was obtained by over-loading cellulase hydrolysis of the solid residues followed by 96% dioxane (*v/v*) extraction. Each lignin fraction was purified according to the milled wood lignin method (Björkman, 1956). The relative quantity of each lignin fraction was 62.6, 35.4, and 2.0%.

Analysis Procedures

Chemical Composition Analysis

Lignin and carbohydrate contents were determined according to the National Renewable Energy Laboratory procedure (Sluiter

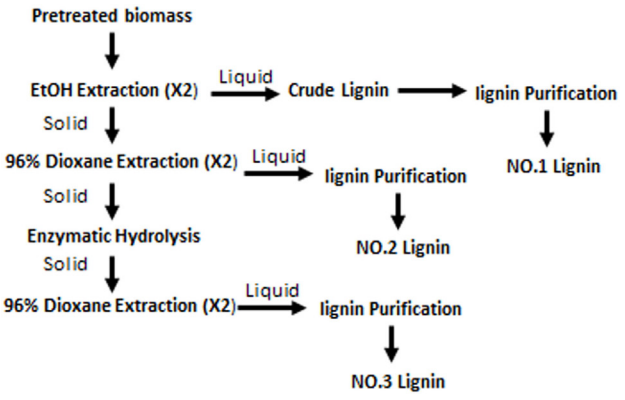

FIGURE 1 | Fractionation of lignin from dilute-acid pretreated switchgrass.

et al., 2008) and analyzed by a high-performance anion exchange chromatography (Dionex, ICS 3000, Sunnyvale, CA, USA), as described in a previous study (Meng et al., 2013).

GPC Analysis

The molecular weights of each lignin fraction was measured with GPC analysis after acetylation of lignins (Kumar et al., 2013). GPC was performed on an Agilent 1200 HPLC system (Agilent Technologies, Inc, Santa Clara, CA, USA).

NMR Analysis

About 50 mg of lignin samples were dissolved in deuterated dimethyl sulfoxide (DMSO-d_6, 0.4 mL). Two-dimensional (2D) ^{1}H–^{13}C HSQC NMR experiment was conducted at 298 K using a Bruker Advance III 400-MHz spectroscopy (5-mm BBO 400 MHz W1 with Z-gradient probe, Bruker). A Bruker standard pulse sequence ("hsqcetgpsi2") was used. Operation parameters were obtained from a previous study (Yoo et al., 2017).

^{31}P NMR spectra were obtained after derivatization of the lignin fractions by 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. Endo N-hydroxy-5-norbene-2,3-dicarboxylic acid imide was prepared as the internal standard. The spectral acquistion parameters for ^{31}P NMR spectra were as follows: a 90° pulse angle, 25-s pulse delay, and 256 transients at room temperature.

Adsorption of CBH to Lignin

Prior to conducting the adsorption test, soluble impurities in the lignin fraction was removed with acetate buffer (50 mM, pH 4.8) at 50°C for 12 h. CBH (Megazyme, Wicklow, Ireland), from *Trichoderma longibrachiatum*, was supplied at 10 mg protein/mL. The adsorption of CBH to the lignins was measured by the Langmuir isotherm. A range concentration of CBH (0.1–10.0 mg/mL) was mixed with lignin fractions (2%, *w/v*) suspended in 50 mM sodium acetate buffer (pH 4.8). The mixture was kept at 50°C with 150 rpm shaking for 4 h to reach the equilibrium. Lignin in acetate buffer (2%, *w/v*) and the same concentration of CBH were used as the controls. The protein concentration was determinated by Pierce™ bicinchoninic acid protein assay from Thermo scientific. The adsorption parameters, such as E_{max} and K_{ads} were obtained by linear regression of the adsorption data by the following Equation.

$$\frac{[E_f]}{[E]} = \frac{1}{K_{ads}[E_{max}]} + \frac{[E_f]}{[E_{max}]}$$

where $[E_f]$ (mg/mL) is the free protein concentration at equilibrium, $[E]$ (mg/mg) is the amount of protein adsorbed by lignin, K_{ads} is Langmuir adsorption constant, and $[E_{max}]$ is the maximum amount of adsorbed protein.

