JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Structural Characterization of Lignin from Triploid of *Populus tomentosa* Carr.

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ABSTRACT: To improve yields while minimizing the extent of mechanical action (just 2 h of planetary ball-milling), the residual wood meal obtained from extraction of milled wood lignin (MWL) was sequentially treated with cellulolytic enzyme and alkali, and the yields of MWL, cellulolytic enzyme lignin (CEL), and alkaline lignin (AL) were 5.4, 23.2, and 16.3%, respectively. The chemical structures of the lignin fractions obtained were characterized by carbohydrate analysis, gel permeation chromatography (GPC), Fourier transform infrared (FT-IR) spectroscopy, and various advanced NMR spectroscopic techniques. The results showed that the lignin isolated as MWL during the early part of ball milling may originate mainly from the middle lamella. This lignin fraction was less degradable and contained more linear hemicelluloses and more C═O in unconjugated groups as well as more phenolic OH groups. Both 1D and 2D NMR spectra analyses confirmed that the lignin in triploid of *Populus tomentosa* Carr. is **GSH**-type and partially acylated at the γ-carbon of the side chain. Two-dimensional heteronuclear single-quantum coherence ($^{13}C-^{1}H$) NMR of MWL, CEL, and AL showed a predominance of β-*O*-4′ aryl ether linkages (81.1−84.5% of total side chains), followed by β-β' resinol-type linkages (12.2−16.4%), and lower amounts of β-5′ phenylcoumaran (2.1−2.6%) and β-1′ spirodienone-type (0.4−1.4%) linkages. The syringyl (**S**)/guaiacyl (**G**) ratios were estimated to be 1.43, 2.29, and 2.83 for MWL, CEL, and AL, respectively.

KEYWORDS: alkaline lignin, cellulolytic enzyme lignin, milled wood lignin, poplar, ^{13}C NMR, quantitative ^{31}P NMR, HSQC

INTRODUCTION

Triploid of *Populus tomentosa* Carr., a type of fast-growing poplar species widely planted in China, was successfully bred by a research group of Beijing Forestry University, China. Currently, it is a very important variety due to its various beneficial characteristics, such as cold resistance, drought tolerance, and pest resistance. Moreover, this new variety is superior in short rotation and high quality to other poplar varieties. Therefore, it is a promising lignocellulosic feedstock for paper, board, and biofuel production. To ensure efficient utilization of this lignocellulosic material, which consists mainly of three groups of polymers, cellulose, hemicelluloses, and lignin, the physicochemical characterization of the three cell wall components is required. Particularly, more attention should be paid to the structural characteristics of lignin, because lignin has a significant impact on the efficient utilization of lignocellulosic materials, especially on the production of biobased materials and biofuel.[1,2]

Generally, lignin is considered as being formed by the dehydrogenative polymerization of three *p*-hydroxycinnamyl alcohol precursors: *p*-coumaryl, coniferyl, and sinapyl alcohols.[3] Each of these monolignols gives rise to different types of lignin units called **H** (*p*-hydroxyphenyl), **G** (guaiacyl), and **S** (syringyl) units, respectively. Although the structure and biosynthetic pathway of lignin have been studied for more than a century, they have not yet been completely elucidated.[4,5] A primary obstacle in elucidating the structure of native lignin is that it cannot be isolated in a chemically unaltered form because of the tight physical binding and chemical linkages between lignin and cell wall polysaccharides.[6]

The first major method for lignin isolation in a relatively unaltered state was proposed by Björkman,[7] who extracted lignin from finely ball-milled wood with aqueous dioxane. The milled wood lignin (MWL) obtained is considered to be a representative source of native lignin and has been extensively used in the elucidation of native lignin structure. However, concerns exist over the similarity between MWL and native lignin based on the low yields.[8] Therefore, many efforts have been made to improve the yield of lignin isolated from ball-milled wood, especially through the use of cellulolytic enzymes.[9−13] In these cases, ball-milled wood meal was treated with cellulolytic enzyme to remove most of the polysaccharides before aqueous dioxane extraction. Cellulolytic enzyme lignin (CEL) was found to be structurally similar to MWL,[9,10] but in a higher yield, and hence it is more representative of the total lignin in wood than MWL. It is well-known that cellulose is highly crystalline and inaccessible, which impairs the physical contact between cellulose and enzyme. To cope with this problem, ball-milled wood has been swelled or dissolved in organic solvents, such as *N*,*N*-dimethylacetamide/lithium chloride or dimethyl sulfoxide/*N*-methylimidazole, then regenerated in water or ethyl acetate, and treated with cellulase.[11,12] The yields of CEL isolated from the regenerated ball-milled wood samples were therefore higher than those obtained directly from the ball-milled wood material, presumably via a decreasing crystallinity of cellulose. Recently, a novel procedure using the combination of enzymatic and mild acidolysis was proposed.[13] It was reported that the enzymatic mild acidolysis lignins (EMAL) was more representative of the total

Received: January 27, 2011
Revised: May 13, 2011
Accepted: May 15, 2011
Published: May 16, 2011

lignin present in milled wood and offered higher yields and purities than those of the corresponding MWL and CEL.[14]

Although all of the above-mentioned lignin isolation approaches have been widely conducted for structural studies of lignins, it is evident that structures of lignin are modified to some extent during the different ball-milling processes. Many efforts have been made to understand the effects of ball milling.[6,8,10,15,16] It was found that ball milling of wood degrades β-O-4′ structures in lignin and that the effect was greater for hardwood than for softwood lignin.[8] Besides, previous studies also indicated that phenolic β-O-4′ structures increased continuously at the expense of etherified β-O-4′ structures during ball milling. Some condensation reactions may occur during the ball-milling process, especially if the ball milling is conducted in a vibratory ball mill without toluene (dry ball milling).[6,15]

It is widely accepted that the complex structure of the lignin−hemicelluloses network is the major reason for the difficulty in isolating the pure lignin in an unaltered form. A cellulolytic enzyme and alkali are usually adopted to cope with this serious difficulty.[8−10,17] The function of the cellulolytic enzyme is to remove most of the polysaccharides from ball-milled wood meal before aqueous dioxane extraction, whereas alkali effectively isolates relatively pure components via the cleavage of alkali-labile linkages between lignin and carbohydrates. On the basis of all the above considerations, it is a potential procedure to combine MWL and CEL or alkaline lignin (AL) for structural characterizations from mild ball-milled wood sample. In this work, to minimize the structural alterations to lignin caused by ball milling, the milled wood was prepared from coarse wood meal by just 2 h of planetary ball milling under the milling conditions employed. CEL, residual enzyme lignin (REL), and AL were isolated from the residual wood meal after extraction of MWL. The structural features of these four lignin fractions were comparatively elucidated by gel permeation chromatography (GPC), carbohydrate analysis, and advanced spectroscopic techniques, including Fourier transform infrared (FT-IR) spectroscopy, quantitative ^{31}P NMR spectroscopy, ^{13}C NMR spectroscopy, and two-dimensional heteronuclear single-quantum coherence (HSQC) NMR spectroscopy.

MATERIALS AND METHODS

Materials. Triploid of *P. tomentosa* Carr., a fast-growing poplar tree, 7 years old, was harvested from the experimental farm of Beijing Forestry University, China. The wood sample was ground to pass through a 0.8 mm size screen and was then extracted with toluene/ethanol (2:1, v/v) in a Soxhlet instrument for 6 h. The Klason lignin content was measured by the TAPPI standard, which amounts to 21.5% of this wood. The air-dried extractive-free wood sample (25 g) was milled using a planetary ball mill (Fritsch, Germany) in a 500 mL ZrO_2 bowl with mixed balls, 10 balls of 2 cm diameter and 25 balls of 1 cm diameter. The milling was conducted under a nitrogen atmosphere at 500 rpm with 10 min of rest after every 10 min of milling. The only 2 h milling time performed was to minimize the structural alternations to lignin caused by ball milling.

The cellulolytic enzyme used in this study was Celluclast 1.5 L, containing hemicellulase activities, kindly supplied by Novozymes. It has a filter paper activity of 70 FPU g^{-1}. All chemicals used were of analytical or reagent grade and directly used as purchased without further purification.

