Supporting Information

Preserving Aryl Ether Linkages and Higher Yields of Isolated Lignin through Biomass Fibrillation

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Experimental

Materials

Reference poplar (GW-9947S) chips were procured from Center for Bioenergy Innovation (CBI) program at the Oak Ridge National Laboratory. The chips were knife-milled in model 4 Wiley mill (Thomas Scientific, Swedesboro, NJ) using 1 mm screen. Soxhlet extraction was carried out for 6 h using 2:1 v/v toluene: ethanol to obtain extractives-free poplar. Biomass composition was determined by two-step acid hydrolysis NREL procedure[12,13]. Accellerase 1500 (cellulases) and Accellerase XY (xylanases) were kind gifts from Dupont Industrial Biosciences. Lyophilized pectinase from *Aspergillus niger* (Alfa Aesar EINECS 232-885-6, 20 U/mg) was purchased through Fisher Scientific and lyophilized non-specific protease (Pronase protease from *Streptomyces griseus*, EC no. 232-909-5, 45 KU/g) was purchased through Sigma Aldrich. All other chemicals were of analytical grade and were purchased through Fisher Scientific and Sigma Aldrich.

Molecular Weight

The purified lignins were vacuum-dried in 20 mL scintillation vials at 40 °C overnight. Acetylation was then carried out in 20 mL vials using 1:1 v/v acetic anhydride: pyridine for 24 h at room temperature with magnetic stirring. Then ethanol was added to stop the reaction followed by rotary evaporation. The residual pyridine was evaporated by vacuum drying at 40 °C overnight. Then, HPLC-grade tetrahydrofuran was added to the vials and kept overnight. The liquids were then transferred to HPLC vials through syringe-filtration using 0.22 µm hydrophobic PTFE filters. Gel permeation chromatography was carried out through Agilent 1260 Infinity II chromatography module with UV detector (Agilent G7114A Variable Wavelength Detector), Agilent PLGel organic GPC 5 µm diameter, 250 mm length and 500, 10^4 and 10^5 Å

individual pore size columns connected in series at 0.5 mL/min tetrahydrofuran mobile phase at 25 °C. The molecular weights were calculated based on polystyrene calibration and softwood kraft lignin was taken through the procedure for validation.

Spectroscopy

NMR

The duplicate purified lignin samples were combined, vacuum dried and then dissolved in DMSO-d_6 and transferred into NMR tubes. The concentrations of lignin samples were ~80 mg/mL (~40 mg purified lignin + 0.5 mL DMSO-d_6). ^{13}C-^{1}H heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectroscopy was carried out through Bruker NMR equipped with a 5 mm Broadband Observe probe at 300 K using standard 'hsqcetgpsisp2.2' pulse program that had a spectral widths of 220 ppm in ^{13}C frequency (256 data points) and 12 ppm (2048 data points) in ^{1}H frequency and 1 sec interscan delay and 64 scans.

Mid-IR

Mid-infrared spectroscopy was carried out on Perkin Elmer Spectrum 100 instrument with attenuated total reflectance (ATR) accessory. 64 scans from 4000 to 600 cm^{-1} were carried out on purified lignins isolated from ball-milled and fibrillated biomass. Atmospheric suppression option was chosen on Spectrum software and the raw data were converted from %transmittance to absorbance. Baseline was correct using rubber band method using the same wavenumbers on both lignins. Peak height at 1505 cm-1 was scaled to 1.0 absorbance to normalize the spectra.

Sugar Quantification

Aliquots were taken at the end of 48 h after the initial and second enzymatic hydrolysis and centrifuged. The supernatants were syringe-filtered and glucose and xylose concentrations were analyzed through high pressure anion exchange chromatography - pulsed amperometric detection (HPAEC-PAD) (Dionex ICS-3000) using CarboPac PA1 column and 2 mM NaOH mobile phase. Fucose was used as an internal standard and glucose and xylose standards for calibration.

Water Retention Value

Water retention values (WRV) were determined as per the procedure described previously[14]. It is the ratio of the weight of water retained in the sample after centrifugation to the oven dry weight.

