CHARACTERIZATION OF FRACTIONATED LIGNINS POLYMERIZED BY FUNGAL LACCASES

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Lignins are important biopolymers that can be converted into value-added materials by enzymatic treatments. However, the heterogeneity of the lignin polymer makes it a challenging material to modify. Thus, chemical fractionation was used to obtain lignins with high homogeneity in order to assess their biotechnological utilization. Commercial Alcell, birch organosolv lignins, and steam-exploded pine and eucalypt lignins were sequentially fractionated by ether, ether/acetone 4:1 (v:v), and acetone. All fractions were structurally characterized prior to treatments with *Thielavia arenaria*, *Trametes hirsuta*, and *Melanocarpus albomyces* laccases. The reactivities of the enzymes towards the lignins were determined by oxygen consumption measurements, and the degree of polymerization was confirmed by size exclusion chromatography. Field emission scanning electron microscopy revealed that the surfaces of the lignin nanoparticles were dispersed in the enzyme treatment, suggesting an increase in hydrophilicity of the surfaces detected as loosened morphology. Hence, it was concluded that enzyme-aided valorization is an attractive means for lignin modification, provided that optimum reaction conditions are employed.

Keywords: Lignin; Laccase; Fractionation; Modification; Characterization; Morphology

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INTRODUCTION

Replacement of petrochemicals with sustainable alternatives is becoming increasingly important due to the escalating costs of fossil fuels and the anticipated reduction in supply worldwide (Metz et al. 2007; Nguyen et al. 2010). Lignin sourced from lignocellulosic biomass is a highly promising renewable alternative for the many aromatic chemicals and polymers that are used as components for plastics and resins (Lora and Glasser 2002; Stewart 2008). However, lignin produced as a by-product of wood processing, e.g. pulp and paper processing or lignocellulosic bioethanol production, is not immediately suitable as a direct replacement of various petrochemicals. Lignin needs to be further refined and functionally modified before it can fulfill its potential as a petrochemical replacement.

The chemical nature of any lignin obtained from wood processing is affected by (i) the lignocellulosic source and (ii) the way the fibers have been processed. Lignins are composed of three different types of phenylpropane units, i.e. *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) groups (Ralph et al. 2007; Argyropoulos and Menachem 1997; Morreel et al. 2010; Vanholme et al. 2010). Softwood lignin isolated from pine

contains mainly G units, whereas lignins isolated from hardwoods (eucalypt and birch) contain mainly S units.

Processing of lignocellulosic fibers by pulping or bioethanol processing therefore affects the chemical composition of the lignin. Thus, it is assumed that lignin may be fractionated into smaller physical units according to their functionality, i.e. guaiacyl, syringyl, condensed, and oxidised groups, as well as by surface area, with varying functionalities. However, this heterogeneity of isolated lignin is probably the most significant challenge currently limiting its application in a wide range of value-added products (Gosselink et al. 2004; Stewart 2008). One way to reduce the complexity of these lignins and to identify fractions with the highest potential as petrochemical replacements is through chemical fractionation (Morck et al. 1986; Thring et al. 1996). Isolated fractions of processed lignins can be expected to be more homogenous in character, typically having distinct molecular mass (MM) distributions and chemical group functionalities.

Laccases (EC 1.10.3.2) are copper-containing enzymes that can oxidize a variety of phenolic compounds including those typically found in lignin (Couto and Herrera 2006; Kunamneni 2008). They provide an attractive means to modify the physical and chemical properties of lignin, *e.g*. by altering solubility, surface properties, and hydrophobicity of the polymer *via* oxidation. Potential options to increase the value of isolated lignin include addition of key functionalities or molecules directly to the lignin, thereby increasing its versatility. The activity of laccases on small lignin model compounds has been reported in several publications (Lahtinen et al. 2009a,b; Uzan et al. 2010; Kudanga et al. 2010a,b). However, the idea that laccases could be used to add different functionalities to lignin *via* small molecules in order to produce value-added materials was demonstrated, e.g. by Mattinen et al. (2009), who linked ferulic acid to polymerized secoisolariciresinol lignan *via* decarboxylation by laccase. In addition Witayakran and Ragauskas (2009) demonstrated modification of high-lignin softwood kraft pulp with various amino acids using laccase. The modification was proposed to occur *via* Michael addition of the amino group of single amino acids to lignin-quinonoid structures. Mattinen et al. (2008) also provided evidence for the polymerization of different technical lignins by high redox potential *Trametes hirsuta* laccase (ThL) without mediators at low pH (5.0), conditions under which lignin forms a dispersion. The results showed that all the lignins tested could be activated and polymerized by laccase to different degrees. Polymerization of lignosulfonates by various laccases with and without mediators was further demonstrated by Areskogh et al. (2010) and Prasetyo et al. (2010). Their results are compatible with the findings of Qiu and Chen (2008) with an alkali-stable enzyme having laccase activity and showing polymerization of low MM lignin in alkaline conditions.

In this paper the abilities of four laccases, of high and low redox potential, to oxidase, polymerize, and modify the surface characteristics of isolated and chemically fractionated lignins were investigated in a quantitative manner. Fractionated lignins were obtained from both hardwood and softwood, using organosolv and steam explosion processes prior to sequential solvent extraction and detailed structural characterization.

