JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Lignin Changes after Steam Explosion and Laccase-Mediator Treatment of Eucalyptus Wood Chips

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ABSTRACT: *Eucalyptus globulus* chips were steam exploded followed by treatment with a laccase-mediator system (LMS) under different experimental conditions. Removal of hemicelluloses and, to a lesser extent, lignin was observed. Thermogravimetic analyses of whole meal obtained from chips before and after steam explosion indicated an increase in lignin degradation temperature due to lignin condensation. In contrast, application of LMS treatment caused a reduction in lignin and polysaccharide degradation temperatures. Lignins were isolated from wood samples before and after each treatment and analyzed by 2D NMR and ^{13}C NMR. An increase in carboxyl and phenolic hydroxyl groups and a significant decrease in β-O-4 structures were found in steam-exploded samples. The most relevant changes observed after laccase treatment were increased secondary OH and degree of condensation.

KEYWORDS: *Eucalyptus globulus*, steam explosion, laccase-mediator system, thermogravimetric analyses (TGA), milled wood lignin (MWL), 2D NMR, HSQC, quantitative ^{13}C NMR, lignin thermal properties, lignin chemistry

INTRODUCTION

In steam explosion, biomass is subjected to high-pressure steam followed by a rapid decompression, which forces the fibrous material to "explode" into component fibers and fiber bundles. Therefore, steam explosion is effective in opening up biomass structure and increases chemical reactivity for further operations.[1] Steam explosion has also been proposed as a pretreatment method to enhance the susceptibility of cellulose fibers to enzymatic hydrolysis to produce fermentable sugars [2,3] and to assist in the fractionation of its components.[4,5]

When no other chemicals are added to the process, the main reaction that takes place is an autohydrolysis of the ether bonds present in polysaccharides and lignin. The high-temperature steam leads to the release of acetic acid from wood components, which catalyze hydrolytic reactions of constituent polymers. These reactions result in a loss of hemicelluloses and amorphous cellulose and decrease the amount of β-O-4 structure in lignin.[6−9]

During steam explosion and other related processes, lignin is one of the most recalcitrant polymers present. Lignin is a macromolecule containing aromatic and aliphatic moieties. Despite extensive investigations, the structure of lignin, especially that in hardwoods, is not completely understood. Lignin isolation from wood and further structural characterization is usually difficult because it is covalently linked to carbohydrates, forming a complex lignin−carbohydrate network.[10,11] Björkman[12] used aqueous dioxane solvent to obtain a milled wood lignin (MWL), which has been used as a reference for native lignin. In general, the most common techniques for lignin isolation are based on extraction of ball-milled wood by neutral solvents.

Changes occurring in MWL from aspen, birch, and pine after steam explosion have been studied by nuclear magnetic resonance (NMR), thioacidolysis, size exclusion chromatography (SEC), nitrobenzene oxidation, and other techniques. A reduction in the content of β-O-4 structures due to depolymerization reactions and an increase in the content of C−C condensed structures have been observed.[6,7,13] Furthermore, Li et al.[13] reported an increase in the average molecular weight and a more heterogeneous lignin structure with the severity of the steam explosion conditions. Such observations were explained by lignin condensation reactions.

The effect of steam explosion on the structure of lignin in wood, its properties, and tolerance for downstream delignification are subjects that have been addressed in the literature. However, systematic studies are still needed to elucidate the effect of wood species, steam explosion severity, method of lignin isolation, etc. Most of the published results have been obtained from alkali-extracted lignins, and data on the structure of the residual lignin are limited. Therefore, the aim of this work is to elucidate the relationship between the intensity of the steam explosion process and key structural features of MWL lignin from *Eucalyptus globulus*, as well as the susceptibility of the exploded material to enzymatic oxidation by laccases. Related research questions are relevant when the use of steam explosion assisted with enzymatic treatment is considered as a choice for effective transformation of lignocellulosics in biorefinery or biopulping platforms. For instance, in the production of bioethanol, the laccase-mediator system (LMS) treatment has been used to remove inhibiting compounds.[14,15] In paper production, LMS treatments between steam explosion and kraft pulping increase the delignification rate,[16] even when applied on substrates with high lignin content.

As such, changes in the structure of lignin isolated from *E. globulus* chips were analyzed by 2D NMR and ^{13}C NMR

Received: April 21, 2011
Revised: July 9, 2011
Accepted: July 12, 2011
Published: July 12, 2011

before and after steam explosion. Also, changes in lignin after laccase treatment were investigated to elucidate the impact of steam explosion in downstream enzymatic treatments. Finally, thermogravimetric analyses (TGA) were carried out on whole meal biomass before and after steam explosion and enzyme treatment to determine the effects of process conditions on the thermal properties of the lignocellulosic material.

MATERIALS AND METHODS

All of the chemicals used were of reagent grade and were obtained from Sigma-Aldrich.

Wood chips of *E. globulus* were kindly supplied by La Montañanesa pulp mill (Torraspapel group, Spain). The laccase used in enzymatic treatments (Novozym 51003) was supplied as a donation by Novozymes (Bagsvaerd, Denmark).