TABLE 1 | Weight-average (M_w), number-average (M_n) molecular weights and polydispersity indexes (M_w/M_n) of the three lignin samples.

Samples	M_n g/mol	M_w g/mol	M_w/M_n
NO.1 lignin	1,511 ± 50	2,277 ± 75	1.51 ± 0.00
NO.2 lignin	2,177 ± 71	3,551 ± 85	1.63 ± 0.01
NO.3 lignin	2,187 ± 74	3,284 ± 93	1.51 ± 0.01

TABLE 2 | Chemical composition of three lignin samples.

Samples	Acid insoluble lignin %	Glucose%	Total%
NO.1 lignin	89.35	0.20	89.55
NO.2 lignin	90.20	0.15	90.35
NO.3 lignin	90.02	0.20	90.22

RESULTS AND DISCUSSION

Molecular Weight and Chemical Composition of Lignin

Table 1 presents molecular weights of three lignin fractions isolated from dilute acid pretreated switchgrass. Molecular weights of these lignins were lower than the values of untreated switchgrass in the previous study [5,000 g/mol of weight average molecular weight (M_w) and 2,940 g/mol of number average molecular weight (M_n), respectively] (Samuel et al., 2010). It indicated that these lignin fractions extracted was depolymerized during the dilute acid pretreatment. In addition, slightly lower polydispersity indexes of the three lignin samples (1.51–1.63) were observed as compared to that of initial lignin from untreated sample (1.70), suggesting a narrower molecular weight distribution of these lignins. In specific, the weight average molecular weight of the NO.1 lignin extracted by ethanol was 2,277 g/mol, which represents the lowest molecular weight among all three fractions, while the NO.2 and NO.3 lignin fractions had higher Mw (3,551 and 3,284 g/mol, respectively).

The carbohydrate and lignin content of the three lignin fractions are presented in **Table 2**. The results showed that the acid insoluble lignin contents were 90% for all three lignin samples with small quantity of glucose. There were no significant compositional differences among the three lignin fractions.

^{13}C NMR Analysis

NMR is a well-established analytical tool to observe lignin structures (Capanema et al., 2004). To compare the characteristic

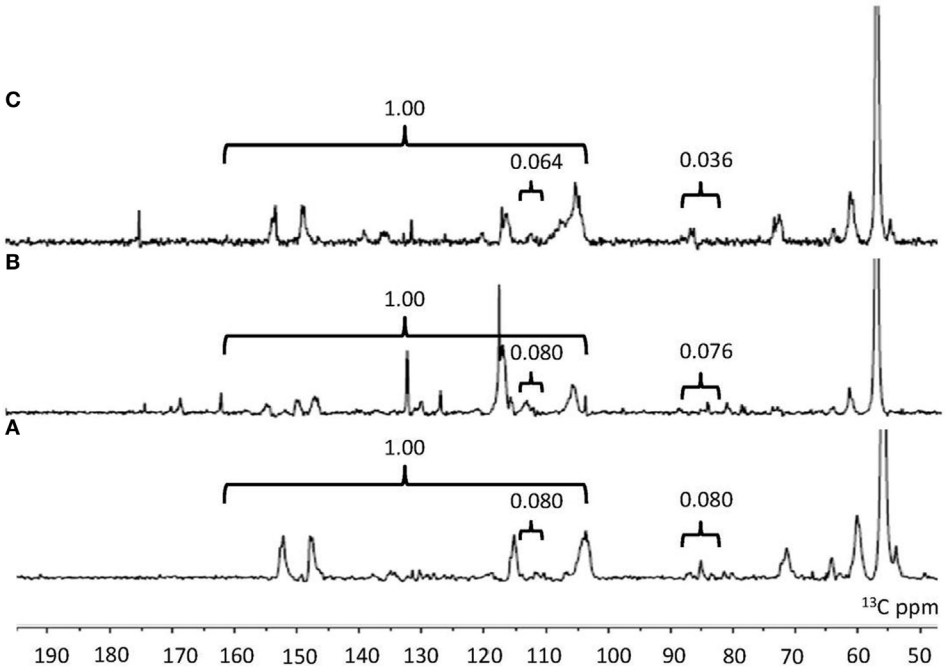

FIGURE 2 | ^{13}C-NMR spectra and integration value of lignin samples **(A)** NO.1 lignin. **(B)** NO.2 lignin. **(C)** NO.3 lignin. 160–102 ppm in the quantitative ^{13}C nuclear magnetic resonance was representative of the total carbon atoms from all aromatic rings within the lignin. 119–113 and 88–82 ppm was used as internal standard in the aromatic and aliphatic region.