Isolation of Lignins. MWL, CEL, REL, and AL were isolated according to Figure 1. MWL was isolated and purified according to the method of Björkman.[7] The residual wood meal (R1) after extraction of MWL was divided into two parts: one was selected to isolate CEL,

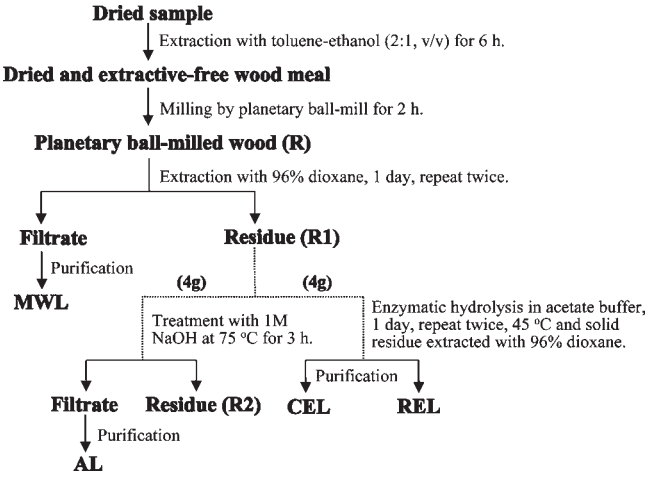

Figure 1. Isolation procedure for milled wood lignin (MWL), cellulolytic enzyme lignin (CEL), residual enzyme lignin (REL), and alkaline lignin (AL).

whereas the others were selected to extract AL. CEL was isolated according to the method of Chang et al.[10] Specifically, the residual wood meal after extraction of MWL (4 g) was suspended in acetate buffer (120 mL, pH 4.8), and 2.4 mL of cellulase was added and incubated at 45 °C for 24 h. The reaction system was centrifuged, the supernatant was removed, and the residue was again suspended in acetate buffer (60 mL, pH 4.8) and treated with cellulase (1.2 mL) for an additional 24 h at 45 °C. The solution was centrifuged and the enzyme-treated residue was washed with water (200 mL), followed by repeated extraction (2 × 24 h) with dioxane/water (40 mL, 96:4, v/v) under nitrogen atmosphere. The solution was collected by centrifugation and concentrated. The crude CEL was freeze-dried and purified as per the MWL. The residue remaining after CEL isolation was freeze-dried to produce the REL sample. AL was isolated by treatment of R1 with 1 M sodium hydroxide with a solid-to-liquid ratio of 1:15 (g mL^{-1}) at 75 °C for 3 h. The purification procedure was performed according to the method of Sun et al.[17]

Characterization of Lignins. The carbohydrate moieties associated with the four lignin fractions were determined by hydrolysis with dilute sulfuric acid according to the procedure described in a previous paper.[18] The weight-average (M_w) and number-average (M_n) molecular weights of these lignin preparations were determined by GPC on a PL-gel 10 mm Mixed-B 7.5 mm i.d. column according to a previous paper.[19] The FT-IR spectra of the lignin preparations were recorded from a KBr disk containing 1% finely ground samples on a Tensor 27 FT-IR spectrophotometer in the range of 4000−400 cm^{-1}. NMR spectra were recorded at 25 °C on a Bruker AVIII 400 MHz spectrometer. Quantitative ^{31}P NMR spectra of MWL, CEL, and AL were recorded according to published procedures.[20−23] A solvent mixture composed of pyridine and deuterated chloroform (1.6:1, v/v) was prepared. An internal standard solution was prepared with cholesterol (400 mg) and chromium(III) acetylacetonate (40 mg) dissolved in 10 mL of the above solvent. Relaxation reagent was prepared with chromium(III) acetylacetonate (27.9 mg) dissolved in 5 mL of the above solvent. Lignin sample (40 mg) and internal standard solution (100 μL) were added into 800 μL of above solvent. The resulting suspension was left at room temperature until the lignin was totally dissolved before 5 mL of relaxation reagent was added. Finally, 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholate (130 μL) was added, and the mixture was transferred into a NMR tube for analysis. ^{13}C NMR spectra and two-dimensional HSQC NMR spectra were also recorded at 25 °C on a Bruker AVIII 400 MHz spectrometer according to a previous paper.[19]

Table 1. Yields and Carbohydrate Contents of Lignin Fractions

	yield (%)			carbohydrate[a] content (%)						
sample	with sugars[b]	without sugars	total sugar content (%)	Rha	Ara	Gal	Glc	Man	Xyl	Uro
MWL	5.4	4.9	9.88	0.08	0.04	0.10	0.43	nd[c]	8.90	0.33
CEL	23.2	21.4	7.74	0.27	0.16	0.85	1.50	0.40	4.16	0.40
REL	88.9	67.0	24.68	0.36	0.46	1.63	11.04	0.61	10.07	0.51
AL	16.3	16.1	1.24	0.01	0.07	0.13	0.23	0.03	0.39	0.38

[a] Rha, rhamnose; Ara, arabinose; Gal, galactose; Glc, glucose; Man, mannose; Xyl, xylose; Uro, uronic acid. [b] Based on Klason lignin of wood. [c] Not detected.

The central solvent (DMSO) peak was used as an internal chemical shift reference point (δ_C/δ_H 39.5/2.49). HSQC cross-signals were assigned by comparison with the published literature.[24−30] A semiquantitative analysis of the intensities of the HSQC cross-signal was performed according to the method given in previous papers.[24−26] In the aliphatic oxygenated region, the relative abundance of the different interunit linkages was estimated from $C_\alpha-H_\alpha$ correlations to avoid possible interference from homonuclear $^1H-^1H$ couplings, and the relative abundances of side chains involved in the different interunit linkages were calculated. In the aromatic region, $C_{2,6}-H_{2,6}$ correlations from **S** units and C_2-H_2 correlation from **G** units were used to estimate the **S**/**G** ratio of lignin.

RESULTS AND DISCUSSION

Lignin Isolation. Traditional methods, both MWL and CEL, for isolating lignin were based on extensive ball milling of plant cell wall. Therefore, some original structural characteristics of the isolated lignin could be altered, at the expense of the integrity of the lignin macromolecule and associated condensation and oxidation reactions during ball-milling process.[6,8,15,16] In this study, to minimize the structural alterations during the ball-milling process, the mild ball-milled wood was prepared from the dried and extractive-free wood sample by just 2 h of planetary ball milling under the milling conditions employed. CEL, REL, and AL were isolated from the residual wood meal after extraction of MWL from the mild ball-milled wood. The yields and carbohydrate contents of these four lignin fractions are given in Table 1.

Obviously, by comparison to the lignin yields of previous papers,[6,8,14] the yield of MWL (w/w, based on the amount of Klason lignin of the starting wood and the isolated lignin) was rather lower, just 5.4%. This low yield is not surprising because the yield of extractable MWL was shown to be dependent upon milling severity.[6,8,15] Furthermore, lignin isolated as MWL during the early part of ball milling (low-yield MWL) may originate mainly from lignin in the middle lamella.[8] It has been reported that lignin in S2 has more linkages with carbohydrates and only becomes extractable as MWL after the carbohydrates are degraded sufficiently by the ball-milling process. This view was further confirmed by the quantitative ^{31}P NMR spectra of the lignin fractions.