Steam Explosion Treatment. The *E. globulus* chips were dried and homogenized at room temperature for 2 weeks, and the moisture content was determined (ISO 638). The size of the chips was around 2 × 3 × 0.5 cm. Before the steam explosion treatment, the chips were separated into eight 500 g batches. Four of them were immersed in distilled water during 16 h at 25 °C; the others were kept at room temperature. These two types of samples were used to evaluate the effect of impregnation on the subsequent steam explosion treatment. The steam explosion treatment was performed in a 26 L stainless steel vessel by charging steam to operation temperature and pressure of 183 °C and 10 kg-f/cm^2, respectively. After these conditions had been reached (typically within 3 min after steam injection), variable residence times were applied. At the end of the steam treatment, the pressure was reduced to 6 kg-f/cm^2, and then the chips were discharged into an expansion (blowing) tank at atmospheric pressure. After discharging, the chips were washed with water. The steam explosion factors evaluated in this work included the use of preimpregnation of wood chips in water, the number of steam explosion cycles (one or two), and the time employed during the first cycle (5 or 10 min). Table 1 summarizes the conditions used and the nomenclature used thereafter to indicate the respective steam-exploded (SE) samples.

The severity factor, S_0, for each treatment was calculated according to eq 1 defined by Overend and Chornet[17] as a function of the temperature (T, °C) and the duration of the treatment (t, min).

$$S_0 = \log(e^{T-100/14.75} \times t) \quad (1)$$

Enzymatic Treatment. The steam-exploded sample denoted SE4′ was treated with a laccase-mediator system. Enzyme treatment was performed in 500 mL flasks with air bubbling placed in a temperature-controlled shaker operating at 100 rev/min. The LMS consisted of the laccase enzyme (30 U of laccase per gram of dry SE wood) and HBT (5 mM), which was used as mediator. The temperature, time, and pH of treatment were 70 °C, 30 min, and 6 (100 mM phosphate buffer), respectively. The final concentration of the substrate (steam-exploded chips) was 7% (w/v). Here, 1 U of laccase is defined as the amount of laccase required to convert 1 μmol/min of ABTS to its cationic radical (0.1 M phosphate buffer, pH 6, at 24 °C). A control experiment with no LMS was carried out (sample SE4′+control-L).

Thermogravimetric Analysis. TGA of wood meal samples (ground to pass a 20 mesh screen) were conducted in a TA Instruments model Q-500. Fifteen milligrams of the respective sample were used, and the thermograms were obtained by ramping the temperature from 30 to 600 °C at a heating rate of 10 °C/min. An air atmosphere was maintained at 60 mL/min flow rate.

Isolation of Milled Wood Lignin. A modified MWL isolation procedure[18] was used to isolate lignin from *E. globulus* wood. The same procedure was employed in the isolation of lignin from wood after steam explosion and enzymatic treatments. The detailed procedure followed in the isolation of MWL is shown schematically in Figure 1. Briefly, chips were ground to pass a 20 mesh screen in a Wiley mill and extracted with an ethanol/toluene mixture 1:2 (v/v). The extractive-free wood meal was submitted to an alkaline extraction mainly to remove tannins. The obtained extracted wood was then thoroughly washed until pH-neutral, dried under vacuum, and subjected to ball-milling in a planetary ball mill (Fritsch Planetary Mill P7). The resulting wood residue was extracted with dioxane/water solution (96:4) during 72 h. The dioxane extracts were combined and concentrated under vacuum. The extracted MWL was dried under vacuum and stored in a desiccator for further analyses.

The liquor from the alkali extraction was acidified at pH 2 by adding H_2SO_4 and the precipitated lignin recovered by filtration, water-washed until pH-neutral, air-dried, and stored for further analyses.

The milling time and intensity (as given by the number of balls per gram of sample) were adjusted to obtain yields between 30 and 50%. This was done to ensure MWL samples that could be taken as close as possible to the native lignin.[11] Also, processing the material to a similar isolation yield facilitates a better comparison between different samples. Acetylation of the MWL, for NMR analysis, was carried out according to a procedure reported earlier by Capanema et al.[19]

Carbohydrate Composition and Klason Lignin. The carbohydrate composition was determined by high-performance liquid chromatography (HPLC) analyses after acid hydrolysis of the wood samples.[20] The equipment used consisted of an Agilent Technology 1200 series RID with an Aminex HPX-87H column operated at 50 °C with a mobile phase consisting of 5 mM sulfuric acid pumped at a rate of 0.6 mL/min. Klason lignin content in the wood samples and solid fractions obtained during the isolation procedure (see Figure 1) were determined according to TAPPI T222 om-88 standard.

NMR Analyses. NMR spectra were recorded on a Bruker Avance 300 MHz spectrometer at 25 °C using DMSO-d_6 as the solvent under conditions optimized and reported earlier.[19,21] Chemical shifts were referenced to TMS (0.0 ppm). Heteronuclear single-quantum coherence (HSQC) analysis was performed with a 5% solution of lignin by applying a 90° pulse width, a 512 ms acquisition time, a 1.2 s pulse delay, and $^1J_{C-H}$ of 147 Hz. For the quantitative ^{13}C NMR, the concentration

Table 1. Summary of Conditions Used in Steam Explosion of *Eucalyptus globulus* Chips

		residence time during steam explosion (min)		
sample	water soaking prior to steam explosion (16 h, 25 °C)	cycle 1	cycle 2	severity factor (S_0)
control	no			
SE1	no	5		3.14
SE1′	yes	5		3.14
SE2	no	10		3.44
SE2′	yes	10		3.44
SE3	no	5	3	3.35
SE3′	yes	5	3	3.35
SE4	no	10	3	3.56
SE4′	yes	10	3	3.56
SE4′+laccase [a]	yes	10	3	3.56
SE4′+control-L [b]	yes	10	3	3.56

[a] SE4′+laccase, same as SE4′ but included a treatment of the chips after the second cycle of steam explosion with laccase-mediator system. [b] SE4′+control-L, a control sample after same treatment used in SE4′ +laccase but with no laccase-mediator system added.

