Molecular and enzymatic characterization of two stilbene synthases from Eastern white pine (*Pinus strobus*) A single Arg/His difference determines the activity and the pH dependence of the enzymes

Sigrid Raiber, Gudrun Schröder, Joachim Schröder

Institut für Biologie II, Universität Freiburg, Schänzlestrasse 1, D-79104 Freiburg, Germany

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Abstract ***Pinus strobus*** **(Eastern white pine) contains stilbenes biosynthetically derived from cinnamoyl-CoA (pinosylvin) or dihydrocinnamoyl-CoA (dihydropinosylvin). We screened a *P. strobus* cDNA library with a stilbene synthase (STS) probe from *Pinus sylvestris*. The eight isolated cDNAs represented two closely related STS genes with five amino acid differences in the proteins. The enzyme properties were investigated after heterologous expression in *Escherichia coli*. Both proteins preferred cinnamoyl-CoA against dihydrocinnamoyl-CoA and thus represented pinosylvin synthases. Otherwise they revealed large differences. STS1 had only 3–5% of the activity of STS2, its pH optimum was shifted to lower values (pH 6), and it synthesized with cinnamoyl-CoA a second unknown product. Site-directed mutagenesis demonstrated that a single Arg-to-His exchange in STS1 was responsible for all of the differences. The proton acceptor properties of His are discussed as the reason for the properties of STS1.**

Key words: Dihydropinosylvin; Pinosylvin; *Pinus strobus*; Polyketide synthase; Stilbene synthase

1. Introduction

Stilbene synthases (STS) catalyze in a complex reaction the backbone of the stilbene phytoalexins which have antifungal properties and contribute to plant defenses against pathogens. The enzymes occur in a limited number of widely unrelated plants, and evidence is accumulating that STS developed from the closely related, ubiquitous chalcone synthases several times in the course of evolution [1]. STS have been cloned sofar only from peanut (*Arachis hypogaea*) [2,3], grape (*Vitis vinifera*) [4,5], and Scots pine (*Pinus sylvestris*) [6], and molecular and biochemical information on STS from other sources would be desirable for a better understanding of the enzymes and the evolutionary aspects.

Stilbenes are wide-spread in gymnosperms, and their distribution in the genus *Pinus* (pines) has been used for chemotaxonomic distinction between different subgenera [7]. The subgenus Haploxylon, with *P. strobus* (Eastern white pine) as a representative member, contains two stilbene types (pinosylvin and dihydropinosylvin) which are biosynthetically derived from cinnamoyl-CoA and dihydrocinnamoyl-CoA (Fig. 1). The subgenus Diploxylon, with *P. sylvestris* (Scots pine) as a well-investigated example, contains only pinosylvin type stilbenes. Previous work with STS cloned from *P. sylvestris* showed that the enzyme accepted both substrates in vitro, but with a preference for cinnamoyl-CoA, and therefore it was classified as pinosylvin synthase [8]. Enzymes reducing the aliphatic double bond in cinnamoyl-CoA or in pinosylvin are not known, suggesting that plants synthesizing both stilbene types may have two STS enzymes with different substrate preferences. We investigated *P. strobus* as representative of the Haploxylon subgenus.

*Corresponding author. Fax: (49) (761) 2032601.

Abbreviations: AA, amino acid(s); STS, stilbene synthase(s).

The nucleotide sequences newly reported in this paper have been deposited in the GenBank/EMBL Data Banks with Accession Nos. Z46914 (STS1) and Z46915 (STS2).

2. Experimental

2.1. Plant material and induction

P. strobus seeds (Fa. Geigle, Nagold, Germany) were germinated and grown in white light (80 W/m^2; 16 h light, 8 h dark) in a phytotron at 25°C and with 50–60% humidity. The plantlets were used three weeks after germination, and they were induced for STS expression by wounding and infiltration with a yeast extract solution as described [6].

2.2. cDNA synthesis, screening, and sequence analysis

The cDNA library was constructed with 5 μg of poly(A)-rich RNA and cDNA synthesis kits from Boehringer (Mannheim, Germany). After addition of *Eco*RI/*Not*I adaptors (Pharmacia Biotech), the cDNAs were ligated to *Eco*RI digested phage lambda gt11 (Pharmacia Biotech) and packaged with a kit from Amersham Corporation. The library was screened with the STS from Scots pine (*P. sylvestris*) [6] according to published methods [10,11].

