To assess the possible effects of lignin on enzymatic hydrolysis, the structural characteristics of milled wood lignin (MWL) isolated from reed straw and corn stover pretreated with liquid hot water (LHW) at different severities were investigated. The changes in the chemical structure of the MWL were characterized by gel permeation chromatography, elemental analysis, differential scanning calorimetry, thermogravimetric analysis, Fourier transform infrared spectroscopy, and \(^1\)H and \(^{13}\)C nuclear magnetic resonance. The results showed that the MWL isolated from reed straw and corn stover pretreated with LHW was more condensed. The non-conjugated and conjugated ketone groups, as well as the ether bonds of the MWL, disappeared after LHW pretreatment, while the phenolic hydroxyl and methoxy groups increased. The carboxyl groups of MWL from reed straw increased after LHW pretreatment, whereas the carboxyl groups of MWL from corn stover decreased. When MWL was added into the enzymatic hydrolysis system, the filter paper activity of cellulase, the protein content, and the conversion of cellulose to glucose all decreased.

Keywords: Milled wood lignin; Liquid hot water pretreatment; Enzymatic hydrolysis; Reed straw; Corn stover

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INTRODUCTION

Bioethanol obtained from lignocellulosic biomass is considered to be a potential alternative energy source that could reduce the current reliance on fossil fuels (Romaní et al. 2012; Tan et al. 2015). A typical process for converting lignocellulosic biomass into bioethanol generally involves three steps: pretreatment, enzymatic hydrolysis, and fermentation. The key step that enables bioconversion of the lignocellulosic biomass is enzymatic hydrolysis, which efficiently converts cellulose into fermentable sugars (Mood et al. 2013; Tan et al. 2015). The efficiency of the enzymatic hydrolysis of untreated lignocellulosic biomass is low because of its firm structure, and thus, the industrialization of bioethanol from lignocellulosic biomass remains restricted. The rate and extent of enzymatic hydrolysis are influenced by the effectiveness of the cellulose and also by the physical, chemical, and morphological characteristics of the heterogeneous lignocellulosic biomass. Therefore, understanding the characteristics of biomass that limit enzymatic hydrolysis is important for academic and practical applications. These characteristics include the fiber surface properties, polymerization degree, cellulose crystallinity, hemicellulose content, lignin content, and lignin structure. Among these characteristics, the content and structure of lignin have a noticeable effect on the enzymatic hydrolysis of various lignocellulosic biomasses (Ju et al. 2013).
Lignin is an aromatic network polymer composed of phenylpropane units connected to one another via ether and carbon–carbon bonds (Ke and Chen 2013). The type, number, and connection methods of the structural units of lignin vary dramatically with the kind of lignocellulosic biomass. The structural units of the lignin of Gramineae lignocellulosic biomass contain guaiacyl, syringyl, and p-hydroxyphenyl units.

Generally, lignin acts as a physical barrier to cellulase, restricting its access to cellulose, and as an attractant of cellulase, thus resulting in non-productive binding (Siqueira et al. 2013). Several reports have shown that the chemical and physical structures of lignin have a notable function in determining the magnitude of inhibition contributed by lignin to enzymatic hydrolysis (Ko et al. 2015; Lu et al. 2016). For example, Sewalt et al. (1997) demonstrated that increasing the phenolic hydroxyl content of lignin also increases its inhibition activity towards enzymatic hydrolysis. Berlin et al. (2006) showed that lignin contains small amounts of carboxyl and aliphatic hydroxyl groups that may produce more hydrophobic lignin, and thus, the hydrophobic interaction between lignin and cellulase occurs easily and causes low efficiency in enzymatic hydrolysis.

