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Inhibitory effects of lignin on enzymatic hydrolysis: The role of lignin chemistry and molecular weight

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ABSTRACT

Lignocellulose is a promising feedstock for biofuel production, while lignin poses a grand challenge on the entire process, especially enzymatic hydrolysis. In this study, different types of lignin inhibited enzymatic hydrolysis by different mechanisms. Organosolv lignin from Loblolly pine adsorbed enzyme nonproductively and reduced the available enzyme for cellulose, therefore decreasing hydrolysis rate and ultimate sugar yield. Kraft pine lignin precipitated on the surface of cellulose, preventing it from contacting with enzyme. The molecular weight influenced the inhibition effect of lignin. Lignin of lower molecular weight could bind enzyme nonproductively and when the molecular weight increased, the steric repulsion caused by lignin deposition on cellulose became more significant. The NMR analysis revealed that lignin structural features, e.g., functional groups, S/G ratio, determined the behaviors of lignin in enzymatic hydrolysis. High content of aliphatic hydroxyl groups, or low content of carboxylic groups led to high surface hydrophobicity, increasing the adsorption between lignin and enzyme. In addition, the substrate reactivity is also an important factor that affects enzymatic hydrolysis. Cellulose with higher crystallinity exhibited slower hydrolysis rate and lower conversion. When the crystallinity index increased from 0.43 to 0.72 and 0.81, the ultimate conversion decreased from 80 to 68% and 57%, respectively.

1. Introduction

Lignocellulose is a promising feedstock for biofuel production, as it is the most abundant and widely distributed biomass material in the world and would not compete with food demand when used for biofuel production [1]. In biological conversion of lignocellulosic biomass, enzymatic hydrolysis is one of the key steps to produce fermentable sugars for downstream conversion. However, pretreatment is needed prior to enzymatic hydrolysis to break down the strong cell wall, partially remove hemicellulose and/or lignin and modify the structure of biomass, making cellulose more amenable to enzyme attack [2]. As a major component of lignocellulose, lignin tends to have a significant negative influence on enzymatic hydrolysis resulting in low sugar yield. As such, high enzyme dosage is usually required to achieve high sugar yield for producing biofuel at decent concentration for recovery [3], which leads to the high production cost of biofuel due to the high cost of enzymes. Therefore, reduction of lignin's inhibition can logically enhance the cost-efficiency of biofuel produced via bioconversion pathway. Extensive studies have been conducted to explore the mechanisms of lignin-enzyme interactions in order to find ways to overcome lignin's inhibition. According to previous results, the most recognized mechanisms include nonproductive adsorption of enzyme to lignin and steric hindrance against enzyme from attaching to cellulose. It was commonly found that lignin appeared to adsorb enzyme more easily than cellulose nonproductively and irreversibly, leading to the reduction of effective enzyme [4,5]. In addition, part of solubilized lignin by pretreatment (e.g., batch dilute sulfuric acid pretreatment) could recondense during pretreatment and deposit on the surface of cellulose, blocking

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enzymatic access to cellulose [6,7]. The nonproductive adsorption and/or steric hindrance between lignin and enzyme impair the cost-efficiency of enzymatic hydrolysis, thus commercial feasibility of biofuels. However, the magnitude of different mechanisms under different conditions is not clear and inconsistent among different research.

Various factors, such as physicochemical properties of lignin and enzymes and environmental conditions (pH and temperature), can influence the interactions between lignin and enzyme. Lignin from different biomass exhibited different extent of inhibition on pure cellulose hydrolysis as well as lignocellulose hydrolysis [4,8], as different structural features would affect the behaviors of lignin when interacting with enzyme. The pretreatment and isolation process would alter lignin structure, thus influencing lignin-enzyme interaction. Lignin isolated by different methods also resulted in different effects during enzymatic hydrolysis [3,9]. Lignin molecules contain multiple functional groups on their surface, including phenolic and aliphatic hydroxyl, carboxyl, methoxyl, and etc., which renders lignin various superficial features such as hydrophobicity and surface charges. These functional groups can interact with enzyme differently and affect the process of adsorption [6,10]. And as an important physical feature of lignin, the molecular weight would affect lignin behaviors. A less inhibitory effect was observed for lignin with a lower molecular weight, and in some cases small lignin molecules can positively affect enzymatic hydrolysis [11,12]. The complexity of lignin structure and composition and the inconsistency of its effects on enzymatic hydrolysis pose challenges on the study of lignin-enzyme interaction mechanisms [13].

The goal of this paper was to study the effect of lignin structure on the lignin-enzyme interaction and the enzymatic hydrolysis of pretreated biomass. Various types of cellulosic substrates were used including pure cellulose and dilute acid pretreated switchgrass. To explore the effect of lignin source, lignin from two biomass materials (aspen and Loblolly pine) were studied and two extraction methods (organosolv and kraft extraction) were compared on their modification on lignin structure. Lignin fractions with different molecular weight derived from Kraft pine lignin (KPL) were also acquired to investigate the relation between lignin inhibition and its molecular weight. NMR analysis was conducted to provide deeper insight into lignin structural features and reveal the correlation between lignin structure and lignin-enzyme interaction. The results would provide useful information for future studies to reduce lignin inhibition on enzymatic hydrolysis and improve the cost-efficiency of the final yield of sugars to be used for downstream fermentation. For example, the results can help with pretreatment method selection and optimization, improvement of hydrolysis and even genetic modification of lignin to mitigate the recalcitrance of lignocellulosic biomass to biodegradation.

2. Materials and methods

2.1. Materials

Three types of cellulosic substrates of different crystallinity were selected including Sigmacell, α-cellulose and Avicel PH 101 with the crystallinity indexes (CrI) of 0.43, 0.72 and 0.81 respectively [14]. Sigmacell and α-cellulose were purchased from Sigma-Aldrich Co. LLC. (MO, USA), and Avicel PH 101 was purchased from Fisher Scientific (PA, USA). Six different lignin samples were used in this study including two organosolv lignins [extracted from aspen (EOL aspen) and Loblolly pine (EOL LP) by ethanol and sulfuric acid], Kraft pine lignin (KPL) and three KPL-derived fractions (i.e., KPL Fr1, KPL Fr2 and KPL Fr3). The KPL was recovered from pine black liquor by SLRP process [15] and separated into three fractions including KPL Fr1 (Mn < 1100 Da), KPL Fr2 (Mn = 1100–2700 Da) and KPL Fr3 (Mn > 2700 Da) based on molecular weight via the method of Aqueous Lignin Purification with Hot Acids (ALPHA) [16]. Therefore, we can study the type of biomass (Aspen vs. LP undergone the same pretreatment), pretreatment method (organosolv vs. alkaline on LP) and molecular weight (lignins from the same biomass and pretreatment) of lignin on affecting the enzymatic hydrolysis. Cellulase used in this study was Cellic CTec 2 which was provided by Novozyme Inc. (Franklinton, NC, USA) as a gift. The protein content and enzyme activity of the cellulase were 150 mg/mL and 119 filter paper unit (FPU)/mL, respectively. Alamo switchgrass was harvested from the farm at Pee Dee Research & Education Center, Clemson University in February 2014. It was first air-dried to moisture content (MC) below 10% and cut into small pieces followed by milling through a 40-mesh sieve with a knife mill (Thomas Wiley Mini-Mill 3383-L10, Thomas Scientific, NJ, USA).