EXPERIMENTAL

Lignins

Birch (hardwood) organosolv (OS) lignin was obtained from a patent pending process (Mikkonen 2009) at VTT (Rajamäki, Finland). In addition, OS lignin from mixed hardwoods (50% maple, 35% birch, 15% poplar) was purchased from Sigma (cat. no. 371017, Milwaukee, WI, USA) and is referred to as Alcell OS lignin.

The steam exploded (SE) lignins were isolated from two New Zealand plantation sources, i.e. from softwood (*Pinus radiata*) and hardwood (mixed *Eucalyptus* species including *E. nitens*, *E. fastigata*, and *E. regnans*). Steam explosion was performed without SO_2 pre-impregnation in order to conserve the reactivity of the lignin. The procedure was based on the method Overend and Chornet (1987). Wood chips were steam-impregnated in an autoclave at 120 °C for 40 min prior to steam explosion at 225 °C for 5 min for softwood and at 210 °C for 9 min for hardwood. After steam explosion the pH of process liquor was 3.3 for *P. radiata* and 3.5 for mixed eucalypt. The SE fibers were then washed thoroughly with water, air dried, and Wiley milled (40 mesh) before extraction with an acetone/water mixture 9:1 (v:v) for 2 h at ambient temperature under constant stirring. The liquor to solids ratio was 9:1 (w:w). The solids were filtered and washed twice with acetone/water. The extracts were concentrated under vacuum, and the resulting aqueous suspensions were freeze-dried. Yields of SE fibers and extracted lignin were calculated as percent by weight of oven-dried/freeze-dried material.

Lignin Fractionation

Isolated lignin samples (4 g) were further fractionated by sequential solvent extraction with ether, followed by ether/acetone 4:1 (v:v), and finally acetone. Lignin fractionation by sequential solvent extraction was based on the method by Thring et al. (1996). Small scale experiments were initially conducted to optimise the ratio of ether and acetone to obtain three fractions with similar yields. For each extraction, lignin was shaken with 40 mL of solvent for 15 min at ambient temperature, the samples were centrifuged, and the supernatants collected. The solids were re-extracted twice with 20 mL of fresh solvent, and the supernatants were combined with the primary extract. The residual fraction, *i.e.* after acetone extraction, was dissolved in acetone/water 9:1 (v:v). All extracts were transferred to round-bottomed flasks, evaporated to dryness under vacuum, and the residues were re-dissolved with a minimum volume of the acetone/water mixture. Water was added to precipitate the lignin, the acetone was evaporated under vacuum, and finally the aqueous suspensions were freeze-dried. Yields of the fractions calculated as percent by weight of freeze-dried material.

Lignin Characterization

Carbohydrate content, Klason and acid-soluble lignin

Wiley milled wood samples were extracted with dichloromethane in a Soxtec apparatus (Tecator Soxtec System, Model HT1043, Hoganas, Sweden) using 1 h boiling and rinsing times. The extracted wood samples were then Wiley milled to pass a 0.25 mm discharge screen. Lignin samples and SE fibers were not pre-extracted with dichloromethane. Total lignin content was determined in duplicate as the sum of Klason

residue and acid-soluble lignin, following the standard methods (TAPPI Standard 1988; TAPPI Useful Method 1991), which were scaled down for the analysis of 0.25 g samples. Monomeric sugars in the filtrates from Klason lignin determinations were analyzed by ion chromatography (Dionex ICS-3000, Sunnyvale CA, USA) as described by Pettersen and Schwandt (1991). The results were expressed as dehydrated sugar units. All carbohydrate and lignin content values were calculated as percent by weight of oven-dried or freeze-dried material.

^{13}C CP/MAS NMR spectroscopy of lignins

Solid-state ^{13}C CP/MAS NMR (nuclear magnetic resonance) spectra were obtained using a Bruker DRX 200 spectrometer (Bruker, Fällanden, Switzerland) operating at 200.13 MHz for ^{1}H and 50.33 MHz for ^{13}C and equipped with a 4 mm doubly tuned H/X MAS probe. Samples were spun at 5 kHz. Each 1.5 s pulse delay was followed by a 5.6 μs proton preparation pulse, a 1 ms contact time and a 30 ms acquisition time, with a ^{1}H decoupling field of 125 kHz. Spectra were compiled from 32k transients and processed with Gaussian line broadening of 25 Hz. The chemical shift scale was calibrated on the basis of the methoxyl signal from lignin at 56.3 ppm.

^{1}H and ^{13}C NMR spectroscopy of ether fractions

Solution state NMR spectra were measured using a Bruker Avance DPX 400 spectrometer (Bruker, Fällanden, Switzerland) equipped with a 5 mm inverse broad-band probe operating at 400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C. ^{1}H spectra were obtained using an acquisition time of 3.96 s and a recycle delay of 1 s, and were compiled from 16 transients. Proton decoupled ^{13}C spectra were obtained using an acquisition time of 1.25 s and a recycle delay of 2 s, and were compiled from at least 256 transients. ^{1}H and ^{13}C spectra were processed with exponential line broadening of 0.3 and 1.0 Hz, respectively. The spectra were calibrated on the basis of the signal from TMS.

