STILBENE SYNTHASE (PINOSYLVINE SYNTHASE) AND ITS INDUCTION BY ULTRAVIOLET LIGHT

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1. Introduction

Stilbenes are constituents of heartwood and roots [1]. Some stilbenes are known to function as fungistatic reagents and phytoalexins [2,3]. In a broader sense, stilbenes might be considered as stress metabolites. Induction of phytoalexin formation can be brought about either by fungal attack or by artificial elicitors, i.e., ultraviolet light [4] or desiccation [5].

Biogenesis of pinosylvin (3,5-dihydroxystilbene, see fig.1) was investigated in vivo by feeding experiments with radioactive precursors [6,7], indicating that a phenylpropane moiety is condensed with 3 acetyl units [1]. Recently we could demonstrate stilbene biosynthesis in vitro using a partially purified enzyme preparation from the rhizome of *Rheum rhaponticum* [8,9]. The enzyme catalyzed the formation of resveratrol (3,5,4′-trihydroxystilbene, see fig.1).

The data presented here describe, for the first time, a different stilbene synthase from *Pinus sylvestris* that is capable of catalyzing the conversion of cinnamoyl-CoA and malonyl-CoA into 3,5-dihydroxystilbene.

2. Materials and methods

2.1. *Preparation of cell extracts*

Pine plants *(Pinus sylvestris)* were grown from seeds obtained from M. Meyer, Bad Vilbel, FRG, in vermiculite under standardized conditions, a 12 h light–12 h dark regime (8000 lux). Seedlings were homogenized in a medium containing 120 mM Hepes–NaOH, (pH 7.5), 5 mM dithiothreitol, and 10% sucrose (extraction buffer). After centrifugation of the extract at 1000 × *g* for 15 min the supernatant liquid was again subjected to centrifugation, at 20 000 × *g* for 15 min. The resuspended pellet was used as enzyme source.

For several experiments an acetone powder was prepared from whole pine seedlings. Acetone powder (2 g) was mixed with 4 ml extraction buffer for several minutes then centrifuged at 20 000 × *g* for 30 min.

2.2. *Enzyme assay and identification of the product*

Cinnamoyl-CoA (10 μM) prepared according to [10] and 10 μM [2-^{14}C]malonyl-CoA (0.1 μCi) were incubated in a buffer solution containing 20 mM

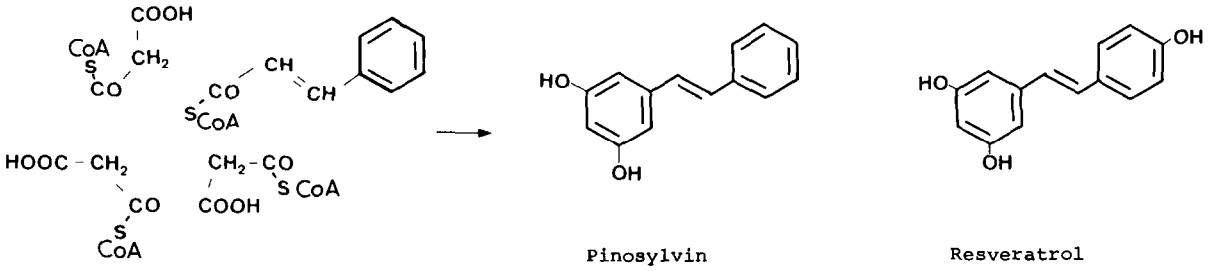


Fig.1. Formation of pinosylvin from cinnamoyl-CoA and malonyl-CoA.

Hepes–NaOH (pH 7.5), 5 mM dithiothreitol and 10% sucrose with 250 μl of the extract in 270 μl total vol. After 30 min at 30°C the reaction was terminated by adding 100 μg pinosylvin in 1 ml H_2O and 2 ml ethyl acetate. After extraction with ethyl acetate the organic layer was evaporated and the residue chromatographed in system L1 (see later).

