

POLYMERIZATION OF DIFFERENT LIGNINS BY LACCASE

Maija-Liisa Mattinen,^{a*} Tapani Suortti,^a Richard Gosselink,^b Dimitris S. Argyropoulos,^c Dmitry Evtuguin,^d Anna Suurnäkki,^a Ed de Jong,^e and Tarja Tamminen^a

In this study the oxidative polymerization of different lignins, i.e. Flax Soda lignin, Spruce EMAL, and *Eucalyptus* Dioxane lignin by *Trametes hirsuta* laccase was compared. Initially the structures of the different lignins were compared by Fourier transform infrared spectroscopy. The reactivity of laccase with the different types of lignins in the absence of mediators was examined and verified by oxygen consumption measurements. The molecular weight distributions of treated and untreated lignins were determined by two different size exclusion chromatography methods. Furthermore, the potential of matrix-assisted laser desorption/ionisation-time of flight-mass spectroscopy for determination of the absolute molecular weights of the different lignins was evaluated. The data showed that all the technical lignins could be activated and polymerized by laccase to different degrees. The efficiency as indicated by measurements of the degree of polymerization was found to increase in the order of Spruce EMAL < *Eucalyptus* Dioxane lignin < Flax Soda lignin. Overall, this data supplies foundations for using enzymes more efficiently in the enzymatic upgrading of lignin.

Keywords: Lignin; Laccase; Molecular weight distribution; Polymerization; SEC; MALDI-TOF MS

Contact information: a: VTT, P.O. Box 1000, FIN-02044 VTT, Finland; b: Wageningen UR, Agrotechnology & Food Innovations BV, Biobased Products, Fibre & Paper Technology, P.O. Box 17, 6700 AA Wageningen, The Netherlands; c: Organic Chemistry of Wood Components Laboratory, North Carolina State University, Department of Forest Biomaterials Science & Engineering 5-80 Raleigh, USA; d: University of Aveiro, Department of Chemistry, Santiago, 3810-193 Aveiro, Portugal; e: Avantium Technologies BV, Zekeringstraat 29, 1014 BV Amsterdam, The Netherlands

*Corresponding author: Maija.Mattinen@vtt.fi

INTRODUCTION

Lignin is one of the most abundant polymers in nature and the second in abundance as constituents of cell walls of plants after cellulose. The chemical composition of lignin varies from plant to plant (Glasser et al. 1981). The chemical structure of lignin cannot be described by a simple structural formula, because it is a statistically amorphous branched biopolymer (Freudenberg and Neish 1968; Nimz 1974; Alder 1977; Sakakibara 1980; Hwang et al. 1989). Lignin is chemically bonded to cell wall polysaccharides, as reviewed by Koshijima and Watanabe (2003). Hence, isolated lignins may contain small amounts of carbohydrate impurities (Koshijima and Watanabe 2003).

Lignins from various plant materials and pulping processes provide an important source of raw material that may be converted into value-added products by chemical or enzymatic means (Sena-Martins et al. 2008). The free phenolic units (Hong et al. 2006)

in lignin can be enzymatically or chemically oxidized, yielding resonance-stabilised phenoxyl radicals via a single electron transfer process. These radicals are potential sites for coupling reactions with reactant phenoxyl radicals, thus providing a route for the addition of desired functionalities to lignin. Laccases can oxidize both polymeric lignin and small molecular reactants via a one-electron oxidation, yielding reactive intermediates. Most studies on radical coupling relate to lignin biosynthesis (Boerjan et al. 2003; Ralph et al. 2004; Davin et al. 2000). Laccase in the presence of mediators oxidizes lignin via hydrogen atom transfer, which is different from the electron transfer mechanism in direct laccase oxidation (Baiocco et al. 2003; Crestini et al. 2003) and typically leads to lignin degradation.

In order to evaluate the reactivity and suitability of various lignins as raw materials for various applications, it is important to be able to characterize the absolute molecular mass (MM), the molecular weight distributions (MWD), and structural features i.e. the number and type of functional groups and the distribution of inter-unit linkages in lignin. Small sized and flexible lignin molecules probably provide better accessibility for laccase and the reactants. Non-covalent hydrophobic interactions between the aromatic rings in lignin (Chen et al. 2003; Sarkanen et al. 2007) may hinder reactions between lignin macromolecules and reactants. The strongest interactions have been found in softwood lignins, less strong in hardwood (*Eucalyptus*), and none in straw lignin (Guerra et al. 2007).

Different spectroscopic methods such as electrospray ionization mass spectrometry (ESI-MS), matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS), laser desorption ionisation-time of flight-mass spectrometry (LDI-TOF-MS), and temperature resolved analytical pyrolysis field ionisation mass spectrometry (Py-FIMS) have been developed for the determination of MM and MWD of lignin. ESI-MS is a promising mass spectrometric technique for the determination of lignin MM and MWD in solution (Evtuguin et al. 1999). MALDI-TOF MS is a relatively new mass spectroscopic technique that has been employed in the absolute molar mass analysis of different natural and synthetic polymers (Metzger et al. 1992; Jacobs and Dahlman 2002; Reale et al. 2004; Hillenkamp and Peter-Katalinić 2007). The main advantages of the technique are a soft ionization technique, high sensitivity, and wide molar mass range. LDI-TOF-MS has been used for birch milled wood lignin (Srzic et al. 1995). Py-FIMS is a technique to extract and ionize monomeric or dimeric subunits from polymers and it has been applied to lignin and to soil and other plant materials (Leinweber and Schulten 1999; Hempfling and Schulten 1990).

