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# Isoenzymes of hydroxycinnamate:CoA ligase from poplar stems properties and tissue distribution

C. Grand, A. Boudet and A.M. Boudet
Centre de Physiologie Végétale, Laboratoire Associé au C.N.R.S. N° 241, Université Paul Sabatier, 118, route de Narbonne, F-31062 Toulouse Cédex, France

**Abstract.** Three different forms of hydroxycinnamate:CoA ligase (EC 6.2.1 –) have been separated by chromatofocusing from poplar stems. These three forms exhibit different substrate specificities and tissue distribution. A correlation was established between the monomeric composition of lignins isolated from xylem and sclerenchyma and the pattern of hydroxycinnamate:CoA ligase isoforms in these tissues. The results obtained indicate that, in poplar, the hydroxycinnamate:CoA ligase isoenzymes could play an important role in the control of the monomeric composition of lignins.

**Key words:** Hydroxycinnamate:CoA ligase (tissue distribution) – Lignification – *Populus*.

## Introduction

Under natural conditions, large differences in the monomeric composition of lignins are observed not only between plant species but also within one and the same plant according to the location in the tissues and cells (Grand et al. 1982). The differences between gymnosperm and angiosperms lignins have been mainly ascribed to the differential ability of these plants to synthesise the three lignin monomers. Several enzymes involved in the synthesis of lignin monomers: S-adenosyl-L-methionine, caffeate 3-O-methyltransferase (EC 2.1.1.6.) (Shimada et al. 1973; Kuroda et al. 1975), hydroxycinnamate:CoA ligase (Lüderitz et al. 1982), cinnamoyl:CoA reductase (EC 1.2.1.44) (Lüderitz and Grisebach 1981), and cinnamyl alcohol dehydrogenase (EC 1.1.1.2.) (Kutsuki et al. 1982a) have been demonstrated to exhibit particular substrate specificities towards their potential substrates according to the plant species from which they were isolated. These characteristics could explain the different production of the basic monomeric units involved in the synthesis of the polymer. In contrast, the biological mechanism responsible for the specific monomeric composition of lignins located in different tissues of one and the same plant is not yet well understood.

In poplar stems, the sclerenchyma is enriched in syringyl units as compared to xylem (Grand and Ranjeva 1979). Previous studies in our laboratory established a correlation between the substrate specificity of the hydroxycinnamate:CoA ligase (EC 6.2.1.–) isolated from these tissues and the monomeric composition of their lignins. The enzyme from sclerenchyma is more active towards sinapate, the precursor of syringyl units, than the enzyme from xylem. These specific activation patterns could be explained by a specific distribution in each tissue of different isoforms of hydroxycinnamate:CoA ligase.

In this work, we separated three hydroxycinnamate:CoA ligases from poplar stems and we studied their properties and their tissue distribution. The results are discussed in relation to the involvement of hydroxycinnamate:CoA ligase in the qualitative control of the lignification process.

## Material and methods

*Plant material. Populus x euramericana* (Dode cv. "I 214") plants were grown in controlled conditions and the tissue fractions (xylem, sclerenchyma and parenchyma) were isolated from the stems according to procedures already described (Grand and Ranjeva 1979). The individual tissue fractions were lyophilised and finely powdered before use.

*Protein extraction and enzyme purification.* All procedures were carried out at 4° C. Fifteen g of the powdered material were homogenized in a Turmix homogenizer (Janke Kunkel, FRG) for four minutes in 200 ml 0.1 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl buffer (pH 7.5) containing 10 g of

Polyclar AT (Sigma Chemical 6., St. Louis, Mo., USA), 5 g of acid-washed quartz sand, 0.5% polyethyleneglycol ($M_r$ 6,000) and 15 mM mercaptoethanol. The crude extract was filtered through a nylon net (pore size 35 µm) and centrifuged at 45,000 *g* for 30 min. The supernatant was fractionated by ammonium-sulphate precipitation in the range of 40–80% saturation. The pellet was dissolved in 10 ml of 25 mM histidine-HCl buffer (pH 6.4) (30% glycerol v/v) and desalted through a Sephadex G 50 "medium" column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with the same buffer.

