Characterization of Residual Lignin after SO_2-Catalyzed Steam Explosion and Enzymatic Hydrolysis of *Eucalyptus viminalis* Wood Chips

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The lignin component found in both water insoluble (WI) and water and alkali insoluble (WIA) fractions derived from SO_2-impregnated steam-exploded eucalyptus chips (SEE) was isolated and characterized. Dioxane lignins with a sugar content lower than 2% (w/w) were obtained after each material was treated with commercial cellulases. The C9 formulas of both SEE-WI and SEE-WIA dioxane lignins were $C_9H_{6.83}N_{0.04}O_{2.24}(OCH_3)_{1.21}(OH_{aro})_{0.56}(OH_{ali})_{0.77}$ and $C_9H_{8.65}N_{0.29}O_{1.97}(OCH_3)_{0.90}(OH_{aro})_{0.46}(OH_{ali})_{1.02}$, respectively. The weight-average molecular weight (M_w) of the SEE-WI lignin corresponded to 3.85 kDa, whereas the SEE-WIA lignin had an M_w of 3.66 kDa for the same polydispersity of 2.4. The SEE-WIA lignin was shown to be more thermally stable than the SEE-WI lignin, requiring temperatures in the range of 520 °C for complete degradation. FTIR and ^1H NMR analyses of both untreated and peracetylated lignin fractions showed that (a) the alkali insoluble lignin contained a relatively higher degree of substitution in aromatic rings per C9 unit and that (b) alkaline extraction removed lignin fragments containing appreciable amounts of phenolic hydroxyl groups.

Keywords: *Eucalyptus viminalis; steam treatment; alkali insoluble lignin; enzymatic hydrolysis*

INTRODUCTION

The utilization of lignocellulosic materials as chemical feedstocks for fuels and chemicals is hindered by the low efficiency that is obtained when these materials are hydrolyzed by chemical and enzymatic treatments. However, several types of pretreatment have been shown to enhance the conversion of wood and agricultural residues (Tassinari et al., 1980; Chum et al., 1988; Gould, 1984; Schwald et al., 1989a,b; Torget et al., 1990; Ramos et al., 1992a). Acid-catalyzed steam treatment appears to be one of the best methods studied to date because it not only provides effective fractionation of the three major wood components but also ensures effective hydrolysis of the cellulose component (Torget et al., 1990; Ramos et al., 1992b; Saddler et al., 1993). Previous results have suggested that the enhanced accessibility of enzymes toward steam-treated substrates is correlated to the relative easiness in the extraction of hemicellulose and lignin after pretreatment (Torget et al., 1990; Ramos et al., 1992a). The extraction results in a substantial increase in the available surface area of the substrate (substrate porosity) (Wong et al., 1988). This in turn leads to a corresponding increase in hydrolysis efficiency. On the other hand, the effect of other structural factors such as cellulose crystallinity and degree of polymerization may also be important in the increase in hydrolysis efficiency (Sinitsyn et al., 1991).

Although several substrate-related factors have been suggested to explain the incomplete enzymatic hydrolysis of cellulosic substrates at high substrate concentrations, enzyme-related factors may also contribute significantly to the incomplete hydrolysis. These include the accumulation of sugars during hydrolysis leading to end-product inhibition (Saddler, 1986), the inactivation of key components of the cellulase system, and the irreversible and/or nonspecific adsorption of cellulases onto the hydrolysis residue (Sinitsyn et al., 1983; Ooshima et al., 1990).

In earlier work with eucalyptus (Ramos et al., 1992a), aspen (Schwald et al., 1989b), spruce (Schwald et al., 1989a), and radiata pine (Clark and Mackie, 1987), the optimum steam pretreatment conditions for enhanced enzymatic hydrolysis and recovery yield of pretreated fractions were determined. It was found, in general, that the more drastic the pretreatment conditions, the lower the recovery yield of the water soluble fraction and the better enzymatic accessibility toward the resulting substrate. A subsequent alkaline extraction of steam-exploded substrates was shown to be beneficial because the alkaline process resulted in (a) better separation of the majority of the lignin component for further processing, (b) increase in the relative cellulose content of the steam-treated material, and (c) improvements in further steps of enzyme recycling (Eklund et al., 1990; Ramos et al., 1993). Although alkaline extraction could remove a substantial amount of the original lignin present in both steam-treated hardwoods (aspen and

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eucalyptus) and softwoods (spruce and radiata pine), the efficiency of enzymatic hydrolysis was only marginally increased for the former and significantly reduced for the latter (Schwald et al., 1989b; Ramos et al., 1992b; Wong et al., 1988) after alkaline extraction. The redistribution of the alkali insoluble lignin was then suggested as being responsible for the relatively lower susceptibility of the alkali-washed, steam-exploded substrates toward the enzymatic hydrolysis (Ramos et al., 1992b). However, these authors have not made further attempts to identify structural features of this modified lignin that could play a role in limiting the efficiency of the enzymatic hydrolysis of cellulose.