2.2. Dilute sulfuric acid (DA) pretreatment

DA pretreatment was conducted in a 1-L Parr reactor (Model 4843, Carpenter 20 Cb-3, Parr Co. Moline, IL) equipped with impeller mixers. A working volume of 700-mL was used to allow space for liquid water expansion at high temperature during pretreatment. Switchgrass particles were pretreated at two temperatures of 140 °C and 160 °C with an agitation speed of 150 rpm for 30 min starting from the time when the mixture of switchgrass, water, and sulfuric acid in the reactor reached the desired reaction temperature. The sulfuric acid concentration was 1% (w/w, biomass dry weight) and the biomass solid loading was controlled at 10% (w/w). The pretreatment was terminated by immersing the reactor into ice water until the internal temperature decreased to below 50 °C. The reactor was opened slowly and the contents were recovered. The pretreated switchgrass slurry was thoroughly washed via vacuum filtration using deionized water until the pH of the filtrate reached about 4–5. A portion of the washed pretreated solid was stored at −20 °C for subsequent research on enzymatic hydrolysis and adsorption. The remaining solid was dried at 45 °C in an oven for chemical composition analysis.

2.3. Lignin structure analysis by nuclear magnetic resonance (NMR) spectroscopy

^{13}C-^{1}H 2D heteronuclear single quantum coherence (HSQC) NMR spectroscopic analysis which combined the sensitivity of ^{1}H NMR with the high resolution of ^{13}C NMR, was applied to quantitative evaluation of different lignin units and inter-unit linkages in samples [17]. About 20 mg of lignin was dissolved in 0.1 mL DMSO-d_6 in micro-NMR tube. NMR spectra of isolated lignin samples were acquired in a Bruker Avance III 400-MHz spectrometer and spectral processing was carried out using a Bruker Topspin 3.5 (Mac) software. A standard Bruker HSQC pulse sequence (hsqcetgpspsi2) was used on a BBFO probe with the following acquisition parameters: spectra width 10 ppm in F2 (^{1}H) dimension with 2048 time of domain (acquisition time 256.1 ms), 210 ppm in F1 (^{13}C) dimension with 256 time of domain (acquisition time 6.1 ms), a 1.5-s delay, a $^{1}J_{C-H}$ of 145 Hz, and 32 scans. The central DMSO solvent peak (δ_C/δ_H at 39.5/2.49) was used for chemical shifts calibration. Assignments of lignin compositional subunits and interunit linkage were based on reported contours in HSQC spectra [18–20].

^{31}P NMR has been used to quantitate the hydroxyl functional groups of lignins. Each lignin sample was phosphitylated with 2-chloro-4,4, 5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) in a solvent of pyridine/deuterated chloroform ($CDCl_3$) (1.6/1.0 *v*/*v*) according to published method [21,22]. In detail, 20.0 mg of lignin sample was accurately weighed into a 4-mL vial sealed with PTFE

cap. A prepared stock solution of pyridine/$CDCl_3$ (500 μL) including 1 mg/mL $Cr_{(acac)3}$ and 4 mg/mL internal standard (endo N-hydroxy-5-norbene-2,3-dicarboxylic acid imide) was added to dissolve lignin. The derivatization was performed by adding 50 μL of the phosphitylative reagent TMDP. Quantitative ^{31}P NMR spectra were acquired on a Bruker Avance 400 MHz spectrometer equipped with a BBO probe using an inverse-gated decoupling pulse sequence (Waltz-16), 90° pulse, 25-s pulse delay with 64 scans. All chemical shifts reported are relative to the product of TMDP with water, which has been observed to give a sharp signal at 132.2 ppm. The contents of hydroxyl groups were quantitated on the basis of the amount of added internal standard.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis was conducted to compare hydrolysis performance (initial rate and ultimate sugar conversion) of different types of cellulosic substrates according to the NREL procedure [23]. The cellulosic substrate loading was 20 mg/mL and the enzyme dosage was 5 FPU/g cellulose with the total reaction volume of 50 mL. The pH was maintained at 4.8 during hydrolysis using sodium citrate buffer (50 mM). To prevent contamination, sodium azide solution was added to the hydrolysis broth as an antimicrobial agent with a concentration of 0.2 mg/mL. Substrate blanks without enzyme and enzyme blanks without cellulosic substrate were run in parallel. To study the effect of lignin on enzymatic hydrolysis, isolated lignin was loaded as 2 mg/mL. All flasks were incubated in shaking incubators at 50 °C and 150 rpm up to 72 h when the increase of glucose concentration became negligible. To evaluate the hydrolysis performance and enzyme adsorption in hydrolysis process, sugar and free protein (not adsorbed by substrates) concentration were measured by withdrawing hydrolysate samples of 1 mL periodically. For sugar measurement, samples were boiled at around 100 °C for 10 min to denature the enzymes and centrifuged at 10,000 *g* for 5 min, and the supernatants were taken for HPLC sugar analysis. When free protein concentration was measured, samples underwent centrifugation only without heating and supernatants were used for protein measurement.

2.5. Enzyme adsorption

Both cellulosic substrate (Sigmacell, α-cellulose, Avicel, and DA pretreated switchgrass) and lignin were used in these enzyme adsorption experiments and the enzyme adsorption affinity and capacity of different cellulosic substrates and isolated lignins were determined and compared among substrates. The total working volume was 10 mL. All other conditions were kept same as enzymatic hydrolysis, i.e., cellulosic substrate loading of 20 mg/mL or lignin loading of 2 mg/mL, 50 °C, 150 rpm, and pH 4.8. Langmuir adsorption isotherm was used to quantitatively describe enzyme adsorption behavior (Eq. (1)).

$$\Gamma = \frac{K_L E \Gamma_{max}}{1 + K_L E} \tag{1}$$

Here, E is the free enzyme protein concentration in bulk solution (mg/mL), Γ is the adsorbed enzyme protein (mg/g substrate), Γ_{max} is the maximum adsorbed enzyme protein (mg/g substrate), and K_L is the Langmuir constant (mL/mg protein). Γ_{max} and K_L reflect the enzyme adsorption capacity and adsorption affinity, respectively and were determined by liner regression of adsorption data to the transformed Langmuir adsorption equation (Eq. (2)). In Eq. (2), the slope of $1/\Gamma_{\max}$ and the intercept of $1/(K_L\Gamma_{max})$ were first obtained through the liner regression, and the values of Γ_{max} and K_L were calculated correspondingly.

$$E/T = E/\Gamma_{max} + 1/(K_L\Gamma_{max}) \tag{2}$$

To develop adsorption isotherm, enzyme loadings were varied from 0 to 0.6 mg/mL. Based on hydrolysis experiment, the free protein concentration became stable after 24 h, indicating the adsorption equilibrium. Samples of 0.5 mL were withdrawn and centrifuged after 24 h incubation. The protein concentration in the supernatant was measured. As cellulose can be hydrolyzed during incubation, the amount of hydrolyzed cellulose was determined by measuring the released sugar in supernatant and the adsorbed protein was calculated based on the remaining substrate solid.