^{31}P NMR spectroscopy of phosphitylated lignins

The ^{31}P NMR method used in this study was adapted from Granata and Argyropoulos (1995). Endo-N-hydroxy-5-norbornene-2,3-dicarboximide (Aldrich, Steinheim, Germany) dissolved in DMF at a concentration of 16.7 mmol L^{-1} was used as the internal standard, and chromium acetyl acetonate (Aldrich, Steinheim, Germany) dissolved in pyridine and $CDCl_3$ (1.6:1 v:v) solution at 16.4 mmol L^{-1} was used as a relaxation agent. An accurately weighed lignin sample (20 to 25 mg) and the internal standard (300 μL) were added to a stirred reaction vial. Once the lignin had dissolved, 400 μL of pyridine and $CDCl_3$ (1.6:1 v:v) solution, 100 μL of the relaxation agent and 100 μL of the phosphitylating agent 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (Aldrich, Milwaukee WI, USA) were added, and the vial was capped. The solution was mixed for several seconds at ambient temperature and then transferred to a 5 mm NMR tube and analysed by ^{31}P NMR in a Bruker Avance DPX 400 spectrometer (Bruker, Fällanden, Switzerland). Spectra were collected using a 5 s delay. Spectra were compiled from 512 transients and processed with exponential line broadening of 2 Hz. The chemical shift scale was calibrated on the basis of the signal from phosphitylated water (132.2 ppm). Integration of the peaks was based on the following chemical shift regions;

internal standard (152.1 - 151.5 ppm), aliphatic OH (149.8 - 144.9 ppm), condensed/S-type phenolic OH (144.9 - 140.2 ppm), G/H-type phenolic OH (140.2 - 136.6 ppm), and carboxylic OH (136.6 - 133.5 ppm).

Size exclusion chromatography of isolated lignins

Alkaline size exclusion chromatography (SEC) was used to determine molecular mass distributions for isolated, chemically fractionated, and enzymatically treated lignins. Analyses were performed using a Waters size-exclusion chromatography M-2690 separation module equipped with an M-996 diode array detector (230 - 500 nm) and three μHydrogel columns (pore size: 2000, 250 and 120 Å) connected in series (Waters, Milford MA, USA). Isocratic chromatography was performed at 60 °C using 50 mM NaOH as eluent at a flow rate of 0.50 mL min^{-1}. The chromatographic system was calibrated with pullulan standards. The system was controlled and data were analysed with Empower software. Freeze-dried lignin samples were dissolved in 0.1 M NaOH at a concentration of 1 mg mL^{-1} for the chromatographic separation. The injection volume was 50 μL.

Field emission scanning electron microscopy of isolated lignins

A field emission scanning electron microscope (FE-SEM) JEOL 6700F (JEOL Ltd. Tokyo, Japan) was used for imaging of surfaces of non-treated and laccase-treated lignin nanoparticles at micrometer and nanometer scales. The instrument was optimised for imaging of biological materials at low kV. Thus, for imaging, non-treated and laccase-treated lignin samples solubilized in 25 mM succinate buffer at pH 6 in 1 mM concentration were precipitated with 1 M HCl down to pH 1.5, centrifuged, washed with Milli-Q water to remove the buffer salts, and finally air dried on glass plates. In addition, salt-free lignin samples were dissolved in acetone and dried on glass plates to reduce hydrophobic aggregation between lignin nanoparticles. For the imaging dried samples were coated with chromium and examined at an accelerating voltage of 3-5 kV.

Enzymes

Laccases

Four fungal laccases, *Trametes hirsuta* (ThL), *Melanocarpus albomyces* (MaL), *Thielavia arenaria* Lcc1 (TaLcc1, patented by AB Enzymes Oy et al. 2006a), and Lcc2 (TaLcc2, patented by AB Enzymes Oy et al. 2006b), were used in this study. Some of the biochemical properties of the enzymes crucial for this investigation are summarized in Table 1. The 3D structure and function of TaLcc1 and TaLcc2 are likely very different, as only 51% full-length sequence of TaLcc2 is similar to that of TaLcc1. TaLcc1 has been previously shown to efficiently bleach, e.g. denim in textile applications (patented by AB Enzymes Oy et al. 2006a), showing the potential of these enzymes for biotechnological applications. MaL, a low redox potential laccase, was overproduced in *Trichoderma reesei* and purified as described by Kiiskinen et al. (2004). ThL, a high redox potential laccase, was produced in its native host and purified as described by Rittstieg et al. (2002). Both laccases were produced and purified at VTT (Espoo, Finland). Recombinant TaLcc1 and TaLcc2 were produced in *T. reesei* at Roal Oy (patented by AB Enzymes Oy et al. 2006a,b). Laccase activities of the enzyme

preparations were determined using 2,2-azino-bis-(3-ethylbenzothiazoline)-6-sulphonate (ABTS) as substrate according to the method developed by Niku-Paavola et al. (1988). The oxidation of ABTS at pH 5 in 25 mM succinate buffer was measured spectrophotometrically at 436 nm (ε = 29 300 M^{-1} cm^{-1}) and used as a reference for dosing of the enzymes.

