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REGULATORY ROLE OF CINNAMYL ALCOHOL DEHYDROGENASE IN THE FORMATION OF GUAIACYL AND SYRINGYL LIGNINS

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Key Word Index—*Pinus thunbergii*; Pinaceae; Japanese black pine; lignin biosynthesis; cinnamyl alcohol dehydrogenase; coniferaldehyde; sinapaldehyde; guaiacyl lignin; syringyl lignin.

Abstract—The substrate specificities of cinnamyl alcohol dehydrogenase (CAD) of angiosperms and gymnosperms were examined using coniferaldehyde and sinapaldehyde as substrates. Angiosperm CADs reduced both aldehydes to the corresponding alcohols almost equally, whereas those of gymnosperms were remarkably specific for the reduction of coniferaldehyde. The purified CAD of Japanese black pine (*Pinus thunbergii*) showed that the K_ms to NADPH and coniferaldehyde were 6.8 and 9.1 μM, respectively and the V_{max} to sinapaldehyde was only 2.2% of that for coniferaldehyde. The MW of the CAD was 67 000 and the optimum pH was 6.8. It is thus presumed that CAD is one of the regulating enzymes which control the formation of guaiacyl and syringyl lignins.

INTRODUCTION

It is well known that the chemical structure of lignins differs between angiosperms and gymnosperms: the former consists of both guaiacyl and syringyl units, whereas the latter is composed mainly of guaiacyl units [1]. Studies of the substrate specificities of *S*-adenosyl-L-methionine:caffeate 3-*O*-methyltransferase (OMT, EC 2.1.1.1.–) which is involved in the formation of guaiacyl and syringyl lignin precursors gave a good biochemical explanation for the difference: angiosperm OMT methylates both caffeic and 5-hydroxyferulic acids, whereas gymnosperm OMT efficiently methylates caffeic acid but is almost inactive with 5-hydroxyferulic acid [2–5]. While in some cases OMT was found not to be a controlling enzyme in the formation of guaiacyl and syringyl lignin, the OMTs of tissue cultures of angiosperms and *Erythrina crista-galli* showed high activities toward 5-hydroxyferulic acid, although syringyl units were almost lacking in their lignins [6, 7]. A similar phenomenon was observed for some exceptional gymnosperms such as *Thuja standishii*, *T. orientalis* [8] and bamboos at different lignification stages. The content of syringyl units in a bamboo shoot was found to increase greatly during growth [9], but the OMT activity to sinapic acid remained constant [4].

Shimada *et al.* suggested that ferulic acid-5-hydroxylase, as well as OMT, might play important roles in determining the formation of syringyl lignin [4]. On the other hand, it was found that angiosperms reduce both ferulic and sinapic acids to the corresponding alcohols, whereas gymnosperms reduce ferulic acid but not sinapic acid [10, 11]. This indicates that gymnosperms lack the enzymes involved in the reduction of sinapic acid. These investigations may lead to the conclusion that the enzymes which potentially regulate syringyl lignin formation are ferulate-5-hydroxylase, OMT, *p*-hydroxycinnamate: CoA ligase (EC 6.2.1.12), cinnamoyl-CoA reductase (EC 1.1.1.–) and cinnamyl alcohol dehydrogenase (EC 1.1.1.–).

In the present paper, cinnamyl alcohol dehydrogenase (CAD), which catalyses the last step of the formation of lignin precursors (monolignols), was selected and the substrate specificities between gymnosperm and angiosperm were investigated. In addition, the CAD of Japanese black pine (*Pinus thunbergii*) was partially purified and characterized.

RESULTS

Substrate specificities of gymnosperm and angiosperm CADs

Substrate specificities of CADs were investigated using crude enzyme solutions after $(NH_4)_2SO_4$ fractionation of the seedlings of Japanese black pine (gymnosperm) and *Zelkova serrata* (angiosperm) as shown in Fig. 1. A marked difference was found between both CADs for the reduction of sinapaldehyde, which was a poor substrate for Japanese black pine CAD, but was a good substrate for *Zelkova serrata* CAD. On the other hand, both coniferaldehyde and *p*-coumaraldehyde were reduced equally well to the corresponding alcohols by both the gymnosperm and angiosperm CADs.

