Bamboo Lignin and Its Biosynthesis*

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樋口隆昌**：タケリグニンおよびその生合成

About 100 species of bamboos are distributed in Japan and 3 species of them (*Phyllostachys heterocycla var. pubescens.*, *P. reticulata* C. KOCH, and *P. nigra Munro var. Henonis* MAKINO) are quite common in central Honshu, Shikoku and Kyushu islands. These bamboos grow up 10-20 m in height (10-20 cm in diameter) and have been used for several kinds of household industrial products for a long time in Japan.

These bamboos begin shooting from subterranean stems from April to June and grow quickly up to full size within 1 month. Thus, these are very suitable plant materials to get tissues with various phases of lignification from the top to lower parts of the same plant. Bamboo shoots are also quite suitable to isolate enzymes because of negligible amounts of phenol oxidases in tissues and therefore no inactivation of enzymes with quinoid compounds produced in a browning reaction ocurring generally on homogenization of plant materials.

In our laboratory, bamboo shoots have been used for elucidation of biochemical pathways leading to lignin and of its related enzymes.

Enzymes in Shikimate Pathway

Since tracer experiments[1,2] have demonstrated an important role of the shikimate-cinnamate pathway in biosynthesis of phenylpropanoids in higher plants, many enzymes[3] involved have been isolated from various plant materials. However, investigations on the enzymes in woody plants are very few and a systematic investigation has been expected in relation to lignin biosynthesis. As shown in Fig. 1 shikimic acid, a precursor of aromatic compound, is synthesized from erythrose-4-phosphate and phosphoenolpyruvate provided through pentose phosphate pathway and glycolytic breakdown of glucose, respectively and thus importance of the pentose phosphate pathway in the biosynthesis of aromatic compounds in higher plants has been demonstrated.

Then, characterization of D-glucose-6-phosphate- and 6-phosphogluconate dehydrogenases which are situated at a divergent point of the pentose phosphate pathway and the glycolysis in sugar metabolism in bamboo shoots was carried out.[4] As shown in Fig. 2 both enzymes were demonstrated to be NADP-specific and activities in moderate

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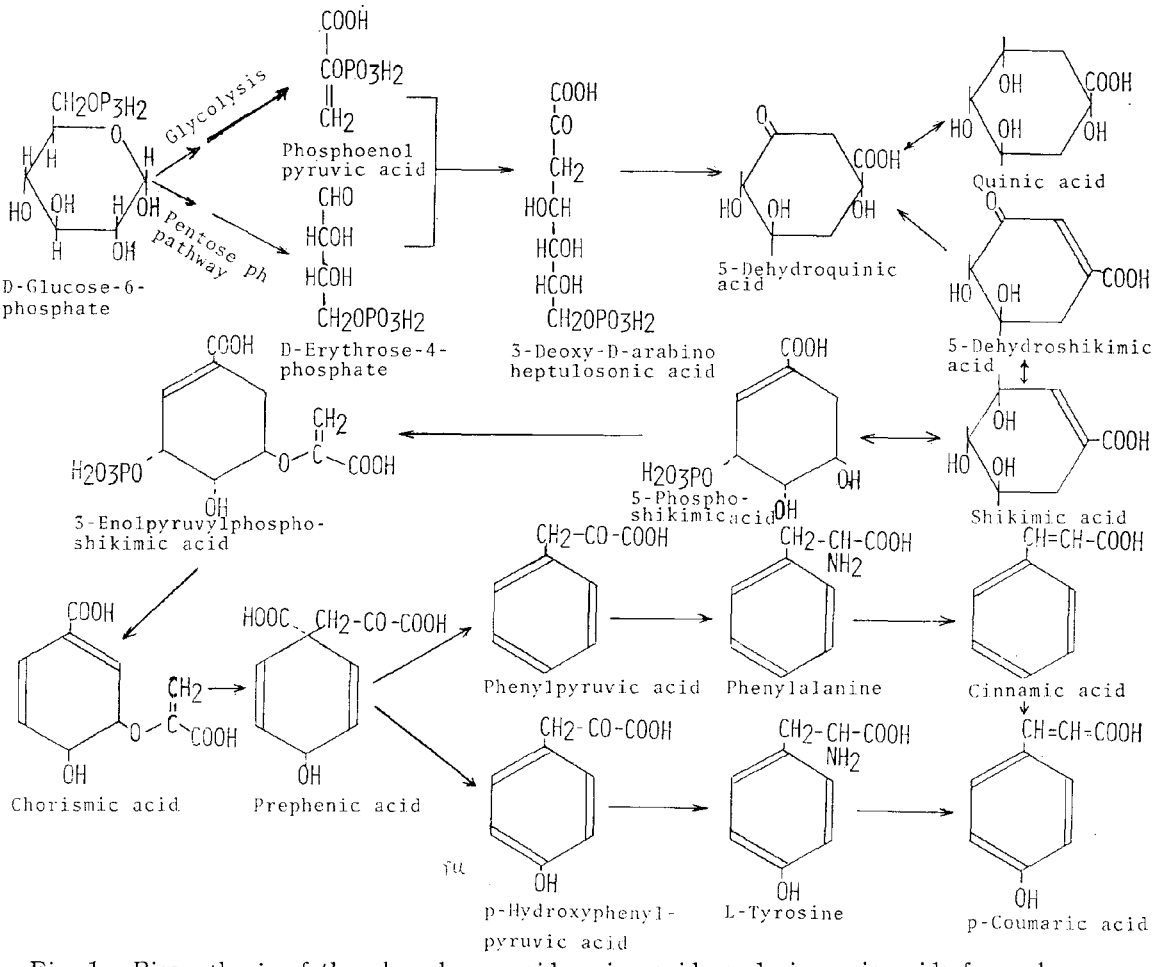

Fig. 1. Biosynthesis of the phenylpropanoid amino acids and cinnamic acids from glucose-6-phosphate.

level of both enzymes in apical part of a bamboo shoot maintained a certain level even in lower parts of the shoot (Fig. 3). And as shown in Fig. 4, C_6/C_1 ratio calculated from $^{14}CO_2$ formed in respiratory breakdown of glucose-1-^{14}C and glucose-6-^{14}C in different parts of a bamboo decreased considerably toward lower parts indicating more contribution of the pentose phosphate pathway in lower parts of the bamboo shoot with progressing lignification.