2.6. Analytical methods

The chemical composition of switchgrass was analyzed by following NREL procedure [24]. The protein content of enzyme was measured using Bradford Protein Assay Kit with bovine serum albumin (BSA) as a standard. Structural carbohydrates of switchgrass and released monosaccharides during enzymatic hydrolysis were determined with a high-performance liquid chromatography (HPLC) (Dionex UltiMate 3000, Thermo Scientific, CA, USA) equipped with a refractive index (RI) detector (Shodex RI-101, Showa Denko America, NY, USA) and a Shodex carbohydrate analytical column (Sugar SP0810, Showa Denko America, NY, USA). Sugars were separated at 85 °C with HPLC grade water as a mobile phase at a flow rate of 0.6 mL/min. A deashing guard column (Shodex Sugar SP-G 6B, Showa Denko America, NY, USA) was installed prior to the analytical column for preventing column from contamination. The cellulase activity was measured as FPU according to the protocol developed by Adney and Baker [25]. The cellulose conversion during enzymatic hydrolysis was calculated as:

$$\%\ \text{cellulose conversion} = \frac{mg\ sugar/ml \times 50\ mL \times 0.90}{g\ cellulose/g\ biomass \times 1.0\ g\ biomass} \times 100$$

where, 0.90 represents the hydrolysis factor specific for glucose production from cellulose.

2.7. Data analysis

Statistical significance was determined by analysis of variance (ANOVA) using JMP Pro 12 (SAS Institute, Cary, NC, USA) with $p_{critical} = 0.05$. Multiple comparisons were performed with Tukey's test with $\alpha = 0.05$. All treatments were performed in two replicates in this study unless specified, otherwise.

3. Results and discussion

3.1. Effects of dilute acid pretreatment on switchgrass composition and digestibility

The composition of raw switchgrass and DA pretreated switchgrass are shown in Table 1. For raw switchgrass, the composition is comparable to that reported in literature [26]. After pretreatment, glucan content increased significantly from 31.05% in raw material, up to 47.02% and 60.53% in the switchgrass pretreated at 140 °C and 160 °C, respectively. Hemicellulose components which include xylan, galactan, arabinan, and mannan, experienced a significant decrease from 39.18 to 23.85% and 15.56% for the pretreatment at 140 °C and 160 °C, respectively. The DA pretreatment reduced acid soluble lignin slightly, while having little effect on acid insoluble lignin content. High temperature (i.e., 160 °C) had

Table 1
Composition of raw and dilute acid pretreated switchgrass.

Switchgrass	Chemical composition (%, dry weight basis)						
	Gluan	Xylan	Galactan	Arabinan+Mannan	Acid soluble lignin	Acid insoluble lignin	Ash
Raw	31.05	26.26	7.02	5.90	1.09	26.69	6.36
140 °C	47.02	20.72	ND	3.13	0.80	26.42	0.77
160 °C	60.53	12.70	ND	2.86	0.69	26.76	1.45

ND: Not detected.

better performance on removing hemicellulose and increasing content of cellulose, compared to low temperature (i.e., 140 °C).

When compared to raw switchgrass, DA pretreatment at 160 °C resulted in remarkable improvement of enzymatic hydrolysis yield from 7 to 27% at 72 h while the enzymatic hydrolysis yield of switchgrass pretreated at 140 °C was only 12% (Fig. 1). Thus, 160 °C was selected to pretreat switchgrass for the following experiments. DA pretreatment has been known to reduce the recalcitrance and improve the digestibility of lignocellulose, mainly by solubilizing hemicellulose and a small part of lignin [27]. Jensen et al. [28] utilized sulfuric acid to pretreat switchgrass under the conditions of 0.75% acid concentration, 160 °C and 24 min, and achieved 24.4% of hydrolysis with enzyme loading of 60 FPU Spezyme CP/g glucan. To achieve high sugar yield, Li et al. [26] presoaked switchgrass in acid for 4 h prior to heating and increased the 24-hr sugar yield of pretreated switchgrass to 48%. Under milder DA pretreatment conditions (temperature = 160 °C, sulfuric acid loading = 0.5% wt and time = 20 min), the 72-hr cellulose conversion of corn stover reached approx. 80% which was much higher than that of switchgrass found in our research with the similar enzyme loading of 6 mg protein/g cellulose [29]. It was found that corn stover lignin from stem and cob residue has an average S/G ratio of 1.4, and the S/G ratio for switchgrass is 0.8 [22,30,31]. Higher S/G ratios have been correlated with less resistance to pretreatment and inhibition on enzymatic hydrolysis [32]. In addition, it appears that cellulose microfibrils in switchgrass are more condensed than those in corn stover and this condensed architecture could render increased resistance to pretreatment, thus requiring higher pretreatment severity to achieve high cellulose conversion [33]. Therefore, switchgrass seems much more recalcitrant than corn stover so that it would require higher pretreatment severity to achieve comparable enzymatic hydrolysis yield.

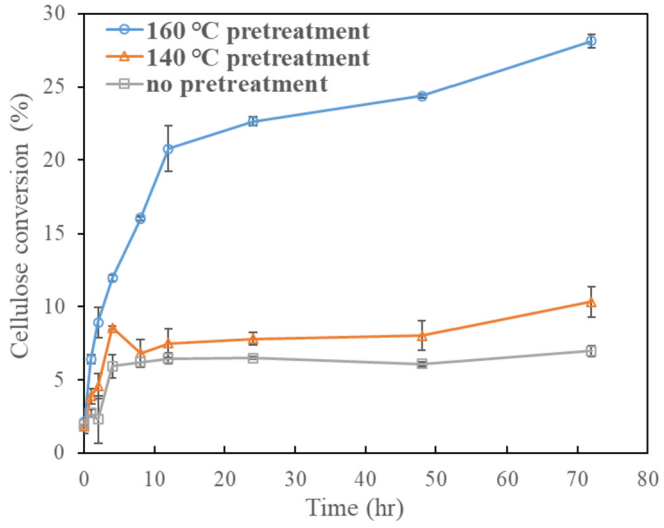

Fig. 1. Cellulose conversion of switchgrass enzymatic hydrolysis.

3.2. Compositional and structural analysis of lignin

Six different types of lignin including EOL aspen, EOL LP, KPL, KPL Fr1, KPL Fr2, and KPL Fr3 were investigated and compared in terms of their effects on enzymatic hydrolysis of pure cellulose substrate and DA pretreated switchgrass. To reveal the fundamentals behind lignin influence, we used NMR to analyze lignin compositions and substructures which determine the behaviors of lignin. It was found that lignins from different biomass or generated by different pretreatment methods presented distinct features (Figs. 2 and 3).