The largest cDNAs were sequenced on both strands by the dideoxy nucleotide chain termination technique [12,13]. The others were analyzed fully on one and partially on the second strand (at least 50%). The vectors, phages, and methods have been described [11]. The pTZ18R and pTZ19R system, helper phage M13K07, *E. coli* strain JM109 (Pharmacia LKB Biotechnology), and the reverse sequencing primer (Boehringer Mannheim) or custom-synthesized oligonucleotides were used with subcloned cDNA fragments. DNA polymerisation reactions were performed with [^{35}S]dAdo*P*[S]*PP* (adenosine 5′[1-thio]triphosphate) (37 TBq/mmol, Amersham Corporation) and modified T7 DNA polymerase (Sequenase, United States Biochemical Corporation).

2.3. Heterologous expression in E. coli

Site-directed mutagenesis with appropriate custom-synthesized oligonucleotides was used to introduce an *Nco*I-site into the start ATGs of the cDNAs and to exchange Arg^{313} in STS2 to His. The mutagenesis was performed with the cDNAs cloned in vector pTZ19R [14], and single-stranded DNA was obtained with helper phage M13K07 in *E. coli* strain RZ1032 [15]. The mutations were verified by DNA sequence analysis. The cDNAs were then recloned as *Nco*I/*Pst*I fragments into expression vector pQE-6 [16]. This joined the protein-coding re-

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gions via the *Nco*I site directly with the optimal promoter-translation-start configuration of the vector. The plasmids were called pQ/STS1$_{Pstr}$ (=STS1), pQ/STS2$_{Pstr}$ (=STS2), and pQ/STS2$_{Pstr}$ (R313→H) (=STS2*).

2.4. Enzyme extracts, assays, and evaluation

The experiments were performed as described [8], with the modification that the lysozyme concentration during lysis of the *E. coli* cells was raised to 2 mg/ml. Standard assays (0.1 ml, duplicates) contained 5–10 μg protein extract, 10 μM starter CoA-ester (cinnamoyl-CoA, dihydrocinnamoyl-CoA or 4-coumaroyl-CoA), 16.5 μM [2-^{14}C]malonyl-CoA (73,000 dpm; 0.78 GBq/mmol, Amersham), and 50 mM HEPES-buffer (*N*-[2-hydroxyethyl]piperazine-*N*'[2-ethanesulfonic acid]) adjusted to pH 7.0. The incubation times and protein concentrations were adjusted for conditions of linear product formation. The K_m determinations with cinnamoyl-CoA and dihydrocinnamoyl-CoA were performed with concentrations ranging from 1 to 20 μM. The pH dependencies were measured with 2-[*N*-morpholino]ethanesulfonic acid (MES), 3-[*N*-morpholino]propanesulfonic acid (MOPS), potassium phosphate, HEPES or Tris-HCl as buffers.

The radioactive products were separated by thin-layer-chromatography (TLC) on cellulose plates with 20% acetic acid as solvent, and they were quantified with a TLC analyzer [17]. The identity of the products pinosylvin, dihydropinosylvin, and resveratrol had been previously established by HPLC chromatography and gas chromatography-mass spectrometry [17]. The products with other starter CoA-esters (10 μM feruloyl-CoA or caffeoyl-CoA) were identified by their R_f-values.

The enzyme activities were not based on total protein in the enzyme extracts, but on the quantity of the STS proteins determined by immunoblotting with the antiserum against the closely related STS from *P. sylvestris* [8,17]. This allowed a direct comparison of the specific enzyme activities, and the determinations were performed with each enzyme extract (soluble proteins after a centrifugation of 15 min at 15,000 × *g*) as described in detail [1].

2.5. Other techniques

Standard procedures of molecular biology were performed according to established techniques [10].