In biosynthesis of aromatic amino acids in germinating shoots of *Phaseolus aureus* MINAMIKAWA[5] could demonstrate a similar pattern of activity of 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) synthetase. The enzyme should be found in bamboo shoots.

5-Dehydroquinate hydro-lyase catalyzing interconversion between 5-dehydroquinic acid and 5-dehydroshikimic acid which is responsible for the shikimate pathway has been isolated from various plant tissues. The enzyme was also isolated from bamboo shoots and characterized.[6] As shown in Fig. 5, the enzyme activity was highest in the top and decreased quite gradually toward lower parts of a bamboo shoot without

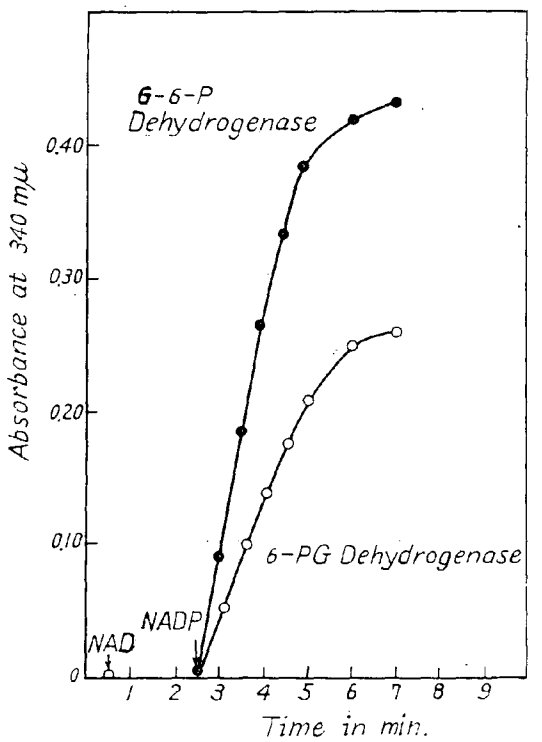

Fig. 2. Specificity of enzyme
Enzyme concentration ; 3.0 mg protein/ml. NAD or NADP was added at the points shown.
(*Phyllostachys pubescens*)

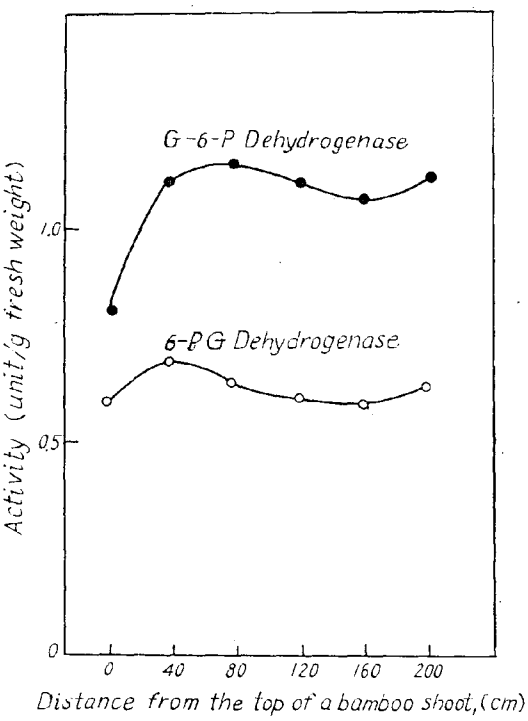

Fig. 3. Changes in activity of G-6-P and 6-PG dehydrogenases in differents parts of bamboo.
(*Phyllostachys pubescens*)

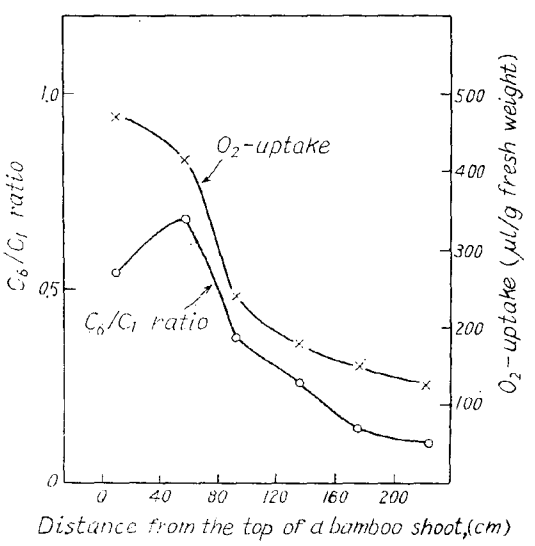

Fig. 4. Pattern of respiratory breakdown of glucose-1-^{14}C and -6-^{14}C.
(*Phyllostachys pubescens*)

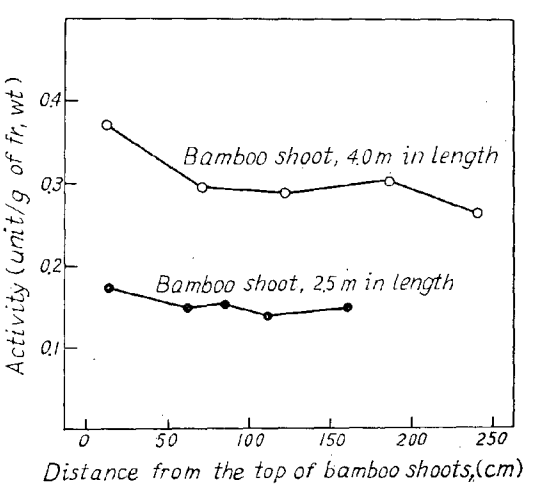

Fig. 5. Activity of 5-Dehydroquinate hydrolyase in different parts of bamboo.
(*Phyllostachys pubescens*)

marked decrease. However, as enzyme preparations from very young bamboo shoot (20 cm in length) which contains a large amount of starch granules gave the quite weak enzyme activity the enzyme seems to be synthesized in an early stage of growth of bamboo shoots and a rather similar pattern of the activity to other enzymes described above might be expected.

