博 士 論 文 の 要 約

博士の専攻分野の名称： 博　士（農学）　　氏名　　Yan LYU

学 位 論 文 題 名

Functions of wood cell wall polysaccharides on lignification *in vitro*
（*in vitro* の木化における木材細胞壁多糖類の機能）

Wood cell walls are mainly composed of cellulose, hemicellulose, and lignin. The monomer composition of lignin differs in wood species: softwood lignin mainly consists of guaiacyl nuclei, while hardwood lignin consists of mainly guaiacyl (G) and syringyl (S) nuclei. Both lignins also comprise of a trace of *p*-hydroxyphenyl (H) nuclei. During tree growth, lignin is biosynthesized by radical coupling of monomers catalyzed by peroxidase/H_2O_2 and/or oxidase/O_2 in swollen polysaccharide matrices assembled with cellulose and hemicelluloses. However, the interaction among these components and effects of the preformed cell wall polysaccharides on lignification, such as, the morphology, substructure, and generated amount of resultant lignin, are still under discussion.

The investigations of lignin-carbohydrate complex (LCC) have suggested contradictory results of hemicelluloses effects on lignification: one reported that xylan promoted to form *β-O-4'* linkage as a predominant interunitary linkage of lignin (Giummarella et al., 2016), but another study reported that xylan facilitated the formation of condensed substructure (Du et al., 2014). To clarify these contradictory results, dehydrogenation polymer (DHP) was synthesized from CA by horseradish peroxidase (HRP) in the polysaccharide matrix containing xylan and bacterial cellulose (BC) film to mimic the lignification in cell wall, and demonstrated that xylan attributed to the increase of both DHP amount and the frequency of aryl ether linkage (Li et al., 2015). However, functions of other polysaccharides are still unknow.

HRP is the enzyme often used for DHP formation. However, HRP is not a tree enzyme, and thus should not involve lignification in tree. By contrast, a cationic cell wall-bound peroxidase (CWPO-C) discovered in poplar (*Populus alba* L.) callus culture can oxidize monolignols, both coniferyl alcohol (CA) and sinapyl alcohol (SA), and also polymeric lignin (Aoyama et al., 2002; Sasaki et al., 2004). However, it is very difficult to obtain a large quantity of CWPO-C from callus culture. Therefore, the procedure of recombinant CWPO-C (rCWPO-C) preparation was established (Shigeto et al., 2012). rCWPO-C was used for catalyzation of monolignol polymerization to elucidate the effects of cell wall polysaccharides on lignification in hardwood in this study.

A main objective of this study is to elucidate the effects of wood cell wall polysaccharides on lignification through the fabrication of artificial cell walls mimicking the process of tree cell wall formation. In addition, clarification of the interaction between the real tree peroxidase (rCWPO-C) and polysaccharides is also an important objective. The artificial cell walls were fabricated by dehydrogenative polymerizations of CA and SA with two enzymes, HRP and rCWPO-C, in polysaccharide matrices comprised of cellulose and several kinds of hemicelluloses, water-soluble fraction of beech xylan (WXY), partially acetylated WXY with a degree of substitute (DS) of 0.50 (AcXY), galactoglucomannan (GGM) isolated from *Picea jezoensis* with hot water, and xyloglucan (XG) from tamarind seeds. XG represents the hemicellulose in the primary wood cell wall of both hardwood and softwood. WXY and GGM are representative hemicelluloses in the secondary cell wall. In hardwood, xylan is partly acetylated with the DS of 0.40−0.75. Thus, AcXY was chemically synthesized as an analog of native xylan in hardwood.

1. Elucidation of polysaccharides effects on HRP-catalyzed dehydrogenative polymerization of CA in BC-based matrices

Li et al. (2015) clearly elucidated the functions of xylan on lignification by using the artificial cell wall model *in vitro*. However, the effects of other hemicelluloses on lignification

are still unclear. The objective of this chapter is to elucidate the effects of hemicelluloses (WXY, GGM and XG) on lignin formation by using the similar method report by Li et al. (2015). Firstly, artificial polysaccharide matrices were fabricated by adsorption of hemicelluloses on BC films. Afterwards, DHP formation from CA as a monolignol of both softwood and hardwood by HRP as an often-used enzyme for the polymerization was attempted in the presence of the polysaccharide matrices.

BC films were immersed into hemicellulose aqueous solutions at different concentration for adsorption. The adsorption amounts were calculated according to the concentrations of hemicellulose solutions before and after adsorption and applied to the Langmuir adsorption isotherm model. After hemicelluloses adsorption, the BC films immersed into the hemicelluloses solution at 1 mg/mL were dried and used as polysaccharide matrices for DHP formation. Dehydrogenative polymerization of CA was then attempted in the presence of matrix, which was catalyzed by HRP by an endwise method (*Zutropfverfahren*, ZT). After DHP formation, the samples were washed with 90% 1,4-dioxiane aqueous solution to remove loosely bound DHP. The samples before and after washing were subjected to the acetyl bromide and alkaline nitrobenzene oxidation to determine the content and substructure of the deposited DHP in/on the matrix. The nitrobenzene oxidation products were determined on a gas chromatograph (GC) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), which give the yields of vanillin, vanillic acid and dehydodivanilline.

