The DFRC Method for Lignin Analysis: Detection and Determination of Acetylated Units in Lignins
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Introduction

Some lignins have long been known to be naturally acylated by various acids, although the biochemistry and even the full role of such acylation remains unresolved. All grasses (both C₃- and C₄-grasses) are p-coumaroylated; some hardwood lignins, notably willow and aspen, and some grass lignins, notably bamboo, are p-hydroxybenzoylated; and acetates have been implicated in many hardwood and some dicot lignins. In the cases of forage grasses and legumes, the implications of these units on digestibility of the plant cell wall, by ruminants, has not yet been fully resolved.

The recently developed DFRC (Derivatization Followed by Reductive Cleavage) method is a degradation procedure that produces analyzable monomers and dimers by cleaving α- and β-ethers in lignins (U.S. Dairy Forage Research Center 1997, 1996). One feature of the method is that γ-ester groups on lignins remain intact. Without modification, however, any information about acetate groups is lost since the DFRC method utilizes various acetate-containing reagents in its protocol. Information on natural lignin acetates has also been scarce from NMR studies, where lignins are frequently acetylated for improved NMR properties, or are purified using acetic acid where acetylation artifacts might arise.

The need for an independent method to determine acetates on native lignin follows from our discovery of high levels of acetate on kenaf bast fiber lignins, and a general disbelief of the result by other researchers. Acetylation in lignins is important as it represents another form of lignin acylation and presumably invokes another transferase enzyme that could be targeted for biogenetic modification to possibly improve fiber degradability.

Methods

Cell wall and lignin isolations. Cell walls were isolated simply by solvent-extracting ground (2 mm, Wiley mill) lyophilized plant-stem material. The kenaf was from the bast fiber fraction only because its core contains lignin of a different composition. Solvent extraction was sequentially with water, methanol, acetone, and chloroform. Lignin isolations were from ball-milled material following standard procedures. No purification steps involving acetic acid were used, and no steps in the extractions or isolations involved any acetyl-containing reagents.

The modified DFRC (DFRC') procedure. Lignin (5 mg) was stirred overnight at room temperature with propionyl bromide in propionic acid (1:2, 3 ml). Alternatively, the solvent-extracted cell wall sample (30 mg) was stirred for 16 h. at 50 °C with 5 ml of the same reagent. The solvents and excess bromide were removed at 50 °C under a stream of nitrogen, and then at high vacuum (~50 mtorr). The product was then dissolved in 8 ml dioxane:propionic acid:water (1:1:0.1), and 100 mg powdered Zn added. After stirring for 30 min, the product was worked up as usual. Propionylation with propionic anhydride in pyridine completed the procedure. Analysis was by GC and/or GC-MS as described previously. Absolute yields were not calculated since response factors for the variously acetylated and propionylated monomers were not determined. Monomer yields from normal DFRC on these materials were approximately 1260 mmole/g for kenaf lignin, and 900 mmole/g for aspen lignin.

Results and Discussion

The modification to the basic DFRC method simply involves replacing all acetate-based reagents with their propionate analogues. It is then a simple matter to differentiate fully propionylated monomers (which come from normal unacetylated lignin units) from any γ-acetylated monomers which must come from γ-acetylated units in the native lignin, Fig. 1. Propionyl bromide in propionic acid readily derivatized and dissolved lignin samples, but was a little less efficient than the normal acetyl bromide system for whole-cell-
Figure 1. Formation and degradation pathways for acylated lignins. Monolignols 1 can be acylated by suitable activated acids (acetic, p-hydroxybenzoic, p-coumaric, and others are known in nature) via transferase enzymes to monolignol esters 2, presumably before diffusing into the cell wall. Esters 2, along with monolignols 1, can be subsequently acylated (pathway b). The normal DFRC protocol cleaves α- and β-ethers (in unacylated lignins 3) releasing, following acetylation, quantifiable monomers 5. Lignin γ-esters (such as in 4) are not cleaved during the normal DFRC protocol and the method can be used for identifying p-coumarate, p-hydroxybenzoate (and presumably other) esters (not specifically shown in this figure). A modification to the DFRC protocol, substituting all acetate-based reagents with their propionate analogues (DFRC’ protocol), allows determination of acetates that are naturally on lignin γ-positions. Thus, normal lignin β-ether units can release the 4,γ-dipropoxy monomers 7, whereas units originally bearing γ-acetates will release 4-propoxy-γ-acetoxy monomers 6. Releasable units originally bearing acetates can then be readily distinguished from normal (unacetylated) units by GC.

wall samples; some finely divided insoluble material remained. Use of fresh propionyl bromide was crucial: reagent from an old bottle of unknown purity and composition did not dissolve even isolated lignin samples.

The GC spectra are easy to interpret, particularly when aided by MS. Fig. 2a shows the monomeric products 6, 7 that resulted from the modified DFRC procedure applied to isolated lignin from kenaf bast fiber; this lignin was never subjected to acetyl reagents in any form. Compounds 7 arise from normal (γ-OH) units in lignins whereas compounds 6 (γ-OAc) arise from originally γ-acetylated units in lignins 4 (R = CH₃). The predominant 4-propoxy-sinapyl-γ-acetate peak S-6 proves that syringyl units were highly acetylated in the isolated lignin. The same procedure applied to whole cell walls from kenaf bast fiber produced the chromatogram in Fig. 1b. This sample had been extracted only with toluene:ethanol to remove extractives. Obviously, the unisolated cell wall lignin also had syringyl units which were heavily acetylated. In both cases, only minor amounts of acetate were on guaiacyl units, G-6. Although the guaiacyl component of kenaf lignins is strikingly low, the preference for acetylation of syringyl components suggests a specific enzymatic process (although chemical acetylation may also be selective). Presumably this is the acetylation of sinapyl alcohol S-1 via a transferase and activated (e.g. S–CoA) acetic acid.

The modified DFRC procedure also allowed detection of minor acetate components in hardwood lignins.
Thus, for example, the chromatogram from isolated aspen lignin also showed a small 4-propoxy-sinapyl-\(\gamma\)-acetate S-6 peak; the labeled peak actually contains two components so the compound really is quite minor but definitely present as confirmed by MS. The analogous guaiacyl G-6 peak could not be detected.

The methods applied here do not exclude acetylation at other positions in hardwoods, but apparently, as in kenaf, a similarly selective transferase enzyme exists in aspen for acetylating sinapyl alcohol prior to its export to the wall for lignification.

**Conclusions**

Modification of the DFRC protocol by use of propionate analogs of normal reagents and solvents allows acetates in lignins to be unambiguously detected and confirms their presence at the \(\gamma\)-positions of lignin side-chains. Kenaf bast fiber lignin is naturally highly acetylated as originally reported, overwhelmingly on syringyl units; releasable sinapyl monomers were over 50% acetylated in Tainung kenaf (reported here) and in 3 other varieties. Supporting literature observations, selected hardwood lignins were only slightly \(\gamma\) acetylated, again predominantly on syringyl units. The results suggest relatively specific acetylation of sinapyl alcohol prior to lignification. The modified DFRC protocol is a powerful new method for analyzing naturally acetylated lignins and pinpointing acetylation sites. It will allow researchers to screen hardwoods and other plant materials for the presence of acetates on lignins, although it is recommended that GC-MS (or LC-MS) be used for product authentication. The propionyl modification described provides a useful complement to the acetyl DFRC method and other lignin analytical methods.

**References**