In this work, we have looked into the properties and chemical composition of the residual lignins isolated from both water-washed (SEE-WI) and water and alkali washed (SEE-WIA) steam-exploded residues obtained from hardwood chips. The lignin component of both SEE-WI and SEE-WIA fractions was isolated and purified after carbohydrates (mostly cellulose) in the SEE-WI and SEE-WIA were almost completely removed by treatment with cellulases. Our goal is to show that the residual lignin present in alkali insoluble residues (alkali-insoluble lignin) is a more recalcitrant lignin type and that its redistribution onto the surface of cellulose fibers may restrict hydrolysis by creating a hydrophobic, physical barrier which limits substrate accessibility and causes an irreversible, nonproductive adsorption of the enzymes.

MATERIALS AND METHODS

Steam Pretreatment. The steam pretreatment of *Eucalyptus viminalis* Labill was performed in a steam gun at 210 °C for 50 s after the wood chips were impregnated with 0.9% SO_2 (w/w) (Ramos et al., 1992a,b). The resulting steam-treated substrate (SEE) was extracted twice with water for 1 h at room temperature (RT) using a substrate consistency of 5% (w/v). The water insoluble fraction (SEE-WI) was then extracted twice with alkali (NaOH 0.4%, w/v, RT) for 1 h at 5% consistency (w/v), and the resulting alkali-washed substrate (SEE-WIA) was washed thoroughly with water until the washing was neutral. Both pretreated substrates were stored at 4 °C for further use with a typical moisture content of 67–75% (w/w).

Substrate Analysis. Lignin content was determined by using TAPPI Standard Method T222 os-74 for acid insoluble lignin (Klason lignin). Acid soluble lignin was determined according to the TAPPI Useful Method 250. After the substrate was hydrolyzed to its component sugars during a typical Klason lignin determination, carbohydrate analysis of the resulting hydrolysates was performed in a Waters 600E high-performance liquid chromatograph (HPLC) using an Aminex HPX-87H column (Bio-Rad, Richmond, CA) at 65 °C (Irick et al., 1988). The column was eluted isocratically with 8 mM H_2SO_4 at 0.6 mL min^{-1}, and the eluent was monitored by differential refractometry. In some cases, the carbohydrates in the acid hydrolysate from a Klason lignin determination were analyzed by capillary gas chromatography (CGC) as the corresponding alditol acetates. The GC analyses of the mixtures of alditol acetates was carried out in a Hewlett-Packard (Miami, FL) gas chromatograph (GC), model 5890 S II, with an OV-225 capillary column (30 m × 0.25 mm i.d.) (J&W Scientific, Folsom, CA), operated at 250 °C with nitrogen as the carrier gas at a flow rate of 10 mL min^{-1}. The injector temperature was maintained at 280 °C, and eluents were monitored by a flame ionization detector. Alternatively, the total sugar contents were determined using the phenol–sulfuric acid method (Dubois et al., 1956).

Steam-treated substrates were also analyzed by Fourier transformed infrared spectroscopy (FTIR). After milling, each cellulosic material was compacted into KBr disks (3 mg in 150 mg of KBr) at 10 ton for 3 min and analyzed using a Bomem FTIR MB-100 spectrophotometer (Hartmann & Braun, Quebec, Canada) within the region of 500–4000 cm^{-1} with a resolution of 4 cm^{-1}, 64 scans, and triangular apodization.

Enzymatic Hydrolysis and Lignin Extraction. The isolation of lignin from steam-treated substrates was carried out after most of the cellulose component had been removed by enzymatic hydrolysis. The enzymatic hydrolyses were performed in two-stages at an initial substrate concentration of 5% (w/w) using a mixture of Celluclast and Novozym (Novo Nordisk, Denmark), a cellulase preparation from *Trichoderma reesei* and a β-glucosidase preparation from *Aspergillus niger,* respectively. The substrates were initially hydrolyzed for 48 h at 45 °C, 145 rpm in 0.05 M sodium acetate buffer containing 60 FPU g^{-1} and 180 CBU g^{-1} of substrate (dry basis) (Ramos et al., 1993). After the hydrolysis, the resulting mixture was cooled in an ice bath and the unhydrolyzed residue was filtered and then washed thoroughly with hydrolysis buffer. The washed residue was resuspended in fresh buffer to which fresh cellobiase was added because this enzyme was not expected to adsorb efficiently to the cellulosic residue. The mixture was then incubated at 45 °C for another 48 h. After completion of the second hydrolysis step, the unhydrolyzed residue was centrifuged and washed with water until no sugars could be detected in the supernatant by the phenol–sulfuric acid method. The lignin component was then extracted overnight with an excess of dioxane/water (9:1, v/v) under continuous stirring at RT. The resulting dioxane solution was centrifuged to remove any insoluble materials. The solvent was also removed from the supernatant by evaporation under reduced pressure at 35–45 °C. The resulting syrup was dropped into deionized water to precipitate the residual lignin, which was separated by centrifuge, then washed twice with deionized water, and finally suspended in deionized water and freeze-dried.

Enzymatic activities in the enzyme mixture were determined against filter paper (filter paper units, FPU) and cellobiose (cellobiase units, CBU) as previously described (Ghose, 1987). The resulting hydrolysates, obtained after each of the hydrolysis stages described above, were initially analyzed by using the DNS method for reducing sugars (Ghose, 1987) and subsequently confirmed by HPLC (Schwald et al., 1988).

