

**Studies on phenyl glycoside-type lignin-carbohydrate complexes (LCCs)  
in *Eucalyptus globulus* wood**

**-Abstract version-**

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## General introduction

Main components of woods are cellulose, hemicelluloses and lignin. They are considered to be not only physically associated with each other (Salmen and Burgert 2008), but also chemically bonded each other to form lignin-carbohydrate complexes (LCCs) (Koshijima and Watanabe 2003; Balakshin et al 2008, 2014). LCCs are thought to prevent complete delignification in chemical pulping (Gierer and Wannström 1986; Iversen and Wannström 1986; Choi et al. 2007) and bioethanol production (Kim et al 2003; Aita et al. 2011; Zeng et al. 2014). Therefore, the direct structural analysis of LCCs, especially lignin-carbohydrate (LC) linkages, is important to establish more efficient delignification methods for biorefinery.

Many previous studies suggested that there are three major LCCs with different LC linkages, that is,  $\alpha$ -ether, ester and phenyl glycoside-type (Fengel and Wegener 1985; Lai 2001; Koshijima and Watanabe 2003; Balakshin et al. 2008; 2014). Among them, phenyl glycoside-type LC linkage is a bond between the phenolic hydroxyl group of lignin and the anomeric hydroxyl group in the reducing-end units of carbohydrates (cellulose and hemicelluloses). The biosynthesis of phenyl glycoside-type LC linkages is unclear, because it could not be explained by the mechanism of dehydrogenative polymerization of monolignol. However, the existence of phenyl glycoside-type LC linkages was indirectly suggested by the previous experimental results. For example, Yaku et al. (1976) have reported that decrease of molecular weight of a LCC fraction, which was extracted with hot water from pine wood, after enzymatic sugar hydrolysis using  $\beta$ -glucosidase. Lawoko et al. (2005a) have also reported that increase of phenolic hydroxyl groups after mild acidolysis in a LCC fraction, which was prepared from spruce wood by the fractionation method using a urea solution and a barium hydroxide solution. Recently, liquid high resolution two-dimensional (2D) nuclear magnetic resonance (NMR) techniques have been applied to the direct analysis of phenyl glycoside-type LC linkages (Balakshin et al. 2007, 2011; Yuan et al. 2011a; Wen et al. 2012, 2013; Du et al. 2014), but the assignment of NMR signals was not performed based on the NMR data with the appropriate LCC model compounds as described below. Therefore, development of a new direct analytical method of phenyl glycoside-type LCCs is needed, because the presence of phenyl glycoside-type LCCs is still speculative.

Generally, two processes are required for direct elucidation of LC linkages in plant cell walls. A first process is a fractionation method of the sample without the modification (in other words, with the retention of the native LC linkages) to isolate LCC-rich fractions, because of a small amount of the LC linkages in plant cell walls. A second process is direct analysis of LC linkages in the LCC-rich fractions prepared by the fractionation method.

As a fractionation method to isolate LCC-rich fractions, several methods have been reported based

on various techniques, such as ball-milling, solvent extraction, gel-filtration and enzymatic hydrolysis (Björkman et al. 1957; Koshijima et al. 1976, Azuma et al. 1981, Watanabe et al. 1987; Lawoko et al. 2003, 2005b, 2006; Henriksson et al. 2007; Li et al. 2011; Du et al. 2013). Our group also proposed a new fractionation method to isolate LCC-rich fractions via multi-step solvent extraction with the solvents for each main wood component (cellulose, hemicelluloses and lignin). However, there is still room for improvement in this fractionation method to isolate the fractionations more enriched with LCCs.

On the other hand, as a powerful direct analytical method for LC linkages in plant cell walls, liquid high resolution two-dimensional (2D) nuclear magnetic resonance (NMR) techniques have been recently developed. 2D NMR techniques [e.g., heteronuclear single quantum coherence (HSQC)] have provided detailed structural information of lignin and carbohydrates (Ralph et al. 1999; Rencoret et al. 2008, 2009; Kim et al. 2008, 2010, 2014; Mansfield et al. 2012) including LC linkages (Balakshin et al. 2007, 2011; Yuan et al. 2011a; Wen et al. 2012, 2013; Du et al. 2014). With regard to phenyl glycoside-type LC linkages, for example, Balakshin et al. (2011) have reported that several correlations assigned to anomeric C<sub>1</sub>-H<sub>1</sub> of phenyl glycoside-type LC linkages were observed in the HSQC NMR spectra of crude milled-wood lignin (cMWL) of pine and birch and a LCC-rich fraction (LCC-AcOH) which was prepared from cMWL by the extraction with acetic acid. Du et al. (2014) have also reported that the several correlation derived from phenyl glycoside-type LC linkages were found in HSQC NMR spectra of LCC-rich fraction by the fractionation method using a barium hydroxide solution. In these literatures, however, the correlations derived from anomeric C<sub>1</sub>-H<sub>1</sub> bonds of phenyl glycoside-type LC linkages in the HSQC spectra of wood samples were assigned based on those from anomeric C<sub>1</sub>-H<sub>1</sub> bonds of only three known LCC model compounds, that is, monolignol glucosides (coniferin, syringin and *p*-glucocoumaryl alcohol), and they were located differing from those of the known LCC model compounds. Therefore, the assignments in these literatures were speculative. A refined NMR database with various phenyl glycoside-type LCC model compounds is required for direct analysis of phenyl glycoside-type LCCs using 2D NMR techniques.