The hemicelluloses adsorption amounts on BC films were in the order of XG > GGM > WXY at each concentration. For Langmuir fitting, the determination coefficients (R^2) were greater than 0.99, indicating the adsorption obey Langmuir theory. In addition, the affinity of hemicelluloses with BC films were in the order of XG > GGM > WXY. After DHP deposited on the hemicelluloses-adsorbed BC films, the presence of hemicelluloses in the artificial cell walls was confirmed by immunolabeling. Among all polysaccharide matrices, the largest amount of DHP was generated in/on the XG-adsorbed BC matrix, indicating that XG facilitated DHP formation. WXY also enhanced DHP formation amount, while GGM slightly

inhibited DHP formation. In nitrobenzene oxidation, yields of vanillin and vanillic acid give information of the frequency of aryl ether (mainly *β-O-4'*) linkage (Meshitsuka et al., 1985), and the yield of dehydrodivanillin indicates the frequency of *5–5'* bond (Katahira et al., 2001; Wang et al., 2020). As the results of nitrobenzene oxidation of DHP generated in different hemicellulose-adsorbed matrices, the DHPs formed in WXY- and GGM-adsorbed matrices showed higher frequency of aryl ether linkages and lower *5–5'* linkages than DHP formed in a BC film without hemicelluloses. By contrast, the DHP generated in the XG-adsorbed matrix showed lowest aryl ether linkage among all samples. In addition, the DHP formed in the XG-contained matrix was found to be enriched in *5–5'* interunitary linkages as one of condensed substructures. Lignin in the primary wall has been reported to be higher concentration and rich in condensed substructures than that in the secondary cell wall (Terashima et al., 2012), therefore, XG must be a key polysaccharide to control the substructure of the lignin located in the primary wall.

2. Elucidation of polysaccharides effects on rCWPO-C-catalyzed dehydrogenative polymerization of CA and SA

The enzyme, HRP, used in last chapter for DHP formation is not a tree enzyme, and thus should not involve lignification in tree. In this chapter, the effects of wood cell wall polysaccharides on lignification were investigated through rCWPO-C-catalyzed dehydrogenative polymerization of CA and SA in the polysaccharide matrices composed of CNFs and hemicelluloses. Due to the limitation of rCWPO-C amount, a quartz crystal microbalance with dissipation (QCM-D) was adopted to monitor the reaction. Native hardwood xylan is partially acetylated (Pawar et al., 2013; Teleman et al., 2000; A. Teleman et al., 2002). The WXY used in the last chapter was not acetylated and the DHP formation in the presence of such acetylated xylan was not reported. Therefore, expect for the hemicelluloses used in chapter 2 (WXY, GGM and XG), partially acetylated WXY (AcXY) was prepared and used for DHP formation in this chapter. The same experiment was performed by using HRP as

a reference enzyme.

AcXY with a DS of 0.50 was synthesized from WXY by full acetylation and deacetylation. Firstly, hardwood cellulose nanofibers (CNFs) were anchored on the QCM-D sensor surface by spin-coating. Afterwards, hemicelluloses and enzymes (HRP or rCWPO-C) were successively deposited on the sensor to prepare hemicelluloses-coated artificial polysaccharide matrices. Finally, CA and SA buffered solutions with H_2O_2 were separately flowed into the polysaccharide-coated sensors to generate DHP. These deposition processes mimicked the cell wall formation process in tree. In addition, the monolignol buffered solution without H_2O_2 were flowed to the CNFs-coated sensor surface to clarify the monolignol adsorption. The DHP deposited sensor surfaces were dried under a N_2 flow and subjected to an atomic force microscope (AFM) observation.

The hemicelluloses adsorption amount on CNFs surfaces was in the order of XG > GGM > WXY > AcXY. The order of XG, GGM and WXY was consistent with that obtained from the equilibrium adsorption experiment using BC, indicating that CNFs behaves similarly to BC at least in the interaction with the hemicelluloses. Among all polysaccharides-coated sensors, the largest amount of rCWPO-C adsorbed on AcXY, whereas HRP adsorbed on CNFs in the largest amount. Therefore, HRP showed the highest affinity with CNFs, whereas rCWPO-C showed the highest affinity with AcXY among all polysaccharides. It is concluded that the high affinity of rCWPO-C on AcXY is attributed to their strong hydrophobic interaction, because rCWPO-C is more hydrophobic than HRP (Aoyama et al., 2002) and the acetylated xylan is more hydrophobic than cellulose (Busse-Wicher et al., 2014).