Chemical and Spectroscopic Analyses of Lignin. Methoxyl content in lignin was determined by GC after treatment of the residual material with iodidric acid (HI) (Silva, 1995). Using this treatment, methoxyl groups (OCH_3) can be quantified as their methyl iodide derivative (CH_3I). The elemental analysis of isolated lignins was determined at the Institute of Chemistry of the State University of Campinas (Campinas, SP, Brazil) with a CHN 2400 Perkin-Elmer analyzer (Perkin-Elmer, Norwalk, CT), and the resulting compositional analysis, corrected for methoxyl content, was used to calculate their theoretical C_9 formula.

The thermodegradation behavior of lignin was measured in a Netzsch (Selb, Germany) STA 409EP thermogravimetric analyzer (TGA). Approximately 3 mg of each lignin preparation was placed in an aluminum oxide (Al_2O_3) crucible and heated from 20 to 950 °C at 5 °C/min.

Analyses by FTIR, gel permeation chromatography (GPC), and ^1H nuclear magnetic resonance (^1H NMR) spectroscopy were also performed after acetylation of each lignin fraction. Acetylation was carried out in pyridine and acetic acid anhydride according to the procedure of Lenz (1968). Complete acetylation was ensured through the complete disappearance of the O–H stretching absorption band in the FTIR spectra of the acetylated derivative.

The molecular weight distribution of each lignin preparation was obtained by GPC of their fully acetylated derivatives. GPC was carried out on a Shimadzu 10AD liquid chromatograph (Shimadzu, Kyoto, Japan). Samples of acetylated lignin in tetrahydrofuran (THF) were filtered through a Teflon membrane with a pore size of 0.45 μm and analyzed in duplicates at 40 °C using a series of four Alltech chromatographic columns (Alltech Associates, Deerfield, IL) with pore diameters

Table 1. Chemical Composition and Hydrolysis Yields of Steam-Treated Substrates Derived from SO_2-Impregnated Eucalyptus Wood Chips

analysis	SEE-WI[a]	SEE-WIA[a]
chemical composition[b]		
cellulose[c] (%)	58.6	84.4
Klason lignin[d] (%)	30.4	5.4
acid soluble lignin (%)	1.2	0.6
hemicelluloses[c] (%)	2.5	2.1
ash (%)	1.1	0.9
total recovery yield (%)	93.8	93.4
enzymatic hydrolysis[e]		
yield in step I (g, %)	3.43 (68.6)	3.66 (73.2)
yield in step II (g, %)	0.07 (1.4)	0.42 (8.4)
dioxane-extracted residue[f] (g, %)	0.33 (6.6)	0.66 (13.1)
dioxane lignin[f] (g, %)	1.21 (24.2)	0.09 (1.8)
total recovery yield (%)	100.8	96.5

[a] SEE, SO_2-impregnated steam-exploded eucalyptus wood chips; WI, water insoluble fraction; WIA, water and alkali insoluble fraction. [b] All figures were expressed in terms of g/100 g of oven-dry wood weight. [c] Determined on Klason lignin filtrates by HPLC (see Materials and Methods). [d] Acid-insoluble lignin. [e] Enzymatic hydrolyses were carried out in two stages of 48 h at 2% (w/w) using an enzyme preparation containing 60 FPU g^{-1} and 180 CBU g^{-1} of substrate; the hydrolysis reaction was monitored by the DNS method for reducing sugars and by HPLC for glucose release; values in parentheses express yields in relation to the substrate dry weight. [f] The hydrolysis residue was extracted with dioxane/water (9:1, w/w) and the dioxane lignins were freeze-dried after precipitation in water.

of 100, 500, 1000, and 10000 Å. THF was used as the eluting solvent at a flow rate of 0.8 mL min^{-1}, and the eluent was monitored by an UV detector at the wavelength 254 nm.

The GPC calibration curve was generated from the elution profile of polystyrene standards with narrow MW distributions (Coll and Gilding, 1970; Valtasaari and Saarela, 1975; Danhelka and Kossler, 1976). Both number-average (M_n) and weight-average molecular weights (M_w) of lignin were calculated as described previously (Yau et al., 1979) and corrected for their theoretical acetyl groups content in relation to the lignin total hydroxyl content. Polydispersity was defined as the ratio between M_w and M_n.

The NMR spectra were recorded in a Varian Gemini 300 MHz spectrometer after ∼5−15 mg of each acetylated lignin had been dissolved in 0.5 mL of $CDCl_3$ containing 1% of tetramethylsilane (TMS) as internal standard (Chen and Robert, 1988). The conditions for analysis included a pulse width of 10.5 μs and an acquisition time of 2.7 s.

The ^{1}H NMR data were used to estimate both aliphatic and phenolic hydroxyl groups in lignin, as well as their methoxyl contents. These latter figures were shown to be in good agreement with those obtained by GC.

