Phytochemistry, Vol. 23, No. 8, pp. 1557–1559, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00

PROPERTIES OF HYDROXYCINNAMATE:CoA LIGASE FROM STEMS OF *SALIX BABYLONICA* CULTURED *IN VITRO*

A. FEUTRY and R. LETOUZE*

Laboratoire de Recherche de Physiologie Végétale, 16 Boulevard Lavoisier, 49000 Angers, Belle Beille, France

(*Revised received* 11 *January* 1984)

Key Word Index—*Salix babylonica*; Salicaceae; weeping willow; *in vitro*; hydroxycinnamate: CoA ligase; lignin biosynthesis.

Abstract—Hydroxycinnamate: CoA ligase was extracted from stems of *in vitro* willow cultures and characterized. One peak of activity was obtained after column chromatography on Sephadex G 100 or DEAE Sephacel. *p*-Coumaric acid gave the highest V_{max} among the cinnamates examined. The K_m values for *p*-coumaric, caffeic and ferulic acid were 31.0, 4.7 and 46 μM, respectively. The MW of the CoA ligase was 57 000 and the pH optimum was 7.0. The characteristics of the enzyme correspond to its physiological role in lignin biosynthesis.

INTRODUCTION

Physiological studies on the lignin pathway have been carried out either with cultivated cells and tissues [1–4] or with developed plants grown in glasshouses or in the field [5, 6]. These different plant materials have been used to obtain some very important results, particularly with regard to enzyme regulation and the relationship between enzyme specificity and lignin composition [7, 8]. However, the environmental conditions and the early stages of lignification are difficult to control with developed plant material.

In vitro cultures of willow (*Salix babylonica* L.) cuttings appear to be an attractive experimental model for studying the lignin pathway, as lignification is a well defined process of differentiation and the use of cuttings should allow the study of the variations of this process with time on a growing plant. Furthermore, the growth parameters of this material are easily controlled.

In the present work, one of the more important enzymes involved in the lignin pathway, hydroxycinnamate: CoA ligase (EC 6.2.1.12.), has been extracted from the stems of willow cuttings and characterized.

RESULTS AND DISCUSSION

Enzyme purification

The CoA ligase of *S. babylonica* stems cultured *in vitro* was purified by precipitation with ammonium sulphate followed by column chromatography of the resulting pellet on a Sephadex G-100 or DEAE Sephacel. The ligase was recovered from the gel filtration step within one peak. Its MW was estimated at about 57 000, a value which is very near to that established for CoA ligase from soybean [9] and *Forsythia* [10].

The desalted ammonium sulphate pellet was chromatographed on DEAE Sephacel using a 0–300 mM potassium chloride gradient. One peak of activity was obtained with *p*-coumaric, caffeic and ferulic acids as substrates (Fig. 1.). Unlike CoA ligase from soybean [9], pea and *Petunia* [12], no multiple forms were detected.

Kinetic properties

The optimal concentrations for *p*-coumaric, caffeic and ferulic acids were 0.6, 0.2 and 0.4 mM respectively. At higher concentrations, these substrates were inhibitory. This inhibition was particularly strong with *p*-coumaric acid (Fig. 2). Substrate inhibition also occurred with CoA (Fig. 3). The optimal concentration was 0.02 mM. However, to obtain an initial linear phase over a long enough period for measurement, it was necessary to use a higher concentration (0.1 mM). Inhibition by an excess of cinnamic acids and CoA have been described for ligase from *Forsythia* [9], soybean [10] and parsley [13] cell culture. The saturation curves for ATP and Mg^{2+} followed the normal Michaelis–Menten kinetics. Concentrations for maximum activity were 1 mM and 10 mM respectively. The K_m determined by Lineweaver and Burk plots for ATP was 32 μM.

pH and buffer effect

The activity of hydroxycinnamate: CoA ligase was optimal at pH 7; the pH values for half maximum activity were 6.2. and 8. For the same pH, the activity was always slightly higher in phosphate buffer. With CoA ligase from soybean and parsley cell cultures and from *Petunia* [9, 12, 13], the optimum pH values were between 7.8. and 8.5. However, with enzymes extracted from lignifying tissues of *Forsythia* [6], pea [10] and *Erythrina cristagalli* [11], the values were lower, 7.2–7.6.

Specificity for different cinnamic acids

Cinnamic and sinapic acids were never activated (Table 1). The highest V_{max} value was obtained with *p*-coumaric acid. For *p*-coumaric, caffeic and ferulic acids,

*To whom correspondence should be addressed.

