Ferulic acid 5-hydroxylase: a new cytochrome P-450-dependent enzyme from higher plant microsomes involved in lignin synthesis

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Ferulic acid 5-hydroxylase, involved in the synthesis of sinapic acid (a precursor of lignin monomers), has been characterized for the first time in plants. This enzyme is localized in the microsomal fraction of the poplar stem and was identified as a cytochrome P-450-dependent mixed-function monooxygenase. Comparison of the regulating properties and tissue distribution of ferulic acid 5-hydroxylase and cinnamic acid 4-hydroxylase suggests that these hydroxylase activities depend on two distinct cytochrome P-450 systems. A correlation was established between the monomeric composition of lignins isolated from xylem and sclerenchyma and the tissue distribution of ferulic acid 5-hydroxylase. The results obtained indicate that this enzyme could be involved, at least partly, in the qualitative control of lignification.

Ferulic acid 5-hydroxylase *Cytochrome P-450* *Lignification* Populus

1. INTRODUCTION

The biosynthetic pathway of lignin monomers is now well characterized [1]. Among the different enzymes involved in this pathway, only the final steps catalysed by cinnamoyl-CoA reductase (EC 1.2.1.44) and cinnamyl alcohol dehydrogenase (EC 1.1.1), respectively, are specific to lignification [2]. The enzymes converting phenylalanine into the cinnamoyl-CoA esters are also involved in the synthesis of a wide range of phenolic compounds (e.g., flavonoids, esters).

From the results of labelling experiments [3] performed along this common pathway, it was assumed that the transformation of ferulic acid into 5-hydroxyferulic acid occurs in plants, yet the corresponding enzyme has never been identified.

However, as stressed in [4] ferulic acid hydroxylase could play a significant role in the regulation of lignification. Owing to its variable activity, this enzyme could control the flow of precursor for sinapyl alcohol synthesis and consequently influence the monomeric composition of lignins.

Ferulic acid 5-hydroxylase has been characterized here for the first time in plants. The main properties of this enzyme were studied and compared to those of cinnamic acid 4-hydroxylase. Finally, an estimate was made of the role of ferulic acid 5-hydroxylase in control of the monomeric composition of lignins in different poplar stem tissues.

2. MATERIALS AND METHODS

2.1. *Plant material*

Populus X *euramericana* (Dode cv. 'I 214') plants were grown under controlled conditions and tissue fractions (xylem, sclerenchyma) isolated from the stems as in [5].

2.2. *Chemicals*

[*methoxy*-^{14}C]Ferulic acid was synthesised enzymatically from caffeic acid and *S*-adenosyl-[*methyl*-^{14}C]methionine (45 mCi/mmol) (CEA) as in [6] but by using porcine liver catechol *O*-methyltransferase (Sigma) as enzyme source. [3-^{14}C]Cinnamic acid (45–55 mCi/mmol) was purchased from CEA.

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5-Hydroxyferulic acid was a generous gift from Professor Higuchi (Japan). Other cinnamic acids and nucleotides were obtained from Sigma.

2.3. *Enzyme sources*

All experiments were carried out at 4°C. Five g of young poplar stems (8 apical internodes) were ground in a Turmix homogenizer for 1 min in 200 ml of 20 mM Tris–HCl buffer (pH 7.5) containing 10 mM mercaptoethanol, 0.5 M mannitol, 0.1% bovine serum albumin, 0.5% poly(ethylene glycol) (M_r 6000) and 2 g of Polyclar AT. The crude extract was filtered through 4 layers of cheesecloth and the filtrate centrifuged at 10000 × *g* for 20 min. The supernatant was then centrifuged at 100000 × *g* for 45 min. The final supernatant and corresponding pellet were assayed for enzyme activity. The pellet, which had previously been resuspended in 5 ml of 25 mM Tris–HCl (pH 7.5) containing 15% ethylene glycol, is referred to as the microsomal fraction.

