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Stilbene synthase from Scots pine (*Pinus sylvestris*)

Sigrid Schanz, Gudrun Schröder and Joachim Schröder

Institut für Biologie II, Universität Freiburg, Schänzlestr. 1, D-7800 Freiburg, Germany

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Stilbene synthases are named according to their substrate preferences. By this definition, enzymes preferring cinnamoyl-CoA are pinosylvin synthases, and proteins with a preference for phenylpropionyl-CoA are dihydropinosylvin synthases. We investigated the assignment of a stilbene synthase cloned from Scots pine (*Pinus sylvestris*) as dihydropinosylvin synthase and the proposal of an additional pinosylvin synthase [1992, Plant Mol. Biol. 18, 489–503]. The results show that the previous interpretation was misled by several unexpected factors. Firstly, we found that the substrate preference and the activity of the plant-specific protein expressed in *E. coli* was influenced by bacterial factors. This was reduced by improvement of the expression system, and the subsequent kinetic analysis revealed that cinnamoyl-CoA rather than phenylpropionyl-CoA is the preferred substrate of the cloned stilbene synthase. Secondly, mixing experiments showed that extracts from *P. sylvestris* contain factor(s) which selectively influenced the substrate preference, i.e. the activity was reduced with phenylpropionyl-CoA, but not with cinnamoyl-CoA. This explained the apparent differences between plant extracts and the cloned enzyme expressed in *E. coli*. Taken together, the results indicate that the cloned enzyme is a pinosylvin synthase, and there is no evidence for a second stilbene synthase. This study cautions that factors in the natural and in new hosts may complicate the functional identification of cloned sequences.

Pinus sylvestris; stilbene synthase; pinosylvin synthase; heterologous expression

1. INTRODUCTION

Stilbene synthases (STS) synthesize the stilbene backbone in one enzymatic reaction from CoA-esters of intermediates of the phenylpropanoid pathway and malonyl-CoA. They may be constitutive, but often they are induced by stress, including pathogen attack, and stilbenes are considered as phytoalexins because of their antibacterial and antifungal activities [1–3]. STS are rare in crop plants [4], and their introduction may contribute to disease resistance. STS clones have been described from groundnut [5,6], grapevine [7], and Scots pine (*Pinus sylvestris*) [8].

Scots pine is an interesting source of STS genes. The stress-induced stilbenes are pinosylvin (from cinnamoyl-CoA, Fig. 1) and derivatives. The literature also cites dihydropinosylvin (from phenylpropionyl-CoA [9], Fig. 1) as common constituent of *Pinus* species [4], but there is no information on the regulation of its biosynthesis. It is therefore an intriguing question whether *P. sylvestris* contains two different types of STS. The functional analysis of a STS cloned from stressed seedlings and expressed in *E. coli* showed that the protein accepted both substrates, a property typical for these enzymes, and it was labelled a dihydropinosylvin synthase, because it preferred phenylpropionyl-CoA against cinnamoyl-CoA [8]. The plant extracts also accepted both substrates, but with a preference of cinnamoyl-CoA, and this suggested a second STS with the substrate preference predicted for pinosylvin synthase [8].

Further investigations, however, raised some doubts on the presence of a second stress-induced STS (unpublished results). An extensive analysis of cDNA libraries from stressed seedlings identified several additional STS clones with slightly different DNA sequences, but the deduced proteins were ≥98% identical with those of the previously identified clones. The few amino acid exchanges were in variable positions and conservative, and it seemed very unlikely that they caused different substrate preferences. The same result was obtained with more than 15 clones obtained from genomic DNA by polymerase chain reactions. These data suggested that *P. sylvestris* contains only one STS gene family, and the correlation between induced pinosylvin synthase activity and cDNAs further suggested that the previously identified clones code for this enzyme rather than for a dihydropinosylvin synthase.

These findings and the lack of direct evidence for dihydropinosylvin in *P. sylvestris* [10] prompted a re-evaluation of the assignment of the cloned STS. The results indicate that bacterial as well as plant extracts contain factor(s) which influence the substrate preference of STS, and that the cloned STS should be defined as pinosylvin synthase. An essential step in this analysis

Correspondence address: J. Schröder, Institut für Biologie II, Universität Freiburg, Schänzlestr. 1, D-7800 Freiburg, Germany. Fax: (49) (761) 2032701.

Abbreviations: IPTG, isopropyl-β-D-thiogalactoside; STS, stilbene synthase; TLC, thin-layer-chromatography.

was the improvement of the expression system for the cloned enzyme.

2. EXPERIMENTAL

2.1. *STS expression in E. coli and preparation of antiserum*

The previously used expression system has been described [8]. It used plasmid pKK233-2[STS] which contains the coding region fused with its start AUG via a *Nco*I restriction site to the inducible promoter in vector pKK233-2. Protein expression was induced at 37°C.