of the isolated lignin samples, quantitative ^{13}C NMR was carried out. ^{13}C-NMR spectra of lignin samples are shown in **Figure 2**. The assignments of signals are listed in **Table 3**. In the aromatic region, the signals were mainly from guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H). It was facile to assign signals for these three substructures in NO.1 and NO.2 lignin at 128.9 ppm ($C_{2/6}$ in *p*-hydroxyphenyl), 115.0 ppm (C_5 in guaiacyl), and 104.0 ppm ($C_{2/6}$ in syringyl) (Yang et al., 2016). The signal at ~173.9 ppm assigned for C = O was absent in NO. 1 and 2 lignin. In the aliphatic region, the signals from $-OCH_3$ at 55.8 ppm, β-*O*-4′ at around 72.3 ppm (Cα in β-*O*-4′), 85.2 ppm (Cβ in S type β-*O*-4′) and 60.1 ppm (Cγ in β-*O*-4′), β-5 at about 86.8 ppm (Cα) and Cγ in β-β′ at ~71.7 ppm (Samuel et al., 2010; Strobel et al., 2016) were readily assigned.

TABLE 3 | Signal assignments for the ^{13}C-NMR spectra of three lignin samples from dilute acid pretreated switchgrass.

Assignments	Chemical shift (ppm)		
	NO.1 lignin	NO.2 lignin	NO.3 lignin
C = O	–	–	173.9
$C_{3/5}$ in syringyl	152.5	152.7	152.2
C_3 in guaiacyl	147.8	148.0	147.5
$C_{2/6}$ in *p*-hydroxyphenyl	128.9	128.9	–
C_6 in guaiacyl	–	111.8	119.1
C_5 in guaiacyl	115.4	115.1	115.9
C_2 in guaiacyl	111.8	111.4	–
$C_{2/6}$ in syringyl	104.1	104.1	104.0
C_α in β-5	86.8	86.8	87.1
C_β in S type β-*O*-4′	85.2	85.1	85.3
C_α in β-*O*-4′	72.3	72.3	72.2
C_γ in β-β′	71.7	71.6	71.4
C_γ in β-*O*-4′	60.1	60.0	59.7

Structural Information of Lignin Fractions using 2D-HSQC NMR Analysis

HSQC is the most applied 2D NMR techniques for the structure determination of lignin samples with enhanced spectral resolution. HSQC spectra of aromatic and aliphatic regions of each lignin fraction from dilute acid pretreated switchgrass are shown in **Figure 3**. The cross peaks were assigned according to previous studies (Samuel et al., 2010; Hu et al., 2012; Zeng et al., 2013; Yang et al., 2016).

In aromatic region, lignin subunits such as G, S, and H units were observed. The spectra indicated that the lignin fractions were mainly enriched in G and S units with small contents of H unit in NO.1 and NO.2 lignin. The ^{13}C–^{1}H correlation for $S_{2/6}$ was found at δ_C/δ_H 103.0/6.6 ppm. Cross peak from $C_{2/6}$/$H_{2/6}$ in oxidized Cα = O was shifted to δ_C/δ_H 106.0/7.3 ppm. Condensed S (δ_C/δ_H106.6/6.5 ppm) unit was observed in NO.1 and NO.3 lignin fractions, while the signal from condensed G (δ_C/δ_H113.4/6.7 ppm) was found only in ethanol-extractable lignin fraction. For relative abundance analysis of the lignin subunits in this region, the range of δ_C/δ_H 113–109/7.6–6.8 ppm was used as the internal standard (Zeng et al., 2013). The ^{13}C–^{1}H correlation for $S_{2/6}$ at δ_C/δ_H 103.0/6.6 ppm, G_2 at δ_C/δ_H 111.0/6.9 ppm, $H_{2/6}$ at δ_C/δ_H 128.0/7.2 ppm, and $pCA_{2/6}$ at δ_C/δ_H130.0/7.4 ppm were used for volume integration of the S,