Table 1. Biochemical Properties of Laccases

Property	ThL	MaL	TaLcc1	TaLcc2
pH optimum*	4.5 – 5	5 – 7.5	6	5.5
$T_{½}$ at 60 °C (h)	4	5	0.5	0.5
Redox potential (V)	0.78 (pH 4.9**)	0.46 (pH 6***)	0.45 (pH 6)	0.45 (pH 5.5)

* Determined with guaiacol as subtrate; **Rebrikov et al. (2006); ***Kiiskinen (2005)

Oxygen consumption measurements

Laccase reactivity on various lignins was determined by measuring the uptake of oxygen associated with the radical-mediated oxidation of the phenolic substrate. The oxygen consumption was measured in a high throughput manner using BD™ Oxygen Biosensor plates (BD Biosciences, Bedford, MA, USA), which enable 96 samples to be analysed simultaneously. For the analyses the fractionated lignins as well as raw lignins were dissolved in 0.1 M NaOH at a concentration of 1 mg mL^{-1}, and the pH values of the solutions were decreased to 5 or 6 using 1 M HCl and 25 mM succinate buffer as described by Mattinen et al. (2008). Prior to actual data collection, each well of the BD Oxygen Biosensor plate was filled (volume: 315 µL) with dissolved lignins (buffer: pH 5 or 6, volume: 285 µL) and enzyme solution (volume: 30 µL) corresponding to an enzyme dosage of 100 nkat g^{-1} based on ABTS activity determined at pH 5. In the case of MaL a fourfold higher enzyme dosage was also used for comparison to ensure end point of the enzyme catalysed reactions. After addition of the enzyme the reaction plate was rapidly closed with an adhesive sealer and incubated at 30 °C with shaking between each measurement in the dark chamber of BMG Polarstar microplate reader (BMG Labtech, Offenburg, Germany).

Fluorescence signals were measured using 485 nm for excitation and 612 nm for emission at 5 minute intervals for 16 h. Reference samples containing lignin but no enzyme were analysed at the same time to account for lignin auto-oxidation during the measurement. Sodium sulphite 1% (w:v) was used as a 'zero oxygen' standard in the calibration of the equipment. All measurements were performed in triplicate. The degree of oxidation of phenolic hydroxyl groups as µmol g^{-1} and percentage of the available phenolic hydroxyl groups was calculated from the amount of oxygen consumed at the end-point of the reaction. After overnight incubations it was confirmed that some of the oxygen remained unreacted. The stoichiometry of the laccase-catalyzed reaction is 4 (Yaropolov et al 1994), *i.e.* one oxygen molecule (O_2) is required for the oxidation of four substrate molecules by laccase. The phenolic content of the lignin was determined by ^{31}P NMR spectroscopy.

RESULTS AND DISCUSSION

Lignin Isolated from Steam Explosion

Steam explosion of the hardwood chips resulted in a fibrous material resembling pulp. However, the corresponding material obtained from softwood was much less fibrous and had a larger amount of unexploded chips, despite having being subjected to steam explosion conditions that were greater in severity. Wood components were dissolved to a greater extent during steam explosion in the case of softwood chips than hardwood chips, as shown in Table 2.

Table 2. Yields of Steam Exploded Fibers and Extracted Lignin along with Steam Explosion Severity Factor

	Wood species	
	Pine	**Mixed eucalypt**
Steam exploded material (% on wood)	56	72
Extracted lignin (% on lignin in SE fibers)	24	53
Extracted lignin (% on starting lignin in wood)	17	43
Severity factor, log(Ro)	4.4	4.2

Almost all of the hemicelluloses present in the wood were removed during steam explosion, as concluded on the basis of the results shown in Table 3. The yield of solvent extractable lignin for the hardwood (mixed eucalypt) was approximately double that for the softwood (*P. radiata*), as calculated by the sum of Klason and acid-soluble lignin. The yields of lignin are within the range obtained by Li et al (2009) for both softwood and hardwood, suggesting that extraction with acetone/water 9:1 (v:v) is similar in efficiency to alkaline extraction. Advantages of using a solvent mixture for lignin extraction rather than alkaline solution is that there is no need for neutralization and no risk of unwanted condensation reactions occurring during the extraction.

Table 3. Extractives, Carbohydrate and Lignin Content by Weight % of Material. A: Arabinan, B: Galactan, C: Glucan, D: Xylan, and E: Mannan

Sample	**Extractives (%)**	**Carbohydrates (%)**					**Lignin (%)**		**Total (%)**
		A	**B**	**C**	**D**	**E**	**Klason**	**Acid-soluble**	
Pine wood	1.4	1.6	2.2	45	5.1	12	28	0.5	95
Mixed eucalypt wood	0.4	0.55	1.2	46	13	1.2	26	3.4	92
Pine SE fibers	-	< 0.01	< 0.01	51	1.4	1.7	35	0.4	90
Eucalypt SE fibers	-	< 0.01	< 0.01	53	2.6	0.72	32	1.3	89
Pine SE lignin	-	0.18	0.40	0.63	0.56	1.9	92	2.1	98
Eucalypt SE lignin	-	0.03	0.06	0.07	0.88	0.02	95	2.7	99
Alcell OS lignin	-	0.01	0.02	0.06	0.12	0.01	92	3.2	95
Birch OS lignin	-	0.04	0.04	0.05	0.18	0.01	91	1.9	93

- Not determined

Purity of Steam Explosion and Organosolv Lignins

The ^{13}C CP/MAS spectra of the SE and OS lignins indicated high purity, with only little contamination from the residual carbohydrates (spectra not shown). Carbohydrate analysis (Table 3) confirmed the qualitative NMR results, with total sugars accounting for 4 % and 1 % for pine SE and eucalypt SE lignin, respectively, and < 0.5 % for the Alcell OS and birch OS lignins. Furthermore, in the case of both OS lignins, small peaks at 172 and 21 ppm were detected in the spectra, suggesting the presence of acetyl groups (data not shown).