3.2.1. ^{13}C-^{1}H HSQC analysis of lignin structures

The aromatic spectra revealed that EOL LP, KPL and its fractions (KPL Frs1-3) featured G-type lignin, which was evidenced by the dominance of guaiacyl (G) units. EOL aspen was GS-type lignin with the presence of both guaiacyl and syringyl (S) units (Fig. 2a). In the regions of aliphatic spectra which showed the main linkages and side chains of lignin (Fig. 2b), β-*O*-4′, β-5′, and β-β′ were observed in the original KPL as well as three KPL fractions. In addition to the difference of lignin molecular weights distribution, the KPL fractions showed substantial differences on certain types of linkages revealed by 2D HSQC NMR. The relative contours intensity of β-*O*-4′ and β-5′ were significantly decreased in KPL Fr1 to Fr3 compared with KPL whereas β-β′ was slightly decreased (Fig. 2b). The total amount of identifiable linkages contained in the Fr1, Fr2 and Fr3 (presented as % of total aromatic units) were 3.27, 3.40 and 4.05%, respectively. This finding is consistent with the distribution of lignin molecular weights in the three fractions from low to high in the order of Fr1, Fr2 and Fr3 [16]. Both derived from the same organosolv pretreatment, the side chain linkages in EOL aspen and EOL LP were strikingly different. For instance, EOL aspen had about 16 times abundance of β-*O*-4′ and 3 times abundance of β-β'linkages than EOL LP whereas the abundance of β-5′ are comparable (Fig. 2b). Each type of linkage appears much more abundant in EOL aspen, especially β-*O*-4′ linkage, compared with other kinds of lignin.

As two of main subunits of lignin macromolecules, variation in ratio between S and G units is believed to affect the recalcitrance of biomass, as different types of subunits could influence the cross-linking between lignin molecules and other constituents of lignocellulose [34]. Lignin with high S/G ratio was reported to have higher proportion of labile β-*O*-4′ bonds [32], which is consistent with our analysis results. Organosolv pretreatment breaks down β-*O*-4′ linkages and ester bonds and imparts significant changes in lignin structure including increases in phenolic and methoxyl groups and decreases in the average molecular weight [35,36], which could influence the behavior of lignin in enzymatic hydrolysis.

3.2.2. ^{31}P NMR analysis of lignin functional groups

^{31}P NMR spectroscopy is an effective tool for differentiating and quantitating the different types of hydroxyl groups in lignin including aliphatic, carboxylic, guaiacyl, syringyl, C_5 substituted

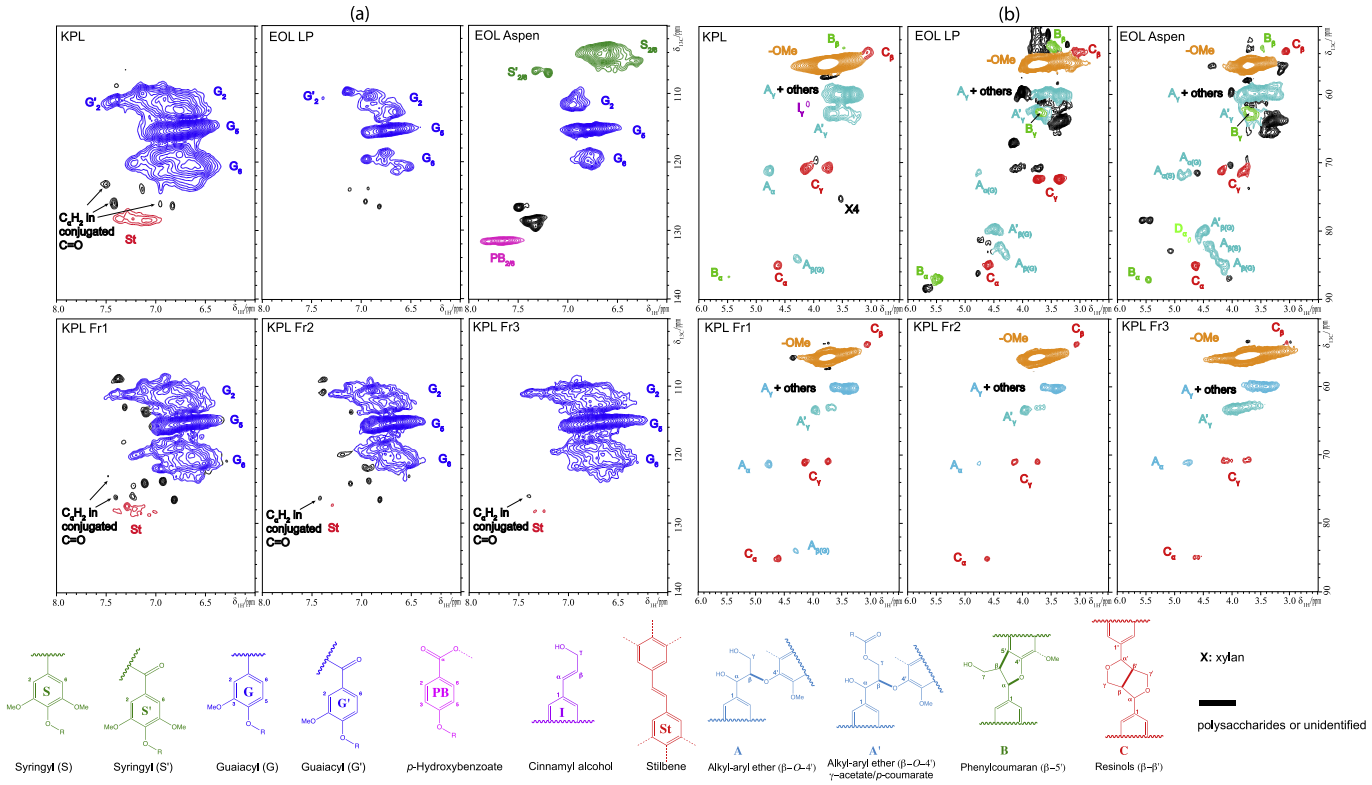

Fig. 2. ^{13}C–^{1}H spectra of aromatic (a) and aliphatic (b) regions of different types of lignin. EOL aspen-ethanol extracted lignin from aspen; EOL LP-ethanol extracted lignin from Loblolly pine; KPL-Kraft pine lignin; KPL Fr1, Fr2 and Fr3-fractions 1, 2 and 3 from Kraft pine lignin with an increasing molecular weight.