RESULTS AND DISCUSSION

Hydrolyses of both pretreated substrates were routinely carried out in two consecutive steps using Celluclast supplemented with an excess of Novozym. This was to ensure that optimum hydrolysis would be achieved while cellobiose concentrations were maintained below inhibitory levels (Sternberg et al., 1977; Ramos et al., 1993). The cellulose component in SEE-WI was shown to be almost completely removed by using this hydrolysis procedure (Table 1), leaving a lignin-rich residue that was easily extracted with dioxane/water (9:1, v/v). The unhydrolyzed, dioxane insoluble residue from SEE-WI was determined to be only 6.6% of its original dry weight, whereas the dioxane lignin fraction was ∼24.2%. The latter figure corresponded to ∼79.6% of the acid insoluble lignin in the SEE-WI fraction, because this fraction had a Klason lignin content of 30.4%.

Table 2. Chemical Characterization of Dioxane Lignin Fractions Derived from SO_2-Impregnated, Steam-Treated Eucalyptus Wood Chips

analysis	SEE-WI lignin[a]	SEE-WIA lignin[a]
carbon (% ± CV)	57.90 ± 0.20	57.89 ± 0.04
hydrogen (% ± CV)	5.60 ± 0.10	6.25 ± 0.05
nitrogen (% ± CV)	0.25 ± 0.01	1.96 ± 0.02
oxygen	36.20	34.01
methoxyl[b] (% ± CV)	17.8 ± 0.90	13.6 ± 2.8
molecular weight distribution[c] (GPC analysis)		
av M_w (kDa)	3.85	3.66
av M_n (kDa)	1.57	1.51
polydispersity	2.45	2.42

[a] SEE, SO_2-impregnated steam-exploded eucalyptus wood chips; WI, water insoluble fraction; WIA, water and alkali insoluble fraction. [b] Lignin methoxyl content was determined by gas chromatography after treatment of the residual material with iodidric acid (Silva, 1995). [c] The average molecular weights of both lignin fractions were corrected for acetyl groups on the basis of their total hydroxyl contents.

By contrast, the dioxane insoluble residue obtained from SEE-WIA after enzymatic hydrolysis corresponded to 13.1% of the original dried substrate weight. The relatively high yield of the residue was probably associated with the relatively lower accessibility of the enzymes to the cellulose component in the SEE-WIA fraction. As a result, the yield of the dioxane lignin fraction (alkali insoluble lignin) was only 1.8%, corresponding to a total recovery of only 33.3% of the original SEE-WIA Klason lignin. The total recovery yield, obtained after hydrolysis and extraction of the SEE-WIA fraction, was also relatively low (96.5%, Table 2), indicating that part of the lignin component in the substrate may have been lost during washing with water as water soluble lignocarbohydrate complexes of low molecular mass.

Alkaline extraction did remove a substantial amount of lignin from the SEE-WI fraction. However, compared to the SEE-WI fraction, alkaline washing decreased the hydrolysis efficiency of cellulose, even though there was an apparent increase in glucose yield when the yields were calculated on the basis of the dried substrate weight (Table 1). Because enzyme loadings in this study were also expressed on the basis of the dried substrate weight, the lower susceptibility of the SEE-WIA fraction toward hydrolysis may have been triggered by its higher cellulose content, which would result in a higher degree of end-product inhibition of the enzymes. These results were consistent with our previous work (Ramos et al., 1992b), in which almost the same hydrolysis efficiencies were obtained only when both substrate and enzyme loadings were expressed on the basis of the total cellulose content. Thus, the redistribution of lignin was considered to be responsible for the reduced degree of cellulose hydrolysis in alkali-washed substrates such as the SEE-WIA fraction (Schwald et al., 1989a; Ramos et al., 1992b).

The effect of alkaline washing on the steam-treated SEE-WI fraction was easily demonstrated by a substantial decrease in the intensity of absorption bands at both 1510−1517 and 1600−1620 cm^{-1} regions in the FTIR spectra (skeletal or carbon-to-carbon axial deformations in aromatic rings) (Figure 1) (Keller, 1986; Faix and Böttcher, 1993). As a result of the lignin removal, the intensities of absorption bands usually associated with carbohydrates (C−O−C axial deformations of ether bonds at 1050−1250 cm^{-1}) were predominant in the FTIR spectrum of the SEE-WIA fraction.

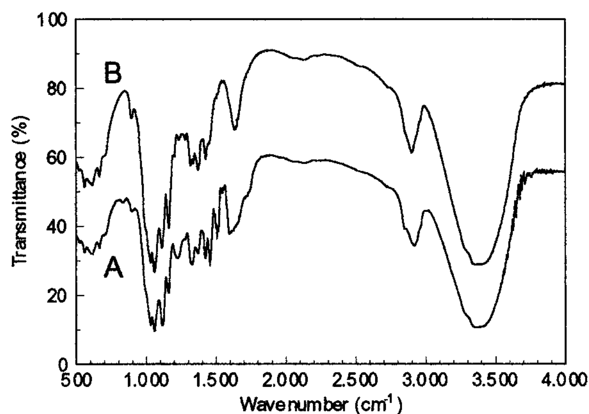


Figure 1. FTIR analysis of steam-treated substrates obtained from SO_2-impregnated eucalyptus wood chips: (A) SEE-WI, steam-exploded eucalyptus, water insoluble fraction; (B) SEE-WIA, steam-exploded eucalyptus, water and alkali insoluble fraction.