5-Dehydroshikimate reductase extracted from bamboo shoots[7] was NADP-specific as shown in Fig. 6 and the enzyme activity was highest at the top, decreased in the tissues just below the top and was maintained at a certain level even in lower parts without significant decrease (Fig. 7). The concentration of shikimic acid in various parts of a bamboo shoot seemed to follow the pattern of the enzyme activity. The maintenance of the certain level of the enzyme activity and an increased specific activity of the enzyme in the lower parts may indicate a significant contribution of the enzyme to synthesis of lignin precursors as well as other phenolic compounds in this stage.

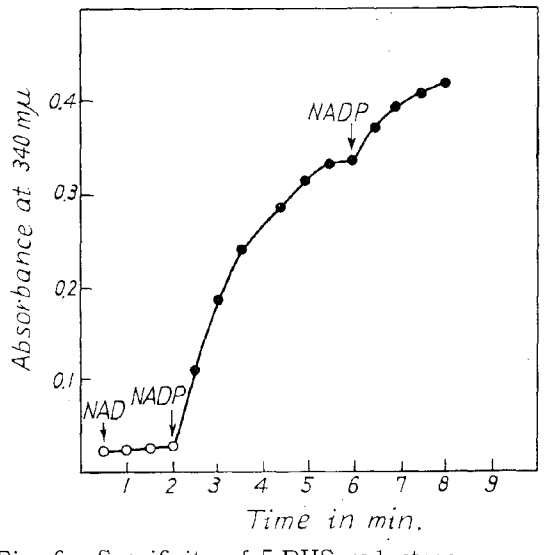

Fig. 6. Specificity of 5-DHS reductase
Enzyme concentration; 13.2mg protein/ml. NAD or NADP was added at the points shown.
(*Phyllostachys pubescens*)

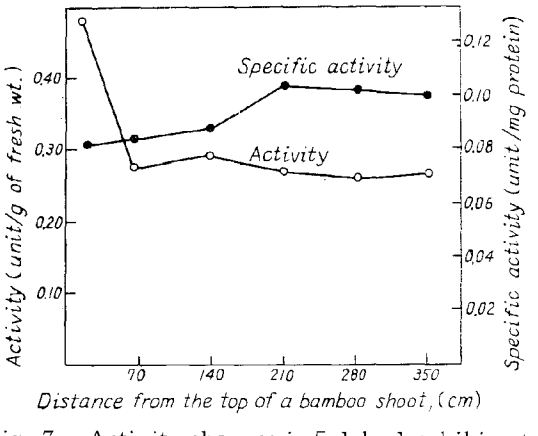

Fig. 7. Activity changes in 5-dehydroshikimate reductase in different parts of bamboo.
(*Phyllostachys pubescens*)

Phenylalanine has been known to be a natural intermediate of phenylpropane constituents of lignins in higher plants.[1] Tyrosine has also been known to be incorporated into lignins of grasses.[2] Then, the pattern of activity in aromatic amino acid transaminase during growth of bamboo shoots was examined using their sliced tissues.[8] As shown in Fig. 8, more phenylpyruvate and *p*-hydroxyphenylpyruvate added as substrates was recovered from the reaction mixture when sliced tissues from upper parts of a bamboo shoot were used, and the results suggested that the tissues of the upper parts have lower transaminase activity than the tissues from lower parts of the

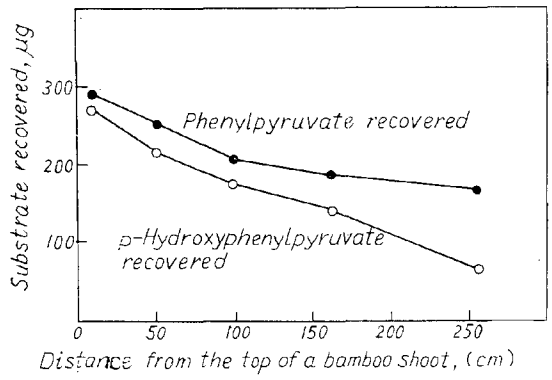

Fig. 8. Consumption pattern of phenylpyruvate and *p*-hydroxyphenylpyruvate incubated with sliced tissue. (*Phyllostachys pubescens*)

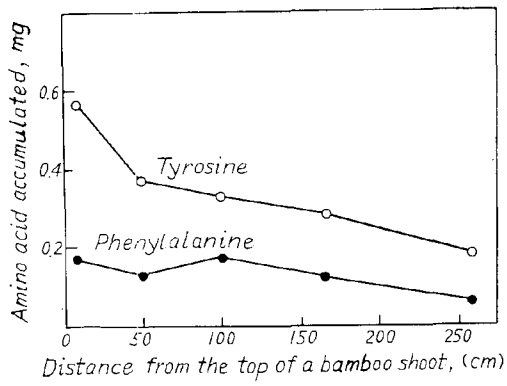

Fig. 9. Accumulation of phenylalanine and tyrosine by sliced tissue (*Phyllostachys pubescens*)

shoot. However, as shown in Fig. 9, apparent amounts of phenylalanine and tyrosine were higher in the upper tissues indicating more rapid conversion of these amino acids formed to other substances, such as cinnamic acids in the lower tissues than the upper tissues. In fact, *p*-coumaric acid was found to be synthesized from both phenylpyruvate and *p*-hydroxyphenylpyruvate, and the amount of *p*-coumaric acid formed from the acids was greatest using lower tissue reaction system.