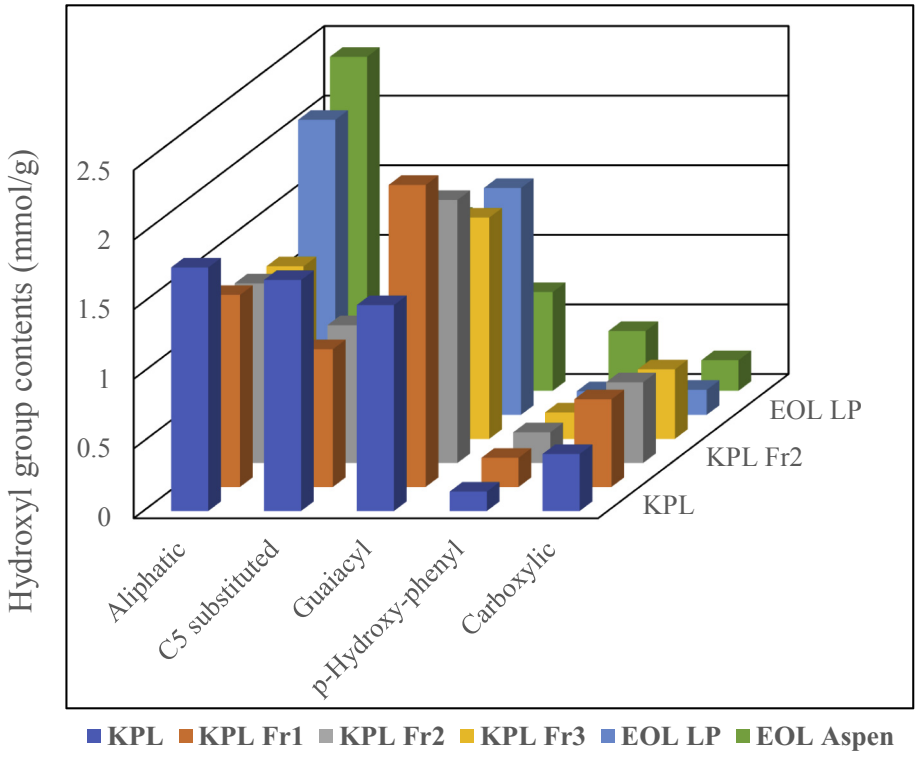

Fig. 3. Hydroxyl group contents in different types of lignin determined by ^{31}P NMR analysis. EOL aspen-ethanol extracted lignin from aspen; EOL LP-ethanol extracted lignin from Loblolly pine; KPL-Kraft pine lignin; KPL Fr1, Fr2 and Fr3-fractions 1, 2 and 3 from Kraft pine lignin with an increasing molecular weight.

phenolic hydroxyls, and *p*-hydroxyphenyls [22]. The contents of these various hydroxyl groups (OHs) play a vital role in contributing biomass recalcitrance and affecting the enzymatic hydrolysis efficiency [13]. The OHs of KPL and its fractions quantitated in our study are consistent with previous results [37]. In comparison to the original KPL sample, the fractionated lignins had slightly less amount of aliphatic OH and comparable amount of phenolic (C_5 substituted, guaiacyl, and *p*-hydroxyphenyl) and carboxylic OHs, which suggests there were.

Some structural degradation occurring on the lignin side chains during fractionation (Fig. 3). As for EOL, it was found that EOL aspen had relatively higher C_5 substitute and *p*-hydroxyphenyl and lower guaiacyl OHs compared with EOL LP. That could be explained by the specific lignin type, i.e., G type lignin for LP and GS type for aspen, and the presence of *p*-hydroxybenzoate (PB) units in EOL aspen. When comparing pine lignin extracted by two different methods, KPL had slightly less aliphatic OH and significantly more contents of four other types of OHs (C_5 substituted, guaiacyl, *p*-hydroxyphenyl, and carboxylic hydroxyls). The higher phenolic and carboxylic OHs in KPL may be partially attributed to the formation of catechol groups during Kraft pulping process [38].

3.3. *Effects of crystallinity on cellulose enzymatic hydrolysis*

Among three different pure cellulose substrates, Avicel has the highest crystallinity (CrI = 0.81) followed by α-cellulose (CrI = 0.72) and Sigmacell (CrI = 0.43) [14]. Crystallinity refers to the degree of structural order, i.e., how the cellulose fibers are organized and how loose the cellulose structure is. When cellulose is less crystalline, cellulose fibers are more randomly distributed and more biodegradable [39]. As shown in Fig. 4a, cellulose with higher crystallinity was more difficult to be hydrolyzed. From Sigmacell to Avicel, the hydrolysis rate decreased gradually, and the maximum sugar conversion at 72 h also decreased from 80.36 to 68.19% and 57.36%. Enzyme adsorbed to cellulose quickly at the beginning, and as the hydrolysis proceeded, it was released back to solution. The adsorption reached equilibrium after around 24 h, and the final enzyme adsorption percentage for all three types of cellulose were similar, i.e., approx. 75% (Fig. 4b). In addition, three cellulose substrates presented different enzyme adsorption affinity and capacity (Table 2) (The curves of Langmuir adsorption isotherm were not shown). The adsorption capacity (Γ_{max}) decreased from 133.33 (Sigmacell) to 68 (α-cellulose) and to 23.26 (Avicel) mg/g cellulose with the increase of cellulose crystallinity, which shows increasing CrI reduced the adsorption capacity of cellulose towards cellulase. However, no direct correlation between CrI and adsorption affinity (K_L) was found, i.e., Avicel had the largest adsorption affinity (K_L) (3.86 mL/mg) followed by Sigmacell and α-cellulose, which is different from the result reported by Lee et al. [40] who studied the enzyme adsorption using various types of cellulose, and found that K_L increased with the increase of CrI. Binding strength ($Q_s = \Gamma_{max} \times K_L$) (or distribution coefficient) was used to estimate the relative affinity of cellulases on substrates [41,42] and appeared to be inversely correlated to the CrI of celluloses in our research, i.e., Sigmacell had the highest binding strength towards cellulase followed by α-cellulose and Avicel (Table 2).

Table 2
Langmuir adsorption isotherm parameters for different types of substrate and lignin.

Substrates[a]	Γ_{max} (mg/g substrate)	K_L (mL/mg protein)	Q_s (mL/g)
KPL	227.27	1.69	384.09
EOL aspen	96.15	3.35	322.10
EOL LP	256.41	2.29	587.18
KPL Fr1	7.37	5.59	41.20
KPL Fr2	185.19	3.18	588.90
KPL Fr3	103.09	4.41	454.63
Sigmacell	133.33	2.37	315.99
α-cellulose	68.03	1.99	135.38
Avicel	23.26	3.86	89.78

[a] EOL aspen-ethanol extracted lignin from aspen; EOL LP-ethanol extracted lignin from Loblolly pine; KPL-Kraft pine lignin; KPL Fr1, Fr2 and Fr3-fractions 1, 2 and 3 from Kraft pine lignin with an increasing molecular weight.