To isolate MWL in higher yields, more extensive milling is required.[6] In the present study only 2 h of ball-milling time was expected to achieve the minimal structural alterations to lignin during the process. However, to maximally isolate the lignin for structural characterization, CEL and AL were obtained by cellulolytic enzyme and sodium hydroxide treatments from the residual wood meal after extraction of MWL, respectively. The yields of CEL, REL, and AL, were 23.2, 88.9, and 16.3%, respectively. It is believed that the majority of the carbohydrates can be removed by the enzymatic treatment, and CEL can be subsequently isolated by aqueous dioxane extraction.[10,31] Besides, alkali treatment of lignocelluloses has been reported to cleave the alkali-labile linkages between lignin and carbohydrates, such as the ester bonds between lignin and/or hemicelluloses.[32]

Carbohydrate Analysis. Although all crude lignin fractions obtained have been purified and substantial amounts of carbohydrates have been removed from them, some carbohydrates remained in the purified lignin preparations. To figure out what kind of association existed between lignin and carbohydrates, the four lignin fractions were prepared for determination of their carbohydrate contents, and the results are also given in Table 1. As can be seen, MWL, CEL, REL, and AL all contained associated carbohydrates, amounting to 9.88, 7.74, 24.68, and 1.24%, respectively. Therefore, after calibration of the amounts of associated carbohydrates, the pure yields of the four lignin fractions were 4.9, 21.4, 67.0, and 16.1%, respectively, as shown in Table 1. Obviously, the difference of carbohydrate contents between MWL and CEL was not significant as compared to the big difference of carbohydrate compositions of these two fractions. MWL, which was extracted with aqueous dioxane from the mild ball-milled wood, contained a larger percentage of xylose (90.1%) among the total sugars and uronic acids. However, in CEL, which was isolated from the residual wood meal after extraction of MWL, xylose was the major component sugar and comprised 53.7% of the total sugars. Glucose and galactose appeared to be the secondary major sugars, comprising 19.4 and 11.0% of the total sugars, respectively. Mannose, rhamnose, and arabinose were observed as noticeable amounts. The results showed that the lignin released by mild/early ball milling may contain more linear hemicelluloses (xylans). Lignin associated with more branched hemicelluloses is hard to isolate from the cell walls during a ball-milling process.

The results also showed that glucose (44.7%) and xylose (40.8%) were demonstrated to be the two main sugars in REL, and this could be explained by the incorporation of the obstinate cellulose and hemicelluloses during the enzymatic treatment. However, AL, which was directly isolated from the residual wood meal after extraction of MWL with 1 M sodium hydroxide at 75 °C for 3 h, had a very low carbohydrate content (1.24%) and a high proportion of uronic acids (30.6%). This phenomenon might be explained by the cleavage of alkali-labile linkages between lignin and carbohydrates during alkali treatment, especially the ester linkages between lignin and hemicelluloses. This was in line with the result of a previous paper obtained during the study of the purity of lignin extracted from *Eucalyptus tereticornis* through a three-step sequential extraction−precipitation method.[33]

Molecular Weight Distributions. The values of the weight-average (M_w) and number-average (M_n) molecular weights,

calculated from the GPC curves (relative values related to polystyrene), and the polydispersity (M_w/M_n) of MWL, CEL, and AL are illustrated in Table 2. As can be seen from Table 2, all three lignin fractions exhibited similar molecular weight distributions, ranging from 4250 to 3200 g mol^{-1}. Specifically, the M_w of MWL isolated after 2 h of planetary ball milling was 4250 g mol^{-1}, whereas the weight-average molecular weights determined for CEL and AL were 3670 and 3200 g mol^{-1}, respectively.

The M_w of MWL was apparently higher than that of CEL in the present study. However, this result was quite different from a previous study of Guerra et al.,[14] in which MWL and CEL were similarly isolated from six different wood species after 28 days of rotary ball milling. For all six studied wood species, the M_w of MWL was significantly lower than that of CEL. This obvious difference might be due to the different ball-milling methods used for preparing the wood meals. Furthermore, a previous paper also indicated that as the ball-milling time is prolonged, the accessibility to higher molecular weight lignin fragments is increased.[16] Another reasonable explanation for this phenomenon might be the different contents of carbohydrates. It has been documented that the carbohydrate chains linked to lignin can increase the hydrodynamic volume of lignin and therefore increase the apparent molar mass of the lignin when it is measured using GPC.[23] This was in line with the results of carbohydrate analysis as shown in Table 1. In addition, all three lignin fractions exhibited relatively narrow molecular weight distributions, as shown by $M_w/M_n < 4$. The polydispersities of MWL (2.27) and AL (2.32) were close to and lower than that of CEL (3.99). These data indicated that extraction of CEL produced the lignin fraction with a broader molecular weight distribution when compared to the extraction of MWL and AL under the conditions given in the present study. These were in accordance with the results obtained from a previous study.[14] The polydispersity of MWL was always lower than that of the corresponding CEL.

Table 2. Weight-Average Molecular Weights (M_w), Number-Average (M_n) Molecular Weights, and Polydispersity (M_w/M_n) of Lignin Fractions

	lignin fraction		
	MWL	CEL	AL
M_w	4250	3670	3200
M_n	1870	920	1380
M_w/M_n	2.27	3.99	2.32

FT-IR Analysis. Figure 2 shows the FT-IR spectra of the four lignin fractions obtained from the mild ball-milled triploid of *P. tomentosa* Carr. Obviously, the spectra of MWL, CEL, and AL were rather similar except for the intensities of the absorption bands. On the contrary, the spectrum of REL was quite different from them. As shown in Figure 2, a wide absorption band at 3448 cm^{-1} originated from the O—H stretching vibration in aromatic and aliphatic OH groups, whereas the bands at 2939, 2882, and 2841 cm^{-1} arise from the C—H asymmetric and symmetrical vibrations in methyl and methylene groups, respectively. The absorption at 1668 cm^{-1} is attributed to the carbonyl stretching in conjugated *p*-substituted aryl ketones. The bands at 1593, 1507, and 1421 cm^{-1}, corresponding to aromatic skeletal vibrations and the C—H deformation combined with aromatic ring vibration at 1462 cm^{-1}, are present in these four spectra.[34] Syringyl and condensed guaiacyl absorptions are obviously observed at 1329 cm^{-1}, whereas guaiacyl ring breathing with C=O stretching appears at 1270 cm^{-1}. The strong band at 1227 cm^{-1} is due to the C—C, C—O, and C=O stretching. Besides, the band at 1032 cm^{-1} is indicative of the aromatic C—H in-plane deformation. The maximum absorption band at 1124 cm^{-1} and the absorption bands at 850 and 832 cm^{-1} in these spectra

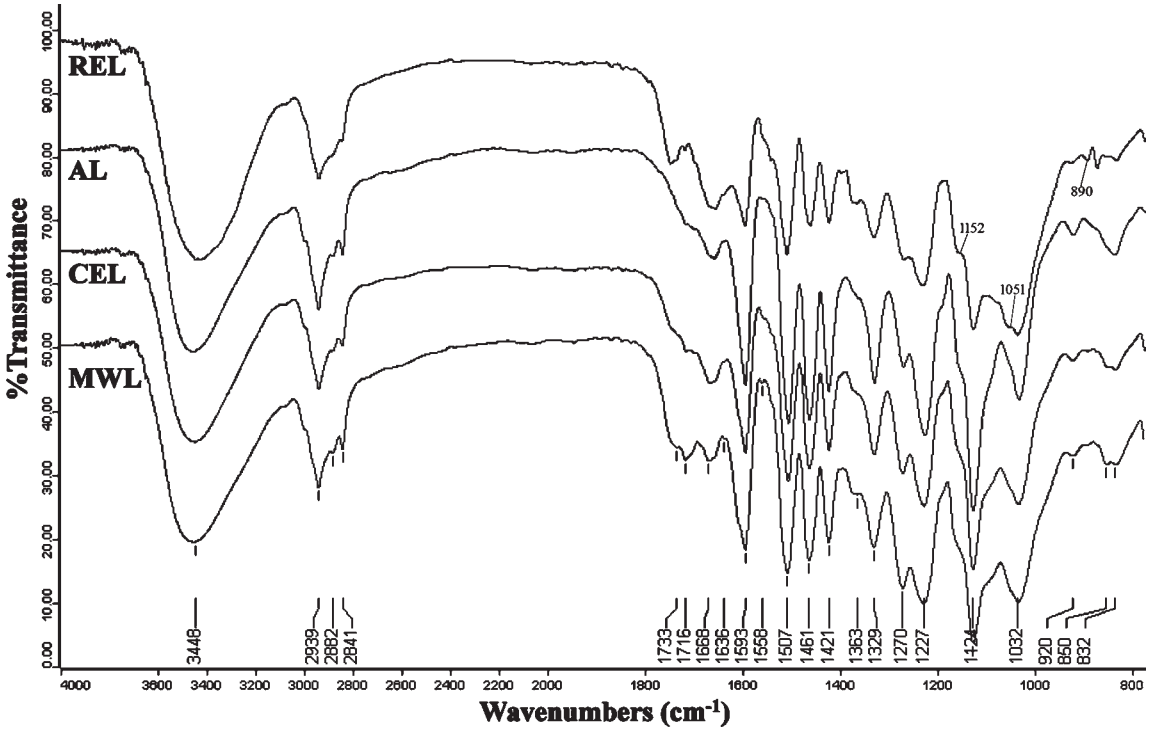

Figure 2. FT-IR spectra of milled wood lignin (MWL), cellulolytic enzyme lignin (CEL), residual enzyme lignin (REL), and alkaline lignin (AL).

