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Dissolving Lignin in Water through Enzymatic Sulfation with Aryl Sulfotransferase

Pepijn Prinsen, Anand Narani, Aloysius F. Hartog, Ron Wever, and Gadi Rothenberg[a]

Introduction

Lignin is nature’s richest store of aromatics, and its valorization can increase the economic viability of tomorrow’s biorefineries.[1] The most important source of commercial lignins today is the pulp and paper industry, mainly from Kraft pulp mills. Lignosulfonates, isolated from waste liquors in sulfite pulp mills, are another significant fraction of the global lignin production. The Kraft delignification process uses a combination of NaOH and Na₂SO₃ at pH 13–14. The former leads to caustic hydrolysis,[2] whereas the latter creates C–SO₃H bonds via nucleophilic attack of HS⁻ ions (Figure 1a). The lignosulfonates isolated from waste liquors in sulfite pulp mills contain C–SO₃H groups on the lignin side chains (Figure 1b).[3] This creates more hydrophilic domains, facilitating fragmentation and delignification of the fiber.

In addition to these, large amounts of acid hydrolysis lignin and organosolv lignin are expected from second-generation bioethanol production plants[4] and biomass pretreatments,[5] respectively. But getting lignins into aqueous solution is no mean task.[6] At room temperature, caustic solutions, ionic liquids,[7] and liquid ammonia[8,9] can be used, yet each of these options has its drawbacks: Caustic solutions must be neutralized after processing, ionic liquids are still relatively costly and using liquid ammonia incurs significant capital investment. Another option is using supercritical solvents: water, CO₂, ethanol, acetonitrile, and mixtures thereof have all been reported.[10] However, such supercritical systems typically require extreme conditions.

We introduce the concept of using site-specific sulfation of various lignins for increasing their aqueous solubility and thereby their processability. Using p-nitrophenylsulfate as a sulfate source and an aryl sulfotransferase enzyme as catalyst, lignins are easily sulfated at ambient conditions. We demonstrate the specific sulfation of phenolic hydroxyl groups on five different lignins: Indulin AT (Kraft softwood), Protobind 1000 (mixed wheat straw/Sarkanda grass soda) and three organosolv lignins. The reaction proceeds smoothly and the increase in solubility is visible to the naked eye. We then examine the reaction kinetics, and show that these are easily monitored qualitatively and quantitatively using UV/Vis spectroscopy. The UV/Vis results are validated with 31P NMR spectroscopy of the lignin phenol groups after derivatization with phosphorylation reagent II. In general, the results are more significant with organosolv lignins, as Kraft and soda lignins are produced from aqueous lignocellulose extraction processes.

Figure 1. Formation of thiol (C–SH) bonds in the side chain of phenolic lignin units during Kraft pulping (a); formation of sulfonate bonds (C–SO₃H) during sulfite pulping (b) and enzymatic sulfation of phenolic groups in mild alkaline aqueous buffer (c).

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Here, we tackle the solubility challenge from another direction, adding hydrophilic sulfate groups to specific positions on the lignin aromatic rings using an enzymatic process. Earlier, we demonstrated the enzymatic sulfation of various phenol model compounds with \( p \)-nitrophenylsulfate as a sulfate donor, catalyzed by an aryl sulfotransferase (AST) enzyme isolated from Desulfitobacterium hafniense.\(^{[11]}\) We now report that this enzyme is also an excellent catalyst for sulfating actual lignins (i.e., not only model compounds, Figure 1c). The reaction is highly selective for phenolic groups, leaving the aliphatic hydroxyl groups in the lignin side chain intact. The resulting sulfated lignins dissolve easily in mildly alkaline solutions. In principle, these could be broken down further using beta-esterases\(^{[12]}\) and laccases.\(^{[13]}\) To the best of our knowledge, this is the first example of enzymatic lignin sulfation (the formation of an aromatic C–O–SO\(_3\)H group, unlike lignin sulfonation, which gives aliphatic C–SO\(_3\)H). The simplicity of the process and the fact that it runs under ambient conditions make it attractive for practical applications across the board, from medicine\(^{[14]}\) to biorefining.