The hydrolysis rate was shown to be proportional to the amount of adsorbed enzyme and crystalline cellulose often has well-organized structure and is poorly accessible to enzymes, leading to low adsorption and slow hydrolysis as a result [43]. Therefore, using cellulosic substrates with different initial crystallinity, amorphous cellulose had faster reaction and higher ultimate sugar yield than the crystalline counterpart [44,45]. However, Hall et al. [43] proposed that the increased hydrolysis rates were likely the results of an increasing substrate reactivity rather than adsorptive capacity. That is consistent with the present results that cellulosic substrates with different crystallinity presented similar enzyme adsorption profile but different saccharification performance during hydrolysis. With relatively low enzyme loading (5 FPU/g cellulose), the active sites on cellulose are enough (unsaturated) for enzymes to bind, thus substrate reactivity (e.g., CrI in this scenario) rather than adsorption capacity becomes a limiting factor for enzymatic hydrolysis rate and yield. Also, less crystalline cellulose could have more open structure, which ensured enough space for enzyme molecules to function completely without hindering each other when residing on neighboring chains [46]. Comparing different types of crystalline cellulose such as triclinic (I_α) and monoclinic (I_β) cellulose, Igarashi et al. [47] reported that the hydrolysis yield of I_α is higher than I_β, although the latter showed a 1.5 times higher maximum adsorption of Cel7A. These researchers

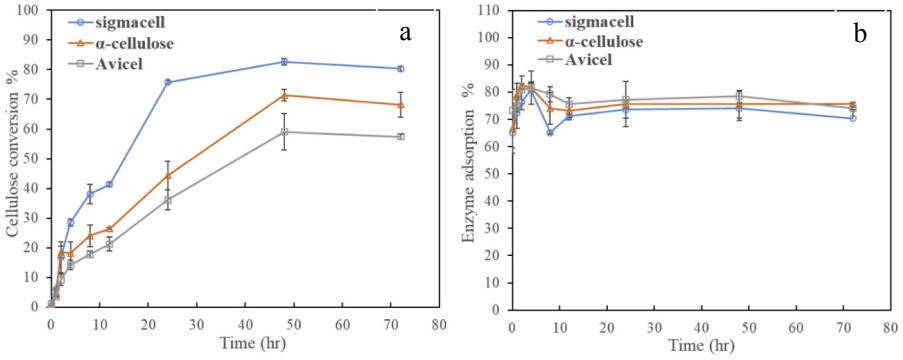

Fig. 4. Hydrolysis of different types of cellulose: (a) Cellulose conversion; (b) Enzyme adsorption.

suggested that overcrowding of enzymes on cellulose surface would reduce its activity significantly [47]. When increasing enzyme loading, the hydrolysis rate and ultimate yield would increase correspondingly, while the benefits from higher enzyme loading appeared to be different for various types of substrate [41]. For examples, high enzyme loading could improve cellulose conversion of high CrI cellulose more than low CrI cellulose since high enzyme dose can overcome the barrier of crystallinity on cellulose conversion [29]. In addition, when enzyme adsorption is monitored during enzymatic hydrolysis in the presence of lignin, high enzyme loading may cause underestimation of lignin effects on enzymatic hydrolysis because enzyme protein itself can act as an additive to reduce enzyme adsorption to lignin so that sufficient effective enzyme remained available for hydrolysis of cellulose. Gao et al. [29] found increasing enzyme loading changed the cellulose hydrolysis of DA pretreated corn stover from lower to higher than that of ionic liquid pretreated corn stover although DA pretreated corn stover contain much higher lignin content.

3.4. Effects of lignin on enzymatic hydrolysis

Three types of lignin (EOL LP, EOL aspen and KPL) were added respectively to enzymatic hydrolysis of different substrates (Sigmacell, α-cellulose, Avicel, and DA pretreated switchgrass) to evaluate the inhibition effects of lignin. Relative inhibition which was defined as the ratio of decreased cellulose conversion percentage to cellulose conversion percentage of hydrolysis without lignin at 72 h, was used to evaluate the inhibition effect of lignin [48]. It was unexpected that all three different types of lignin reduced cellulose conversion in a similar pattern and their relative inhibitions did not show significant difference. For Sigmacell, α-cellulose, and Avicel, their cellulose conversions decreased from 80 to 64%, from 68 to 45%, and from 57 to 35% in the presence of lignin (Fig. 5a–c). However, the relative inhibitions of lignin on cellulose saccharification varied on the cellulose sources (i.e., 20%, 34%, and 39% for Sigmacell, α-cellulose and Avicel, respectively). The relative inhibition caused by lignin increased upon the crystallinity of pure cellulose. It has been indicated that the recalcitrance of highly crystalline cellulose was due to both its low accessibility and strong association with lignin [49]. The maximum enzyme adsorption capacity of Avicel was significantly reduced with the addition of lignin [50]. In addition, the molecular dynamics simulations indicated that the noncrystalline regions had a lower tendency to associate with lignin compared with crystalline cellulose chains which arises from stronger hydration and higher hydrophilicity on the surface [49]. Therefore, our results suggest that a stronger adsorption between lignin and Avicel reduced their surface areas available for cellulase and inhibited saccharification to a larger degree than the Sigmacell and α-cellulose. The inhibition effect of lignin on pretreated biomass has also been observed. As switchgrass was hydrolyzed in the presence of externally added lignin, cellulose conversion at 72 h decreased from 27 to approximately 20% (Fig. 5d). Also using EOL LP, Lai et al. [6] determined that addition of EOL LP decreased the 72 hr-hydrolysis yields of organosolv pretreated sugarcane and LP from 49 to 41–42% and 38%, respectively.

The effect of lignin on enzyme adsorption were also evaluated to reveal the impacts of lignin on cellulose hydrolysis. The lignin source was found to exhibit different effects on the enzyme adsorption properties. Compared with the controls (hydrolysis without lignin), KPL resulted in reduction of enzyme adsorption for all three types of cellulosic substrates, while EOL LP enhanced the amount of adsorbed enzyme. Distinctively, the role of EOL aspen appeared to be influenced by cellulosic substrate sources. For instance, the addition of EOL aspen significantly increased enzyme

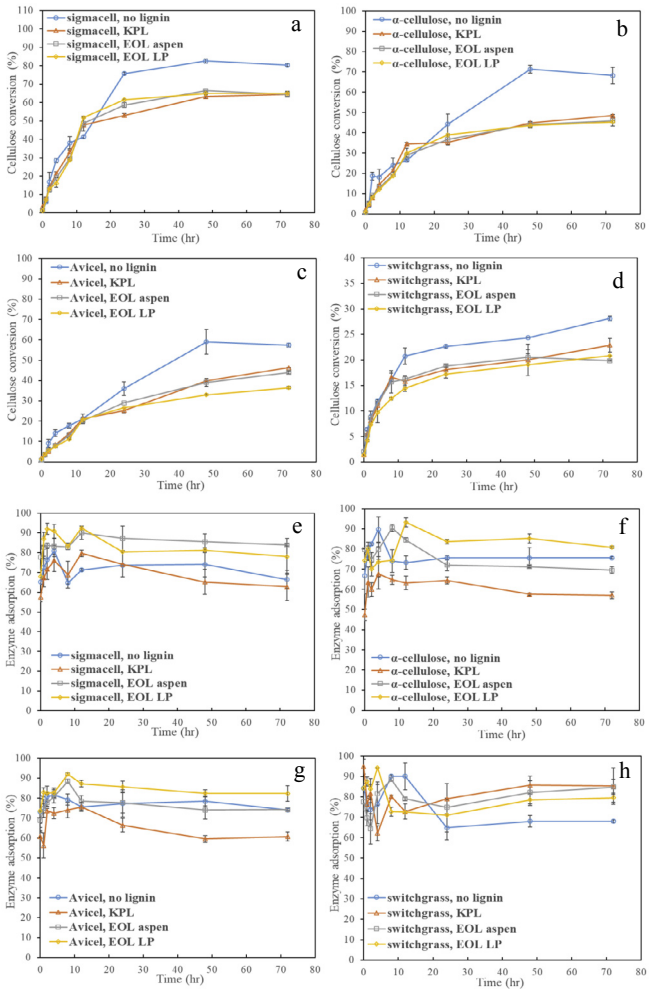

Fig. 5. Enzymatic hydrolysis (a, b, c, and d) and enzyme adsorption (e, f, g, and h) in the presence of lignin. EOL aspen-ethanol extracted lignin from aspen; EOL LP-ethanol extracted lignin from Loblolly pine; KPL-Kraft pine lignin.