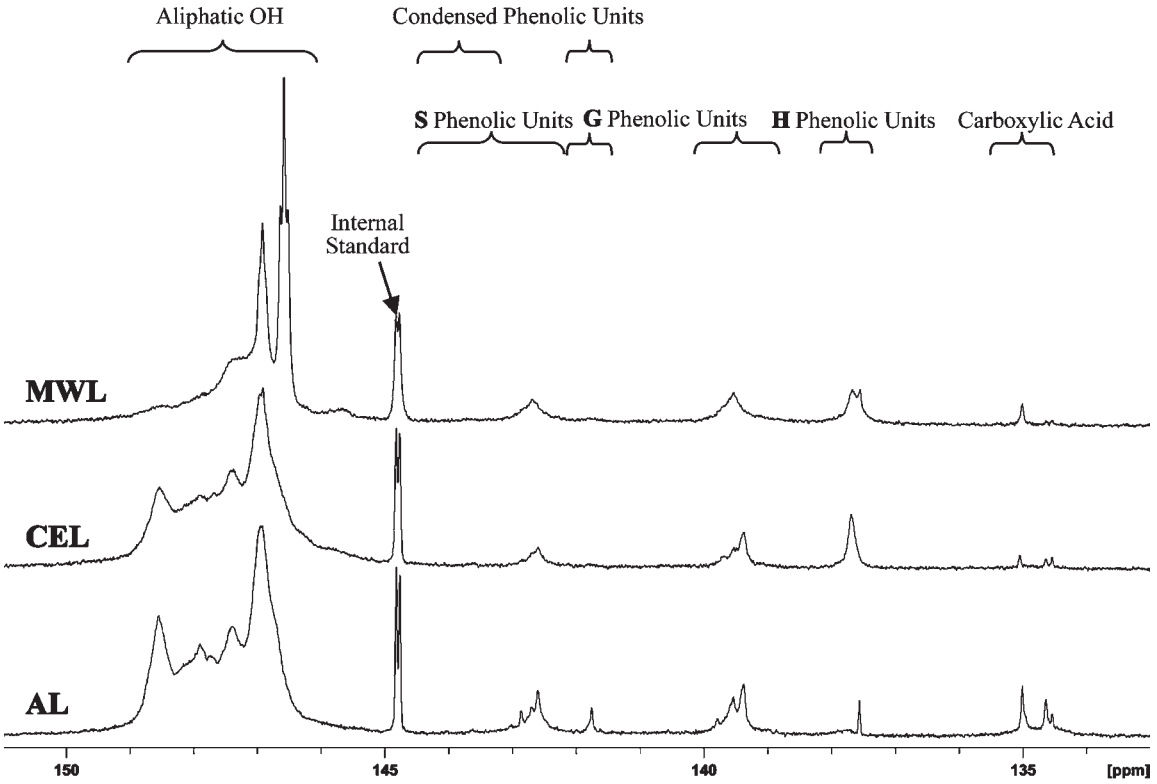

Figure 3. Quantitative ^{31}P NMR spectra of milled wood lignin (MWL), cellulolytic enzyme lignin (CEL), and alkaline lignin (AL).

Table 3. Content and Distribution of the Various Hydroxyl Groups (Millimoles per Gram) Quantified by ^{31}P NMR in the Lignin Fractions

		phenolic						
sample	aliphatic	condensed **S**	noncondensed **S**	condensed **G**	noncondensed **G**	noncondensed **H**	total phenolic	carboxylic acid
MWL	2.72	0.10	0.21	0.06	0.27	0.23	0.87	0.04
CEL	3.83	0.05	0.11	0.01	0.18	0.15	0.50	0.02
AL	4.41	0.04	0.24	0.06	0.30	0.02	0.66	0.18

indicated that all four lignin fractions are **GSH**-type lignins.[34] This result has also been confirmed by the following NMR analysis.

Other absorptions discussed herein were different in these four FT-IR spectra. A sharp signal at 1827 cm^{-1} was observed in the spectrum of REL, which is due to the incorporation of the enzyme. The bands at 1733 and 1716 cm^{-1} originated from the C═O stretch in unconjugated ketone, carbonyl, and ester groups.[34] The intensity of these two bands decreased from the spectra of MWL to CEL and to AL, and almost disappeared in that of REL. This means that the lignin fraction containing more C═O in unconjugated groups is preferentially extracted from the ball-milled meal. Furthermore, the intensity of the band at 1363 cm^{-1} showed the same tendency. It originated from the aliphatic C—H stretch in methyl (not in methoxyl) and phenolic hydroxyl groups. According to the lignin classification system of Faix,[34] the intensity ratio of 1507/1461 cm^{-1} increased from AL to CEL to MWL and to REL, indicating that the content of **S** units in these four lignin fractions decreased accordingly. All of these were well in line with the results obtained in the following NMR analysis of the corresponding spectra. In addition, as mentioned under Carbohydrate Analysis, REL contained the highest amount of carbohydrates. These associated carbohydrates also exhibited typical FT-IR absorptions, such as the obvious shoulder absorption at 1152 cm^{-1}, which is attributed to the association of xyloglucan.[35] The obstinate cellulose during the enzymatic treatment gives two typical FT-IR absorptions at 1051 and 890 cm^{-1}, which are assigned to the ν_{C-O-C} mode of the pyranose ring and β-glycosidic linkages, respectively.[35,36]

Quantitative ^{31}P NMR Spectra. To further investigate the structural features of the lignin fractions obtained, MWL, CEL, and AL were comparatively studied by quantitative ^{31}P NMR spectrometry (Figure 3). The aliphatic hydroxyls, condensed and uncondensed phenolic hydroxyls, and carboxylic acids were determined by phosphitylation of the lignins with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane.[20−23] Quantification was carried out via peak integration using cholesterol as an internal standard. Details of signal acquisition, assignment, and integration can be found elsewhere.[20−23] Table 3 lists the quantitative data on the distribution of the various OH groups of these three lignin fractions.