Since the control reactions containing no aldehydes as substrate showed a little oxidation of NADPH, a possible involvement of peroxidase in the oxidation of NADPH was examined [12]. Mn^{2+} and/or *p*-coumaric acid, which are known to activate peroxidase [13], were added to the reaction mixture of CAD or the control reaction. The results (Table 1) showed that oxidation of NADPH was not activated in either case but somewhat inhibited. The addition of Mn^{2+} and *p*-coumaric acid to control reactions also showed no effect, indicating that peroxidase had no influence on the CAD reactions in both the gymnosperm and angiosperm. Control reactions were

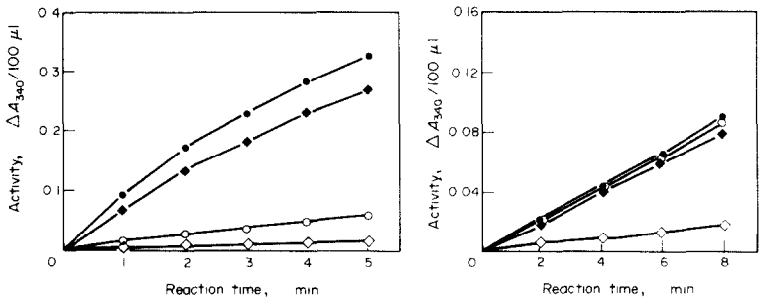

Fig. 1. Reduction of *p*-hydroxycinnamaldehydes by the CADs of Japanese black pine (left) and *Zelkova serrata* (right). ● Coniferaldehyde as substrate. ○ Sinapaldehyde. ♦ *p*-Coumaraldehyde. ◇ Control.

always run, and the activity of CAD was calculated by subtracting the amounts of oxidized NADPH in the control.

To elucidate the substrate specificities of the gymnosperm and angiosperm enzymes, CADs were extracted from xylems, seedlings or shoots of 16 plant species and the reducing activities for coniferaldehyde (Calc) and sinapaldehyde (Salc) assayed. The results (Table 2) showed that gymnosperm CADs are notably more active with coniferaldehyde than with sinapaldehyde, and the ratios of Salc activity to Calc activity (Salc/Calc) ranged from 0.05 to 0.55 (average 0.22), whereas in angiosperm CADs Salc activity was almost equal to that of Calc and the ratio of both activities are in the range of 0.65–1.53 (average 1.09). These findings suggest that CAD is another enzyme involved in the regulation of the formation of guaiacyl and syringyl lignins.

Nature of Japanese black pine CAD

Japanese black pine CAD was purified and characterized to elucidate further the guaiacyl specific nature of gymnosperm CAD. 2-Mercaptoethanol (5 mM) was always added to the CAD solutions. The activity of the crude CAD after $(NH_4)_2SO_4$ fractionation was lost in 5 days at 0°, whereas the CAD activity containing 10% glycerol under the same conditions retained *ca* 30% of the original activity after 5 days. Therefore, 10% glycerol was added to all the buffers except the one used for enzyme assay.

Reduction of coniferaldehyde by the CAD proceeded optimally at pH 6.8 in 80 mM Tris–HCl and 40 mM K-Pi buffers and the half-maximal activity was observed at pH 6 and 7.7.

Table 3 summarizes the results of purification procedures. 20- and 5-fold purification was achieved for Calc and Salc activities of the CAD, respectively. Through all the steps Salc/Calc values remained low (average 0.069), indicating that Japanese black pine CAD is much more specific to coniferaldehyde than sinapaldehyde. The elution patterns of the CAD on DEAE-cellulose and hydroxyapatite chromatography are shown in Figs. 2 and 3. Multiple forms, which have been found in several plant CADs [14–17], were not observed for Japanese black pine CAD, and Calc and Salc activities were eluted as the same fraction from the columns. Calc and Salc activities were eluted as a single peak from Sephadex G-100 chromatography. The MW was estimated to be *ca* 67000 using bovine serum albumin, hen egg albumin, chymotrypsinogen A and cytochrome *c* as references.