adsorption for Sigmacell but decreased the adsorption for α-cellulose. However, no obvious effect of EOL aspen was observed for Avicel (Fig. 5e–g). During switchgrass hydrolysis, all three types of lignin increased enzyme adsorption, from 65 to 78% for EOL LP and 82% for KPL and EOL aspen. When comparing enzyme adsorption capacity and affinity of different lignin samples (Table 2), EOL LP showed the highest Γ_{max} of 256.41 mg/g, followed by KPL (227.27 mg/g) and EOL aspen (96.15 mg/g). The value of Q_s for EOL LP was the highest (587.18 mL/g), followed by KPL (384.09 mL/g) and EOL aspen (322.10 mL/g), indicating the strongest adsorption affinity between EOL LP and enzyme. That is because EOL LP has relatively high content of guaiacyl and aliphatic OH which has been proven to adsorb enzyme easily [4]. In addition, the lower carboxylic groups in EOL LP leads to more relatively higher hydrophobicity and less repulsive charges in lignin. The high adsorption capacity and affinity of KPL could be possibly explained by its high content of phenolic OHs, low content of carboxylic groups, and consequently high surface hydrophobicity [7].

Lignin can inhibit enzymatic hydrolysis via various mechanisms such as nonproductive adsorption and steric hindrance. The relative importance of mechanisms varies depending on the compositional and structural characteristics of lignin derived from different raw and/or pretreated biomass materials [51]. During enzymatic hydrolysis, EOL LP was anticipated to increase enzyme

adsorption, which could indicate that EOL LP inhibits enzymatic hydrolysis mainly by non-productive adsorption of enzyme. EOL LP outcompeted substrate for enzyme due to its strong binding with enzyme and reduced the effective concentration of enzyme, resulting in decreased hydrolysis rate and final yield. However, KPL and EOL aspen mostly reduced enzyme adsorption during hydrolysis of pure cellulose and steric hindrance seemed to be the main mechanism for its inhibition on enzymatic hydrolysis. KPL and organosolv lignins were not soluble in the hydrolysis mixture at pH 4.8, and would precipitate on the cellulose surface, which is similar to that occurred during washing of Kraft or organosolv pulps, i.e. washed Kraft or organosolv fiber surfaces were covered by an adsorbed layer of reprecipitated lignin [8,52]. The lignin layer on cellulose surface prevented enzyme from being adsorbed onto cellulose, decreased the contact between celluloses and enzyme, and ultimately lowered the hydrolysis yield.

In addition, these inhibitory effects were complicated by the substrate properties. For cellulose with higher crystallinity, tight and highly organized structure led to less surface area available, which made the steric hindrance caused by lignin deposition more significant on blocking enzyme adsorption to cellulose. Especially, EOL aspen increased enzyme adsorption remarkably with Sigmacell as substrate. The low crystallinity of Sigmacell possibly made it difficult to associate with EOL aspen (with a high content of S unit), which highlighted nonproductive adsorption as the main mechanism. This hypothesis still needs further investigation. During hydrolysis of DA pretreated switchgrass it became more complicated as the lignin effects were comprised by the properties of other biopolymers remained in the biomass. Enzyme adsorption was increased with the addition of all lignins, indicating the main interaction in system was lignin-enzyme interaction. Since DA pretreatment can only remove lignin slightly, most lignin still remained in pretreated switchgrass and may partially cover the surface of cellulose. The externally added lignin could not contact cellulose as much as when pure cellulose was used as a substrate, making steric hindrance minimal. Thus, nonproductive adsorption of enzymes to lignin became the dominant mechanism, resulting in the increase of enzyme adsorption in general.

3.5. Effect of lignin molecular weight on enzymatic hydrolysis

Three fractions of KPL with low (KPL Fr1), medium (KPL Fr2) and high (KPL Fr3) molecular weight, were added to cellulose hydrolysis respectively, to explore how is the influential performance of the lignin molecular weight. KPL Fr1 had the least inhibition effect, with relative inhibition of 7%, 14%, and 17% for Sigmacell, α-cellulose, and Avicel, respectively, while there was no significant difference between KPL Fr2 and KPL Fr3, especially for Sigmacell and Avicel (Fig. 6a–c). Unlike the lignin inhibition on pure cellulose hydrolysis, the lignin molecular weight showed insignificant effect on its inhibition on switchgrass hydrolysis (Fig. 6d), i.e., the cellulose conversion at 72 h decreased from 27 to 23% with the relative inhibition of 15% in the presence of any of the KPL fractions.

When KPL Fr1 was added to enzymatic hydrolysis, enzyme adsorption percentage was reduced significantly, from approximately 75% (pure cellulose) and 60% (pretreated switchgrass) to 50% (Fig. 6e–h). However, still no significant difference was observed between KPL Fr2 and KPL Fr3. Both of them had negligible effect on enzyme adsorption during enzymatic hydrolysis of pure cellulose, while slightly increased enzyme adsorption from 70 to 78% for pretreated switchgrass (Fig. 6e–h). When the enzyme adsorption characteristics were evaluated on KPL Frs 1–3, KPL Fr1 exhibited the lowest adsorption capacility and affinity, with the Γ_{max} of only 7.37 mg/g and Q_S of 41.20 mL/g (Table 2). It was also found that lignin molecular weight was a critical factor affecting enzyme adsorption affinity, but there was no direct correlation between lignin molecular weight and its enzyme adsorption affinity. Li et al. [53] determined that alkali lignin with higher molecular weight showed stronger adsorption capacity and affinity to enzyme. In the present study, however, KPL Fr2 which had the medium molecular weight exhibited the maximum *Qs* (588.90 mL/g). Further research is needed to explore the mechanisms behind this phenomenon.