To improve the expression, the STS sequence was excised as *Nco*I/*Hin*dIII fragment and inserted in vector pQE-6. This placed the expression under control of the strong pQE-6 promoter which is regulated by the lacI repressor encoded in pREP4 [11]. Both plasmids were obtained from DIAGEN, Hilden, FRG. For enzyme assays, protein expression with pQE-6[STS] was induced with 2 mM IPTG for 3 h at 28°C.

Plasmid pQE-6[STS] was also used for the isolation of STS to raise antibodies. Protein expression was induced at 37°C in this case. Under these conditions ≥50% of the STS was present in insoluble aggregates. The purification of the protein and the raising of antiserum in rabbits followed established procedures [12].

2.2. *STS assays*

Standard assays contained in a final volume of 0.1 ml: 0.5–1 μg *E. coli* or 20–30 μg plant protein, 10 μM cinnamoyl-CoA or phenylpropionyl-CoA, 15 μM [2-^{14}C]malonyl-CoA (60,000 cpm; 0.78 GBq/mmol, Amersham), and 50 mM HEPES-NaOH adjusted to pH 7 with HCl. The incubations with plant extracts were supplemented with 5 mM EDTA and 1 mM dithiothreitol to stabilize the enzyme activity. These additions were omitted with *E. coli* extracts, because they had no significant effects. After 10 min at 37°C, the incubation mixtures were extracted twice with ethylacetate, and the reaction products were analyzed by TLC with 20% acetic acid as solvent. The radioactive products were quantified with a TLC analyzer [8]. Their identity was established by high-performance liquid chromatography and gas chromatography/mass spectrometry as described [13].

2.3. *Other techniques*

The preparation of enzyme extracts from *E. coli* and *P. sylvestris*, and the immunoblots have been described [8].

3. RESULTS

3.1. *Improved expression of STS in E. coli*

This was achieved in two steps. First, the STS was recloned into expression vector pQE-6 which possesses a stronger promoter than pKK233-2. Estimated from immunoblots and enzyme assays, this increased the amount of enzyme protein and activity at least five-fold. Second, the temperature during protein induction was reduced from 37°C to 28°C. This drastically improved the distribution of STS in the 15,000 × *g* supernatant (soluble) and pellet (aggregates) from about 1:1 to 15:1 (Fig. 2). The immunoblots were performed with a new antiserum prepared against the cloned enzyme (see Section 2), and lane 5 shows that the protein band recognized in the plant had the same size as in the bacterial extracts. The expression at 28°C in *E. coli* led to at least eight-fold increase of STS activity. Similar improvements by reducing the temperature during protein expression have been observed by others [14]. The combination of the two steps improved the yield of active STS

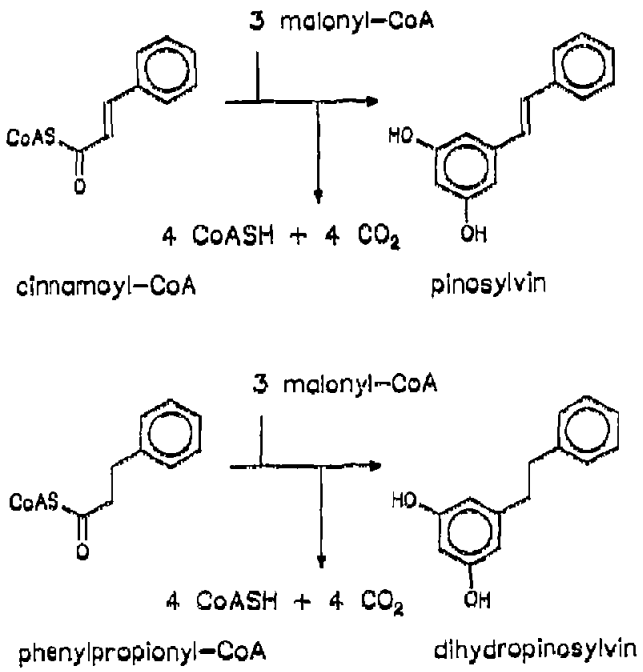


Fig. 1. Synthesis of pinosylvin and dihydropinosylvin with STS from *P. sylvestris*. Pinosylvin and its methylated derivative are the stress-induced stilbenes.

at least 40-fold, i.e. 0.5–1 μg protein in the crude extracts now contained the same activity as 30–40 μg from the previous expression system.