Thus, it might be understood that in early stage of the growth of bamboo shoot, the enzymes in the shikimate pathway and the aromatic amino acid transaminases may participate dominantly in the synthesis of phenylalanine and tyrosine incorporated into protein but with onset of lignification metabolic regulation systems may be transformed to accelerate the synthesis of a series of the related enzymes such as phenylalanine deaminase, to supply lignin precursors.

Enzymes in Cinnamate Pathway

Since the finding of phenylalanine deaminase and tyrase by KOUKOL and CONN,[9] and NEISH,[10] respectively several papers on the possible role of these enzymes in phenol metabolism have been reported. YOSHIDA and SHIMOKORIYAMA[11] found that phenylalanine deaminase activity develops in parallel with lignification in stems of a buckwheat plant.

The variation pattern in activity of both phenylalanine deaminase and tyrase of bamboo shoots during lignification was investigated.[12] In quite young shoots (length, about 1 m) the activity of these enzymes increased from the top to the basal part of the shoots progressively. However, in the more matured shoots (about 5 m in length) the activity of both enzymes increased progressively from the top to lower parts, but at the basal part of the shoots, where the tissue was quite hard, the vessels gave strong lignin reactions and the parenchyma tissue also gave weak lignin reactions, the enzyme activity decreased (Fig. 10). In the most matured bamboo (length, 8.8 m) of which

branches were begining to develop and lower 2/3 of the stem was quite hard and lignified, the activity of both enzymes increased from the top to adjacent parts but the activity decreased at the part of 1/3 of the stem length where the tissue was quite hard and parenchyma was lignified. In other words, the pattern of the enzyme activity moved toward the tissue of upper parts, where the lignification was taking place most actively during maturation of the bamboo. The amount of cinnamic acids in the respective parts was found to be in good accordance with the pattern of activity of both enzymes.

Phenylalanine deaminase and tyrase are both key enzymes in the synthesis of of phenolic compounds, because these enzymes irreversibly divert phenylalanine and tyrosine in the protein metabolism into the synthesis of phenolic compounds during lignification of the bamboo.

Cinnamic acid-4-hydroxylase[13,14] which mediates conversion of cinnamic acid to *p*-coumaric acid was also found in bamboo shoots.[15] Since cell-free extraction of cinnamic acid hydroxylase from bamboo shoots was found to be difficult in spite of the high productivity for *p*-coumaric acid of the bamboo shoots, the sliced tissues were employed tentatively for the assay of enzyme activity. As shown in Fig. 11, the amount of *p*-coumaric acid formed from cinnamic acid was quite low around the apical part of a shoot and increased markedly toward lower parts reaching the maximum value and

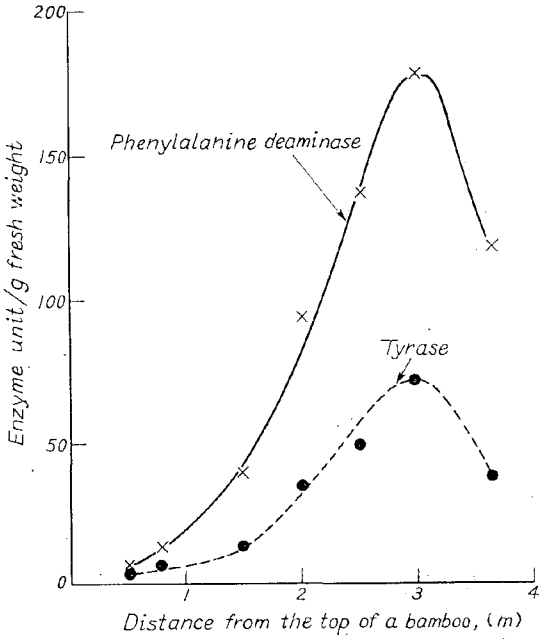

Fig. 10. Activity changes in phenylalanine deaminase and tyrase of different portions of a bamboo. (*Phyllostachys pubescens*)

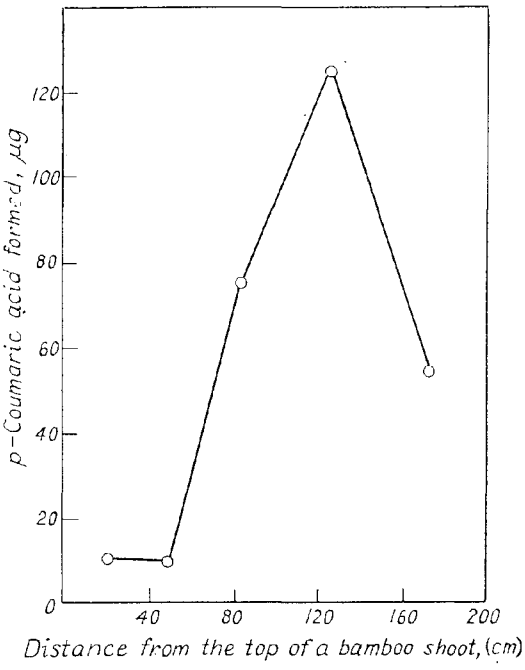

Fig. 11. Pattern of cinnamic acid hydroxylating activity in different tissue samples. (*Phyllostachys pubescens*)

then decreased toward further lower parts of the shoot. These results may indicate that cinnamic acid hydroxylase activity increases from the top toward lower parts of the shoot.

Cinnamic acid-4-hydroxylase should be quite important as an enzyme mediating first hydroxylation reaction occurring at aromatic ring and therefore it should be one of key enzymes regulating the formation of lignin precursors.