MWD of lignin is generally evaluated by size exclusion chromatography (SEC) (Gellerstedt 1992). Several modifications of alkaline and organic SEC have been developed for lignin. The choice of a suitable solvent to avoid complex interaction phenomena between lignin molecules or between lignin and the column packing material is critical for the success of the separation (Chum 1987). The interpretation of SEC data is based on comparison with calibration standards (Faix and Beinhoff 1992), assuming that the ratio between the weight of the molecule and its hydrodynamic radius is the same in the sample and in the calibration standards, which is not necessarily the case. Typically linear polystyrene standards have been used for calibration, since amorphous standards better representing lignin macromolecules are not yet available. Bayerbach et al. (2006)

and Baumberger et al. (2007), compared different analytical methods for the characterization of MM and MWD. In general, the mass spectrometric techniques are attractive over the SEC methods, because in these methods the actual weight of the lignin is measured instead of the hydrodynamic radius of the molecules.

In this work, three different types of isolated lignins were used as model compounds to study the efficiency of laccase-catalyzed oxidation of lignin in the absence of mediators. The tested lignins were of different botanical origins i.e flax, softwood, and hardwood. The flax lignin was isolated from soda pulping black liquor (Lora and Glasser 2002). Wood lignins were isolated from *Eucalyptus globulus* (Evtuguin et al., 2001) and Norway Spruce TMP pulp (Guerra et al. 2006, 2008) by the methods that gave the highest yield and thus most representative lignin sample. Although the chemical and mechanical separation of lignin may alter its structure, it is probable that the isolated lignin still possesses the same morphology as the native lignin in the cell walls. FTIR spectroscopy was used to identify certain important functional groups in the model lignins. The *Trametes hirsuta* laccase (ThL) of high redox-potential (0.78 V) was used for lignin activation and to alter the MWD and chemical structure of the model lignins. Two different alkaline SEC methods were compared and used to analyse the polymerization products. The potential of MALDI-TOF MS for high molecular weight (HMW) lignin characterization was also tested.

EXPERIMENTAL

Materials

Enzyme

Laccase from the filamentous fungus *Trametes hirsuta* was purified and characterized at VTT (Rittstieg et al. 2002). Enzyme activity was determined using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) as substrate (Niku-Paavola et al. 1988). The pH optimum of laccase application was 4.5.

Substrates

Lignins used in the ThL treatments were soda lignin from flax from Granit (Flax Soda lignin), enzymatic mild acidolysis lignin from *Picea abies* (Spruce EMAL) and Dioxan lignin from *Eucalyptus globulus* (*Eucalyptus* Dioxane lignin). The chemical properties of these lignins are described in the final report of COST Action E41 (<http://www.vtt.fi/proj/coste41/>).

Methods

Solubilization of lignins

Most industrial lignins are soluble at high pH. However, at alkaline pH laccases lose their activity. Hence, before the ThL treatments, the lignins were solubilized by weighing 50 mg of dry lignin into a 50 ml measuring flask and mixed with 35 ml 100 mM NaOH and 3.5 ml 1 M HCl. Thereafter the measuring flasks were filled to the mark with 50 mM sodium citrate buffer, pH 5.0. The pH of the final true solution was 5.0 (Fig. 1).

Enzyme treatments

ThL-catalyzed oxidation of solubilised lignins was monitored by measurement of oxygen consumption. Before adding the enzyme (100 nkat g⁻¹) into the reaction vessel, lignin solutions (32 ml) were stabilized for 20 minutes at room temperature. After ThL addition the consumption of dissolved oxygen was monitored for 3 h using an oxygen electrode (PCM800 Orion Sensor link, Finland). The measurements were carried out under constant mixing in completely filled and sealed glass flasks in order to avoid entry of oxygen into the reaction mixture during the experiments. After the oxygen consumption measurement the reaction vessels were left open and ThL treatments were continued overnight (Fig 1).

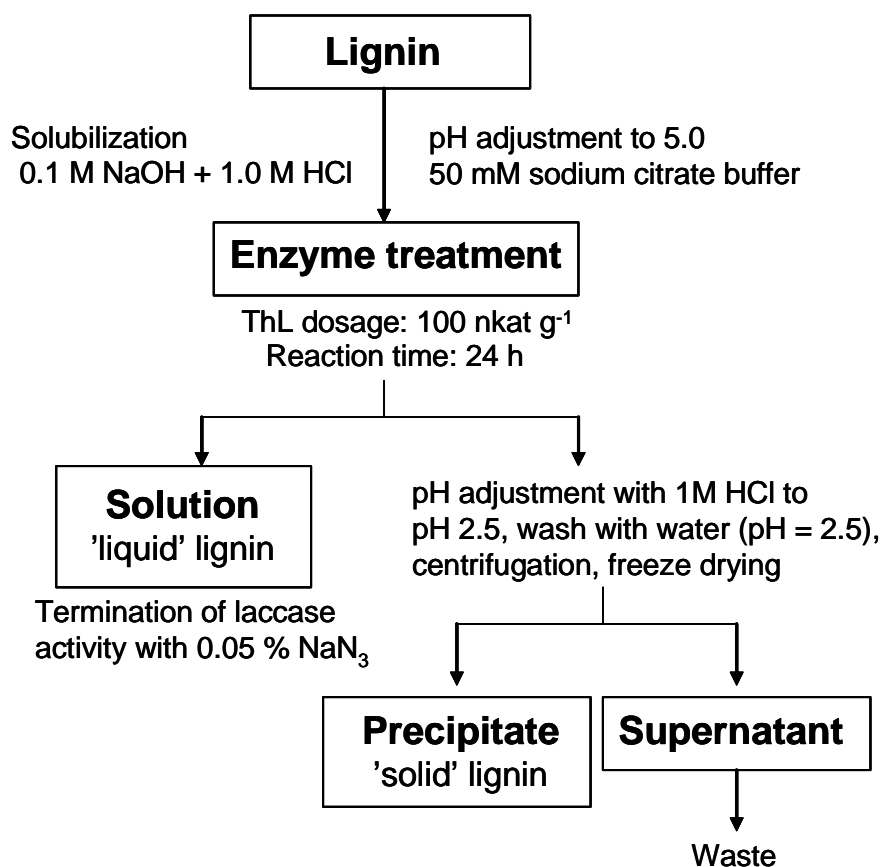


Fig. 1. Treatment scheme of lignin and ThL treatment prior to FTIR, MALDI-TOF MS and SEC analyses