As can be seen from Table 3, MWL was observed to contain higher amounts of total phenolic OH, condensed phenolic OH,

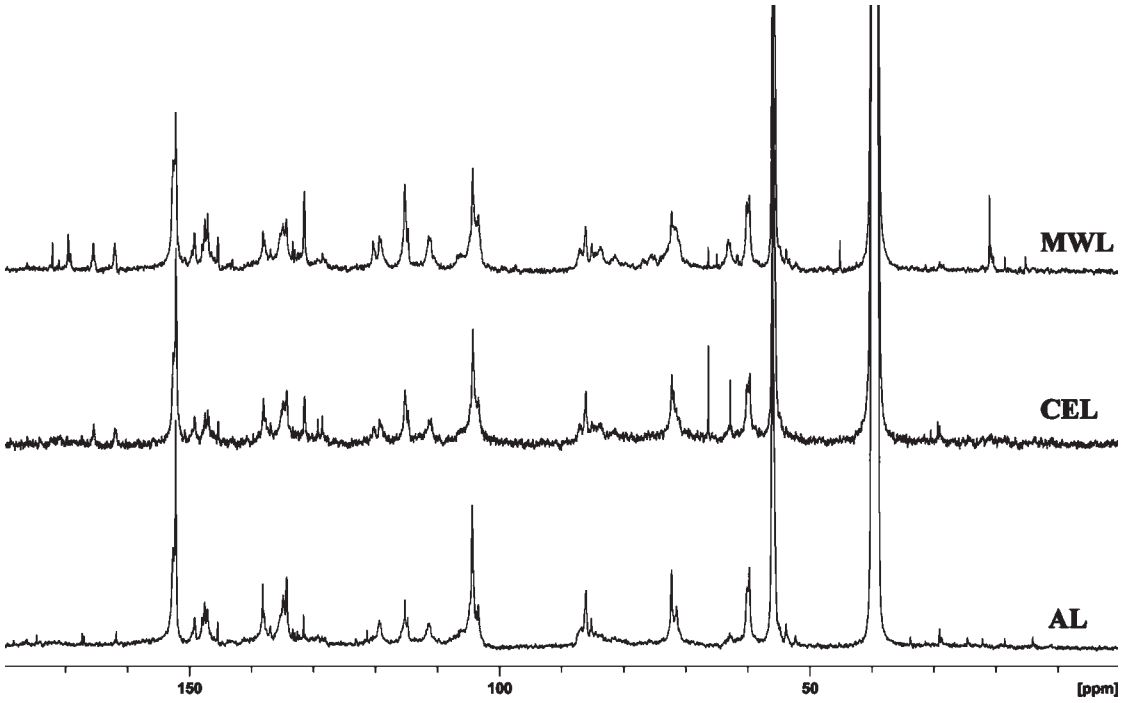

Figure 4. ^{31}C NMR spectra of milled wood lignin (MWL), cellulolytic enzyme lignin (CEL), and alkaline lignin (AL).

and noncondensed H units than CEL and AL. It appears that phenolic-rich lignin structures are preferentially isolated in the 96% dioxane extraction step.[6,14] This selective fractionation might explain the much lower contents of such phenolic OH observed for CEL and AL, because in the present work CEL and AL were isolated from the residue of MWL isolation. This result was consistent with the recent reports of Holtman et al.[31] and Guerra et al.[14] This means that the lignin isolated as MWL during the early part of ball milling (low-yield MWL) may originate mainly from lignin in the middle lamella.[8] Lignin in the compound middle lamella (CML) was found to have a higher degree of condensation and contain many more H units than lignin in the S2.[37,38] It is worth noting that *p*-hydroxyphenyl structures characterized herein essentially belong to *p*-hydroxybenzoic esters specifically linked to the lignin fractions.[22] Therefore, the value of noncondensed **H** units in AL was rather low, as in the case of alkali extraction the ester bonds were significantly cleaved.

Clearly, the content of carboxylic acids of AL, which was released from alkaline hydrolysis, was much higher than those of MWL and CEL. In addition, various aliphatic OH moieties can be observed in the quantitative ^{31}P NMR spectra of the lignins (Figure 3). In the spectra of MWL and CEL, a clear signal for 146−145.5 ppm appeared, whereas this signal disappeared from the spectrum of AL. This region was known to originate from carbohydrates,[23] and it was in accordance with the results obtained under Carbohydrate Analysis. Another remarkable difference in the aliphatic OH region of the ^{31}P NMR spectra was at 149−148 ppm. The intensity of the signals in this region increased from MWL to CEL and to AL. It has been shown that secondary hydroxyl groups in lignin, such as α-hydroxyl groups in *β*-aryl ether structures, give signal at this position (*erythro* at 148.2 ppm and *threo* at 147.8 ppm).[39] The high intensity of the signal at this region in AL can be explained by the cleavage of lignin−carbohydrate linkages during the alkaline treatment. Because the carbohydrates are most probably connected to the α-carbon of the lignin phenyl propane unit, the cleavage of this lignin−carbohydrate bond liberates a new α-hydroxyl group.[23]

^{31}C NMR Spectra. ^{13}C NMR spectroscopy is a reliable method to investigate the structure of the carbon skeleton in lignin, which provides a more comprehensive view of the entire lignin macromolecule. The ^{13}C NMR spectra for MWL, CEL, and AL are shown in Figure 4. Most of the observed signals have been previously assigned in wood lignin spectra.[19,40,41] The peaks between 172.1 and 162.0 ppm are attributed to C═O in carboxyl groups, which may originate from aliphatic carboxyl and aliphatic esters. The intensity of these signals decreased from the spectra of MWL to CEL and to AL. The same results were also observed in the FT-IR analysis.

In the aromatic region (153−103 ppm), the **S** units are detected by signals at 152.2 ppm (C-3/C-5, etherified) and 147.1 ppm (C-3/C-5, nonetherified), 138.1 ppm (C-4, etherified), 134.9 and 134.4 ppm (C-1, etherified), and 104.3 ppm (C-2/C-6). The **G** units produce signals at 149.2 ppm (C-3, etherified), 147.1 ppm (C-4, etherified), 145.4 ppm (C-4, nonetherified), 134.9 and 134.4 ppm (C-1, etherified), 119.4 (C-6), 115.3 ppm (C-5), and 111.5 ppm (C-2). The **H** units appear as two weak signals at 128.6 ppm (C-2/C-6) and 120.4 ppm (C-1). These results further confirmed that the lignin in triploid of *P. tomentosa* Carr. is **GSH**-type. Furthermore, the signals arising from the esterified *p*-hydroxybenzonic acid could be clearly noted in the spectra of MWL and CEL; however, they are sharply decreased in the spectrum of AL. This structural moiety is made evident by signals at 165.5 ppm (C═O), 162.0 ppm (C-4), 131.4 ppm (C-2/C-6), 121.3 ppm (C-1), and 114.9 ppm (C-3/C-5). These results were concordant with those from quantitative ^{31}P NMR spectra. All of these results showed that lignin in triploid of *P. tomentosa* Carr. contains a certain amount of esterified *p*-hydroxybenzonic acids.

In the oxygenated and nonoxygenated interunit linkages region of MWL, the *β*-*O*-4′ linkages were separately detected

