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DISTRIBUTION AND ROLES OF *p*-HYDROXYCINNAMATE:CoA LIGASE IN LIGNIN BIOSYNTHESIS

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Key Word Index—*Erythrina crista-galli*; Leguminosae; lignin biosynthesis; *p*-hydroxycinnamate:CoA ligase; ferulic acid; sinapic acid; guaiacyl lignin; syringyl lignin.

Abstract—*p*-Hydroxycinnamate:CoA ligases were extracted from the xylems of angiosperms and gymnosperms, and the substrate specificities toward ferulate and sinapate were examined. Most of angiosperm and gymnosperm CoA ligases examined were active with ferulate but not with sinapate; however, the enzymes of *Erythrina crista-galli*, *Robinia pseudoacacia* and bamboo showed considerable activity with sinapate. The other enzymes, although inactive with sinapate, showed no inhibitory effect on the *Erythrina* CoA ligase reaction with sinapate. The K_ms for sinapate and ferulate of the *Erythrina* enzyme were 1.0 and 2.1 μM, respectively, and *p*-hydroxycinnamate was the best substrate among cinnamates examined. The MW of the CoA ligase was 40 000 and the pH optimum was between 7.2 and 7.6. The possible roles of *p*-hydroxycinnamate:CoA ligase in lignin biosynthesis are discussed.

INTRODUCTION

Biochemical differences in lignin formation between gymnosperm and angiosperm have been mainly ascribed to the different substrate specificities of *S*-adenosyl-L-methionine:caffeate 3-*O*-methyltransferase (EC 2.1.1.-). In a previous report, we showed that cinnamyl alcohol dehydrogenase (EC 1.1.1.-) is another possible enzyme which regulates the formation of gymnosperm and angiosperm lignins; angiosperm enzyme reduced both coniferaldehyde and sinapaldehyde almost equally to the corresponding alcohols, whereas the gymnosperm enzyme acted preferentially on coniferaldehyde [1].

In the present paper, in a search for enzymes involved in the regulation of the formation of gymnosperm and angiosperm lignins, *p*-hydroxycinnamate:CoA ligase (EC 6.2.1.12) has been characterized. The enzyme was extracted from the xylems of angiosperm and gymnosperm and the substrate specificities were investigated using ferulate and sinapate, which are precursors of guaiacyl and syringyl lignin, respectively. We focused on sinapate:CoA ligase which is presumed to be indispensable to the biosynthesis of syringyl lignin but has not hitherto been characterized from a woody plant. The CoA ligase active with sinapate was extracted from the xylem of *Erythrina cristagalli*, partially purified and characterized.

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RESULTS

Substrate specificity of hydroxycinnamate:CoA ligase within different taxonomic groups

Since the activity of the CoA ligase was found to be consistently high in the xylem as shown in Table 1, the xylem of woody plants was always used as a source of the enzyme. Table 2 shows the substrate specificity of the CoA ligases extracted from the xylems of eight angiosperms, four gymnosperms and

Table 1. *p*-Hydroxycinnamate:CoA ligase activities of different tissues with ferulic acid (FA) and sinapic acid (SA)

Species	Substrate	Activity (pkat/mg)			
		Xylem	Phloem	Leaf	Petiole
Erythrina crista-galli	FA	497	51.5	0	37.7
	SA	189	19.8	0	17.9
Populus euramericana	FA	61	0	—	—
	SA	0	0	—	—

Table 2. Activities of *p*-hydroxycinnamate:CoA ligases with *p*-hydroxycinnamic acid (PA), ferulic acid (FA), sinapic acid (SA), caffeic acid (CA) and 5-hydroxyferulic acid (5HFA)