Following the hydroxylation step at aromatic ring of cinnamic acids, transmethylation reaction should participate in the formation of lignin precursors. FINKLE et al[16,17] reported on the occurrence of S-adenosylmethionine : catechol O-methyltransferase in cambial tissues of an apple tree and a pampas grass.

The enzyme was also isolated from bamboo shoots and characterized.[18] As shown in Table 1, when caffeic acid and S-adenosylmethionine were used as substrates only ferulic acid was formed and no formation of isoferulic and 3, 4-dimethoxycinnamic acid was recognized. When 5-hydroxyferulic and 3, 4, 5-trihydroxycinnamic acids were added in the reaction mixture instead of caffeic acid, only sinapic acid was formed from the former and both 5-hydroxyferulic and sinapic acids were formed from the latter system. The enzymic formation of ferulic and sinapic acids is in good accordance with the fact that both acids are efficient lignin precursors.

Although 3, 4, 5-trihydroxycinnamic acid was found to be a fairly good methyl acceptor *in vitro*, it is still obscure whether or not the acid acts as an obligatory pre-

Table 1. Substrate specificity of bamboo O-methyltransferase.

Substrate	Product expected	Result
Caffeic acid	Ferulic acid	+
5-Hydroxyferulic acid	Sinapic acid	+
3, 4, 5-Trihydroxycinnamic acid	5-Hydroxyferulic acid	+
	Sinapic acid	+
Chlorogenic acid	Feruloylquinic acid	+
p-Coumaric acid	*p*-Methoxycinnamic acid	−
Caffeic acid	iso-Ferulic acid	−
iso-Ferulic acid	3, 4-Dimethoxycinnamic acid	−
p-Hydroxybenzoic acid	*p*-Methoxybenzoic acid	−
Protocatechuic acid	Vanillic acid	−
Gallic acid	Syringic acid	−
p-Hydroxybenzaldehyde	Anisaldehyde	−
Protocatechuic aldehyde	Vanillin	−
	iso-Vanillin	−
iso-Vanillin	Veratric aldehyde	−
5-Hydroxyvanillin	Syringic aldehyde	−
Gallic aldehyde	5-Hydroxyvanillin	−
Pinosylvin	Pinosylvin monomethyl ether	−

cursor in the biosynthesis of lignin *in vivo*. Because the acid has not yet been detected in nature.

Caffeoylquinic acid was converted to feruloylquinic acid by this enzyme. However, any information of these depsides as intermediates in lignin formation has not yet been obtained. *p*-Coumaric acid, isoferulic acid, benzoic acid derivatives, benzaldehyde derivatives and pinosylvin were recognized not to be utilized as methyl acceptors by this enzyme.

These results indicate that O-methyltransferase of the bamboo is *meta*-specific which is consistent with results obtained by FINKLE et al. The results further suggest that the methyl acceptors for this bamboo enzyme are necessary to have hydroxyl group on *para*- position and acryl group on the side chain at the same time.[19]

The enzyme isolated from yound shoots of a poplar tree could also catalyze methylation of caffeic, 5-hydroxyferulic and 3, 4, 5-trihydroxycinnamic acids to ferulic, sinapic, and 5-hydroxyferulic and sinapic acids, respectively.[19]

In connection with the study on O-methyltransferase of bamboo shoots our interest has been focused on metabolic differences between soft and hard woods, the former mainly synthesize guaiacyl lignin and the latter does both guaiacyl and syringyl lignins. Trials to isolate O-methyltransferase from various conifers were failed. However, as shown in Fig. 12 sliced tissue of young shoots of a gingko tree was proved to contain O-methyltransferase activity, since caffeic acid administered to the sliced tissue was

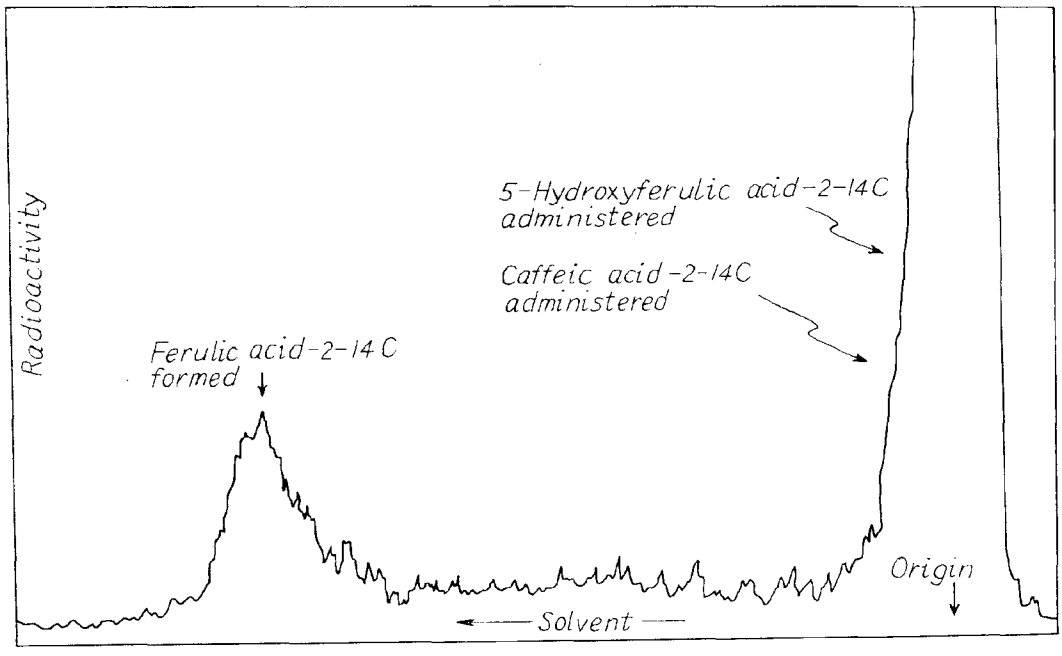

Fig. 12. Selective methylation for dihydroxycinnamic acid by *Gingko biloba*. Solvent, toluene, acetic acid and water (4 : 1 : 5, v/v, organic layer).

converted to ferulic acid. Unexpectedly, 5-hydroxyferulic and 3, 4, 5-trihydroxycinnamic acids did not act as a methyl acceptor, for no corresponding products such as sinapic and 5-hydroxyferulic acids were formed. Although it is difficult to discuss the substrate specificity of O-methyltransferase of the gingko tree only from the results obtained with sliced tissue, it will be postulated probably that O-methyltransferase of gymnospermous trees including a gingko tree will have high specificity for the methylation of caffeic acid.