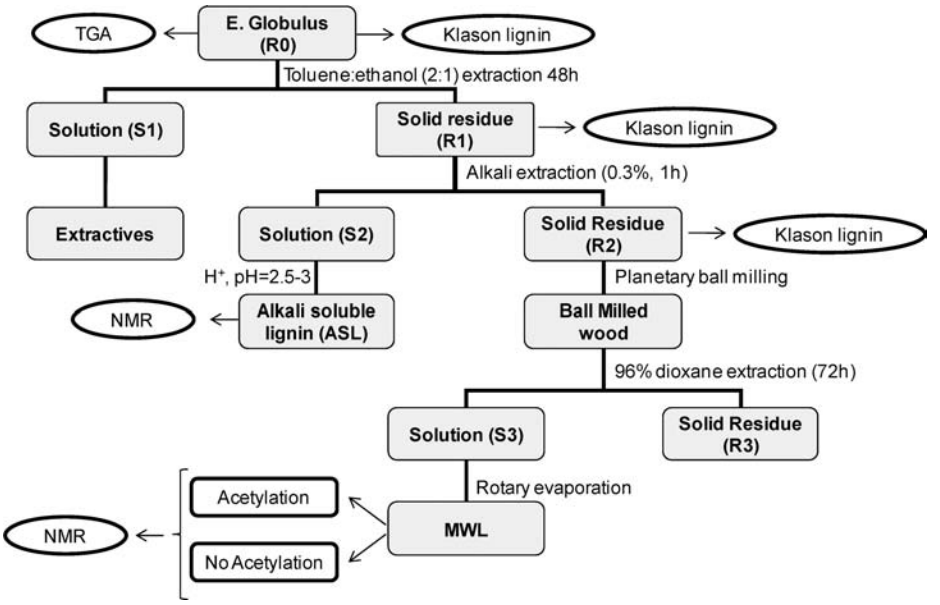

Figure 1. Schematic block diagram of milled wood lignin isolation indicating main analyses performed such as Klason lignin, TGA, 2D and ^{13}C NMR (ovals), and samples with respective nomenclature (squares).

of lignin was ~20%; a 90° pulse width, 1.4 s acquisition time, and 1.7 s relaxation delay were used. Chromium(III) acetylacetonate (0.01 M) was added to the lignin solutions to provide complete relaxation of all nuclei. A total of 20000 scans were collected. A quantitative ^{1}H NMR spectrum of acetylated MWL was recorded at a lignin concentration of ~20% in DMSO, with a 90° pulse width and a 1.3 s acquisition time.

RESULTS AND DISCUSSION

Thermal Properties. Themogravimetric analyses were used to characterize the different wood chip samples. The analysis was based on the fact that each of the fiber cell wall polymers has a distinctive degradation temperature and rate of energy release upon thermal breaking and combustion. During TGA the lignocellulosic samples were kept under air atmosphere, and two main temperature ranges of degradation were observed, 250−350 and 400−500 °C. These are attributed to degradation of polysaccharides and lignin, respectively.[22] Relative to cellulose and hemicelluloses, which are aliphatic structures, the higher degradation temperature for lignin is ascribed to its aromatic structure. For hemicelluloses and cellulose two consecutive processes have been determined during biomass combustion. The first one corresponds to a thermal decomposition (volatilization) that produces volatiles and char. The second reaction involves char oxidation in the presence of oxygen. Volatilization and oxidation of lignin-derived char are considered to be concurrent processes.[23]

TGA spectra for samples before (control) and after the most intense steam explosion treatment (SE4′) were analyzed, and the first derivate of the thermograms is shown in Figure 2A. Shifts in degradation temperature were taken as indirect indication of collective changes that occurred in wood components during steam explosion. After steam explosion, an increase by 20 °C in the second degradation temperature (at about 490 °C), corresponding to lignin, was observed, whereas the degradation temperature for polysaccharides (at about 330 °C) remained approximately constant. Generally, the lower the molecular weight and/or crystallinity, the lower the temperature for polymer degradation.[24] Therefore, the observed increase in lignin degradation temperature suggests possible condensation reactions during steam explosion. Furthermore, a shoulder around 280 °C was observed in the control sample (untreated wood). This shoulder can be attributed to the degradation of xylans, which are the main hemicelluloses in the hardwood considered in this study[25] but are extensively reduced upon steam explosion.[13] This observation was corroborated by carbohydrate analyses of related samples that showed a progressive reduction of xylans in the exploded samples (see Table 2). A reduction of the amount of lignin was also observed upon steam explosion treatment.

The degradation temperatures attributed to polysaccharides and lignin after the different steam explosion treatments are shown in Figure 2B. It can be observed that after steam explosion the degradation temperature corresponding to polysaccharides remained approximately constant, at about 333 °C, whereas the temperature for lignin degradation increased with the severity of the treatment. For instance, compared with steam explosion in a single cycle, two cycles raised the lignin degradation temperature (see data for SE1−SE3, SE1′−SE3′, SE2−SE4, or SE2′−SE4′ pairs). Except for sample pair SE3−SE4, the same observation applies when the residence time during steam treatment was increased (see data for SE1−SE2, SE1′−SE2′, and SE3′−SE4′ pairs). The higher TGA degradation temperature observed with the number of cycles or with the residence time is hypothesized to be the result of lignin condensation. It is also possible that the strong conditions used during steam explosion may promote removal of carbohydrates linked to lignin (lignin−carbohydrate complexes, LCC), as has been reported by others.[13] This hypothesis is supported by the decrease in intensity of the NMR signal at 81.25/5.1 ppm assigned to benzyl ether linkages between lignin and xylan.[26]