			Activity (pkat/mg)				
	Taxonomic group	Species	PA	FA	SA	CA	5HFA
Gymnosperm	Cupressaceae	*Chamaecyparis psifera*	—	73	0	—	—
		Juniperus chinensis	—	51	0	—	—
		Thuja orientalis	206	213	0	144	100
	Taxodiaceae	*Metasequoia glyptostroboides*	337	289	0	179	91
Angiosperm	Leguminosae	*Erythrina crista-galli*	128	70	30	55	15
		Robinia pseudoacacia	301	143	57	112	49
		Sophora japonica	—	44	0	—	—
	Salicaceae	*Populus euramericana*	155	137	0	95	51
	Bignoniaceae	*Paulownia tomentosa*	190	141	0	110	48
	Magnoliaceae	*Liriodendron tulipifera*	—	63	0	—	—
	Aceraceae	*Acer buergeriana*	—	260	0	—	—
	Rosaceae	*Prunus yedoensis*	—	43	0	—	—
Monocotyledon	Gramineae	*Phyllostachys bambusoides*	27	34	13	—	—

one monocotyledon. *p*-Hydroxycinnamate was almost always the best substrate among the five examined. Ferulate was converted into feruloyl-CoA by all the CoA ligases examined. In contrast, the CoA ligases were completely inactive with sinapate except those of *Erythrina*, *Acacia* and bamboo, in which the ratio of sinapate to ferulate activity was 0.40 on average. As expected no CoA ligase activities with sinapate were observed in gymnosperm enzymes, presumably because gymnosperm lignin lacks syringyl units [2]. However, since sinapate:CoA ligase is indispensable for the biosynthesis of syringyl lignin, the apparent absence of sinapate:CoA ligase activity in most of the angiosperms examined is surprising. The absence from poplar xylem was further tested by improving the extraction method, by looking for inhibitors of sinapate:CoA ligase and by varying the season when the plant was harvested. As shown in Table 3, the enzyme from the xylem powder prepared at 4°, under liquid N_2 or under ultrasonic was found to be inactive with sinapate. Besides adding ethylene glycol, mercaptoethanol and Polyclar AT to the standard extraction method, the effect of other additives was examined (Table 3), namely enzyme stabilizers (polyethylene glycol, sucrose, albumin), reagents to extract membrane-bound enzymes (Triton

Table 3. Effect of extraction methods and additives on the activity of poplar CoA ligase

		Activity (ΔA/min/mg)	
Extraction method	Additive	SA	FA
Mortar (4°)	None	0	0.044
	Sucrose 0.25 M	0	0.056
	PEG (6000)*0.5%	0	0.025
	Albumin	0	+
	Triton X-100 1%	0	+
	EDTA	0	+
Mortar (liq N_2)	None	0	0.036
	NaCl 0.6 M	0	0.096
	NaSCN 1 M	0	0
	Triton X-100 1%	0	0.067
Ultrasonic	None	0	+
	Triton X-100 1%	0	0.175

Additives were added into standard extraction mixture containing 10% ethylene glycol, 10 mM mercaptoethanol and Polyclar AT. Sinapic acid (SA) or ferulic acid (FA) was used as substrate.

*PEG: polyethylene glycol.

X-100, NaSCN), and reagents to separate extrinsic enzymes (EDTA, NaCl). In no case was there any sinapate:CoA ligase activity; however, the extraction of ferulate:CoA ligase was greatly improved by the addition of NaCl or Triton X-100.

When *Erythrina* CoA ligase active with sinapate was added to the reaction mixture of poplar CoA ligase after 3 min, the same amount of sinapoyl-CoA was newly formed as in control (Fig. 1). The same result was obtained by similar experiments using the CoA ligase of *Paulownia* or *Metasequoia* which are inactive with sinapate. These results show that interfering materials such as sinapoyl-CoA hydrolase, apyrase [3] have little effect on the activity of sinapate:CoA ligase. The xylem of poplar was harvested periodically from May to June when lignin formation was proceeding most actively. The CoA ligase extracted using standard extraction systems at every season was completely inactive with sinapate. These results suggest that the participation of sinapate:CoA ligase in syringyl lignin formation in poplar is doubtful.