2.4. *Enzyme assays*

The ferulic acid 5-hydroxylase activity was assayed at 30°C using a radiochemical method. The reaction mixture in a final volume of 1 ml usually contained 1 mM ferulic acid, 0.5 μCi [*methoxy*-^{14}C]ferulic acid, 1.5 mM $NADP^+$, 2 mM glucose 6-phosphate, 0.1 units glucose-6-phosphate dehydrogenase, 1 mM mercaptoethanol, 500 μl microsomal fraction containing 1 mg/ml bovine serum albumin and 25 mM Tris–HCl (pH 7.5) containing 15% ethylene glycol.

After 20 min, the enzymatic reaction was stopped by adding 3 ml of 3 N HCl. Precipitated proteins were removed by centrifugation and the supernatant was then extracted 3 times with 10 ml ethyl acetate. This extract was evaporated to dryness and the residue, dissolved in 0.2 ml ethanol, was deposited onto Whatman 1MM paper before descending chromatography (chloroform–acetic acid–H_2O, 2:1:1, by vol.).

The chromatograms were then scanned for radioactivity (LB 2723 Berthold scanner) and the labelled zone corresponding to 5-hydroxyferulic acid cut out and dipped into 10 ml Ready-solve MP liquid scintillation cocktail (Beckman). Radioactivity was measured with a liquid scintillation counter (CLS Packard 460c).

Cinnamic acid 4-hydroxylase activity was estimated as above except that ferulic acid (unlabelled and labelled) was replaced by the same proportions of cinnamic acid. The radioactivity corresponding to the reaction product, *p*-coumaric acid, was measured as described for 5-hydroxyferulic acid.

Proteins were assessed with the Biorad assay [7].

3. RESULTS

Ferulic acid 5-hydroxylase activity was only detected in the subcellular fraction which sedimented between 10000 and 100000 × *g*. For substrate saturating concentrations, the rate of hydroxylation was constant for 40 min and then decreased. The activity was very unstable, 90% of it being lost during storage for 24 h at 4°C and even at −20°C. However, ethylene glycol at 15% concentration stabilized the enzyme and microsomal fractions stored for 1 week at −20°C in its presence retained 45% of their original activity.

3.1. *Product identification*

The reaction product, tentatively proposed as 5-hydroxyferulic acid, was identified by ultraviolet spectroscopy and thin-layer chromatography on silica gel in the following solvents: (1) chloroform–acetic acid–H_2O, 2:1:1, by vol.; (2) *n*-butanol–H_2O–acetic acid, 6:2:1, by vol.; (3) benzene–methanol–acetic acid, 11:2:1, by vol. The properties of the reaction product (R_{f1} = 0.32, R_{f2} = 0.75, R_{f3} = 0.46; maximum = 307 nm) were identical to those of the reference compound.

In addition the reaction product was identified as hydroxyferulic acid by HPLC (according to [8]; identity of retention time and cochromatography experiment) and by electronic impact mass spectroscopy (Varian Mat 311A).

3.2. *Enzyme activity dependence*

Molecular oxygen was essential for activity. 5-Hydroxyferulic acid was not identified as a reaction product when the microsomal fraction was incubated under a nitrogen atmosphere.

The optimum pH value for activity was 7.5 in either 25 mM Tris–HCl or 20 mM KH_2PO_4 buffers. pH values for half-maximal activity were 6.7 and 8.

Thiol reagents stimulated ferulic acid 5-hydroxylase activity, glutathione being the most efficient (table 1). A sulphydryl agent such as *p*-chloromer-

Table 1

Effect of various compounds on ferulic acid 5-hydroxylase activity

Additions	Concentration (mM)	Relative activity
None	–	100
Mercaptoethanol	1	136
	2	129
	3	42
Dithiothreitol	1	149
Glutathione	1	198
p-Chloromercuribenzoate	1	65
1,10-Phenanthroline	1	100

curibenzoate inhibited the enzyme by 35%. Moreover, a metal-chelating agent such as 1,10-phenanthroline had no effect on the hydroxylase activity.