This crude extract was then chromatofocused on a polybuffer exchanger column PBE 94 (Pharmacia) (equilibrated in 25 mM histidine buffer, pH 6.5) with 250 ml of polybuffer 74 (Pharmacia) as the elution buffer. Fractions of 5 ml were collected and assayed for activities.

*Assay for hydroxycinnamate:CoA-ligase activities.* The enzyme activities were determined at 30° C by a direct spectrophotometric method (Ranjeva et al. 1976). The reactions mixtures, which contained 30% glycerol (v/v) in a final volume of 1.5 ml included 0.5 mM hydroxycinnamic acid, 1.5 mM ATP, 3.5 mM $MgCl_2$, 0.15 mM CoA, 0.5 ml of protein extract containing 1 mg $ml^{-1}$ bovine serum albumin and 0.1 M Tris-HCl buffer (pH 7.8). The esterification of each acid was determined by the increase in absorbance caused by the formation of the corresponding thioester. Measurements were performed at the wavelengths given by Stöckigt and Zenk (1975).
The protein was estimated with the Biorad assay (Bradford 1976).

## Results

The hydroxycinnamate:CoA-ligase activity was very unstable in 0.1 M Tris-HCl buffer (pH 7.5) but was stabilized by the presence of 30% glycerol or 15% ethylene glycol. Under these conditions it was possible to store the enzyme for two days at 0° C or for several weeks at −20° C without appreciable loss of activity. All of the following steps were therefore carried out in buffer containing 30% glycerol.

*Purification and separation of hydroxycinnamate: CoA ligase isoenzymes.* After precipitation of proteins by ammonium sulphate in the range of 40–80% saturation, the enzyme extract obtained from poplar stem was chromatofocused. This procedure allowed separation of three forms of hydroxycinnamate:CoA ligase when *p*-coumaric acid was used as substrate (Fig. 1). These forms which differed in their isoelectric point, were purified 15- to 70-fold (based on activity in 40–80% cut; Table 1) with a yield of 85% calculated from the sum of the activities of three forms.

In order to determine whether artefactual modifications of a single form had occurred during the purification procedure, form I, which was present in the largest quantities, was chromatofocused a second time under the same conditions. The enzyme activity was eluted as a single peak at the same pH as previously.

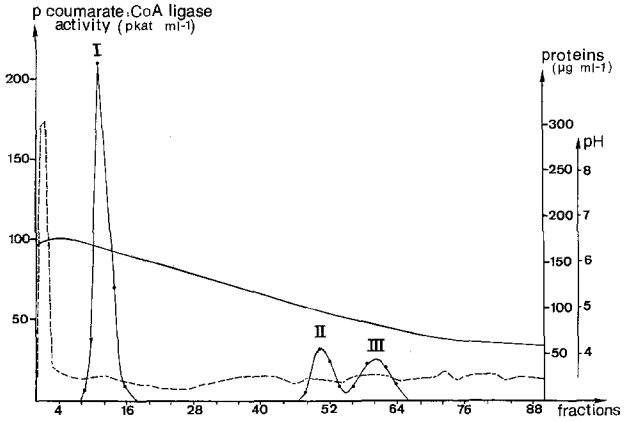


**Fig. 1.** Chromatofocusing elution profile of hydroxycinnamate:CoA ligase isoenzymes. -●—●-: p-coumarate:CoA-ligase activity; --------: proteins; ——: pH gradient

**Table 1.** Characteristics of hydroxycinnamate:CoA-ligase isoenzymes after chromatofocusing. The enzyme assays were carried out with *p*-coumaric acid

| | Isoelectric point | Specific acitivity nkat $g^{-1}$ protein | Purification factor |
|---|---|---|---|
| Form I | 6.35 | 1,928 | 72 |
| Form II | 5 | 247 | 14 |
| Form III | 4.4 | 295 | 16 |

**Table 3.** Tissue distribution of hydroxycinnamate:CoA-ligase isoenzymes

| Enzymatic forms | *p*-coumarate:CoA-ligase activity (nkat $g^{-1}$ protein) | | |
|---|---|---|---|
| | Xylem | Sclerenchyma | Parenchyma |
| Form I | 2,240 | 1,280 | 25 |
| Form II | 358 | n.c.[a] | 72 |
| Form III | 385 | n.d. | 147 |

[a] n.c. = not detectable

*Product identification.* The products, resulting from the incubation of the enzymes for 1 h with different cinnamic acids, were isolated as described by Ranjeva et al. (1976) and identified by paper chromatography [*n*-butanol/acetic acid/$H_2O$ 5/2/3; by vol.] and ultraviolet spectroscopy ($\lambda$ max). The properties of these reaction products were shown to be identical to those of the synthetic cinnamoyl-:CoA's corresponding to the cinnamic-acid substrates.

