Green Fractionation and Structural Characterization of Lignin Nanoparticles via Carboxylic-Acid-Based Deep Eutectic Solvent (DES) Pretreatment

Meng-Kai Qin †, Cheng Zuo †, Yi-Ting Yang, Yi-Hui Liu, Cheng-Ye Ma * and Jia-Long Wen *

[1] Beijing Key Laboratory of Lignocellulosic Chemistry, Beijing Forestry University, No. 35 Tsinghua East Road, Haidian District, Beijing 100083, China

* Correspondence: Tel./Fax: +86-10-62336903; chengye.ma@foxmail.com (C.-Y.M.); wenjialong@bjfu.edu.cn (J.-L.W.)

† These authors contributed equally to this work.

1.1 Preparation of double enzymatic lignin (DEL)

The ball-milled powder (5 g) was mixed with the desired amounts of sodium acetate buffer (pH 4.8) with a solid-to-liquid ratio of 1:20 (g/mL) and cellulase (35 FPU/g substrate). Then the mixture was incubated at 50 ºC in a rotary shaker with a rotational velocity of 150 rpm for 48 h. Next, the mixture was centrifuged and the residue was washed thoroughly with sodium acetate buffer (pH 4.8) to remove the hydrolyzed carbohydrates, and then freeze-dried. Finally, the dried residual solid was repeatedly subjected to ball-milling for 2 h and enzymatic hydrolysis again as above-mentioned processes. After washing with acidic water (pH 2.0) and freeze-drying, DEL sample was obtained. To increase the solubility of the lignin in tetrahydrofuran (THF) for the molecular weights determination by GPC technique, the acetylation of lignin was performed.

1.2 Characterization of the lignin fractions

The weight-average (M_w) and number-average (M_n) molecular weights of the lignin samples were determined by gel permeation chromatography (GPC) (Agilent 1200, Agilent Technologies, USA) with an ultraviolet detector (UV) at 240 nm. The column used was a PL-gel 10 mm mixed-B 7.5 mm i.d. column, which was calibrated with PL polystyrene standards according to a previous report (Shen et al., 2016a). NMR spectra of lignin samples were recorded on a Bruker AVIII 400 MHz spectrometer at 25 ºC in DMSO-*d*6. The quantitative 2D-HSQC experiments were conducted according to previous literatures (Crestini & Argyropoulos, 1997; Wen et al., 2015). Quantitative ^{31}P

NMR spectra were acquired according to previous literatures (Crestini & Argyropoulos, 1997; Pu et al.,2011; Wen et al., 2015). In detail, 20 mg of lignin was dissolved in 500 μL of anhydrous pyridine and deuterated chloroform (1.6:1, v/v) under stirring. This was followed by the addition 100 μL of cyclohexanol (10.85 mg/mL in anhydrous pyridine and deuterated chloroform 1.6:1, v/v) as an internal standard and 100 μL of chromium(III) acetylacetonate solution (5 mg/mL in anhydrous pyridine and deuterated chloroform 1.6:1, v/v) as the relaxation reagent. After that, the mixture was reacted with 100 μL of phosphitylating reagent (2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholate, TMDP) for about 10 min and was transferred into a 5 mm NMR tube for subsequent ^{31}P NMR analysis. FT-IR spectra of lignin preparations were obtained using a Thermo Scientific Nicolet iN10 FT-IR microscope (Thermo Nicolet Corp., Madison, WI, USA) equipped with liquid nitrogen cooled MCT detector. The SEM analysis of lignin were performed using a S-3400N II SEM operating at 10 kV acceleration voltages. All samples were coated with gold prior to acquiring images. The nanoparticle size analysis of DES lignin was determined by dynamic light scattering technique (Anton Paar Litesizer 500, Italy) using water as the dispersing medium. The Litesizer 500 has a 40 mW semiconductor red laser with a wavelength of 658 nm. A total of 1 mg of the DES lignin was added to 10 mL of water, and then placed in the cuvette for a light scattering determination of particle size.

1.3 Antioxidant activities

The antioxidant activities of lignins were estimated using 2,2-diphenyl-1-picryl-

hydrazyl (DPPH) free radical in methanol solution. Concretely, 0.64 mL of lignin solutions (0.01-1.00 mg/mL) in dioxane-water (9/1, v/v) was mixed with 2.36 mL of a 24.5 mg/L DPPH methanol solution at 25 ºC for 16 min. The concentrations of DPPH radicals at 0 and 16 min were monitored at 515 nm (λ max) using an ultraviolet/visible spectrophotometer (Tec comp, UV 2300). The inhibition percentage (IP) of the DPPH radical was calculated using the following equation. The inhibition percentage was plotted as a function of the lignin concentration. In the graph the 50% IP concentration of the lignin needed to obtain was determined and defined as IC_{50}. The radical scavenging activity of the lignin was characterized using the term of radical scavenging index (RSI), which was defined as the inverse of IC_{50}. According to the definitions, higher antioxidant activity results in a lower value of IC_{50} and higher value of RSI. Moreover, the antioxidant activities of the two typical commercial antioxidants, butylated hydroxyanisole (BHA) and 2,6-di-tert-bntyl-p-cresol (BHT), were determined using the same method.

