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Structural Investigation of Lignins in Three Different Ferns (*Pteridophytes*)*1

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ABSTRACT

This paper examines the structural characteristics of fern lignins (deer fern (*Blechnum spicant*), sword fern (*Polystichum munitum*) and maidenhair fern (*Adiantum pedatum*)) by chemical degradation methods of thioacidolysis and nitrobenzene oxidation as well as 13C NMR. Phloroglucinol-HCl staining indicates that the lignins are specifically accumulated at the sclerenchyma cells beneath the epidermis and vascular bundles. The fern lignins consist of only guaiacyl units. Remarkably, the frequency of the -O-4 linkages is extremely low in fern lignins (only 9 to 11%). Furthermore, the presence of lignin is ambiguous in maidenhair fern, due to very rare amount of -O-4 linkage. Biphenyl (5-5) and 1,2 bis arylpropane (-1) are main condensed dimeric substructures in fern lignins over 70%. In addition, 13C NMR analysis strongly evidenced the integration of phenolics or their derivatives into the fern lignins.

Keywords : Fern lignins, Guaiacyl units, -O-4 linkage, Wienser reaction, Thioacidolysis, 13C NMR

1. INTRODUCTION

The polymerization of lignin is initiated via oxidative coupling of three phenyl propane monomers of p-coumaryl (H), coniferyl (G) and sinapyl alcohol (S), which are synthesized from amino acids (phenyl alanine and tyrosine) through complex phenylpropanoid pathway (Freudenberg, 1968). During the coupling reactions, *arlyglycerol--aryl ether* (-O-4 substructure, ca. 40 to 60%) is the most abundant coupling mode in all plant lignins (Higuchi, 1985). In general, the lignin can be divided into four categories, G-lignin (normal gymnosperm lignins), H-G-lignin (compression wood lignin), G-S-lignin (angiosperm lignins) and H-G-S-lignin (grass and annul plant lignins) (Freudenberg, 1968).

The lignin may first emerge in the ferns (*Pteridophyte)* and there is no evidence for presence of lignins in algae, mosses and fungi (Lewis and Yamamoto, 1990; Zinsmeister and Mues, 1987). In mature ferns, stems, leaves and root are individually developed and true vascular bundles are present in the stems (Ogura, 1972). Sclerenchyma cells, which are reported to have thick walls in *blechnum*, are located just beneath the epidermis and contribute to the mechanical support in ferns. It is expected that the lignins should be distributed in these cells in fern stems.

To date, little is known about the lignin structures in extant ferns. It is reported on the

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basis of the results from alkaline nitrobenzene oxidation that ferns are lignified mainly with G monomer. However, high proportion of S monomer is also identified in the lignins of some fern species (*Dennstaedtia bipinnata* and *Lygodium circinatum*) (Bohm *et al.*, 1967, Gross, 1980, Logan *et al.*, 1985).

The goals of this work are to confirm the existence of lignin in ferns and to further evaluate the monomeric composition of H, G and S units, as well as the bonding patterns of fern lignins, i.e. quantitative -O-4 linkage and C-C condensed units. For this purpose, nitrobenzene oxidation and thioacidolysis followed by desulphuration are employed. In addition, enzyme alkali lignins are purified from fern cell wall residues by mild alkaline treatment and subjected to 13C NMR measurement.

2. MATERIAL and METHODS

2.1 Sampling and lignin assay

Three different ferns (deer fern (*Blechnum spicant*), sword fern (*Polystichum munitum*) and maidenhair fern (*Adiantum pedatum*)) were collected in northeast region of Washington State in USA. After harvesting, the samples were put in ice bag immediately and stored in 20℃ before using. Cell wall residues (CWRs) were prepared from the dried stem of ferns after fine grinding and successive extraction with ethanol/toluene, ethanol and water, respectively. The lignin contents were determined by spectroscopic procedures by acetyl bromide method (Ikiyama *et al.*, 1990). Samples (4 to 5mg) were individually placed in 5ml glass vials, containing a solution of 25% (w/w) acetyl bromide in glacial acetic acid (2.5ml) and perchloric acid (70%, 0.1ml). Lignin contents were determined by using an absorptivity value for lignin of $20.09 g^{-1} \cdot litre \cdot cm^{-1}$ for the absorbance at 280nm. All data were mean values for three replicates.

