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Lignin characteristics of peculiar vascular plants

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Abstract *Trochodendron aralioides* Sieb. et Zucc. and *Chloranthus glaber* (Thunb.) Makino [*Sarcandra globra* (Thunb.) Nakai] belong to primitive angiosperms, and are characterized by the absence of vessels. *Ephedra sinica* Stapf, which is classified in the gymnosperms, contains vessel elements and fiber together with tracheids. *Podocarpus macrophyllus* (Thunb. Ex Murray) D. Don var. macrophyllus is reported to give a weak positive Mäule test, although it is classified as a gymnosperm. *Podocarpus macrophyllus* gave only vanillin on alkaline nitrobenzene oxidation, while *E. sinica*, *T. aralioides*, and *C. glaber* gave both vanillin and syringaldehyde. The molar ratio of syringyl to guaiacyl nuclei (S/V ratio) of *C. glaber* was significantly low (S/V = 0.08) in comparison with the value for *T. aralioides* (S/V = 1.56). The *erythro* form is the predominant diasteromeric form of *β*-*O*-4 intermonomer linkages of *T. aralioides* and *E. sinica* [molar ratio of *erythro* to *threo* forms (E/T ratio): 1.93 and 1.67, respectively]; however, *P. macrophyllus* and *C. glaber* lignins, of which S/V ratios were 0 and 0.08, gave approximately equal amounts of *erythro* and *threo* forms (E/T ratio: 1.01 and 1.14). Results from ^{1}H NMR spectroscopy agree well with S/V ratios of alkaline nitrobenzene oxidation and E/T ratios of ozonation products. In conclusion, differences in guaiacyl lignin and guaiacyl–syringyl lignin do not exactly reflect taxonomical differences, as reported in previous articles. It was found that guaiacyl–syringyl lignin is not necessarily linked to the presence of vessels.

Key words Primitive vascular plants · Tracheids · Vessels · Lignin characteristics

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Introduction

Lignin is a substance unique to vascular plants, and it is well recognized that the evolution of lignin deposition is closely associated with vascular plant evolution.[1,2] In general, the vascular systems of primitive vascular plants such as pteridophytes and gymnosperms are composed of tracheids, whereas that of angiosperm is composed of vessels. In parallel with the differences of the vascular systems, the lignin of pteridophytes and gymnosperms gives vanillin as a major product by alkaline nitrobenzene oxidation, whereas vanillin and syringaldehyde are obtained from angiosperm lignin.[3,4] However, some species of *Selaginella* and *Gnetales*, which belong to the pteridophytes and gymnosperms, respectively, contain vessel elements, and have been found to give not only vanillin but also high yields of syringaldehyde.[3–7] Species of *Magnoliaceae*, *Winteraceae*, and *Trochodendraceae*, all of which are classified as primitive angiosperms, are characterized by the absence of vessels and the yield of syringaldehyde was relatively low in comparison with typical angiosperms.[3]

Trochodendron aralioides and *Chloranthus glaber* (Thunb.) Makino [*Sarcandra globra* (Thunb.) Nakai] (local name: senryou) belong to the primitive angiosperms, and are characterized by the absence of vessels. *Ephedra sinica*, which is classified in Ephedrales of gymnospermae, contains vessel elements and fiber together with tracheids. *Ephedra sinica* gave a positive color to the Mäule test.[3,8] *Podocarpus macrophyllus* was reported to give a very weak positive result to the Mäule test,[3,8] although it belongs to Podocarpaceae of gymnospermae.

In this study, *T. aralioides*, *C. glaber*, *E. sinica*, and *P. macrophyllus* were subjected to alkaline nitrobenzene oxidation for aromatic composition of lignin and ozonation to investigate intermonomer linkages and their diastereomeric structure of lignin of these peculiar plant species. Björkman lignins were isolated and structural characteristics were analyzed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy.

Materials and methods

Samples

Trochodendron aralioides and *Chloranthus glaber* were collected from the University Forest at Chichibu of the University of Tokyo and a private garden at Ichikawa, Chiba Prefecture, respectively. *Podocarpus macrophyllus* was harvested from the University Forest at Chiba. *Ephedra sinica* was purchased (Hyakka-en Chinese Pharmacy, Osaka, Japan). After being freeze-dried, samples were ground in a Wiley mill to pass a 420-μm sieve. The ground samples except *E. sinica* were extracted with ethanol–benzene (1:2, v/v) using a Soxhlet extractor; *E. sinica* was extracted with boiling 80% (v/v) ethanol for 1h (three times), then with hot water overnight at 40°C with shaking. The extract-free samples were further ground using a vibratory ball mill MM200 (Retsch, Germany) for 15min at a vibration rate of $30 s^{-1}$ to use in chemical analyses.