The structure of lignin is heavily dependent on pretreatment conditions. Pretreatment is essential to achieving high-efficiency conversion of bioethanol with lignocellulosic biomass. Among the different types of pretreatment, liquid hot water (LHW) pretreatment has been demonstrated to be an effective method for several lignocellulosic biomass types (Kou et al. 2013; Lu et al. 2013). After LHW pretreatment, the lignocellulosic biomass is divided into prehydrolysates that contain a large number of five-carbon sugars and water-insoluble solids (WIS), which mainly consist of glucan and lignin. The WIS are used in the subsequent enzymatic reaction. Therefore, lignin can be regarded an important factor that limits the enzymatic hydrolysis reaction of WIS. Many researchers have been searching for ways to prevent lignin inhibition of enzymatic hydrolysis (Yang and Wyman 2004; Chandra et al. 2007; Lou et al. 2013; Rahikainen et al. 2013; Li et al. 2015).

Milled wood lignin (MWL) is regarded as one of the best samples to represent natural lignin. To investigate and compare structural variations during LHW pretreatment, MWL fraction was extracted from reed straw and corn stover in the present study before and after LHW pretreatment by using the Bjorkman procedure, which has minimal effect on the structure of lignin. This work aimed at elucidating factors that influence cellulase and lignin interactions during enzymatic hydrolysis when an MWL fraction isolated from reed straw and corn stover pretreated with LHW at different severities was used.

The changes in the chemical structure of the MWL were comprehensively characterized by Fourier transform infrared (FTIR) spectroscopy, and 1H and 13C nuclear magnetic resonance (NMR). Moreover, differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), and differential thermal analysis (DTA) techniques were employed for the thermal analysis of the MWL fractions. Gel permeation chromatography (GPC) and elemental analyses were conducted for the molecular weight and functional group analyses. The MWL fraction was added into the enzymatic hydrolysis system. The filter paper activity (FPA), protein content, and conversion of cellulose to glucose were investigated to assess the effect of MWL on enzymatic hydrolysis.

In view of the described progress in the field, in the present work the inhibition of byproducts were studied in-depth in the present work, with LHW pretreatment to reveal the inhibition of byproduct effect on enzymatic hydrolysis. This work was aimed at addressing the toxicity of the byproducts. It will have important value in promoting the industrial development with straw feedstock for cellulosic ethanol. It is important to note that the economic necessity for a lignocellulosic biorefinery to produce biofuels has been advocated. Indeed, the production of fuels is necessary to justify construction of the
biorefinery in order to achieve a high energy impact and proper return on investment. In addition, valorization of biofuels of lignocellulosic biomass is essential for an economically viable biorefinery.

EXPERIMENTAL

Materials
Reed straw was provided by the Yingkou Papermaking Mill (Yingkou, China). Corn stover was collected from a field near the new district of Jinzhou, Dalian, China. The reed straw and corn stover were milled to a particle size of 40 to 60 meshes using a laboratory ball mill (Taijihuan Nanometer Limited Company, Qinhuangdao, China) and stored in a plastic bag until they were used. The commercial cellulase used in enzymatic hydrolysis was purchased from Imperial JADE Biotechnology Ltd. Co. (Ningxia, China).

LHW Pretreatment
The LHW pretreatment was conducted in a 15-L digester with four small tanks (Mechanical Mill of the Shanxi University of Science and Technology, China). Approximately 40 g of the materials and 800 mL of deionized water were loaded into the small tanks. The pretreatment temperature was controlled from 180 °C to 210 °C, and the pretreatment time was set to 20 min. After pretreatment, the WIS and the prehydrolysates were separated by filtration with a Büchner funnel. The WIS were washed with deionized water to obtain a pH of approximately 7 and used for subsequent enzymatic hydrolysis and MWL preparation.

Preparing MWL

Isolating raw MWL
The WIS of reed straw and corn stover pretreated with LHW under different conditions were successively extracted with benzene/alcohol 2:1 (v/v) for 24 h in a Soxhlet extractor. The extract-free WIS were finely ball-milled continuously for 72 h. The milled samples were extracted (3 × 24 h) with dioxane/water 96:4 (v/v) (20 mL solvent/g of milled samples). The solution was centrifuged, and the supernatant was subsequently evaporated at 40 °C at reduced pressure until it dried completely. The raw MWL was then obtained.