3.3. *Cofactor requirements for ferulic acid 5-hydroxylase activity*

The results in table 2 show that, in absence of external cofactors, microsomes do not catalyse hydroxylation of ferulic acid. The enzyme requires NADPH for activity. With NADH alone no activity was observed. However, in the presence of NADPH, NADH exerted a synergistic effect on the hydroxylation reaction.

Table 2

Cofactor requirement for ferulic acid 5-hydroxylase activity

Cofactor	Concentration (mM)	Enzyme activity (pkat/mg protein)
None	–	0
NADPH	0.01	1.5
NADPH	0.1	5.6
NADPH	0.5	11
NADPH	1	11.1
NADPH[a]	0.5	18.7
NADH	0.5	0
NADPH	0.1	8.1
+ NADH	0.1	
NADPH	0.5	13.4
+ NADH	0.1	
NADPH	0.5	18.2
+ NADH	0.5	

[a] In this assay NADPH was supplied through a regenerating system (glucose-6-phosphate dehydrogenase, glucose 6-phosphate and $NADP^+$) so that its potential concentration was 0.5 mM

This effect, observed for non-saturating (0.1 mM) and saturating (0.5 mM) concentrations of NADPH, was not due to a transhydrogenation process of NADH to $NADP^+$.

As shown in table 2, when NADPH was supplied along with an NADPH-regenerating system (glucose-6-phosphate dehydrogenase, glucose 6-phosphate and $NADP^+$) the hydroxylase activity was stimulated by 70%.

3.4. *Substrate specificity*

In addition to ferulic acid (apparent K_m 6.3×10^{-5} M) cinnamic acid also acted as a substrate and was converted to *p*-coumaric acid (apparent K_m 1.9×10^{-5} M). No hydroxylation of *p*-coumaric acid was detected.

3.5. *Effect of carbon monoxide and light on the hydroxylase activities*

Microsomal fractions were incubated under controlled atmospheres containing CO, O_2 and N_2 (the $CO:O_2$ ratio was kept constant at 1). The results (table 3) show that in darkness 10% CO causes 41% inhibition of ferulic acid 5-hydroxylase and 52% inhibition of cinnamic acid 4-hydroxylase. In both cases, the inhibitory effect of CO was partially reversed by light. Under air atmosphere, light had no effect on hydroxylase activities.

3.6. *Action of different effectors on hydroxylase activities*

As the hydroxylase associated with the microsomal fraction no longer had any activity shortly after solubilization we did not try to purify the ferulic 5-hydroxylase further. However, to check whether it represented a specific enzyme distinct from cinnamic acid 4-hydroxylase, which has been characterized in many plants [9], we studied the action of different effectors on the two activities.

Ferulic acid 5-hydroxylase activity was inhibited by cinnamic acid and conversely cinnamic acid 4-hydroxylase activity was inhibited to a lesser extent by ferulic acid (table 4). The kinetic studies showed that these inhibitors act in a non-competitive manner. Moreover, the reaction products of both hydroxylases, 5-hydroxyferulic acid and *p*-coumaric acid, inhibit the enzyme reaction

Table 3
Effect of carbon monoxide and light on hydroxylase activities

Assay	Ferulic acid hydroxylase		Cinnamic acid hydroxylase	
	Activity (pkat/mg protein)	% inhibition	Activity (pkat/mg protein)	% inhibition
Air, control, dark	32.5	0	71	0
5% CO–5% O_2, dark[a]	24	26	42.6	40
10% CO–10% O_2, dark[a]	19.1	41	34	52
Air, control, dark	29	0	61.3	0
Air, control, light	28.7	0	61.8	0
10% CO–10% O_2, dark[a]	18.4	36	30.6	50
10% CO–10% O_2, light[a]	25.2	12	43.7	29

[a] In these experiments, complement to 100% was provided by adding N_2

Table 4
Action of various effectors on hydroxylase activities

Enzymatic reaction	Effector (0.5 mM)	Activity (pkat/mg protein)
Ferulic acid 5-hydroxylase	none	22.3
	cinnamic acid	11.5
	5-hydroxyferulic acid	18.1
	p-coumaric acid	23
Cinnamic acid 4-hydroxylase	none	54.1
	ferulic acid	41.7
	5-hydroxyferulic acid	53.7
	p-coumaric acid	25.9

Each substrate was used at a concentration of 1 mM in the incubation mixtures

catalysing their own synthesis in a non-competitive manner.

