

p-HYDROXYPHENYLPROPANE COMPONENT OF GRASS LIGNIN AND ROLE OF TYROSINE-AMMONIA LYASE IN ITS FORMATION

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Abstract—It is shown that a large proportion of the *p*-hydroxyphenylpropane moiety of grass lignin can be ascribed to *p*-coumaric acid esterified with the lignin. There may therefore be little fundamental difference between the polymeric systems of grass and hardwood lignins, although a small amount of *p*-hydroxyphenylglycerol- β -aryl ether structure is contained in the former. The results of incorporation of tyrosine- $G-^{14}C$ indicate that tyrosine is utilized for the synthesis of both the monomers of the grass lignin and the esterified *p*-coumaric acid by action of tyrosine-ammonia lyase.

INTRODUCTION

IT HAS been generally believed that grass lignins are co-polymers composed of *p*-hydroxyphenyl-, guaiacyl- and syringylpropanes. However, previous papers^{1,2} have indicated that a large proportion of *p*-hydroxybenzaldehyde produced in the alkaline nitrobenzene oxidation of the grass lignin is formed from *p*-coumaric acid esterified with the lignin, and not from a *p*-hydroxyphenyl moiety in the lignin polymers itself. The main polymeric systems of the grass lignin are therefore not very different from hardwood lignins.

On the other hand, it has been shown that tyrosine-ammonia lyase, which mediates the formation of *trans-p*-coumaric acid from tyrosine, participates in the synthesis of the grass lignin.^{3,4}

The present paper provides evidence to confirm these conclusions and also demonstrates the participation of tyrosine-ammonia lyase in the formation of both the esterified *p*-coumaric acid and the lignin monomers of grasses.

RESULT AND DISCUSSION

The lignin content, the methoxyl content of the lignin and the yield of each aldehyde in the lignin oxidation products, determined by gas-liquid chromatography, are shown in Table 1. As seen in the table the amount of total aldehydes based on the lignin content was between 15–26 per cent in grasses, and 17–50 per cent in other monocotyledons which were used for comparison, except for *Dendrobium* and asparagus. In the case of the latter, the stems contained a large amount of proteinaceous substances and polysaccharides, and their alkali-extracted residues gave rather more aldehydes on nitrobenzene oxidation than unextracted stem powder (Table 2).

¹ T. HIGUCHI and I. KAWAMURA, *Holzforschung* **20**, 16 (1966).

² T. HIGUCHI, N. KIMURA and I. KAWAMURA, *J. Japan Wood Res. Soc.* **12**, 173 (1966).

³ S. A. BROWN, D. WRIGHT and A. C. NEISH, *Can. J. Biochem. Physiol.* **37**, 25 (1959).

⁴ A. C. NEISH, *Phytochem.* **1**, 1 (1961).

TABLE 1. YIELD OF LIGNIN OXIDATION PRODUCTS

	Lignin (%)	Methoxyl of lignin (%)	Lignin oxidation products*			Molecular ratio†	
			V (%)	S (%)	p-HB (%)	S	p-HB
<i>Miscanthus sacchariflooyus</i>	21.4	13.4	11.5	8.1	4.9	0.59	0.53
<i>M. sinensis</i>	23.5	6.4	7.7	4.2	3.0	0.46	0.49
<i>M. condensatus</i> 1	17.4	11.8	10.9	7.7	6.1	0.58	0.69
<i>M. condensatus</i> 2	18.3	11.7	6.6	5.2	4.0	0.66	0.75
<i>M. tinctorius</i>	22.7	8.3	6.0	2.8	2.4	0.37	0.51
<i>Coix lachryma</i>	20.6	10.3	9.5	9.5	6.7	0.84	0.93
<i>Phragmites communis</i>	22.5	12.8	8.4	6.6	5.3	0.66	0.99
<i>Arundo donax</i>	22.7	15.0	8.2	11.2	4.1	1.05	0.57
<i>Imperata cylindrica</i>	22.0	6.9	4.4	1.9	1.9	0.34	0.54
<i>Dendrobium nobile</i>	23.6	9.8	2.7	5.5	†	1.80	
<i>Asparagus officinalis</i>	18.8	13.3	8.1	6.9	2.0	0.61	0.24
<i>Trachycarpus excelsa</i>	19.0	15.4	14.3	19.2	†	1.12	
<i>Smilax china</i>	24.9	13.5	5.9	11.6	1.8	1.63	0.37
<i>Pandanus tectorius</i>	19.8	19.3	13.6	33.5	2.7	2.05	0.25

* Per cent of lignin. V: vanillin; S: syringaldehyde; p-HB: p-hydroxybenzaldehyde.