After ThL treatments, the reaction mixtures were divided into two parts. In one half of the reaction mixture ThL activity was terminated with 0.05 % sodium azide (hereafter called 'liquid' lignin). In the other half of the reaction mixture the pH of the solution was adjusted to 2.5 with 1 M HCl. The precipitated lignin was centrifuged and washed with water (pH 2.5). After centrifugation, the precipitated lignin was freeze dried (hereafter called 'solid' lignin) for further analyses. The supernatants were discarded.

FTIR spectroscopy

FTIR spectra of different enzymatically treated and untreated lignins were measured using a Bruker Equinox 55 FTIR spectrometer equipped with an IR-microscope and MCT detector (Karlsruhe, Germany). For the analysis, a few milligrams of freeze dried lignin were applied to a diamond cell and the transmission spectra (4000 to 600 cm^{-1}) of the treated and untreated lignins were measured at room temperature. The spectral resolution was typically 4 cm^{-1} and the number of scans was typically 100. The collected transmission spectra were baseline corrected and normalised according to the highest band using Opus software.

MALDI-TOF mass spectroscopy

MALDI-TOF MS spectra were obtained using a Bruker Ultraflex II instrument (Bremen, Germany). The mass spectrometer was equipped with an N_2 -laser (337 nm / 100 μJ). The matrix compound was 2,5-dihydroxy benzoic acid (DHB) (Leipzig, Germany) dissolved in N,N-dimethylformamide (DMF, peptide synthesis grade, Biosolve Ltd, Valkenswaard, The Netherlands) with a concentration of 10 g L^{-1} . 'Solid' lignins were dissolved in DMF with a concentration of 1 g L^{-1} . 'Liquid' lignins were first dried overnight under N_2 -flow before dissolving in DMF with a concentration of 1 g L^{-1} . For the MALDI-TOF MS analyses solubilised lignins were mixed with DHB solution 1:2 (v/v) and 1 μL of the mixture was placed on the golden MALDI target plate and dried under air flow. The positive ion MALDI-TOF MS spectra were collected in the linear mode. Maltoheptaose and maltodextrin (Sigma–Aldrich GmbH, Schnelldorf, Germany) solubilised in DMF were used for the molecular mass calibration.

Size exclusion chromatography

SEC analyses of lignins were carried out using two different alkaline elution methods. The first method is generally accepted to determine MWD of different lignins (Baumberger et al., 2007). When using this method the treated and untreated lignins were separated using a manually packed TSK gel Toyopearl HW-55F column (length 30 cm, i.d. 7.8 mm, stainless steel, Tokyo, Japan). Toyopearl is a rigid methacrylic polymer incorporating hydrophilic matrices with high mechanical and chemical stability. It is a chromatographic resin that is specially designed for aqueous SEC. The mean pore diameter of the resin is 500 Å and particle size distribution (> 80 %) is within the range 30 – 60 μm . Isocratic analyses were performed using a chromatography system from Waters (717plus autosampler, 1515 isocratic pump, 410 differential refractometer and 2487 dual absorbance detector) at 30 °C, using 50 mM NaOH as eluent. The running time was 15 min at a flow rate of 1 ml min^{-1} . For the analyses the lignin samples were dissolved overnight under gentle stirring in 50 mM NaOH. The injection volume was 100 μL . Breeze software (version 3.20) was used for data analysis.

In the second SEC method a Waters size exclusion chromatography apparatus equipped with three $\mu\text{Hydrogel}$ columns (pore size 2000 Å, 250 Å and 120 Å) in series (Milford MA, USA) was used to separate the treated and untreated lignin macromolecules. Ultrahydrogel™ columns packed with cross-linked hydroxylated polymethacrylate gels (particle size: 13 μm) are ideal for the analysis of water-soluble samples. The chromatographic instrument consisted of M-2690 separation module, M-996 diode

array detector (detection range 230 – 500 nm) and M-2410 refractive index detector. Isocratic chromatography was performed at 60 °C using 50 mM NaOH as the eluent. The flow rate was 0.50 ml min⁻¹. The system was controlled and data was analysed with Empower software. Before the analyses lignin samples were dissolved in 100 mM NaOH at a concentration of 1mg ml⁻¹. Injection volume was 50 µL.

RESULTS AND DISCUSSION

The aim of the ThL treatments of different types of isolated lignins was to study whether it is possible to alter the functionality and MWD of lignin in order to alter its physical properties and/or enable further chemo-enzymatic upgrading.