Laccase treatment of chips after steam explosion under the most severe conditions (sample SE4′+laccase) changed further the respective degradation temperatures of the polysaccharides and lignin, as observed in Figure 3. In fact, a reduction in the degradation temperature by 19 and 31 °C for lignin and

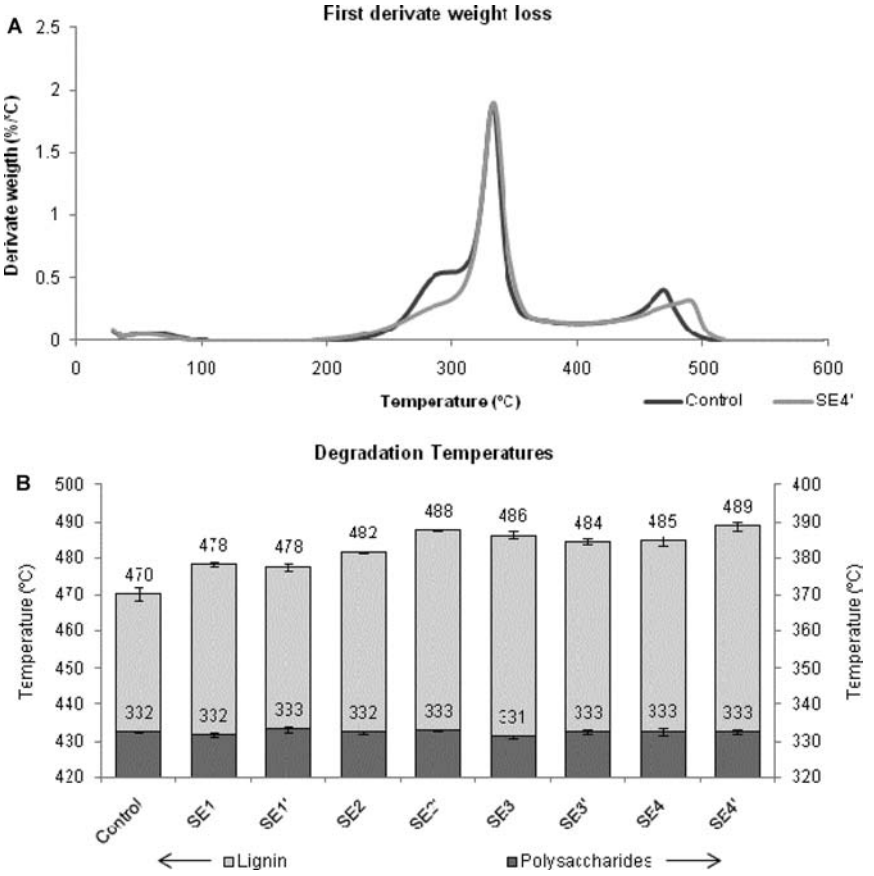

Figure 2. (A) Thermal properties of steam-exploded samples: first derivate of thermograms of *Eucalyptus globulus* wood before (control sample, black profile) and after steam explosion under conditions denoted S4′ in Table 1 (gray profile). (B) Lignin and polysaccharide degradation temperatures for the control and steam-exploded samples.

Table 2. Lignin, Glucan, Xylan, Arabinan, and Acetyl Group Content in Exploded Wood Samples, Calculated as the Relative Percent Amount of Each Compound Relative to the Amount in the Original Wood Sample (Control)

	% lignin	% glucan	% xylan	% arabinan	% acetyl groups
control	100	100	100	100	100
SE1	88	100	91	38	81
SE1′	88	100	94	42	82
SE2	90	100	91	41	79
SE2′	80	100	57	14	40
SE3	88	100	82	14	70
SE3′	86	100	66	9	64
SE4	83	100	76	20	89
SE4′	81	96	53	7	77
SE4′+laccase	74	93	46	5	32
SE4′+control-L	75	97	43	6	31

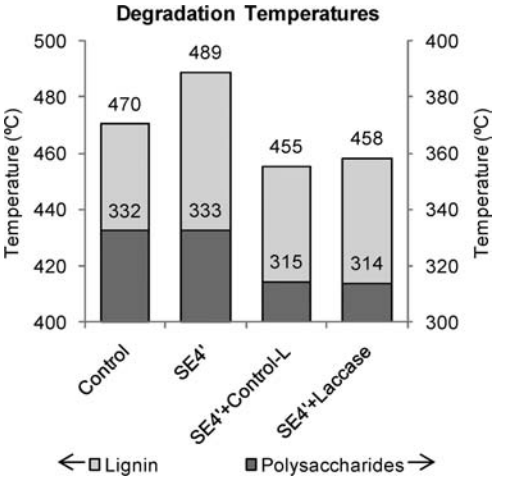

Figure 3. Polysaccharide and lignin degradation temperatures for samples before and after steam explosion and after laccase-mediator treatment (see Table 1 for nomenclature).

polysaccharide fractions, respectively, was observed after enzyme treatment of the steam-exploded substrates (compare data for SE4′ and SE4′+laccase). Importantly, these changes cannot be ascribed to a change in composition due to the addition of the enzyme because no significant differences were observed when a control sample (with no laccase and no mediator, SE4′+control-L) and the laccase-treated sample (SE4′+laccase) were compared. Thus, it is possible that the shifts in degradation temperature are related to changes that occurred in the molecular structure and/or bonding features of lignin and cellulose due to relatively high temperatures (70 °C) and the presence of oxygen (air bubbling); for example, depolymerization reactions were possible. However, another plausible explanation is that during enzyme treatment some low molecular weight lignin fractions