Purifying raw MWL
The raw MWL was redissolved in a solution of acetic acid/water 9:1 (v/v) (15 mL solvent/g of raw MWL). The solution was then precipitated in 150 mL of water. The precipitated residue was separated by centrifugation and subsequently dissolved in a solution of 1,2-dicloromethane/ethanol 2:1 (v/v). The mixture was centrifuged to eliminate insoluble residues. The supernatant was precipitated three times in diethyl ether, and the obtained residue was separated by centrifugation. This residue was re-suspended in petroleum ether and re-centrifuged to obtain the final purified MWL fraction, which was dried under a P2O5 current.

Acetylating purified MWL
The 100 mg of purified MWL was dissolved in 5 mL of pyridine-acetic anhydride 1:2 (v/v) in an Erlenmeyer flask. The flask was purged with nitrogen to drive out air, quickly corked securely, placed in the dark at room temperature for 72 h, and shaken continuously to facilitate a uniform reaction. The solution was gradually added to a stirred mixture of ether (200 mL) to precipitate the acetylated lignin sample. The sample was
washed several times with ether until no pyridine odor was detected. The lignin sample was placed in a vacuum oven with P₂O₅ and dried at 40 °C to obtain the acetylated MWL sample.

The Effect of MWL on FPA and Protein Content

The experiment was performed at 50 °C for 72 h in 100-mL Erlenmeyer flasks. Each flask contained 50 mL of cellulase solution diluted to a certain concentration with 0.05 M sodium citrate buffer (pH 4.8). A certain quality non-acetylated MWL of corn stover pretreated at 190 °C was added into the Erlenmeyer flasks. The MWL loadings were 0.15 g and 0.3 g. The samples were collected at 0.25, 0.5, 1, 2, 4, 7, 12, 24, 36, 48, and 72 h to determine FPA and protein content.

The Effect of MWL on the Enzymatic Hydrolysis of WIS

The WIS of corn stover pretreated at 190 °C were the substrates for enzymatic hydrolysis. This process was performed at 50 °C for 72 h in 100-mL Erlenmeyer flasks. Each flask contained 50 mL of 0.05 M sodium citrate buffer (pH 4.8) and had a solid-to-liquid ratio of 1:50 weight per volume (w/v) of WIS. The enzyme loading was 10 FPU per g of oven-dried WIS. Non-acetylated and acetylated MWL isolated from the WIS of corn stover were added into the Erlenmeyer flasks. The MWL loading was 0.2 g. The samples were collected at 1, 5, 9, 12, 24, 36, 48, and 72 h to determine glucose concentration.

Analysis Methods

The methoxyl groups of the MWL were determined via the improved ViebÖck-Schwappach method. The phenolic hydroxyl and carboxyl groups of the MWL were measured by non-electric conductivity titration. The carbonyl groups of the MWL were determined via the reaction of lignins with hydroxylamine hydrochloride (Chen 1992). The FTIR spectra of the MWL were obtained on an FTIR spectrometer (Spectrum One-B, PerkinElmer Inc., Waltham, USA) by using KBr discs with approximately 1% finely ground samples within the range of 800 cm⁻¹ to 2000 cm⁻¹. Thermogravimetric analysis (TGA) of the MWL was performed on a TGA system (TGA-Q50, TA Instruments, New Castle, USA). A sample weighing between 3 mg and 7 mg was heated from room temperature to 600 °C at a rate of 20 °C/min. Differential thermal analysis (DTA) was performed on a simultaneous thermal analyzer. The apparatus was continually flushed with a nitrogen flow of 30 mL/min at a rate of 20 °C/min. The weight-average (Mₙ) and number-average (Mₙ) of the MWL fractions were determined by GPC on GMHx1 and GMHLx1 columns in a GPC instrument with an interface (PE Series 900, PerkinElmer Inc.).

