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Activities of Some Enzymes of Lignin Formation in Reaction Wood of *Thuja orientalis* and *Metasequoia glyptostroboides* 2 *

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Summary. To elucidate biochemical features leading to p-hydroxyphenyl-rich lignin in gymnosperm reaction wood the activities of the following five enzymes involved in the biosynthesis of p-hydroxyphenyl lignin were compared in reaction and opposite woods: phenylalanine ammonia-lyase (EC 4.3.1.5), cinnamate 4-hydroxylase (EC 1.14.13.11), p-hydroxycinnamate: CoA ligase (EC 6.2.1.12), cinnamyl alcohol dehydrogenase (EC 1.1.1.-) and peroxidase (EC 1.11.1.7). The enzyme activities in the reaction woods of *Thuja orientalis* and *Metasequoia glyptostroboides* were remarkably higher than those in the opposite woods, reflecting the higher contents of p-hydroxyphenyl lignin in reaction wood.

Introduction

In a previous paper two aspects of the characteristics of the reaction wood lignin of gymnosperm were investigated enzymatically (Kutsuki and Higuchi 1981a): high lignin and condensed unit contents in reaction wood were mainly ascribed to the higher enzyme activity involved in their guaiacyl lignin formation than those in opposite wood.

Since the synthesis of p-hydroxyphenyl lignin proceeds by the mediation of phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (CAH), p-hydroxycinnamate: CoA ligase (PCL), p-hydroxycinnamoyl-CoA reductase, cinnamyl alcohol dehydrogenase (CAD) and peroxidase (PO), successively, the activities of these enzymes of reaction and opposite woods were compared in relation to the occurrence of p-hydroxyphenyl-rich lignin in gymnosperm reaction wood (Bland 1961; Morohoshi and Sakakibara 1971b; Yasuda and Sakakibara 1975).

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Materials and Methods

Several-year-old branches of *Thuja orientalis* and *Metasequoia glyptostroboides* were collected on the campus of the Wood Research Institute, Kyoto University, in July, and the xylems were used as source of enzymes. Cinnamic acid 3-^{14}C was purchased form Service des Molecules Marquees, France. p-Hydroxycinnamic acid was synthesized from p-hydroxybenzaldehyde and malonate. p-Hydroxycinnamaldehyde was synthesized as reported elsewhere (Kutsuki et al. 1981 b). Other chemicals were purchased from Nakarai Chemical Co. Ltd., Japan.

The extraction of enzymes except CAH was performed in the same way as reported earlier (Kutsuki and Higuchi 1981 a). In the case of CAH, microsomal fraction was prepared by the method of Russel (1971). The assay methods and the substrates are outlined in Table 1. Protein concentration was determined by the Lowry method using bovine serum as standard.

Results and Discussion

The activities of PAL, PCL, CAD and PO, which are involved in the formation of p-hydroxyphenyl lignin, were compared between reaction and opposite woods. The results obtained with *Thuja orientalis* are shown in Fig. 1. It appeared that the activities of the enzymes except PO in reaction wood were considerably higher in comparison with those in opposite wood, and the ratio of the activities in both woods ranged from 1.1 (PAL) to 1.6 (PCL). On the other hand, the activity of PO remained constant, suggesting the occurrence of non specific PO for lignification. The same tendency was observed in *Metasequoia glyptostroboides* although PO activity was somewhat higher in the reaction wood (Fig. 2). The activity ratio of the enzymes in reaction wood ranged from 1.3-fold (PO) to 2.9-fold (CAD).