Rechromatography was done in system L2, and finally the pinosylvin zone was eluted from the silicagel and subjected to scintillation counting. In addition to this routine procedure, pinosylvin was identified, during the course of the first successive preparations, by recrystallization to constant specific radioactivity. To this end, 50 mg authentic pinosylvin was added to the reaction mixture, and several times recrystallized from water and subsequently from benzene. Pinosylvin was also transformed, with dimethyl sulfate [6], into the dimethylether which was chromatographed on silicagel using system L3 and subjected to $KMnO_4$ oxidation in acetone at room temperature. The 3,5-dimethoxybenzoic acid [8] formed was purified by paper chromatography in system L4.

When *p*-coumaroyl-CoA was used instead of cinnamoyl-CoA as substrate, the resveratrol formed was identified chromatographically.

L1: Benzene–methanol (9:1) R_F values: pinosylvin 0.30; monomethyl ether 0.66

L2: Chloroform–ethyl acetate–formic acid (5:4:1) R_F value: pinosylvin 0.78; resveratrol 0.59

L3: Benzene-petrolether (9:1) R_F values: monomethyl ether 0.08; dimethyl ether 0.62

L4: *n*-Butanol–ammonia–ethanol–benzene (5:3:2:1)

3. Results

3.1. *Stilbene synthase: Detection and identification of the reaction product*

During attempts to detect stilbene synthase in preliminary trials the following parameters were considered: Season, age of seedlings, roots and autotrophic parts, inducing agents, thioesterases destroying the

Table 1
Specific activities of pinosylvin synthase extractable from seedlings

Exp. no.	Preparation source	Fraction	Additions	Pinosylvin formed pmol × [mg protein]$^{-1}$
1	Seedlings, 3 week old	20 P		< 0.01
	6 week old	20 P		1.00
	6 week old	20 P	+*p*-Coumaroyl-CoA −Cinnamoyl-CoA	0.20[a]
	9 week old	20 P		4.50
	9 week old	AP		45.90
2	Seedlings, 6 week old			
	needles	20 P		< 0.01
	roots	20 P		0.93
3	Seedlings, 6 week old			
	roots	1 P		< 0.01
		20 P		1.05
		20 S		< 0.01

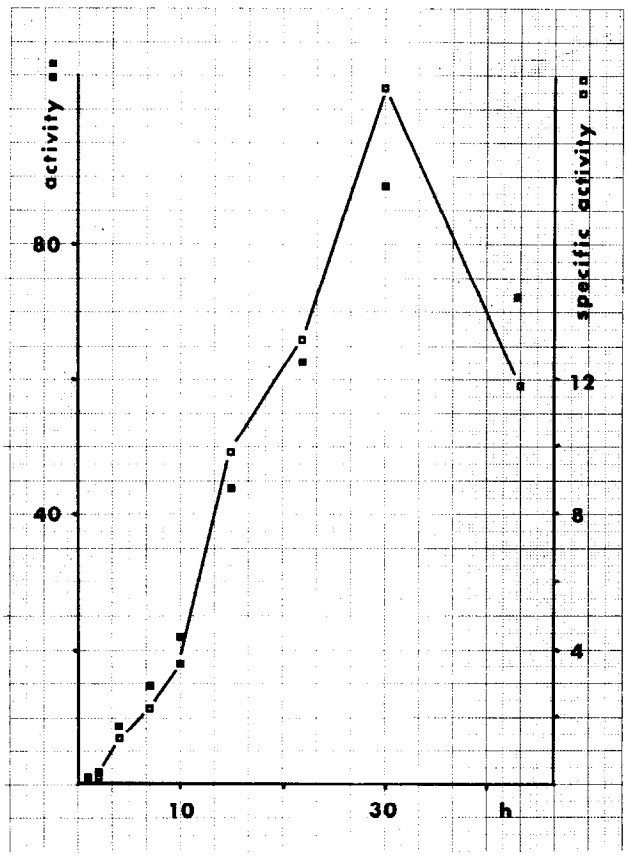
[a] Resveratrol formed

The suspension of 20 000 × *g* pellet (20 P) was routinely used for assays, as neither larger particles (1000 × *g* pellet (1 P) nor the 20 000 × *g* supernatant (20 S) were enzymatically active. Soluble preparations could be obtained when the enzyme was extracted from acetone powders (AP)