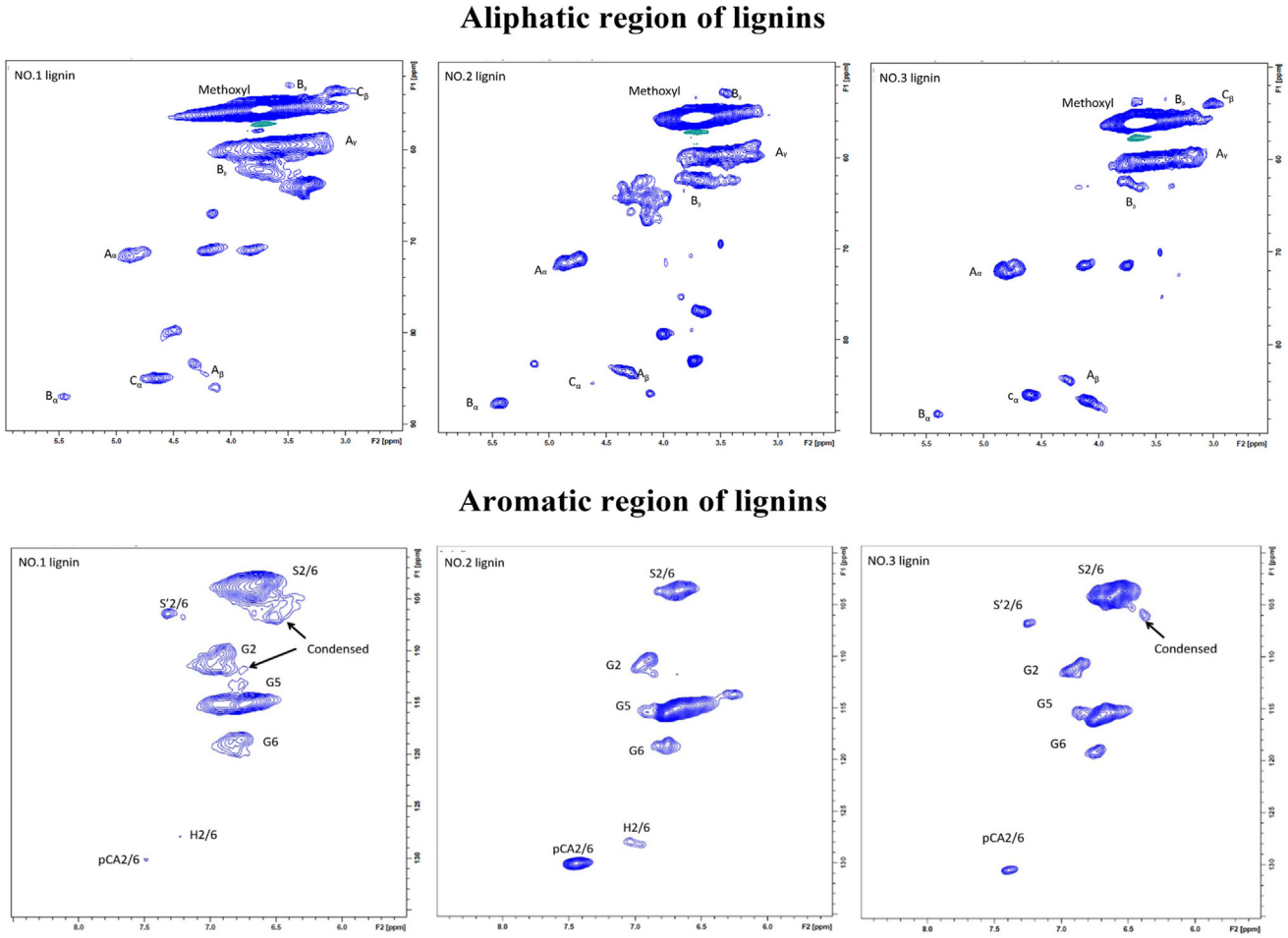

FIGURE 3 | Aromatic regions (δ_C/δ_H 160–90/8.0–5.5) and aliphatic regions (δ_C/δ_H 90–45/6.0–2.0) in heteronuclear single quantum coherence (HSQC) spectra of the isolated lignin samples from switchgrass: (A) β-*O*-4 alkyl-aryl ethers; (B) phenylcoumarane; (C) resinols; (*p*CA) *p*-coumarates; (G) guaiacyl units; (S) syringyl units; (S′) oxidized syringyl units with a Cα ketone.

G, H, and *p*-coumaric acid (*p*CA) moieties. Analysis results by a combination of ^{13}C NMR and 2D-HSQC NMR were also shown and the results were expressed as number of specific subunit per 100 aromaitc rings (Ar). As presented in **Table 4**, the relative content of S unit was the highest in NO.3 lignin (62.8/100 Ar and 74.8%) and the lowest in NO.2 lignin (22.0/100 Ar and 36.6%), while the relative content of G unit in those samples was decreased in the order of NO.1 lignin (34.6/100 Ar) > NO.2 lignin (28.2/100 Ar) > NO.3 lignin (23.1/100 Ar). The S/G ratios of the three lignin fractions were 1.4, 0.7, and 2.9 for NO.1 lignin, NO.2 lignin, and NO.3 lignin, respectively. H units were mainly found in NO.2 lignin fraction (96% dioxane extraction). Previous studies showed that *p*CA was formed *via* lignification of *p*-coumarolyated monolignols and acylated at γ-OH (Del et al., 2012; Zeng et al., 2013), which was mostly contained in NO.2 lignin (12.0/100 Ar), followed by NO.3 (3.1/100 Ar) and NO.1 (0.4/100 Ar) lignin.

