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Structural variations of lignin macromolecule from different growth years of Triploid of *Populus tomentosa* Carr.

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ABSTRACT

To better understand the variations of structural characteristics of lignin macromolecules during different growth years of Triploid of *Populus tomentosa* Carr, a novel lignin isolation procedure based on double ball-milling and enzymatic hydrolysis (DEL) was proposed in this study. The morphological distributions of lignin in the plant cell wall of these poplar wood samples were monitored by Confocal Raman Microscopy (CRM). The ultrahigh yields (105.1%–111.2%) of DELs were significantly higher than those (24.4–31.8%) of corresponding cellulolytic enzyme lignins (CELs). DELs and CELs were elaborately characterized by HPAEC, GPC, 2D-HSQC NMR and ^{31}P NMR techniques, and NMR results showed that DEL samples possess similar structural features as compared to CEL counterparts except for the decreased S/G ratio and *p*-hydroxybenzoate (PB) as well as increased *p*-hydroxyphenyl units (H). There are no obvious differences in the structural characteristics except for high contents of PB and H units in DEL-1, as well as high S/G ratio and β-*O*-4′ linkages in DEL-5. It is believed that the DEL proposed in the present study can be used for characterizing the entire structural features of lignin macromolecules in the plant cell wall of different kinds of lignocellulosic biomass.

1. Introduction

Carbohydrates and lignin are natural macromolecules existing in biomass cell walls, in which carbohydrates mainly consist of cellulose and hemicelluloses [1]. Cellulose is a linear homogenous polysaccharide linked by (1,4)-β-glucopyranoside. By contrast, hemicelluloses are kinds of heterogeneous polysaccharides, containing diverse monosaccharides, such as arabinose, galactose, glucose, xylose, mannose, glucuronic acid, and galacturonic acid, which depend on the plant species [2]. Lignin, the third most abundant biopolymer on earth after cellulose and hemicelluloses, is composed of three units: guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H), which are linked by aryl ether and carbon–carbon linkages [3]. Besides the lignin linkages, lignin and carbohydrates (mainly hemicelluloses) are also linked by different chemical linkages (ether and ester bonds), forming three main lignin-carbohydrate complexes (LCC), such as phenyl glycoside, benzyl ether, and γ-ester [4]. In fact, the existence of the LCC linkages in the plant cell wall mostly impedes the isolation of lignin macromolecule from lignocellulosic biomass.

In general, it is very important to obtain a more representative lignin sample prior to detailed analysis of its structural features. However, a principal question in elucidating the structural characteristics of native lignin is that it cannot be isolated without any chemical and physical alteration form [5]. Traditionally, the pioneers adopt ball-milling to squash the plant tissue and then resulted in the dissociation of lignin macromolecules. The fragmented lignin is extracted with aqueous dioxane (96%) from the ball-milled wood, which is named milled wood lignin (MWL). MWL is considered to be the first advance towards isolating lignin in a relatively unaltered state [6]. Subsequently, other method used enzymatic treatment to remove the majority of carbohydrates, prior to extracting with aqueous dioxane, then a native lignin with a high yield is produced, which called cellulolytic enzyme lignin (CEL) [7]. However, the yield is still low (more than 20% based on the total lignin) and often relates to ball-milling and subsequent purification process. In fact, the lower yield of CEL is mostly ascribed to the existence of highly crystalline and stubborn cellulose, which is also embedded in a matrix of wood polysaccharides and lignin [8]. The enzymatic hydrolysis efficiency of plant cell wall can be improved to some

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degree after different dissolution or swelling process (DMSO/NMI, DMSO/LiCl) and the CEL yield can also increased correspondingly [9,10]. Recently, swollen residual enzyme lignin (SREL) was isolated with high yield and purity based on mild NaOH preswollen and enzymatic hydrolysis from most of hardwood species. However, the applicability of this isolated method for some specific plants with sensitive structures in lignin, such as *p*-hydroxybenzoate substructures (PB) from poplar wood and *p*-coumarate from grass species, still needs to be improved [11]. It was reported that the lignin-rich residue after enzymatic hydrolysis was not easily dissolved in DMSO-d_6 for direct structural characterization of lignin macromolecule by NMR technique [12]. The poor solubility was mainly due to the existence of stubborn cellulose, which restricts the dissolution of lignin-rich residue for direct characterization. In addition, because of the esterase activity of cellulose, some LCC linkages can be degraded after the cellulase treatment [13]. In fact, the residual cellulose in the lignin-rich residue can be hydrolyzed with an appropriate pretreatment based on the existing knowledge. It was reported that ball-milling process could facilitate the decrystallization of crystalline structure of cellulose and increases the accessibility of cellulose to cellulases [14]. Thus, after double ball-milling and enzymatic hydrolysis, a novel lignin isolation procedure based on double ball-milling and enzymatic hydrolysis (Double enzymatic lignin, DEL) was proposed in this study to improve the yield and structural integrity of the native lignin in the plant cell wall.

