NMR study on enzymatic polymerization of spruce lignosulfonate

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1. Introduction

Lignosulfonates, isolated from the black liquors of sulfite cooking to obtain cellulose pulp (Sjöström 1993), are the only available commercial lignins nowadays, with different industrial and agricultural applications. This is because the lignin released during the kraft cooking of wood, largely the process most widely used for paper pulp manufacture, is burnt for recovery of chemicals and energy supply to the mill. The commercial utilization of lignosulfonates largely depends on tailoring their physico-chemical properties for the different applications intended, including molecular weight distribution and presence of functional groups (Lebo et al. 2008). Different chemical treatments, including oxidative modifications, can be used to change these lignosulfonate properties, but the economic costs of some of them limit their industrial applicability, especially when low-value products are to be produced.

Oxidative enzymes, including laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) in the presence of redox mediators, have been largely investigated for the removal of lignin-derived compounds in paper pulp manufacture (Call et al. 1997) and more recently also for pitch control (Gutiérrez et al. 2009) and multipurpose functionalization of pulp fibers and other wood products (Widsten et al. 2008). Very recently the potential of the above enzymatic system to modify lignosulfonates is being investigated as a potential alternative to the chemical treatments, which often require harsh application conditions (Prasetyo et al. 2010).

Despite sulfite cooking historically preceded kraft cooking, the chemical structure of lignosulfonates is not known into detail. This is because some of the most powerful tools for the chemical characterization of complex macromolecules, such as multidimensional nuclear magnetic resonance (NMR) that enabled the discovery of new substructures in wood lignin (Zhang et al. 2006), are only recently being applied to lignosulfonates (Lutnaes et al. 2008). In the present study we used two different NMR techniques - namely solution ^{1}H-^{13}C correlation 2D-NMR in HSQC (heteronuclear single quantum correlation) experiments and solid-state CPMAS (cross-polarization and magic angle spinning) ^{13}C NMR - to investigate the nature and extent of the chemical changes produced by the laccase-mediator system when applied on a softwood lignosulfonate.

2. Materials and methods

2.1 Enzymatic treatment of lignosulfonate

Fifty mL of a 2% water solution of spruce lignosulfonate from Borregaard (Sarpsborg, Norway) were treated with 30 nkat mL^{-1} of *Trametes villosa* laccase (reference NS51002) from Novozymes (Bagsvaerd, Denmark) in the presence of 1 mM 1-hydroxybenzotriazole (HBT) as redox mediator, at 30ºC, and 150 rev min^{-1} shaking. The laccase activity was estimated by oxidation of ABTS (2,2'-azinobis-(3-ethylbenzthiazoline)-6-sulfonate) to its cation radical (ε_{436} 29 300 M^{-1} cm^{-1}) at 30ºC in 50 mM sodium succinate, pH 4.5. An initial control, and two samples collected after short (30 min) and long (83 h) incubation periods were frozen, freeze-dried and stored at 4ºC until analysis.

2.2 Analysis of the treated lignosulfonate

Changes in the lignosulfonate molecular mass during the enzymatic treatment were analyzed by size-exclusion chromatography using three TSK-gel columns (3000 PW, 4000 PW and 3000 PW) coupled in series, 0.1 M sodium hydroxide as the mobile phase (at a flow rate of 1 mL min^{-1}), and UV (280 nm) detection. Solution ^{1}H NMR, ^{13}C NMR and HSQC 2D-NMR spectra were recorded on 40 mg of lignosulfonate dissolved in 0.75 mL of deuterated dimethylsulfoxide (DMSO-d_6) using a Bruker AVANCE 500 MHz as previously described (Ibarra et al. 2007). A semiquantitative analysis of some HSQC cross-peaks was performed including volume integrations and comparison in each of the regions of the spectrum (that include correlations of chemically analogous carbon-proton pairs). CPMAS ^{13}C NMR spectra of solid lignosulfonate samples were recorded for 9 h on a Bruker AVANCE DSX 300 using the standard pulse sequence, a time domain of 4 K, a spectral width of 41666 Hz, a contact time of 2 ms, and an interpulse delay of 4 s. Signals were assigned by comparison with the literature (Bardet et al. 2006; Capanema et al. 2004; Lebo et al. 2008; Liitiä et al. 2003; Lundquist 1981; Lutnaes et al. 2008; Martínez et al. 1999; Ralph et al. 1999; 2004; Robert 1992).

3. Results and Discussion

Treatment of spruce lignosulfonate with *Trametes villosa* laccase in the presence of mediators (HBT in the present study) appeared as a procedure to significantly improve its dispersion properties (Prasetyo et al. 2010), at the same time that a significant polymerization is produced. The lignosulfonate molecular mass estimated by SEC passed from 28 kDa to 191 kDa at the end of the treatment with the laccase-mediator system (**Figure 1**).

