Evaluating Lignins as Enzyme Substrates: Insights and Methodological Recommendations from a Study of Laccase-Catalyzed Lignin Polymerization

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Lignin preparations from kraft and sulfite pulping, steam explosion, and enzyme saccharification processes were assessed as substrates for lignin polymerization catalyzed by *Trametes hirsuta* laccase (ThL). Oxygen consumption associated with laccase catalyzed oxidation of the selected lignins was measured using a microplate-based oxygen assay. Laccase-induced changes in the molecular masses of the lignin polymers were assessed with aqueous-alkaline size exclusion chromatography (SEC) and changes in monomeric phenolics by reverse-phase high pressure liquid chromatography (HPLC). Obtaining consistent results in the lignin-laccase assay system required careful pH monitoring and control. All lignin preparations were oxidized by ThL, the rate being highest for steam-exploded eucalypt and lowest for enzyme-saccharified lignin. Comparing lignins, higher lignin-laccase reactivity was correlated with lower lignin molecular mass and higher amounts of monomeric phenolics. Solubility was not an indicator of reactivity. Steam-exploded and lignosulfonate-treated pine preparations were further fractionated by ultrafiltration to determine what molecular mass fractions were the most reactive in ThL catalyzed oxidation. Both retentate (> 3kDa), and to a lesser degree permeate (< 3kDa), fractions were reactive.

Keywords: lignin; Laccase; Mediator; Polymerization; Ultrafiltration; Phenolic; Oxygen sensor

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INTRODUCTION

Lignin constitutes up to 30% of plant biomass and is an abundant low-value byproduct of lignocellulosic material processing. There is much interest in developing high-value products from this material as an alternative to burning it as fuel (da Silva *et al.* 2013; Hüttermann *et al.* 2001; Wyman 2007). Purified industrial lignins can be classified by the type of wood they have been derived from and the processes used to extract and purify them. Their origin and processing determine their chemical and physical properties. Kraft lignin and lignosulfonate, which are byproducts of kraft and sulfite pulping, respectively, are the predominant industrial lignins (Chakar and Ragauskas 2004; Voitl *et al.* 2010). Others are more recent, originating from processes used in the emerging industry of biorefineries (Alriols *et al.* 2010; Mansouri and Salvadó 2006; Martin-Sampedro *et al.* 2011b; Zhao *et al.* 2009).

Using lignin in high-value products will require alteration of its physical and/or chemical properties compared to the original starting lignin (Azadi *et al.* 2013). Enzyme-

catalyzed reactions could potentially be one step of a process to create these new products from lignin substrates. A key step in developing successful enzymatic alteration of lignin will be to generate lignin-based substrates and measure their amenability to enzyme modification. However, measuring enzymatic action on lignin substrates is not straightforward; lignins are well-known enzyme inactivators (Berlin *et al.* 2006; Lange *et al.* 2013; Moilanen *et al.* 2011; Rahikainen *et al.* 2013), and there is no simple and universal way to characterize and quantitate substrate-to-product changes (González Arzola *et al.* 2006; Kim *et al.* 2009; Li *et al.* 2009; Zhou *et al.* 2012). Laccases are a possible model enzyme; their ability to bring about lignin polymerization is well documented (Bollag *et al.* 1982; Madad *et al.* 2013; Mattinen *et al.* 2008; Moya *et al.* 2011; Rittstieg *et al.* 2002; van de Pas *et al.* 2011). They have also been used to graft small phenolic compounds onto biopolymers such as lignin and proteins, and could be used to tailor-make polymers by introducing novel functionalities onto the lignin polymer (Chandra and Ragauskas 2002; Hüttermann *et al.* 2001; Kim *et al.* 2009; Kudanga *et al.* 2010a; Kudanga *et al.* 2010b; Mattinen *et al.* 2008; Mikolasch and Schauer 2009). In this study we have evaluated, using relatively simple techniques, the reactivity of five "industrial lignins" with the enzyme laccase, comparing two lignins derived from standard large-scale production methods such as kraft and sulfite (Voitl *et al.* 2010) with three produced by alternative methods using harsher (steam explosion) and milder (enzyme saccharification) conditions. In addition to presenting these results, we highlight a number of methodological constraints which researchers need to be aware of when planning experimental work for other studies using lignin as an enzyme substrate.

METHODS

Lignins

The kraft lignin product Indulin AT was purchased from MeadWestvaco (Richmond VA, USA) and lignosulfonic acid sodium salt (lignosulfonate) from Sigma (St. Louis MO, USA). The steam exploded (SE) lignins were prepared as described previously (van de Pas *et al.* 2011). Briefly, wood chips were steam-impregnated at 120 °C for 40 min and steam exploded through rapid pressure release at 225 °C for 5 min (softwood) or 210 °C for 9 min (hardwood). Fibres were washed, dried, and milled before extracting with acetone/water 9:1 v:v, washing and freeze-drying. Enzyme saccharification (ES) lignin was prepared as described by Bridson *et al.* (2013). Briefly, *Pinus radiata* wood pulp prepared by a proprietary process was digested with a cocktail of cellulase and cellobiose. The ES lignin was extracted from the undigested material using 1% NaOH at 121 °C for 90 min and dried under vacuum.