The C–H correlations of methoxyl group (–OCH_3), β-*O*-4, β-5, and β-β were observed in the aliphatic region. For the quantitative analysis of the lignin subunits in this region, the range of δ_C/δ_H 88–82/5.6–3.9 ppm was used as the internal standard (Zeng et al., 2013). Signals from Cα/Hα in β-*O*-4 linkage at δ_C/δ_H 72.0/4.8 ppm, Cα/Hα in phenylcoumaran (β-5) substructure at δ_C/δ_H 87.0/5.4 ppm, Cα/Hα in resinol (β-β) substructure at δ_C/δ_H 85.0/4.6 ppm were used to represent the total linkages. The analysis results showed that β-*O*-4 was the dominant interunit linkages in the lignin fractions, which accounted for 61.7, 73.1, and 76.4% for NO.1, NO.2, and NO.3 lignin fraction, respectively. NO.2 lignin had the highest β-*O*-4 content (18.6/100 Ar), while NO.3 lignin had the lowest (8.6/100 Ar). The same trend could also be found in the content of phenylcoumaran linkages. More resinol linkages were observed than phenylcoumaran linkages in NO.1 and NO.3 lignin fractions, while phenylcoumaran linkages were more abundant than resinol in NO.2 lignin.

^{31}P-NMR Determination

For the quantitative analysis of hydroxyl group (–OH) in each lignin fraction, ^{31}P NMR analysis was conducted as described in previous study (Pu et al., 2011). Quantitative analysis results of

TABLE 4 | Nuclear magnetic resonance analysis results of three lignin samples fractionated from dilute acid pretreated switchgrass.