Table 2
Induction of stilbene synthase

Exp. no.	Target, Enzyme source	Treatment	Pinosylvin formed	
			pmol	pmol × [mg protein]$^{-1}$
1	Whole plant	–	1.14	0.29
	Whole plant	Wounding	1.83	0.25
	Whole plant	Desiccation	1.90	0.18
	Whole plant	Ultraviolet light	4.17	0.33
2	Isolated roots	–	17.5	8.5
	Isolated needles	–	4.1	0.5
	Isolated roots	Ultraviolet light	55.9	28.6
	Isolated needles	Ultraviolet light	24.9	3.1
	Roots from plants with needles the only tissue being exposed	Ultraviolet light	21.6	25.4

Experiment 1 was carried out with 5.5 week old seedlings. For expt 2, 10 week old seedlings were divided into green parts and roots. In the last experiment (lower-most row) intact plants were used, but roots were thoroughly covered with aluminium foil during exposure



substrates, and inhibitors. Having examined plants of different age, we finally used roots of 6 week old seedlings as enzyme source. After incubation of cinnamoyl-CoA and malonyl-CoA with the crude extracts, the chromatography in system L1 showed a main peak at R_F 0.30 which coincided with the pinosylvin marker. In several cases minor amounts of pinosylvin monomethyl ether were also observed. The pinosylvin zone was scraped off and the compound rechromatographed in system L2. The product thus obtained was radiochemically pure as could be demonstrated by recrystallization to constant specific activity. A portion of pinosylvin was converted into pinosylvin dimethylether, the purity of which was checked. Finally, dimethylpinosylvin was oxidized, and the formation of radioactive 3,5-dimethoxybenzoic acid was demonstrated. As outlined in table 1, most stilbene synthase activity appeared to be bound to structures sedimentable at 20 000 × *g*. Soluble

Fig.2. Induction of stilbene synthase activity in whole seedlings exposed to ultraviolet light for 10 min. The enzyme activity (pmol pinosylvin formed/30 min) from 2 g plants was plotted against the induction period; this is the time following the exposure to ultraviolet light before the enzyme was prepared. A Camag inspection lamp, without filter, was used at 12 cm.

stilbene synthase, however, was obtained when acetone powder was used as starting material. The stilbene synthase from *Pinus sylvestris* exhibited specificity towards cinnamoyl-CoA (versus *p*-coumaroyl-CoA).

3.2. *Induction of stilbene synthase by ultraviolet light*

Stilbene synthase was shown to be present in roots of at least 6 week old plants (table 1). By contrast, green needles contained stilbene synthase only when its formation was induced by a 10 min exposure to ultraviolet light of 254 nm (table 2).

A 10 min exposure to ultraviolet light greatly increased the stilbene synthase activity during an induction period of 30 h (fig.2). One of the most intriguing observations was the increase of stilbene synthase in the roots when only needles of the intact plants were exposed to ultraviolet light (table 2).

4. Discussion

The pinosylvin synthase which could be demonstrated for the first time showed specificity towards cinnamoyl-CoA as substrate (table 1). The enzyme was therefore clearly distinguished from the resveratrol synthase which converted *p*-coumaroyl-CoA 5 times better than cinnamoyl-CoA into the respective stilbene [9]. These two enzymes are the only examples of stilbene synthases active in vitro.

A few papers [3,5] reported an increase in stilbene concentration, elicited in plants by various treatments. However, it was so far unknown which enzyme activity in the chain of metabolic steps between L-phenylalanine and stilbenes was changed and was thus responsible for the enhanced stilbene formation. It becomes now evident that the enzyme responsible for the condensation of a C_6C_3-unit with activated C_2-units is induced to a high extent upon stress.

Acknowledgements

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