**Results and Discussion**

Building on our earlier work using polyphenols as lignin model compounds,\(^{[11, 15]}\) we applied here the enzymatic sulfation using \( p \)-NPS and AST to actual lignins. Our hypothesis was that a selective sulfation of the aromatic OH groups would allow the lignins to dissolve in water more easily as sulfate esters. Note that such esters differ from lignosulfonates, which contain mainly aliphatic sulfonated groups on the carbon skeleton.\(^{[3]}\)

First, we tested the spruce organosolv lignin (Table 1, entry 1; control experiments shown in entries 2 and 3). The control experiments confirmed that both the enzyme and the \( p \)-NPS are essential for the sulfation. When both are present, a peak appears (\( \lambda_{\text{max}} = 399 \text{ nm} \) Figure 2) corresponding to \( p \)-nitrophenol (\( p \)-NP, Figure S1 in the Supporting Information). After correcting for the control experiments, we found that approximately 30–32% of the initial lignin is solubilized as a result of this enzymatic sulfation. This corresponds to 32% conversion of the initial load, as observed by UV/Vis spectrosopy. The darker color of the solution after removing the insoluble fraction reflects the improved lignin solubility (Figure 2, inset). The enzyme transfers sulfate groups from the \( p \)-NPS to the lignin, increasing its solubility as hypothesized. Further control experiments confirmed that the amount of dissolved lignin (DL, quantified by HCl precipitation followed by centrifuging) differed between sulfated lignins and non-sulfated ones. The sulfated lignins, when incubated with enzyme, yielded a precipitated fraction (PDL) and a non-precipitated (NPDL) fraction. The non-sulfated lignins were precipitated almost quantitatively leaving behind a supernatant with only \( p \)-NP, \( p \)-NPS, buffer, and chloride salts.

Monitoring the reaction over time of 7.9 g L\(^{-1}\) wheat straw organosolv using 2.0 U mL\(^{-1}\) AST showed that all of the 5 mM \( p \)-NPS was consumed after 96 h, whereas only 24% were consumed in the control without AST. We therefore increased the concentration up to 15 mM, in which some \( p \)-NPS remained, indicating that the sulfation of the lignin phenol groups reached equilibrium (some of the lignin hydroxyl groups remain unsulfated). Similarly, using more enzyme increased \( p \)-NPS conversion (Figure 3). During the course of the reaction, the maximum at 278 nm (corresponding to residual \( p \)-NPS) shifts towards lower wavelengths (see Figure S2a). This is caused by the lignin getting into solution, as seen from the

**Table 1. Yields of residual lignin (RL).\(^{[4]}\)**

<table>
<thead>
<tr>
<th>Entry</th>
<th>( p )-NPS (max)</th>
<th>AST (U mL(^{-1}))</th>
<th>( p )-NPS conversion [%]</th>
<th>RL [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.2</td>
<td>2</td>
<td>38</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>89</td>
</tr>
<tr>
<td>3</td>
<td>16.2</td>
<td>0</td>
<td>6</td>
<td>102</td>
</tr>
</tbody>
</table>

\([a]\) Isolated after 96 h stirring of 8.3 g L\(^{-1}\) dry spruce organosolv lignin in 0.1 M TG buffer (pH 9.1) at rt, with/without \( p \)-NPS as sulfate donor and AST enzyme as catalyst.
local absorbance maximum at 275 nm when dissolving isolated PDL fractions in Tris-glycin (TG) buffer (Figure S2b). Plotting the difference of the absorbance between 275 and 278 nm (Figure S2c) shows that most of the lignin is dissolved as the sulfation occurs. In principle, the sulfation degree of the lignin can be calculated from the p-NPS conversion determined by UV/Vis analysis. However, the individual sulfation values of the two lignin fractions could not be determined using only UV/Vis spectroscopy.

To quantify the sulfation of each lignin fraction, we ran a $^{31}$P NMR analysis on the parent lignin, the residual (RL) and the precipitated dissolved lignin (PDL; for details see Table S1 in the Supporting Information). These $^{31}$P NMR spectra are shown in Figure 4. The NMR analysis requires a derivatization of the remaining aromatic OH groups with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (phosphorylating reagent II).[17] There are three possible scenarios for $^{31}$P derivatization of (partially) sulfated lignins (Figure S3). In the first one, the sulfate groups may remain on the lignin, precluding any lignin $^{31}$P NMR signal. In the second, the sulfated groups themselves would be also substituted by the phosphorous derivatizing agent forming HSO$_3$Cl instead of HCl. This would make quantification impossible, as the reagent would not distinguish between the sulfated phenolic groups and the remaining free phenolic groups. In the third case, the derivatizing agent would react with the sulfate oxygen instead of with the phenolic oxygen. This would result in a $^{31}$P NMR shift, which may overlap with other signals. Here, the first scenario dominated (the sulfate groups remain on the lignin), enabling us to quantify the phenolic groups and the sulfated groups. Comparing the $^{31}$P NMR spectra of the post-reaction lignins with that of the parent lignin, we see that the phenolic group signals are much lower.