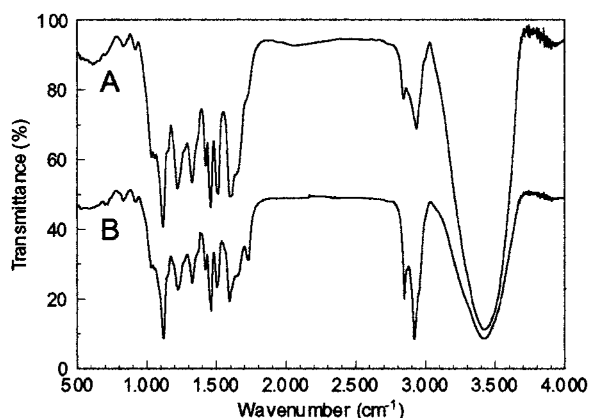


Figure 2. FTIR analysis of dioxane lignins isolated from SO_2-impregnated, steam-treated eucalyptus wood chips after extensive enzymatic hydrolysis using a mixture of Celluclast 1.5L and Novozym 188: (A) SEE-WI lignin; (B) SEE-WIA lignin.

Compared to the SEE-WI lignin, the FTIR spectrum also indicated that the SEE-WIA lignin absorbed more intensively in the 1510−1517 cm^{-1} range (main skeletal deformation of aromatic rings) (Figure 2). Because this indicated a relative loss in the amount of aromatic hydrogens per C_9 unit in the lignin structure (Morais et al., 1994), the degree of condensation of the SEE-WIA lignin appeared to be greater than that of the corresponding SEE-WI lignin.

The relatively predominant absorption bands at 1140 and 1240 cm^{-1} in the spectra of both dioxane lignins (Figure 2) indicated their higher methoxyl content, compared to that observed in grass and softwood lignins (Fengel and Wegener, 1989). These two bands have been attributed to C−H and C−O−ϕ axial deformations in the syringyl nucleus, respectively (Faix et al., 1992). A third band of considerable intensity (1340 cm^{-1}) also appeared to be characteristic of hardwood lignins (syringyl lignins) and was centered in the region classically assigned to C−H deformation modes.

Both FTIR spectra of SEE lignins (WI and WIA) further showed that the intensities of absorption bands for C−O−C axial deformations (ether and/or glycosydic bonds) are nearly equal to the intensities of absorption bands for the aforementioned skeletal deformations of aromatic rings (Figure 2) (Keller, 1986). Considering that most, but not all, of the aromatic rings in hardwood lignins are methoxylated at C-5, this result indicated

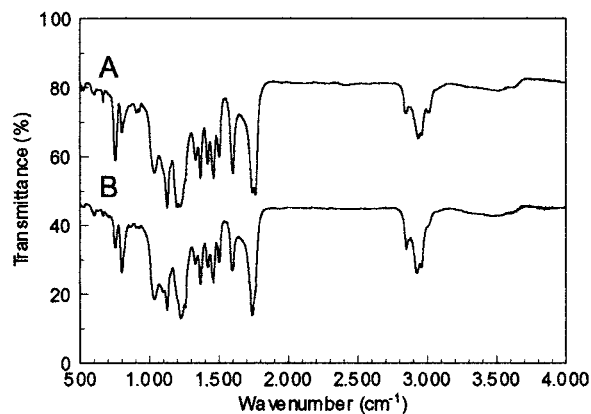


Figure 3. FTIR analysis of acetylated SEE-WI and SEE-WIA dioxane lignins prepared from SO_2-impregnated, steam-treated eucalyptus wood chips (SEE) after extensive enzymatic hydrolysis using a mixture of Celluclast 1.5L and Novozym 188: (A) acetylated SEE-WI lignin; (B) acetylated SEE-WIA lignin.

that the dioxane lignins obtained from SEE have a relatively low carbohydrate content. To verify this postulation, each lignin preparation was subjected to a Klason lignin procedure and sugar contents in the acidic hydrolysates were determined according to the phenol−sulfuric method (Dubois et al., 1956). Indeed, the SEE-WI lignin hydrolysate contained only 0.99% (w/w) of sugars, whereas the SEE-WIA lignin hydrolysate contained twice as much sugars (1.98%) on the basis of the dry weight of the lignin. To better characterize these fractions, both acid hydrolysates were analyzed by CGC after their sugar components had been converted to the corresponding alditol acetates. Small amounts of xylose, glucose, and mannose were identified in the SEE-WI lignin hydrolysate in an aproximate molar ratio of 3:2:1 (Xyl/Glc/Man). Similarly, the carbohydrate content of the SEE-WIA lignin was shown to consist of the same sugars, even though in a slightly different molar ratio of 3:2:2 (Xyl/Glc/Man).