Lignin Solubilization

Alkali-solubilized lignins were prepared according to the method described by Mattinen *et al.* (2008), which involved suspending 1.43 mg/mL of dry powdered lignins in 0.1 M NaOH and adding 5 M NaOH dropwise until lignin was dissolved. Acidification of the solution to pH 5.5 was carried out by combining 30 mL of the alkali-solubilized lignin with 10.7 mL of 0.1 M sodium phosphate buffer pH 5.5, and adding 10% (v/v) HCl to bring the final pH to 5.5. These preparations were left overnight at room temperature to stabilize, re-adjusted the following day to pH 5.5 with HCl, made up to 50 mL (final

buffer concentration 21.4 mM and initial lignin concentration 0.86 mg/mL) with water, and clarified by centrifugation (5000 *g* / 5 min). These lignins are referred to as pH-adjusted lignins.

Ultrafiltration

Ultrafiltration of pH-adjusted lignins to generate low (permeate) and high (retentate) molecular mass fractions was carried out using Vivaspin 20 devices with a 3 kDa molecular mass cutoff (GE Healthcare, Chalfont St Giles, UK), centrifuged at 8000 *g* for 60 min. The permeate fraction was collected after one ultrafiltration round of 10 mL of pH-adjusted lignin preparation. The retentate was diluted to 20 mL with 50 mM sodium phosphate pH 5.5, ultrafiltered and this process repeated once more, to remove as much remaining low molecular mass material as possible from this fraction. The original retentate liquid was diluted at least 35-fold by this process.

Laccase Enzyme Activity

Trametes hirsuta laccase (ThL) produced according to Rittstieg *et al.* (2002) was a gift from Dr. K. Kruus, VTT (Espoo, Finland). Reactivity of this high redox potential (780 mV) laccase (Shleev *et al.* 2004) towards 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was measured at room temperature (21 ± 1 °C) in 50 mM phthalate buffer pH 5.0 with 0.2 mg/mL ABTS as the substrate. An extinction coefficient (ε) = 29,000 Mcm^{-1} at 436 nm was used to calculate activity for determining enzyme dose in oxygen uptake assays. Laccase-catalyzed oxidation of lignin was determined by measuring oxygen uptake from air-saturated assay medium using OxoPlate 96-well microplates (PreSens, Regensburg, Germany) according to the manufacturer's instructions. Each well in the microplate has a sensor film on the bottom which fluoresces (excitation 540 nm, emission 650 nm) in proportion to the oxygen concentration of the solution in the well. For the lignin oxidation assay, each well contained 48 mM sodium phosphate buffer pH 5.5 (previously air-saturated by vigorous shaking for several seconds), ThL (0.0057 U) and pH-adjusted lignin in a final volume of 380 µL. The final amount of pH-adjusted lignin per well was 0.19 mg of alkali-solubilized lignin multiplied by the percent recovery of each lignin type during pH adjustment (*i.e.* a constant laccase:lignin ratio of 30 U laccase/g lignin based on the initial alkali-soluble amount). The assay was started by addition of ThL, the plate immediately sealed with an opaque cover, and fluorescence read at 62 s intervals for sixty readings. Non-enzymatic auto-oxidation of lignin was measured in the same assay by adding heat-denatured ThL (10 min at 100 °C) to each lignin. Auto-oxidation rates were subtracted from the total (active enzyme) rate to give an enzymatic oxidation rate. Each sample was assayed in quadruplicate.

At the end of the reaction time a 1.0 mL sample from the combined quadruplicates was taken, 10 µL of 100 mM sodium azide added to stop the reaction, and the sample analyzed by reverse phase high pressure liquid chromatography (RP-HPLC) and size exclusion chromatography (SEC)**.**

Size Exclusion Chromatography (SEC)

Aqueous-alkaline SEC of lignin preparations was based on the method described by Rittstieg *et al.* (2002). A TSKgel $GMPW_{XL}$ SEC column (TOSOH Corporation, Tokyo, Japan) was used to determine molecular mass distributions (MMD) of lignin preparations. The column was equilibrated and run in 50 mM NaOH at 0.5 mL/min and

40 °C on an Agilent 1290 HPLC system (Agilent, Santa Clara CA, USA) with a diode array detector. The injection volume was 20 µL for standard solutions and 5 µL for lignin samples. Samples and standards were adjusted to 50 mM NaOH with 1% (v/v) 5M NaOH. A Varian carbohydrate calibration kit PL2090-0100 (Agilent, Santa Clara CA, USA) was used to calibrate the column over the range 0.18 to 708 kDa.