Characterization of Fractionated Lignins

The isolated SE and OS lignins were successfully extracted with organic solvents of increasing polarity, *i.e.* ether < ether/acetone 4:1 (v:v) < acetone. In all cases the residual fraction after acetone extraction was soluble in acetone/water 9:1 (v:v) solution. A visual examination of the freeze-dried fractions showed that the fractions obtained with higher-polarity solvents were generally darker in color. In general, the colors of the fractions varied from pale yellow to dark brown across the series from the ether fraction to the residual fraction. The fractions of the birch OS lignin showed the most pronounced variations in color (data not shown).

Yields of the various lignin fractions are summarized in Table 4. Fractions had different average MM and polarity, as they contained different proportions of key functional groups. The acetone fraction yielded the greatest proportion of extracted material in the case of all lignins except the birch OS lignin, of which the residual fraction had the highest yield. The amount of lignin extracted into ether was relatively low, especially for the Alcell lignin, although it has been reported to give an ether fraction containing 27% of the initial lignin (Thring, et al. 1996). The ether fractions of birch OS and pine SE lignins were obtained as sticky residues rather than as free-flowing powders as was the case for the other fractions. NMR analysis of the ether fractions revealed that they were contaminated by wood extractives such as fatty acids (data not shown). For this reason, in addition to their low yields, the ether extracts were disregarded as meaningful fractions and omitted from further analyses.

Table 4. Yields of Lignin Fractions after Sequential Solvent Fractionation

Lignin	Ether (%)	Ether/acetone (%)	Acetone (%)	Residual (%)	Total (%)
Pine SE	10	24	50	14	98
Eucalypt SE	4.7	12	48	34	99
Alcell OS	2.8	27	57	12	99
Birch OS	8.1	4.9	27	56	96

The amounts of various hydroxyl functionalities in the starting lignins and in all their fractions, as determined by ^{31}P NMR are summarized in Table 5. The fraction with the highest phenolic content was the ether/acetone 4:1 (v:v) fraction. The phenolic content decreased with increasing polarity of the extraction solvents, *i.e.* ether/acetone 4:1 (v:v) > acetone > residue. This trend was opposite for the amount of aliphatic hydroxyl groups, which was highest in the residues. The phenolic content of the

unfractionated lignins was similar for both the SE lignins and the Alcell OS lignin. The phenolic content of the birch OS lignin was approximately 30% lower than that of the others. As a higher phenolic content indicates a more depolymerised lignin, this suggests that the molecular mass values were lowest for those fractions obtained using extraction solvents of lowest polarity. Analysis by SEC was used to confirm this hypothesis.

Table 5. ^{31}P NMR Analysis of Phosphitylated Lignins

Lignin	Aliphatic -OH (mmol g^{-1})	Cond./S Phenolic -OH (mmol g^{-1}/%)	H/G Phenolic -OH (mmol g^{-1}/%)	Acidic -OH (mmol g^{-1})	Total Phenolic -OH (mmol g^{-1})	Total -OH (mmol g^{-1})
Pine SE	3.4	1.1 / 30	2.6 / 70	0.28	3.7	7.4
Ether/acetone	1.9	0.95 / 21	3.8 / 79	0.47	4.7	7.1
Acetone	4.0	1.3 / 36	2.3 / 64	0.14	3.6	7.7
Residual	4.9	1.0 / 40	1.5 / 60	0.10	2.5	7.5
Eucalypt SE	2.5	2.5 / 68	1.2 / 32	0.12	3.7	6.4
Ether/acetone	1.3	3.3 / 63	1.9 / 37	0.28	5.2	6.8
Acetone	2.6	2.7 / 68	1.3 / 33	0.14	4.0	6.7
Residual	3.3	2.1 / 70	0.92 / 30	0.08	3.0	6.4
Alcell OS	1.7	2.5 / 69	1.1 / 31	0.31	3.7	5.7
Ether/acetone	0.84	2.9 / 67	1.4 / 33	0.42	4.2	5.5
Acetone	2.0	2.5 / 71	1.0 / 29	0.21	3.5	5.7
Residual	2.7	1.8 / 69	0.81 / 31	0.16	2.6	5.5
Birch OS	1.2	2.1 / 78	0.61 / 22	0.35	2.7	4.2
Ether/acetone	0.75	3.1 / 77	0.92 / 23	0.30	4.0	5.0
Acetone	1.0	2.8 / 80	0.71 / 20	0.16	3.5	4.7
Residual	1.3	1.9 / 76	0.56 / 24	0.21	2.4	4.0

The molecular mass of each lignin and their fractions are shown in Table 6, and the SEC profiles of the fractionated birch OS lignins are shown in Fig. 1a as an example of the chromatographic resolution. As pullulan standards with varying molecular mass were used for calibration of the chromatographic system, the numerical data presented in the table should be considered on a relative rather than an absolute basis, due to the lack of suitable calibration standards (Baumberger et al. 2007; Mattinen et al. 2008). The average molecular mass of the starting lignins increased in the order pine SE < Alcell OS < eucalypt SE < birch OS. The birch OS lignin had a much higher molecular mass than the other lignins, which probably explains why most of the fractionated lignin was recovered in the residual fraction rather than in the acetone fraction. This relationship could also be depicted rather well from the chromatograms, as the SEC profile determined for the unfractionated lignin nearly overlapped with the profiles of the residue (Fig. 1a). As predicted by the ^{31}P NMR data, the molecular mass increased with decreasing phenolic content.