Preparation of Björkman lignin

The extract-free sample was dried in a vacuum oven overnight at 40°C, and then was finely ground with a vibratory ball mill VS-2 (Irie Shokai, Tokyo, Japan) for 72h with cooling by tap water flow. Dispersing solvent was not used during milling.[9] Björkman lignin was extracted from the finely ground sample with dioxane–water (9:1, v/v) and purified according to the procedure of Björkman.[10] The yields of purified Björkman lignins isolated from *T. aralioides, C. glaber*, *P. macrophyllus*, and *E. sinica* were 34.6%, 36.2%, 29.8%, and 19.5% (w/w), respectively.

Chemical analyses

Lignin content of the extract-free ground sample was determined according to the Klason procedure (Tappi Standard T222 om-88) with minor modifications. The sample was treated with 72% (w/w) H_2SO_4 for 3h at room temperature. The reaction mixture was diluted with water to 3% (w/w) concentration of H_2SO_4, and then digested at 121°C for 30min. After cooling, the insoluble fraction (Klason lignin) was separated by filtration using a glass filter (G4). Klason lignin was measured gravimetrically after drying overnight at 105°C. Acid-soluble lignin was calculated by measuring the UV absorption at 205nm and using a known extinction coefficient of $110 l \cdot g^{-1} \cdot cm^{-1}$.[11]

The aromatic composition of lignin of the extract-free samples was examined by an alkaline nitrobenzene oxidation.[12] The reaction products were trimethylsilylated with *N,O*-bis(trimethylsilyl)acetamide (BSA). The products were analyzed by a Shimadzu GC-17A gas chromatograph using a capillary column (NB1; 25 m × 0.25 mm i.d.) equipped with a flame ionization detector (FID). Both injector and detector temperatures were 280°C. The column temperature was kept at 150°C for 10min, and was programmed to rise at $5°C min^{-1}$ to 250°C. Ethyl vanillin was used as an internal standard.

Ozonation analysis was carried out according to the scheme of Akiyama et al.[13] for the extract-free samples. The ozonation products were trimethylsilylated with hexamethyldisilazane and trimethylchlorosilane for 30min at 60°C in dimethylsulfoxide, then analyzed by a Shimadzu GC-17A gas chromatograph using an NB1 capillary column (25 m × 0.25 mm i.d.) equipped with FID. Both injector and detector temperatures were 280°C. The column temperature was kept at 120°C for 5min, and was programmed to increase at $4°C min^{-1}$ to 170°C and then by $10°C min^{-1}$ to 280°C. Erythritol was used as an internal standard.

Spectroscopy analyses

The Björkman lignin was acetylated with acetic anhydride with a catalytic amount of pyridine overnight at room temperature with stirring. The acetylated sample was dissolved in chloroform-*d* and the ^{1}H NMR spectrum was recorded using a Jeol JNM-A 500 NMR spectrometer (Jeol, Japan).

Results and discussion

Podocarpus macrophyllus and *Ephedra sinica* belong to the gymnosperms, and *Trochodendron aralioides* and *Chloranthus glaber* are classified in the angiosperms. Lignin contents of *P. macrophyllus* and *E. sinica* were higher than those of *T. aralioides* and *C. glaber* (Table 1) and the result was in agreement with general accepted fact that gymnosperms give higher lignin content than angiosperms.[14,15] Acid-soluble lignin contents of *T. aralioides* and *C. glaber* were 2.3% and 0.9%, respectively. The acid-soluble lignin formed during Klason lignin preparation is significantly different among wood species.[14]

Podocarpus macrophyllus gave only vanillin on alkaline nitrobenzene oxidation, while *E. sinica*, *T. aralioides*, and *C. glaber* gave both vanillin and syringaldehyde (Table 1). *Podocarpus macrophyllus* was found to give a very weak positive Mäule test,[3,8] while syringaldehyde could not be detected.[3] On the other hand, *E. sinica* gave a strong positive Mäule test,[3,8] and the molar ratio of syringaldehyde to vanillin (S/V) was 1.04. The lignin content of *E. sinica* was 36.2%, while the total yield of aldehydes and acids from alkaline nitrobenzene oxidation was as low as $79.4 mmol \cdot (200 g\ lignin)^{-1}$. These results suggest that Klason residue of *E. sinica* would be expected to contain substances other than lignin, such as proteins, lipids as com-