Reverse Phase Liquid Chromatography (RP-HPLC)

RP-HPLC was carried out with a Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 µm) (Agilent, Santa Clara CA, USA) on an Agilent 1290 HPLC with diode array detection, at a flow rate of 0.5 mL/min using a gradient of buffer A (0.1% formic acid) and buffer B (acetonitrile + 0.1% formic acid) as follows: 100–90% A over 1.6 min, 90–83% A over 4.9 min, 83–60% A over 0.2 min, 60–0% A over 0.5 min, 0% A for 1 min, 0–100% A over 0.1 min, and 100% A for 1.4 min. A 2 µL injection volume was used for samples and monomeric phenolic compound standards.

RESULTS AND DISCUSSION

Solubilization and pH Adjustment of Lignin Preparations

Initial laccase oxidation assays with different lignin substrates (data not presented) did not give reproducible results. On closer investigation, it was observed that over time, the pH of the lignin preparations drifted, usually upwards. Both enzyme activity and lignin solubility are pH-dependent, making the assay very sensitive to this factor. It was found that it was necessary to check and adjust (if necessary) the pH immediately before assaying to obtain good reproducibility, even when using buffered lignin solutions.

The rate of ThL-catalyzed oxidation of lignin preparations was optimal at pH 5.5, and this was used as the working pH. During acidification of the lignins to this pH the colour of the solutions lightened as a proportion of the lignin became insoluble and precipitated. This was particularly noticeable with SE and ES lignins. Kraft lignin was much less precipitated during acidification, and lignosulfonate remained almost completely soluble.

The non-precipitated portion will be a mixture of lower molecular mass phenolic compounds (soluble molecules), and higher molecular mass compounds (soluble molecules and/or suspended intermediate sized colloids) (Kurek *et al.* 1990). Absorbance spectra were obtained for the five alkali-solubilized lignins before and after pH adjustment, and presented as a plot of the ratio of absorbance before:after adjustment (Fig. 1).

The percentage of lignin remaining in solution/suspension after acidification-centrifugation was calculated by averaging these absorbance ratios at 5 nm intervals over the range 250 to 300 nm; the averaged ratio varied from 20% (ES) to 95% (lignosulfonate) (Table 1). This method of estimating lignin recovery was used due to the small amounts of lignins available, which precluded a gravimetric analysis. The validity of this approach was corroborated by SEC analysis of the lignins; the percent recoveries of each lignin by absorbance corresponded to the peak heights and areas of each lignin by SEC.

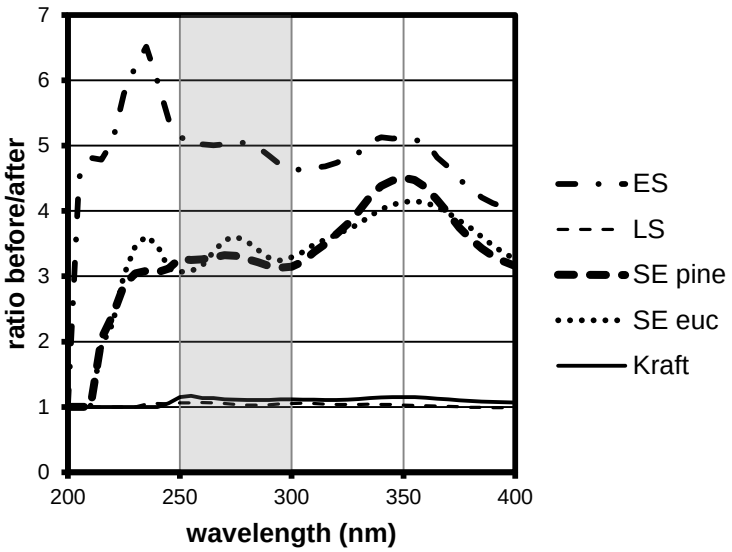

Fig. 1. Ratio of absorbance before and after pH-adjustment of five alkali-solubilized technical lignins. ES, enzyme saccharified; LS, lignosulfonate; SE, steam exploded. The average ratio for each lignin given in Table 1 was calculated using the ratio values between 250 and 300 nm as indicated by the shaded area on the graph.