Triploid of *Populus tomentosa* Carr. is a kind of fast-growing poplar species widely planted in China, and it has various particular characteristics, such as cold resistance, drought tolerance, and pest resistance [15]. To reveal the differences in the distribution of lignin in the plant cell wall, the original distributions of lignin in different growth years (1–5 years) of Triploid of *Populus tomentosa* Carr were revealed by the Confocal Raman spectroscopy. Additionally, to better understand the structural variations of lignin macromolecule during its growth process, DEL and CEL were isolated from different growth years of Triploid of *Populus tomentosa* Carr., respectively. The yields, associated carbohydrates (lignin-carbohydrate complexes, LCC), molecular weights, structural characteristics, and functional groups of the isolated DELs were comprehensively investigated and compared with those of the corresponding CELs. It is believed that the investigation of lignin–lignin and lignin-carbohydrate linkages in different growth years of Triploid of *Populus tomentosa* Carr., will facilitate the understanding of the structural variations of lignin macromolecule in the plant cell wall during its growth process. Meanwhile, the better understanding of lignin structures in different growth years of poplar wood will also facilitate the development of a more efficient deconstruction strategy for the poplar wood.

2. Materials and methods

2.1. Materials

Poplar wood samples (Triploid of *Populus tomentosa* Carr., 1–5-year-old) were collected from Shandong, China, in March 2015, and more details related to the chemical composition of the wood are shown in Table 1. Prior to using, all wood samples were smashed and screened to obtain the section of sized between 450 and 900 μm (20–40 mesh) and then extracted with ethanol/benzene (1:2, v/v) using a soxhlet extractor, until the siphoned liquid colorless. The extractive free poplar woods were air-dried at 60 °C for 16 h. The dewaxed poplar wood powders (25 g, 1–5 years) were subjected to mill in a planetary ball mill (Fritsch GmbH, Idar-Oberstein, Germany) at a fixed frequency of 500 rpm for 5 h under N_2 (room temperature) as previously [16]. All chemicals used in this study were purchased from Sigma Chemical Co. (Beijing, China), except for cellulase. Commercial cellulase (Cellic® CTec2, 100 FPU/mL) was kindly provided from Novozymes (Beijing, China).

2.2. Characterization of lignin distribution in different growth years of poplar wood

To detect the changes in lignin distribution in different growth years of poplar wood, Confocal Raman Microscopy (CRM) was used to achieve this purpose. CRM (Horiba Jobin Yvon, Longjumeau, France) is a LabRam XploRa equipped with a confocal microscope (Olympus BX51, Tokyo, Japan) and a motorized x, y stage. The high spatial resolution of CRM was ascribed to an MPlan 100 × oil immersion microscope objective (Olympus, NA = 1.40) and a linear-polarized laser (532 nm), which possessed of a diffraction-limited spot about 1.22/NA. An air-cooled back-illuminated CCD behind the spectrograph was used to detect the Raman light. The detailed method of making up cross section was followed the pattern as previously [17]. The spectra were obtained from the secondary wall (S), compound middle lamella (CML), and cell corner middle lamella (CCML), respectively.

2.3. Preparation of DELs and CELs

The overall schematic illustration for the isolation of double enzymatic lignin (DEL) is presented in Fig. 1. The 1–5 years of ball-milled poplar wood powders (10 g) were suspended in acetate buffer (0.05 mM, 200 mL, pH = 4.8) with a loading of 5.0 mL of Cellic® CTec2 (100 FPU/mL), respectively. The reaction mixtures were incubated at 50 °C in a rotary shaker (150 rpm) for 48 h. After enzymatic hydrolysis, the mixture was centrifuged and the residual lignin was washed extensively with acetate buffer (pH = 4.8), and then freeze-dried. The dried residual lignin undergone ball-milling for 2 h and enzymatic hydrolysis once again as above-mentioned procedures. After purification and freeze-drying process, five DEL samples were obtained, named as DEL-1, DEL-2, DEL-3, DEL-4, and DEL-5, respectively. For CEL extraction, the ball-milled poplar wood was suspended in an acetate buffer solution (pH = 4.8) with 5% solid loading. Cellulase was added to the suspension at the dose of 50 FPU/g substrate, the mixture was then incubated at 50 °C for 48 h. After enzymatic hydrolysis, the solid was separated by centrifugating and washing with buffer solution and deionized water. Then the residual residue was extracted twice (24 h*2) with 96% aqueous dioxane (v/v) with a solid to liquid ratio of 1:40 (g/mL). All supernatants were collected, concentrated, regenerated in acidic water (pH = 2.0), centrifuged, and freeze-dried to obtain crude CEL fractions. The crude CEL samples were further dissolved in 90% acetic acid, and then regenerated into acidic water (pH = 2.0), after washing and freeze-drying, the purified CEL samples were obtained (labeled as CEL-1, CEL-2, CEL-3, CEL-4, and CEL-5, respectively).

2.4. Characterization of the CELs and DELs

The associated carbohydrates in these lignins were analyzed by high-performance anion exchange chromatography (HPAEC) according to a previous article [18]. In detail, the lignin samples were oven dried at 105 °C until a constant weight. A lignin sample of 5 mg was treated with 0.125 mL of 72% H_2SO_4 for 5 min at room temperature. Ultrapure water was added to the solution to dilute the H_2SO_4 into 4% and the reaction continued at 105 °C for 150 min to determine the carbohydrates in the lignin samples. A sample of liquor obtained by acid hydrolysis was diluted and filtered for further analysis. The weight average (M_w) and number-average (M_n) molecular weights of the lignin preparations were determined by gel permeation chromatography (GPC, Agilent 1200, USA) after acetylation in DMSO/NMI dissolution system (Supporting Informa-