Table 3. Weight and Lignin Content of Residues Obtained in the MWL Isolation Procedure (See also Figure 1 for the Nomenclature Used)

		control	SE4′	SE4′+laccase
R0	yield (%)	100.0	100.0	100.0
	Klason lignin (%)	21.1	23.8	22.5
	acid-soluble lignin (%)	2.5	1.7	1.5
	total lignin content relative to R0	23.6	25.5	24.0
S1	extractives %	2.7	9.6	4.5
R1	yield (%) respect R0	97.3	90.4	93.3
	Klason lignin (%)	20.3	20.1	20.8
	acid-soluble lignin (%)	2.3	1.5	1.4
	total lignin content relative to R0	22.1	19.5	21.2
	lignin yield (%) relative to lignin R0	93.6	76.5	88.3
ASL	yield % relative to R0	2.3	6.9	6.0
	yield % relative to lignin content R0	9.6	26.9	25.0
R2	yield (%) relative to R0	89.1	70.3	87.9
	Klason lignin (%)	20.4	16.8	15.9
	acid-soluble lignin (%)	2.0	1.2	1.2
	total lignin content relative to R0	20.0	12.7	15.7
	lignin yield (%) relative to lignin R0	84.8	49.7	62.6
MWL	yield % relative to lignin content R2	40.5	44.8	42.4
	yield % relative to lignin content R0	34.3	22.2	26.5

and/or small lignin fragments attached to carbohydrates were solubilized in the aqueous solution. This would imply not only a decrease in the degree of polymerization (DP) of the carbohydrates but also the loss of some lignin, which all together can cause a reduction in the degradation temperature.

Isolation of MWL. Lignin was isolated from the untreated wood chips (before steam explosion), after the most intense steam explosion treatment (SE4′), and from a sample subjected to the same steam explosion followed by application of a LMS. The optimization of the milling process that led to the selection of conditions to obtain similar yields, as explained under Materials and Methods, indicated that steam-exploded wood chips (sample SE4′) required more intensive milling compared to the original wood. On the other hand, a less intense milling was required in the case of the sample obtained after treatment with LMS (SE4′+laccase). Under the hypothesis that noncondensed lignin is more easily removed in isolation procedure, the results just described for the milling intensity are in support of the TGA that suggested that steam explosion produced lignin condensation reactions. On the other hand, laccase treatment was expected to mainly cause some depolymerization reactions that could reduce the milling intensity required compared to SE4′ sample.

After steam explosion of the wood chips the amount of organic extractives and alkali-soluble lignin (ASL in Figure 1 and Table 3) increased. Furthermore, the total lignin content with respect to the original sample (R0) after each extraction was lower for samples after steam explosion than for untreated samples. This observation is in support of conclusions from other studies that indicated that aqueous alkaline or organic solvent extraction

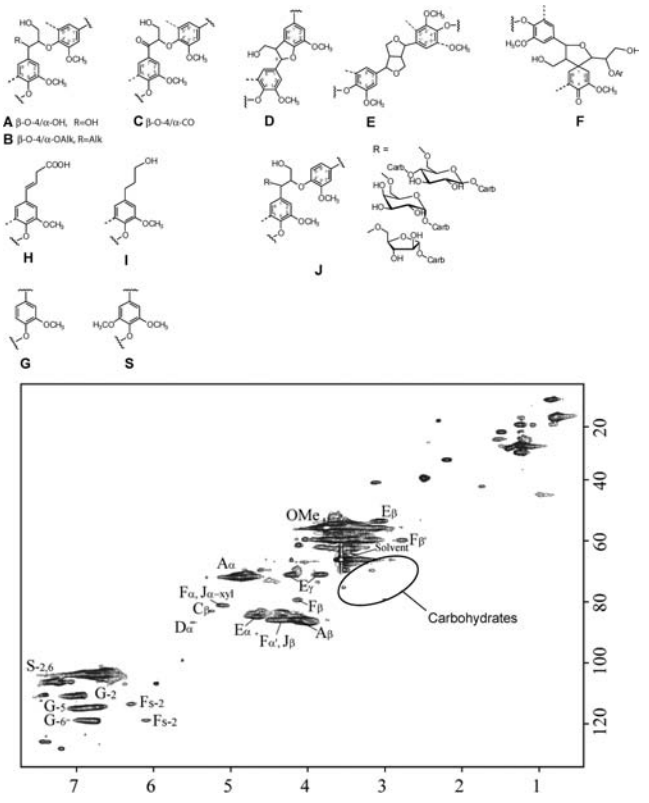

Figure 4. HSQC spectrum of MWL from *Eucalyptus globulus* with the respective lignin structures indicated in the upper panel.

resulted in an appreciable delignification of steam-exploded aspen.[9,27] Robert et al.[27] and Lora and Wayman [28] have reported that most of the lignin in steam-exploded aspen can subsequently be removed either by extraction with an organic solvent, such as ethanol, acetone, or dioxane, or by extraction with aqueous alkali. However, in the present case the degree of delignification after alkali extraction was comparatively lower than that observed in these studies (26.9 vs 98%). Such a difference can be explained by the fact that milder steam explosion conditions were used in the present study (severity factor of 3.56 vs 4.32). Finally, it is worth mentioning that lignin from aspen, used in the cited references, was found to be the most readily extractable compared with lignin from other hardwoods and also from softwoods.[28,29]

The ASL yield and extractive percentage for the SE4′+laccase sample were higher than those for the control sample, but lower than those for sample SE4′. This fact could be due to the removal of lignin during laccase-mediator treatment, as Table 2 indicates (7% lignin reduction with respect to control sample).

