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CHARACTERIZATION AND BIOSYNTHESIS OF MISTLETOE LIGNIN

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Key Word Index—*Viscum album*; Loranthaceae; parasitic plant; mistletoe lignin; ^{13}C-NMR; lignin biosynthesis; enzymes in cinnamate pathway; inhibitor of *O*-methyltransferase.

Abstract—Mistletoe lignin was a typical angiosperm one based on the spectral (UV, IR, ^{13}C-NMR) and functional group analyses, and on degradation products (nitrobenzene oxidation and acidolysis), the analytical results of which were compared with those of the host lignin. L-Phenylalanine-[U-^{14}C] was efficiently incorporated into mistletoe lignin. Phenylalanine ammonia-lyase and cinnamate-4-hydroxylase were detected by incubation of the tissue slices under illumination. It was also found that *O*-methyltransferase activity of the crude homogenate catalyzed the methylation of 5-hydroxyferulic but not the methylation of caffeic acid. However, the latter methylation activity could be recovered by purification. These results indicate that mistletoe lignin is synthesized independently from that of its host.

INTRODUCTION

Mistletoe (*Viscum album*) is an evergreen dicotyledon which is usually parasitic on *Celtis sinensis* and occasionally on cherry trees in Japan. Several characteristic compounds, such as physiologically active proteins [1–3], viscotoxin [4,5], acetylcholine [6], verazine [7], a cyclic peptide [8] and flavonoids [9–11], have already been isolated. On the other hand, few studies have been reported on the nutritional dependence of mistletoe on the hosts [12–14]. It is known that mistletoe usually obtains water from its host, and that some species also obtain carbon [12–14]. Freudenberg reported that European mistletoe grown on either gymnopserm or angiosperm tree gave the corresponding type of lignins [15]. However, it has been recently demonstrated that lignins of European mistletoe were of the angiosperm type and different from those of the host trees [16–18]. It has been further reported that the flavonoids in a parasitic plant are quite different from those of the host plants [9,11]. These observations may indicate that the biosynthesis of mistletoe lignin is genetically controlled by the parasitic plant itself and not by host trees. However, the direct demonstration of such nutritional independency for lignin biosynthesis is still missing. The present paper reports chemical properties of parasitic and host lignins, and the presence of enzymes of the cinnamate pathway in the parasitic plant.

RESULTS AND DISCUSSION

Characterization of mistletoe lignin

Analytical results of mistletoe lignin were compared with those of the host (*Celtis sinensis*) and the data obtained are summarized in Table 1. Lignin contents in the mistletoe and host woods determined by the Klason method were 22.1 and 21.6%, respectively. The values are in good agreement with those of typical angiosperm woods. Empirical formulae of the milled wood lignins (MWLs) which were purified by the standard method of Björkman [19], show rather high oxygen contents which may be ascribed to contaminants such as sugar in comparison with Freudenberg's results [15].

IR spectra of the mistletoe and host MWLs were almost identical. The main bands were at 1325, 1235, 1130 cm^{-1} (syringyl ring); 1275, 1145, 1030 cm^{-1} (guaiacyl ring); 1595, 1505, 1425 cm^{-1} (aromatic skeletal bands) and the bands assigned to aliphatic and aromatic bonds [20]. Comparison of the most important bands showed that the host had rather high relative intensities (1665/1505, 1325/1505, 1230/1275 and 1130/1030) except the ratio (1735/1505) which shows the amount of unconjugated ester carbonyl groups [21]. Since the relative ratio (1600:1505) is known to be increased by the presence of syringyl component, *p*-hydroxyphenyl esters, carboxylate ions in carbohydrates and condensed tannin impurities [20], the higher value observed in mistletoe MWL may be ascribed to the predominance of syringyl component which is reflected by OMe content and the ratios (1325/1505, 1230/1275, 1130/1030). The presence of conjugated ester groups, such as *p*-coumaryl esters observed in the UV spectrum of bamboo lignin [22], was negligible in the MWLs, although the ratio (1660/1505) was rather high in mistletoe MWL. The UV spectra of the MWL of mistletoe and the host showed maxima at 277.5 nm (absorptivity 15.3 l/gcm) and 278.0 nm (absorptivity 14.4 l/gcm), respectively. No absorption was observed in the region *ca* 350 nm, absorption at which is due to the presence of conjugated ester groups, and the spectra in this region showed a general absorption curve. These observations indicated that both mistletoe and host lignins are typical angiosperm types, although some variations were observed in the IR spectra.