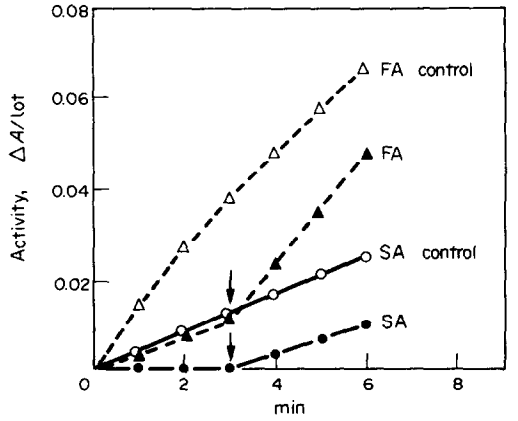

Fig. 1. Differences in *p*-hydroxycinnamate: CoA ligase reactions between *Populus euramericana* and *Erythrina cristagalli*. After the reaction of poplar enzyme for 3 min, *Erythrina* enzyme was added at the arrow. *Erythrina* enzyme was used as control reaction. Sinapic acid (SA) or ferulic acid (FA) was used as substrate.

Purification of hydroxycinnamate:CoA ligase of Erythrina crista-galli *using sinapate as substrate*

Enzyme stability. The crude CoA ligase solution containing 10 mM mercaptoethanol at 0° was completely inactivated in 10 days, whereas half of the activity was retained in the same period when stored at −20°. By the addition of 10% or 30% glycerol, or 10% ethylene glycol, the enzyme could be stored at −20° for 10 days without appreciable loss of activity.

Optimum pH. The activity of sinapate:CoA ligase proceeded optimally between pH 7.2 and 7.6 in 100 mM KP and 100 mM Tris–HCl buffers, and the half-maximal activity was observed at pH 6.6 and 8.3.

Purification. By $(NH_4)_2SO_4$ precipitation and Sephacryl S-200 chromatography, the CoA ligase activity was inactivated to 40% and 20% of the original activity, respectively. Therefore, ultrafiltration was commonly used to concentrate the enzyme, and the gel chromatography by a long column was omitted. Instead, a short Sepharose 4B column was used to remove the enzymes of low MW. The results for *Erythrina* CoA ligase are summarized in Table 4. The CoA ligase was purified 48- and 47-fold using sinapate and ferulate as substrate, respectively. Chromatography on DEAE-Sephacel (Fig. 2), hydroxyapatite and Sepharose 4B showed no multiple forms of the CoA ligase.

Molecular weight. The CoA ligase active with both sinapate and ferulate was eluted as one peak from the Sephacryl S-200 column calibrated with BSA, ovalbumin, chymotrypsinogen and cytochrome *c*. The estimated MW was about 40 000.

Cofactor requirements. Table 5 demonstrates the cofactor requirement of the CoA ligase reaction with sinapate as substrate. Co^{2+}, Mn^{2+} and Ni^{2+} were remarkably efficient next to Mg^{2+}, whereas Zn^{2+} caused complete inhibition.

Substrate specificity. The sinapate:CoA ligase of *Erythrina* was specific for ATP, and the other

Table 4. Purification of *p*-hydroxycinnamate:CoA ligase of *Erythrina*

Purification step	Substrate	Protein (mg)	Specific activity (pkat/mg)	Purification (fold)	Recovery (%)	SA/FA
Crude extract	SA	235	47	1	100	0.36
	FA		130	1	100	
Concentrated solution	SA	113	93	2	96	0.33
	FA		280	2	108	
DEAE-Sephacel	SA	20.8	490	11	92	0.33
	FA		1500	12	104	
Hydroxyapatite	SA	7.7	930	20	65	0.34
	FA		2700	21	69	
Sepharose 4B	SA	2.4	2300	48	47	0.38
	FA		6100	47	48	

SA: sinapic acid, FA: ferulic acid

nucleotides such as UTP, CTP, GTP and TTP did not substitute for ATP. As listed in Table 6, *p*-coumarate was the best substrate, followed by ferulate and isoferulate, and the activity for sinapate was 48% that of ferulate. The ligase had no activity with cinnamate, which suggests that hydroxyl substituents *para* and/or *meta* to the side-chain of cinnamate seem to be necessary for CoA ligase activity.