2.2 Phloroglucinol-HCl staining (Wiesner reaction)

Lignin was stained with Wiesner reagents (Geiger and Fuggerer, 1979). Phloroglucinol (0.1g) was dissolved to ethanol (95%, 100ml). Hydrochloric acid (16ml) was added to the phloroglucinol solution. The fern stems were put in 70% ethanol for overnight and embedded into the paraffin after fixation with FAA (Formaldehyde-Alcohol-Acetic acid). The Wiesner reagent was dropped directly onto the mounted paraffin block after trimming (Kim *et al.*, 2001). After 30min, the color of sample turned to red and the stained sample was observed with light microscope.

2.3 Chemical degradation analyses of cell wall residues

2.3.1 Thioacidolysis

Samples (14 to 16mg) were dispersed with thioacidolysis reagent (15ml, 0.2M BF3 etherate in a 8.75:1 (v/v) dioxane:ethanethiol) in a 20ml glass tube fitted with Teflon-lined screwcap under nitrogen atmosphere. Thioacidolysis was performed by placing each sample in a heating block held at 100℃ for 4hrs. After cooling the reaction tubes in ice-water, each reaction mixture was individually poured into dichloromethane (20ml). The reaction tube was then rinsed with water and added to the reaction mixtures. After addition of internal standard (tetracosane C24, ca 0.8mg), the reaction mixtures were adjusted to pH 3 to 4 with sodium bicarbonate (0.4M). Each organic phase was extracted with dichloromethane (3 30ml); the combined organic phases were dried over anhydrous sodium sulfate and evaporated to dryness. Each thioacidolysis residue was silylated at

room temperature with N,O bis (trimethylsilyl) trifluoroacetamide (100l) in pyridine (40l) in a reaction vial (200l). The resulting derivatives were applied to a HP 6890 Series GC System equipped with a HP-5 (5% phenyl methyl siloxane, 30m 250m) column; identification was established by reference to retention times of authentic silylated standards, and by mass spectrometric analyses using the HP 5973 MS detector (EI mode, 70eV). Column condition was 160 to 250℃ at 2℃/min until the final temperature, this being held at 250℃ for a further 5min. For quantitative analyses, authentic standards (G-CHSEt-CHSEt-CH2SEt and S-CHSEt-CHSEt-CH2SEt) were used to calculate the response factors for both monomeric G and S units, respectively. All samples were analyzed in duplicate.

2.3.2 Desulphuration

The thioacidolysis products in dichloromethane were reduced in the presence of raney nickel aq. slurry (2ml) and methanol (5ml). The reaction was run at 80℃ for 4 hrs. After cooling the reaction mixtures, the pH was adjusted to 3 to 4 with diluted HCl. The whole mixtures were extracted with dichloromethane (20ml 3) and evaporated to dryness. The final dried residues were silylated and injected to GC-MS. The GC-MS condition was the same to thioacidolysis experiments.