The MWLs were degraded by acidolysis and the monomeric products analyzed to confirm the spectral analyses. The yields of acidolysis oils were 58 and 61%

for the mistletoe and host MWLs, respectively. The ratios of syringyl acetone, (vanilloyl methyl ketone and syringoyl methyl ketone) to guaiacyl acetone in the acidolysis of mistletoe and host lignins were 0.92 (0.38 and 0.18) and 0.73 (0.27 and 0.11), respectively, the values of which showed that both lignins are typically angiosperm (the corresponding values of beech wood were 0.71, 0.38 and 0.15). The following compounds were identified as TMSi-derivatives by GC–MS analysis, guaiacyl acetone, vanilloyl methyl ketone, syringyl acetone and syringoyl methyl ketone. Other peaks of shorter R_t than the guaiacyl and syringyl derivatives were observed by GLC in all cases. These peaks were very small except one, (M^+ 220) which coincided with TMSi *p*-coumaryl aldehyde but showed different fragment ions. *p*-Hydroxyphenyl derivatives cannot be ascribed to these peaks, because they were not observed in the acidolysis products of beech wood, and the MS of the *p*-hydroxyphenyl monomers [23], such as *p*-hydroxybenzaldehyde, *p*-hydroxyphenylacetone, 1-hydroxy-1-(4-hydroxyphenyl)-2-propanone and *p*-hydroxybenzoyl methyl ketone, were quite different from those of these peaks. The analytical results indicated that both mistletoe and host lignins are typical of the angiosperm type.

Recently, ^{13}C-NMR has been used in structural studies of lignins and shown to give much useful information [18,24–28]. The method was applied to mistletoe and host MWL and spectra gave ester carbonyl carbon (172.4 ppm from TMS) [25] only in the host MWL in accordance with the absorption ratio (1735:1505) by IR. The signals for guaiacyl and syringyl ring carbons were observed in both mistletoe and host MWL. The peaks at 154.3, 139.3, 135.9, 107.7 and 105.7 ppm observed in these MWL may be assigned to the absorption of syringyl ring carbons on the position 3 and 5, 1 and 4, 4, 2 and 6, 2 and 6, respectively [25,26]. No distinct peaks appeared in the region of *p*-hydroxyphenyl ring carbons (160.9, 131.3 and 117.0 ppm control: bamboo MWL). [29]. Thus, the mistletoe lignin as well as the host lignin were confirmed to be typical normal angiosperm types.

Mistletoe O-methyltransferase and lignin biosynthesis

O-methyltransferases (OMTs) involving in the biosynthesis of angiosperm lignin catalyze the formation of ferulic (FA) and sinapic acids (SA) from caffeic and 5-hydroxyferulic acids, respectively, [30] while the corresponding gymnosperm enzyme only weakly catalyzes the formation of SA [31]. The ratio of SA- to FA-activities (SA:FA) is very useful as an indicator for the ability to form syringyl lignin [32]. Thus, the phylogenic distribution of gymnosperm and angiosperm lignins is partially ascribed to the SA:FA ratio of these OMTs.

Table 1. Chemical properties of mistletoe lignin

	Viscum	*Celtis*
Lignin content	22.0%	21.6%
Empirical formulae of MWL*	$C_9H_{5.77}O_3(H_2O)_{1.44}(OMe)_{1.33}$	$C_9H_{5.44}O_2(H_2O)_{1.45}(OMe)_{1.29}$
UV λ_{max} (nm)	277.5	278.0
A (1/g cm)	15.3	14.4
IR	Typical angiosperm type	
^{13}C-NMR	Typical angiosperm type	
Nitrobenzene oxidation†	1.7	—
Acidolysis‡	0.92	0.73

* Upper: *Viscum*, lower: *Celtis*.
† The ratio, syringaldehyde:vanillin.
‡ The ratio, syringyl acetone:guaiacyl acetone.