Lignin Structure

Untreated and ThL-treated ‘solid’ lignins as well as corresponding references were first analysed by FTIR spectroscopy (Fig. 2) and interpreted according to Hergert 1971. The Spruce EMAL and *Eucalyptus* Dioxane lignins showed the predominance of bands typical for G- and S-type lignins, respectively. By contrast, the Flax Soda lignin appeared to be a mixture of these two types. The typical aromatic lignin bands (ca. 1505, 1420 and 1600 cm⁻¹) were evident in all samples. The FTIR spectra measured from the reference lignins were almost identical with the spectra measured from the untreated lignins, confirming that the solubilisation did not affect the structure of lignins. Only in the case of Flax Soda lignin (Fig. 2 A) did the carbonyl band at 1670 cm⁻¹, typical for lignin impurities or moisture associated with lignin, decrease after the treatments, suggesting that the original lignin was purified in the precipitation.

Activation of Lignins

The reactivities of ThL with different lignins were analysed by measurement of oxygen consumption. Before the enzyme addition, lignin solutions were stabilized at room temperature. On the basis of the oxygen consumption curves (Fig. 3), clear oxidation of lignins by ThL was observed already in three hours. The oxidation of the lignins decreased in the order of Flax Soda lignin > *Eucalyptus* Dioxane lignin > Spruce EMAL. All the studied lignins constituted of G- and S-type lignins with redox potentials of 0.73 V and 0.58 V, respectively, well below the redox potential of ThL (0.78 V). The slight oxidation of the Spruce EMAL by ThL was probably due to the low solubility of this lignin, which hindered the laccase – substrate interaction, rather than caused by the chemical structure of the lignin.

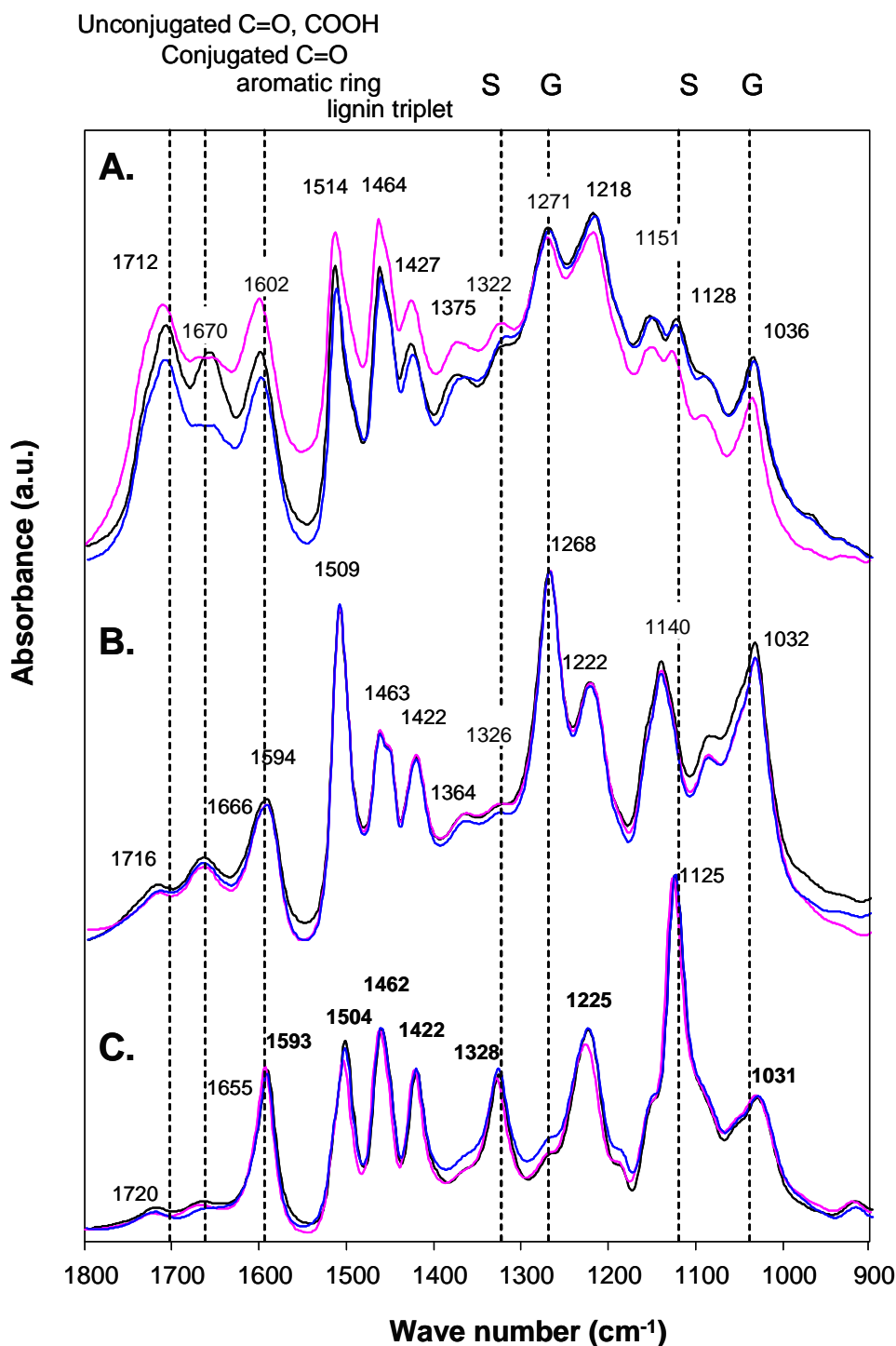


Fig. 2. FTIR spectra measured from (A) Flax Soda lignin, (B) Spruce EMAL and (C) *Eucalyptus* Dioxane lignin. Pink = ThL-treated lignin spectra and blue = the corresponding reference spectra. Black = spectra measured from untreated lignins. Abbreviations: G = guaiacyl and S = syringyl type lignin