The K_m values of sinapate and ferulate were 1.0 and 2.1 μM, respectively, i.e. the affinity of sinapate for the CoA ligase was twice as high as that of ferulate. On the other hand, V_{max} values of sinapate (570 pkat/mg) were only 0.22 times that of ferulate (2600 pkat/mg). The ratio of V_{max}/K_m of sinapate to ferulate was 0.48, indicating that *Erythrina* CoA ligase has a high activity for sinapate.

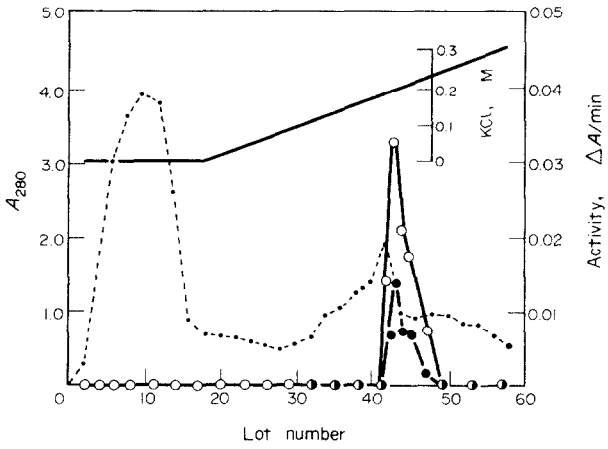

Fig. 2. DEAE-Sephacel CC of *Erythrina* CoA ligase; ○ ferulic acid as substrate, ● sinapic acid as substrate, · · · A_{280}, —— KCl gradient.

Table 5. Cofactor requirement of purified *Erythrina* CoA ligase

Metal ions*	Activity (pkat/mg)	Relative activity (%)
Mg^{2+}	740	100
Co^{2+}	720	97
Mn^{2+}	630	85
Ni^{2+}	600	81
Ba^{2+}	470	64
Ca^{2+}	420	57
K^{+}	370	50
Zn^{2+}	0	0

*Final concentration was 1.5 mM.

DISCUSSION

p-Hydroxycinnamate:CoA ligase has been found to be widely distributed among many different plants, using ferulate as substrate [4]. However, no experiments have hitherto been done on the distribution of CoA ligase specific to sinapate among woody plants. We found that sinapate:CoA ligase could not be detected in the gymnosperms or in most of the angiosperms examined, although activity was found in *Erythrina*, *Acacia* and bamboo. Sinapate CoA ligases have been reported in swede root [5], soya bean [6, 7], petunia [8], pea, runner bean, leek [7] and poplar [9], while no activities were observed in *Forsythia* [10], *Acer*, yew [4], tomato [11], potato [3] and carrot [12]. It is interesting that most of the species containing sinapate:CoA ligase belong to Leguminosae. Grand and Ranjeva reported that poplar CoA ligase was active with sinapate [9], but in our experience this activity could not be found under any conditions. This could be partly due to differences in the variety of poplar examined.

Since angiosperm lignin is rich in syringyl units [2], the CoA ligase has to be active with sinapate. However, the fact that such activities were not always found in angiosperms suggests another possible pathway: sinapate is converted into sinapaldehyde via some intermediate other than sinapoyl-CoA; without passing through sinapate, sinapaldehyde is formed via, for example, 5-hydroxyferulate, 5-hydroxyferuloyl-CoA and 5-hydroxyconiferaldehyde. As for the latter possibility, the substrate specificity of CoA ligase in Table 2 showed that 5-hydroxyferulate was readily converted into its CoA ester by both angiosperm and gymnosperm CoA ligases. The involvement of sinapate:CoA ligase in syringyl lignin formation, however, cannot be ruled out completely since some plants have sinapate:CoA ligase, as mentioned above.