3. Results

3.1. STS cDNAs from P. strobus

The screening of the cDNA library with the STS from *P. sylvestris* identified eight cross-hybridizing cDNAs ranging

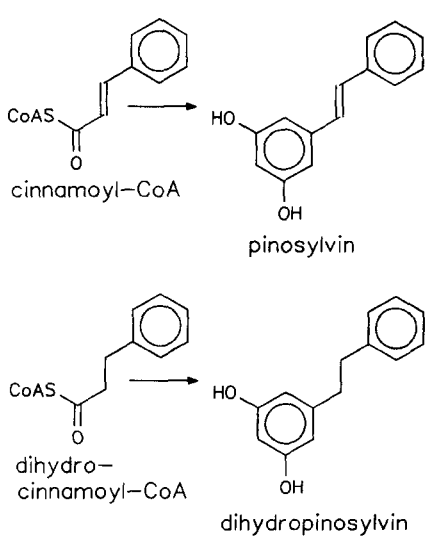

Fig. 1. Biosynthetic origin of pinosylvin and dihydropinosylvin from cinnamoyl-CoA and dihydrocinnamoyl-CoA. The reactions involve a sequential addition of three acetate units to the starter CoA-ester, formation of a new aromatic ring system, and the removal of a carboxyl group [9]. The stilbenes shown and their derivatives are natural constituents of *P. strobus*.

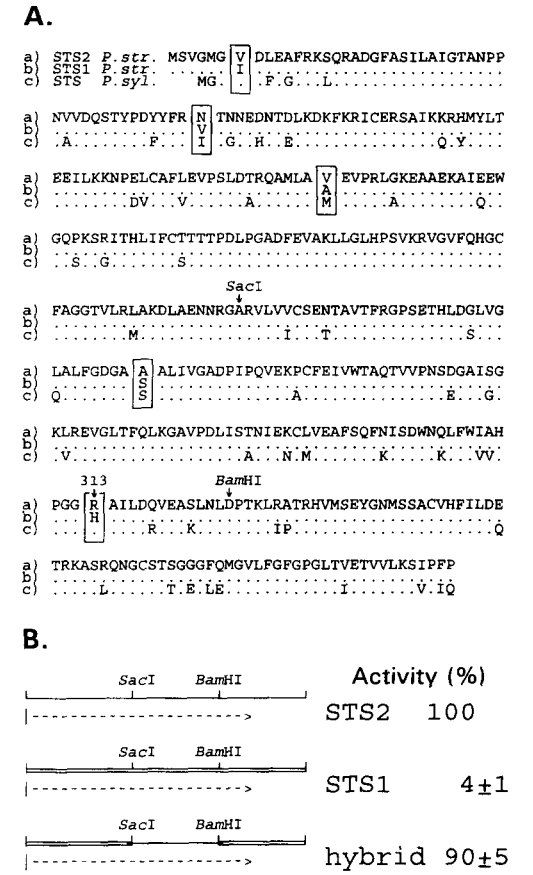

Fig. 2. A. Protein sequences of STS2 and STS1 from *P. strobus*, and of the STS from *P. sylvestris*. Only the differences to STS2 are shown in the latter two proteins; the dots indicate sequence identity. *Sac*I and *Bam*HI, position of the two restriction sites shared by STS2 and STS1 from *P. strobus* and used for construction of the protein hybrid. B. Scheme of the cDNAs and position of the restriction sites used to create a hybrid cDNA of STS1 and STS2 for expression of a protein chimera (hybrid). |- - ->, protein-coding region.

in size from 0.7 to 1.47 kbp. The sequences indicated that they fell into two classes. STS1 was represented by a single cDNA of 1,411 bp. The seven others (STS2, largest cDNA 1,470 bp) were sequence-identical but different in size. The sequences of STS1 and STS2 were closely related (DNA identity 98.8%), and the Poly(A) addition sites were in identical positions. The largest difference was in the 3'-nontranslated regions where STS1 lacked a stretch of 21 bp that was present in STS2. Both cDNAs coded for proteins of 396 AA (calculated size 43.1 kDa). The protein-coding regions contained 12 differences scattered throughout the sequence, and five led to AA exchanges (Fig. 2A). The two proteins shared 86% identity with the STS from *P. sylvestris* (Fig. 2A). The antiserum against that enzyme [8] cross-reacted with both STS from *P. strobus*, and this allowed the quantification of the two enzymes expressed in *E. coli* by immunoblotting. The relationship with the STS from peanut and grape was less pronounced (67% and 65% identity, respectively).