The MWL samples were dissolved in tetrahydrofuran and injected into the GPC columns after filtration. The injection volume was 100 μL. The columns were operated at ambient temperature and eluted with tetrahydrofuran at a flow rate of 1 mL/min. A refractometric detector (Series 200) was employed, and polystyrene was used as the standard for the molecular weight. The Mₙ and Mₙ of the MWL fractions were computed from their chromatograms. Elemental analyses (C, H, and N) were performed on a Vario EL III elemental analyzer (Bruker Elemental GmbH, Karlsruhe, Germany). Samples were ground to a fine powder, and each sample (2 mg to 3 mg, dry basis) was weighed on tin foil, placed in an elemental furnace, and subjected to complete combustion in a pure oxygen environment. The oxygen content was obtained by calculating the difference in C, H, and N contents according to Eq. 1.

\[ \text{O wt.%} = 100 - (\text{C wt. %} + \text{H wt. %} + \text{N wt. %}) \]  

\[ (1) \]
A 910S differential scanning calorimeter (TA Instruments, USA) was used to characterize the thermal behavior of the solid fractions. A total of 18 mg to 22 mg of each sample was placed in an aluminum sample pan and sealed. The samples were subjected to thermal treatment at a scan rate of 20 °C/min, and pure nitrogen (99.9%) was used as the carrier gas at 30 mL/min. The $^1$H and $^{13}$C NMR spectra were obtained on a Varian Inova-400 MHz NMR system (Varian Inc., USA). The 25-mg MWL sample was dissolved in CDCl$_3$, and the 250-mg of MWL was dissolved in dimethyl-d$_6$ sulfoxide.

RESULTS AND DISCUSSION

Analyzing Molecular Weight and Distribution of MWL Fractions

Gel permeation chromatography was performed to determine the molecular weight and distribution of the MWL fractions. To investigate the changes in molecular weight before and after LHW pretreatment, the $M_n$, $M_w$, and polydispersity ($M_w/M_n$) of the MWL fractions were determined (Table 1).

<table>
<thead>
<tr>
<th>MWL</th>
<th>$M_n$ (kDa)</th>
<th>$M_w$ (kDa)</th>
<th>$M_w/M_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reed Straw</td>
<td>54.71</td>
<td>50.68</td>
<td>1.079</td>
</tr>
<tr>
<td>Reed Straw - 180 °C</td>
<td>417.21</td>
<td>413.48</td>
<td>1.009</td>
</tr>
<tr>
<td>Corn Stover</td>
<td>19.74</td>
<td>18.03</td>
<td>1.095</td>
</tr>
<tr>
<td>Corn Stover - 190 °C</td>
<td>1778</td>
<td>1457</td>
<td>1.220</td>
</tr>
</tbody>
</table>

The molecular weights of the MWL of reed straw pretreated at 180 °C and that of corn stover pretreated at 190 °C were remarkably higher than those of untreated reed straw and untreated corn stover. This result indicated that the molecular weight of lignin increased during LHW pretreatment. The increase in molecular weight could be attributed to lignin condensing in the macromolecular structure to a noticeable extent during LHW pretreatment.

The dissolution of lignin with low molecular weight also resulted in an increase in the molecular weight of the remaining insoluble lignin. In contrast, the $M_w/M_n$ of the MWL fraction of pretreated reed straw was lower than that of the untreated reed straw, indicating that the MWL fraction of pretreated reed straw was more disperse. This probably resulted from the recondensation of several linkages during LHW pretreatment.
The low $M_w/M_n$ value of the lignin fraction indicated a narrow molecular weight distribution on the analyzed section of the lignin. However, the $M_w/M_n$ of the MWL fraction of pretreated corn stover was higher than that of the untreated corn stover, indicating that the $M_w/M_n$ values of the MWL fractions isolated from different raw materials before and after LHW pretreatment were different. The molecular weight distributions (MWDs) of the MWL fractions isolated from the original material and the two pretreated WIS detected by the GPC curves are illustrated in Fig. 1. The MWDs generally followed a normal distribution with a symmetrical curve. The MWL of untreated corn stover had one wider peak and exhibited a wider molecular size distribution than the MWL of pretreated corn stover. The presence of the peak with lower molecular size in the latter could indicate modification of the macromolecular structure of lignin (Bu et al. 2011).