Japanese black pine CAD was exclusively dependent on NADPH. K_m and V_{max} values for NADPH and cinnamaldehyde derivatives were obtained from Lineweaver-Burk plots as listed in Table 4. NADPH and coniferaldehyde had the strongest affinities for the CAD, whereas the K_m of sinapaldehyde was too large to obtain reproducible results. On the other hand, both cinnamaldehyde and *p*-coumaraldehyde gave rather higher V_{max} values than NADPH and coniferaldehyde, whereas that of sinapaldehyde was extremely low. These

Table 1. The effects of peroxidase on CAD reaction

Phenolic substrate	Activity (ΔA_{340}/100 µl.min)	
	Liriodendron tulipifera	*Thuja orientalis*
Cald*	0.048	0.16
Cald + $MnCl_2$	0.037	0.15
Sald†	0.035	0.047
Sald + $MnCl_2$	0.031	0.040
Control	0.0038	0.0040
Control + $MnCl_2$	0.0028	0.0040
PA‡ + $MnCl_2$	0.0035	0.0040

* Coniferaldehyde.
† Sinapaldehyde.
‡ *p*-Coumaric acid.
0.1 mol of $MnCl_2$, 0.15 mol of PA and 100 µl of enzyme solution were used.

Table 2. Reduction of coniferaldehyde and sinapaldehyde to conferyl alcohol (Calc) and sinapyl alcohol (Salc) by different CADs

	Taxonomic group	Species	Activity (pkat/mg) Calc	Salc	Salc/Calc
Gymnosperm	Pinaceae	*Pinus thunbergii*	330	37	0.11
			450	70	0.16*
		Pinus densiflora	—	—	0.06*
		Larix leptolepis	—	—	0.22*
	Ginkgoaceae	*Ginkgo biloba*	300	15	0.05
	Cupressaceae	*Chamaecyparis obtusa*	—	—	0.07
		Thuja orientalis	1500	500	0.33
	Taxodiaceae	*Cryptomeria japonica*	680	290	0.43
		Metasequoia glyptostroboides	3300	1800	0.55
Angiosperm	Salicaceae	*Populus euramericana*	29	43	1.50
			20	23	1.15†
	Magnoliaceae	*Liriodendron tulipifera*	460	380	0.83
	Leguminosae	*Robinia pseudoacacia*	1600	1900	1.20
		Erythrina crista-galli	200	130	0.65†
	Rosaceae	*Prunus yedoensis*	860	1320	1.53
		Prunus persica	1300	840	0.65
	Ulmaceae	*Zelkova serrata*	—	—	1.20*
Monocotyledon	Gramineae	*Phyllostachys bambusoides*	—	—	1.69†

Enzymes were extracted from xylems except four seedlings* and three shoots†.

findings seem to indicate that Japanese black pine CAD possibly regulates the lignin biosynthesis toward preferential productions of guaiacyl lignin.

DISCUSSION

CAD involved in the reversible conversion of cinnamaldehydes to the corresponding alcohols catalyses the last step of the formation of lignin precursors. CAD was found to be distributed throughout a wide variety of land plants [14, 15] and has been characterized from potato [18], *Forsythia suspensa* [15], soybean [16] and swede root [17]. However, no *in vitro* experiments of CADs have been conducted to examine the differences in substrate specificities between gymnosperms and angiosperms. The results of the present investigation show that the reducing activities of angiosperm and gymnosperm CADs for coniferaldehyde and sinapaldehyde differ markedly: the CAD of gymnosperm has a great affinity for coniferaldehyde but a low activity for sinapaldehyde, whereas for angiosperm CAD both aldehydes were good substrates. These findings may indicate that CAD is another enzyme which regulates the formation of guaiacyl and syringyl lignins in addition to *O*-methyltransferase (OMT).

