On the Structure of Lignin from Soybean Cell Suspension Cultures

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Treatment of soybean cell cultures according to the Björkman-procedure yields a "milled wood lignin" (MWL), which has been shown to be a hardwood lignin by thermofractography and carbon-13-NMR spectroscopy. The ^{13}C-NMR spectrum of this acetylated lignin indicates that this lignin contains relatively high amounts of cinnamyl alcohol and *p*-hydroxyphenyl groups compared with other typical hardwood lignins. The ^{13}C-spectrum of the acetylated MWL also indicates the presence of carbohydrate components, which are apparently very tightly bound to the lignin.

A number of enzymes involved in the biosynthesis of lignin precursors have recently been isolated from soybean *(Glycine max)* cell suspension cultures and some of their properties have been studied [1–4]. The insoluble residue of the cell cultures obtained by a modified Klason procedure [5] was subjected to nitrobenzene oxidation [6]. Subsequent separation of the oxidation products on silica gel plates with benzene-acetic acid (8 : 2) yielded two substances which gave a yellow color with 2,4-dinitrophenylhydrazine and had the same R_F-values as *p*-hydroxybenzaldehyde and vanillin [7]. The insoluble residue could therefore be described as lignin-like material. In view of the above mentioned work on the enzymology of lignin biosynthesis in the soybean cell cultures it was, however, necessary to obtain more precise information on the structure of the lignin formed by the cell cultures. Since ^{13}C-NMR-spectroscopy has been shown to be a valuable method for the classification of lignins of different origin [8, 9] we have now applied this method for the characterization of the lignin from soybean cell cultures. Evidence is presented that this lignin is similar to hardwood lignin.

Materials and Methods

Cell cultures

Soybean cells *(Glycine max)* were propagated at 27 °C in a fermenter containing modified B5 medium as described previously [4]. Cells used for lignin preparation were of different age.

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Preparation of acetylated lignin

The washed cells were extracted twice for 6 h with water in a boiling water bath. The dried residue was then extracted under reflux with ethanol-benzene (1:2, v/v) for 24 h. The dried material was ground in a vibratory ball mill under toluene and nitrogen for 48 hours and then extracted with dioxane/water (9:1, v/v), according to the Björkman procedure [10]. Most of the lignin carbohydrate complexes were precipitated from the solution of the crude material in dioxane/water (9:1, v/v) with benzene [11] and the clear solution was freeze-dried to give a pale yellow milled wood lignin (MWL), which was then acetylated with pyridine/acetic anhydride.

Thermofractography

About 2 mg of the acetylated MWL were heated in the pyrolysator tube of a TASOMAT (Desaga, Heidelberg, Germany) as described earlier [12]. The thin layer plate was developed twice with a chloroform/ethylacetate mixture (95:5, v/v) and sprayed either with fast-blue-salt reagent or anisaldehyde-sulfuric acid reagent [12]. Identification of the colored spots was achieved by comparison with authentic substances.

^{13}C-NMR spectroscopy

The ^{13}C-spectrum was taken of an approximately 15% (w/w) solution of the MWL-acetate in hexadeuteroacetone, after some undissolved particles had been filtered off. A Varian XL-100-15 spec-

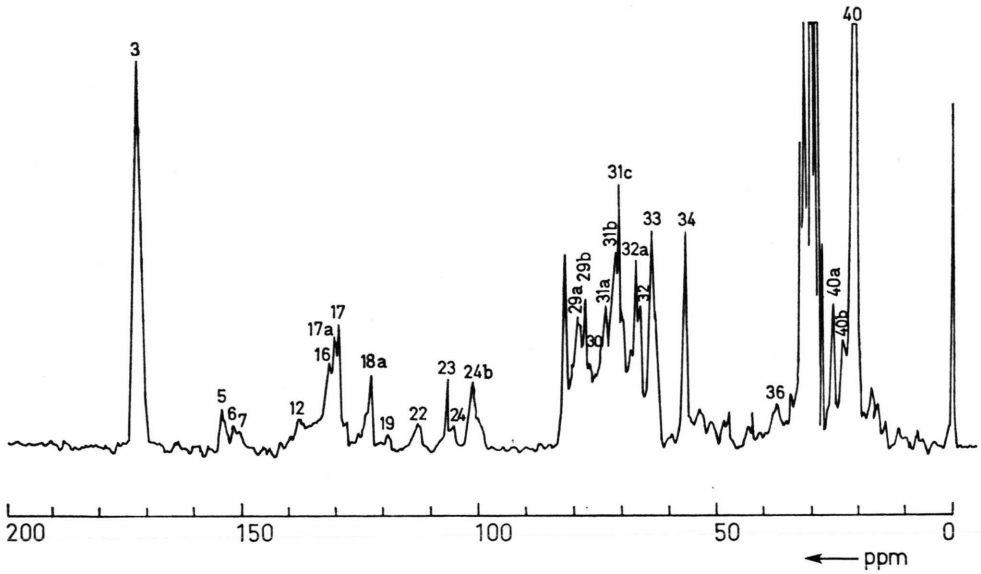
trometer was used, operating at 25.2 MHz by the Fourier-transform-technique. 100,000 transients were accumulated at a pulse delay time of 0.5 sec and 0.1 sec acquisition time. The spectrometer was locked to the $-CD_3$ groups of the acetone. TMS was added to the solution as an internal standard.