2.3.3 Nitrobenzene oxidation

Samples (30 to 35mg) were placed in a stainless steel bombs containing 2M NaOH (4ml) and nitrobenzene (250l). The reaction was held at 170℃ for 2hrs. After cooling in ice-water, 3-ethoxy-4-hydroxybenzaldehyde was added as an internal standard. The aqueous reaction mixture was extracted with CH_2Cl_2 (3 30ml) and the resulting aqueous phases were acidified to pH 1 to 2 using 4M HCl. Each acidified solution was next extracted with CH_2Cl_2 (2 30ml), and then diethyl ether (30ml). The combined organic phases were then washed with distilled water (50ml), dried (anhydrous Na_2SO_4) and evaporated to dryness. The dried residue was silylated with N,O bis (trimethylsilyl) trifluoroacetamide (100l) in pyridine (40l). Oxidation products were analyzed using an HP 6890 Series GC System equipped with a HP-5 (5% phenyl methyl siloxane, 30m 250m) column. Products were identified by comparison of retention times with those of authentic standards, and by mass-spectrometry analysis, using a HP 5973 MS detector in the EI mode (70eV). Initial oven temperature holds for 5min at 120℃, then rises to 260℃ at 5℃/min and maintain for 5min at 260℃. Both vanillin and vanillic acid were combined for total G unit analysis and both syringaldehyde and syringic acid were combined for S units. Each sample was analyzed in duplicate.

2.4 Purification of enzyme alkali lignins

The extractive free CWR (10g) was ground very finely with vibratory ball miller and hydrolyzed with enzyme cocktails with cellulase (Sigma, 4000units) and hemicellulase (Sigma, 4000units) in sodium acetate buffer (100ml) for 72hrs at 37℃. After centrifuge the insoluble lignin rich residues were treated with 2M NaOH at 37℃ for overnight with stirring. The alkaline filtrate was separated from insoluble material by centrifuge and acidified to pH 1 to 2 with HCl. The resultant precipitate was recovered by centrifuge (enzyme alkali lignin). The enzyme alkali lignin was washed with distilled water and lyophilized.

2.5 13C NMR analysis of fern lignins (Robert, 1992)

13C NMR spectra were taken with 300 MHz Varian instrument. The enzyme alkali lignin (ca. 50mg) was dissolved in dimethylsulfoxide-d6 (1ml) and recorded at the temperature of 80℃ for overnight (30000 scans).

3. RESULTS and DISCUSSION

3.1 Lignin determination and localization

In Table 1, the analysis data of three different ferns are summarized. In fern stems of mature stage, the lignin amounts to around 24 to 26%. The results of phloroglucinol-HCl staining of sword fern stem are depicted in Figure 1. This staining method is widely used for lignin detection. The acid catalyzed condensation of phloroglucinol with coniferaldehyde units in lignin gives rise to color change to red (Pew, 1951, Clifford, 1974, Geiger and Fuggerer, 1979). Figure 1(A) is transverse section before staining, and (B) and (C) are transverse and radial section of sword fern stem after staining, respectively. In fern stem sclenchyma cells beneath the epidermis and vascular bundles

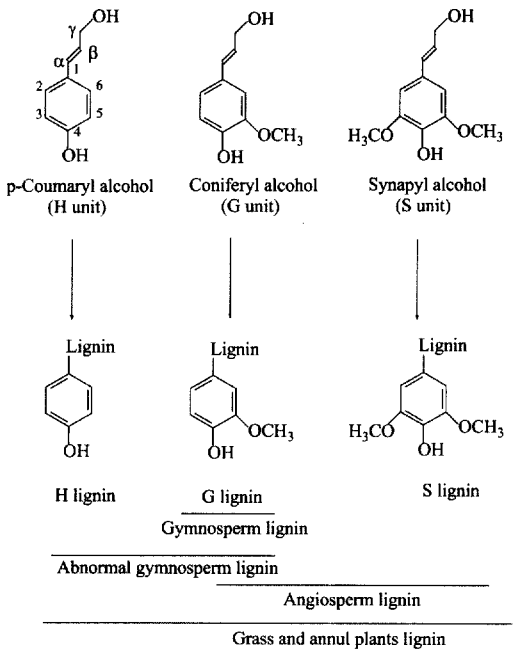


Fig. 1. Structures of three precursors of lignin macromolecules and the classification of lignins (Freundenberg 1965).

Table 1. Analytical data of three different fern CWRs by acetyl bromide, thioacidolysis and nitrobenzene oxidation.