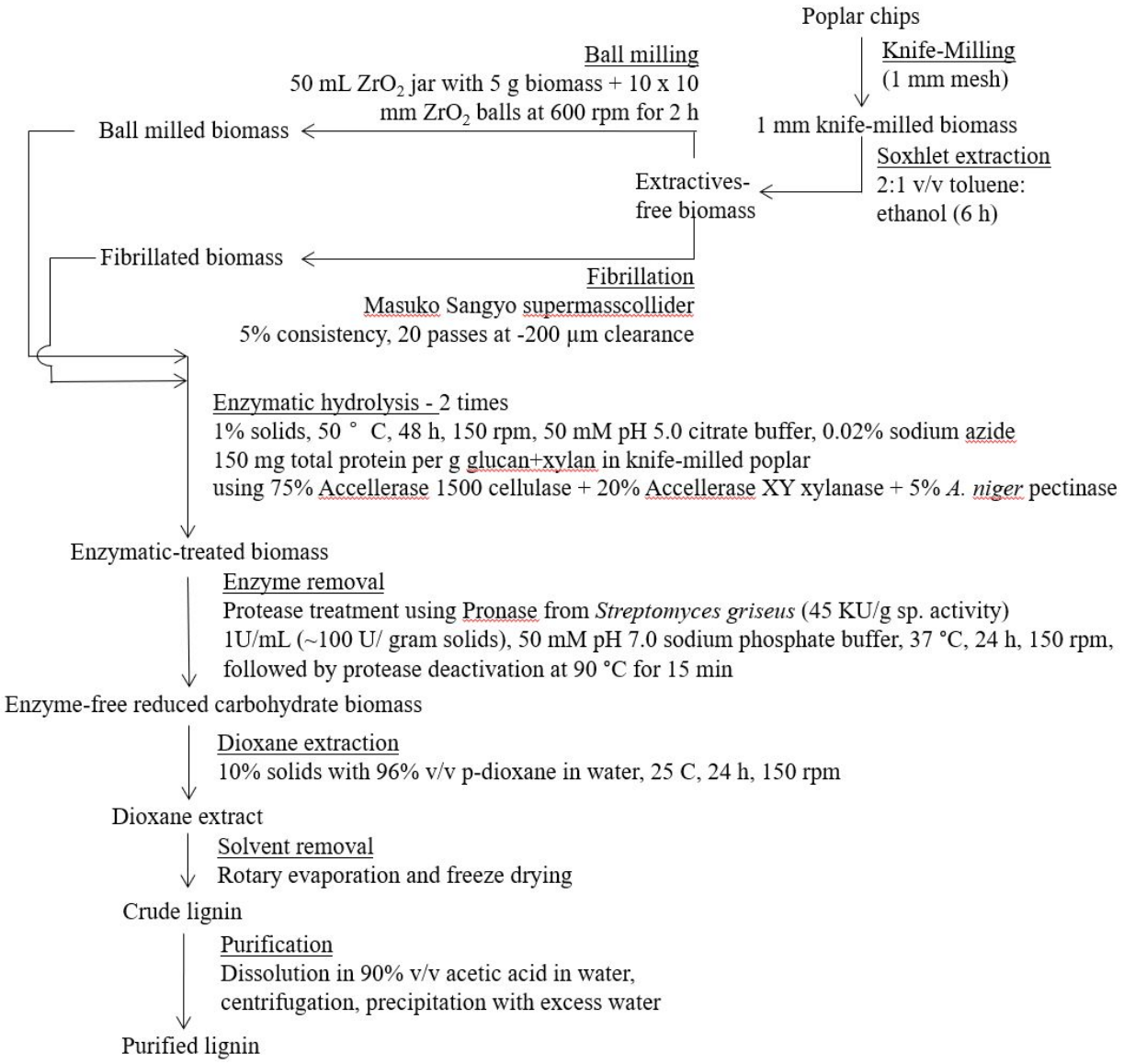

Fig S1. Experimental strategy for recovering cellulolytic enzyme lignin (CEL) from biomass