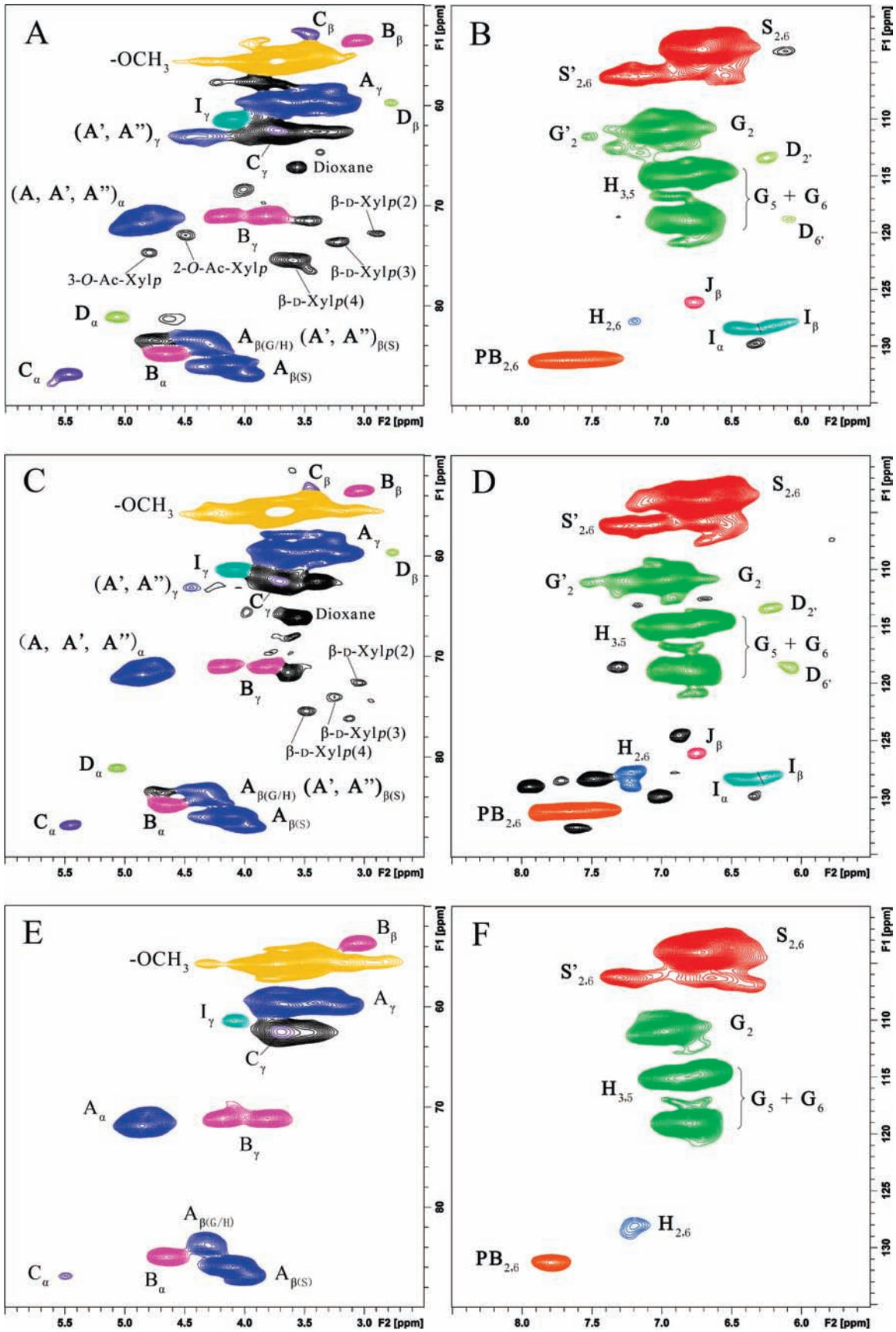

Figure 5. Side-chain (left column) and aromatic regions (right column) in the 2D HSQC NMR spectrum, δ_C/δ_H 50−90/2.5−6.0 and δ_C/δ_H 100−135/5.5−8.5, respectively: (A, B) milled wood lignin (MWL); (C, D) cellulolytic enzyme lignin (CEL); (E, F) alkaline lignin (AL). Symbols are taken from Figure 6. See Table 4 for signal assignment.

dx.doi.org/10.1021/jf2003865 |J. Agric. Food Chem. 2011, 59, 6605–6615

Table 4. Assignment of Main Lignin $^{13}C-^{1}H$ Cross-Signals in the HSQC Spectra of the Lignin Fractions

label	δ_C/δ_H	assignment
$\mathbf{C}_\beta$	52.9/3.73	$C_\beta-H_\beta$ in phenylcoumaran substructures (**C**)
$\mathbf{B}_\beta$	53.5/3.06	$C_\beta-H_\beta$ in resinol substructures (**B**)
$-OCH_3$	55.9/3.73	C−H in methoxyls
$\mathbf{D}_\beta$	59.6/2.78	$C_\beta-H_\beta$ in spirodienone substructures (**D**)
$\mathbf{A}_\gamma$	59.5−59.7/3.41−3.63	$C_\gamma-H_\gamma$ in β-*O*-4′ substructures (**A**)
$\mathbf{I}_\gamma$	61.4/4.10	$C_\gamma-H_\gamma$ in *p*-hydroxycinnamyl alcohol end groups (**I**)
$(\mathbf{A}', \mathbf{A}'')_\gamma$	63.2/4.33−4.46	$C_\gamma-H_\gamma$ in γ-acylated β-*O*-4′ substructures (**A**′ and **A**″)
$\mathbf{B}_\gamma$	71.1/3.82	$C_\gamma-H_\gamma$ in resinol substructures (**B**)
$\mathbf{B}_\gamma$	71.1/4.19	$C_\gamma-H_\gamma$ in resinol substructures (**B**)
$(\mathbf{A}, \mathbf{A}', \mathbf{A}'')_\alpha$	71.8/4.86	$C_\alpha-H_\alpha$ in β-*O*-4′ substructures (**A**) and γ-acylated β-*O*-4′ substructures (**A**′ and **A**″)
$\mathbf{D}_\alpha$	81.1/5.07	$C_\alpha-H_\alpha$ in spirodienone substructures (**D**)
$\mathbf{A}_{\beta(G/H)}$	83.9/4.29	$C_\beta-H_\beta$ in β-*O*-4′ substructures linked to **G** and **H** units (**A**)
$(\mathbf{A}', \mathbf{A}'')_{\beta(S)}$	83.9/4.29	$C_\beta-H_\beta$ in γ-acylated β-*O*-4′ substructures linked to **S** units (**A**′ and **A**″)
$\mathbf{B}_\alpha$	84.8/4.66	$C_\alpha-H_\alpha$ in resinol substructures (**B**)
$\mathbf{A}_{\beta(S)}$	85.8/4.12	$C_\beta-H_\beta$ in β-*O*-4′ substructures linked to **S** units (**A**)
$\mathbf{C}_\alpha$	86.8/5.47	$C_\alpha-H_\alpha$ in phenylcoumaran substructures (**C**)
$\mathbf{S}_{2,6}$	103.8/6.71	$C_{2,6}-H_{2,6}$ in etherified syringyl units (**S**)
$\mathbf{S}'_{2,6}$	106.2/7.27	$C_{2,6}-H_{2,6}$ in oxidized (C_α=O) syringyl units (**S**′)
$\mathbf{G}_2$	110.9/6.98	C_2-H_2 in guaiacyl units (**G**)
$\mathbf{G}'_2$	111.5/7.52	C_2-H_2 in oxidized (C_α=O) guaiacyl units (**G**′)
$\mathbf{D}_{2'}$	113.4/6.25	$C_{2'}-H_{2'}$ in spirodienone substructures (**D**)
$\mathbf{G}_5$	114.9/6.77	C_2-H_2 in guaiacyl units (**G**)
$\mathbf{D}_{6'}$	118.8/6.09	$C_{6'}-H_{6'}$ in spirodienone substructures (**D**)
$\mathbf{G}_6$	119.0/6.79	C_6-H_6 in guaiacyl units (**G**)
$\mathbf{J}_\beta$	126.1/6.77	$C_\beta-H_\beta$ in cinnamaldehyde end groups (**J**)
$\mathbf{H}_{2,6}$	127.8/7.19	$C_{2,6}-H_{2,6}$ in *p*-hydroxyphenyl units (**H**)
$\mathbf{I}_\beta$	128.3/6.22	$C_\beta-H_\beta$ in *p*-hydroxycinnamyl alcohol end groups (**I**)
$\mathbf{I}_\alpha$	128.5/6.45	$C_\alpha-H_\alpha$ in *p*-hydroxycinnamyl alcohol end groups (**I**)
$\mathbf{PB}_{2,6}$	131.2/7.67	$C_{2,6}-H_{2,6}$ in *p*-hydroxybenzoate substructures (**PB**)

by signals at 87.1 (C_β in **S** β-*O*-4′ *threo*), 86.1 (C_β in **S** β-*O*-4′ *erythro*), 85.1 (C_β in **G**/**H** β-*O*-4′ *threo*), 83.8 (C_β in **G**/**H** β-*O*-4′ *erythro*), 72.3 (C_α in β-*O*-4′ **G** and **S** *erythro*), 71.5 (C_α in β-*O*-4′ **G** and **S** *threo*), 63.2 (C_γ in γ-acylated β-*O*-4′), and 59.7 (C_γ in β-*O*-4′) ppm. It has been reported that in the degradation of β-*O*-4′ structures, preferential *erythro* degradation proceeded under all milling conditions.[15] However, in this work the *erythro* form of β-*O*-4′ structures in MWL was clearly observed. On the basis of these results, it was deduced that the mild ball-milling conducted in the present work had slight effect on the degradation of the macromolecular lignin. Moreover, the weak signal at 61.7 ppm is assigned to C_γ in the *p*-hydroxycinnamyl alcohol end group. The strong signal at 55.9 ppm is attributed to the $-OCH_3$ groups in **S** and **G** units. The two small signals at 53.8 and 52.2 ppm belong to the C_β in β-β' and β-5′ structures, respectively. In addition, the signals between 15.2 and 29.1 ppm represent the γ-methyl, as well as the α- and β-methylene, groups in *n*-propyl side chains of the lignin preparations.