Table 1. Lignin Properties Before and After Laccase Treatment

Lignin	% sol	M_n		M_w		P_i		Oxygen uptake (μM/min)
		-L	+L	-L	+L	-L	+L	
SE Pine	31	1721	2574 (∧50%)	2751	5023 (∧83%)	1.60	1.95 (∧22%)	1.4 ± 0.10
ES	20	2235	3032 (∧36%)	3993	6115 (∧53%)	1.79	2.02 (∧13%)	0.4 ± 0.04
Kraft	89	2500	2824 (∧13%)	5325	6453 (∧21%)	2.13	2.29 (∧8%)	1.7 ± 0.09
Lignosulfonate	95	6723	8000 (∧19%)	26442	27557 (∧4%)	3.93	3.44 (∨12%)	0.6 ± 0.03
SE Eucalypt	30	1865	2883 (∧55%)	3244	6705 (∧107%)	1.74	2.33 (∧34%)	2.1 ± 0.05

% sol = percentage of alkali-solubilized lignin remaining in solution/suspension after pH adjustment, estimated spectrophotometrically; M_n = number-weighted molecular mass; M_w = mass-weighted molecular mass; P_i = polydispersity index; number in brackets is percentage increase (∧) or decrease (∨) in M_w, M_n or P_i; -L = incubation with denatured laccase; +L = incubation with active laccase. Oxygen uptake is expressed as the reduction in oxygen concentration in the assay mixture per $minute_i$.

Physicochemical Characteristics of Lignin Preparations

The molecular mass distributions of the pH-adjusted lignin preparations, as determined by aqueous SEC calibrated with carbohydrate standards (Chen and Li 2000; Kukkola *et al.* 2011; Rittstieg *et al.* 2002), are presented in Table 1. SE pine and SE eucalypt had the smallest mass-weighted average molecular mass, ES and kraft lignins were intermediate, and lignosulfonate had a very much larger mass. SEC chromatograms of four of the lignins are shown in Figs. 2A-2D; the SEC chromatogram of SE eucalypt (not shown) was similar to that of SE pine.

SEC data can be expressed as number average molecular mass (M_n), weight average molecular mass (M_w), and the ratio M_w/M_n, *i.e.* the polydispersity index (P_i). Small molecules in the < 1 kDa region of the chromatogram potentially have different 280 nm extinction coefficients and anomalous retention times (Rittstieg *et al.* 2002). Because of this, only chromatographic molecular mass data above 0.6 kDa were used to calculate M_w and M_n. However, M_w still can be regarded as a better indicator of changes in polymerization than M_n because M_w is less influenced by low molecular mass compound anomalies.

Molecular mass values determined by aqueous-alkaline SEC were useful for making comparisons between lignins and treatments within the same experiment. Comparing published absolute values is not recommended, given the diversity of experimental conditions, analytical techniques, and sources of the same type of lignin. For example, lignosulfonate M_w values in six publications varied from 7000 to 60,000 (Braaten *et al.* 2003; Chen and Li 2000; Kim *et al.* 2009; Madad *et al.* 2013; Nugroho Prasetyo *et al.* 2010; Voitl *et al.* 2010).

Treatment of Lignin Preparations with Laccase

Laccase activity measurement by oxygen uptake

To determine reactivity of the lignin preparations to laccase, measurements were made of the decrease in concentration of oxygen, one of the laccase substrates, over time in the assay mixture. According to Frasconi *et al.* (2010) laccase will be almost saturated with oxygen at the concentrations present over the time course of the present assay (initially saturated at ~ 260 µM and dropping to no less than approx. 100 µM at the end of the linear phase); thus any reaction rate decrease due to the drop in oxygen concentration during the assay will be minimal.

There are disadvantages to measurement of substrate decrease rather than product formation; to get acceptable constant rates, the rate of oxygen concentration decrease must be above the background noise of the assay system, and the measurement must be done over a short time period to minimize enzyme inactivation and lignin substrate concentration change. It was found that quadruplicate assays and high enzyme doses were necessary to get reliable data from the OxoPlate microplate assay, compared to spectrophotometric assays using ABTS or dimethoxyphenol (DMP) as substrates, where duplicates and ten- to hundred-fold less enzyme were sufficient. Measurements were taken over 60 to 80 minutes, but the linear portion of the reaction was shorter, typically the first 10 to 30 minutes of the assay (Fig. 3, A and B).

The ranking of lignin reactivity in the laccase incubation was found to be SE eucalyptus > kraft > SE pine > lignosulfonate > ES. The SE eucalyptus and Kraft lignins were approximately three to four times more rapidly oxidized than lignosulfonate and ES lignin (Table 1, oxygen uptake). Each laccase assay was dosed with an amount of lignin based on its alkali-soluble concentration (0.5 mg/mL final concentration for each assay). However, as noted in Methods, the process of pH adjustment of the lignins for the assay caused a varying proportion of each lignin to precipitate (Table 1, % sol); therefore the concentration of lignin in the assay will be lower than 0.5 mg/mL, by an amount proportional to the solubility of each lignin at the pH of the assay. ThL activity against the pH-adjusted preparations of the less soluble lignins (SE and ES lignins) in the present study was similar whether or not the precipitated lignin was present; therefore any reaction with the precipitated lignin fraction must be minimal.