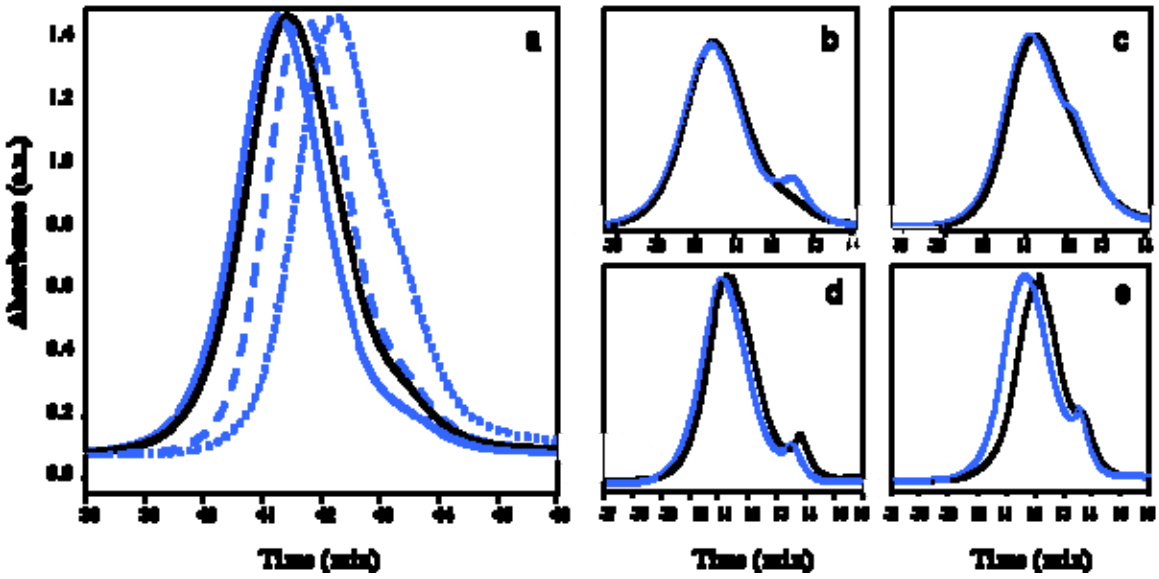

Fig. 1. Normalized size exclusion chromatograms of the chemically fractionated and ThL-treated birch OS lignin. (a) Unfractionated (black solid line), ether fraction (dotted blue line), ether/acetone 4:1 (v:v) fraction (blue broken line), and residue (blue solid line). Unfractionated and ether/acetone fraction 4:1 (v:v) with (blue) and without (black) ThL-treatment at pH 5 (b-c) and at pH 6 (d-e).

Table 6. Molecular Masses of the Chemically Fractionated Lignins and Differences Compared to those of Enzymatically Treated Lignins

Lignin	Treatment (pH 6)					
	Reference	ThL (pH5 and 6)		MaL	TaLcc1	TaLcc2
	MM ($g\ mol^{-1}$)	ΔMM ($g\ mol^{-1}$)		ΔMM ($g\ mol^{-1}$)	ΔMM ($g\ mol^{-1}$)	ΔMM ($g\ mol^{-1}$)
Pine SE	5760	780	2500	2900	2000	1500
Ether/acetone	4200	230	2300	1000	700	1900
Acetone	6110	770	-	-	-	-
Residual	8930	280	-	-	-	-
Eucalypt SE	8330	810	6200	10700	5300	5600
Ether/acetone	4430	630	2200	3900	1000	2700
Acetone	6620	480	-	-	-	-
Residual	14800	1270	-	-	-	-
Alcell OS	7170	420	4200	6300	5500	3700
Ether/acetone	4790	510	2400	-	2700	3100
Acetone	7520	670	-	-	-	-
Residual	13100	1170	-	-	-	-
Birch OS	12200	430	4100	7200	3500	3100
Ether/acetone	5860	340	2300	4100	4300	1800
Acetone	8840	< 100	-	-	-	-
Residual	14200	< 100	-	-	-	-

- Not determined

Laccase Treatments

Reactivity of laccases on lignins

High throughput activity measurements of oxygen-consuming enzymes active on aromatics such as oxygenases (Tizzard and Lloyd-Jones 2006) and Cytochrome P450 (Olry et al 2007) have been used to demonstrate the applicability of the BD Oxygen Biosensor plates in biochemical applications related to monitoring of enzymatic catalysis. Hence, in this study these plates were exploited to measure simultaneously the reactivity of four different laccases towards sixteen lignin samples with varying phenolic contents and MM, as summarized in Table 7. On the basis of triplicate measurements the error of the method was found to be less than 5%.

Table 7. Yield of Oxidation of Various Lignins after Laccase Treatments

Lignin	ThL (pH 5/pH 6)		MaL (pH 6)*		TaLcc1 (pH 6)		TaLcc2 (pH 6)	
	(μmol g^{-1})	(%)	(μmol g^{-1})	(%)	(μmol g^{-1})	(%)	(μmol g^{-1})	(%)
Pine SE	878/201	24/5	365	10	200	5	147	4
Ether/acetone	966/282	20/6	-	-	352	7	194	4
Acetone	721/77	20/2	289	8	138	4	69	2
Residual	409/50	16/2	157	6	108	4	102	4
Eucalypt SE	1029/264	28/7	899	24	618	17	501	14
Ether/acetone	891/437	24/8	918	18	652	13	702	13
Acetone	933/275	26/7	945	24	592	15	482	12
Residual	565/39	16/1	806	27	388	13	375	13
Alcell OS	1143/339	32/9	1011	28	676	19	580	16
Ether/acetone	1059/379	25/9	981	23	851	20	723	17
Acetone	838/219	24/6	922	26	672	19	572	16
Residual	704/40	27/2	-	-	367	14	343	13
Birch OS	623/250	17/9	644	24	307	11	316	12
Ether/acetone	924/348	25/9	805	21	572	15	475	12
Acetone	417/142	12/4	609	17	396	11	310	9
Residual	213/73	6/3	492	20	294	12	158	6

* Experiments performed with fourfold higher MaL dosage gave comparable results within error of the method (data not shown).