**Elemental and Unit Molecular Weight Analyses of MWL Fractions**

Carbon, hydrogen, and nitrogen in the MWL isolated from the WIS were measured with a Vario EL III CHN element analyzer. The oxygen content and the methoxyl group content were measured. The C$_9$ formula and the unit molecular weight were calculated from the composition of the elements of the MWL and the methoxyl content of the C$_9$ lignin structural unit, as shown in Table 2. The methoxyl content of the MWL increased after LHW pretreatment, whereas the proportion of oxygen and hydrogen decreased. This result was caused by the disappearance of the hydroxyl structure of the MWL, whereas alkyl ether caused the rupture of the MWL after LHW pretreatment. Alkyl ether then became reconnected to the lignin structure to increase the unit molecular weight. This result indicated that the molecular structure of lignin caused condensation. In particular, the unit molecular weight of the MWL of reed straw increased from 194.51 g/mol to 243.35 g/mol, indicating that the condensation of reed straw lignin pretreated with LHW was obvious. However, the unit molecular weight of corn stover lignin did not increase as much. This finding confirmed the results of the GPC and DTG analyses. The increase in the molecular weight of the dissolved lignin did not only indicate low molecular weight, but also implied that the lignin structural unit generated condensation.
**Table 2. Elements of MWL Isolated from WIS Before and After LHW Pretreatment, with C₉ Formula and Unit Molecular Weight**

<table>
<thead>
<tr>
<th>MWL</th>
<th>C (%)</th>
<th>H (%)</th>
<th>N (%)</th>
<th>O (%)</th>
<th>OCH₃ (%)</th>
<th>C₉Formula</th>
<th>Unit Molecular Weight (Mw/C₉)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reed Straw</td>
<td>59.37</td>
<td>5.931</td>
<td>0.229</td>
<td>34.47</td>
<td>10.39</td>
<td>C₇H₅O₃.53 (OCH₃)₀.66</td>
<td>194.51</td>
</tr>
<tr>
<td>Reed Straw-180 °C</td>
<td>59.92</td>
<td>5.924</td>
<td>0.214</td>
<td>33.94</td>
<td>40.39</td>
<td>C₇H₄O₂.00 (OCH₃)₂.18</td>
<td>243.35</td>
</tr>
<tr>
<td>Corn Stover</td>
<td>57.45</td>
<td>6.028</td>
<td>0.106</td>
<td>36.42</td>
<td>16.27</td>
<td>C₇H₄O₂.70 (OCH₃)₂.11</td>
<td>210.91</td>
</tr>
<tr>
<td>Corn Stover-190 °C</td>
<td>61.02</td>
<td>5.769</td>
<td>0.584</td>
<td>32.63</td>
<td>32.31</td>
<td>C₇H₆O₂.22 (OCH₃)₂.32</td>
<td>221.32</td>
</tr>
</tbody>
</table>

**Quantitative Analysis of the Functional Groups of MWL Fractions**

The functional groups of the MWL fractions isolated from reed straw and corn stover before and after LHW pretreatment are shown in Table 3. The changes in functional group contents were different in reed straw and corn stover. For reed straw, the carboxyl, phenolic hydroxyl, methoxyl, and carbonyl contents all increased after LHW pretreatment. For corn stover, the phenolic hydroxyl and methoxyl contents increased after LHW pretreatment, but the carboxyl content decreased. The carbonyl content differed according to type. Phenol type-α and phenol type-γ carbonyls increased, whereas non-phenolic-α and non-phenolic-γ carbonyls decreased. Total carbonyl content decreased. The differences in the functional groups of the MWL fractions might have caused various effects.