Table 2. Purification of mistletoe *O*-methyltransferase

Purification procedure	Protein (%)	Recovery (%) FA	Recovery (%) SA	Fold FA	Fold SA	SA:FA Ratio
1. Crude juice	100.0	100	100.0	1.0	1.0	46.7
2. Centrifugation	71.8	86	92.0	1.2	1.3	50.0
3. Ion-exchange	10.8	1020	56.9	94.4	5.3	2.6
4. $(NH_4)_2SO_4$ ppt.	7.5	1060	51.2	141.0	6.9	2.3
5. Gel filtration	3.8	944	44.1	247.0	11.5	2.2

The SA:FA ratio of mistletoe homogenate was remarkably high as a result of negligible FA formation. On the basis of the biosynthesis of lignin monomers in mistletoe, the absence of FA formation contradicts the previous results which showed it to be typical of angiosperms irrespective of the host species [17,18]. The absence of FA formation may be caused by (1) the presence of a particular OMT which is unable to catalyze the FA formation (2) normal angiosperm type OMT is present but FA activity is inhibited by some causes. In the case of (1), mistletoe may have no ability to synthesize lignin itself, or be able to synthesize lignin monomers, *via* a different biosynthetic pathway from other angiosperms. In the case of (2) mistletoe may have no ability to synthesize lignin because FA activity is inhibited or that normal angiosperm type lignin biosynthesis is present but that the inhibition of FA activity is caused by some inhibitor(s) which may be liberated by cell rupture during extraction of OMT.

Mistletoe OMT was purified in order to elucidate the cause of the lack of FA activity. The SA activity of mistletoe OMT in crude homogenates showed an optimum pH *ca* 7.2, FA activity at which was not detected. However, weak FA activity was observed in the lower pH (optimum pH 5.3). FA activity was detected even at pH 7.5 after ammonium sulfate precipitation followed by dialysis of the crude extract. The purification results of the mistletoe OMT are shown in Table 2. FA activity was considerably activated by dialysis following separation on DEAE–cellulose, and the ratio SA:FA (2.6) became small as is common in angiosperms (cf. ratio of the host, 2.7).

Since the presence of specific substance(s) inhibiting FA activity suggested earlier, the DEAE–cellulose fraction after elution of the OMT was treated with 0.05 M K Pi buffer (pH 5.2) and the eluate adjusted to pH 7. This fraction inhibited not only the FA activity of mistletoe enzyme but also that of gymnosperm (*Pinus thunbergii*) and angiosperm (*Pueraria thunbergiana*) OMTs, although SA activities in these plants were gradually inhibited by ageing of the fraction. The aged fraction was able to inhibit both activities even after heat treatment (100°, 5 min) or after pronase treatment. No inhibition was observed in the inhibitor fraction after dialysis against 0.025 M K Pi buffer (pH 7.4) for 8 hr. It is interesting that β-glucosidase [33] and glucan phospholyrase [34,35] activities were also inhibited by addition of crude mistletoe juice, and the latter inhibition was suggested to be caused by some phenols [35]. The inhibition of OMT is not caused by an *o*-phenol which is a Me acceptor of *S*-adenosyl-L-methionine and competes with caffeic or 5-hydroxyferulic acids, because no methylation was found in control assay (minus substrate) in these exper-

Table 3. Incorporation of L-phenylalanine-[U-^{14}C] into mistletoe lignin

Nitrobenzene oxidation products†	Yield % of KL‡	Yield (ratio)	Specific activity cpm/μmol	Dilution value
p-Hydroxybenzaldehyde	3.0	(0.7)	570	1070
Vanillin	4.4	(1.0)	1580	386
Syringaldehyde	8.1	(1.7)	1090	560

* *Viscum album* 24.5 g (shoot 13.4 g): L-phenylalanine 6.67 μCi/24.2 μmol.
† m-Nitrobenzhydrazone derivatives.
‡ Klason lignin.

iments. Although the characterization of the inhibitor(s) is incomplete, it seems that mistletoe OMT is a normal angiosperm one and that the methylation of caffeic to ferulic acids is not blocked *in vivo*.