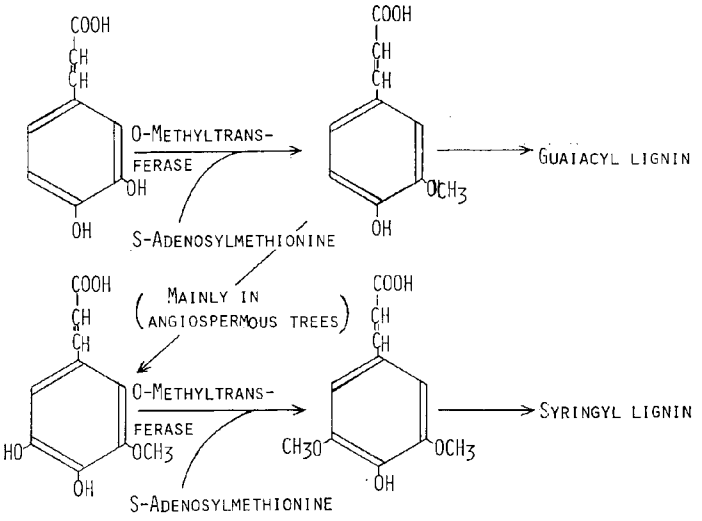

Fig. 13. Metabolic differences in formation of guaiacyl- and syringyl lignins in gymnospermous- and angiospermous trees.

These results suggest that O-methyltransferase of conifers may differ from that of broad-leaved trees and bamboos in its substrate specificity and its function. Therefore, even if 5-hydroxyferulic acid is administered to conifers, few syringyl nuclei will be formed. Thus the metabolic differences in formation of guaiacyl- and syringyl lignins in gymnospermous and angiospermous trees seems to be ascribed to the difference of specificity of O-methyltransferase and to the possible absence of ferulic acid-5-hydroxylating enzyme in gymnospermous trees as illustrated in Fig. 13.

O-methyltransferase at the top of a

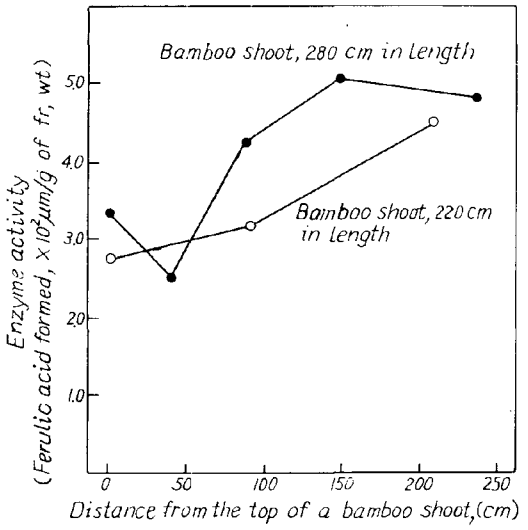

Fig. 14. Changes in activity of O-methyltransferase.
(*Phyllostachys reticulata*)

bamboo shoot was moderately active and the activity increased from the top to lower parts of the shoot as shown in Fig. 14. The variation pattern of enzyme activity was quite similar to those of phenylalanine deaminase and tyrase during growth of bamboo shoots. It may be thought, therefore, that the contribution of O-methyltransferase, cinnamic acid 4-hydroxylase and aromatic acid ammonia-lyases is more directly related than the enzymes in the shikimate pathway for the production of lignin precursors. Because the activity levels of the latter enzymes were almost constantly maintained from the top to lower parts of bamboo shoots and it is reasonable to consider that these enzymes are responsible for protein metabolism as well as the formation of lignin precursors.

The methionine activating enzyme which catalyze the formation of S-adenosylmethionine from ATP and L-methionine was also isolated from bamboo shoots, and formation of ferulic acid from L-methionine-$^{14}CH_3$, ATP and caffeic acid by the mediation of this enzyme and O-methyltransferase was established.[18]

Metabolic Regulation of Lignification

Although the physiological nature of the trigger for lignification is still obscure, the results obtained seem to indicate that the formation of enzymes in cinnamate pathway, phenylalanine deaminase in particular, is one of main factors to induce lignification. ZUCKER[20] reported that phenylalanine deaminase was induced by light in relation to chlorogenic acid synthesis. YOSHIDA[21] also reported recently that in germinating pea seedlings, the formation of phenylananine deaminase was greatly enhanced by light, and that phytochrome and related pigments participated as an inducer of the enzyme.

A similar inducer may participate in the formation of phenylalanine deaminase in bamboo shoots.

As another regulating factor for lignification, NADP level in tissues should be considered. As described above, enzymes in pentose phosphate and shikimate pathways are NADP-specific and therefore low level of NADP should repress the function of these enzymes. As factors to increase NADP level in plant tissues, oxygen, red light and kinetin have been known.[22]

Such many factors are involved in initiation of lignification and thus further investigations on regulating mechanism for induction of enzyme synthesis and on genetic controls for phenol metabolisms during plant development are needed.