3.2. Enzyme properties of STS2 and STS1

Both proteins were cloned to expression in *E. coli* to investigate whether they represented pinosylvin or dihydropinosylvin

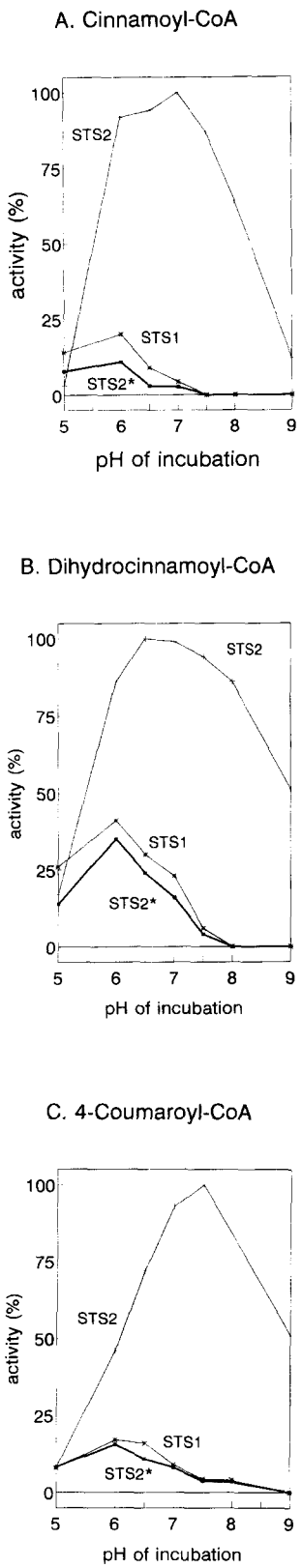

Fig. 3. Activities and pH dependencies of the *P. strobus* enzymes STS2, STS1, and of the mutant STS2* (Arg^{313} in STS2 exchanged to the His of STS1) with three starter CoA-esters. For each substrate, the specific activities of STS1 and STS2* were normalized to the highest values obtained with STS2 (= 100%). The ratio of the specific activities of STS2 with cinnamoyl-CoA, dihydrocinnamoyl-CoA, and 4-coumaroyl-CoA was 1:0.2:0.24.

synthases. STS2 revealed high activity (comparable to the STS from *P. sylvestris* [8]) with cinnamoyl-CoA as starter substrate (K_m 3.6 ± 1.5 μM). The activity with dihydrocinnamoyl-CoA was five-fold lower under the standard assay conditions (pH 7), and the K_m of 22 ± 10 μM was higher than with cinnamoyl-CoA. The substrate preference indicated that STS2 should be classified as pinosylvin synthase. The enzyme also accepted 4-coumaroyl-CoA (product resveratrol), feruloyl-CoA (product rhapontigenin), and caffeoyl-CoA (product piceatannol) with 4–8-fold lower efficiency than cinnamoyl-CoA. The reaction products or their derivatives are not known in *P. strobus*, and the activities therefore reflect the wide substrate specificities that were described also for other cloned STS [1,6,8,18].

The activity of STS1 was very low when compared to STS2. The specific activities (based on equivalent protein amounts in immunoblots) were with all starter CoA-esters only 3–5% of those detected with STS2 under the standard assay conditions. This hindered a detailed kinetic analysis, but the data allowed the conclusion that cinnamoyl-CoA was the preferred substrate (five-fold higher activity than with dihydrocinnamoyl-CoA), and thus STS1 should also be defined as pinosylvin synthase. Another difference to STS2 was that STS1 synthesized with cinnamoyl-CoA in addition to pinosylvin (R_f = 0.15 in the TLC system used) a second radioactive product (R_f = 0.3), and the rates of synthesis with respect to incorporation of radioactivity were similar for both products. This product was not observed with STS2, and the low activity of STS1 sofar precluded its identification.

The drastic differences between the two closely related STS were unexpected. Additional experiments (details in the next chapter) indicated that they were partly due to different pH dependencies. The unusual properties of STS1 must reflect differences in the protein sequences, and the experiments described below were designed to identify the AA responsible for these effects.