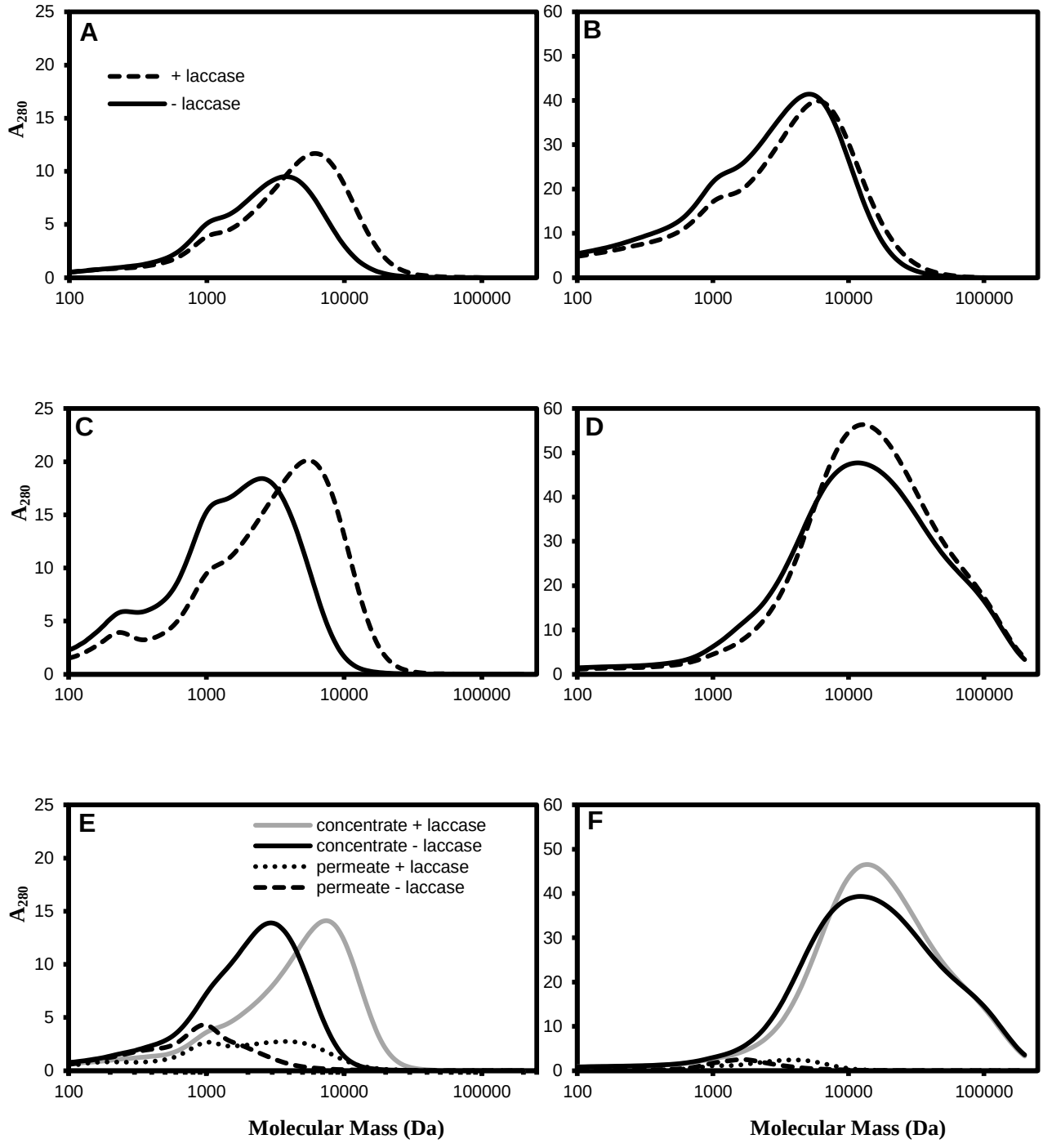

Fig. 2. A-D: size exclusion chromatograms of four lignin preparations before and after laccase treatment. A, ES; B, Kraft; C, SE pine; D, lignosulfonate; E and F: size exclusion chromatograms of SE pine (E) and lignosulfonate (F) ultrafiltration fractions before and after laccase treatment.