Bamboo Lignin

The bamboo lignin is one of typical grass lignins and gives a large amount of *p*-hydroxybenzaldehyde as well as vanillin and syringaldehyde on alkaline nitrobenzene oxidation. However, *p*-hydroxybenzaldehyde produced is mainly due to the *p*-coumaric

acid esterified with lignin polymer and the amount of the aldehyde was reduced to to about 1/3 by previous alkaline hydrolysis of the lignin. Almost calculated amount of *p*-coumaric acid was obtained from the alkaline hydrolyzate of bamboo lignin and the ester linkage of *p*-coumaric acid with the lignin polymer was confirmed by UV, IR and NMR spectra.[23~26]

The fact that oxidation of the ethanolysis oils from bamboo lignin to diketones gave reasonable amounts of vanilloyl- and syringoyl acetyls but a very small amount of *p*-hydroxybenzoyl acetyl suggests the occurrence of quite a low amount of *p*-hydroxyphenylglycerol-β-aryl ether structure in the bamboo lignin.[25]

As shown in Table 2, L-phenylalanine-G-^{14}C and L-tyrosine-G-^{14}C administered into young branches of *P. pubescens* and *P. reticulata* were well incorporated into vanilloyl- and syringoyl acetyls obtained on the ethanolysis of the fed plant.[12,23] These results are in good accordance with the role of phenylalanine deaminase and tyrase in lignification of the bamboos. Thus it is thought that the main polymeric systems of the bamboo lignin may not be very different from those of the hard wood lignin. However, the fact that about 1/3 of the amount of *p*-hydroxybenzaldehyde was still produced on the alkaline nitrobenzene oxidation even after the previous hydrolysis of the bamboo lignin shows that considerable amounts of *p*-hydroxyphenyl moiety other than *p*-coumaric acid ester are composed of the polymeric systems of the lignin.

Table 2. Incorporation of L-phenylalanine-G-^{14}C and tyrosine-G-^{14}C into lignins of bamboo.

	Compound administered	Dose, μM/g Fresh wt.	Specific activity of diketones from lignin, μC/mM		Dilution	
			VA*	SA**	VA	SA
Phyllostachys pubescens	Phenylalanine	3.5	1.10	1.30	319	270
	Tyrosine	3.6	0.80	0.93	381	328
Phyllostachys reticulata	Phenylalanine	3.5	1.25	1.21	280	290
	Tyrosine	3.6	0.95	1.16	321	263

*VA ; vanilloyl acetyl. ** SA ; syringoyl acetyl

Then, bamboo MWL was thoroughly methylated and subjected to permanganate oxidation and several degradation products were identified and determined by gas-liquid chromatography.[26] Bamboo MWL previously hydrolyzed with alkali and a dehydrogenation polymer (DHP) prepared from a mixture solution of coniferyl and *p*-coumaryl alcohols (1 : 1 M/M) were treated in the same way for comparison.

As shown in Table 3, the yield of *p*-anisic, veratric and trimethyl gallic acids formed from the open type units were reasonably high and the molecular ratios were apparently in accordance with the results on alkaline nitrobenzene oxidation of the

Table 3. Permanganate oxidation products of methylated lignins.

Product (%)*	Bamboo MWL	Bamboo MWL, NaOH treated	Grass (*Coix lachryma*)	DHP**	Red pine	Beech	Poplar
p-Anisic acid	1.77	0.58	4.60	4.93	+	+	0.24
Veratric acid	1.46	1.98	5.51	2.41	3.65	1.34	1.26
Trimethylgallic acid	1.14	2.33	5.75	—	+	2.45	0.95
4-Methoxyisophthalic acid	0.42	0.55	0.40	1.50	0.35	0.45	0.73
Isohemipinic acid	0.32	0.22	0.54	0.56	0.73	0.18	0.19
Metahemipinic acid	?	?	0.20	0.47	?	?	?
Methoxytrimesic acid	—	—	?	—	?	—	—

* Based on lignin weight, ** Prepared from a mixture solution of coniferyl alcohol and *p*-coumaryl alcohol (1 : 1, M/M)

lignin.

The yield of *p*-anisic acid from the bamboo MWL hydrolyzed previously with alkali gave about 1/3 of the amount of the acid from untreated bamboo MWL and the results was also comparable to that of aromatic aldehydes found on alkaline nitrobenzene oxidation of the hydrolyzed MWL. However, again about 1/3 of the amount of the structure producing *p*-anisic acid still remained in the hydrolyzed bomboo MWL. The amounts of *p*-anisic and veratric acids from the DHP were much lower than those from bamboo MWL.

As the structures producing *p*-anisic acid, dehydrodi-*p*-coumaryl alcohol, *p*-coumarylresinol and similar compounds composed of *p*-coumaryl alcohol and coniferyl alcohol or *p*-coumaryl alcohol and sinapyl alcohol are suggested. The possibility of participation of *p*-hydroxyphenylglycerol-*β*-aryl ether is minor as stated above.

The acids which should be produced from the condensed type units of lignins such as isohemipinic and 4-methoxyisophthalic acids were obtained in reasonable amounts from both bamboo MWL and DHP. Methoxytrimesic acid probably ascribed to the polymers double condensed by biphenyl linkages and to a phenylcoumarane of biphenyl compounds was scarecely detected.

BLAND[27,28] reported that the *p*-coumaric lignin formed from *p*-coumaric acid on potato parenchyma and the MWL of Sphagnum gave hydroxytrimesic acid as well as 4-hydroxyisophthalic acid by permanganate oxidation. He suggested that these lignins were basically a high condensed C-C linked polymer of *p*-hydroxyphenyl moiety through double condensation at C-5.