We then calculated the sulfation degree from the $^{31}$P NMR spectra by comparing the number of remaining phenol groups with the number of phenol groups in the original lignin (see the Supporting Information for details). These groups are present in syringyl, guaiacyl, p-hydroxyphenyl, p-coumarate, and tricin units, totaling 3.2 mmol per gram of parent lignin (Table 2, entry 1). The sulfation degree of the RL is lower than that of the PDL (Table 2, entries 2 and 3). This may be the reason for their difference in solubility. Looking closer at the $^{31}$P NMR spectra of the PDL fraction, we see that the syringyl (A, 142.6 ppm) and guaiacyl (C, 139.7 ppm) phenol signals diminish, leaving the condensed guaiacyl phenol units (B, 141.3–143.8 ppm excluding syringyl units) unaltered. This shows that the sulfation of the condensed guaiacyl phenol groups is more difficult. Another signal appears at 140.7 ppm in all three spectra. This signal does not come from p-NP nor from one of the amino acids of the enzyme.[16] This was also observed by King et al.[17] and may reflect the de-fragmentation of low-molecular-weight (LMW) lignin.

By subtracting the content of the remaining phenol groups in the RL, PDL, and NPDL fractions from the initial content in the parent lignin, we see that according to $^{31}$P NMR, approximately 0.085 mmol phenolic groups are sulfated (Table 3). This

![Precipitated dissolved lignin, PDL](image)

![Parent lignin](image)

![Residual lignin, RL](image)

![Figure 4. $^{31}$P NMR spectra of the parent wheat straw organosolv and the corresponding residual lignin (RL) and precipitated dissolved lignin (PDL; Table S1, entry 3). A = syringyl, B = 5-substituted guaiacyl, C = guaiacyl and catechol, D = p-hydroxy coumaric acid (PCA) and free phenol, E = tricin.](image)

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</thead>
<tbody>
<tr>
<td>1</td>
<td>Parent (79.9)</td>
<td>1.6</td>
<td>3.2</td>
<td>1.3</td>
<td>1.1</td>
<td>0.3</td>
<td>0.5</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>RL (34.8)</td>
<td>2.8</td>
<td>2.5</td>
<td>1.1</td>
<td>0.8</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>PDL (28.2)</td>
<td>1.5</td>
<td>1.7</td>
<td>0.7</td>
<td>0.5</td>
<td>0.2</td>
<td>0.2</td>
<td>0.5</td>
<td>47</td>
</tr>
</tbody>
</table>

[a] Determined by $^{31}$P NMR of the parent (100 wt%), RL (54 wt%), and PDL (35 wt%) wheat straw organosolv lignin. The remaining fraction consisted of NPDL (10 wt%). The phenolic groups are the sum of syringyl (S–OH), condensed and non-condensed guaiacyl (G–OH), p-hydroxyphenyl (H–OH), p-coumarate (PCA–OH) and tricin hydroxyl groups.
value agrees well with the amount of $p$-NP present after 96 h, as determined from UV/Vis analysis (0.086 mmol). Indeed, this means that the UV/Vis analysis is a valid method for quantifying the amount of sulfate groups that were transferred to the lignin. This is important, because running UV/Vis analyses is much quicker, simpler and cheaper than $^{31}$P NMR. The latter is needed if one wants also to calculate the sulfation degree of individual fractions (RL and DL).

Figure 5 shows the time-resolved $p$-NP yield for several lignins at different concentrations. Initially, $p$-NP formation is rapid, followed by a much slower phase. At higher concentrations of lignin more $p$-NP forms, whereas at lower concentrations the equilibrium is reached earlier (Figure 5). Figure 5c,d shows that the LMW fraction had a much higher sulfation degree compared to the high-molecular-weight (HMW) fraction. This is expected, as the phenol content in the LMW fraction is much higher. Surprisingly, the solubility of the HMW fraction was higher than the LMW fraction. Control experiments showed that lignins from soda-derived pulping processes show a good intrinsic solubility (Figure S5). Note that the HMW fraction is a fine powder whereas the LMW fraction contains coarser particles and tends to aggregate more (as a result of the fractionation method). The HMW fraction also contained some non-lignin extractives, which may also explain the difference in sulfation and solubilization degree. As judged from the formation of $p$-NP, soda-derived lignins react faster than

| Table 3. Amount of aliphatic, phenolic, and carboxylic OH groups.[$^{a}$] |
|-------------------|-------------------|-------------------|
| Lignin            | Aliphatic OH [mmol] | Phenolic OH [mmol] | COOH [mmol] |
| parent            | 0.125             | 0.252             | 0.028       |
| RL                | 0.122             | 0.108             | 0.014       |
| PDL               | 0.042             | 0.047             | 0.013       |
| NPDL              | 0.011             | 0.013             | 0.004       |
| parent–RL–PDL–NPDL| –0.050            | 0.085             | –0.003      |
| $p$-NP yield UV/Vis [mmol][c] | 0.086          |

[a] Calculated as weight (g)×OH content (mmol g$^{-1}$) from Table 2. [b] Calculated assuming that the NPDL phenol group content was the same as in the PDL fraction (independent of this assumption, the overall $^{13}$C NMR does not vary significantly as the NPDL mass fraction was only 10 wt %). [c] $p$-NP yield calculated from UV/Vis analysis shown for comparison.