During monolignol solutions flowed into the enzyme adsorbed surface, DHP formed from CA catalyzed by adsorbed HRP and rCWPO-C and from SA by adsorbed rCWPO-C. There are no DHP formation from SA by HRP. In addition, the amount of DHP formed from CA by rCWPO-C was much more than by HRP. For rCWPO-C adsorbed sensor surfaces, the mount of formed DHP from SA was much larger than from CA due to the high activity of rCWPO-C to SA. Among all polysaccharides, the amount of DHP formed from both CA and

SA by rCWPO-C and from CA by HRP on the AcXY-adsorbed sensor were much larger than other polysaccharides coated surfaces. As shown in the AFM images, there are a small amount of DHP particles formed from CA by HRP. For DHP formed from CA by rCWPO-C, a larger number of DHP particles formed and adsorbed along the CNFs fibers. After DHP formation form SA by rCWPO-C, AFM image shows many particles with a larger diameter than DHP from CA and the particles completely covered the CNFs fibers. Furthermore, the size of DHP particles formed on AcXY coated surface were much larger than on other polysaccharide coated surfaces, indicating that AcXY facilitated to grow DHP particles. These results obviously revealed the significance of the acetylated xylan for lignification in hardwood. In addition, WXY did not facilitated DHP formation, from SA in particular, thus, the acetyl group of AcXY should be a key functional group to facilitate lignin formation in hardwood.

In conclusion, this study elucidated the significance of acetyl group in native glucuronoxylan for hardwood lignification by monitoring dehydrogenative polymerization of monolignols catalyzed by a hardwood-derived peroxidase, rCWPO-C, in artificial polysaccharide matrices.

3. Conclusions

The existences and types of polysaccharides in plant cell wall are well known, but the functions of them have not been directly proved so far. In this thesis, the influence of five kinds cell wall polysaccharides, cellulose, WXY, AcXY, GGM, and XG, on lignification *in vitro* was demonstrated. In particular, XG facilitated much amount of DHP formation and *5−5'* linkage formation. Since characteristics of lignin in primary cell wall is higher concentration and high frequency of condensed substructure than those in the secondary cell wall, my result suggests that XG is a key compound to control lignification in the primary cell wall. In addition, AcXY promoted DHP formation, whereas WXY did not, indicating that the acetyl group in xylan must regulate lignification in the secondary wall of hardwood. I convinced that these findings will contribute to understanding the functions of hemicelluloses

on wood lignification.

4. Reference

Aoyama W., Sasaki S., Matsumura S., Mitsunaga T., Hirai H., Tsutsumi Y., Nishida T. (2002). Sinapyl alcohol-specific peroxidase isoenzyme catalyzes the formation of the dehydrogenative polymer from sinapyl alcohol. *Journal of Wood Science*. 48(6), 497-504.

Busse-Wicher M., Gomes T. C., Tryfona T., Nikolovski N., Stott K., Grantham N. J., Bolam D. N., Skaf M. S., Dupree P. (2014). The pattern of xylan acetylation suggests xylan may interact with cellulose microfibrils as a twofold helical screw in the secondary plant cell wall of *Arabidopsis thaliana*. *the plant journal*. 79(3), 492-506.

Du X., Perez-Boada M., Fernandez C., Rencoret J., del Rio J. C., Jimenez-Barbero J., Li J., Gutierrez A., Martinez A. T. (2014). Analysis of lignin-carbohydrate and lignin-lignin linkages after hydrolase treatment of xylan-lignin, glucomannan-lignin and glucan-lignin complexes from spruce wood. *Planta*. 239(5), 1079-1090.

Giummarella N., Zhang L., Henriksson G., Lawoko M. (2016). Structural features of mildly fractionated lignin carbohydrate complexes (LCC) from spruce. *RSC Advances*. 6(48), 42120-42131.

Katahira R., Nakatsubo F. (2001). Determination of nitrobenzene oxidation products by GC and1H-NMR spectroscopy using 5-iodovanillin as a new internal standard. *Journal of Wood Science*. 47, 378-382.

Li Q., Koda K., Yoshinaga A., Takabe K., Shimomura M., Hirai Y., Tamai Y., Uraki Y. (2015). Dehydrogenative polymerization of coniferyl alcohol in artificial polysaccharides matrices: effects of xylan on the polymerization. *Journal of Agricultural and Food Chemistry*. 63(18), 4613-4620.

Meshitsuka G., Nakano J. (1985). Structural characteristics of compound middle lamella lignin. *Journal of Wood Chemistry and Technology*. 5(3), 391-404.

Sasaki S., Nishida T., Tsutsumi Y., Kondo R. (2004). Lignin dehydrogenative polymerization mechanism: a poplar cell wall peroxidase directly oxidizes polymer lignin and produces in vitro dehydrogenative polymer rich in β-*O*-4 linkage. *FEBS Letters*. 562(1-3), 197-201.

Shigeto J., Itoh Y., Tsutsumi Y., Kondo R. (2012). Identification of Tyr74 and Tyr177 as substrate oxidation sites in cationic cell wall-bound peroxidase from *Populus alba* L. *the FEBS Journal*. 279(2), 348-357.

Terashima N., Yoshida M., Hafrén J., Fukushima K., Westermark U. (2012). Proposed supramolecular structure of lignin in softwood tracheid compound middle lamella regions. *Holzforschung*. 66(8), 907-915.

Wang L., Shigetomi K., Koda K., Gele A., Uraki Y. (2020). A branched structure provides kraft lignins a denser morphology and a high molar mass for a given hydrodynamic radius. *Holzforschung*. 74(6), 551-558.