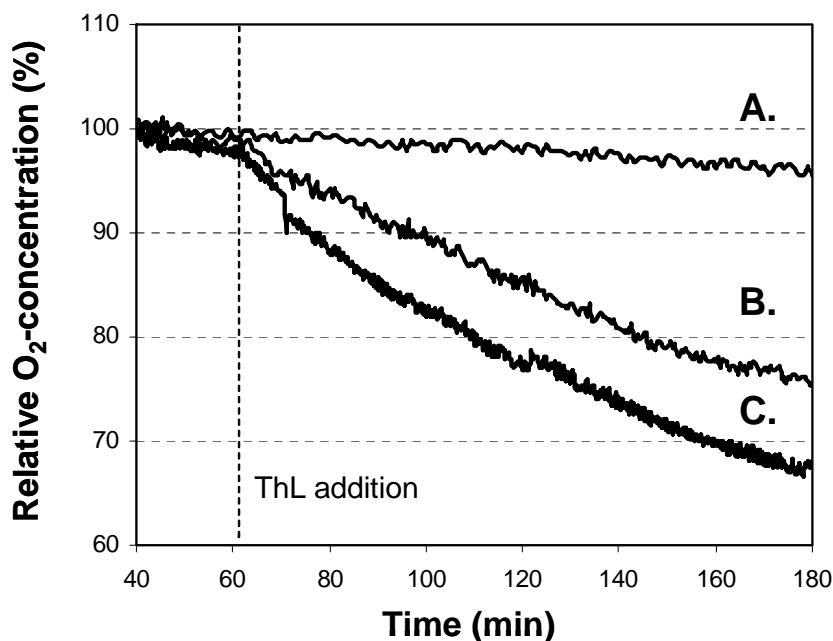


Fig. 3. Oxygen concentration vs. time. Oxidation of (A) Spruce EMAL, (B) *Eucalyptus* Dioxane lignin and (C) Flax Soda lignin by ThL

Polymerization of Lignins

After ThL treatments, the lignins were studied by FTIR spectroscopy, by two different alkaline SEC methods and by MALDI-TOF MS. The FTIR spectra measured from ThL-treated lignins are shown in Fig. 2. When the spectra are compared to those measured from the corresponding reference sample (blank) as well as to the spectra measured from the untreated lignins, they are surprisingly similar to each other. Clearly, the sensitivity of the method was not high enough to be able to detect changes in the structure of different lignins after ThL treatments. On the other hand, the precipitated ‘solid’ lignins contain only part of the whole lignin and hence the samples are not as representative as ‘liquid’ lignin samples. Because the recoveries of the ‘solid’ lignins varied to some extent (Table 1), both fractions were analysed by size exclusion chromatography.

SEC is a straightforward and widely accepted method to determine the relative MWD of lignins and thus it is often used as a routine method. Due to the lack of suitable calibration standards, numerical parameters were not determined from the measured chromatograms. Hence, the analysis of the data is based on visual comparison of the elution time and shape of the chromatograms. In this study, two different SEC methods were tested and compared with different ThL-treated lignins. The high-resolution columns offer many advantages over conventional SEC columns, because of the minimal non-size exclusion effects. In Figs 4 - 6 the size exclusion chromatograms measured from ThL-treated Flax Soda lignin, Spruce EMAL and *Eucalyptus* Dioxane lignin are shown, as well as chromatograms measured from the corresponding ‘solid’ references and untreated lignins using μ Hydrogel columns from Waters. In the inserts of these figures, the corresponding chromatograms measured using HW-55F from Toyopearl are shown.

Most of the lignins had good solubility in 50 mM NaOH and all lignin samples could be examined without problems.