*Enzyme activity dependence.* No significant difference in optimum pH was observed between the isoenzymes. For all three forms, the *p*-coumarate:

**Table 2.** Substrate specificity of the separate forms of hydroxycinnamate:CoA ligase[a]

| Substrate | Form I | | | Form II | | | Form II | | |
|---|---|---|---|---|---|---|---|---|---|
| | $10^5$ Km (mol $l^{-1}$) | V (pkat $mg^{-1}$ protein) | $10^{-5}$ V/Km ((pkat $mg^{-1}$ protein)/(mol $l^{-1}$)) | $10^5$ Km (mol $l^{-1}$) | V (pkat $mg^{-1}$ protein) | $10^{-5}$ V/Km ((pkat $mg^{-1}$ protein)/(mol $l^{-1}$)) | $10^5$ Km (mol $l^{-1}$) | V (pkat $mg^{-1}$ protein) | $10^{-5}$ V/Km ((pkat $mg^{-1}$ protein)/(mol $l^{-1}$)) |
| *p*-Coumaric acid | 4.2 | 2,240 | 533 | 1 | 275 | 275 | 1.3 | 315 | 242 |
| Caffeic acid | n.c[b] | — | — | n.c | — | — | 1 | 540 | 540 |
| Ferulic acid | 2 | 936 | 468 | 1.1 | 435 | 395 | 1.5 | 450 | 300 |
| 5-OH-Ferulic acid | 1.7 | 1,120 | 658 | n.c | — | — | n.c | — | — |
| Sinapic acid | 2.2 | 560 | 254 | n.c | — | — | n.c | — | — |

[a] Hydroxycinnamate:CoA ligase is the general name for the enzyme involved in the esterification of different cinnamic acids into their thioesters of CoA. However, for specific esterification of eg. *p*-coumaric, ferulic, sinapic acids, enzyme activities are referred to as respectively *p*-coumarate:CoA ligase, ferulate:CoA ligase, and sinapate:CoA ligase, etc.

[b] n.c = no conversion detectable

CoA-ligase activity was optimal between pH 7.6 and 8.0 in 0.1 M Tris-HCl or sodium-potassium phosphate buffer. However, in the latter buffer the activity was 20% lower. These results are in agreement with other data reported in the literature (Ranjeva et al. 1976; Knobloch and Hahlbrock 1977).

*Molecular weight.* The different forms of hydroxycinnamate:CoA ligase were not separated by chromatography on a Sephacryl S 200 column and were eluted as one single peak. The apparent molecular weight, determined after calibration of the column with bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome C, was about 41,000.

This value is similar to that already obtained for the enzyme of *Erythrina crista-galli* ($M_r$ 40,000) but is lower than the values determined for hydroxycinnamate:CoA ligase, from other plants (Kutsuki et al. 1982b).

*Substrate specificity.* Apparent $K_m$ values and maximal velocities of the three enzyme forms with different cinnamic acids were determined from Lineweaver-Burk plots (Table 2).

All the isoenzymes reacted with *p*-coumaric and ferulic acids. Form II was only efficient towards these two substrates, but both other forms reacted more specifically with the other cinnamic acids. Form I was the only isoenzyme able to esterify 5-hydroxyferulic and sinapic acids, and form II specifically activated caffeic acid. Cinnamic acid itself was not esterified by any of the forms.

*Tissue distribution of hydroxycinnamate:CoA-ligase isoenzymes.* In a previous work we observed that in poplar stems the *p*-coumarate:CoA-ligase activity was essentially localized in the lignified tissues, i.e. xylem and sclerenchyma (Grand and Ranjeva 1979). Moreover, the ability of hydroxycinnamate:CoA ligase to catalyse the esterification of ferulic and sinapic acids was different according to the tissue from which it was extracted. In order to find out whether this specific activation pattern was related to a specific distribution of the three isoenzymes in the different tissues, we chromatofocused enzyme extracts from xylem, sclerenchyma and parenchyma independently (Table 3).