NMR Analysis. 2D NMR spectra of isolated lignin from *E. globulus* before and after steam explosion, and also after steam explosion and laccase treatment, are shown in Figures 4, 5, and 6, respectively, and their signal assignments were made according to previous studies.[19,21]

A wide variety of saturated aliphatic moieties with chemical shifts of about δ_H 0.8 and δ_H 1.2−1.3 was observed in the HSQC spectrum. They corresponded to extractives, which were likely chemically bonded to lignin.[30] The cross-signal in the lower field (δ_H 1.5−2.5 and δ_C 20−43) corresponded to aliphatic groups neighboring alkene and oxygen-containing groups such as alcohol, carbonyl, and ethers. Furthermore, some minor lignin structures, such as Ar−$COCH_2$−CH_2OH type moieties and

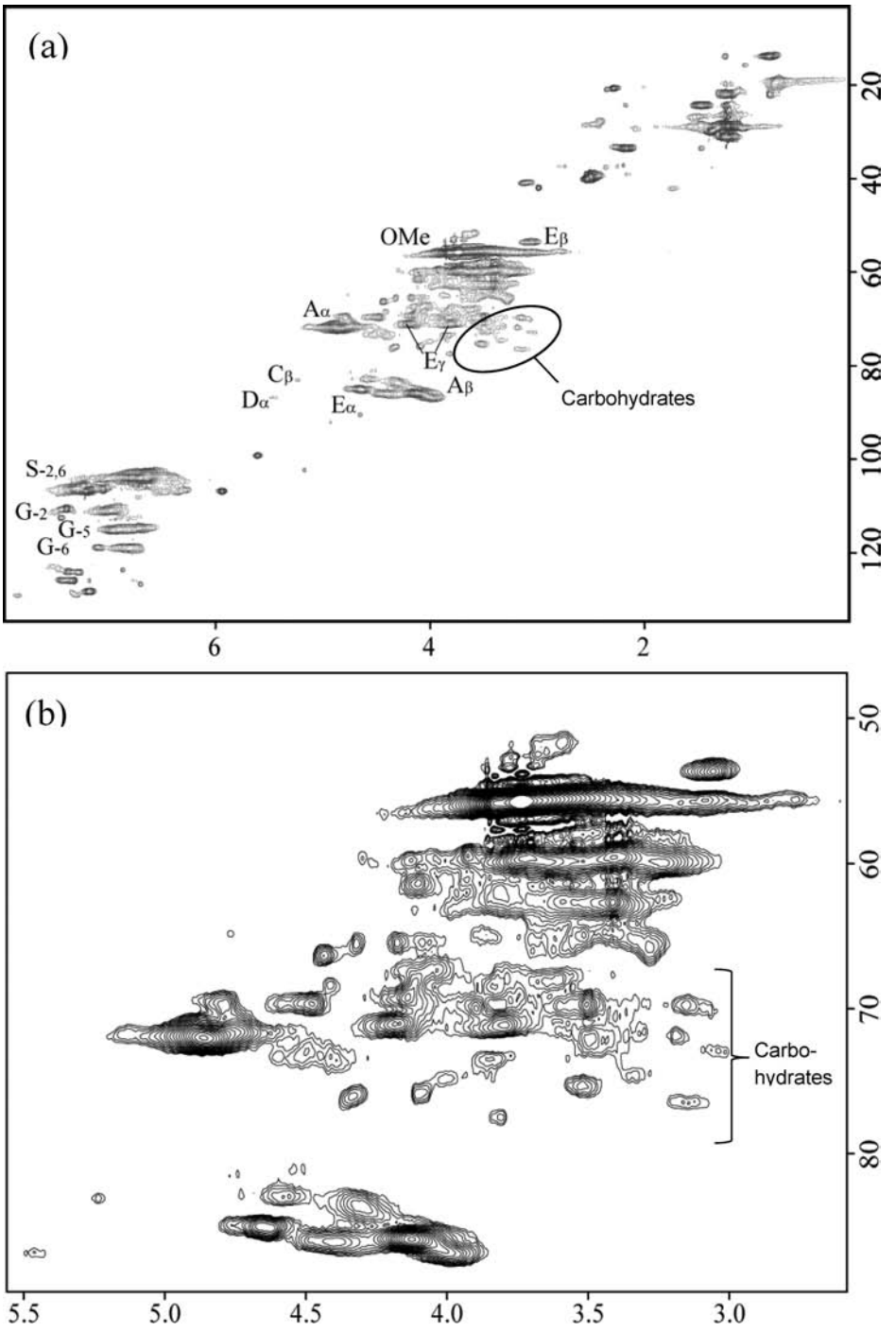

Figure 5. HSQC spectrum of isolated lignin from *Eucalyptus globulus* after steam explosion treatment (SE4′) (a). The detailed oxygenated aliphatic region is included in panel b.

dihydroferulic acid type units (Ar−CH_2−CH_2−COOH), were identified on the basis of the reported chemical shifts. Weak signals of dihydrocinnamyl alcohol type units (Ar−CH_2−CH_2−CH_2OH) (I) were detected in MWL from control *E. globulus* wood, but not in lignin isolated from *E. globulus* after steam explosion and laccase treatments.

