A PRELIMINARY INVESTIGATION ON ENZYMATIC OXIDATIVE POLYMERIZATION OF LIGNIN

Hongli Zhu¹, Dimitri Areskogh¹, Mikaela Helander², and Gunnar Henriksson¹,²*

1. Division of Wood Chemistry and Pulp Technology, Department of Fibre and Polymer Technology, Royal Institute of Technology, KTH, Stockholm, Sweden; 2. Wallenberg Wood Science Center, Royal Institute of Technology, KTH, Stockholm, Sweden.

*Corresponding author: ghenrik@pmt.kth.se

ABSTRACT

Enzyme catalyzed oxidative polymerization of technical bagasse lignin and low-molecular-weight ultra-filtered kraft pulp lignin (UFL) were studied in methanol-water solution. Lignin was dissolved in methanol-water solution at pH 13 in steam heated autoclave at 130°C for 2h. The polymerization reaction was conducted at 40°C with a commercial laccase under oxygen saturation. The weight-average molecular weight (Mw) of original macromonomer and polymerized lignin were characterized with alkaline size exclusion chromatograph (SEC) system. Enzyme treatment increased the molecular weight of both technical bagasse lignin and ultra-filtered lignin up to 20 times. The reaction time and the enzyme dosage were studied to obtain the maximal molecular weight.

Keywords: laccase, polymerization, technical bagasse lignin, ultra-filtered lignin

INTRODUCTION

Lignin, 30 wt% in wood, represents enormous under-utilized natural biopolymer generated in the sulfate (Kraft), soda and sulphite pulping procedure. In the Kraft process, the majority of the lignin in pulp mills is burned during the chemical recovery process. In the sulphite process, the lignin is converted to a soluble derivate, lignolsulphonate which is solubilized in water, extracted and used for different products such as plasticizer in concrete, surfactants, binders and dispersants. The possible applications of lignins from sulphate and soda processes are fewer, but there are efficient process developed for the preparation of lignin from process liquids. These types of technical lignins have less good solubility properties and often a lower molecular weight. Structural modification such as increase of molecular weight may increase the applicability of these materials. Enzymatic strategies can be interesting here.

Laccase is a cupper protein that oxidizes a large variety of aromatic substrates. It is widely distributed in white rot fungi and it is believed to possess a key role in the degradation of lignin[1]. Laccase has also been shown to carry out the oxidation of free phenolic end groups in lignin, and it was recently shown that it can be used for increasing the molecular weight of lignosulphonates [2-4] by radical coupling reactions (Fig. 1). The technical lignins obtained from soda and kraft pulping processes [5,6] presents a more difficult challenge since their solubility in aqueous solutions are poor. Organic solvents dissolve the material, but may denaturate the enzyme catalyst. A balance between solubility and enzyme stability must therefore be found.

Fig. 1. Mechanism of Laccase Oxidative Polymerization of Lignin

In this study, technical bagasse lignin and ultrafiltrated kraft lignin has been incubated with a commercially available laccase in 35% methanol solvent, using oxygen as an oxidant. The increase of molecular weight has been investigated by alkaline SEC. UFL, a low molecular weight ultra-filtered lignin, was isolated by ultra-filtration from the kraft cooking black liquor obtained from softwood pulping. The ultra-filtration equipment used for extracting this lignin consisted of a mixing tank, a gear pump and a cross-flow filtration unit and a ceramic membrane with 1000Da pore size. This by-product from the pulp industry contains an oligomeric lignin with almost twice the amount of free phenolic moieties than residual kraft pulp lignin[7].

EXPERIMENTAL

Materials

A thermally stable laccase labeled as Myceliophthora thermophila, Novozymes NS51003 was kindly supplied from Novozymes (Bagsvaerd, Denmark) and used without any further purification. The pH and temperature optimal were stated by the supplier to 7–9 and 40–50 °C, respectively.

Technical soda bagasse lignin with 3.41% moisture content was provided from Granit Corp. (Lausanne, Switzerland). Ultra-filtered lignin was prepared in within the department. Analytical reagent grade methanol was purchased from Fisher Scientific (Stockholm, Sweden). 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was purchased from Sigma-Aldrich.

Methods

5g dry content soda-bagasse lignin or ultra-filtered lignin was dissolved in 100ml 35% methanol solvent with pH ca. 13.0 in steam heated autoclave under the temperature...
130°C for 2h.