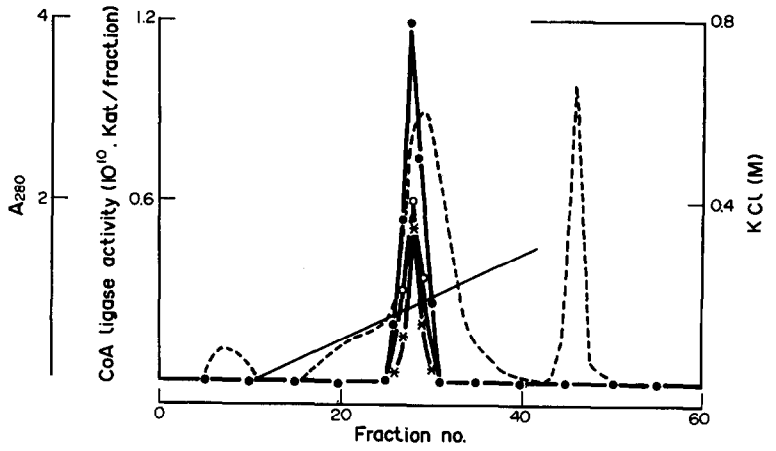

Fig. 1. Elution profile of willow CoA ligase activity against *p*-coumaric (●), caffeic acid (*) and ferulic acid (○) after chromatography on a DEAE Sephacel column; ---, A_{280}; ——, KCl gradient.

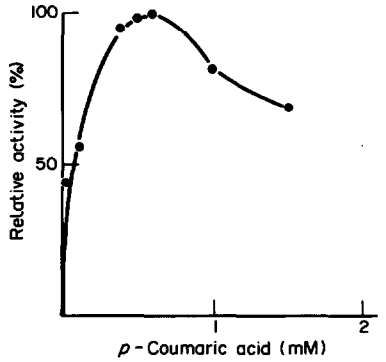

Fig. 2. Effect of *p*-coumaric acid on the activity of hydroxycinnamate: CoA ligase extracted from willow stems.

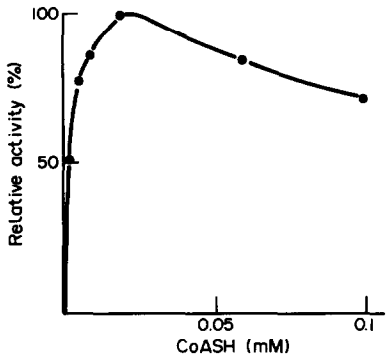

Fig. 3. Effect of CoA on the activity of *p*-hydroxycinnamate: CoA ligase extracted from willow stems.

K_m values were determined by Lineweaver and Burk plots (Table 1). These values showed a clear-cut specificity of willow CoA ligase for hydroxylated cinnamic acids.

Hydroxycinnamate: CoA ligase is the last of the enzymes of the general pathway of phenylpropanoid metabolism [14]. After activation, cinnamic acids can enter different pathways, the main ones being those leading to esters, flavonoids and lignin pathways [15]. Different studies have shown the occurence of CoA ligase isoenzymes [9, 11, 12]. These isoenzymes could control the direction of phenylpropanoid metabolism. In willow stems, no multiple forms of hydroxycinnamate: CoA ligase were observed during purification.

Two facts testify to a physiological role of the enzyme studied in lignin biosynthesis. First, it was extracted from stems. Second, it presented similar properties (optimal pH

Table 1. Substrate specificity of willow CoA ligase

	V		
Substrates	Activity (10^{10}. kat/g fr. wt.)	Relative activity (%, *p*-coum A = 100%)	K_m (μM)
Cinnamic acid	0	—	—
p-Coumaric acid	5.0	100	31
Caffeic acid	2.17	43	4.7
Ferulic acid	3.05	61	46
Sinapic acid	0	—	—

value, substrate specificity) to hydroxycinnamate: CoA ligase from lignifying tissue. The high affinity for hydroxylated cinnamic acids only could be explained by the early stage of lignification of our material [8].

As shown by the K_m values, the willow enzyme shows a high affinity for caffeate. This result may be linked to the high rate of pigment synthesis in willow cuttings placed in a medium rich in sugar (> 20 g/l.) or in strong light (20 Wm^{-2} continuous lighting).

In conclusion, the results reported here confirm the usefulness of willow cuttings cultured *in vitro* as a model for studying the lignification process. The work is being continued with the characterisation of cinnamyl alcohol dehydrogenase and the qualitative and quantitative assays of lignin under various conditions of culture.

EXPERIMENTAL

Plant material. Cuttings of willow cultured *in vitro* were used. The origin of the clone and its culture conditions have been previously described [18]. The properties of hydroxycinnamate: CoA ligase were investigated on stems of a 49-day-old plant.