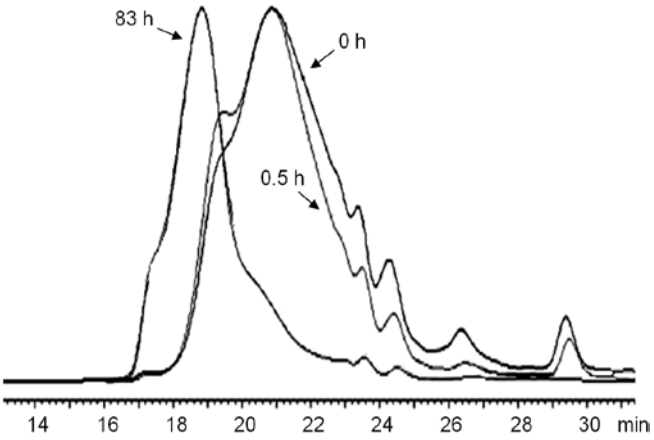

Figure 1. SEC of lignosulfonate (1 g in 50 mL of distilled water) treated with *T. villosa* laccase (30 nkat mL^{-1}) and HBT (1 mM) for 0, 0.5 and 83 h, at 30ºC and 150 rpm.

The structural changes produced during the above treatment were analyzed by a combination of solution (1D and 2D) and solid-state NMR spectroscopy. The ^{1}H-^{13}C correlations in the HSQC spectra (**Figure 2**) showed the aromatic moiety of the lignosulfonate and the predominant α-sulfonated β-O-4' substructures, the latter together with some polysaccharides and an important amount of other aliphatic oxygenated structures. The former (lignin) HSQC signals near completely disappeared at the end of the enzymatic treatment (over 3 days) while the latter ones were not affected by the enzyme.