Among gymnosperms, OMTs of *Thuja* species which contain unique lignans with pyrogallol-1-methyl ether groups or syringyl groups [19] showed relatively high activity with 5-hydroxyferulic acid although the lignin was found to be composed entirely of guaiacyl units [8]. These findings suggest that some regulations after the OMT reaction are indispensable to explain why *Thuja* lignins are of the guaiacyl type. The regulation seems to be operated partly by the CAD, which reduces conifer-

Table 3. Purification of Japanese black pine CAD

Purification step	Product	Protein (mg)	Sp. act. (nkat/mg)	Purification (fold)	Recovery (%)	Salc/Calc
Crude extract	Salc	560	0.041	1	100	0.079
	Calc		0.52	1	100	
$(NH_4)_2SO_4$ 30–70%	Salc	160	0.070	1.7	50	0.16
	Calc		0.45	0.86	25	
DEAE-cellulose	Salc	63	0.059	1.4	16	0.055
	Calc		1.1	2.0	23	
Sephadex G-100	Salc	15	0.056	1.4	3.7	0.027
	Calc		2.1	3.9	11	
Hydroxyapatite	Salc	2.0	0.22	5.4	2.0	0.022
	Calc		10	19	7.0	

Salc: sinapyl alcohol, Calc: coniferyl alcohol.

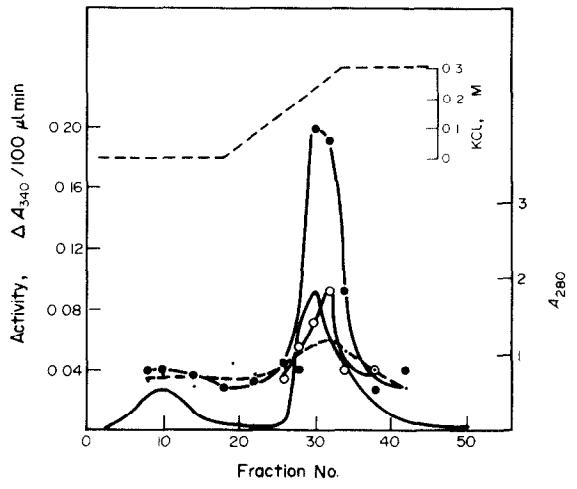

Fig. 2. DEAE-cellulose chromatography. ● Coniferaldehyde as substrate. ○ Sinapaldehyde. Control. A_{280}.

aldehyde more specifically than sinapaldehyde (Salc/Calc = 0.33).

It has been found that the ratios of syringaldehyde to vanillin (S/V) after alkaline nitrobenzene oxidation of lignin and those of sinapic acid to ferulic acid (SA/FA) in the OMT reaction were parallel in different families of plants [4]. Average SA/FA ratios [20] are almost as high as average S/V ratios [21]: 3.0 in angiosperm, 1.0 in grass and bamboo (monocotyledon), and 0.1 in gymnosperm. However, the ratios of guaiacyl to syringyl units in angiosperm lignins calculated on the basis of the methoxyl content is *ca* 1.0 [21] which is in good accordance with the Salc/Calc ratios in angiosperm CADs (average 1.09). Lignin formation in angiosperms is therefore considered to be regulated by the CAD.

Erythrina crista-galli (Leguminosae) contains a typical guaiacyl lignin, although the OMT belongs to the angiosperm type [6, 7]. The Salc/Calc value of *Erythrina* CAD was relatively low among angiosperms (0.65), but the contribution of the CAD to the regulation of lignin formation seems to be minor. The guaiacyl lignin formation in *Erythrina* could be ascribed to the low activity of ferulic acid-5-hydroxylase as shown by a tracer experiment [7].

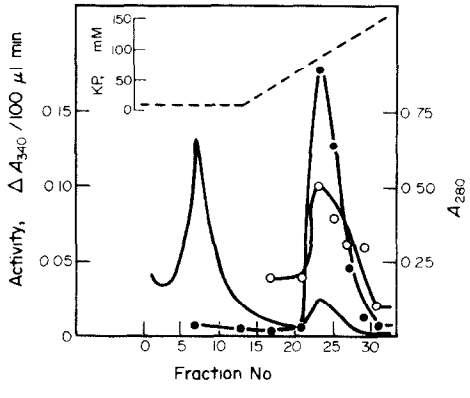

Fig. 3. Hydroxyapatite chromatography. ● Coniferaldehyde as substrate. ○ Sinapaldehyde (activity × 10). — · A_{280}.

Nakamura *et al.* [11] reported that both angiosperm and gymnosperm tissues reduce coniferaldehyde and sinapaldehyde to the corresponding alcohols by feeding experiments, which seems very likely because even gymnosperm CADs could reduce sinapaldehyde to 0.22-fold of coniferaldehyde on average.