Results and Discussions

Besides 4-vinyl-guaiacol, guaiacol, vanillin, coniferaldehyde and coniferyl alcohol, which are typical for all lignins, the thermofractogrammes of the acetylated MWL from soybean indicate 4-vinyl-2,6-dimethoxy-phenol, 2,6-dimethoxy-phenol, syringaldehyde, sinapaldehyde and sinaply alcohol. The latter compounds are obtained from hardwood lignins in visible amounts only. Therefore, the lignin from soybean cultures is classified by thermofractography as a hardwood lignin or, according to Gibbs [13], as a guaiacyl-syringyl lignin. The name implies that hardwood lignins are composed of both guaiacyl and syringyl C_9-units, whereas softwood lignins contain mainly guaiacyl units and only very low amounts of syringyl units, which are not detectable in thermofractography experiments.

Syringlys units are also indicated by the signals 5, 23 and 24 in the carbon-13 NMR spectrum (Fig. 1), which can be assigned according to Table I. Besides these three signals, all the other main signals in the absorption region of the aromatic and olefinic carbon atoms (104 – 154 ppm) typical for a lignin are present. The relatively low intensity of these signals can be ascribed to a lower solubility of the MWL-acetate from soybean cultures in acetone compared with other lignin acetates. However, deviations from the ^{13}C-spectra of other hardwood lignin acetates exist in the relative signal intensities. This is mainly true for the relatively high signals 17a, 17 and 18a. The last signal can be assigned to the β-carbon atom in acetylated cinnamyl alcohols (*cf.* Table I). Further proof for the presence of these groups comes from the signal 32, which is caused by the γ-carbon atom. Signals 17a and 17 can be assigned to carbon atoms α and β in nonacetylated cinnamylic alcohol groups. However, assuming that all γ-hydroxy groups are acetylated, these signals should rather indicate *p*-hydroxyphenyl groups.

In the aliphatic range of the spectrum (56 – 82 ppm) signals 29, 30 and 33 can be assigned to carbon atoms β, α, and γ in "β-arylether units", respectively, which are the main linkages in all lignins. These signals therefore can be regarded as "finger print" signals for lignin. In our case, the three signals 29, 30 and 33 provide definite evidence for the lignin-like structure of the MWL obtained from soybean cultures. Signals 32 and 34 further support this assumption. On the other hand,



Carbon-13 NMR spectrum of acetylated milled wood lignin from soybean cultures. δ-values in ppm from TMS. Solvent: hexadeuteroacetone.

Table I. Assignments of the signals in Fig. 1. δ-values in ppm from TMS.

Signal-No.	δ [ppm]	Rel. intensity	Assignements
(3)	171.9	92	CO in acetyl
(5)	153.9		C-3/5 in syringyl, etherified or acetylated
(6)	151.7		C-3 in guaiacyl, acetylated; C-4 in guaiacyl, etherified
(7)	150.1		C-3 in guaiacyl, etherified
(12)	138.0		C-1 in guaiacyl, etherified, with α-CHO or α-CH_2-R
(16)	131.3		C-1 in guaiacyl, with α-CO; C-β in cinnamaldehyde
(17a)	130.2	14	C-2/6 in *p*-hydroxyphenyl, with unsaturated sidechain; C-α in cinnamyl alcohols
(17)	129.3	18	C-2/6 in *p*-hydroxyphenyl; C-β in cinnamyl alcohols
(18a)	122.5		C-6 in guaiacyl, with α-CH_3 or α-CH_2-R; C-β in acetylated cinnamyl alcohols
(19)	119.1		C-6 in guaiacyl
(22)	112.7		C-2 in guaiacyl
(23)	106.6		C-2/6 in syringyl, acetylated or with α-carbonyl
(24)	104.1		C-2/6 in syringyl, etherified
(24b)	101.3		
(29)	81.9	45	C-β in β-aryl ethers
(29a)	79.3	27	
(29b)	77.9	32	
(30)	75.5		C-α in β-aryl ethers
(31a)	73.6	30	
(31b)	71.4	45	
(31c)	70.7	65	
(32a)	67.1	43	
(32)	66.4	30	C-γ in acetylated cinnamyl alcohols and in phenylcoumarans
(33)	63.8	51	C-γ in β-aryl ethers
(34)	56.7	51	OCH_3
(36)	37.4		
(40a)	25.5	39	
(40b)	23.3	29	
(40)	20.5	156	CH_3 in acetyl

signals 29a, 29b, 31a, 31b, 31c and 32a are absent from the spectra of other acetylated lignins. They probably indicate carbohydrate compounds that are not separated from the lignin by the Björkman-procedure. A further peak, which cannot be assigned, appears at 101.3 ppm (24b). It is also present in the spectra of some other hardwood lignins [14], but with a lower relative intensity.

From these results it can be concluded, that soybean cultures contain a hardwood lignin. The carbon-13 NMR spectrum further provides some indications that the lignin contains relatively high amounts of cinnamyl alcohol and *p*-hydroxyphenyl groups. Carbohydrates seem to be tightly bound to the lignin and cannot be wholly removed by the Björkman-procedure.

The structure of the lignin from soybean cultures is also consistent with the substrate specificity of one of the two cinnamyl alcohol dehydrogenase isoenzymes present in these cultures. It was found that this enzyme catalyses the reversible NADPH-dependent reduction of cinnamaldehyde, *p*-coumaraldehyde, coniferaldehyde and sinapaldehyde to the corresponding cinnamyl alcohols (D. Wyrambik and H. Grisebach, unpublished results).

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