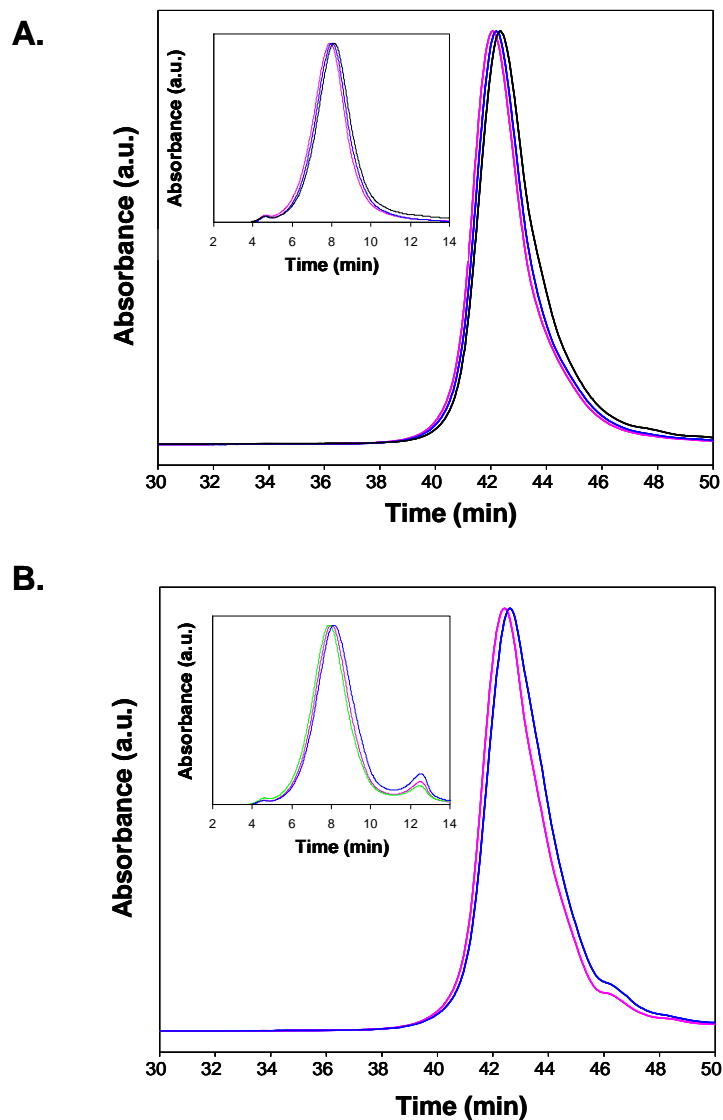


Fig. 4. Size exclusion chromatograms measured from Flax Soda lignin. (A) ‘Solid’ and (B) ‘liquid’ lignin. Pink = ThL-treated lignin, blue = the corresponding reference lignin, black = untreated lignin and green = ThL-treated lignin without terminating laccase reactivity with NaN_3 and measured using Waters μ Hydrogel columns. In the inserts the chromatograms measured using the Toyopearl HW-55F column are shown.

In the case of Flax Soda lignin (Fig. 4), it is clear that ThL could polymerize this lignin to some extent. In the size exclusion chromatograms measured from the ‘liquid’ and ‘solid’ lignin the lignin band was clearly shifted towards the higher molecular weight area, showing that the MM of lignin increased after ThL treatment. In the absence of NaN_3 , polymerization of lignin proceeded further (Fig. 4 B, insert).

When the chromatograms measured with two different SEC methods from the treated and untreated lignins are compared they are almost identical to each other. Rather well-defined Gaussian distribution lignin bands were obtained with both SEC methods. However, in the case of the ‘liquid’ lignin sample, small molecules from the system (eluting between 12 and 13 min) could be separated from the main lignin component when using the Toyopearl HW-55F column. In the other method in which Waters μ Hydrogel columns were used for lignin separation these impurities could not be separated from the main lignin band. Instead, a small shoulder band eluting at around 46 - 48 min was detected from the chromatogram.

The recovery of ThL-treated Flax Soda lignin as well as the corresponding reference ‘solid’ lignin is shown in Table 1. The recovery of ThL-treated lignin was ca. 20 % higher than that of the ‘solid’ reference sample due to decreased solubility as a consequence of polymerization after ThL treatment.

On the basis of the oxygen consumption measurements (Fig. 3 C), two different SEC methods (Fig. 4) and yield of precipitation (Table 1), ThL could easily oxidize this type of lignin.

Table 1. Recoveries (%) of Model Lignins after Different Treatments.

Lignin	Reference (%)	ThL-treated (%)
Flax Soda lignin	52	72
Spruce EMAL	96	92
<i>Eucalyptus</i> Dioxane lignin	81	88

Size exclusion chromatograms analysed from ThL-treated Spruce EMAL as well as from the corresponding reference samples are shown in Fig. 5. The chromatograms measured from ThL-treated ‘solid’ and ‘liquid’ lignins (Figs. 5 A and B) are almost identical with the references, revealing that no lignin polymerization occurred during ThL treatment as could be expected on the basis of the oxygen consumption measurement (Fig. 3 A). However, when the chromatogram measured from the untreated lignin is compared to those measured from the precipitated ‘solid’ lignin, it is clear that lignin was purified in this treatment. The dissolution/precipitation changed the MWD of the lignin probably by breaking up aggregates, as detected by the slight shift of the main lignin band to the lower MWD area.

On the other hand, when the chromatograms measured with two different SEC methods from the treated and untreated lignins are compared, they were clearly different from each other. When the Toyopearl HW-55F column was used some of the lignin eluted already in the void volume of the column (c.a. 5 min). This was obvious especially in the case of untreated Spruce EMAL (Fig. 5 A, insert). Because a smaller amount of lignin was eluted in the void volume of the column in the case of the ThL-treated and ‘solid’ lignin reference samples, it appears that precipitation breaks up aggregates and narrows the MWD of lignin. When using Waters μ Hydrogel columns the main lignin band had an almost perfect Gaussian shape for ‘solid’ lignins.