Lignin substructure	NO.1 lignin		NO.2 lignin		NO.3 lignin	
	Ar[a]	%[b]	Ar[a]	%[b]	Ar[a]	%[b]
S	48.5 ± 0.01	58.0 ± 0.03	22.0 ± 0.04	36.6 ± 0.02	64.8 ± 0.24	74.8 ± 0.02
G	34.6 ± 0.05	41.7 ± 0.03	28.2 ± 0.03	53.2 ± 0.04	23.1 ± 0.63	25.2 ± 0.02
H	0.3 ± 0.01	0.3 ± 0.00	6.4 ± 0.04	0.11 ± 0.01	0	0
p-coumaric acid (*p*CA)	0.4 ± 0.00	0.7 ± 0.00	12.0 ± 0.08	21.9 ± 0.01	3.1 ± 0.02	3.7 ± 0.00
S/G	1.4 ± 0.01	1.4 ± 0.08	0.8 ± 0.01	0.7 ± 0.00	2.8 ± 0.06	2.9 ± 0.07
β-*O*-4	10.4 ± 0.01	61.7 ± 0.01	18.6 ± 0.04	73.1 ± 0.04	8.6 ± 0.10	76.4 ± 0.02
β-5	1.7 ± 0.03	11.0 ± 0.01	8.3 ± 0.01	25.4 ± 0.02	1.1 ± 0.04	9.1 ± 0.02
β-β	4.5 ± 0.01	27.3 ± 0.01	0.6 ± 0.03	1.5 ± 0.01	1.7 ± 0.03	14.5 ± 0.01

[a]*Amount of specific functional group was expressed as number per 100 Ar.*

[b]*Amount of specific functional group was expressed as percentage of S + G + H for S, G, H, and pCA, of total side chain for* β-*O*-4, β-5, *and* β-β.

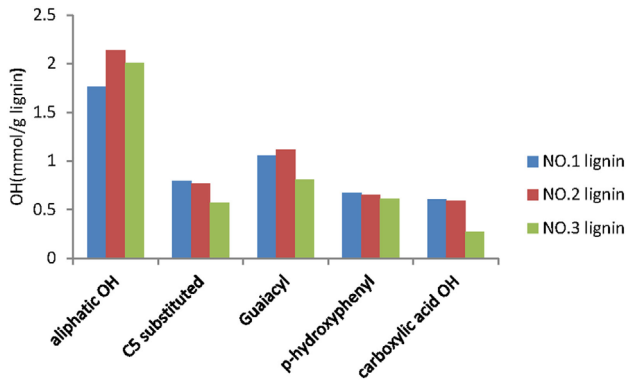

FIGURE 4 | ^{31}P-NMR spectra analysis results of lignin fractions from dilute acid pretreated switchgrass.

OH groups in the lignin fractions were presented in **Figure 4**. The results clearly showed that the aliphatic hydroxyl group was a major hydroxyl group in the three lignin fractions. This was in accordance with the previously reported results, which stated that the dominant hydroxyl groups from lignin of switchgrass after acidic pretreatment were at aliphatic site (Samuel et al., 2010). The NO.2 lignin fraction has the most aliphatic OH content (2.14 mmol/g) and the least C5 substituted OH (0.57 mmol/g), while the NO.1 fraction has the least aliphatic OH content (1.76 mmol/g) and the most C5 substituted OH content (0.79 mmol/g). The contents of other groups from the three lignin fractions were similar, except that the carboxylic acid OH in the NO.3 lignin was only half of that in the other two lignin fractions. Similar results have been previously reported, which indicated that lignin fraction with high molecular weight contains high aliphatic OH and low phenolic OH (Sadeghifar et al., 2017).

CBH Adsorption to Lignins by Langmuir Equation

It was reported that binding ability of lignin to enzymes can represent the binding capacity of lignin in the biomass (Sun et al., 2016). In this study, the binding ability between the isolated lignin fractions and CBH was measured by determining the Langmuir adsorption isotherms of CBH onto the isolated lignins. The results are shown in **Table 5**. The adsorption data fitted well with the Langmuir isotherm. The maximum adsorptions of CBH were 7.63, 5.53, and 12.32 mg/g for the three lignin fractions, which were similar to the literature (Pareek et al., 2013; Zheng et al., 2013; Machado et al., 2015; Lu et al., 2016; Sun et al., 2016). It should be noted that the binding results could be affected by the different lignin isolation/pretreatment conditions, enzyme and test conditions (temperatures, lignin loading) used for determination. The highest binding strength was observed for NO.3 lignin (60.61 ml/g), followed by NO.1 (31.13 ml/g) lignin and NO.2 lignin (18.47 ml/g). The variations in the adsorption results for the lignin faction samples might be due to the different structural characteristics.