3.3. *A single AA exchange is responsible for the different properties of STS1 and STS2*

We first constructed an STS1/STS2 hybrid protein to test whether a combination of the five AA exchanges was responsible for the different properties or whether it would be possible to reduce the number of candidate AA. The cDNAs had two unique restriction enzyme sites in common (*Sac*I and *Bam*HI, Fig. 2). We used these to exchange the *Sac*I/*Bam*HI fragment in STS1 against the corresponding fragment from STS2, thus creating a hybrid in which most of the C-terminal half of STS1 was replaced by STS2 sequences (Fig. 2B). The enzyme assays revealed that the specific activity of the hybrid protein corresponded to STS2, not to STS1. Fig. 2A shows that the part introduced from STS2 into STS1 contained two of the five AA differences (Ala → Ser and Arg → His), and the results therefore indicated that either one or both exchanges were responsible for the different properties of STS1.

The highly active STS from *P. sylvestris* also contained Ser instead of Ala (Fig. 2A), suggesting that this exchange had no important functional significance. That left the Arg > His exchange in position 313 as the most likely candidate. Its role was investigated by changing the Arg in STS2 to His by site-directed mutagenesis (protein = STS2*). Fig. 3 summarizes the results of a comparison of the activities of STS2, STS1, and STS2* with three starter CoA-esters tested at different pH in the incuba-

tions. With all three substrates the data showed that STS1 differed from STS2 not only by its low activity, but also in the pH dependence, because STS1 was more active at pH 6 than at pH 7. The substrate preference for cinnamoyl-CoA was not significantly changed at the lower pH. The formation of the unidentified STS1 product with cinnamoyl-CoA followed the same kinetics as the synthesis of pinosylvin (not shown). The most important result of these experiments was that the exchange of Arg to His in STS2 (STS2*) converted the enzyme to a protein with the properties of STS1 in all tested aspects, i.e. low activity and changed pH dependence with all three starter CoA-esters (Fig. 3). Like STS1, the mutant STS2* also synthesized from cinnamoyl-CoA the unknown product with the same kinetics (not shown).

4. Discussion

P. strobus is the fourth plant from which cloned STS sequences are now available. The proteins share the highest identity with the *P. sylvestris* enzyme, and this is not unexpected in view of the close relationship of the plants. *P. sylvestris* contains a single STS identified as pinosylvin synthase [8], and this corresponds to the stilbene type (pinosylvin). Since *P. strobus* is known to contain two types of stilbenes, it was hoped that STS1 and STS2 represented two enzymes differing in the their substrate preferences. However, both preferred cinnamoyl-CoA and therefore should be classified as pinosylvin synthases. The question of an additional dihydropinosylvin synthase remains open. In view of the considerable STS2 activity with dihydrocinnamoyl-CoA it is also possible that this enzyme serves both reactions, and that the synthesis of the two stilbene types is controlled at the level of substrate availability. These questions can only be resolved by an additional exhaustive search of cDNA and genomic libraries and the demonstration that the cloned protein has the expected substrate preference.

From the enzymatical point of view the most interesting findings were that two STS differing in only five AA are so different in their properties, and the demonstration that a single Arg to His exchange is responsible for the low activity of STS1, the changed pH dependence, and the formation of a second product from cinnamoyl-CoA. The STS reactions are complex (three sequential condensation reactions, stabilization of polyketide intermediates, ring closure, and removal of a carboxyl group), and it is difficult to define precisely which aspect is affected. It is noteworthy, however, that all other sequenced STS contain either Arg or Pro in position 313, and that all known chalcone synthases (which are closely related to STS in structure and function [1]) contain Pro in the corresponding position. This suggests that a property unique to His may be responsible. In contrast to Pro and Arg, His at pH 7 is at least partly in a proton acceptor form because of the secondary amino group (pK = 6.5). It is tempting to speculate that a proton acceptor in position 313 disturbs some aspect of the STS reaction. Shifting the pH to lower values reduces the proton acceptor capacity by converting His into the protonated form, and this could explain the higher activity of the enzyme at pH 6. The reduction of activity at even lower pH may reflect a general negative effect on the overall reaction. It should be noted, however, that the interpretation is based on the properties of the free amino acid. The micro-environment in the protein may have decisive effects, and other explanations are not excluded. At the present state of knowledge it is not possible to speculate on the physiological role of an enzyme with the properties of STS1. The few differences between STS2 and STS1 suggest that the genes diverged fairly recently.

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