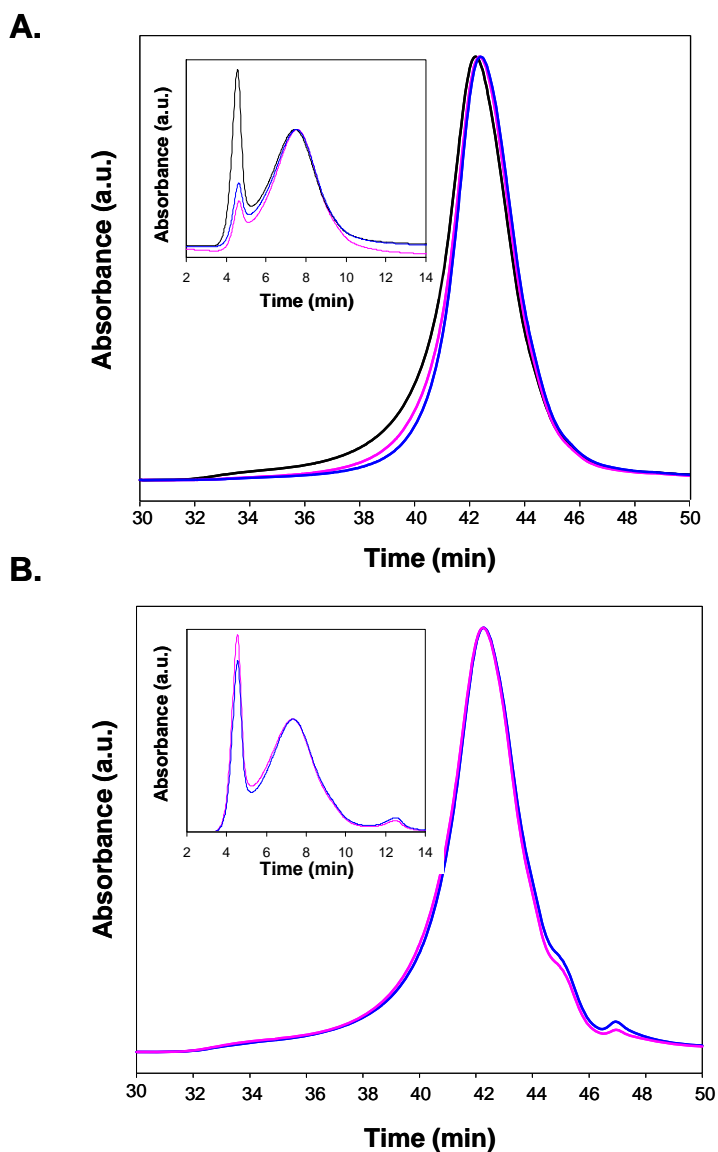


Fig. 5. Size exclusion chromatograms measured from Spruce EMAL. (A) ‘Solid’ and (B) ‘liquid’ lignin. Pink = ThL-treated lignin, blue = the corresponding reference lignin and black = untreated lignin measured using Waters μ Hydrogel columns. In the inserts the corresponding chromatograms measured using the Toyopearl HW-55F column are shown.

The recovery of ThL-treated Spruce EMAL, as well as the recovery of the ‘solid’ reference lignin, is shown in Table 1. The recovery of ThL-treated lignin was almost the same as that of the reference sample, suggesting that it hardly reacted or polymerized during the ThL treatment.

On the basis of the oxygen consumption measurement (Fig. 2 A), two different SEC methods (Fig. 5) and precipitation (Table 1), oxidation of this lignin by ThL was weak. The apparent explanation is the low solubility of lignin that could be observed already in the sample preparation, since the lignin tended to precipitate during the pH adjustment after solubilisation.

Size exclusion chromatograms measured from ThL-treated *Eucalyptus* Dioxane lignin and the corresponding reference chromatograms are shown in Fig. 6. The shape of the lignin band measured from the ‘solid’ untreated and treated lignins was almost Gaussian when Waters μ Hydrogel columns were used for separation (Fig. 6 A). Although the position of the top of the lignin band was not affected by the treatments, the width of the ThL-treated lignin band was much broader than that of the corresponding reference sample and shifted towards high MWD, suggesting that it had reacted and polymerized at least to some extent. Polymerization of *Eucalyptus* Dioxane lignin by ThL was clear in the case of ‘liquid’ lignin (Fig. 6 B), as could be expected on the basis of the oxygen consumption measurement (Fig. 3 B).

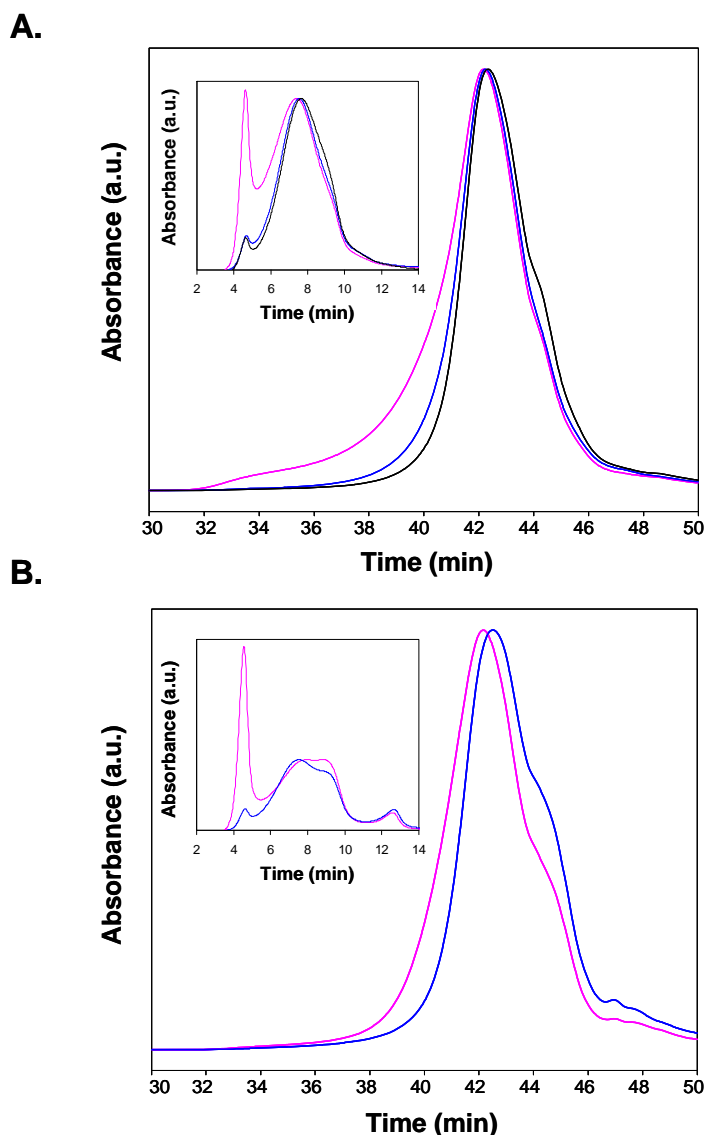


Fig. 6. Size exclusion chromatograms measured from *Eucalyptus* Dioxane lignin. (A) ‘Solid’ and (B) ‘liquid’ lignin. Pink = ThL-treated lignin, blue = the corresponding reference lignin and black = untreated lignin measured measured using Waters μ Hydrogel columns. In the inserts the corresponding chromatograms measured using the Toyopearl HW-55F column are shown.