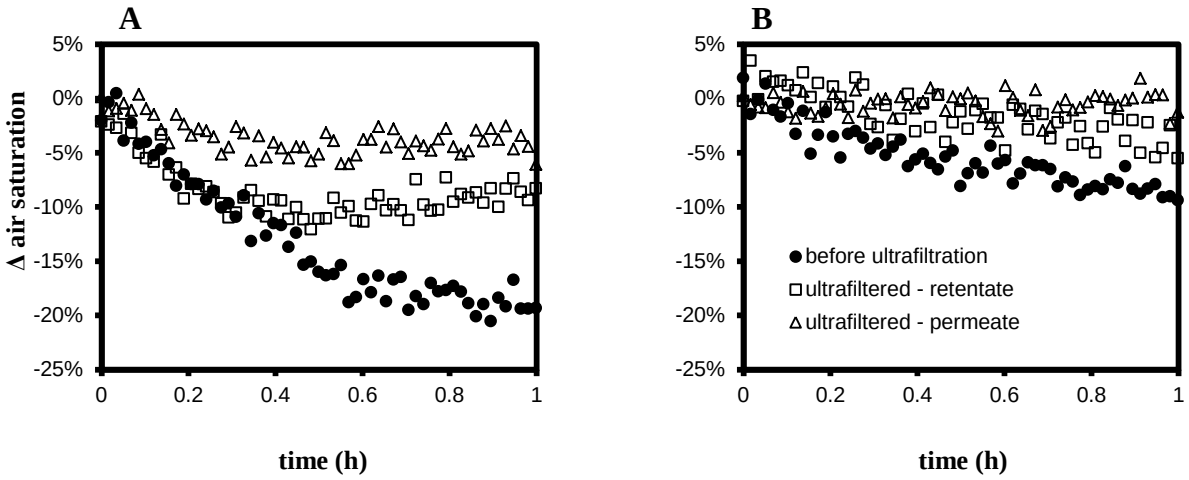

Fig. 3. Oxygen depletion of assay medium during laccase treatment of SE pine lignin (A) and lignosulfonate (B) before and after ultrafiltration.

Lignin concentration will affect reactivity, but there is no simple way of calculating this effect without knowing the kinetics of the reaction of each lignin with the enzyme. The technical lignins in this study are heterogeneous mixtures of polymers, oligomers, and monomers due to degradation during processing. Some of the low molecular mass components will be direct substrates of laccase. Others could act as mediators, being oxidized by laccase and in turn oxidising larger oligomers and polymers that cannot interact directly with the enzyme (Cañas and Camarero 2010; Suurnäkki *et al.* 2010). This non-classical activity of laccase with these lignins makes any meaningful measurement of kinetics difficult if not impossible.

Identification of monomeric phenolic compounds

Monomeric phenolic compounds, which are known to be generated from degradation of phenylpropanoid units of lignin during pulping/extraction (Chum *et al.* 1999; Reiter *et al.* 2013), were identified and quantified in the lignin preparations by RP-HPLC on the basis of comparison of retention times and absorbance spectra (from diode array data) with a set of phenolics standards (Table 2). Laccase treatment of the preparations resulted in a reduction or disappearance of some chromatogram peaks, no change in most peaks, and occasionally the appearance of new peaks. The disappearing phenolic compounds may have undergone laccase-catalyzed oxidation, and some of these oxidised phenolics could have polymerized, either with other phenolic compounds or with larger lignin-derived oligomers. There were higher levels and a greater variety of low molecular weight phenolics in more reactive preparations (steam exploded pine/eucalypt and kraft preparations), less in ES lignin, and negligible amounts in lignosulfonate. Compounds identified were vanillic acid, 4-hydroxybenzaldehyde, vanillin, ferulic acid, 4-hydroxybenzoic acid, and in steam exploded eucalypt, syringaldehyde. Chromatograms of all the lignins except for lignosulfonate contained many other unidentified peaks with retention times within the range (2 to 10 min) of the standards and with absorption spectra characteristics of phenolics; some of these peaks are likely to be monomeric phenolic compounds.

Table 2. Free Monomeric Phenolic Compounds Identified in Technical Lignins Before and After Laccase Treatment

Compound	SE pine		SE eucalypt		Ligno-sulfonate		Kraft		ES	
	-L	+L	-L	+L	-L	+L	-L	+L	-L	+L
Ferulic acid	0.36	0	tr	0	0	0	tr	0	0	0
4-Hydroxybenzaldehyde	0.14	0.13	tr	tr	0	0	0.12	0.13	0.07	0.07
4-Hydroxybenzoic acid	0.09	0.05	1.15	1.27	0	0	tr	tr	0	0
Sinapic acid	0	0	0	tr	0	0	0	0	0	0
Syringaldehyde	0	0	2.83	1.60	0	0	0	0	0	0
Syringic acid	0	0	1.00	tr	0	0	0	0	0	0
Vanillic acid	1.56	1.02	0.60	0	0	0	0.90	0.70	0.58	0.34
Vanillin	3.91	3.86	1.42	1.05	0	0	2.85	2.90	1.93	1.92
Total identified	6.06	5.06	7	3.92	0	0	3.87	3.73	2.58	2.33
% decrease after laccase		17		44		-		4		10

Amounts of monomeric phenolics are in mg/L; tr = trace; -L = lignin treated with denatured laccase, +L = lignin treated with active laccase.