**Table 3. Functional Group Contents of MWL Before and After LHW Pretreatment**

<table>
<thead>
<tr>
<th>MWL</th>
<th>COOH (%)</th>
<th>Phenol -OH (%)</th>
<th>-OCH₃ (%)</th>
<th>Phenol Type-α /100C₉</th>
<th>Phenol Type-γ /100C₉</th>
<th>Non-Phenolic-α /100C₉</th>
<th>Non-Phenolic-γ /100C₉</th>
<th>-C=O (%)</th>
<th>Total Carbonyl /100C₉</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reed Straw</td>
<td>1.64</td>
<td>5.90</td>
<td>10.30</td>
<td>0.40</td>
<td>0.64</td>
<td>2.88</td>
<td>0</td>
<td>3.92</td>
<td>3.92</td>
</tr>
<tr>
<td>Reed Straw-180 °C</td>
<td>2.78</td>
<td>6.13</td>
<td>40.39</td>
<td>1.69</td>
<td>0.86</td>
<td>3.41</td>
<td>0.83</td>
<td>6.79</td>
<td>6.79</td>
</tr>
<tr>
<td>Corn Stover</td>
<td>1.79</td>
<td>3.20</td>
<td>16.27</td>
<td>1.24</td>
<td>0.66</td>
<td>4.06</td>
<td>1.15</td>
<td>7.11</td>
<td>7.11</td>
</tr>
<tr>
<td>Corn Stover-190 °C</td>
<td>1.26</td>
<td>5.48</td>
<td>32.31</td>
<td>1.55</td>
<td>0.82</td>
<td>2.50</td>
<td>0.38</td>
<td>5.25</td>
<td>5.25</td>
</tr>
</tbody>
</table>

**FTIR Analysis of MWL Fractions**

The FTIR spectra of the MWL fractions isolated from the untreated original material and the WIS of reed straw and corn stover pretreated at different temperatures are presented in Fig. 2.
Fig. 2. FTIR spectra of MWL isolated from the original material and the two pretreated WIS. (a) Untreated reed straw, (b) reed straw pretreated at 180 °C, (c) reed straw pretreated at 210 °C, (d) untreated corn stover, (e) corn stover pretreated at 190 °C, and (f) corn stover pretreated at 210 °C.

The bands at 1732 cm\(^{-1}\) were assigned to the carbonyl stretching in unconjugated ketones and aliphatic acetate. The band at 1659 cm\(^{-1}\) was assigned to the conjugated carbonyls that can be mainly attributed to the coumaryl ester group. Moreover, the fingerprint regions of the FTIR spectra of the lignin preparations exhibited typical grass lignin patterns at 830 cm\(^{-1}\) and 1120 cm\(^{-1}\), and a shoulder at 1159 cm\(^{-1}\) indicated the presence of G, H, and S units (Faix 1991; Seca et al. 2000). A comparison of the MWL samples showed that the bands (and their relative intensities) of the fingerprint region were similar, indicating similar chemical structures of the lignin fractions. This observation highlighted the fact that LHW pretreatment did not remarkably change the core of the lignin structure. However, differences in the carbonyl region were observed with the disappearance of the bands at 1732, 1659, 1385, 1167, and 1087 cm\(^{-1}\) in the MWL spectrum assigned to the damage of the conjugated ketone C=O group and the cleavage of the ether bond.