Molecular weight appears to be a factor that influences how lignin interacts with cellulase during enzymatic hydrolysis. The NMR results showed that all three fractions of KPL had relatively high content of carboxylic OH. After dissociation, carboxylic OHs could make lignin molecules negatively charged. Most kinds of cellulase own pI value less than 5 (common pH for the enzymatic hydrolysis experiment) and also negatively charged under hydrolysis conditions. As a result, higher content of carboxylic OHs in lignin can lead to more repulsion force between lignin and enzyme and reduce enzyme adsorption [54]. According to Pareek et al. [7], carboxylic OHs can decrease hydrophobicity, which in turn negatively affects the nonproductive binding of cellulase to lignin. Nakagame et al. [10] also showed that the hydrolysis yield of Avicel exhibited a positive correlation with the carboxylic OH content of lignin. This could explain why the three fractions of KPL with relatively high amount of carboxylic OHs barely caused significant enzyme adsorption increase when they were added to the hydrolysis of both pure cellulose and pretreated switchgrass. In addition, the contents of total phenolic OHs (C_5 substituted, guaiacyl and *p*-hydroxyphenyl), which has been suggested to have negative effect on enzymatic hydrolysis, were also high for all fractions. Phenolic groups were believed to be able to deactivate enzyme by reversible or irreversible complexing according to the results that external addition of phenolic compounds (model lignin compounds) increased the inhibition and/or deactivation of enzymes [55]. Pan [56] also found that chemical blocking of phenolic OHs by hydroxypropylation can eliminate the lignin inhibition effects completely.

Among three fractions, KPL Fr1 reduced enzyme adsorption significantly and exhibited extremely low adsorption capability and affinity towards enzyme. Considering its solubility and low molecular weight, KPL Fr1 could combine with enzyme but the complexes remained in aqueous phase, leading to a relatively high protein concentration in supernatant. The formation of lignin-enzyme complex only resulted in partial loss of enzyme activity (10% reduction of enzyme activity shown by our measurement), which possibly explained its limited inhibition on pure cellulose hydrolysis. KPL Fr2 and KPL Fr3 did not have significant difference from each other on the effect of enzymatic hydrolysis and adsorption. Lignin can inhibit enzymatic hydrolysis by various mechanisms, including inhibitory binding (e.g., competitive, noncompetitive or uncompetitive inhibition) by small molecules, nonproductive adsorption and/or steric repulsion by bulk part. With higher molecular weight, KPL Fr3 may not access the catalytic tunnel in cellulase or complex with it effectively [10], while the steric repulsion caused by lignin precipitation on cellulose surface could be more pronounced. The combined action of both effects possibly leads to insignificant difference between KPL Fr2 and KPL Fr3.

4. Conclusions

Crystallinity exhibited a negative impact on enzymatic hydrolysis of pure cellulose, as cellulose with higher crystallinity index showed lower conversion during enzymatic hydrolysis. Compared with adsorption capacity, substrate reactivity appears to be a more important factor affecting enzymatic hydrolysis. For different types

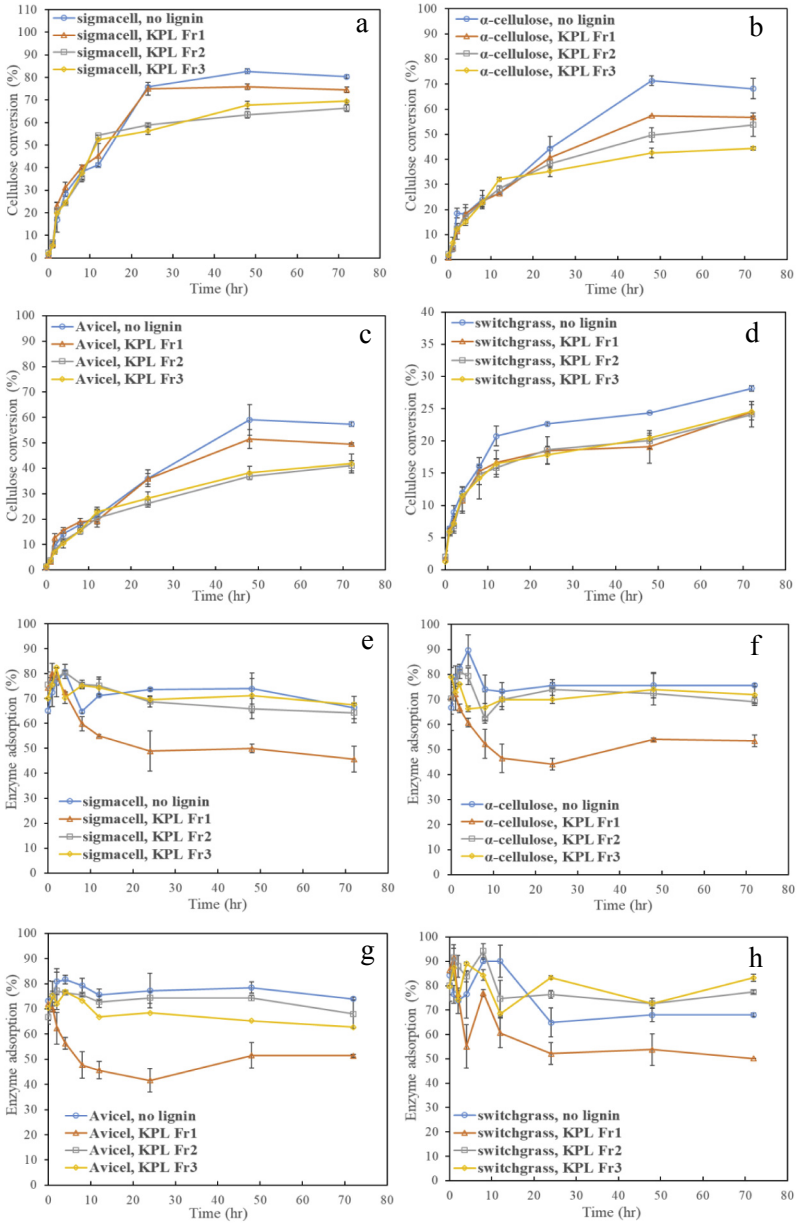

Fig. 6. Enzymatic hydrolysis (a, b, c, and d) and enzyme adsorption (e, f, g, and h) in the presence of KPLs with different molecular weight. KPL Fr1, Fr2, and Fr3-fraction 1, 2, 3 from Kraft pine lignin with an increasing molecular weight.

of lignin used in this study, both EOL and KPL inhibited cellulose hydrolysis, and their inhibition behavior was influenced by lignin features (e.g., source, composition, functional groups, and molecular weight) as well as cellulosic substrates. EOL LP caused inhibition on enzymatic hydrolysis of various pure cellulose mainly by nonproductive binding, supported by its high adsorption capacity and affinity to enzyme. KPL mainly precipitated on the surface of cellulose and negatively affected hydrolysis by steric repulsion, which limited the productive contact between cellulose and enzyme. EOL aspen affected enzymatic hydrolysis differently when different types of substrate were used. In contrast to pure cellulose hydrolysis, all three types of lignin (EOL aspen, EOL LP and KPL) inhibited the hydrolysis of DA pretreated switchgrass mainly by nonproductive adsorption of enzymes. The results on the effect of lignin molecular weight showed that lignin of lower molecular weight presented less inhibition on hydrolysis.

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Abbreviations

CrI	crystallinity index
DA	dilute sulfuric acid pretreatment
EOL aspen	lignin extracted from aspen by ethanol and sulfuric acid

EOL LP lignin extracted from Loblolly pine by ethanol and sulfuric acid
KPL Kraft pine lignin
KPL Fr1, Fr2 and Fr3 fractions 1, 2 and 3 of Kraft pine lignin based on molecular weight

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