Biosynthesis of mistletoe lignin

The ability of mistletoe to synthesize lignin was evaluated by other enzyme reactions related to lignin biosynthesis. Phenylalanine ammonia-lyase (PAL) was not detected in the intact mistletoe plant even in early July in which the high activity was expected. Figure 1 shows the development of enzyme activities during the incubation of the sliced mistletoe tissues under illumination. PAL activity, the product of which was identified by GC–MS, was gradually activated but the SA activity of the OMT was not detected during incubation, showing that the inhibitor(s) of OMT did not affect PAL activity. In addition, it has been suggested that OMT and PAL may be under different genetic control because of the different activation pattern similar to those of parsley cell suspension culture [36].

Cinnamate-4-hydroxylase activity, which was very low in this plant, was also detected and the product formed was also identified by MS, although *p*-coumarate was not detected in control assay (minus substrate).

Overall enzyme reactions in lignin biosynthsis were examined by tracer experiment using phenylalanine-[U^{14}C]. Plants were allowed to metabolize the compound for 24 hr and then tissues were oxidized after extraction. The results shown in Table 3 indicate that mistletoe is able to synthesize both guaiacyl and syringyl nuclei, and that the ratio of the oxidation products (syringaldehyde/vanillin) is in accord with that of the products of normal angiosperm lignin. A high yield of *p*-hydroxybenzaldehyde as an oxidation products was found in the mistletoe. However, this aldehyde may not be derived from the lignin, because of its low sp act and from the lack of evidence of a *p*-hydroxyphenyl nucleus in the lignin.

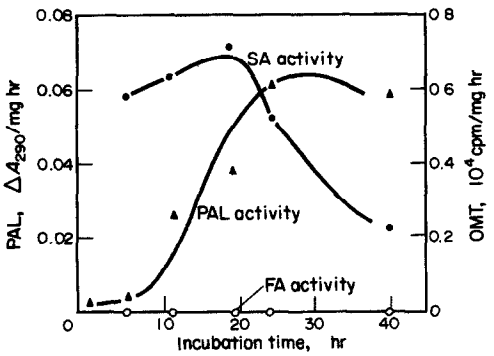

Fig. 1.

EXPERIMENTAL

Plant material. Lignified wood tissues of mistletoe (*Viscum album*) and the host (*Celtic sinensis*), which were grown in Uji city, were used for preparation of milled wood lignin. Fresh shoots of mistletoe were used for the enzyme assay and shoots after removal of leaves for the tracer experiment.

Preparation of milled wood lignin (MWL). Extractive free-wood (40 mesh) was dried over P_2O_5 *in vacuo*, and milled in toleune for 100 hr using a ball mill [19]. Milled powder was extracted with dioxane–H_2O (9:1), and MWL extracted was dissolved in HOAc–H_2O (9:1), and then the soluble portion poured into H_2O. MWL thus obtained was further purified by pouring it into Et_2O after dissolving in dichloroethane–EtOH (2:1). MWL was then collected by centrifugation and dried over P_2O_5 *in vacuo*.

Estimation of methoxyl groups of lignin. A conventional volumetric method was employed [37].

Spectral analyses. UV and IR spectra were measured in dioxane–H_2O (9:1) and in KBr discs, respectively. FT-^{13}C-NMR spectra were measured in $(CD_3)CO$–D_2O (9:1) by scanning 5.3 × 10^4 times (100 MHz).

Acidolysis of MWL. The MWL (10 mg) was dissolved in 1 ml of 0.2 N HCl–dioxane (1:9) and the soln was sealed in a glass tube under N_2. The reaction mixture was kept at 120° for 20 hr, and then poured into H_2O (15 ml) and extracted with Et_2O. The acidolysis oil thus obtained was silylated in the usual way [38] and analyzed by GC–MS, column: 3% SE-52 on chromosorb W, 2 m, temp 195°.