Table 1. Enzyme assays used

Enzyme	Phenolic substrates	Method [a]	Reference
PAL	Phenylalanine	290 nm	Zucker (1975)
CAH	Cinnamic acid 3-^{14}C	^{14}C [b]	Russell (1971)
PCL	p-Hydroxycinnamic acid	333 nm	Kutsuki (1981 c)
CAD	p-Hydroxycinnamaldehyde	340 nm	Kutsuki (1982)
PO	Phenol	510 nm	[c]

[a] Enzyme assays were performed at 35 °C and activities were measured spectrophotometrically at the wave length shown

[b] Cinnamic acid 3-^{14}C (6.8 Ci/mol) was used as substrate and p-hydroxycinnamic acid 3-^{14}C formed was measured radiochemically by liquid scintillation counter after separation by TLC (benzene-AcOH-H_2O 6/7/3 v/v organic layer)

[c] PO was assayed with 0.85 mmol H_2O_2, 1.3 mmol 4-aminoantipirine and 85 mmol phenol in 100 mmol potassium-phosphate buffer, pH 6.5

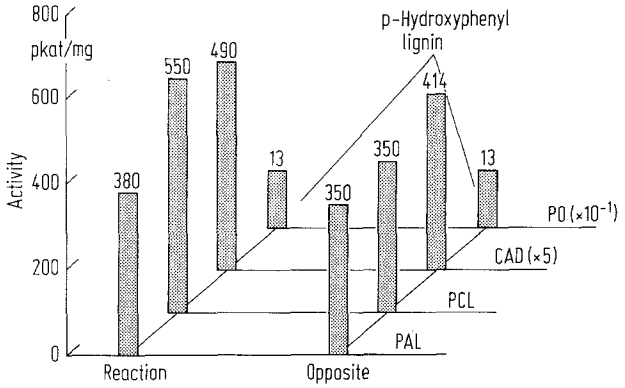

Fig. 1. Enzyme activities of reaction and opposite woods in *Thuja orientalis.* PO activity was defined as ΔA_{510} min^{-1} mg^{-1} and the other activities are expressed as pkat/mg. *PAL* phenylalanine ammonia-lyase; *PCL* p-hydroxycinnamate: CoA ligase; *CAD* cinnamyl alcohol dehydrogenase; *PO* peroxidase

The activity of CAH was also assayed using the microsomal fraction of *Metasequoia glyptostroboides.* The time course of the CAD reaction of reaction and opposite woods is shown in Fig. 3. The reaction proceeded linearly for at least 30 min and the activity of reaction wood was about 1.4 times higher than that of opposite wood. The enzymatically obtained product was identified by radio-TLC, using an authentic compound as reference (Fig. 4). p-Hydroxycinnamic acid (Rf 0.42) was shown to be formed exclusively from cinnamic acid (Rf 0.87) by the enzyme reaction. However, the activity of CAH of *Thuja orientalis* could not be measured owing to the low level and instability of the enzyme.

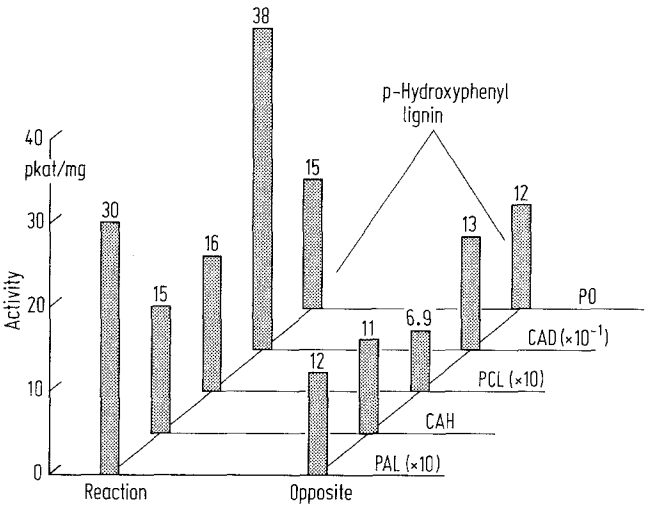

Fig. 2. Enzyme activities of reaction and opposite woods in *Metasequoia glyptostroboides. CAH* cinnaic acid 4-hydroxylase. Activities and the other abbreviations as in Fig. 1