Fig. 3. \(^1\)H NMR (a, b) spectra of MWL fractions isolated from the original material and the two pretreated WIS. (a) Untreated reed (black line) and WIS pretreated at 180 °C (red line), (b) untreated corn stover (black line), and WIS pretreated at 190 °C (red line).
1H NMR Spectra Analysis of MWL Fractions

Analyzing the 1H NMR signal intensity provides an indirect method of monitoring the level of substitution of aromatic rings in lignin (Zhou et al. 2012). The 1H NMR spectra of acetylated MWL are shown in Fig. 3. The integral of all of the signals at 6.0 ppm to 8.0 ppm in the 1H NMR spectra was amenable to assignments as aromatic protons of three kinds of lignin units. The shifts of the signals between 6.1 ppm and 6.8 ppm were attributed to the aromatic protons in the syringyl units (S), whereas the shifts of the signals between 6.8 ppm to 7.2 ppm were related to the aromatic protons in the guaiacyl units (G) (Xu et al. 2008). The integral signals of the G units were more prominent than those of the S units. Therefore, the G and S units were the main structural components in the lignin of reed straw and corn stover. The peaks that appeared between 4.1 ppm and 6.3 ppm were attributed to the protons in the side chain of the aromatic ring, and they were generally too weak to be detected in the spectra. The 1H NMR spectroscopic analysis clearly confirmed the methoxylation with the strengthened signal intensity for methoxyl groups at 3.7 ppm, compared with that of the MWL of the untreated corn stover. The two strong signals at 2.2 ppm and 1.9 ppm were the characteristic absorptions of the protons in the acetyl groups connected to the benzene ring and the aliphatic side chains, respectively. These acetyl groups originated from the acetylated phenolic hydroxyl and the alcoholic hydroxyl in the MWL samples.

13C NMR Spectra Analysis of MWL Fractions

To investigate the structural features of the lignin fractions further, the MWL samples before and after LHW pretreatment were investigated by 13C NMR spectrometry. The 13C NMR spectra of the MWL fractions isolated from the original material and the two pretreated WIS are given in Figs. 4(a) and 4(b), respectively. Most of the observed signals were previously assigned in the lignocellulose material spectra (Sun et al. 2005; Vivas et al. 2006; Xu et al. 2007; Baptista et al. 2008). The peaks at 160 ppm to 170 ppm, which originated from the carbon in the carbonyl groups, indicated abundant carbonyls in the MWL samples. The region from 102 ppm to 157 ppm was assigned as the aromatic part of the MWL. The aromatic region was divided into three regions of interest: protonated aromatics (102 ppm to 123 ppm), condensed aromatics (123 ppm to 140 ppm), and oxygenated aromatics (140 ppm to 157 ppm). The oxygenated aromatic region contained C-3, C-4, and C-5 carbons on the aromatic ring. The condensed aromatic region consisted of C-1 carbons, plus any ring carbons involved in cross-linking, such as the 5-5 or β-5 substructures. The S residues were indicated by signals at 152 ppm. The signals appearing in the 13C NMR spectra at 122.3 ppm and 114.3 ppm all originated from G units. Moreover, the p-hydroxyphenyl (H) residues appeared as signals at 129.7 ppm. These results demonstrated that the lignin of the MWL samples contained three basic structural units, namely, H, G, and S. However, the distinction among absorption peak intensities suggested that the proportion of the three units in the MWL was different.
The etherified ferulic acid was observed with a small signal at 169.6 ppm. Therefore, the majority of the ferulic acids were linked to lignin by ether bonds. The side chain carbons of Cγ in the β-5 substructures was at 61 ppm to 64 ppm. These observations demonstrated that the carbon-carbon linkages in the lignin fractions might be the dominant connection styles. The differences in the shape and strength of the peaks in the two spectra at 61 ppm to 64 ppm demonstrated that several linkages of the lignin fractions from the two materials were different from one another. The strong signal at 55.9 ppm was attributed to the –OCH3 in the S and G units. The signals of dimethyl sulfoxide, which was the solvent of the MWL, showed the strongest absorption at 39 ppm to 40 ppm. The strong absorption at 19 ppm to 21 ppm was the characteristic absorption of the carbons in the acetyl groups introduced during the acetylation of MWL.