The characteristics of the purified *Erythrina* CoA ligase active with sinapate were almost the same as those of the CoA ligases reported earlier: the enzymes are most specific for *p*-hydroxycinnamate, and cinnamate is one of the poorest sub-

Table 6. Substrate specificity of purified *Erythrina* CoA ligase

Substrates	Activity (nkat/mg)	Relative activity (%, FA = 100%)
p-Coumaric acid	3.5	140
Ferulic acid	2.5	100
Isoferulic acid	2.5	100
3, 4-Dimethoxycinnamic acid	2.1	84
p-Methoxycinnamic acid	1.7	68
Sinapic acid	1.2	48
Caffeic acid	1.2	48
5-Hydroxyferulic acid	0.42	17
Cinnamic acid	0	0

strates; they require ATP and Mg^{2+} which can be replaced by Co^{2+}, Mn^{2+}, or Ni^{2+}; and the addition of glycerol or ethylene glycol stabilizes them. On the other hand, no multiple forms of *Erythrina* CoA ligase were observed during purification, unlike soya bean [6], pea [7] and petunia [8] CoA ligases. The MW of *Erythrina* CoA ligase (40 000) was less than that of soya bean (55 000) [13], *Forsythia* (55 000) [10] and pea (75 000) [6]. The remarkably high affinity for sinapate of *Erythrina* CoA ligase was found from the K_m value (1 μM) in comparison with 11 μM (soya bean, isoenzyme 1) [6], and 170 μM (petunia) [8].

Erythrina is known to contain guaiacyl-rich lignin although it is an angiosperm, and has angiosperm-type *O*-methyltransferase [14] and cinnamyl alcohol dehydrogenase [1], i.e. the transferase methylates 5-hydroxyferulate to sinapate and the dehydrogenase reduces sinapaldehyde to sinapyl alcohol efficiently. Moreover, *Erythrina* CoA ligase was found to have great activity with sinapate. These results suggest that the syringyl deficient nature of *Erythrina* lignin is due to the low activity of ferulate-5-hydroxylase, which has not so far been identified [15].

EXPERIMENTAL

Material. All the cinnamate derivatives used were synthesized from the corresponding benzaldehydes and malonic acid in the conventional way [16]. Authentic feruloyl-CoA and sinapoyl-CoA were synthesized according to ref. [17] Woody plant materials except *Erythrina* were collected in the campus of our institute in May and June. *Erythrina* was harvested at the Botanic Garden, Kansai Branch Office of Forestry Experiment Station, Kyoto.

Extraction and purification of enzyme. All the buffers used contained 10% ethylene glycol and 10 mM mercaptoethanol. The shoots of *Erythrina* were freed of extracambial tissue and the xylem was cut into pieces (150 g). The plant material was homogenized in a chilled mortar with 300 ml Tris, pH 7.3, Polyclar AT (15 g) and sea sand (75 g). The homogenate was squeezed with cheese-cloth and centrifuged at 10000 *g* for 15 min. The supernatant (240 ml) was concentrated to 83 ml by ultrafiltration and the solution was applied to a DEAE-Sephacel column (1.5 × 11 cm). The protein fractions were eluted from the DEAE-Sephacel by a linear gradient of KCl (0–0.3 M) in 100 mM Tris, pH 7.3, in a total vol. of 350 ml. After the enzyme activity of the fractions was tested with ferulate and sinapate, the active fractions (50 ml) were collected and concentrated to 2.5 ml by ultrafiltration. The protein solution was layered on a hydroxyapatite column (1.5 × 8.5 cm) and eluted by a linear gradient of KPi (10–250 mM), pH 7.3, in a total vol. of 180 ml. The active solution (34 ml) was concentrated in the same way and passed through a Sepharose 4B column (1.5 × 5.5 cm) with 10 mM KPi, pH 7.3. The fractions containing the highest enzyme activity (25 ml) were pooled and used for the characterization of the CoA ligase of *Erythrina*.