Table 1. Klason lignin contents and yield of alkaline nitrobenzene oxidation and ozonation products

	Trochodendron aralioides	*Chloranthus glaber*	*Podocarpus macrophyllus*	*Ephedra sinica*
Lignin content[a]				
KR	26.2	25.6	34.8	31.7
ASL	2.3	0.9	0.4	4.5
Total	28.5	26.5	35.2	36.2
Alkaline nitrobenzene oxidation products[b]				
H	nd	nd	nd	5.6
HA	nd	nd	nd	2.3
V	127.3	250.1	282.6	32.8
VA	4.8	10.4	12.3	2.0
S	199.1	19.7	trace	34.3
SA	14.7	1.1	nd	2.4
Total	345.9	281.3	294.9	79.4
S/V	1.62	0.08	–	1.05
Ozonation products[b]				
E	133.8	43.3	93.5	14.8
T	69.2	37.8	92.9	8.9
Total	203.0	81.1	186.4	33.7
E/T	1.93	1.15	1.01	1.66

KR, Klason lignin; ASL, acid-soluble lignin; H, *p*-hydroxybenzaldehyde; HA, *p*-hydroxybenzoic acid; V, vanillin; VA, vanillic acid; S, syringaldehyde; SA, syringic acid; S/V, molar ratio of syringaldehyde to vanillin; E, erythronic acid; T, threonic acid; E/T, molar ratio of erythronic acid to threonic acid; nd, not detected

[a] Weight percent based on extract-free sample

[b] Units of mmol per 200 g lignin

ponents of plasma membranes, and nonlignin polyphenols, and/or lignin of *E. sinica* may be rich in condensed units. Although *C. glaber* and *T. aralioides* are characterized by the absence of vessels,[3] the S/V ratio of *C. glaber* was significantly low (S/V = 0.08) compared with the value for *T. aralioides* (S/V = 1.56), which agreed well with the result of the previous study.[16]

The vascular system of *E. sinica* is composed of vessels and fiber together with tracheids, although *E. sinica* is classified as a gymnosperm. *Trochodendron aralioides* and *C. glaber* belong to the primitive angiosperms in the families of Trochodendraceae and Chloranthaceae, respectively, and are characterized by the absence of vessels. Both species gave syringaldehyde together with vanillin by alkaline nitrobenzene oxidation.

A detailed spectral analysis of the various types of cells by UV spectroscopy revealed that the cell walls of vessels contain guaiacyl lignin and those of fibers are of guaiacyl–syringyl composition.[17,18] Yoshinaga et al.[19,20] also reported that the cell walls of vessel elements and tracheids were rich in guaiacyl lignin. On the other hand, fibers were rich in guaiacyl–syringyl lignin. Guaiacyl–syringyl lignin is found in heterophyllous species of *Selaginella*, which have no vessels.[6,7] These results show that guaiacyl–syringyl lignin is not necessarily linked to the presence of vessels.

The *β*-*O*-4 intermonomer linkage is used to define lignin structure, which can be either in the *erythro* or *threo* forms. The molar ratio of *erythro* to *threo* forms of *β*-*O*-4 intermonomer linkages (E/T ratio) is an important structural characteristic of lignin.[13] The *erythro* form is the predominant diasteromeric form of *β*-*O*-4 intermonomer linkages in angiosperm lignins, while there are approximately equal amounts of *erythro* and *threo* forms in gymnosperm lignins.[15] Ozone selectively decomposes aromatic nuclei of lignin and releases erythronic and threonic acids from *erythro* and *threo* forms of *β*-*O*-4 intermonomer linkages, respectively.[21] The E/T ratios of ozonation products of *T. aralioides* and *E. sinica* were 1.93 and 1.67, respectively. Both species were found to contain large amounts of syringyl lignin (Table 1). However, *P. macrophyllus* and *C. glaber* lignins, of which S/V ratios were 0 and 0.08, gave approximately equal amounts of *erythro* and *threo* forms of *β*-*O*-4 intermonomer linkages (1.01 and 1.14, respectively). These results support the suggestion by Akiyama et al.[15] that the E/T ratio depends on the aromatic characteristics of lignin.