The elemental analysis (C, H, N) of both lignin preparations provided further evidence to elucidate their chemical composition and properties. For instance, the total hydrogen content of the SEE-WIA lignin was higher than that of the SEE-WI lignin. Because the carbon contents of both lignins are similar, the SEE-WIA lignin must have correspondingly lower oxygen content than the SEE-WI lignin. The SEE-WIA lignin also contained at least 7 times more nitrogen than the SEE-WI lignin (Table 2). These results imply strongly that, during the hydrolysis, some enzyme components may have associated with lignin through physicochemical interactions which could not be easily broken by extensive washing of the hydrolysis residue with water. The chromatographic determination of methoxyl groups after the reaction of both lignin fractions with HI (Silva, 1995) showed that the SEE-WI lignin contains nearly 31% more methoxyl groups than the SEE-WIA lignin (Table 2). This result was further confirmed by the ^1H NMR spectrum of the lignin as will be discussed later.

The chemical characteristics of both lignin preparations were also investigated by FTIR and ^1H NMR after acetylation. Complete acetylation was ensured through the complete disappearance of the O−H stretching absorption band in the FTIR spectra of both lignin preparations (Figure 3). The FTIR spectra of the acetylated SEE-WI lignin showed two distinct C=O stretching bands at 1735 and 1765 cm^{-1} corresponding to

aliphatic and phenolic acetates, respectively (Figure 3A). By contrast, only one of these bands at 1735 cm^{-1} was clearly resolved in the FTIR spectrum of the acetylated SEE-WIA lignin. In fact, the 1765 cm^{-1} band appeared as a shoulder of the predominant 1735 cm^{-1} band in this spectrum. Because the location of the acetyl C=O stretching band is indicative of the type of acetyl substitution (Faix et al., 1992), these results showed that the acetylated SEE-WI lignin contains a greater amount of phenolic (or conjugated) hydroxyl groups than the SEE-WIA lignin. In addition, the acetylated SEE-WI lignin absorbed more intensively at 3000 cm^{-1} (aromatic C−H deformations) than the acetylated SEE-WIA lignin. These data were in good agreement with the FTIR analysis of the corresponding nonacetylated lignins (Figure 2) and collectively showed that (a) alkaline extraction removed lignin fragments containing appreciable amounts of phenolic hydroxyl groups and (b) the alkali insoluble lignin contained a relatively higher degree of substitution in aromatic rings (higher degree of condensation).

The molecular weight distribution of both lignin fractions was determined by GPC of the acetylated polymers (Table 2). The SEE-WI lignin fraction was shown to have an average M_w of 3.85 kDa, whereas the average M_w of the alkali insoluble lignin (SEE-WIA lignin fraction) corresponded to 3.66 kDa. Therefore, despite chemical differences, both lignin fractions have very similar molecular weight distribution patterns and polydispersities (2.45 and 2.42, respectively).

The thermal degradation pattern of both lignin preparations gave additional evidence to the relatively higher recalcitrancy of the alkali insoluble lignin. The TGA of both lignin preparations indicated that nearly 40% of their weight is lost at temperatures below 335 °C (Figure 4, primary ordinate). The degradation of the SEE-WI lignin (Figure 4A) began earlier and was smoother than that observed for the SEE-WIA lignin (Figure 4B), suggesting that the degradation rate of the former was faster than that of the latter at this temperature range. Both lignin preparations were totally consumed when temperatures around 520 °C were reached.

The energy liberated during thermal degradation was then calculated from the differential scanning calorimetry of each lignin sample (Figure 4, secondary ordinate). The thermal decomposition of the SEE-WI lignin released a total of 6476.5 μVs/mg, whereas that of the SEE-WIA lignin liberated only 4791.6 μVs/mg. Considering that most of the SEE-WI lignin was decomposed at the lower temperature range (below 400 °C), it seemed that the chemical bonds present in this lignin fraction have a greater energy content.

At temperatures <400 °C, the thermal decomposition of both SEE-WI and SEE-WIA lignins was shown to reach a maximum at 327−330 °C. This suggested a bulk cleavage of chemical bonds of similar types in both lignins. However, at temperatures >400 °C, the fragmentation pattern of the SEE-WI lignin was shown to have a second maximum at 472 °C, a temperature 16 °C lower than that observed for the SEE-WIA lignin (488 °C). Thus, two populations of organic molecules were identified, one thermolabile (327−330 °C) and another thermostable (472−488 °C) (Figure 4). As the SEE-WIA lignin required higher temperatures for to reach complete degradation, it seemed that the higher thermostability of this lignin was due to the occurrence