The CoA ligase of other plants were extracted in the same way on 1/15 scale and the $(NH_4)_2SO_4$ precipitates (75% saturation) were used for the enzyme assay after desalting through Sephadex G-25.

Enzyme assay. The incubation mixture contained 500 nmol cinnamic acids, 2 μmol ATP and $MgSO_4$, 50 nmol CoASH (starter) and 10–100 μg enzyme in 2.74 ml of 100 mM Tris pH 7.3. The reaction mixture was incubated at 30° for 5 min in UV cuvettes and the increase in absorbance of λ_{max} values of cinnapoyl-CoA esters was measured. The incubation mixture without CoASH was used as reference. ϵ and λ_{max} values of cinnamoyl-CoA derivatives in refs. [17] and [18] were employed for calculation of the activity.

Protein determination. Protein concentration was determined by the Lowry method using BSA as standard [19].

Product identification. Standard assays were done using ferulate-2[^{14}C] and sinapate-2-[^{14}C] as substrate. The product separated on PC by the method of ref. [6] was identified in comparison with R_f and λ_{max} values with those of authentic feruloyl-CoA and sinapyl-CoA.

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REFERENCES

1. Kutsuki, H., Shimada, M. and Higuchi, T. (1982) *Phytochemistry* **21**, 19.
2. Creighton, R. H. J., Gibbs, R. D. and Hibbert, H. (1944) *J. Am. Chem. Soc.* **66**, 32.
3. Rhodes, M. J. C. and Wooltorton, L. S. C. (1975) *Phytochemistry* **14**, 2161.
4. Gross, G. G., Mansell, R. L. and Zenk, M. H. (1975) *Biochem. Physiol. Pflanzen* **168**, 41.
5. Rhodes, M. J. C. and Wooltorton, L. S. C. (1973) *Phytochemistry* **12**, 2381.
6. Knobloch, C. -H. and Hahlbrock, K. (1975) *Eur. J. Biochem.* **52**, 311.
7. Wallis, P. J. and Rhodes, M. J. C. (1977) *Phytochemistry* **16**, 1891.
8. Ranjeva, R., Boudet, A. M. and Faggion, R. (1976) *Biochimie* **58**, 1255.
9. Grand, C. and Ranjeva, R. (1979) *Physiol. Vég.* **17**, 433.
10. Gross, G. G. and Zenk, M. H. (1974) *Eur. J. Biochem.* **42**, 453.
11. Rhodes, M. J. C. and Wooltorton, L. S. C. (1976) *Phytochemistry* **15**, 947.
12. Heinzmann, U., Seitz, U. and Seitz, U. (1977) *Planta* **135**, 313.
13. Lindl, T., Kreuzaler, F. and Hahlbrock, K. (1973) *Biochim. Biophys. Acta* **302**, 457.
14. Kawamura, I., Shinoda, Y., Tran Van Ai and Tanada, T. (1977) *Mokuzai Gakkaishi* (*J. Jpn Wood Res. Soc. Tokyo*) **23**, 400.
15. Kutsuki, H. and Higuchi, T. (1978) *Mokuzai Gakkaishi* (*J. Jpn Wood Res. Soc. Tokyo*) **24**, 625.
16. Neish, A. C. (1959) *Can. J. Biochem. Physiol.* **87**, 1431.
17. Stöckigt, J. and Zenk, M. H. (1975) *Z. Naturforsch.* **30c**, 352.
18. Hrazdina, G., Kreuzaler, F., Hahlbrock, K. and Grisebach, H. (1976) *Arch. Biochem. Biophys.* **175**, 392.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.