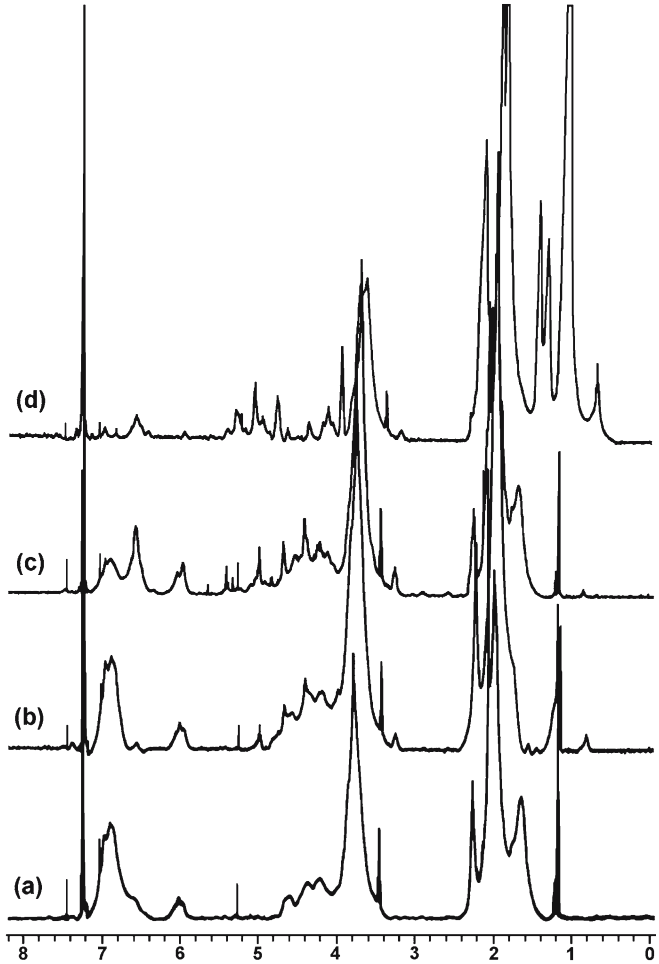

Fig. 1a–d. ^{1}H Nuclear magnetic resonance spectra of acetylated Björkman lignin prepared from **a** *Podocarpus macrophyllus* (Inumaki), **b** *Chloranthus glaber* (Senryou), **c** *Trochodendron aralioides* (Yamaguruma), and **d** *Ephedra sinica* (Maou)

In ^{1}H NMR spectra of acetylated lignins in chloroform-*d*, signals assigned to aromatic protons appeared at about δ 6.90 for gymnosperm lignin (guaiacyl protons), and at δ 6.90 and 6.65 (syringyl protons) for angiosperm lignin.[22] The acetylated Björkman lignin of *T. aralioides* exhibited two strong signals in the aromatic region at δ 6.65 and 6.90 in the ^{1}H NMR spectra (Fig. 1c), and *E. sinica* Björkman lignin showed a strong signal at δ 6.65 and a weak signal at δ 6.90 (Fig. 1d). Björkman lignins of *C. glaber* (Fig. 1b) and

P. macrophyllus (Fig. 1a) gave a very weak signal at δ 6.65. Results from ^{1}H NMR spectroscopy were in close agreement with the S/V ratios of alkaline nitrobenzene oxidation and E/T ratios of ozonation products (Table 1).

Signals at δ 6.01 and 6.06 of the ^{1}H NMR spectrum are assigned to H_α of the *erythro* and *threo* forms of arylglycerol-β-aryl ether intermonomer linkages, respectively.[23] In the spectra of acetylated Björkman lignins of *T. aralioides*, the signal at δ 6.01 was stronger than that at δ 6.06 (Fig. 1c), suggesting that the *erythro* form was the predominant form of arylglycerol-β-aryl ether intermonomer linkages. On the other hand, the signal at δ 6.01 was of near-equal intensity with the signal at δ 6.06 for *P. macrophyllus* (Fig. 1a) and *C. glaber* (Fig. 1b) Björkman lignin. The conclusions drawn from the ^{1}H NMR spectra coincided with the results of the ozonation study. Björkman lignin from of *E. sinica* gave signals around δ 6.01, but the intensities were low. In addition, the ^{1}H NMR spectrum of acetylated *E. sinica* Björkman lignin showed strong signals around δ 1.0 to 1.4, which are probably due to methylene protons of lipids.

Conclusions

Modern analytical procedures, including alkaline nitrobenzene oxidation, ozonation, and ^{1}H NMR spectroscopy, were used to confirm the previous suggestion that the composition of guaiacyl and syringyl nuclei in lignin does not always reflect taxonomical classification. It was also concluded that guaiacyl–syringyl lignin is not necessarily linked to the presence of vessels.

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