The incorporation of carbohydrates in the lignin fractions could also be detected in Figure 4. Specifically, the signals between 90 and 102 ppm represent the anomeric region of carbohydrates. The signal at 81.6 ppm originated from C-4 of uronic acid units in pentosans. Besides, two signals at 76.9 and 75.5 ppm are due to the C-4 and C-3 of xylopyranoside units in xylan, respectively. The changing of the intensity of all above-mentioned signals in these three spectra could be easily distinguished. The reasonable explanations for these changes have been given elsewhere in this paper.

2D HSQC NMR Spectra. Two-dimensional $^{1}H-^{13}C$ NMR (2D NMR) has been able to provide important structural information and has allowed for the resolution of otherwise overlapping resonances observed in either the ^{1}H or ^{13}C NMR spectra.[26] In the present study, to understand the detailed structures of these lignin fractions, MWL, CEL, and AL were characterized by 2D HSQC NMR techniques. A HSQC NMR spectrum of lignin shows three regions corresponding to aliphatic, side chain, and aromatic $^{13}C-^{1}H$ correlations. The aliphatic (nonoxygenated) region shows signals with no structural information and therefore is not discussed here. The side-chain (δ_C/δ_H 50−90/2.5−6.0) and the aromatic (δ_C/δ_H 100−135/5.5−8.5) regions of the HSQC spectra of these three lignin fractions are shown in Figure 5. The main lignin cross-signals assigned in the HSQC spectra are listed in Table 4, and the main substructures are depicted in Figure 6.

Clearly, the HSQC spectra of MWL and CEL were similar. In the side-chain regions of the HSQC spectra of these two lignin fractions, cross-signals of methoxyls (δ_C/δ_H 55.9/3.73) and side chains in β-*O*-4′ aryl ether linkages were the most prominent. The $C_\alpha-H_\alpha$ correlations in β-*O*-4′ substructures were observed at δ_C/δ_H 72.2/4.86 (structures **A**, **A**′ and **A**″). The $C_\beta-H_\beta$ correlations corresponding to the *erythro* and *threo* forms of the **S**-type β-*O*-4′ substructures can be distinguished at

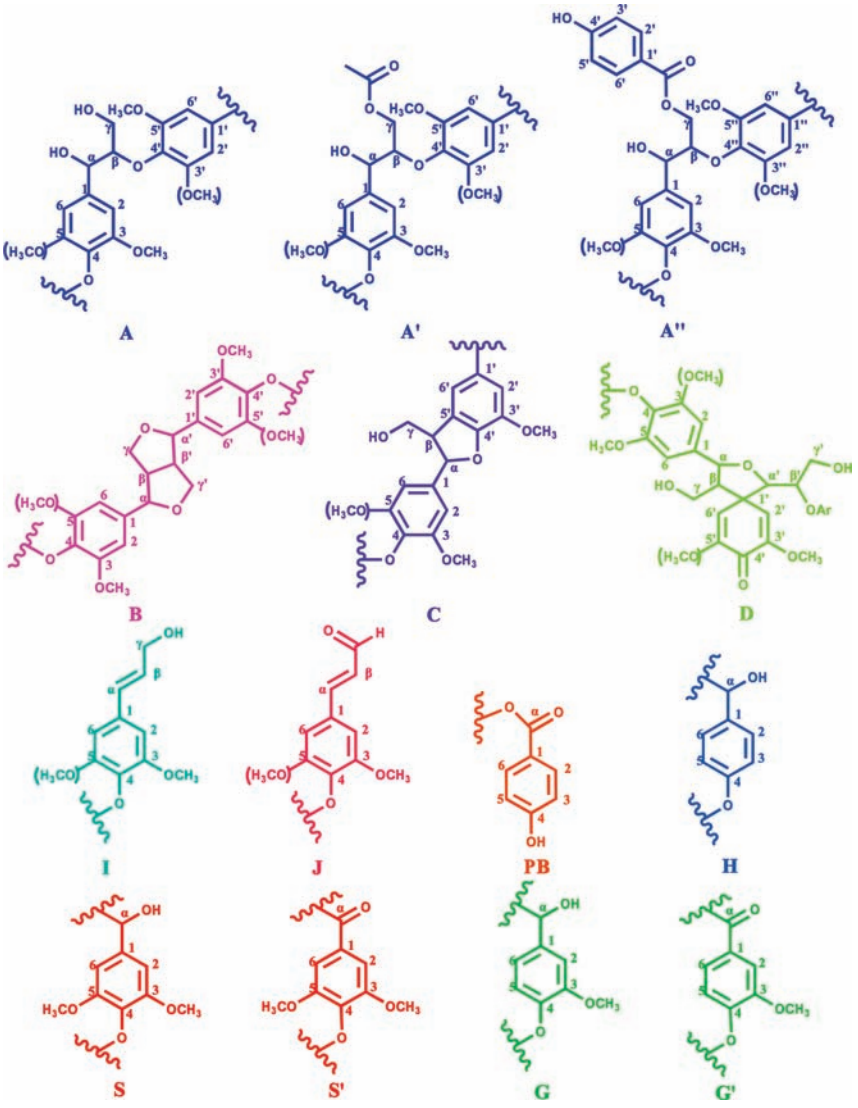

Figure 6. Main classical and acylated substructures, involving different side-chain linkages, and aromatic units identified by 2D NMR of milled wood lignin (MWL), cellulolytic enzyme lignin (CEL), and alkaline lignin (AL): (**A**) *β-O*-4′ aryl ether linkages with a free −OH at the *γ*-carbon; (**A′**) *β-O*-4′ aryl ether linkages with acetylated −OH at *γ*-carbon; (**A″**) *β-O*-4′ aryl ether linkages with *p*-hydroxybenzoated −OH at *γ*-carbon; (**B**) resinol substructures formed by *β-β′*, α-*O*-*γ′*, and *γ-O*-α′ linkages; (**C**) phenylcoumaran substructures formed by *β*-5′ and α-*O*-4′ linkages; (**D**) spirodienone substructures formed by *β*-1′ and α-*O*-α′ linkages; (**I**) *p*-hydroxycinnamyl alcohol end groups; (**J**) cinnamaldehyde end groups; (**PB**) *p*-hydroxybenzoate substructures; (**H**) *p*-hydroxyphenyl units; (**G**) guaiacyl units; (**S**) syringyl units; (**S′**) oxidized syringyl units with a C_α ketone; (**G′**) oxidized guaiacyl units with an α-ketone.

δ_C/δ_H 85.8/4.12 and 86.8/3.99, respectively. These correlations shifted to δ_C/δ_H 83.9/4.29 in structure **A** linked to **G**/**H** lignin units and *γ*-acylated *β-O*-4′ aryl ether substructures (**A′** and **A″**) linked to **S** lignin unit. The $C_\gamma-H_\gamma$ correlations in structure **A** were observed at δ_C/δ_H 59.5−59.7/3.41−3.64. Moreover, the $C_\gamma-H_\gamma$ correlations in *γ*-acylated lignin units (**A′** and **A″**) were also observed at δ_C/δ_H 63.2/4.33−4.46. These signals indicated that lignin in triploid of *P. tomentosa* Carr. is partially acylated at the *γ*-carbon in *β-O*-4′ aryl ether linkages of the side chains. It should be mentioned that *γ*-acylated **G** and **S** units have been detected in the lignins of many species.[19] For instance, sinapyl acetate is implicated similarly as a monomer in lignification in kenaf bast fibers.[42]

Besides *β-O*-4′ ether substructures, the other linkages observed were *β-β′* (resinol, **B**), *β*-5′ (phenylcoumaran, **C**), and *β*-1′ (spirodienone, **D**) linkages. Strong signals for resinol substructures **B** were observed with their $C_\alpha-H_\alpha$, $C_\beta-H_\beta$, and the double $C_\gamma-H_\gamma$ correlations at δ_C/δ_H 84.8/4.66, 53.6/3.06, and 71.1/4.19 and/3.82, respectively. Phenylcoumaran substructures **C** were found in lower amounts. The signals for their $C_\alpha-H_\alpha$ and $C_\beta-H_\beta$ correlations were discovered at δ_C/δ_H 86.8/5.47 and 52.9/3.47, respectively, whereas the $C_\gamma-H_\gamma$ correlations were overlapped with other signals around δ_C/δ_H 62.5/3.73. Moreover, small signals corresponding to spirodienone substructures **D** could also be observed; their $C_\alpha-H_\alpha$ and $C_\beta-H_\beta$ correlations were detected at δ_C/δ_H 81.1/5.07 and 59.6/2.78, respectively.