Table S1. Enzymatic hydrolysis yields

Time	Substrate	Glucan %	Xylan %	Glucan+Xylan %
1st 48 h	ball-milled	44.5 (0.4)	33.6 (2.3)	40.9 (0.9)
	fibrillated	53.0 (0.8)	33.6 (1.4)	46.7 (1.0)
2nd 48 h	ball-milled	8.7 (0.8)	3.4 (2.2)	7.0 (1.3)
	fibrillated	15.0 (1.0)	9.7 (1.1)	13.3 (1.0)

values in parentheses represent standard deviation

Table S2. Mid-infrared spectra band assignments

Wavenumber (cm^{-1})	Assignment
3430	O-H stretch
2937	C- H stretch in methyl and methylene
1705	C=O stretch in unconjugated ketones, conjugated aldehydes and carboxylic acids
1660	C=O stretch in conjugated p- substituted aryl ketones
1591	aromatic skeletal vibrations + C=O stretch
1505	aromatic skeletal vibrations
1459	C-H deformations
1420	C-H deformations
1326	Syringyl ring + condensed guaiacyl
1270	Guaiacyl ring + C=O stretch
1240	O=C-O in acetylated xylan and in uronic acids
1220	C-C + C-O + C=O stretch
1185	C-O of polysaccharides
1150	C-O-C vibrations
1120	C-O-C vibrations
1027	aromatic in plane C-H deformation
920	C-H out of plane deformation
885	C-H deformations
834	trisubstituted aromatic ring
771	C-H out-of-plane bending of aromatic ring