SEC characterization of polymerization

Oxygen uptake during the reaction of laccase with lignin preparations is an indirect (but not definitive) indicator that polymerization reactions are taking place (Mattinen *et al.* 2008; van de Pas *et al.* 2011). In our work, clear shifts in SEC chromatograms towards higher molecular mass were seen after laccase treatment of SE pine and ES preparations, and to a lesser degree, the kraft preparation (Fig. 2A to 2C). These chromatograms also show a lower molecular mass 'shoulder' on the main peak, which after laccase treatment is significantly reduced. Lower molecular mass shoulders (and reduction in their size after laccase treatment) are frequently observed on chromatograms of lignin (Elegir *et al.* 2007; Koda *et al.* 2005; Kukkola *et al.* 2011; Kurek *et al.* 1990; Mattinen *et al.* 2008). The lignosulfonate chromatogram (Fig. 2D) shows only a slight shift to higher MWD after laccase treatment, and a very small lower molecular mass shoulder. The ranking of lignin preparations based on a qualitative assessment of shoulder prominence (steam exploded eucalypt (data not shown) > steam exploded pine > kraft > enzyme saccharified lignin > lignosulfonate) is the same as the ranking of lignin preparations on their monomeric phenolic content (Table 2), and similar to the ranking for reactivity as measured by oxygen uptake. ES lignin is somewhat of an exception; however its apparently low reactivity may be due in part to its very low solubility at pH 5.5.

Lignin fractionation by ultrafiltration

To further explore the role that the lower molecular mass compounds play in laccase polymerization reactions, separation was carried out by ultrafiltration of the components of steam exploded pine lignin and lignosulfonate preparations (representing high and low reactivity preparations respectively) to produce retentate (>3 kDa) and permeate (<3 kDa) fractions. Both fractions were then incubated with laccase. SEC chromatograms of these fractions before and after laccase treatment are shown in Figs. 2E and 2F. Lower molecular mass molecules (permeate) make up a larger proportion and total amount of the steam exploded pine lignin than lignosulfonate. In both lignin

chromatograms, the permeate fraction peaks in Figs. 2E and 2F correspond to the shoulders at approximately 1000 kDa in Figs. 2C and 2D of the pre-ultrafiltered preparations. Laccase treatment of retentate and permeate fractions increased their molecular mass, particularly of the SE pine fractions. Oxygen uptake assay data (Figs. 3A and 3B) show that both steam-exploded pine and lignosulfonate retentate fractions had a higher oxidation rate than permeate fractions; this may be due to the higher concentration of substrate in the retentate fractions relative to the permeate fractions (Figs. 2E and 2F, compare areas under the retentate and permeate 280 nm traces). There does not appear to have been any synergistic effects of the two fractions on each other; adding together the individual retentate + permeate oxidation rates gave a rate equal to, not less than, that for the pre-ultrafiltered preparation.

General Discussion

Lignins are relatively soluble in strongly alkaline conditions, and they progressively precipitate as the pH is lowered from alkaline through neutral to acidic. Most laccases, however, have acidic pH optima in the range where many lignin fractions are precipitating. As previously mentioned, ThL does not appear to react with precipitated lignin. The pH of the reaction therefore affects both substrate availability to the enzyme and enzyme activity *per se*, and small changes in pH can cause very large changes in reaction rates (Gouveia *et al.* 2013). Maintaining a stable pH is therefore vital if consistent assay data is to be obtained.

Two of the lignin preparations examined, kraft lignin and lignosulfonate, were very soluble at pH 5.5, yet the kraft sample had around three times higher activity with laccase than lignosulfonate. Kraft lignin is a lower molecular mass lignin, and its high water solubility is due to the introduction of phenolic hydroxyl groups during the pulping process (Chakar and Ragauskas 2004; Koda *et al.* 2005). Lignosulfonates, by comparison, are high molecular mass lignins. Their water-solubility comes from hydrophilic sulfonate groups introduced onto the lignin polymer during pulping (Braaten *et al.* 2003; Nugroho Prasetyo *et al.* 2010; Vishtal and Kraslawski 2011). Laccase activity may be inhibited by the sulfonate groups in lignosulfonate, and promoted by the phenolic hydroxyls in kraft lignin. Alternatively, lignosulfonate may lack monomeric phenolics to act as mediators of the laccase reaction; only trace amounts of these were detected in lignosulfonate, whereas in kraft lignin they were present at levels similar to those of the other lignins examined (Table 2). Addition of mediators to lignin-laccase reactions can have a range of effects. Nugroho Prasetyo *et al.* (2010) obtained an increase in polymerization of lignosulfonate when the mediator HBT was added to the laccase reaction. Gouveia *et al.* (2012) observed polymerization of kraft lignin using a laccase-mediator system, but polymerization was greater without the mediator. Others (Bourbonnais *et al.* 1995; González Arzola *et al.* 2006) observed depolymerization of kraft lignin when ABTS was used as a mediator in the laccase reaction, and polymerization when the mediator was absent. Surprisingly, in this study high molecular weight retentate fractions from which low molecular weight compounds had been removed by ultrafiltration still promoted oxygen consumption by laccase (Fig. 3A and 3B, retentate fraction). It is speculated that residual amounts of low molecular weight compounds persisting in the retentate fractions are sufficient to mediate the observed oxygen consumption. Much remains to be done in this area to get a clearer picture of the