3.7. *Hydroxylase activities in microsomal fractions isolated from xylem and sclerenchyma*

Cinnamic acid 4-hydroxylase and ferulic acid 5-hydroxylase activities were estimated in xylem and sclerenchyma, the two lignified tissues of poplar stem.

The results (table 5), expressed as a ratio of the enzyme activities, show that they both exhibit different relative activities in these specific tissues. In particular, ferulic acid 5-hydroxylase is preferentially localized in the sclerenchyma fraction.

Table 5
Tissue distribution of cinnamic acid 4-hydroxylase and ferulic acid 5-hydroxylase activity in poplar stems

Tissue	Cinnamic acid 4-hydroxylase (pkat/mg protein)	Ferulic acid 5-hydroxylase (pkat/mg protein)	Ratio of activities (cinnamic acid 4-hydroxylase/ ferulic acid 5-hydroxylase)
Xylem	95	28	3.4
Sclerenchyma	83	52	1.6

4. DISCUSSION

Ferulic acid 5-hydroxylase activity, involved in the synthesis of sinapic acid, a precursor of one lignin monomer (sinapyl alcohol), has been characterized in poplar. The enzyme which is associated with the microsomal fraction, requires O_2 and NADPH and can be identified as a mixed-function monooxygenase [10]. The enzyme activity is stimulated by thiol reagents and inhibited by *p*-chloromercuribenzoate, which suggests the involvement of SH groups in the catalytic process. When NADH is used as cofactor no activity is measured; however, in the presence of NADPH it exerts a synergistic effect on hydroxylation. Moreover, the ferulic acid 5-hydroxylase activity, as well as that of cinnamic acid 4-hydroxylase, is inhibited by CO; this inhibition is partially reversed by light. These results suggest that ferulic acid 5-hydroxylase is a cytochrome P-450-dependent enzyme [11].

It has been demonstrated that multiple forms of cytochrome P-450 with a different substrate specificity exist in plant microsomes [9,12].

In the synthesis of phenolics the hydroxylation of cinnamic acid has been shown to be cytochrome P-450 dependent [9].

To determine whether cinnamic acid 4-hydroxylase and ferulic acid 5-hydroxylase are two different enzymes, we compared their regulative properties and tissue distribution.

p-Coumaric acid, which is a negative effector of cinnamic acid 4-hydroxylase, has no effect on ferulic acid 5-hydroxylase. In addition, 5-hydroxyferulic acid, which inhibits the ferulic acid 5-hydroxylase, has no effect on cinnamic acid 4-hydroxylase. Moreover, the study of the tissue distribution of these activities showed that the ratio of cinnamic acid 4-hydroxylase: ferulic acid 5-hydroxylase activities differs between xylem and sclerenchyma fractions.

These results strongly suggest that the two hydroxylase activities depend on two distinct cytochrome P-450 systems.

One of the goals of this study was to check if, in addition to hydroxycinnamate: CoA ligase [13], specific tissue distribution of ferulic acid 5-hydroxylase could explain the different patterns in lignin monomers occurring in xylem and sclerenchyma [5].

Ferulic acid 5-hydroxylase is preferentially localised in sclerenchyma which contains lignins enriched in syringyl units [5]. Therefore ferulic acid 5-hydroxylase could, at least partly, be involved in the control of the monomeric composition of lignins in the various tissues of the poplar stem. These results are in agreement with those of authors in [14] who have suggested, on the basis of labelling experiments, that this enzymatic step controls the monomeric composition of lignins in *Erythrina crista galli*.

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