$$IP(\%)=\frac{\text{absorbance}_{t=0min}-\text{absorbance}_{t=16min}}{\text{absorbance}_{t=0min}}\times 100\%$$

Table S1. Pretreatment performance analysis (%)

Sample	Dehemicellulose ratio	Delignification ratio
R-SA120	74.93	70.40
R-SA130	77.48	74.19
R-SA140	85.30	82.07
R-SA150	87.44	86.42
R-PA140	88.86	88.98
R-GA140	74.05	72.59
R-MA140	88.15	85.30
R-TA140	91.34	90.93

Table S2. Quantification of lignin fractions by quantitative 2D-HSQC NMR spectroscopy.

Sample	β-*O*-4[a]	β-β	β-5	PB	S/G[b]
DEL	59.86	6.71	1.51	19.67	2.75
L-SA120	35.91	7.22	4.8	21.83	1.18
L-SA130	31.88	6.21	4.44	21.51	1.24
L-SA140	18.32	4.95	3.84	19.36	1.44
L-SA150	15.87	3.84	4.71	18.23	1.49
L-PA140	18.14	5.02	3.85	18.86	1.46
L-GA140	38.17	4.89	3.90	23.63	1.35
L-MA140	17.65	7.01	4.26	15.58	1.65
L-TA140	9.53	3.57	3.14	13.77	2.20

[a] Result expressed per 100 Ar based on quantitative 2D-HSQC spectra.
[b] S/G ratio obtained by this equation: S/G ratio = $0.5\times I(S_{2,6})/I(G_2)$.

Table S3. Molecular weights of the lignin fractions

Sample	M_w	M_n	PDI
DEL	7230±60	3820±40	1.89
L-SA120	1980±20	1250±20	1.58
L-SA130	1510±20	1010±20	1.50
L-SA140	1290±20	980±10	1.32
L-SA150	1450±30	1020±20	1.42
L-PA140	1210±20	980±10	1.23
L-GA140	1570±30	1060±20	1.48
L-MA140	1240±20	970±10	1.28
L-TA140	1280±20	970±10	1.32

Table S4. The antioxidant activity of the lignin preparations, as well as that of 2,6-di-tert-bntylp-cresol (BHT).

Sample	EC_{50} (μg/mL)	RSI
L_{-SA120}	80.26±2.9	12.46
L_{-SA130}	67.52±2.1	14.81
L_{-SA140}	52.85±2.4	18.92
L_{-SA150}	49.41±1.9	20.24
L_{-PA140}	52.33±2.1	19.11
L_{-GA140}	65.27±1.8	15.32
L_{-MA140}	51.68±2.0	19.35
L_{-TA140}	46.4±2.2	21.55
BHT	505.05±1.9	1.98

Table S5. Main assignments of lignin fractions in FT-IR bands

Frequency (cm^{-1})	Assignment	Comments
3000-2840	ν (C-H)	C-H stretching vibrations in methyl and methylene of side chains
1738-1709	ν (C=O)	C=O stretching in unconjugated ketone, carbonyl and ester; C=O stretching in conjugated aldehydes and carboxylic acids absorb around and below 1700 cm^{-1}
1699-1633	ν (C=O)	C=O stretching in conjugated aldehydes and carboxylic acids absorb around and below 1700 cm^{-1}
1684	ν (C=O)	*β*-enone carbonyl stretching modes
1655-1675	ν (C=O)	C=O stretching in conjugated para-substitute aryl ketones
1593-1605	ν (Ar), ν(C=O)	Aromatic skeletal vibrations of S and G (S>G) plus C=O Stretching; S > G and G Condensed > G etherified
1510	ν (Ar)	Aromatic skeletal vibrations of S and G (G > S)
1460	ν (C-H)	Asymmetric C-H deformations in $-CH_3$ and $-CH_2$
1420	ν (Ar)	Aromatic skeletal vibrations combined with C-H in plane deform
1365-1370	ν (C-H), ν (O-H)	Aliphatic C-H stretching in CH_3 and phenolic OH
1267	ν (Ar), ν(C=O)	G ring and C=O stretching
1221-1230	ν (C=O), ν (C-O), ν (C-C)	C-C, C-O and C=O stretching (G condensed > G etherified)
1167	ν (C=O)	Typical for HGS type lignin; C=O stretching in conjugated ketone, ester groups
1124	ν (C-H)	Aromatic C-H bending in-plane (typical for S units)
1030-1035	ν (C-H)	The aromatic C-H deformation acting as a complex vibration associated with the C-O, C-C stretching and C-OH bending
966-990	ν (-HC=CH-)	-HC=CH- out of plane deformation, (trans)
853-858	ν (C-H)	C-H out of plane in position 2, 5, and 6 of G units
834	ν (C-H)	C-H out of plane in position 2 and 6 of S units, and in all positions of H units