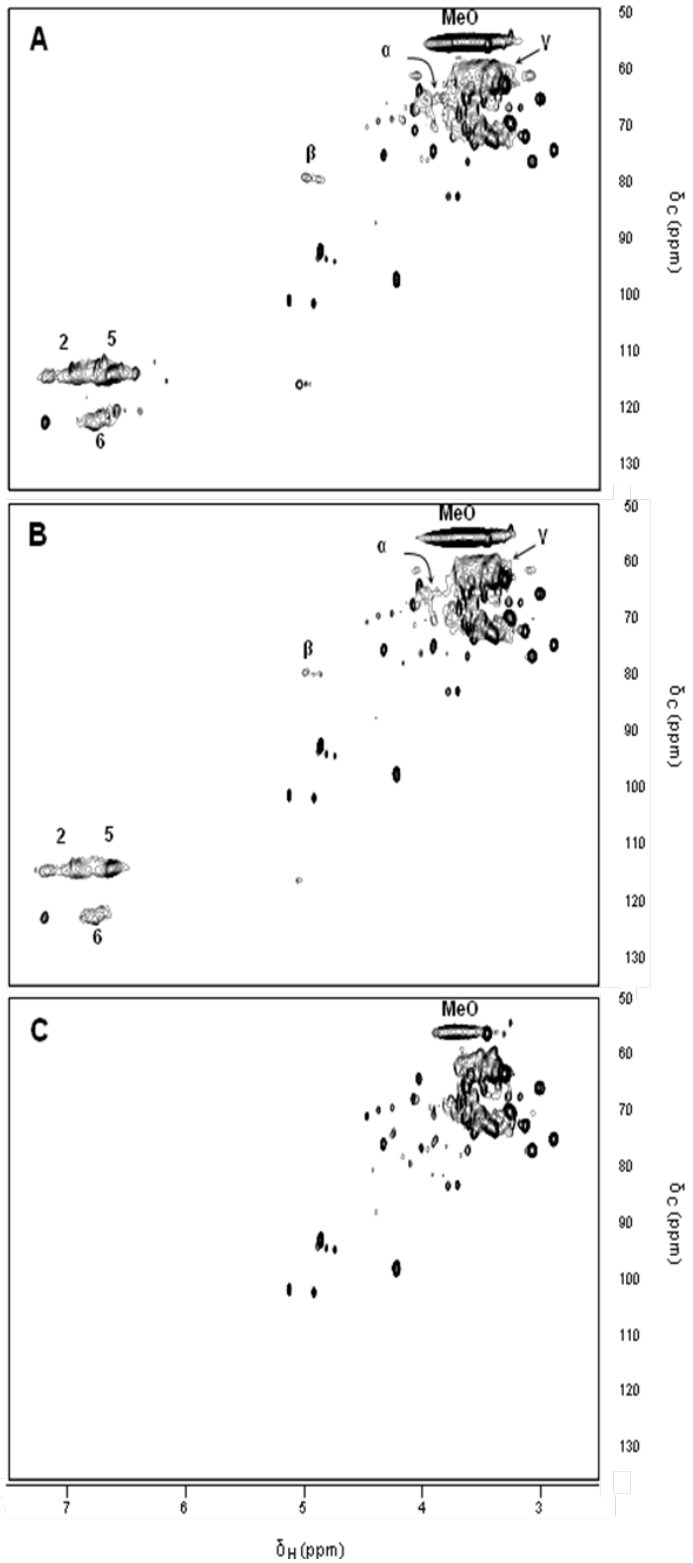

Figure 2. Expanded aromatic and aliphatic-oxygenated regions, δ_H/δ_C 2,5-7.5/50-135 ppm, of the HSQC spectra of spruce lignosulfonate treated with *T. villosa* laccase and HBT for 0 (**A**), 0.5 (**B**) and 83 h (**C**) showing, among others, lignin methoxyl (MeO), side-chain (α, β and γ) and aromatic (2, 5 and 6) ^{1}H-^{13}C correlation signals.

The above changes were correlated with the complete disappearance of the aromatic proton signals in the ^{1}H NMR spectra (not shown). Although the lignosulfonate solutions yielded considerably noisy ^{13}C NMR spectra (**Figure 3**), they clearly showed that the total percentage of aromatic carbon was maintained at the end of the enzymatic treatment, in spite of the lack of aromatic correlation peaks in the HSQC spectrum. A displacement of the main ^{13}C signals was observed, which is discussed below after the solid-state NMR analyses.

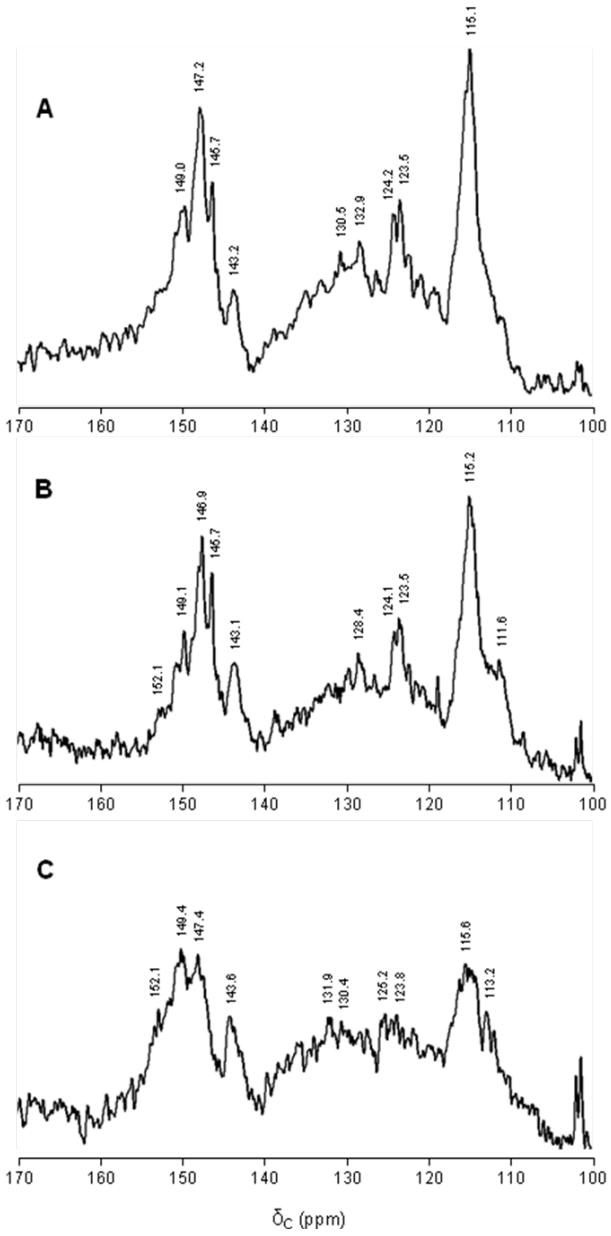

Figure 3. Expanded aromatic region, δ_C 100-170 ppm, of the ^{13}C NMR spectra of spruce lignosulfonate treated with *T. villosa* laccase and HBT for 0 (**A**), 0.5 (**B**) and 83 h (**C**). See **Figure 4** for assignment of the main signals.