Samples	Lignin contents**	Thioacidolysis (umol/g CWR)			Nitrobenzene oxidation (umol/g CWR)		
		G[1]	S[2]	G (%)*/g lignin	G[3]	S[4]	G (%)*/g lignin
Deer fern	24.1±0.5	114.7	0	9.13	189.6	0	12.0
Sword fern	26.1±0.5	152.1	0	1.2	254.5	0	14.6
Maidenhair fern	26.9±0.4	tr	0	1tr	9.7	0	0.55

[1]: G-CHSEt-CHSEt-CH_2SEt

[2]: S-CHSEt-CHSEt-CH_2SEt

[3]: G units is the sum of vanillin and vanillic acid

[4]: S units is the sum of syringaldehyde and syringic acid

*: G % is quantitative frequency of β-O-4 linkage, normalized by lignin contents

** : determined by acetyl bromide method

tr: detected but not quantified amount

(xylem and phloem) are obviously positive and no coloration is seen in parenchyma cells in the pith region. This result means that lignins are likely to be present in those cell walls.

1.2 Characterization of fern CWRs by chemical degradation methods

Thioacidolysis is a widely used method for quantification of alkyl-aryl ether linkages in lignin macromolecules (Lapierre *et al.*, 1986 and Rolando, 1992). The -O-4 linkage can be easily cleaved by the action of Lewis acid (BF3 etherate) and mild nucleophile (EtSH) and C6C3 trithioethylated monomers (G-CHSEt-CHSEt-CH2SEt and S-CHSEt-CHSEt-CH2SEt) are formed as main products from the degradation of uncondensed -O-4 linkage (Figure 2B). Due to stereoisomers of the C6C3 monomers, each monomer is separated very closely to twin peaks approximately with 50:50 ratios on the chromatogram in Figure 2. (Lappiere *et al.*, 1986).

The thioacidolysis results and chromatographic separation of fern CWRs are presented in Table 1 and Figure 2A, respectively. In the Fern CWRs, guaiacyl monomer is only identified and the yields are very low (Table 1). In maidenhair fern CWR, guaiacyl monomer is just detectable. Syringyl unit is not detected in three ferns CWRs by means of thioacidolysis. The thioacidolytic yields are normalized based on the lignin content determined by acetyl bromide. The G% values after normalization (Table 1) represent the essential frequency of -O-4 linkage of guaiacyl unit in fern lignins. In comparison to woody plants, in which the lignin is polymerized by -O-4 bonding type up to over 40 to 50% (Higuchi 1985), the frequency of this subunit is considerably low in fern lignins, only 9 to 11%.

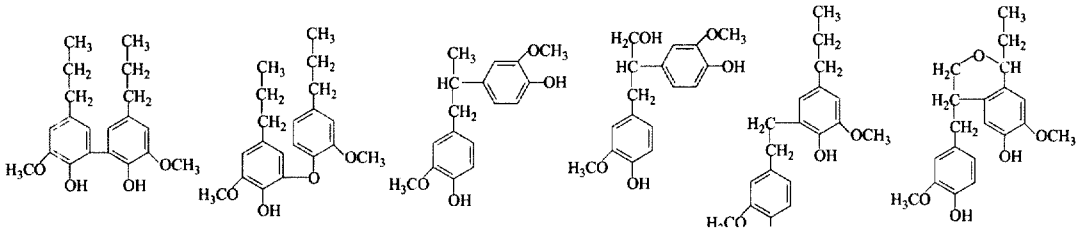
Fig. 2. Phloroglucinol-HCl staining of lignin in fern stem (sword fern). A: control, B: after staining, transverse section, C: after staining, radial section.