Figure 5. Time-resolved plots of the $p$-NP yield at several lignin concentrations and in the control (no AST) with a) Indulin AT, b) wheat straw organosolv lignin, c) Protobind 1000 HMW, d) ProtobindP 1000 LMW.
organosolv ones (Figure 6). This is also why the solubility of the sulfated soda-derived lignins was only about 10% higher than that of the non-sulfated lignins (14.8 g L\(^{-1}\)), compared with about 25% in the case of sulfated wheat straw organosolv lignin. In the initial phase, p-NP yields obtained with Indulin AT were much higher than with wheat straw organosolv lignin, in agreement with their respective total phenol content (3.37 and 2.72 mmol g\(^{-1}\), respectively).\(^{19}\) Indeed, Indulin AT has no syringyl units, and therefore less steric hindrance and better access to the AST enzyme. At equilibrium, however, the p-NP yields were similar (62 and 56%, respectively). This may reflect the higher content of condensed phenol groups in Indulin AT (ca. 44%) as compared to wheat straw organosolv lignin (ca. 9%).

Organosolv lignin is less soluble than Indulin AT and Protobind 1000, and therefore more of a challenge. We ran experiments with organosolv lignins from poplar, spruce, and wheat straw (20.3 g L\(^{-1}\) for 24 h, Figure 7a). After correcting for the non-enzymatic solubility in the control experiments, the percentage of sulfate groups transferred from p-NPS to these samples were 31, 36, and 59%, respectively. Indeed, our previous studies on model compounds affirm this. For example, p-coumaric acid (which is present only in wheat straw lignin)\(^{20}\) is an excellent sulfate acceptor.\(^{11}\) Conversely, p-hydroxy-benzoic acid (present only in poplar lignin)\(^{21}\) is a poor sulfate acceptor.\(^{11}\) A similar effect can be expected for ferulates, which are present only in wheat straw lignin.\(^{20, 22}\) This can explain the sulfation and solubility differences in these three lignins.

Other structural characteristics such as the syringyl/guaiacyl/hydroxyphenyl ratio and steric hindrance may also influence the sulfation degree. Tricin, a flavone that is etherified in position 4’ in native wheat straw lignin,\(^{22}\) is mainly present in its free form in wheat straw organosolv lignin.\(^{19}\) This can be seen in the \(^{31}\)P NMR spectrum (cf. Figure 4, the signal at 141.9 ppm

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**Figure 6.** Time-resolved plots of the p-NP yield at several lignin concentrations during the first 4 h.

**Figure 7.** Experiments with organosolv lignins (20.3 g L\(^{-1}\)) in a 1st and 2nd run (using the RL of the 1st run with new load of 15 mm p-NPS and 3 U mL\(^{-1}\) AST) showing a) p-NP yield as a function of time, and the lignin solubility after 24 h for b) spruce organosolv lignin, c) poplar organosolv lignin, and d) wheat straw organosolv lignin.
corresponding to the 4’ OH group). This OH group and the one in position 7 of tricin (136.5 ppm) are significantly sulfated. Conversely, the OH group in position 5 remains unaltered. This sulfation order is in agreement with the results found for quercetin,[15] which has the same structure as tricin with two additional OH groups in positions 3 and 3’. After 24 h, a lot of lignin still remained insoluble (Figure 7b–d). Therefore, the insoluble fraction (RL) was subjected to a second run with the same reaction conditions as the first. The percentage of sulfate groups transferred to wheat straw, poplar, and spruce organosolv RL were 11, 12, and 13%, respectively (after correcting for the control experiments). These results show that the remaining unsulfated OH groups in wheat straw RL resist further sulfation. After two runs, the solubility was improved, yet 45–65% of the initial lignin remained.