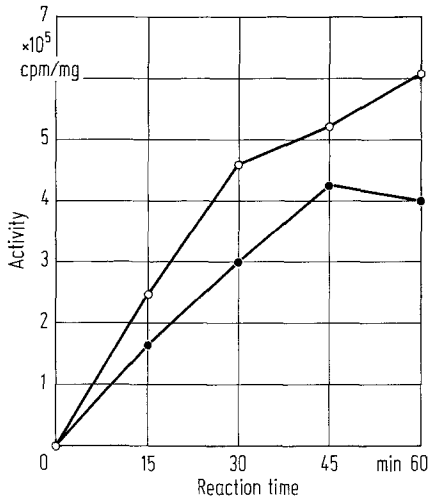

Fig. 3. Time course of p-hydroxycinnamic acid formation by the enzymes from reaction or opposite wood of *Metasequoia glyptostroboides.* ○ Reaction ● Opposite

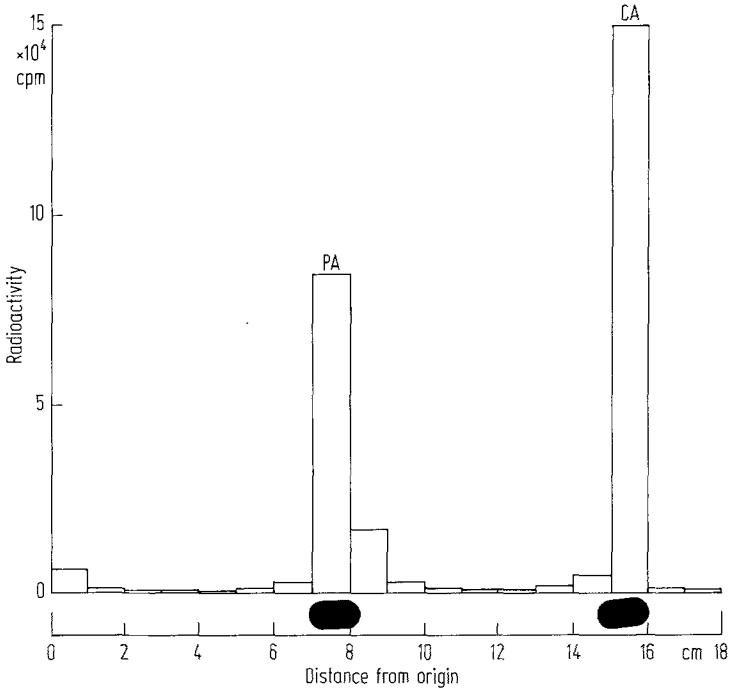

Fig. 4. Radio-thin layer chromatogram of p-hydroxycinnamic acid 3-^{14}C (PA) formed by cinnamate 4-hydroxylase of *Metasequoia glyptostroboides* using cinnamic acid 3-^{14}C (CA) as substrate. Solvent system: benzene-AcOH-H_2O 6/7/3 v/v organic layer

It is likely that the increased activities of these enzymes in reaction wood of gymnosperms lead to the p-hydroxyphenyl-rich lignin. It has been reported that the lignin in reaction wood contains less methoxyl groups and more p-hydroxyphenyl units than that of opposite wood (Bland 1961; Morohoshi and Sakakibara 1971 b; Yasuda and Sakakibara 1975). The low methoxyl content of reaction wood lignin would be explained by the lesser activity of caffeate: O-methyltransferase (EC 2.1.1.-) which mediates the methylation of caffeate to ferulate. However, our previous investigation showed that this was not the case, and the transferase activity was much higher in reaction wood than in opposite wood (Kutsuki and Higuchi 1981 a). It could thus be concluded that the increased supply of p-hydroxyphenyl lignin precursors by the increased activity of the enzymes might be responsible for the occurrence of a p-hydroxyphenyl-rich lignin in gymnosperm reaction wood: it is known that the increased supply of p-hydroxycinnamyl alcohols in dehydrogenation reaction by PO gives a bulk polymer containing more condensed units (Sarkanen 1971). It is conceivable that p-hydroxycinnamyl alcohol, the ortho positions of which are not substituted, gives more condensed units on dehydrogenative polymerization by PO.

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