On nitrobenzene oxidation of lignin, the cleavage of - and -aryl ether bonds occurs and C-C bonds are cleaved. As a result, vanillin and vanillic acid are derived from guaiacyl unit and syringaldehyde and syringic acid from syringyl unit (Chen, 1992). From nitrobenzene oxidation no evidence for syringyl unit is also found in the fern CWRs (Table 1). The yields of G unit (sum of vanillin and vanillic acid) and G% values after normalization are increased in deer and sword ferns rather than those of thioacidolysis. Low amount of vanillin/vanillic acid is just quantified in maidenhair fern CWR. The increased yields may be due to critical drawback of nitrobenzene oxidation methodology. That is, the main products above described can be formed from not only lignin specifically but also non lignin phenolics (p-coumarate or ferulate) by nitrobenzene oxidation (Lapierre *et al.*, 1993).

To get detailed knowledge about condensed dimeric substructures in fern lignins, thioacidolytic products were subjected to desulphuration reaction under the presence of raney nickel and MeOH (Lapierre *et al.*, 1991a,b). Due to high molecular weights and many isomers the thioethylated dimers could not be evaluated in the thioacidolytic products by GC/MS (Lappiere *et al.*, 1991b). 6 dimers are identified in fern CWRs and the total yields, relative distribution and the structures are depicted in Table 2. In maidenhair fern CWR, the total yield of dimers is also low. As major dimeric subunits, biphenyl

Table 2. Total yields of condensed dimeric substructures and their relative distribution recovered by thioacidolysis and consecutive desuphurization of fern CWRs. (dimeric structures below from Lappiere et al. 1991)

Samples	Total yield (μmol/g CWR)	Relative distribution of dimeric subunits (%)					
		D1	D2	D3	D4	D5	D6
Deer fern	99.1	50.5	5.8	14.6	10.3	12.3	6.6
Sword fern	83.9	33.9	4.3	14.5	19.9	20.1	7.3
Maidenhair fern	22.3	59.4	0	20.7	11.2	8.7	0



(D1 (5-5)) (D2 (4-O-5)) (D3 (β-1)) (D4 (β-1)) (D5 (β-5)) (D6 (β-6))

(5-5, D1) and 1,2 bis arylpropane (-1, D3 and D4) bonding patterns are over 70% in fern lignins. In addition, 4-O-5 (D2), -5 (D5) and -6 (D6) forms are to some extents constituted in ferns lignins.

1.3 13C NMR studies of enzyme alkali lignins

Figure 3 shows the 13C NMR spectrum of enzyme alkali lignin purified from deer fern CWR by enzymatic hydrolysis and 2M NaOH treatment and the assignments of significant signals are listed in Table 3. Signals for guaiacyl nucleus are visible in the aromatic region (155 to 105ppm). Strong methoxyl signal is assigned between 55 to 58ppm. The signals of propane side chain (C, C and C) appear with the signals of residual carbohydrates between 100 to 60ppm.

The most prominent feature is the sharp signal (No. 1) in 170 to 175ppm and the signals (No. 6,7,8) at 130 125ppm. The former signal is assigned to carboxyl group and the latter arise from olefinic carbons (C and C with double bond). Tentatively, it is concluded that cinnamate (p-coumaric, ferulic or caffeic acid) or their derivatives are assumed to be attributable to those signals.

In general, phenolic acids above mentioned play a bridge-role between lignin and polysaccharides in cell walls of annual plants (Fry, 1982, Scalbert *et al.*, 1985, Lam *et al.*, 1992). That is, these phenolics are esterified to cell wall polysaccharides and etherified to lignins. From this point of view, the carboxyl groups may be released from the cleavage of ester bonds during lignin purification under mild alkaline condition. Another possible explanation

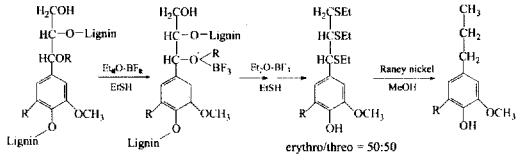


Fig. 3. Reaction of thioacidolysis and consecutive desulphuration of lignins (Rolando *et al.* 1992, R: OCH_3 or H).

Table 3. Assignments of ^{13}C NMR signals of enzyme alkali lignin purified from deer fern *(Blechnum spicant)* (Nimz et al. 1989, Robert 1992).