When the chromatograms measured with two different SEC methods from the treated and untreated *Eucalyptus* Dioxane lignin are compared, they are completely different from each other. As in the case of Spruce EMAL, when the Toyopearl HW-55F column was used some of the lignin eluted in the void volume of the column, in ca. 5 min (Figs. 6 A and B, insert).

Especially in the case of ThL-treated lignin this was very clear. This is also an indication that MWD of lignin was increased after ThL treatment, since some of the polymerized lignin eluted out of the column without separation. Furthermore, when using this kind of column the shape of the main lignin band was very broad, suggesting that lignin was adsorbed to some extent on the column resin. Clearly, in the case of *Eucalyptus* Dioxane lignin, more reliable separation of the molecules based on molecular size could be obtained by using Waters μ Hydrogel columns than by using the Toyopearl HW-55F column. The recovery of ThL-treated *Eucalyptus* Dioxane lignin as well as the recovery of 'solid' reference lignin is shown in Table 1. The recovery of ThL-treated lignin was 7 % higher than that of the corresponding reference sample, suggesting that lignin is polymerised during ThL treatment.

The MALDI-TOF mass spectra measured from treated and untreated 'liquid' and 'solid' lignins are shown in Fig. 7. In general, the spectra are rather weak and somewhat noisy. The broad bands detected between 30 and 50 kDa in all other cases except Flax Soda lignin 'liquid' lignin were presumably caused by poorly ionized lignin. Thus, on the basis of this method, it appears that the MWD of these types of isolated lignins were around 40 kDa. However, using this method and sample preparation technique, it was impossible to determine differences in MWD between ThL-treated and untreated lignins.

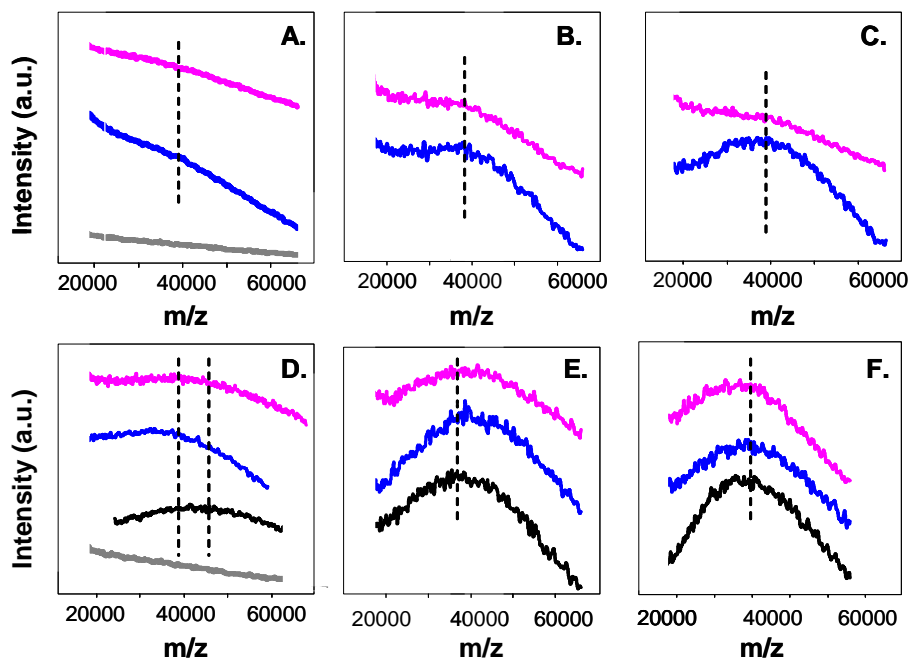


Fig. 7. MALDI-TOF mass spectra of ThL-treated and untreated lignins. (A) 'liquid' and (D) 'solid' Flax Soda lignin; (B) 'liquid' and (E) 'solid' Spruce EMAL lignin; (C) 'liquid' and (F) 'solid' *Eucalyptus* Dioxane lignin. Pink = ThL-treated lignin, blue = the corresponding reference lignin and black = untreated lignin. The grey spectra in (A) and (D) were measured from the pure matrix.

CONCLUSIONS

1. All lignins used in the study, i.e. Flax Soda lignin, Spruce EMAL and *Eucalyptus* Dioxane lignin could be oxidized by *Trametes hirsuta* laccase without any mediators. The oxidation increased in the order of Spruce EMAL < Eucalyptus Dioxane lignin < Flax Soda lignin. Solubility of the lignin at the treatment pH appears to be an important criterion for reactivity.
2. The results provide a basis for enzymatic valorization of lignin. Lignins from various plant materials and pulping processes provide an important source of raw material that may be converted into value-added products by utilizing this technology.
3. On the basis of the SEC comparison presented in this paper, selection of the SEC method depends largely on the type of lignin under study. Special attention should be paid to the selection of the separation columns. Ultrahydrogel separation is based on the hydrodynamic volume representing the molecular size, whereas other phenomena are also important in separation on the Toyopearl gel. No column interaction was observed for Flax Soda lignin, whereas *Eucalyptus* Dioxane lignin was adsorbed. The Toyopearl column used was not able to separate the largest molecules.
4. MALDI-TOF MS appears to be attractive for determination of the absolute molecular weight of lignin. However, ionization of this kind of high molecular weight polymer must be considerably improved to enable successful analysis.

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