interactions between enzyme, mediator and high molecular mass substrate (Cañas and Camarero 2010; Shleev *et al.* 2006).

Members of the laccase enzyme family have widely differing properties such as pH optima, redox potential, and substrate specificity (Giardina *et al.* 2010; Leonowicz *et al.* 2001; Madhavi and Lele 2009; Rivera-Hoyos *et al.* 2013), and not surprisingly, different reactivities towards the same lignin substrate, as illustrated by a couple of examples. Nugroho Prasetyo *et al.* (2010) used SEC to compare polymerization of lignosulfonate by laccases from *Trametes hirsuta* and *Trametes villosa*; after 0.5 h of reaction there was virtually no polymerization by the *T. hursuta* laccase but clear evidence of formation of higher molecular mass molecules by the *T. villosa* laccase. Van de Pas *et al.* (2011) measured oxidation of four lignins by four laccases; ThL and MaL (*Melanocarpus albomyces* laccase) oxidized two steam-exploded hardwood lignins to similar degrees, but ThL was twice as active as MaL towards steam-exploded pine lignin. As is the case with other enzymes, results with a specific laccase cannot necessarily be extrapolated to laccases in general.

Beyond solubility and pH effects a number of other properties have been correlated with reactivity to laccase treatment and consequent polymerization. These include molecular mass, phenolic content, source, isolation, extraction, and solubilization. Mattinen *et al.* (2008) found that for three lignins (Spruce EMAL, Eucalyptus Dioxane and Flax Soda lignins) lower molecular weight correlated with higher solubility and higher oxidation by laccase. Mansouri and Salvadó (2006) examined several lignins (kraft, lignosulfonate, soda-anthaquinone, organosolv, ethanol process), determining molecular mass by SEC and functional groups by UV spectroscopy and ^{1}H NMR. They found that the lower molecular mass lignins had more potentially reactive functional groups (particularly phenolic hydroxyls) than higher molecular weight ones. Elegir *et al.* (2007) determined phenolic hydroxyl groups for two different kraft lignins (one from a low molecular mass ultrafiltered black liquor, the other from a high molecular mass dioxane-extracted liner pulp); the lower molecular mass lignin had significantly more phenolic OH. In a study of kraft pulp preparation by Koda *et al.* (2005), as the severity of pulping conditions was increased, the molecular weight of isolated lignin decreased and there was a concomitant increase in phenolic hydroxyl content and decrease in β-*O*-4 linkages.

Steam explosion treatment of pine (Martin-Sampedro *et al.* 2011a) decreased molecular weight and β-*O*-4 linkages, and increased molecular weight heterogeneity and phenolic hydroxyl groups. Li *et al.* (2009) report similar findings for steam-exploded lignins of both softwoods and hardwoods. Kurek *et al.* (1990) compared spruce milled wood lignin and a colloidal lignin produced from it by repeated precipitation; the colloidal lignin had a lower molecular mass and higher reactivity with lignin peroxidase than the original lignin.

Summarizing, a common observation with lignins is that treatments which decrease the molecular mass of a lignin increase its reactivity and make it a better enzyme substrate. A related observation is that presence of lower molecular mass components (from around 500 to 3000 kDa) and monomeric phenolics in a lignin preparation may be a "marker" of higher reactivity without these components being directly responsible for the reactivity. The results reported in this paper fit well with these published observations.

CONCLUSIONS

1. The pH of the laccase-lignin reaction is critical, as both lignin solubility and enzyme activity are pH dependent. It must be monitored carefully during substrate preparation and in the assay system.
2. Lignins with a low and broad MW distribution and high monomeric phenolic compound content, particularly the two SE lignins and to a lesser degree kraft, were the most reactive overall with laccase.
3. Although ES lignin has low oxygen uptake reactivity, this is largely due to its poor solubility. On a molecular mass increase basis, ES lignin is more reactive than kraft lignin.

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