Protein amino acid residues and a new monolignol conjugate in lignins and their interference with p-hydroxyphenyl (H) unit estimation

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ABSTRACT

We elucidated the detailed structures of the residual protein peaks, phenylalanine and tyrosine, in 2D NMR spectra from corn cob and kenaf samples. Phenylalanine’s 3/5 correlation peak is superimposed on the peak from typical lignin p-hydroxyphenyl (H-unit) structures, causing an overestimation of the H units. We used a protease to remove the protein residues from the ball-milled cell walls. Additionally, we also identified a new monolignol conjugate, ML-benzoate (BA), in the cell wall samples of leaf tissues from Canary Island date palm (Phoenix canariensis) and also a small amount from macaúba (Acrocomia aculeata (Jacq.) Lodd. ex Mart.) endocarp (and a trace in the stem) along with stilbenes, also causing an overestimation of the H units.

Keywords: 2D NMR, H-lignin, Protein, Klason lignin, benzoates

INTRODUCTION

Plant cell walls are complex systems made of a number of components, such as cellulose, hemicelluloses, lignin, structural proteins, enzymes, suberin, waxes, water, and other extraneous components, depending on the species and tissue. One of the most important analytical methods developed for the analysis of lignin and biomass structural characterization over the last several years is the 2D gel-NMR method for whole cell wall profiling and analysis (Kim et al., 2008, 2010). Regarding lignin aromatic compositions, the H-units generated from p-coumaryl alcohol are typically only present at low levels in many plant species (Ralph et al. 2006). As studies relating to H-units are common in transgenic and mutant plant studies, it is important to accurately measure and identify authentic H-units (Ralph et al., 2006; Vanholme et al., 2013). We found new
unknown peaks around the typical H-unit at $\delta_{c}/\delta_{n} 127.3/7.2$ (2 & 6), which is the correlation peak used for the H-unit quantification, in 2D HSQC NMR data of several plant samples (Kim et al., 2008). In this study, we revealed the specific structural information on the newly found aromatic peaks in 2D HSQC NMR data from corn cob, kenaf bast fiber, Canary Island date palm, and macaúba endocarp.

**EXPERIMENTAL**

We examined whole cell walls and lignins primarily by gel-state 2D NMR method (Kim et al., 2008, 2010). The NMR spectra were acquired on a Bruker Biospin (Billerica, MA) Avance 700 MHz spectrometer equipped with a 5 mm QCI $^{1}H/^{31}P/^{13}C/^{15}N$ cryoprobe. Corn cob was mainly used in 2008, 2010. The NMR spectra were acquired on a Bruker Biospin (Billerica, MA) Avance 700 MHz spectrometer equipped with a 5 mm QCI $^{1}H/^{31}P/^{13}C/^{15}N$ cryoprobe. Corn cob was mainly used in this research. Examining the model compounds for the possible structures was crucial to revealing the unknown structures. To confirm the structural and compositional identification results, we used common analytical assays for lignin such as the DFRC method and nitrobenzene oxidation (NBO).

**RESULTS AND DISCUSSION**

Identification of aromatic protein residues and structural elucidation of amino acid residues

**Figure 1.** Lignin aromatic regions of 2D HSQC NMR spectra (DMSO-$d_{6}$:pyridine-$d_{5}$, 4:1, v/v) of ball-milled whole cell walls from corn leaf A, corn cob B.
The aromatic amino acid residues: tyrosine, phenylalanine, and tryptophan, are commonly found in the protein as well (Laidlaw et al., 1965). Those unique peaks are actually a combination of the two different amino acid residues (Figure 1). The three peaks at δ_C/δ_H 129.1/7.2, 127.9/7.2, and 126.1/7.2 ppm belong to phenylalanine residue: 2 & 6; 3 & 5; and 4 respectively, and the other peak at the δ_C/δ_H 129.9/7.1 ppm belongs to tyrosine residue (2 & 6). Another peak from tyrosine (3 & 5) at δ_C/δ_H 115.0/6.7 ppm is superimposed on a massive peak from normal G-units (5 & 6).

Identification of lignin-bound benzoate

We also discovered a new monolignol conjugate, ML-benzoate (BA), in the cell wall samples of macaúba (Acrocomia aculeata (Jacq.) Lodd. ex Mart.) endocarp (and a trace in the stem) (Figure 2) (del Rio et al., 2017) and leaf tissues from Canary Island date palm (Phoenix canariensis) (Figure 3) (Karlen et al., 2017). These NMR peaks have been observed first time. The presence of lignin-bound benzoates BA in the lignins is very distinguishable. However, the benzoate’s 3/5 correlation peak is superimposed on the peak from typical lignin p-hydroxyphenyl (H-unit) structures, also causing an overestimation of the H units.

**Figure 2.** Aromatic regions of 2D HSQC NMR spectra (DMSO-d_6-pyridine-d_5, 4:1, v/v) of milled wood lignin (MWL) from carnauba A, macaúba B, coconut C.

**CONCLUSIONS**

Through this study we have identified and characterized aromatic amino acid residues and benzoate, which are considered to be newly found unknown components in 2D NMR data of plant cell walls. The evidence from this study leads us to firmly conclude that the H-units and lignin contents of such plants have been overestimated in huge numbers of previous studies.
ACKNOWLEDGMENTS

This work was supported by grants from the DOE Great Lakes Bioenergy Research Center (DOE BER Office of Science DE-FC02-07ER64494), and Spanish projects CTQ2014-60764-JIN and AGL2017-83036-R (financed by Agencia Estatal de Investigación, AEI, and Fondo Europeo de Desarrollo Regional, FEDER).

REFERENCES