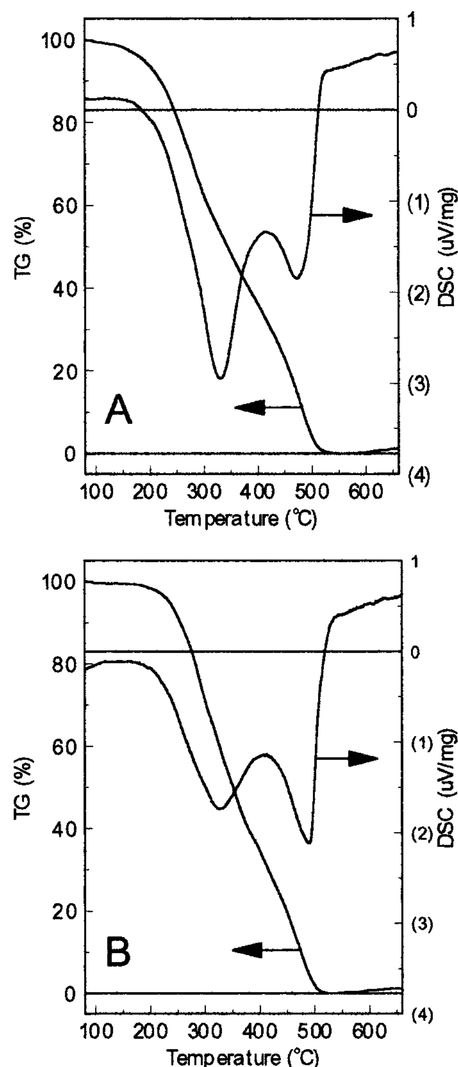
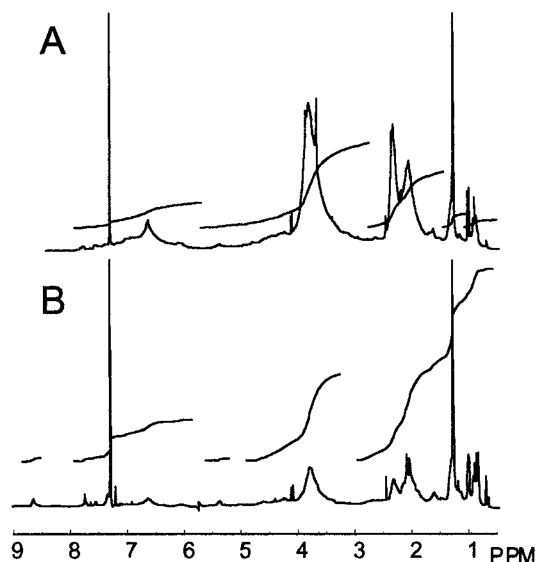


Figure 4. Thermal degradation of SEE-WI and SEE-WIA dioxane lignins prepared from SO_2-impregnated, steam-treated eucalyptus wood chips after extensive enzymatic hydrolysis using a mixture of Celluclast 1.5L and Novozym 188: (A) SEE-WI lignin; (B) SEE-WIA lignin. The primary ordinate indicates the TGA profile of each lignin sample, whereas the secondary ordinate indicates their differential scanning calorimetry.

of more condensed polymeric structures in its composition. Therefore, the SEE-WIA lignin must correspond to a fraction of the SEE-WI lignin that was more extensively modified during pretreatment through mechanisms involving hydrolysis of aryl ether linkages, fragmentation, and condensation.

The methoxyl contents determined by chemical method in both SEE-WI and SEE-WIA lignins were very similar to those obtained by integration of their ^1H NMR spectra. Whereas the chemical method yielded 1.21 and 0.90 mol equiv of methoxyl groups per C_9 unit, the spectroscopic method resulted in approximately 1.15 and 0.76 mol equiv, respectively (Figure 5). Therefore, the ^1H NMR spectra were considered reliable for quantitative analysis of other functional groups in lignin such as contents of both aliphatic and aromatic hydroxyl groups.

Total hydroxyl groups were determined in both acetylated lignin preparations by integrating the signals in the δ range 1.75−2.50 in their ^1H NMR spectra (δ_H for CH_3 in aliphatic and aromatic acetate groups). The total hydrogen content was estimated by integrating the

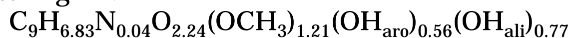


PPM	SEE-WI lignin (A)	SEE-WIA lignin (B)
7.90 - 7.25	0.28	0.40
7.15 - 6.25	1.22	0.76
6.25 - 5.75	0.24	0.20
5.75 - 5.20	0.33	0.20
5.20 - 4.50	0.57	0.48
4.50 - 3.95	1.02	1.01
3.95 - 3.55	3.45	2.29
3.55 - 2.50	1.83	1.55
2.50 - 2.20	0.56	0.46
2.20 - 1.50	0.77	1.02
1.50 - 1.10	1.02	2.58
1.10 - 0.75	0.49	1.49

Figure 5. ^{1}H NMR of acetylated dioxane lignins prepared from SO_2-impregnated, steam-treated eucalyptus wood chips: (A) SEE-WI lignin; (B) SEE-WIA lignin. Values shown in the included table correspond to the integration of the spectra for each of the appointed chemical shift regions.

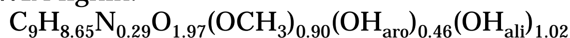
whole spectra. The integration within δ_H for CH_3 in acetyl groups was divided by 3 to account for a 3-fold increase in hydrogens from the original free hydroxyl group. The ratio between aliphatic and aromatic hydroxyl groups was obtained by comparing the integration values between δ 1.75−2.20 and 2.20−2.50, respectively. These observations, together with both the C, H, N analysis and the methoxyl content of both lignin fractions (Table 2), allowed for the calculation of the following hypothetical C_9 formula. It must be noted that technical lignins such as SEE-WI and SEE-WIA lignins do not have a real C_9 unit. However it is customary to expressed their chemical composition in C_9 units to compare their chemical compositions as well as relative amounts of their functional groups.