TABLE 5 | Langmuir adsorption isotherm parameters from CBH adsorption to lignins.

	E_{max} mg/g	K_{ads} ml/mg	Binding strength ml/g lignin	R^2
NO.1 lignin	7.63	4.08	31.13	0.94
NO.2 lignin	5.53	3.34	18.47	0.99
NO.3 lignin	12.32	4.92	60.61	0.99

The Relationship between Lignin Structural Characteristic and Non-Productive Enzyme Adsorption Factors

In order to identify the responsible functional groups for the adsorption ability of different lignin samples, the content of different specific groups in lignins was correlated with the corresponding adsorption results. Positive associations were observed between the percentage of S units and the adsorption capacity ($y = 0.1493x + 2.8231$, $R^2 = 0.99$), K_{ads} ($y = 0.0509x + 1.4104$, $R^2 = 0.90$) and binding strength ($y = 1.2773x - 31.129$, $R^2 = 0.76$). Similar correlations was also found between number of S units/100 Ar and binding parameters, suggesting that higher content of S unit in isolated lignin resulted in more adsorbed CBH. In contrast, negative correlations were observed between the content of G unit and the adsorption parameters (E_{max}: $y = -0.725x + 28.745$, $R^2 = 0.92$; K_{ads}: $y = -0.056x + 6.357$, $R^2 = 0.99$; binding

strength: $y = -1.5246x + 97.77$, $R^2 = 0.98$). For the S/G ratio, we also observed positive correlations with maximum adsorption capacity ($y = 3.1233x + 3.7043$, $R^2 = 0.99$), K_{ads} ($y = 0.6931x + 2.9581$, $R^2 = 0.97$), and binding strength ($y = 19.234x + 4.6808$, $R^2 = 0.99$). Similarly, Tan and coworkers (Tan et al., 2015) observed that the milled wood lignin from bisulfite pretreated oil palm empty fruit bunch with a higher S/G ratio showed higher enzyme adsorption. In addition, negative correlations of guaiacyl OH content with maximum adsorption capacity ($y = -21.135x + 29.475$, $R^2 = 0.98$), K_{ads} ($y = -4.6209x + 8.7006$, $R^2 = 0.91$) and binding strength ($y = -131.61x + 167.39$, $R^2 = 0.99$) were found.

The effect of molecular weights and uniformity in lignin fragment size on the cellulase binding has been studied before (Guo et al., 2014; Tan et al., 2015). Among the three lignin fractions from dilute acid pretreated switchgrass, lignin with higher PDI absorbed less CBH during the binding process. It indicated that lignin with more uniform fragment size tends to have stronger binding ability on cellulase. Previous research also reported that PDI was inversely related to the interaction of the lignin with the cellulase (Berlin et al., 2006; Pareek et al., 2013).

Better understanding of the mechanism of cellulase adsorption to lignin is beneficial to the bioethanol production process. It is possible to make modification to lignin accordingly and choose proper pretreatment techniques to reduce the non-productive binding of cellulase to lignin and make the whole process economically feasible. The results indicates that lower S/G ratio in the the pretreated switchgrass lignin has lower non-productive enzyme binding, which could be favorable for enzymatic hydrolysis.

CONCLUSION

To obtain lignin with different physicochemical characteristics, three lignin fractions were extracted from diluted acid pretreated switchgrass. Each lignin fraction was found to have different structural characteristic and adsorption ability to CBH from *Trichoderma longibrachiatum*. It was found that the monolignol composition of syringyl, guaiacyl units, and S/G ratio in switchgrass lignin had correlation with the adsorption ability of lignin and binding strength of CBH. Lignin with lower S/G ratio appeared to have less effect on the CBH adsorption.

AUTHOR CONTRIBUTIONS

LY and HY performed the research, data analysis and drafted the manuscript. CY, XM, ML, and NH carried out the NMR experiments and revised the manuscript draft. YP and AR analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

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