† Trace.

‡ Vanillin = 1.

TABLE 2. YIELD OF LIGNIN OXIDATION PRODUCTS FROM ALKALI EXTRACTED MATERIAL

	Lignin oxidation products*			Molecular ratio†	
	V (%)	S (%)	p-HB (%)	S	p-HB
<i>Miscanthus sacchariflooyus</i>	9.7	8.5	1.8	0.75	0.22
<i>M. sinensis</i>	6.9	4.1	1.5	0.49	0.27
<i>M. condensatus</i> 1	9.9	7.5	3.4	0.63	0.43
<i>M. condensatus</i> 2	5.9	5.0	2.3	0.70	0.48
<i>M. tinctorius</i>	5.2	2.7	1.6	0.43	0.38
<i>Coix lachryma</i>	8.8	8.9	3.1	0.85	0.44
<i>Phragmites communis</i>	7.1	7.2	2.6	0.85	0.46
<i>Arundo donax</i>	7.5	12.7	2.9	1.41	0.48
<i>Imperata cylindrica</i>	4.0	2.2	1.1	0.46	0.34
<i>Dendrobium nobile</i>	3.4	6.8	1.0	1.68	0.40
<i>Asparagus officinalis</i>	13.0	10.3	1.9	0.66	0.18
<i>Trachycarpus excelsa</i>	13.5	17.9	1.3	1.11	0.12
<i>Smilax china</i>	5.8	10.5	2.9	1.50	0.61
<i>Pandanus tectorius</i>	13.9	33.5	1.0	2.02	0.09

* Per cent of lignin. V: vanillin; S: syringaldehyde; p-HB: p-hydroxybenzaldehyde.

† Vanillin = 1.

In the grasses the ratios of p-hydroxybenzaldehyde and syringaldehyde to vanillin were between 0.5–1.0 and 0.4–1.1, respectively. On the other hand, in other monocotyledons a much smaller amount of p-hydroxybenzaldehyde was produced and the ratios were between 0–0.2 and 0.6–2.1, respectively.

Table 2 shows the yield of each aldehyde obtained by nitrobenzene oxidation of plant material previously extracted by 1 N sodium hydroxide. Although the yield of syringaldehyde was scarcely affected by alkali extraction the yield of *p*-hydroxybenzaldehyde decreased remarkably compared with vanillin, especially in grasses. Thus the ratios of *p*-hydroxybenzaldehyde and syringaldehyde to vanillin increased to 0.2–0.5 and 0.5–1.5 respectively in grasses. These results indicate that the decrease of the yields of *p*-hydroxybenzaldehyde and vanillin in the oxidation of the alkali-extracted residues is due to the removal of an alkali-soluble fraction of the lignins, most probably to *p*-coumaric and ferulic acids bound by ester links.

As expected, considerable amounts of both *p*-coumaric and ferulic acids (especially the former) but no appreciable amounts of sinapic acid were found in the ether extracts of the alkali-soluble fraction of the grass stem powder. *p*-Coumaric acid was identified by mixed melting point and paper chromatography. Ferulic acid was identified by paper chromatography.⁵ However, other monocotyledons gave only one-fiftieth or one-hundredth the amounts of *p*-coumaric and ferulic acids as were obtained from grasses.