Meanwhile, *E. globulus* is one of the important fast growing trees planted in the world for pulp and paper industry (Rencoret et al. 2008; Domingues et al. 2011; Gonzalez et al. 2011; Romani et al. 2011; Myburg et al. 2014; Rico et al 2014). The chemical structures of LCCs in *E. globulus* are important for biorefinery, as described above. However, there are only a few reports concerning LCCs in *E. globulus* wood cell walls (Henriksson et al. 2007; Li et al. 2011). In particular, no reports have been focused on phenyl glycoside-type LC linkages in *E. globulus* wood cell walls.

In this thesis, the author describes development and the application of new direct analytical methods for phenyl glycoside-type LCCs in *E. globulus* wood.

## **Abstract**

### **Chapter 1**

#### **A new fractionation method for isolation of LCC-rich fractions in *Eucalyptus globulus* wood**

To obtain LCC-rich fractions our fractionation method for MWLR of *E. globulus* (Hirosawa et al. 2002, Furuno et al. 2006) was improved by further multi-step extraction and new selective xylan acetate fractionation method. Consequently, the comprehensive fractionation method to give total eleven fractions was established by the combination of the known method of cMWL and our new method of MWLR. In the fractionation method, total eleven fractions were obtained. The four fractions were found to be considered as promising LCC condensed fractions for 2D NMR analysis from the following reasons; (1) The fractions are dissolved in DMSO which is a common NMR solvent, (2) Balakshin et al. (2011) have reported the signals assigned to phenyl glycoside-type LC linkages were observed in 2D NMR spectrum of LCC-AcOH, (3) It was suggested from the combination of enzymatic treatment with glycoside hydrolase and SEC analysis that the fractions contained lignin-xylan LCCs.

Miyagawa, Y., Kamitakahara, H., Takano, T. and Nakatsubo, F. (2012) Fractionation and characterization of lignin carbohydrate complexes (LCCs) of *Eucalyptus globulus* in residues left after MWL isolation. Part I: Analyses of hemicellulose-lignin fraction (HC-L). *Holzforschung*, 66, 459-465.

Miyagawa, Y., Kamitakahara, H. and Takano, T. (2013) Fractionation and characterization of lignin-carbohydrate complexes (LCCs) of *Eucalyptus globulus* in residues left after MWL isolation. Part II: Analyses of xylan-lignin fraction (X-L). *Holzforschung*, 67, 629-642.

### **Chapter 2**

#### **Preparation of synthetic lignins containing phenyl glycoside-type LC linkages via *in vitro* lignin polymerization**

It will be published latter.

## Chapter 3

### Synthesis of new phenyl glycoside-type LCC model compounds

Monolignol  $\beta$ -D-galactosides and  $\beta$ -D-xylosides were prepared according to the method for  $\beta$ -D-glucosides (Terashima et al. 1996), and the monolignol  $\beta$ -D-mannosides were also prepared based on Mitsunobu glycosylation (Cocinero et al. 2008) to obtain fundamental NMR data for phenyl glycoside-type LCC analysis using 2D NMR techniques. In addition, dihydromonolignol  $\beta$ -D-glycosides and *p*-hydroxybenzaldehyde derivative  $\beta$ -D-glycosides were also prepared from corresponding monolignol  $\beta$ -D-glycosides and the synthetic intermediates, respectively. Consequently, 36  $\beta$ -D-glycosides with different lignin moieties (monolignol, dihydromonolignol and *p*-hydroxybenzaldehyde derivative) and sugar moieties (D-glucose, D-galactose, D-xylose and D-mannose) were obtained as phenyl glycoside type LCC model compounds. These LCC model compounds, and additional 10 LCC model compounds synthesized by our group ( $\alpha$ -hydroxyl- $\beta$ -O-4 dilignol  $\beta$ -D-glycosides and  $\alpha$ -keto- $\beta$ -O-4 dilignol  $\beta$ -D-glycosides), were subjected to HSQC NMR measurements. And the NMR database map which shows the correlations derived from  $C_{1\beta}$ - $H_{1\beta}$  of sugar moieties of the LCC model compounds was prepared. It was found that the anomeric correlations of the all  $\beta$ -D-glycosides varied and were in the range of  $\delta_C/\delta_H$  96-104/4.7-5.4 ppm. Furthermore, it was newly found that the correlations derived from  $C_{1\beta}$ - $H_{1\beta}$  bonds of guaiacyl and *p*-hydroxyphenyl  $\beta$ -D-mannosides are close to those derived from the  $C_{1\alpha}$ - $H_{1\alpha}$  bonds of 4-O-methyl- $\alpha$ -D-glucuronic acid moieties described in the literature (Teleman et al. 2002), although the NMR solvent is different.

Miyagawa, Y., Mizukami, T., Kamitakahara, H. and Takano, T. (2014) Synthesis and fundamental HSQC NMR data of monolignol  $\beta$ -glycosides, dihydromonolignol  $\beta$ -glycosides and *p*-hydroxybenzaldehyde derivative  $\beta$ -glycosides for the analysis of phenyl glycoside type lignin-carbohydrate complexes (LCCs). *Holzforschung*, 68, 747-760.

## Chapter 4

### 2D NMR analysis of the LCC-rich fractions from *E. globulus* wood

The author described application of the new LCC analytical method to *E. globulus* wood to delineate

phenyl glycoside-type LCCs in Eucalyptus wood cell walls. The fractions extracted from *E. globulus* wood samples according to our new fractionation method (Chapter 1) were subjected to 2D HSQC NMR analyses to investigate detailed chemical structures. In particular, phenyl glycoside-type LC linkages were profiled more precisely than in the previous LCC studies using our refined NMR database of various phenyl glycoside-type LCC model compounds (Chapter 3). The profiling results suggested the possibilities of several types of phenyl glycoside-type LC linkages in the fractions, although further investigation is required to prove their presence.

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