Conclusions

Enzymatic sulfation of lignins using aryl sulfotransferase (AST) in combination with a sulfate donor such as p-nitrophenylsulfate (p-NPS) is a simple and efficient method for increasing their solubility in water. This method is robust and applies to both technical and organosolv lignins. Remarkably, the kinetics of the sulfation reaction can be easily monitored qualitatively and quantitatively using UV/Vis spectroscopy, as validated here with 31P NMR after derivation of the lignin phenol groups with phosphorylating reagent II. The simple quantification technique, combined with the fact that these enzymatic sulfation reactions run under ambient conditions, make this method a practical and promising tool in biomass valorization.

Experimental Section

Materials and instrumentation: Unless otherwise noted, all chemicals were purchased from Sigma–Aldrich and used as received. Commercial lignins (Indulin AT from Kraft softwood and Protobind 1000 from mixed wheat straw/Sarkanda grass soda) were purchased from Meadwestvaco (USA) and GreenValue S.A. (Switzerland), respectively. The Protobind 1000 lignin was separated into HMW and LMW fractions by extraction at 20 °C in dichloromethane (3 × 12 h). The three organosolv lignins were extracted from wheat straw (Spain), poplar (Kenniscentrum Papier en Karton, The Netherlands), and spruce (Denmark) using the acid-catalyzed ethanol-based organosolv process developed by the Energy research Center of the Netherlands (ECN).[19] Further details on the isolation and characterization of these lignins are reported elsewhere.[18] Prior to use, the humidity of the lignins was determined gravimetrically in threefold (all the results are reported based on initial dry lignin). UV/Vis analysis was performed on a Varian UV/Vis spectrometer using quartz tubes with 1 cm light path length in the 200–480 nm region. 31P NMR spectra were recorded at 25 °C on a Bruker AVANCE III 400 MHz instrument. Lignin phenolic groups were identified and quantified using the procedures published by King et al.[17] and Constant et al.[18] Around 25 mg of lignin was used for 31P NMR analysis after derivatization with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphosphepine (the so-called phosphorylating reagent II). Prior to 31P NMR analysis, samples were first extracted with diethyl ether to remove p-NPS and p-NP residues, followed by drying under a nitrogen stream. The detailed processing methods of the 31P NMR spectra are described elsewhere.[19] The AST enzyme was expressed and purified following a published procedure[15] and stored at −20 °C. Its activity was determined as 47 U mL−1, and its specific activity was 11 U mL−1. The TG buffer was prepared by dissolving 2-amino-2-(hydroxymethyl)-1,3-propanediol (3.03 g) and of glycine (1.88 g) in demineralized water. Then, water was added until reaching 250 mL (pH 9.1).

Procedure for enzymatic lignin sulfation: All experiments were run at room temperature in 16 mL glass vials and 500 rpm magnetic stirring (see workflow in Scheme 1). Example: dry Indulin AT (41.7 mg), p-NPS (19.3 mg, 15 mM), and AST (0.32 mL, 3 U mL) were added to 100 mL TG buffer (4.7 mL, pH 9.1) and stirred. After 96 h, the stirring was stopped, water (50 mL) was added and the mixture was transferred to centrifuge tubes (final pH 8.4). The tubes were stored overnight at 4 °C, and then centrifuged for 50 min (4 °C, 10000 rpm; JA 10 fixed angle rotor). Subsequently, the remaining solid (RL) was taken up with water and dried at 70 °C until reaching constant weight. The supernatant (containing the DL) was filtered on paper, and the very fine solids were recovered and added to the RL fraction. The filtrate was then acidified to pH 2.4 with 6 M HCl under stirring and stored overnight at 4 °C to allow further precipitation. The suspension was then centrifuged and the solids (PDL) recovered with water and weighed. The remaining supernatant was then evaporated at 50 °C/80 mbar and further dried at 70 °C until reaching constant weight. This fraction consisted of NPDL and leftover reactants. The NPDL content was calculated as the difference of the experimental weight of the dry residual aqueous phase with the theoretical content of the sum of p-NPS, p-NP, buffer, and KCl/NaCl salts.

Procedure for monitoring reaction kinetics: Samples were taken at different time intervals in 25 μL aliquots, added to TG buffer (10 mL) and filtered on 0.22 μm PVDF syringe filters, removing any lignin solids. UV/Vis spectra of the filtrates were then recorded in the 200–480 nm region. The p-NP concentration was calculated by measuring the absorbance at 399 nm (maximum) and applying the
Beer–Lambert law. The molar extinction coefficient of \( p \)-NP was calculated from a dilution set in 100 mM TG buffer and was 19,340 M\(^{-1}\) cm\(^{-1}\) (pH dependent). The \( p \)-NP yield (%) was calculated with respect to the initial \( p \)-NPS load. Further details are included in the Supporting Information.

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Keywords: aryl sulfotransferase · biomass · lignin · solubility · sulfation