TABLE 3. YIELD OF *p*-COUMARIC AND FERULIC ACIDS OBTAINED BY ALKALI EXTRACTION

	<i>p</i> -Coumaric acid (%)*	Ferulic acid (%)*
<i>Miscanthus sacchariflorus</i>	4.9	1.9
<i>M. sinensis</i>	4.0	2.0
<i>M. condensatus</i> 1	5.7	2.4
<i>M. condensatus</i> 2	6.1	2.5
<i>M. tinctorius</i>	3.9	2.9
<i>Coix lachryma</i>	6.2	1.9
<i>Phragmites communis</i>	6.0	1.9
<i>Arundo donax</i>	4.1	1.8
<i>Imperata cylindrica</i>	2.7	1.3
<i>Dendrobium nobile</i>	†	0.02
<i>Asparagus officinalis</i>	1.5	0.03
<i>Trachycarpus excelsa</i>	0.03	0.03
<i>Pandanus tectorius</i>	0.13	0.02

* Per cent of lignin.

† Trace.

Table 3 shows the amount of *p*-coumaric and ferulic acids obtained by alkali extraction. Since alkaline nitrobenzene oxidation of authentic *p*-coumaric and ferulic acids gave 40 per cent of the theoretical yields of *p*-hydroxybenzaldehyde and vanillin, respectively, the yields of these acids shown in Table 3 were multiplied by 0.4 and the corrected values compared with the decreased amounts of both aldehydes obtained by oxidation of the alkali-extracted residue. The results are shown in Table 4. As seen in the table there is a relatively good agreement between the calculated values and decreased amounts of both aldehydes and the results indicate that more than half of the *p*-hydroxyphenylpropane portion which has been believed to be a representative component of the grass lignin is due to the *p*-coumaric acid combined with lignin by an ester linkage. Although even after alkali extraction grass lignins give a little more *p*-hydroxybenzaldehyde than conifer or hardwood lignins do, there does not seem to be much fundamental difference between grass lignins and hardwood lignins.

⁵ T. HIGUCHI and S. A. BROWN, *Can. J. Biochem. Physiol.* 41, 65 (1963).

TABLE 4. RELATION BETWEEN THE DECREASED AMOUNT OF *p*-HYDROXYBENZALDEHYDE AND VANILLIN AND THE CALCULATED AMOUNT OF BOTH ALDEHYDES FROM *p*-COUMARIC AND FERULIC ACIDS

	Decreased amount		Calculated amount	
	V (%)*	<i>p</i> -HB (%)*	V (%)*	<i>p</i> -HB (%)*
<i>Miscanthus sacchariflopus</i>	1.8	3.1	0.59	1.48
<i>M. sinensis</i>	0.8	1.5	0.63	1.16
<i>M. condensatus</i> 1	1.8	2.7	0.75	1.72
<i>M. condensatus</i> 2	0.7	1.7	0.78	1.80
<i>M. tinctorius</i>	0.8	0.8	0.91	0.96
<i>Coix lachryma</i>	0.7	3.6	0.59	1.84
<i>Phragmites communis</i>	1.3	2.7	0.59	1.80
<i>Arundo donax</i>	0.7	1.2	0.57	1.20
<i>Imperata cylindrica</i>	0.4	0.8	0.41	0.80
<i>Dendrobium nobile</i>	-0.7	-1.0	†	†
<i>Asparagus officinalis</i>	-4.9	0.1	†	0.52
<i>Trachycarpus excelsa</i>	0.8	-1.3	†	0.01
<i>Pandanus tectorius</i>	-0.3	1.7	†	0.02

* Per cent of lignin. V: vanillin; *p*-HB: *p*-hydroxybenzaldehyde.

† Trace.

Quantitative analysis of the diketones obtained by ethanolysis of the stem powder is shown in Table 5. The ratio of syringoyl acetyl to vanilloyl acetyl was a little higher than that of syringaldehyde to vanillin in the nitrobenzene oxidation but there was a parallelism between the two ratios. However, the amount of *p*-hydroxybenzoyl acetyl which is expected from the grass lignins was so small it could only be detected by paper- and thin-layer chromatography and could not be estimated by gas-liquid chromatography.¹ The results of ethanolysis again support the view that the *p*-hydroxyphenylpropane portion of the grass lignin is mainly due to structures other than *p*-hydroxyphenylglycerol- β -aryl ether, most probably to the esterified *p*-coumaric acid.