Enzyme extraction. Stems were ground at 3° with quartz sand in 0.1 M KH_2PO_4–NaOH buffer (1 ml/0.15 g fr. wt tissue), pH 7, containing 15 mM mercaptoethanol and 30% glycerol. Polyclar AT (water-insoluble polyvinylprolidone for binding phenols) was added in the proportion of 80% of fr. wt. The homogenate was centrifuged at 80000 *g* for 20 min. The supernatant, to which BSA was added to obtain a final concentration of 0.1%, was used for enzyme assay after Sephadex G 25 filtration.

Enzyme assay. The assay mixture contained: 0.2–0.6 μmol cinnamic acid, 1 μmol ATP, 3,5 μmol $MgCl_2$, 0.1 μmol CoA (starter), 35 μmol Pi buffer (pH 7) and 0.5 ml of enzyme soln in a total vol. of 1 ml. The reaction mixture was incubated up to 30°. Then, after CoA addition, the increase in absorbance of λ_{max} values of cinnamoyl ester was measured. A control incubation contained no CoA. Appropriate λ_{max} and ε values for the different products were used according to refs [19, 20].

Column chromatography. All the buffers used contained 15 mM mercaptoethanol and 10% glycerol. For Sephadex G 100 filtration, after extraction and centrifugation in 0.02 M Na, K-Pi buffer, pH 7, the soluble proteins were precipitated by $(NH_4)_2SO_4$ (75% saturation). After centrifugation (15 min, 20000 *g*) the precipitate was redissolved in 8 ml 0.1 M of the same buffer at 0.1 M. This soln was filtered through a Sephadex G 100 column (2.6 × 40 cm). All fractions containing proteins were tested for hydroxycinnamate: CoA ligase activity.

For DEAE Sephacel CC, 2.5 g fr. wt of plant material was ground in 0.1 M Tris–HCl, pH 7.3. The precipitate obtained on treatment with $(NH_4)_2SO_4$ was redissolved in 0.05 M Tris–HCl, pH 7.3, and then desalted on a Sephadex G 25 column. The protein extract was layered on the top of a DEAE Sephacel column (1.6 × 15 cm) previously equilibrated with the same buffer, and eluted by a linear gradient of KCl (0–0.3 M KCl in 0.05 M Tris–HCl, pH 7.3). Five-ml fractions were collected and tested for CoA ligase activity.

REFERENCES

1. Rhodes, M. J. C. and Wooltorton, L. S. C. (1973) *Phytochemistry* **12**, 2381.
2. Yamada, Y. and Kuboi, T. (1976) *Phytochemistry* **15**, 395.
3. Hahlbrock, K. and Grisebach, H. (1979). *Ann. Rev. Plant Physiol.* **30**, 105.
4. Hahlbrock, K., Schroder, J. and Vieregge J. (1980) *Adv. Biochem. Eng.* **18**, 39.
5. Grand, C. and Ranjeva, R. (1979) *Physiol. Vég.* **17**, 433.
6. Kutsuki, H., Shimada, M. and Higuchi, T. (1982) *Phytochemistry* **21**, 267.
7. Grisebach, H. (1977) *Naturwissenschaften* **64**, 619.
8. Gross, G. G. (1980) *Adv. Bot. Res.* **8**, 25.
9. Knobloch, K. H. and Hahlbrock, K. (1975) *Eur. J. Biochem.* **52**, 311.
10. Gross, G. G. and Zenk, M. H. (1974) *Eur. J. Biochem.* **42**, 453.
11. Wallis, P. J. and Rhodes, M. J. C. (1977) *Phytochemistry* **16**, 1891.
12. Ranjeva, R. Boudet, A. M. and Faggion, R. (1976) *Biochimie* **58**, 1255.
13. Knobloch, K. H. and Hahlbrock, K. (1977) *Arch. Biochem. Biophys.* **184**, 237.
14. Ebel, J., Schaller-Hekeler, B., Knobloch, K. H., Wellmann, E., Grisebach, H. and Hahlbrock, K. H. (1974) *Biochim. Biophys. Acta* **362**, 417.
15. Hahlbrock, K. and Grisebach, H. (1979) *Ann. Rev. Plant Physiol.* **30**, 105.
16. Letouzé, R. (1975) *Planta* **123**, 155.
17. Letouzé, R. and Daguin, F. (1983) *Rev. Can. Biol. Exptl.* **42**, 23.
18. Letouzé, R. (1970) *C.R. Acad. Sci. Paris* **2309**, 272.
19. Gross, G. G. and Zenk, M. H. (1966) *Z. Naturforsch.* **21**, 683.
20. Gross, G. G., Mansell, R. L. and Zenk, M. H., (1975) *Biochem. Physiol. Pflanzen* **168**, 41.