In addition to these linkages, the $C_\gamma-H_\gamma$ correlations (at δ_C/δ_H 61.4/4.10) in *p*-hydroxycinnamyl alcohol end groups (substructure **I**) and various signals from the associated carbohydrates were also found in the side-chain regions of the HSQC spectra of MWL and CEL. It is well-known that acetylated 4-*O*-methylgluconoxylan is a major hemicellulosic component in hardwoods, and acetyl groups frequently acylate the C-2 and

dx.doi.org/10.1021/jf2003865 |*J. Agric. Food Chem.* 2011, 59, 6605–6615

Table 5. Percentage of the Main Interunit Linkages (Referred to as the Total Side Chains) and S/G Ratio of the Lignin Fractions

	linkage relative abundance				
sample	*β*-*O*-4′	*β*-*β*′	*β*-5′	*β*-1′	S/G
MWL	83.2	12.7	2.6	1.4	1.43
CEL	84.5	12.2	2.1	1.2	2.29
AL	81.1	16.4	2.1	0.4	2.83

C-3 positions.[43] The side-chain regions of the HSQC spectrum of MWL showed a strong 2-*O*-Ac-*β*-D-Xyl*p* C_2-H_2 correlation at δ_C/δ_H 73.0/4.49 and a 3-*O*-Ac-*β*-D-Xyl*p* C_3-H_3 correlation at δ_C/δ_H 74.7/4.79. Their corresponding anomeric correlations (C_1-H_1) were found at δ_C/δ_H 99.4/4.53 and 101.6/4.34, respectively (not shown). However, these signals disappeared in the HSQC spectra of CEL and AL. It could be deduced that the lignin fraction containing acetylated 4-*O*-methylgluconoxylan is preferentially isolated in the 96% dioxane extraction step. Furthermore, in the HSQC spectra of both MWL and CEL the signals from *β*-D-Xyl*p* were evidently noted, with its C_2-H_2, C_3-H_3, and C_4-H_4 correlations at δ_C/δ_H 72.7/2.89, 73.6/3.22, and 75.5/3.59, respectively. Its corresponding anomeric correlation (C_1-H_1) was found at δ_C/δ_H 103.2/4.21 (not shown). It should be noted that all of the substructures observed in the side-chain regions of the HSQC spectra of MWL and CEL were also clearly distinguished in that of AL, except for the signals from associated carbohydrates and the spirodienone substructures **D**. Strictly speaking, the correlations from spirodienone substructures **D** could only be seen at lower contour levels (not shown). The results observed by HSQC herein were also well consistent with these obtained in carbohydrate analysis.

In the aromatic regions of the HSQC spectra of MWL, CEL, and AL, cross-signals from *p*-hydroxyphenyl (**H**), syringyl (**S**), and guaiacyl (**G**) lignin units could be observed. The **S**-lignin units showed a prominent signal for the $C_{2,6}-H_{2,6}$ correlation at δ_C/δ_H 103.8/6.71, whereas the **G** units showed different correlations for C_2-H_2, C_5-H_5, and C_6-H_6 at δ_C/δ_H 110.9/6.99, 114.9/6.77, and 119.0/6.79, respectively. Signals corresponding to $C_{2,6}-H_{2,6}$ correlations in C_α-oxidized **S** units (**S**′) (δ_C/δ_H 106.2/7.27) were present in all of the HSQC spectra of these three lignin fractions. The correlation for the C_2-H_2 in oxidized α-ketone structures **G**′ was observed in the spectra of MWL and CEL but disappeared in that of AL. The $C_{2,6}-H_{2,6}$ aromatic correlations from **H** units were clearly observed at δ_C/δ_H 127.8/7.19, but the $C_{3,5}-H_{3,5}$ position correlations were overlapped with those from guaiacyl 5-positions.

Other significant signals in the aromatic regions of the HSQC spectra of these three fractions were assigned to *p*-hydroxybenzoate substructures (**PB**), *p*-hydroxycinnamyl alcohol end groups (**I**), and cinnamaldehyde end groups (**J**), as well as spirodienone substructures (**D**). The $C_{2,6}-H_{2,6}$ correlations of **PB** were observed as a strong signal at δ_C/δ_H 131.2/7.67. Actually, **PB** is considered to exclusively acylate the *γ*-position of lignin side chains, analogously with *p*-coumarates (pCA) in grasses.[44] Morreel et al.[45] had found that only **S** units are *γ*-*p*-hydroxybenzoylated in poplar lignin and that the sinapyl *p*-hydroxybenzoate is produced enzymatically and used as an authentic monomer for lignification in poplar.

In the aromatic regions of the HSQC spectra of MWL and CEL, the signals for the $C_{2'}-H_{2'}$ and $C_{6'}-H_{6'}$ correlations of spirodienone substructure **D** were clearly discovered at δ_C/δ_H 113.4/6.25 and 118.8/6.09, respectively. However, these signals could only be seen at lower contour levels in the spectrum of AL (not shown). The signals for the $C_\alpha-H_\alpha$ and $C_\beta-H_\beta$ correlations of substructures **I** were clearly discovered at δ_C/δ_H 128.5/6.45 and 128.3/6.22, whereas these correlations were found at δ_C/δ_H 126.1/6.77 and 153.4/7.60 (not shown) for substructure **J**, respectively, in the HSQC spectra of MWL and CEL. On the contrary, even at lower contour levels only $C_\alpha-H_\alpha$ correlations of substructure **I** could be detected in the spectrum of AL (not shown).

The different structural features among these three lignin fractions were quantitatively investigated. The percentages of lignin side chains involved in the primary substructures **A**−**D** and the **S**/**G** ratios were calculated from the corresponding HSQC spectra, and the results are listed in Table 5. As expected, the main substructures present in all of these lignin fractions were the *β*-*O*-4′ linked ones (**A**, **A**′, and **A**″), which ranged from 81.1 to 84.5%. The *β*-*β*′ resinol substructure (**B**) appeared to be the secondary major substructure, comprising from 12.2 to 16.4%. The *β*-5′ phenylcoumaran substructure (**C**) and *β*-1′ spirodienone substructure (**D**) were calculated as minor amounts, ranging from 2.1 to 2.6% and from 0.4 to 1.4%, respectively. It is worth noting that the percentages of lignin side chains involved in the primary substructures **A**−**D** were very similar for MWL and CEL. However, the **S**/**G** ratios were different from each other and were estimated to be 1.43, 2.29, and 2.83 for MWL, CEL, and AL, respectively.

In summary, CEL and AL have been sequentially isolated from the residual wood meal after extraction of MWL from the mild ball-milled triploid of *P. tomentosa* Carr. The results indicated that the combination of MWL and CEL isolated from the mild ball-milled wood potentially represented the source of native lignin, which has typical structural features of lignin. It has also been found that lignins in different layers of the plant cell walls of triploid of *P. tomentosa* Carr. varied in both interunit linkages and chemical structures.

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Funding Sources

We are grateful for the financial support of this research from the Specific Programs in Graduate Science and Technology Innovation of Beijing Forestry University (No. BLYJ201113), State Forestry Administration (200804015, 20100400706), Major State Basic Research Projects of China (973-2010CB732204), National Science Foundation of China (30930073), and China Ministry of Education (111).

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