TABLE 5. QUANTITATIVE ANALYSES OF ETHANOLYSIS PRODUCTS OF GRASS LIGNINS

	Nickel glyoxime (%)*	Molecular ratio		
		Vanilloyl acetyl	Syringoyl acetyl	<i>p</i> -Hydroxy- benzoyl acetyl
<i>Miscanthus sinensis</i>	7.1	1	1.4	†
<i>M. condensatus</i> 2	9.2	1	1.3	†
<i>Coix lachryma</i>	9.5	1	1.3	†
<i>Phragmites communis</i>	8.0	1	1.4	†
<i>Arundo donax</i>	9.3	1	1.5	†
<i>Dendrobium nobile</i>	8.4	1	1.4	—
<i>Asparagus officinalis</i>	4.8	1	†	†
<i>Smilax china</i>	6.6	1	2.7	—
<i>Pandanus tectorius</i>	13.7	1	2.2	†

* Per cent of lignin.

† Trace.

The results of the incorporation of L-phenylalanine-G-¹⁴C and L-tyrosine-G-¹⁴C into the grass lignins and the *p*-coumaric acid esterified with the lignins are shown in Table 6. The incorporation of both compounds into the *p*-hydroxybenzaldehyde, vanillin and syringaldehyde obtained by oxidation of the plant material was quite good, and the conversion of L-tyrosine to the aromatic aldehydes was comparable to that of L-phenylalanine.

The specific activity of *p*-hydroxybenzaldehyde was much higher than that of the other aldehydes, especially when tyrosine was administered.

TABLE 6. INCORPORATION OF L-PHENYLALANINE-G-¹⁴C AND L-TYROSINE-G-¹⁴C INTO GRASS LIGNINS AND THE *p*-COUMARIC ACID ESTERIFIED WITH LIGNIN

	Compound administered			
	Phenylalanine*		Tyrosine*	
	Unextd.	Extd. with NaOH	Unextd.	Extd. with NaOH
<i>Miscanthus sinensis</i>	V 0.81 μ C/mM S 0.91 P 1.63 <i>p</i> -C 2.10	0.32 μ C/mM 0.40 0.54	0.84 μ C/mM 0.68 3.01 3.20	0.32 μ C/mM 0.38 1.03
<i>M. condensatus</i> 1	V 1.52 S 1.48 P 2.40 <i>p</i> -C 2.53	0.54 0.40 0.58	0.90 1.10 3.25 3.60	0.40 0.37 0.98
<i>Arundo donax</i>	V 2.02 S 1.50 P 2.85 <i>p</i> -C 2.75	0.83 0.64 0.95	1.29 1.51 4.17 4.28	0.45 0.40 0.95
<i>Coix lachryma</i>	V 2.92 S 2.12 P 3.35 <i>p</i> -C 3.28	1.26 0.80 1.03	0.93 1.20 4.55 4.20	0.36 0.47 1.65
<i>Phragmites communis</i>	V 0.99 S 0.89 P 1.54 <i>p</i> -C 2.20	0.38 0.30 0.48	1.17 1.01 3.56 3.40	0.60 0.45 0.92

V: vanillin; S: syringaldehyde; P: *p*-hydroxybenzaldehyde; *p*-C: *p*-coumaric acid.

* 5 μ C of L-phenylalanine-G-¹⁴C (400 μ C/mM) and L-tyrosine-G-¹⁴C (400 μ C/mM) were fed, respectively.

The specific activity of the respective aromatic aldehydes from the alkali-extracted fed plants, from which the esterified *p*-coumaric and ferulic acids were previously removed, was about one-third of that of the aldehydes from the unextracted fed plants, and further the specific activity of the *p*-hydroxybenzaldehyde in this case was only a little higher or almost the same as that of vanillin and syringaldehyde. The results indicate that the lignin was newly formed on the surface of the pre-formed lignin, and the newly formed less-polymerized lignin and *p*-coumaric acid esterified with the lignin were easily dissolved out by the alkali.

The specific activity of *p*-coumaric acid esterified with the lignin was about the same as that of *p*-hydroxybenzaldehyde from the unextracted fed plants, and the incorporation of

tyrosine into the acid was about three times more than into the monomers of lignins whereas phenylalanine was incorporated into the acid about one and a half times more than into the lignin monomers.