The high redox potential laccase (ThL) was able to oxidize on average 25% of the phenolics of pine SE, eucalypt SE, and commercial Alcell OS lignins at the optimum pH 5 of the enzyme, as shown in Table 7. The oxidation of birch OS lignin was somewhat lower, probably due to the slightly higher MM and tighter cross-linked structure of the polymer. The type of the lignin, *i.e.* G in pine SE or S in eucalypt SE, did not appear to have a major effect on the reactivity. At the higher pH of 6, the degree of oxidation of each lignin was surprisingly low, even though the solubility of lignin was better.

In the case of low redox potential MaL the degree of lignin oxidation was as high as in the case of high redox potential ThL for the S type hardwood lignins (eucalypt SE,

birch OS, Alcell OS). Oxidation of pine SE, which is G type lignin, was much lower, *i.e.* less than 10%. The two other low redox potential laccases (TaLcc1 and TaLcc2) showed similar oxidation patterns to that of the MaL and were more capable to oxidize the hardwood lignin than the softwood lignin.

Molecular mass determination of laccase treated lignins

ThL treatment of isolated technical lignins such as flax soda lignin, spruce EMAL, and eucalyptus dioxane has previously been shown to increase the average molecular mass due to radical-radical mediated polymerization in acidic (pH 5.0) reaction conditions (Mattinen et al. 2008). However, in this study, as shown in Table 6 for selected lignins, the average molecular mass increase after laccase treatment ranged from a few percent to over 100% at pH 6. Surprisingly, the highest polymerization was obtained in the case of low redox potential MaL, *i.e*. when the lignins were better dissolved. In the case of high redox potential ThL the polymerization in pH 6 was also unexpectedly high compared to that obtained at the optimum pH 5. In general, in both cases the polymerization of hardwood lignin was higher than that of softwood lignin. The results are in good agreement with the data obtained by Lahtinen *et al*. (2009a,b), as they also reported MaL to be less effective in the oxidation of softwood lignin model compounds. TaLcc1 and TaLcc2 were less effective than MaL and ThL. Recently Qiu and Chen (2008) also reported polymerization of spruce alkali lignin and steam exploded wheat straw lignin in alkaline solution (pH 10) by *Mycelia Sterilia* YY-5, an entophytic fungus isolated from *Rhus chinensis Mill* (MS-Lac). However, the molecular mass of these lignins was less than 2000 kDa, much lower than the mass of the lignins used in this study. In addition the molecular mass increase after MS-Lac treatment remained less than or equal to 30%, even though the lignins were in a fully dissolved state. Polymerization of lignosulfonates by laccases with and without mediators was also confirmed recently under varying reaction conditions (Areskogh et al. 2010; Prasetyo et al. 2010).

Clearly, selection of the buffer, *i.e*. pH 5 or pH 6, may be exploited in lignin modification, depending on the research goals: lignin may be polymerized to a large extent in dissolved state at pH 6, whereas extensive oxidation of the lignin surfaces may be obtained in lignin dispersion at pH 5 when the optimum pH of the high redox potential ThL is employed.

Morphology of isolated lignin

Microscopic analyses of lignins by FESEM revealed the morphology of the isolated lignins not evidenced before. The dried hydrophobic polymer consists of nanoscaled spherical particles, as shown in Figs. 2 a-d as an example for the Alcell OS ether/acetone 4:1 (v:v) fraction. When the dissolved lignin was purified from the buffer salts with Milli-Q water and dried in the ambient air on the glass plate, rather large lignin particles, *ca*. 100-500 μm, were formed (Fig. 2 a). No film formation was detected. On the basis of the magnification of the image (Fig. 2 b), the morphology of the isolated lignin was relatively uniform but it was extensively aggregated by hydrophobic interactions. However, when the lignin was dissolved in acetone and then dried on the glass plate, better dispersion between the individual lignin nanoparticles was obtained (Fig. 2 c). In addition to lignin nanoparticles some residual buffer salts were detected

from the picture. In order to study changes on the surface of the lignin nanoparticles after laccase treatment the same sample preparation method was used for MaL-treated Alcell OS ether/acetone 4:1 (v:v) fraction prior to analysis by FESEM (Figs. 2 e,f). Clearly, when the selected lignin fraction was treated with MaL, dispersing of the surfaces of the nanoparticles was detected when compared to those of non-treated lignin. After laccase treatments the surfaces of hydrophobically aggregated lignin nanoparticles were broken down and re-assimilated. As the morphology and structure of laccase-treated lignin was clearly loosened, increased hydrophilicity of the surfaces of individual lignin nanoparticles was proposed. In addition, polymerization of lignin by laccase may be explained by the formation of large, linear lignin nanofibers not detected before (Fig. 2 f). However, atomic force microscopy (AFM) tapping mode images of wheat straw parenchyma cell walls after partial de-lignification showed lignin deposits as spherical shapes, rather similar to those detected by FESEM from the isolated lignin, on top of the cellulose macrofibrils (Kristensen 2009). Interestingly, FTIR spectroscopy, ^{13}C NMR, and pyrolysis GC/MS analyses of the laccase-treated lignosulfonates revealed no substantial changes in the aromatic structure of the lignosulfonates (Prasetyo et al. 2010).