The pH of the dissolved lignin was reduced to 7.5 and NS51003 was added to 10ml lignin solution under temperature 40°C. The vessels were capped to prevent evaporation and oxygen was flushed during the oxidation reaction. Samples were withdrawn from the reaction at 0, 6, and 24 h of reaction time and analyzed with SEC.

**Characterization**

The activity of laccase in methanol/buffer solution was measured using the ABTS assay based on oxidation of ABTS to its cation radical with molecular oxygen as the electron acceptor. In a quartz cuvette (10mm), 240 μL ABTS solution (2mM ABTS in 100mM phosphate buffer, pH 7.5), 750uL methanol solution with different concentration were mixed. An appropriate amount of laccase was added to the mixture and the absorbance increase at 418nm (ε418 nm = 36 000 M⁻¹ cm⁻¹) was recorded using a Cary 100 UV/VIS spectrophotometer (Varian, Palo Alto, CA, USA). The enzyme activity was expressed in units (1 U = 1 μmol ABTS catalyzed per minute). An activity of 240 U/ml was determined for the NS51003.

The weight average molecular weight of polymerized lignin was determined by alkaline size exclusion chromatography system, consisting of a Rheodyne 7725i (Rohnert Park, CA, USA) manual injector, Waters 515 HPLC pump (Milford, MA, USA), three TSK gel columns (Tosoh Bioscience, Tokyo, Japan) connected in series, G3000PW (7.5mm×300mm, 10μm particle size), G4000PW (7.5mm×300mm, 17μm particle size), and G3000PW. A detection setup consisting of a Waters (Milford, MA, USA) 2487 dual λ and a Waters 410 refractive index detector was used. The mobile phase during the analysis was 10mM NaOH. For calibration, a series of polyethylene oxide (PEO) and polyoxide glycol (PEG) standards ranging from 1 500 to 250 000 Da were used. A volume of 100 uL was withdrawn from the reaction vessel with concentration 5% and diluted with 400uL deionized water to ca. 1%. A volume of 20 uL was injected and the absorbance at 280 nm was recorded. The data were processed with Millenium 2 Software.

Mechanical properties were determined following ASTM(American Society for Testing and Materials, 2000) standard method D1037-99 using Instron 5566 testing system(Instron Corp.). Tensile strength was obtained by applying the load continuously throughout the test at a uniform rate of motion of the movable crosshead of 2mm/min. For the internal bonding strength, the alumina alloy was firstly mechanically abraded by sand and then the specimen were bonded to the alumina alloy by Araldite 420 A/B epoxy adhesive and was clamped as soon as the adhesive has been applied.

**RESULTS AND DISCUSSION**

In these experiments enzyme-catalyzed oxidative cross-linking polymerization of lignin by an commercial laccase (Novozym 51003) in methanol solution was investigated. From Fig. 2 it is evident that the activity of the laccase is reduced by increased methanol concentration in a linear fashion. These findings suggest that enzyme deactivation occurs by the presence of large amounts of methanol in solution.

To enable laccase polymerization, the pH of the lignin solution had to be reduced from 13 to 7.5 after steam heating in the autoclave. The lignin solution remained stable after this drop with no visible precipitation. From that we deduced there is some reaction between lignin and methanol under high temperature and high pressure in the autoclave, which enables the lignin to stay in solution at neutral pH. These findings are worth further research.
Table 1. $M_w$ Averages of Technical Bagasse Lignin (TBL) and Ultra-filtrated Lignin (UFL) Samples after Oxidation by Various Activity Units of NS51003

<table>
<thead>
<tr>
<th>Time</th>
<th>78U TBL</th>
<th>78U UFL</th>
<th>39U TBL</th>
<th>39U UFL</th>
<th>19.5U TBL</th>
<th>19.5U UFL</th>
<th>9.75U TBL</th>
<th>9.75U UFL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>21 400</td>
<td>2 600</td>
<td>21 400</td>
<td>2 600</td>
<td>21 200</td>
<td>2 600</td>
<td>23 200</td>
<td>2 600</td>
</tr>
<tr>
<td>6h</td>
<td>103 900</td>
<td>46 200</td>
<td>105 000</td>
<td>44 600</td>
<td>99 600</td>
<td>37 000</td>
<td>64 400</td>
<td>29 700</td>
</tr>
<tr>
<td>24h</td>
<td>195 000</td>
<td>504 000</td>
<td>190 000</td>
<td>480 000</td>
<td>148 200</td>
<td>36 000</td>
<td>95 500</td>
<td>23 800</td>
</tr>
</tbody>
</table>

Fig. 3 displays SEC chromatographs of a selected sample from oxidation of bagasse and ultra-filtrated lignins by equal amounts of NS51003. All oxidation experiments showed that the average molecular weight increased both with the dosage of added laccase and treatment time (Fig. 4, 5). The corresponding data is also listed in Table 1.