In a series of tracer experiments Brown *et al.*³ have found that tyrosine is incorporated much more readily into *p*-hydroxybenzaldehyde than into vanillin and syringaldehyde from wheat lignin. They explained the result as being due to the *p*-hydroxybenzaldehyde produced in the oxidation of tyrosine incorporated as such into protein. However, the results of present experiments now clearly indicate that high incorporation of tyrosine into *p*-hydroxybenzaldehyde is due to *p*-coumaric acid, esterified with the lignin, which can be formed through mediation by tyrosine-ammonia lyase.

Since the finding of tyrosine-ammonia lyase in grasses, the utilization of tyrosine for the synthesis of lignin only by grasses has a reasonable explanation. Present experiments suggest the participation of tyrosine-ammonia lyase in the synthesis of the esterified *p*-coumaric acid which is mainly found in grass lignin.

EXPERIMENTAL

The stems of mature plants of the following grasses; ogi (*Miscanthus sacchariflopus*), susuki (*M. sinensis*), yorebasusuki (*M. condensatus* 1), hachijosusuki (*M. condensatus* 2), kariyasu (*M. tinctorius*), jyuzudama (*Coix lachryma*), ashi (*Phragmites communis*), danchiku (*Arundo donax*), and chigaya (*Imperata cylindrica*), and other monocotyledons; dendrobium (*Dendrobium nobile*), asparagus (*Asparagus officinalis*), shuro (*Trachycarpus excelsa*), sarutoriibara (*Smilax china*), and adan (*Pandanus tectorius*) were used in these experiments.

Stem powder (60–90 mesh) was extracted with ethanol–benzene (1:1) for 10 hr and hot water for 4 hr, successively, and then lignin and methoxyl of the lignins were determined, respectively, by the TAPPI standard method.

Nitrobenzene Oxidation

The extractive-free stem powder (1 g) was subjected to alkaline nitrobenzene oxidation as described previously, and the aromatic aldehydes formed were analysed by gas–liquid, paper- and thin-layer chromatography.¹

Ethanolysis

The extractive free stem powder was subjected to ethanolysis and the ethanolysis oils containing “Hibbert’s lignin monomers” were oxidized into the diketones and the diketones were analysed by gas–liquid, paper- and thin-layer chromatography.¹

Saponification of Stem Powder

The extractive free stem powder (500 mg) was mixed with *N* NaOH (10 ml) and the reaction mixture was kept for 24 hr at 20°. The mixture was filtered, washed well with warm water and acidified (pH 2) with HCl. As a considerable amount of hemicellulose precipitated sometimes, the solution was filtered again, if necessary, and the filtrate was extracted continuously for 8 hr with ether. The ether extract was dried (Na₂SO₄) and evaporated. The residues were dissolved in 0.5 ml of acetone and analysed by gas–liquid and paper-chromatography.² For gas–liquid chromatography Apiezon N on Fluoropak was used at 215° column temperature, and for quantitative determination of *p*-coumaric and ferulic acids the standard curves relating the amount of each authentic acid injected to its peak area on chromatogram were constructed.

Administration of Labelled Compounds

L-Phenylalanine-G-¹⁴C and L-tyrosine-G-¹⁴C were administered through the cut ends of the plant stems in aqueous solution. After the absorption of the radio-active solution, the plants were allowed to metabolize for 24 hr at room temperature. The plants were disintegrated in a homogenizer with hot 80% ethanol, and the residue was separated by filtration and washed with hot ethanol and air dried. The plant material was pulverized, extracted with ethanol–benzene, 1:1, and water, successively. The extractive-free plant material was saponified with *N* NaOH for 24 hr at room temperature and the alkali extract was filtered, acidified and extracted with ether. Ether was evaporated and *p*-coumaric acid in the residues was purified by vacuum sublimation. Both alkali-extracted and unextracted plant materials were oxidized by alkaline nitrobenzene as described above and the resulting lignin degradation products—*p*-hydroxybenzaldehyde, vanillin and

syringaldehyde—were isolated by a Celite column chromatography, and the aldehydes were converted to the *m*-nitrobenzoylhydrazones.³

Measurement of Radioactivity

The radioactivity of the compounds was measured by a gas flow counter.

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