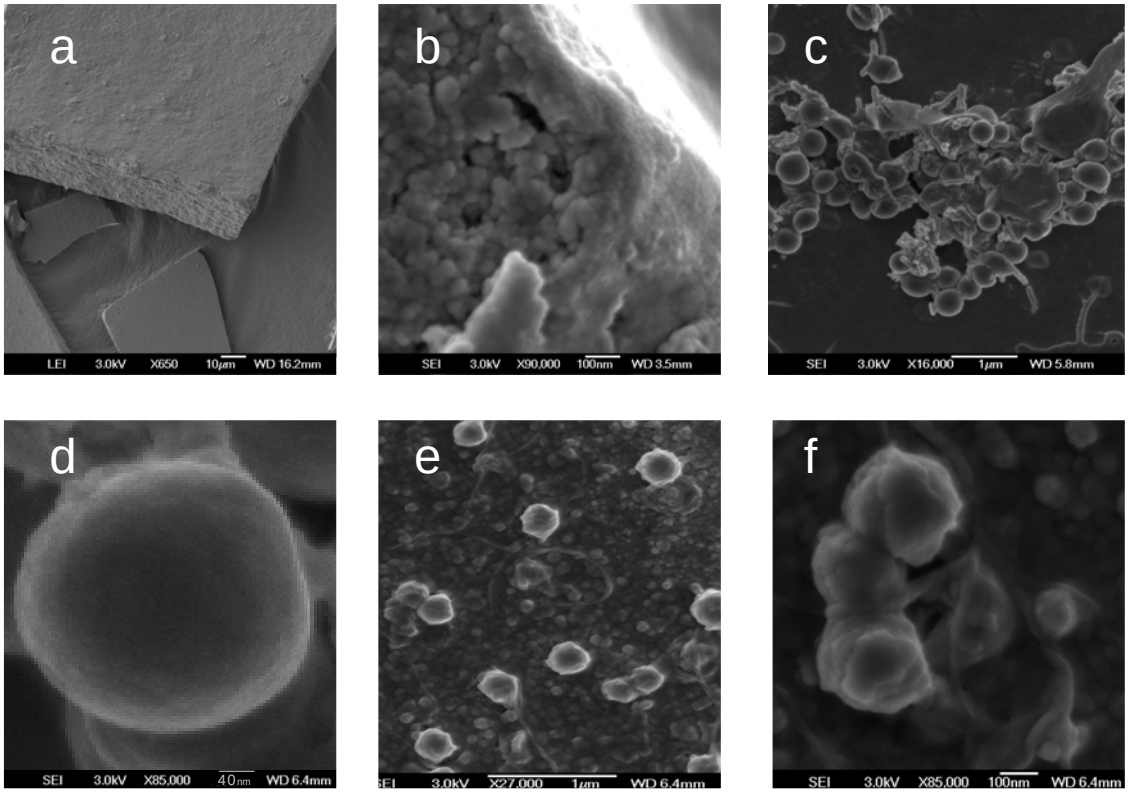

Fig. 2. Morphology and polymerization of isolated Alcell OS lignin by MaL as analysed by FESEM. (a) Precipitated, washed and dried lignin from succinate buffer pH 6. (b) Magnification of dried lignin particle. (c) Individual lignin nanoparticles. (d) Magnification of individual lignin nanoparticle. (e) Individual laccase - treated lignin nanoparticles. (f) Enzymatically polymerized lignin nanoparticles.

CONCLUSIONS

1. The aim of the present study was to evaluate the potential of laccase as a green tool to modify lignins *via* polymerization or as a pretreatment, yielding phenoxy radicals for further enzymatic and chemical functionalization. The results obtained in this study were encouraging, suggesting that enzyme-aided modification of lignin is feasible, provided that proper combinations of lignin and laccase are applied under optimized reaction conditions.
2. In the fractionated lignins the trend of the aliphatic hydroxyl groups was opposite to that of the phenolic hydroxyls, suggesting that fractionation provides an attractive way of refining lignins to suit particular applications, *e.g.* for enzymatic modification by laccases.
3. Enzymes reacted differently towards hardwood and softwood lignins, showing that for effective valorization of lignin, enzymatic cocktails containing different lignin-degrading enzymes must be developed. This motivates continuing screening and production of novel enzymes.

ACKNOWLEDGEMENTS

In New Zealand the study was carried out with financial support from capability funding through MoRST, and *via* the International Investments Opportunity Fund (IIOF) through FRST. In Finland the study is part of the LigniVal project in the Tekes BioRefine program. Funding from Tekes, Metso Power Oy, Oy Metsä-Botnia Ab, Stora Enso Oyj, and Roal Oy is acknowledged. In addition the authors acknowledge Ian Suckling (Scion, New Zealand) for useful lignin-related discussions, and Kristiina Kruus (VTT, Finland) and Marja Paloheimo (Roal Oy, Finland) for providing various laccases for the study. Tapani Suortti and Päivi Matikainen (VTT, Finland) are thanked for technical assistance relating to the SEC analyses.

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Article submitted: December 17, 2010; Peer review completed: February 13, 2011;
Revised version received and accepted: February 18, 2011; Published: February 20, 2011.