NMR spectra of the acetylated conifers and hard wood MWLs gave broad peaks

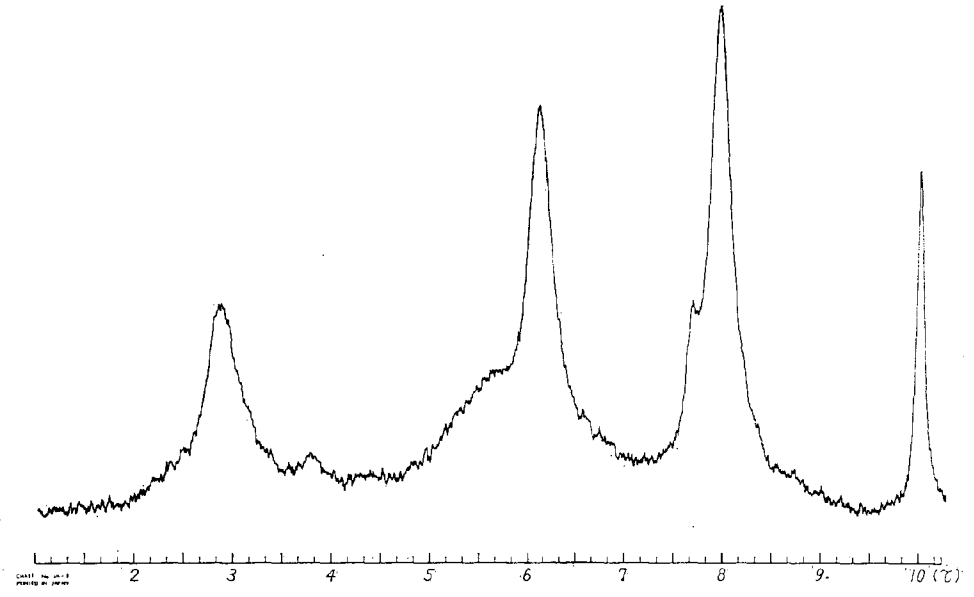

Fig 15. NMR spectrum of the acetylated MWL of Japanese red pine (*Pinus densiflora*).

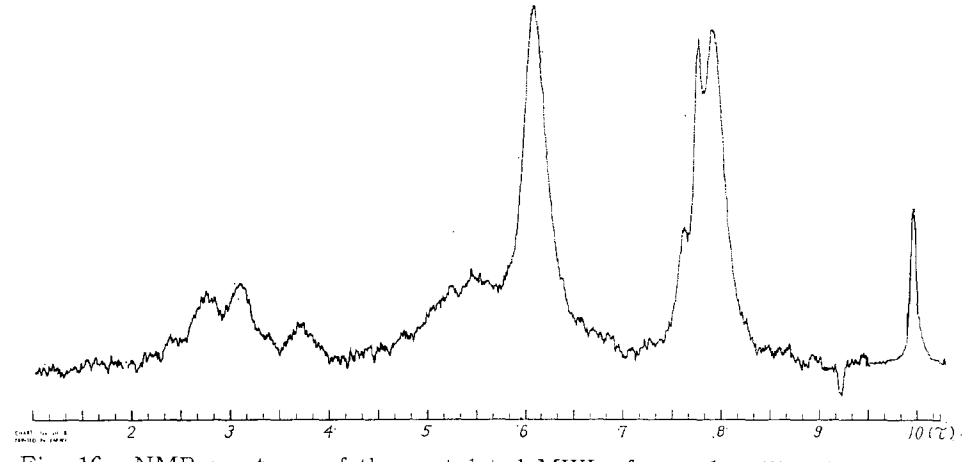

Fig 16. NMR spectrum of the acetylated MWL of a poplar (*Populus nigra*).

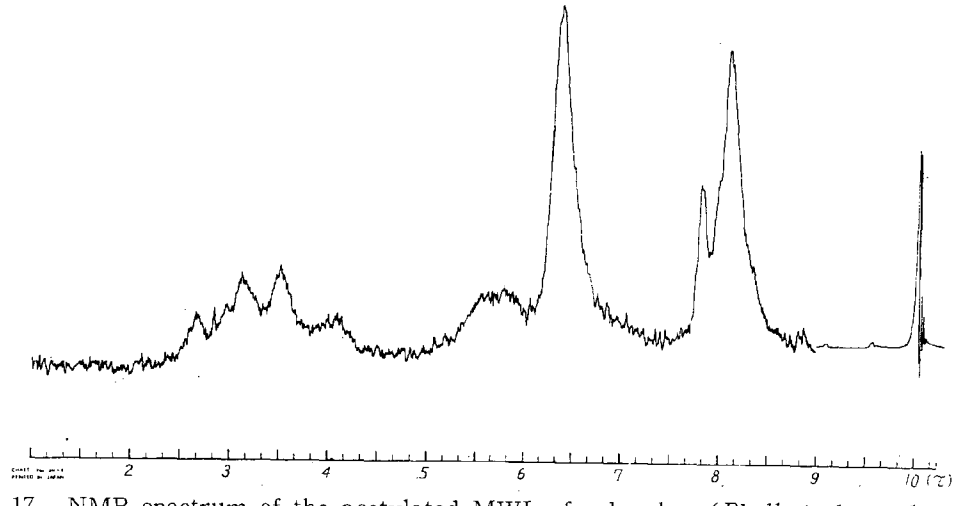

Fig. 17. NMR spectrum of the acetylated MWL of a bamboo (*Phyllostachys pubescens*).

aroun τ 2.5, and 2.5 and 3.0 by aromatic protons, respectively. The acetylated bamboo MWL gave three broad peaks around τ 2.2, 2.5 and 3.0, whereas the hydrolyzed bamboo MWL gave two peaks at 2.5 and 3.0 and the first peak disappeared. As the first peak at τ 2.2 is corresponding to that of aromatic and α protons at the ethyl *p*-coumarate, the peak is probably ascribed to *p*-coumaric acid esterified with lignin polymer. Thus it appears that the condensed type unit at C-5 of the aromatic ring of *p*-hydroxyphenyl moiety is prevalent in bamboo lignin.

In conclusion, the bamboo lignin is composed of guaiacyl, syringyl and *p*-hydroxyphenyl moieties of open type and C-5 condensed type units and *p*-coumaric acid is esterified with the polymeric systems.

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