Signal No.	Chemical shift (ppm)	Assignments
1	175 - 170	C=O carboxyl groups
2	150 - 149	C3 in G units (etherified)
3	148 - 147	C4 in G units (etherified)
4	145 - 143	C4 in β-O-4, 5-5, β-5
5	135 - 133	C1 in G units (β-O-4)
6-7	130 - 128	Cα and Cβ in Ar-CH=CH-
8	126 - 127	Cβ in cinnamaldehyde
9	120 - 119	C6 in G units
10	116 - 115	C5 in G units
11	112 - 111	C2 in G units
12	85 - 84	Cβ in β-O-4 (threo & erythro)
13	73 - 71	Cα in β-O-4 (threo & erythro)
14	63 - 60	Cγ in β-O-4 and β-5
	55 - 58	OCH_3

is that these phenolics may be incorporated into the lignin macromolecules by means of oxidative coupling during lignin polymerization.

4. CONCLUSION

The phloroglucinol-HCl staining and acetyl bromide method provide unambiguous evidence for the presence of lignin in fern stems. Based on the data obtained from chemical degradation methods (thioacidolysis and nitrobenzene oxidation) as well as 13C NMR, the fern lignin seems to be classified into G-type lignin, like gymnosperm lignins. However, in comparison to normal lignin of woody plants, the frequency of -O-4 linkage (major bonding type of lignin) is significantly low in fern lignins (9 to 11%). In maidenhair fern case, -O-4 linkage is hardly to be detected. As condensed dimeric subunits biphenyl (5-5) and 1,2 bis-arylpropane (-1) are mainly identified in the fern lignins. 13C NMR study shows the possibility that phenolic acids

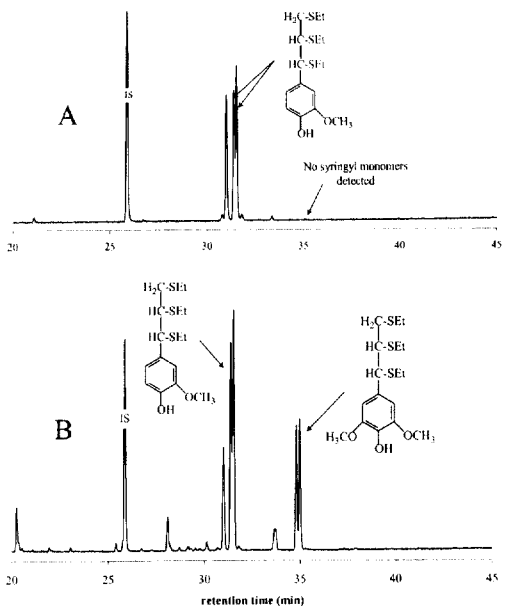


Fig. 4. Gaschromatographic separation of main thioacidolysis products (C6C3 monomers). A: deer fern CWR, and B: model plant CWR, which lignin is composed of G and S units. Due to streoisomers of C6C3 monomers two peaks are separated on the chromatogram.

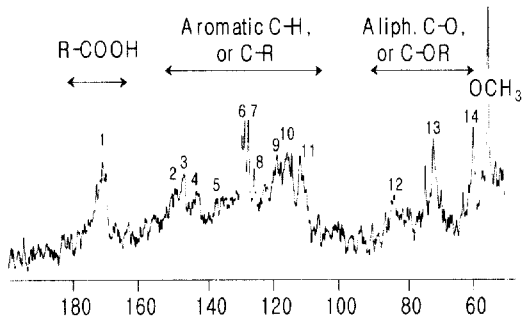


Fig. 5. ^{13}C NMR spectrum of enzyme alkali lignin purified from deer fern (*Blechnum spicant*).

or their derivatives may be involved into the fern lignins.

The ongoing work is the fractionation of lignin to several different molecular sizes by means of size exclusion chromatography with sephadex G-100 in order to observe the

influences of degree of lignification to the structures of lignin in plants.

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