The CPMAS ^{13}C NMR spectra, obtained in the solid state to prevent solubility problems due to lignin polymerization (**Figure 4**), confirmed the above results (i.e. that the lignosulfonate benzenic nuclei are not degraded during the enzymatic treatment). Moreover, they revealed a decrease of

the signals around 114 and 147 ppm (the latter being displaced to around 148 ppm) and an increase of the shoulder around 152 ppm (being specially evident after peak deconvolution). The two former signals correspond to free C5 and methoxylated C3 in normal G-type units (being modified during the treatment), while the 152 ppm shoulder was assigned to C3 in new 5-5' or C3 (and C4/C5) in new 4-O-5' structures formed during the enzymatic treatment.

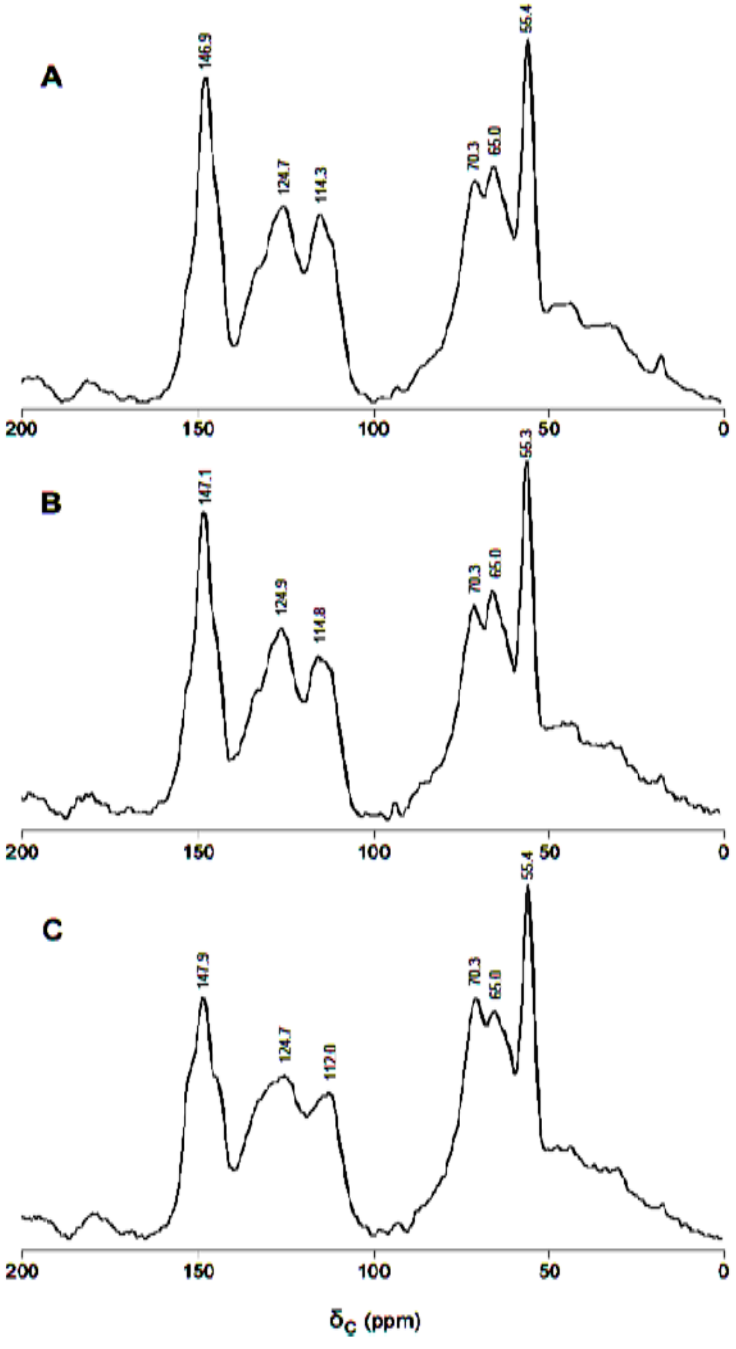

Figure 4. CPMAS ^{13}C NMR spectra of spruce lignosulfonate treated with *T. villosa* laccase and HBT for 0 (**A**), 0.5 (**B**) and 83 h (**C**). The main signals were assigned to C3/C4 (~147 ppm), C1/C6 (~125 ppm), C2/C5 (~114 ppm) and methoxyls (~55 ppm) in the lignosulfonate units, while the signals around 70 and 65 ppm correspond to lignin side-chains overlapping with those from carbohydrates and other oxygenated contaminants.

The latter results, together with those from HSQC NMR, suggest that the laccase-mediator treatment oxidizes lignin units, the phenolic content being significantly reduced (Prasetyo et al. 2010), and promotes condensation reactions between the aromatic radicals formed resulting in new 5-5' and 4-O-5' linkages, and the near 7-fold polymerization observed. At the end of the treatment, a wide oxidative attack on the lignin benzenic ring will be produced causing the loss of aromatic ^{1}H-^{13}C correlations in the HSQC NMR spectra.

4. Acknowledgements

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