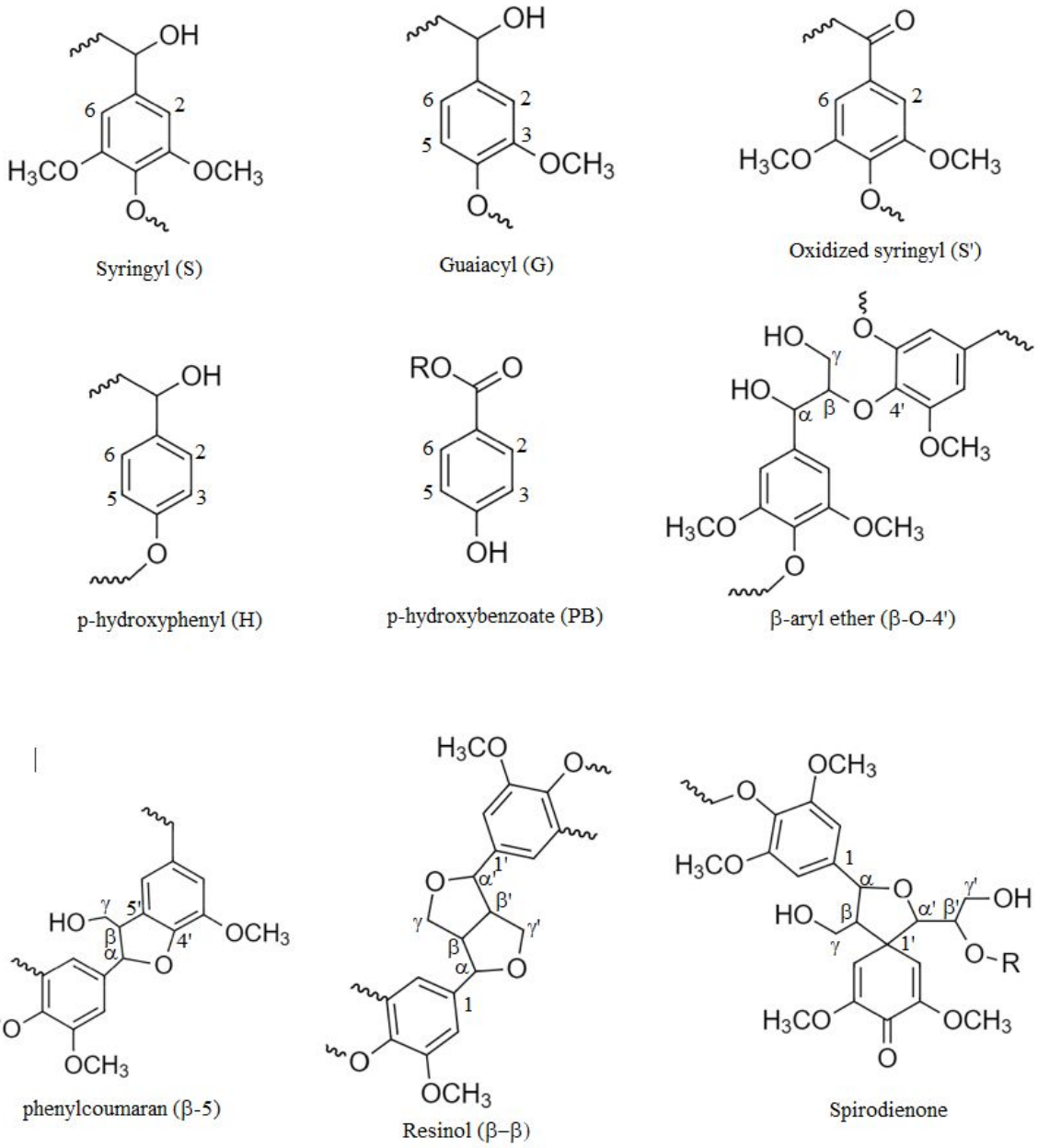

Fig S2. Lignin substructures

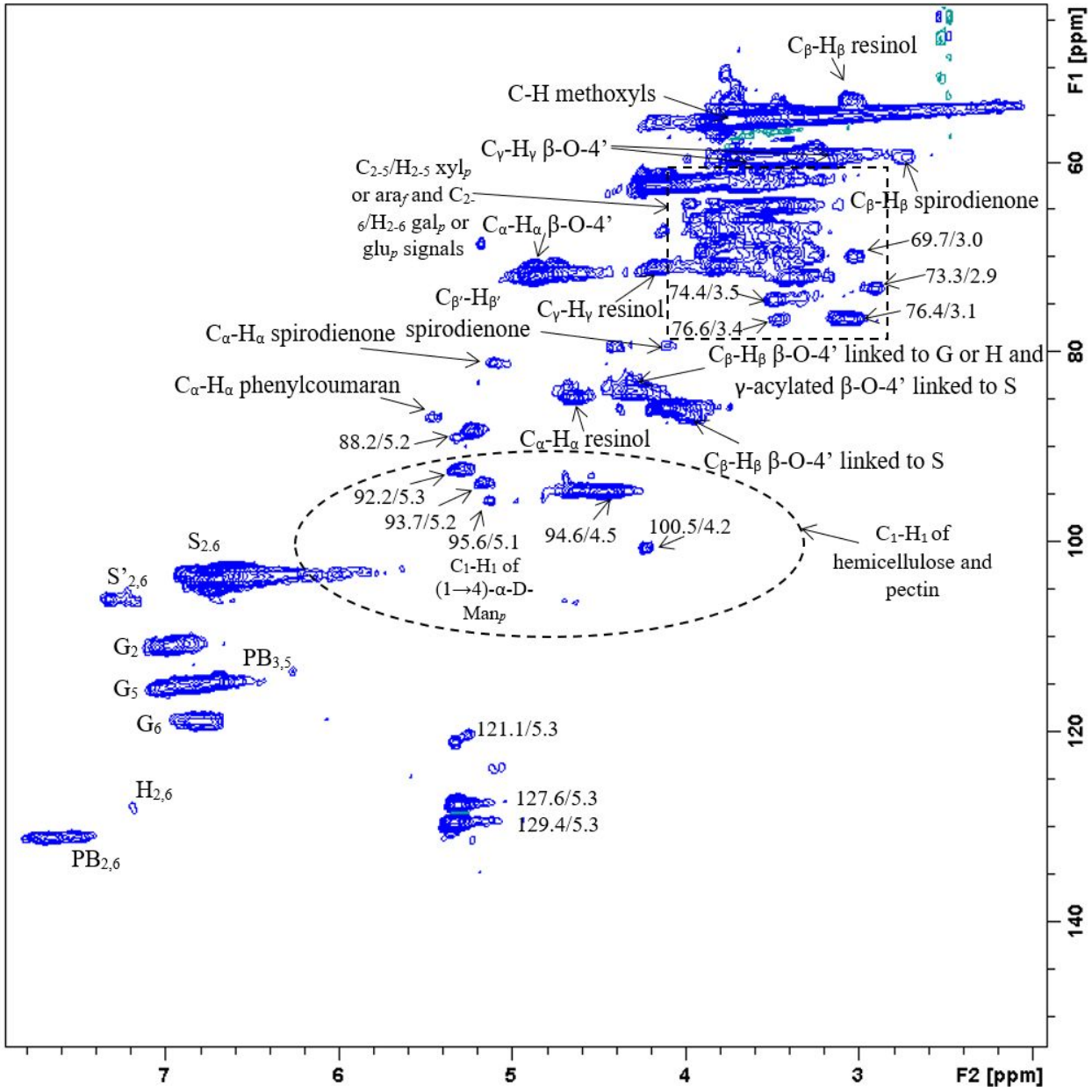

Fig S3. ^{13}C-^{1}H HSQC NMR spectra of cellulolytic enzyme lignin isolated from ball-milled poplar