As visible in Fig. 3, the lignin reference peak was separated into two fractions after 24 hours of laccase oxidation. Before testing, the sample was treated under 90°C to inactive the residual laccase, and then went through 0.45um PTFE syringe filter to purify the sample. Under above treatments, these two peaks distinctively represent two different fractions with different molecular weights ranging from 195 000 to 500 000 Da. Compared to the initial value, the $M_w$ was increased more than 20 times.

It should be noted however that obtained molecular weight values are based on calibration by PEO/PEG standards up to 250 000 Da. It is therefore highly likely that the high-$M_w$ value of 504 000 is less reliable. It does however not change the fact that the overall increase of Mw from the starting material is indeed in the region of 20 times.

Laccase was also shown to polymerize ultra-filtered lignin (Table 1, Fig. 5). The data showed that the weight of average molecular weight of ultra-filtered lignin is increased both with the laccase dosage and treatment time. The average molecular weight of the ultra-filtered lignin increased after 6 hours from 2 600 to no less than 30 000 Da in all dosages of laccase. Not surprisingly, a higher amount of laccase (39U and above) yielded higher molecular weights. Compared to the technical bagasse lignin, the polymerization rate of the ultra-filtered lignin dropped after the initial 6 hours suggesting either enzyme deactivation or consumption of available phenols.

The polymerization is likely to be explained by the mode of action of the laccase where the enzyme initiates oxidation of phenolic end groups into stabilized radicals that subsequently undergo radical-radical coupling through which phenyl ether-carbon and carbon-carbon bonds are formed (Fig. 1) [4, 8, 9].

In the natural world, plant biomass consists of 50 wt% of cellulose, 20 wt% of hemicellulose and 30 wt% of lignin, approximately, and lignin works as a curing agent making these component together [10]. Lignin is constituted by hydroxyphenylpropane units which connect with each other by ether and partial carbon–carbon bonds. It is a natural phenolic resin adhesive. The polymerized lignin was later used as a binder to make particle board consisting of 10% lignin and 90% bleached CTMP (Table 3). The tensile and internal bond strength of the final product was shown to be 9.84 MPa and 0.99 MPa respectively. Compared to the sample made with untreated lignin, the tensile strength was reduced nearly 40%. The reason for this is the viscosity of polymerized lignin is no increase, from which we presume the bonds to link one
macromonomer to another is in the way of crosslinking but not linear.

| Table 2. Mechanical strength of particle boards produced with bagasse lignin |
|-------------------|------------------|------------------|------------------|------------------|
| Composition       | Hot press parameters | Mean Tensile Stress at Max. load/MPa | Mean Tensile strain at max. load/% | Internal bonding strength/MPa |
| Sample A          | 90% TMP+10% Lignin  | 180°C 5min 115bar | 14.36 | 1.49 | - |
| Sample B          | 90% CTMP+10% polymerized lignin | 180°C 5min 115bar | 9.84 | 1.27 | 0.99 |

CONCLUSIONS

Both technical bagasse lignin and low molecular ultra-filtered lignin can be polymerized by laccase to increase their molecular weight. The molecular weight of technical bagasse lignin with large initial molecular weight increased 20 times after 24h treatment with a significant laccase dosage. Similar dosages of laccases yielded similar rate of increase of ultra-filtrated lignin during the first 6 hours but the reaction slowed down the last 18 hours, in contrast to bagasse lignin where the rate appeared to be essentially similar for the complete duration of the oxidation reaction.

Contrary to what was expected, the positive polymerization results could not be translated to the end product of these lignins. Particle boards produced with polymerized lignins displayed less than 40% tensile strength and nearly 20% less tensile strain. These results clearly highlight the importance of further research into the mechanisms of lignin and fibre interactions that occur during particle board manufacturing.

REFERENCES