In the oxygenated aliphatic region, the signals of the major structures of native lignin such as (β-O-4′) (A), phenylcoumaran (β-5′) (D), and pino/syringyresinol (β-β′) (E) were observed in the three studied lignins (Figures 4, 5, and 6), although the amounts of β-O-4′ and β-β′ structures decreased after steam explosion treatment (Table 4).

The cross-peak at δ_C/ δ_H 83.5/5.28 was assigned to β-CH in β-O-4′ moieties of S-type with α-carbonyl groups according to Capanema et al.[21] The signals of the γ-CH in coniferyl alcohol type units were observed at 61.9/4.12 ppm. These signals were observed in the three isolated lignins.

Weak signals of spirodienone structures (F) were observed in the HSQC spectrum of the MWL from control *E. globulus* wood. The signals at 79.5/4.12 and 81.2/5.1 ppm were assigned to

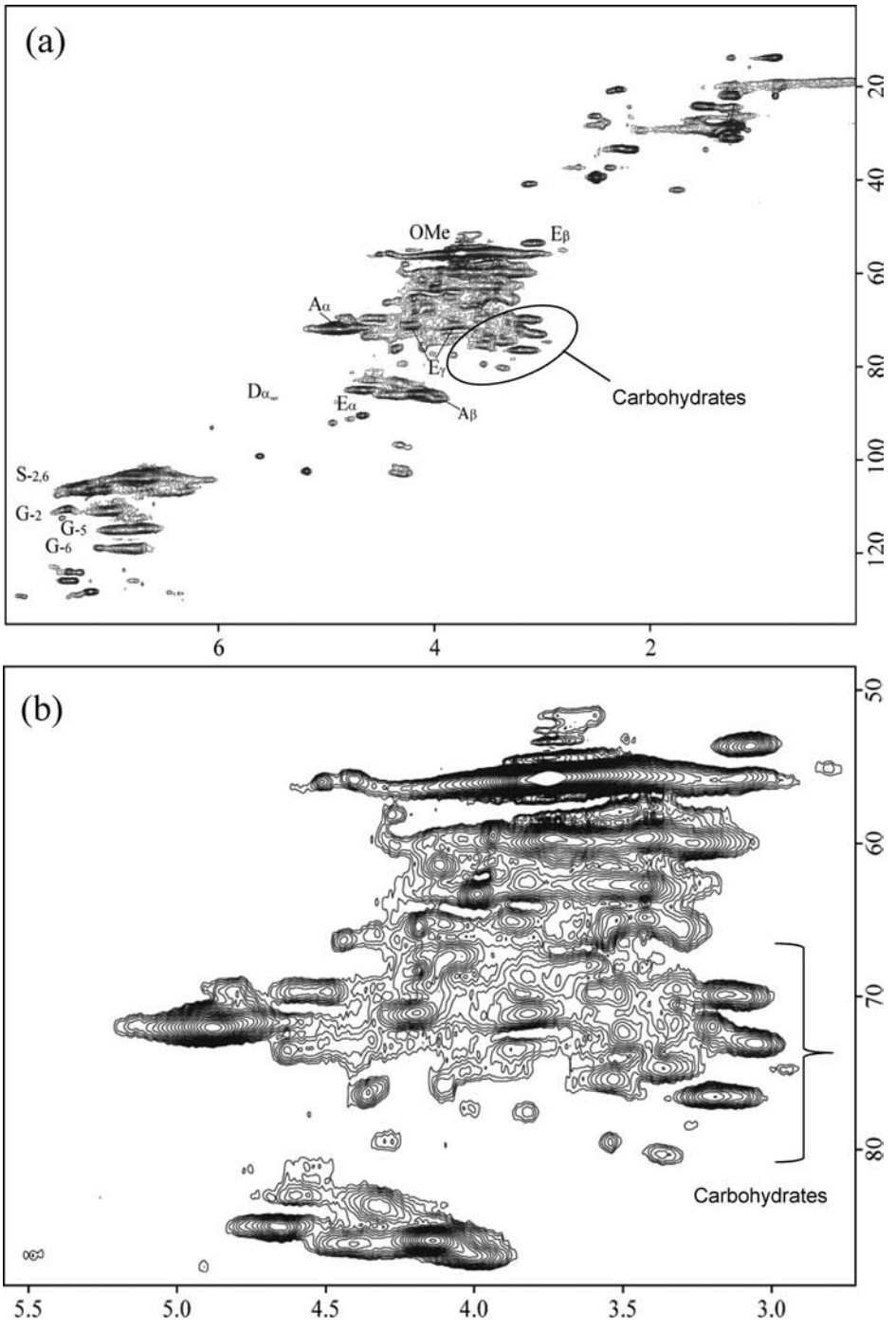

Figure 6. HSQC spectrum of isolated lignin from *Eucalyptus globulus* after steam explosion and laccase-mediator treatment (SE4′+laccase sample) (a). The detailed oxygenated aliphatic region is included in panel b.

β'-CH in the second side chain in the spirodienone and α-CH in spirodienone plus lignin xylan moieties, respectively.[21,26] These signals were very weak or even disappeared in lignin isolated from wood after steam explosion and steam explosion plus laccase treatment.

The oxygenated aliphatic region also contained cross-signals for xylans and other polysaccharides ($\delta_C/\ \delta_H$ 63−75/3.2−3.8) less abundant in MWL from control *E. globulus* wood than in the other lignins analyzed. Lignin carbohydrate linkages were revealed by cross-signals for some β-O-4′ substructures that are α-C or γ-C-etherified with carbohydrates (J).[31]

In the aromatic region, a predominance of syringyl (S) over guaiacyl (G) units was observed in the three lignins studied, which is in concordance with the high proportion of S units in hardwood.