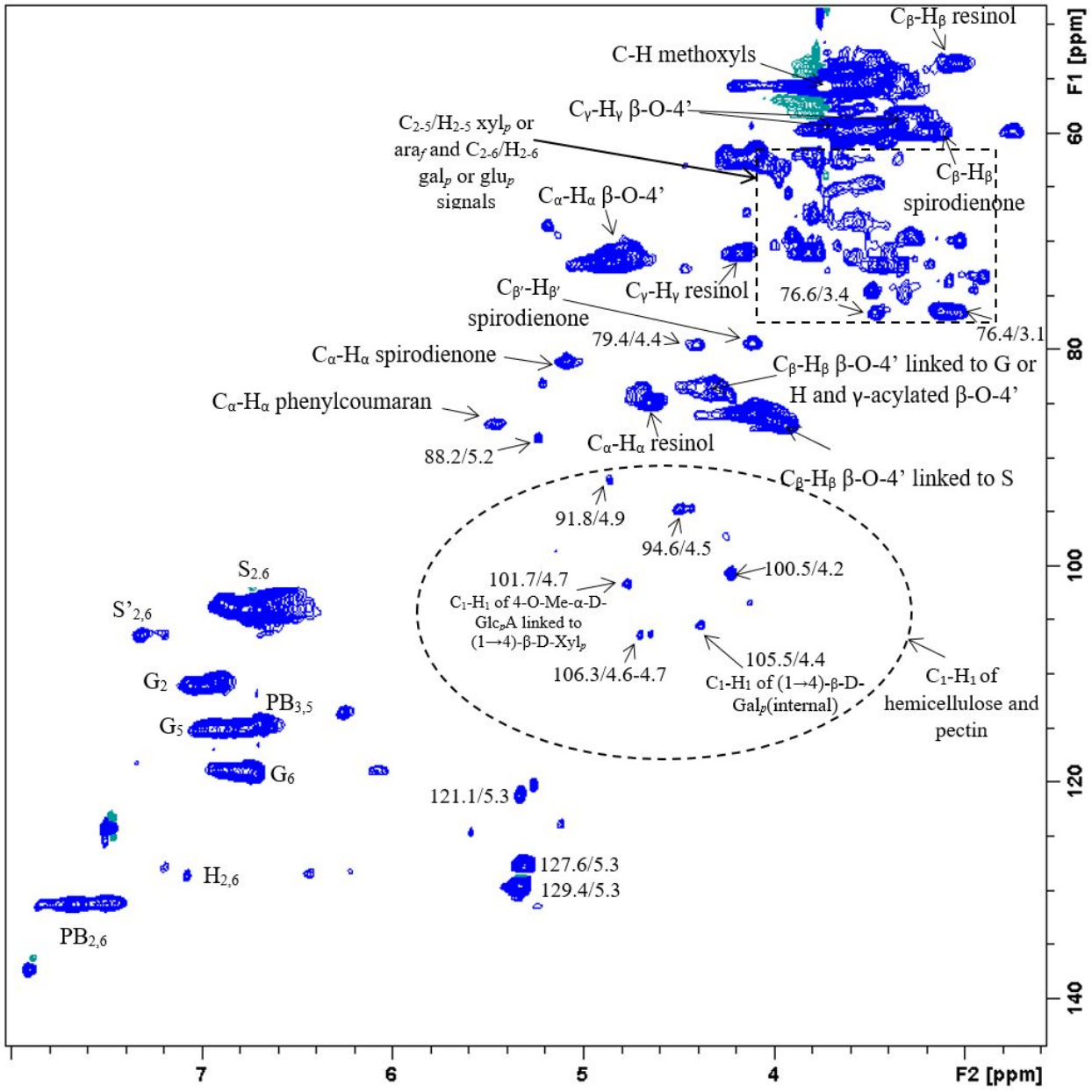

Fig S4. ^{13}C-^{1}H HSQC NMR spectra of cellulolytic enzyme lignin isolated from fibrillated poplar

Table S3. ^{13}C-^{1}H HSQC NMR chemical shifts and assignments of lignin

^{13}C (ppm)	^{1}H (ppm)	Assignment
53.55	3.07	C_β-H_β of resinol (β-β)
55.44	3.71	C-H of methoxyls
59.24	3.71	C_γ-H_γ of β-O-4'
59.31	3.39	C_γ-H_γ of β-O-4'
59.46	2.73	C_β-H_β of spirodienone
70.99	4.18	C_γ-H_γ of resinol (β-β)
71.86	4.87	C_α-H_α of β-O-4'
81.16	5.1	C_α-H_α of spirodienone
79.4	4.1	$C_{\beta'}$-$H_{\beta'}$ of spirodienone
83.89	4.3	C_β-H_β of β-O-4' linked to G and H units and γ-acylated β-O-4' linked to S units
84.7	4.66	C_α-H_α of resinol (β-β')
85.77	4.11	C_β-H_β of β-O-4' linked to S unit
86.75	5.47	C_α-H_α of phenylcoumaran (β-5')
103.75	6.69	$C_{2,6}$-$H_{2,6}$ of syringyl (S) unit
105.94	7.23	$C_{2,6}$-$H_{2,6}$ of oxidized S (S') unit
106.07	7.32	$C_{2,6}$-$H_{2,6}$ of oxidized S (S') unit
110.99	6.97	C_2-H_2 of guaiacyl (G) unit
114.84	6.74	$C_{3,5}$-$H_{3,5}$ of p-hydroxybenzoate
115.06	6.97	C_5-H_5 of G unit
118.84	6.83	C_6-H_6 of G unit
127.8	7.19	$C_{2,6}$-$H_{2,6}$ of p-hydroxyphenyl (H) unit
131.11	7.65	$C_{2,6}$-$H_{2,6}$ of p-hydroxybenzoate

Calculations:

Volume integral of syringyl C_2-H_2 and C_6-H_6 ($S_{2/6}$ @ 103.75/6.69 ppm) = S

Volume integral of guaiacyl C_2-H_2 (G_2 @ 110.99/6.97 ppm) = G

Volume integral of p-hydroxyphenyl C_2-H_2 and C_6-H_6 ($H_{2/6}$ @ 127.8/7.19 ppm) = H

Volume integral of p-hydroxybenzoate C_2-H_2 and C_6-H_6 ($PB_{2/6}$ @ 131.11/7.65 ppm) = PB

Volume integral of C_α-H_α of β-O-4' (β-O-4' @ 71.86/4.87 ppm) = β-O-4'

Volume integral of C_α-H_α of phenylcoumaran (β-5' @ 86.75/5.47 ppm) = β-5'

Volume integral of C_α-H_α and $C_{\alpha'}$-$H_{\alpha'}$ of resinol (β-β' @ 84.7/4.66 ppm) = β-β'

Volume integral can be calculated by first assigning a value of 100 to $S_{2/6}$ signal and then measuring the integrals for all other lignin C-H lignin signals relative to $S_{2/6}$ signal of the ^{13}C-^{1}H HSQC spectra using TopSpin software (Bruker).