It is thus concluded that both OMT and CAD play regulatory roles in the formation of guaiacyl and syringyl lignins. Ferulate-5-hydroxylase may also be involved in the regulation, although attempts to isolate the enzyme have not been successful. The functions of *p*-hydroxycinnamate:CoA ligase and cinnamoyl-CoA reductase in the regulation remain obscure.

The CAD of Japanese black pine was partially purified and characterized in the present investigation for the first time for a gymnosperm. Through all the steps 10% glycerol and 5 mM 2-mercaptoethanol were added to stabilize the enzyme and to avoid possible oxidation of NADPH by peroxidase. Multiple forms of CADs have been reported in soybean [16] and swede root [17] using DEAE cellulose CC, and in *Forsythia suspensa* [15], *Salix* species [14], etc. by electrophoresis. However, no multiple forms were observed in Japanese black pine CAD, which was eluted as a single peak containing both Calc and Salc activities through all the steps. The MW of Japanese black pine CAD (67000) was close to that of isoenzyme 2 of soybean CAD (69000), but different from those of isoenzyme 1 of soybean CAD [16] and *Forsythia suspensa* CAD [15] (43000 and 80000, respectively).

Partially purified Japanese black pine CAD showed the most affinity for coniferaldehyde among the aldehyde substrates examined. The same result was reported for soybean [16] and *Forsythia suspensa* [15] CADs. The ratio of Salc/Calc on the V_{max} values of Japanese black pine was 0.022 in contrast to 0.84 for isoenzyme 2 of soybean [16], which confirms that Japanese black pine CAD is much less active for sinapaldehyde than for coniferaldehyde.

EXPERIMENTAL

Materials. Cinnamaldehydes were synthesized by a new method via oxidation of the corresponding tetrahydropyranyl cinnamyl alcohols using activated MnO_2 as reported elsewhere [22]. Coniferyl and sinapyl alcohols were synthesized in the conventional way [23]. Seedlings of Japanese black pine, *Larix leptolepis* and *Zelkova serrata* were germinated at 27° on wet vermiculite for *ca* 1 month before harvest. The other plants were collected in the campus of our institute in May and June.

Enzyme extraction and purification. All buffers used for enzyme extraction and purification contained 10% glycerol and 5 mM 2-mercaptoethanol. Seedlings of Japanese black pine (100 g) were homogenized in a Waring blender and then in a mortar with 200 ml of 100 mM K-Pi buffer (pH 7.6), Polyclar AT (10 g), and sea sand (20 g, in mortar only). The homogenate was squeezed through gauze and the filtrate centrifuged at 10000 *g* for 30 min. The purification procedure was carried out by a modified method of ref. [8]: 30–70% $(NH_4)_2SO_4$ fractionation, Sephadex G-25, DEAE cellulose, Sephadex G-100 and hydroxyapatite columns; concn of enzyme soln after each chromatographic separation was performed by ultrafiltration instead of $(NH_4)_2SO_4$ pption. CADs of other plants were extrd using the same method on a 1/10 scale and the $(NH_4)_2SO_4$ ppts used for CAD assays after desalting through Sephadex G-25.

Enzyme assay. The incubation mixture contained 150 nmol cinnamaldehydes, 300 nmol NADPH (starter), 100 μl enzyme

Table 4. Substrate specificities of Japanese black pine CAD

Substrate	$K_M(\mu M)$	V_{max}(nkat/mg)	V_{max}/K_M (nkat/μM/mg)
NADPH	6.8	3.9	0.57
Coniferaldehyde	9.1	3.3	0.36
Cinnamaldehyde	14	7.6	0.53
p-Coumaraldehyde	30	5.7	0.19
3,4-Dimethoxycinnamaldehyde	43	0.46	0.011
Sinapaldehyde	—*	0.073	—

*The activity was too low to calculate the K_m.

soln in 2.7 ml, 200 mM K-Pi buffer (pH 6.5). Incubations were carried out at 30° for 5 min in UV cuvettes and the decrease in A_{340nm} was measured continuously. The incubation mixture without cinnamaldehydes was used as ref. A control reaction was run under the same condition using a sample soln and the ref. soln containing enzyme and NADPH, and enzyme only, respectively. The ΔA_{340} of the control reaction was subtracted from the apparent ΔA_{340} of the CAD reaction in question to obtain a net ΔA_{340}. The rates of formation of the cinnamyl alcohols were calculated from the average net ΔA_{340} divided by the $\varepsilon_{aldehyde}$ plus ε_{NADPH} according to the method of ref. [16].