3.2. *Analysis of the cloned STS expressed in E. coli*

The first experiments showed similar activities with cinnamoyl-CoA and phenylpropionyl-CoA in the standard assays, and this was in contrast to the previous expression system which indicated higher activity with phenylpropionyl-CoA [8]. The difference between the two systems is the relative amount of STS in the extracts, and therefore we investigated the effect of *E. coli* proteins on the activity of the enzyme. The activity with cinnamoyl-CoA was not significantly changed by adding a twenty-fold excess of proteins from control *E. coli* cells. The data with phenylpropionyl-CoA were complex: a five-fold excess stimulated slightly (10–20%), while a twenty-fold excess led to 30% inhibition. The results indicated that bacterial proteins influenced the activity and also the apparent substrate preference of the cloned STS. This unexpected effect could not have been detected with the previous expression system. The mechanisms are not clear. STS is a plant-specific enzyme which is not known to be present in *E. coli* [13], and neither cinnamoyl-CoA nor phenylpropionyl-CoA are substrates in pathways of primary metabolism.

STS obtained in the improved expression system was then used for a kinetic analysis of the substrate preference. Initial experiments showed that the enzyme activities dropped to very low levels below 1 μM cinnamoyl-CoA and 2 μM phenylpropionyl-CoA. This indicated

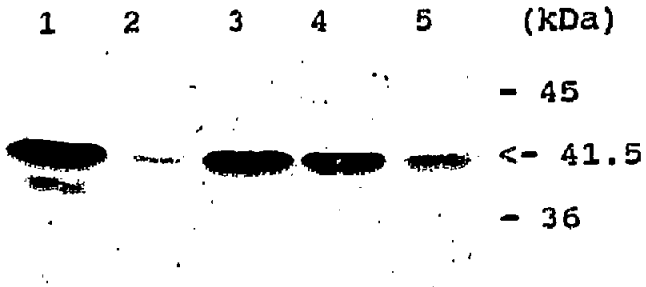


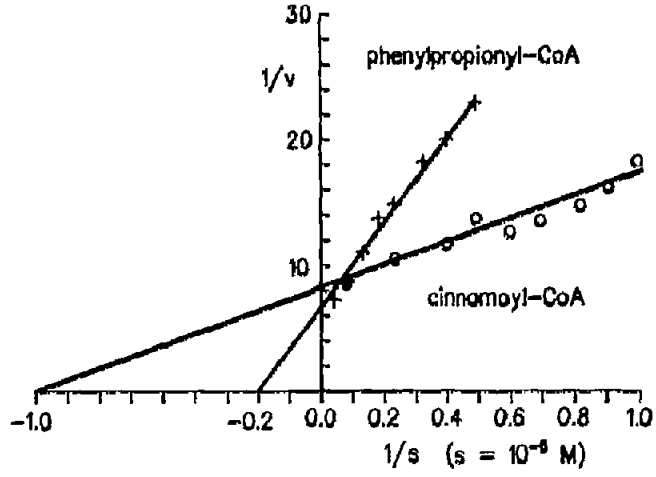
Fig. 2. Immunoblots of the STS expressed in *E. coli* with pQE-6[STS] (lanes 1–4) and in extracts from *P. sylvestris* (lane 5). Lanes 1 and 2: protein induction at 28°C; lanes 3 and 4: induction at 37°C. 1,3 = supernatants; 2,4 = pellets from a 10 min centrifugation at 15,000 × *g*. The size markers are at the right side.

that the substrate dependence did not follow Michaelis–Menten kinetics below these concentrations, and therefore the incubations were performed in the range from 1–10 μM with cinnamoyl-CoA and 2–20 μM with phenylpropionyl-CoA. The results (Fig. 3) showed a five-fold lower K_m with cinnamoyl-CoA (range 0.5–2 μM) than with phenylpropionyl-CoA (range 3–8 μM), while the V_{max} with phenylpropionyl-CoA was slightly higher than with cinnamoyl-CoA. The K_m and the V_{max}/K_m ratio indicate that the cloned STS should be defined as pinosylvin synthase.

3.3. *Plant extracts reduce the activity of STS with phenylpropionyl-CoA, but not with cinnamoyl-CoA*

The kinetic analysis indicated that the cloned enzyme is a pinosylvin synthase with similar reaction rates for both starter CoA-esters at standard substrate concentrations (10 μM; see Fig. 3). This was not the case with the STS activities in *P. sylvestris*, because these had shown a clear preference for cinnamoyl-CoA [8], and this would argue for a second pinosylvin synthase. The results with the *E. coli* extracts, however, cautioned that unexpected factors may influence the apparent substrate preference, and therefore we investigated whether similar effects were detectable with plant extracts. This was tested by experiments in which the plant enzyme preparations were mixed either with extracts from control *E. coli* cells (no STS activity [13]) or with various amounts of the enzyme expressed in the bacteria.