Syringyl (S) % = $\frac{S/2}{S/2+G+H/2}$ *100

Guaiacyl (G) % = $\frac{G}{S/2+G+H/2}$ *100

p-hydroxyphenyl (H) % = $\frac{H/2}{S/2+G+H/2}$ *100

p-hydroxybenzoate (PB) % = $\frac{PB/2}{S/2+G+H/2}$ *100

β-β' % = $\frac{\beta-\beta/2}{S/2+G+H/2}$ *100

β-O-4' % = $\frac{\beta-O-4}{S/2+G+H/2}$ *100

β-5' % = = $\frac{\beta-5}{S/2+G+H/2}$ *100

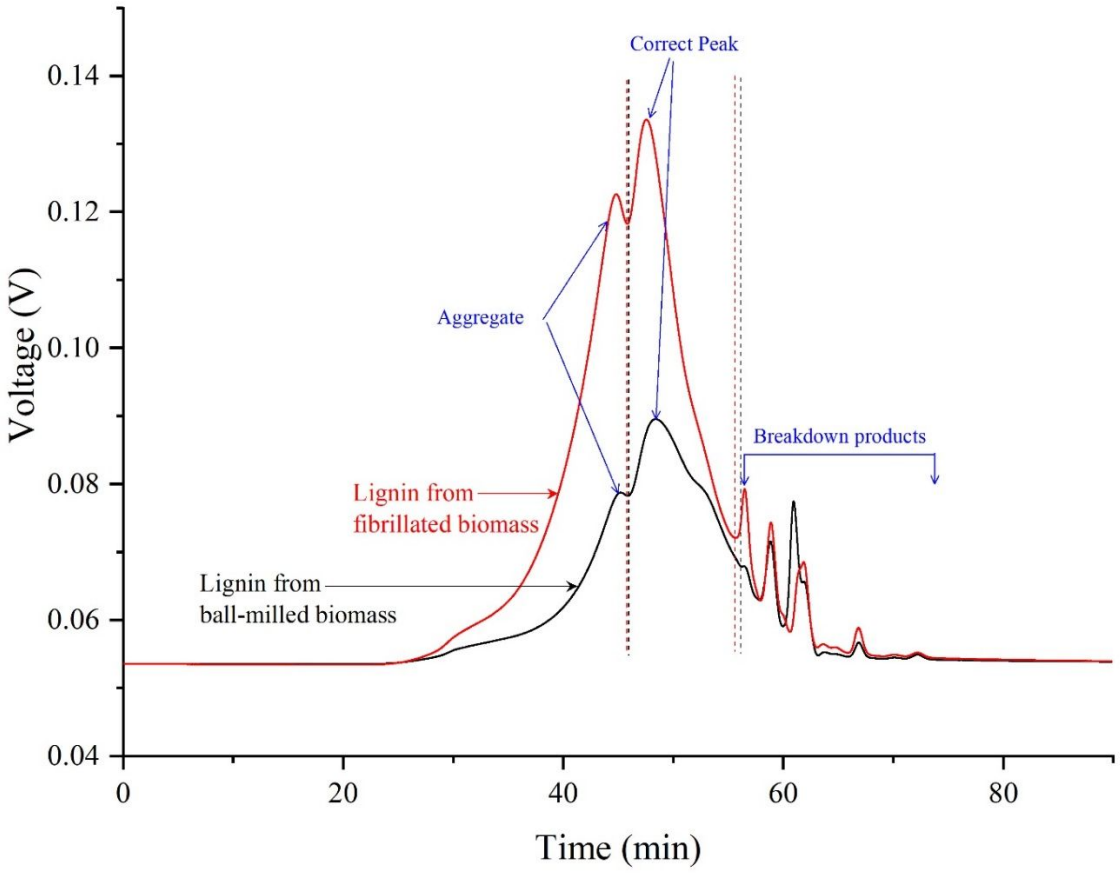

Fig S5. Gel permeation chromatographs of purified lignins isolated from ball-milled and fibrillated poplar