Extraction and purification of OMT. Young mistletoe shoots (815 g) were homogenized in the presence of Polyclar AT (15 g) and 0.25 M K Pi buffer (pH 7.4; 1540 ml) containing 1 mM EDTA, 3 mM mercaptoethanol, 3 mM NaN_3 and 5 mM Na-ascorbate at 0°. The homogenate was filtered through 3 layers of cheese-cloth and the crude juice (1640 ml) centrifuged at 17000 *g* for 30 min (supernatant 1585 ml). After dialysis of the supernatant two times against 25 mM K Pi buffer (pH 7.5) containing 3 mM mercaptoethanol (5 l.) for 10 hr, the dialyzate was treated with DEAE–cellulose (50 g) buffered previously with 25 mM K Pi buffer for 10 hr. The absorbed fraction was eluted with 25 mM K Pi buffer containing 0.25 M KCl and the eluate (1060 ml) containing 3 mM EDTA was precipitated by the addition of $(NH_4)_2SO_4$ (547 g) adjusted to pH 7.2–7.4 with 1% NH_4OH soln. The ppt. was collected by centrifugation at 5600 *g* for 30 min and dissolved in 50% satd $(NH_4)_2SO_4$ soln (100 ml). The undissolved fraction was collected in the same way and the ppt dissolved in 15 ml of 25 mM K Pi buffer (16.4 ml). The soln was applied to a Sephadex G100 column (2.7 × 100 cm, upward flow rate 10.7 ml/hr) which was equilibrated with 25 mM K Pi buffer and the active fraction was collected (146 ml). Protein content was determined by the method of ref. [39].

Tracer experiments and nitrobenzene oxidation. Phenylalanine-[U-^{14}C] (6.67 μCi, 24.2 μmol) was fed to a young shoot (23.4 g) of mistletoe and allowed to metabolize for 24 hr. After removal of leaves (12 g) the shoot was homogenized with hot 80% EtOH and the residue extracted with EtOH–C_6H_6 (1:2) for 9 hr. The sample thus obtained was oxidized with nitrobenzene: sample (3 g) was added in a mixture of 2 N NaOH (60 ml) and nitrobenzene (4.2 ml) and kept at 170° for 2 hr in a rotating autoclave. The aldehyde fraction was extracted by a conventional method and each aldehyde was separated by preparative-TLC (solvent: H_2O saturated *iso*-propylether). Aldehyde were then converted to *m*-nitrobenzoylhydrazone derivatives (each aldehyde 15 mg was dissolved in H_2O (4 ml) and a 5% soln of the hydrazide (3 ml) was added) which were recrystallized twice from H_2O.

Enzyme assay. Sliced tissue of the mistletoe was incubated under light and a 5 g portion of the tissue was homogenized every 6 hr with 5 ml of 0.2 M Tris–HCl buffer (pH 7.5) con-

taining 3 mM mercaptoethanol, sea sand 2.5 g and Polyclar AT 0.7 g in a cold mortar. The homogenate was squeezed by cheese cloth and centrifuged at 17000 *g* for 10 min. The supernatant was used for OMT assay by the procedure previously reported [31]. R_f values of the products formed were found to be identical with those of authentic compounds by paper chromatoscanning (solvent: toluene–HOAc–H_2O, 4:1:5, organic layer). Alternatively, the supernatant was treated with Dowex 1 × 4 (1 g) for 40 min at 0°. After centrifugation (at 1500 *g* for 10 min), the supernatant thus obtained was assayed for PAL activity by a conventional method [40]. Extraction of cinnamate-4-hydroxylase was carried out in a similar fashion except that the tissue was incubated for 20 hr in 0.2 M K Pi buffer. The reaction mixture for the enzyme assay contained cinnamic acid (4.4 μmol), $NADPH_2$ (2 μmol), glucose-6-phosphate (5 μmol), KPi (150 μmol), 2-mercaptoethanol (3 μmol) and enzyme soln (0.5 g fr wt), in a total vol of 3 ml. The product which was purified twice by preparative-TLC ($CHCl_3$–HOAc–H_2O, 4:1:1) was found to be identical with that of authentic p-coumaric acid by MS.

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