E. coli proteins inhibited STS in the plant extracts, and 20 μg in a standard assay with 30 μg plant protein reduced the activity by 60–80%. The improved expression system was therefore essential to minimize these effects. The results of the mixing experiments are summarized in Table I. They confirmed that in the plant extracts cinnamoyl-CoA was a better substrate than phenylpropionyl-CoA (Table I, No. 4,11), and they showed that addition of 1 μg *E. coli* control protein reduced the activity by an acceptable level of about 10% with both starter CoA-esters (Table I, No. 5,12). Addition of the cloned pinosylvin synthase to the plant extracts revealed an unexpected effect: within the limits of



starter CoA	K_m (10^{-6} M)	V_{max} (nkat/mg)	V_{max}/K_m
cinnamoyl-CoA	1	0.12	0.12
phenylpropionyl-CoA	5	0.16	0.03

Fig. 3. Kinetic analysis of STS from *P. sylvestris* expressed in *E. coli*: *K_m determinations for the starter CoA-esters cinnamoyl-CoA and phenylpropionyl-CoA. V* = nkat/mg protein.

assay accuracy, the activity with cinnamoyl-CoA was as predicted from the sum of both extracts (Table I, No. 6,7), but the activity with phenylpropionyl-CoA was much lower than expected (Table I, No. 13,14). This indicated that the plant extracts contained factor(s) which selectively reduced the activity with phenylpropionyl-CoA, but not with cinnamoyl-CoA. This explained

Table I

STS activities in extracts from *P. sylvestris*, *E. coli* expressing the cloned STS, and mixtures of both. The incubations were performed under the standard conditions for plant extracts for 10 min at 37°C. pQE-6: control extracts from *E. coli* containing the vector plasmid; pQE-6[STS]: cloned STS.

no.	extract from plant	extract from *E. coli*	(a) product (cpm)	(b) expected sum (cpm)	a/b (%)
cinnamoyl-CoA					
1	–	1.0 μg pQE-6	<2		
2	–	0.5 μg pQE-6 [STS]	1880		
3	–	1.0 μg pQE-6 [STS]	4620		
4	24 μg	–	5260		
5	24 μg +	1.0 μg pQE-6	4640	5260	88
6	24 μg +	0.5 μg pQE-6 [STS]	6660	7140	93
7	24 μg +	1.0 μg pQE-6 [STS]	8120	9880	82
phenylpropionyl-CoA					
8	–	1.0 μg pQE-6	<2		
9	–	0.5 μg pQE-6 [STS]	2520		
10	–	1.0 μg pQE-6 [STS]	3280		
11	24 μg	–	580		
12	24 μg +	1.0 μg pQE-6	520	580	90
13	24 μg +	0.5 μg pQE-6 [STS]	700	3100	23
14	24 μg +	1.0 μg pQE-6 [STS]	1200	3860	31

the apparent difference in substrate preference between the cloned pinosylvin synthase and the STS activities in plant extracts, and it eliminated the experimental basis for assuming a second STS. The results were reproducible in several independent experiments. The mechanisms are not understood. Dialysis of the plant extracts did not eliminate this effect, but instability of the enzyme activity precluded a more detailed analysis.

4. DISCUSSION

One aim of biotechnology is the improvement of crop plants by introducing new genes which contribute to useful properties, e.g. resistance against disease. STS appears to be a good candidate, because it is rare in crop plants and produces in one step a phytoalexin-active substance. A prerequisite is the unambiguous identification of the enzyme activity, and a standard criterium is expression and functional analysis in a heterologous system. Our study cautions that this may be more difficult than expected, because both *E. coli* and *P. sylvestris* extracts contained factors which influenced the enzyme activity and, more importantly, the apparent substrate preference of STS. It would be interesting whether similar effects can be observed in transgenic plants.

The results indicate that the previous identification of the cloned STS and the proposal of a second STS type was misled by these factors. The cloned enzyme should correctly be labelled as pinosylvin synthase, and neither the enzyme activity data nor extensive cloning experiments (see Introduction) provide any evidence for a second type of STS in *P. sylvestris*. This conclusion is consistent with pinosylvin and its derivatives being the stress-induced stilbenes in *P. sylvestris* and also with the absence of direct evidence for dihydropinosylvin in this plant [10]. The conclusion is also consistent with a recent report which described a *P. sylvestris* cDNA identified by hybridization with heterologous probes from a resveratrol-forming STS from grapevine [15]. Unfortunately, functional assays were not performed in this case, and therefore the assignment as STS was tentative, but the similarity with the previously published sequences [8] (≥98.5% in DNA and deduced protein, 100% in the 3′ non-coding region) indicate that the cDNA belongs to the same STS family. This seems significant, because the heterologous probe shared only about 65% identity with the sequences cloned from *P. sylvestris*.

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