Protein was determined by the Lowry method [24] using BSA as standard.

Product identification. The enzyme assay was scaled up five-fold and incubation was carried out for 1 hr at 30°. The product of CAD reaction using coniferaldehyde or sinapaldehyde was extrd with EtOAc and the alcohols identified by comparison with the corresponding authentic samples by HPLC using a μBondapak-C_{18} column (MeOH–H_2O, 1:1, 133 kg/cm^2 flow rate 2 ml/min) and by TLC (MeOH–$CHCl_3$, 1:19; hexane–EtOAc, 1:1). On TLC the products were detected by UV (254 nm) and by spraying with $FeCl_3$–$K_3Fe(CN)_6$ reagent [25].

REFERENCES

1. Creighton, R. H. J., Gibbs, R. D. and Hibbert, H. (1944) *J. Am. Chem. Soc.* **66**, 32.
2. Shimada, M., Fushiki, H. and Higuchi, T. (1972) *Phytochemistry* **11**, 2657.
3. Shimada, M., Kuroda, H. and Higuchi, T. (1973) *Phytochemistry* **12**, 2873.
4. Shimada, M., Fushiki, H. and Higuchi, T. (1973) *Mokuzai Gakkaishi* **19**, 13.
5. Kuroda, H., Shimada, M. and Higuchi, T. (1975) *Phytochemistry* **14**, 1759.
6. Kawamura, I., Shinoda, Y., Tran Van A. and Tanada, T. (1977) *Mokuzai Gakkaishi* **23**, 400.
7. Kutsuki, H. and Higuchi, T. (1978) *Mokuzai Gakkaishi* **24**, 625.
8. Kutsuki, H., Shimada, M. and Higuchi, T. (1981) *Mokuzai Gakkaishi* **27**, 39.
9. Higuchi, T. (1957) *Physiol. Plant.* **10**, 633.
10. Higuchi, T. (1962) *Can. J. Biochem. Physiol.* **40**, 31.
11. Nakamura, Y., Fushiki, H. and Higuchi, T. (1974) *Phytochemistry* **13**, 1777.
12. Conn, E. E., Kraemer, L. M., Liu, P.-N. and Vennesland, B. (1952) *J. Biol. Chem.* **194**, 143.
13. Elstner, E. F. and Heupel, A. (1976) *Planta* **130**, 175.
14. Mansell, R. L., Babbel, G. R. and Zenk, M. H. (1976) *Phytochemistry* **15**, 1849.
15. Mansell, R. L., Gross, G. G., Stöckigt, J., Franke, H. and Zenk, M. H. (1974) *Phytochemistry* **13**, 2427.
16. Wyrambik, D. and Grisebach, H. (1975) *Eur. J. Biochem.* **59**, 9.
17. Rhodes, M. J. C. and Wooltorton, L. S. C. (1975) *Phytochemistry* **14**, 1235.
18. Davies, D. D., Ugochukwu, E. N., Patil, K. D. and Towers, G. H. N. (1973) *Phytochemistry* **12**, 531.
19. Swan, E. P., Jiang, K. S. and Gardner, J. A. F. (1969) *Phytochemistry* **8**, 345.
20. Kuroda, H. and Higuchi, T. *Phytochemistry* (in press).
21. Sarkanen, K. V. and Hergert, H. L. (1971) *Lignins* (Sarkanen, K. V. and Ludwig, C. H., eds.) pp. 43–94. Wiley–Interscience, New York.
22. Kutsuki, H., Nakatsubo, F. and Higuchi, T. (1981) *Mokuzai Gakkaishi* **27**, 520.
23. Freudenberg, K. and Hubner, H. H. (1952) *Chem. Ber.* **85**, 1181.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randell, R. J. (1951) *J. Biol. Chem.* **193**, 265.
25. Barton, G. M., Evans, R. S. and Gardner, J. A. F. (1952) *Nature (London)* **170**, 249.