Comparison of 2D NMR spectra before and after steam explosion followed or not by laccase-mediator treatment made it evident that lignin preparation isolated from SE4′ and SE4′+laccase had higher sugar content than lignin isolated from original wood at the same yield, as observed in the oxygenated aliphatic region (Figures 4, 5b, and 6b). Additionally, 2D NMR spectra qualitatively indicated a very similar observation. Similar

Table 4. ^{13}C NMR Data of the Most Important Lignin Functionalities (per 100 Ar Units)

isolated lignin	COOR	OHpr[a]	OHsec[a]	OHph	β-O-4	OMe	S/G	DC
original (control)	5	69	57	23	64	177	3.2	13
SE4′	16	66	54	47	36	170	3.1	14
SE4′+laccase	17	63	70	53	36	161	3.0	19

[a] Includes sugar OH groups.

results were found by Balakshin et al.[30] and Ibarra et al.,[31] who studied the residual lignin after LMS-E treatment and found the HSQC NMR spectra very similar to those of the original residual kraft lignin. They ascribed their observations to a removal of the altered lignin from the pulp during the alkaline extraction, subsequent to the LMS treatment; the residual lignin that remained in the pulp did not undergo substantial changes. In our case, the oxidized lignin could be removed during the alkali extraction performed in the lignin isolation procedure used to remove tannins.

A quantitative study based on ^{13}C NMR was carried out. The quantification of the most important lignin functionalities is indicated in Table 4. The calculations were performed according to the method suggested by Capanema et al.[21]

The amount of aliphatic and conjugated COOH groups estimated from the spectrum of the nonacetylated MWL from control *E. globulus* was found similar to that previously reported.[32] However, when wood was subjected to steam explosion, the amount of carboxyl groups increased significantly.

The amount of primary hydroxyl groups observed in SE4′ and SE4′+laccase lignin was slightly lower than in the lignin isolated from original milled *E. globulus* wood. However, the amount of secondary OH increased significantly after the laccase treatment. In addition, the quantity of phenolic OH increased significantly after the steam explosion treatment, which agrees with results found by other authors.[27,33]

The total amount of β-O-4 structures was significantly reduced after steam explosion treatment, which was in agreement with other papers.[6,7,13] It is well-known that steam explosion carried out only in the presence of steam is an autohydrolytic process catalyzed by the organic acids formed from the acetylated wood components. The predominant reactions in lignin are fragmentation by acidolysis of β-O-4 linkages and polymerization by acid-catalyzed condensation between the aromatic C_6 or C_5 and a carbonium ion, normally located at α-C of the side chain.[13] As a result of the substantial cleavage of β-O-4 linkages, the molecular weight of lignin would be expected to decrease sharply as a result of the steam explosion treatment. However, Li et al.[13] observed a gradual increase in molecular weight with increasing severity of the treatment. Thus, they postulated that the cleavage of β-O-4 linkages in lignin during the steam explosion process was accompanied by a comprehensive repolymerization, resulting in an increase in molecular size and a more heterogeneous lignin structure. In the present study, the steam explosion conditions were milder than those reported before. The repolymerization reaction may not be dominant, and no increase in the degree of condensation (DC) after steam explosion treatment was observed.

After the laccase-mediator treatment, no variation in the total amount of β-O-4 structures was observed, but an increase in the DC was found. In contrast, TGA results indicated a decrease in lignin degradation temperature, which correlates with less condensed lignin structures. However, it should be noted that milled wood samples and not isolated lignin was used in TGA measurements. Therefore, indirect and collective effects of lignin and other components are factored in the thermal analyses (structural changes). These results indicate a decrease in lignin molecular weight during steam explosion followed by laccase-mediator treatment. This is also supported by the observed higher extractives yield and ASL yield (Table 3) compared to the control sample; also, the lignin isolated from steam-exploded samples had a more condensed structure.

The amount of methoxyl groups (OMe) was reduced from 1.77/Ar to 1.70/Ar after steam explosion treatment and from 1.70/Ar to 1.61/Ar after a subsequent laccase treatment. A similar reduction in methoxyl groups has been observed by Chua and Wayman.[29]

A slight reduction in S/G ratio was observed after steam explosion treatment and also after laccase-mediator treatment. Chua and Wayman[29] explained this reduction in S/G ratio during mild steam explosion condition as being due to an initial preferential removal of syringyl to guaiacyl units at short autohydrolysis times. The decrase in S/G ratio after laccase-mediator treatment was also observed by Ibarra et al.[31]

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Funding Sources

Financial support from the Spanish Ministry of Science and Innovation (FPI fellowship BES2007-14528) is gratefully acknowledged.

ACKNOWLEDGMENT

We thank Professor M. J. Diaz of Universidad de Huelva (Spain) for assistance with HPLC carbohydrate analyses. We acknowledge the contributions of Prof. F. Somolinos of Universidad Complutense de Madrid (Spain).

ABBREVIATIONS USED

MWL, milled wood lignin; NMR, nuclear magnetic resonance; SEC, size exclusion chromatography; TGA, thermogravimetric analyses; SE, steam exploded; LMS, laccase-mediator system; HBT, 1-hydroxybenzotriazole; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate); HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; HSQC, heteronuclear single-quantum coherence; LMS-E, laccase-mediator system treatment followed by alkaline extraction.

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