The three forms are present in xylem in relative ratios similar to those observed for the entire stems, but only form I was detectable in the sclerenchyma. In addition the parenchyma contained the three forms at a very low level of activity and, in this tissue, form II was the major isoenzyme.

## Discussion

Only one form of hydroxycinnamate:CoA ligase has been characterized in parsley (Knobloch and Hahlbrock 1977), *Erythrina crista-galli* (Kutsuki et al. 1982b) and spruce (Lüderitz et al. 1982). In contrast, the occurrence of multiple forms of this enzyme has been demonstrated in cell suspension cultures of soybean (Knobloch and Hahlbrock 1975) in *Petunia* tissues (Ranjeva et al. 1976) and in etiolated pea seedlings (Wallis and Rhodes 1977).

In the present study, three hydroxycinnamate:CoA ligases were separated from poplar stems, and several lines of evidence indicate that they are true isoenzymes. They differ in ionic properties but exhibit a similar molecular weight and are not inter-

convertible. Moreover, they differ in substrate specificity and tissue distribution.

The specific potential for the activation of different hydroxycinnamic acids in each tissue can be directly related to the proportion of complex phenols (compare xylem and parenchyma) and to the nature of the end products accumulated. Form III, which reacts with caffeic acid, is the most active form in the parenchyma, a tissue usually containing cinnamoyl esters and flavonoids. In contrast, form I, involved in the esterification of methoxylated cinnamic acids (precursors of lignin monomers), is essentially located in the xylem and sclerenchyma.

The polymorphism observed in the poplar stem further substantiates the hypothesis put forward by various authors (Ranjeva et al. 1976; Heinzmann et al. 1977) concerning the involvement of several forms of hydroxycinnamate:CoA ligase in specific pathways which use cinnamoyl:CoA as precursors. Moreover, our results are in agreement with the recent data of Knogge et al. (1981) who observed, in oat leaves, differences in substrate specificity of hydroxycinnamate:CoA ligase according to the tissues. However, in this material no attempts have been made to identify the isoforms.

Taken together, these results strongly indicate that isoenzymes of hydrocinnamate:CoA ligase initiate the distribution of hydroxycinnamic acids towards the synthesis of different polyphenols (eg. flavonoids and lignins).

If we consider the lignification process in more detail, the absence from the sclerenchyma of forms II and III, which are involved in the esterification of ferulic acid, could explain the low proportion of guaïacyl units in this tissue. In contrast, all three forms of hydroxycinnamate:CoA ligase are present in the xylem. So, in agreement with the monomeric composition of lignins in this tissue (guaïacyl-rich lignins) the esterification potential for ferulic acid is higher than in sclerenchyma.

Consequently, if we consider both the substrate specificity and the tissue distribution of the hydroxycinnamate:CoA-ligase isoenzymes, the esterification step could play an important role in the control of the monomeric composition of lignins in poplar. The regulatory functions of this enzymatic step is strengthened by the fact that polymorphism associated with substrate specificity was not detected in poplar for O-methyltransferase (Kuroda et al. 1981), cinnamoyl:CoA reductase and cinnamyl alcohol dehydrogenase (data not shown) which could also be involved, on a theoretical basis, in the regulation of the monomeric composition of lignins.

Moreover, we must emphasize that we detected sinapate and 5-hydroxyferulate:CoA-ligase activities in poplar. Sinapate:CoA ligase had previously been reported to be absent from different plants including poplar (Gross et al. 1975; Kutsuki et al. 1982b). The general assumption that sinapate:CoA ligase has a restricted distribution must, in our opinion be critically examined. On the other hand, the physiological significance of the 5-hydroxyferulate:CoA ligase could be related, at least in part, to the formation of syringyl lignin units through the methylation of the reaction product, 5-hydroxyferuloyl:CoA, to sinapoyl:CoA (Kutsuki et al. 1982b).

In conclusion, our results demonstrate for the first time that hydroxycinnamate:CoA ligase exhibits a specific isoenzyme pattern according to the nature of the tissue in a woody plant. This observation, associated with the different substrate specificities of each isoform, leads to the conclusion that these catalysts are, at least in part, responsible for the relative abundance of each monomer in the lignins of the various tissues.

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