**Effects of MWL on FPA and Protein Content**

Lignin restricts enzymatic hydrolysis by unproductively binding cellulases. To evaluate the relative effect of lignin on cellulase activity, the MWL isolated from the WIS pretreated with LHW was placed in the enzymatic hydrolysis system. The protein content was investigated to evaluate its relation with cellulase activity. The effects of the MWL on FPA and protein content are shown in Fig. 5. In Fig. 5(a), FPA decreased slightly when the MWL was added, indicating that the MWL decreased FPA, although the effect was not obvious. The effect of MWL on FPA was divided into three stages. The initial stage was 4 h before enzymatic hydrolysis. The second stage then followed, in which FPA decreased. During the third stage, FPA decreased gradually until it reached the pretreatment time of up to 48 h. When the enzymatic hydrolysis was terminated after more than 48 h, FPA decreased and remained nearly constant, indicating that the decrease in FPA mainly occurred during the early stage of enzymatic hydrolysis. The protein content is shown in Fig. 5(b). The decrease in protein content was obvious when the MWL was added during enzymatic hydrolysis.
Effects of MWL on the Enzymatic Hydrolysis of Microcrystalline Cellulose and WIS

From the preceding analysis, adding MWL decreased FPA and protein content. Further discussion will be provided to determine whether such decreases can affect the enzymatic hydrolysis of lignocellulosic biomass. The microcrystalline cellulose and WIS of the corn stover pretreated by LHW were the substrates. The MWL isolated from the WIS of the corn stover pretreated with LHW was added to the enzymatic hydrolysis system. The effect of the MWL on enzymatic hydrolysis was analyzed by measuring changes in glucose content (Fig. 6).

Glucose contents were nearly the same whether or not MWL was added during the enzymatic hydrolysis of microcrystalline cellulose. This result indicated that MWL did not affect the enzymatic hydrolysis of microcrystalline cellulose. Figure 6 also shows that the glucose content was lower when the MWL was added, indicating that the MWL affected the enzymatic hydrolysis of WIS. The difference in glucose content between the addition of acetylated and non-acetylated MWL was minimal, indicating that acetylating the MWL had minimal effect on the enzymatic hydrolysis. The phenolic hydroxyl and methoxy of MWL increased in the LHW pretreatment. When MWL was added to the enzymatic hydrolysis system, the FPA of cellulase, protein content, and the conversion of cellulose to
glucose decreased. It appears that the phenolic hydroxyl and methoxy have a certain inhibitory on the enzymatic hydrolysis. The phenolic hydroxyl and methoxy group proportions of MWL were increased in the LHW pretreatment. When MWL was added to the enzymatic hydrolysis system, the FPA of cellulase, protein content, and the conversion of cellulose to glucose decreased. This suggests that the phenolic hydroxyl and methoxy have a certain inhibitory on the enzymatic hydrolysis.

CONCLUSIONS

1. Liquid hot water (LHW) pretreatment affected the milled wood lignin (MWL) of reed straw and corn stover through depolymerization and recondensation reactions. MWL samples isolated from reed straw and corn stover that were pretreated with LHW became more condensed.

2. The non-conjugated and conjugated ketone groups, as well as the ether bonds of the MWL, disappeared after LHW pretreatment. The phenolic hydroxyl and methoxy groups of the MWL increased. The carboxyl groups of the MWL from reed straw increased after LHW pretreatment, whereas those from corn stover decreased.

3. When MWL was added to the enzymatic hydrolysis system, the filter paper activity (FPA) of cellulase, protein content, and the conversion of cellulose to glucose decreased. The MWL only had a minimal effect on the enzymatic hydrolysis of microcrystalline cellulose, whereas it significantly affected the enzymatic hydrolysis of water-insoluble solids (WIS).

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