SEE-WI lignin:



$$C_9H_{6.83}N_{0.04}O_{2.24}(OCH_3)_{1.21}(OH_{aro})_{0.56}(OH_{ali})_{0.77}$$

SEE-WIA lignin:



$$C_9H_{8.65}N_{0.29}O_{1.97}(OCH_3)_{0.90}(OH_{aro})_{0.46}(OH_{ali})_{1.02}$$

These C_9 formulas clearly indicated that both lignin preparations are structurally different. Alkaline washing seemed to remove a lignin fraction with a higher methoxyl and phenolic hydroxyl content, leaving a residual lignin (alkali insoluble lignin) with a lower methoxyl and higher aliphatic hydroxyl content.

According to the ^{1}H NMR spectra (Figure 5), both SEE-WI and SEE-WIA lignins were shown to have a total number of 1.50 and 1.16 aromatic hydrogens (arHs, 6.25−7.90 ppm) per C_9 unit, respectively. On the basis of the OCH_3/C_9 ratio of these lignins, there should be a total of approximately 2.79 and 3.10 arHs/C_9 in each one of these fractions, respectively. Hence, the number of additional substitutions to the aromatic ring can be obtained in each case by subtracting the above-mentioned values. By a simple calculation of this nature, a total of approximately 1.29 and 1.94 additional substitutions of aromatic ring/C_9 unit are obtained for the SEE-WI lignin and the SEE-WIA lignin, respectively. Hence, this extra 0.65 substitution per C_9 unit suggested that the SEE-WIA lignin is a more condensed lignin type than the SEE-WI lignin.

It was also apparent from the ^{1}H NMR spectra that the total H/C_9 ratio in the acetylated SEE-WIA lignin was the same or even lower than that observed for the corresponding acetylated SEE-WI lignin, except for the chemical shift range of δ 1.75−2.20. This region corresponds to the chemical shifts of CH_3 hydrogens in aliphatic acetate groups (Figure 5) (Chen and Robert, 1988; Piló-Veloso et al., 1993). Considering that the chemical shifts of hydrogens in acetate groups of 5-5′ structures fall within this same region, it is possible that the aliphatic hydroxyl contents in both lignin fractions were nearly the same, ~0.77 OH mol equiv/C_9, and that the appearance of a greater amount of hydrogens in the chemical shift range δ 1.75−2.20 may be additional evidence for the higher degree of condensation of the SEE-WIA lignin. However, on the basis of the higher nitrogen content found in this alkali insoluble lignin, part of the signals in the aforementioned range are due to protons linked to aliphatic side chains of amino acids which, themselves, are building blocks of the enzymes and remained irreversibly adsorbed onto this lignin fraction. Alternatively, these signals could partly arise from fatty acid contaminations that commonly occur within technical lignin preparations (Piló-Veloso et al., 1993).

Several hypotheses have been proposed in the literature to explain the role of lignin during the enzymatic hydrolysis of steam-treated substrates. Schwald and coworkers (Schwald et al., 1989a,b) suggested that lignin coats the surface of the steam-treated fibers and hinders the accessibility of the cellulolytic enzymes to the target substrate. Sutcliffe and Saddler (1986) suggested that the adsorption pattern of β-glucosidases onto steam-exploded lignin increases with pretreatment severity, even though the simple entrapment of these enzymes within the three-dimensional matrix of lignin was not totally discarded. Other workers have reported a partial loss of both cellulase and β-glucosidase activities when these enzymes were placed in contact with lignin-containing residues derived from steam-treated wood, and this observation was readily attributed to enzyme inactivation and/or nonspecific binding (adsorption) onto lignin fragments (Clesceri et al., 1985; Ooshima et al., 1990). In addition, there has been some evidence that enzyme recycling proceeds better if lignin has been previously removed from the substrate (Tanaka et al., 1988; Ramos et al., 1993; Ramos and Saddler, 1994).

Our results have shown that the SEE-WIA substrate was not as susceptible to hydrolysis as the corresponding lignin-rich SEE-WI fraction. Therefore, it is probable that part of the enzyme activity was lost due to a nonproductive association between cellulases and the alkali insoluble lignin. Indeed, after complete hydrolysis of the pretreated substrates, the SEE-WIA lignin retained a greater amount of nitrogen than the SEE-WI lignin in its chemical composition. This nitrogen content was certainly associated with the enzymes that remained adsorbed onto lignin, even after extensive water washing of the insoluble residues. Again, mechanisms such as irreversible adsorption or simple entrapment of the enzymes within the three-dimensional lignin matrix may have been involved.

We have also shown that the alkali insoluble lignin of SO_2-catalyzed steam-exploded hardwoods is a more recalcitrant lignin type than the corresponding water insoluble lignin. However, it is important to point out that this heavily condensed polymeric structure was already present as a minor component in the unextracted material (SEE-WI) and that alkali washing caused the accumulation of the modified lignin through the effective removal of the more accessible lignin type. Considering that the conditions used for alkali washing (0.4% NaOH at RT for 1 h) were not drastic enough to result in any chemical modification of the steam-treated lignin, it seemed that the relatively high degree of condensation of the alkali insoluble lignin was a direct result of pretreatment. Once the alkali insoluble lignin became a predominant lignin type in the substrate, its detrimental effects on the enzymatic hydrolysis were more easily and readily observed.

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