# Cross-reaction of chalcone synthase and stilbene synthase overexpressed in *Escherichia coli*

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Received 20 August 1999; received in revised form 4 October 1999

**Abstract Chalcone synthase (CHS) and stilbene synthase (STS) are related plant polyketide synthases belonging to the CHS superfamily. CHS and STS catalyze common condensation reactions of *p*-coumaroyl-CoA and three $C_2$-units from malonyl-CoA but different cyclization reactions to produce naringenin chalcone and resveratrol, respectively. Using purified *Pueraria lobata* CHS and *Arachis hypogaea* STS overexpressed in *Escherichia coli*, bisnoryangonin (BNY, the derailed lactone after two condensations) and *p*-coumaroyltriacetic acid lactone (the derailed lactone after three condensations) were detected from the reaction products. More importantly, we found a cross-reaction between CHS and STS, i.e. resveratrol production by CHS (2.7–4.2% of naringenin) and naringenin production by STS (1.4–2.3% of resveratrol), possibly due to the conformational flexibility of their active sites.**



*Key words:* Chalcone synthase; Stilbene synthase; Bisnoryangonin; *p*-Coumaroyltriacetic acid lactone; Cross-reaction

## 1. Introduction

Chalcone synthase (CHS, E.C. 2.3.1.74) catalyzes the first reaction of flavonoid biosynthesis and stilbene synthase (STS, E.C. 2.3.1.95) catalyzes biosynthesis of stilbene backbone of stilbene-type phytoalexins. CHS and STS are similar both in mechanistic and structural aspects and are representative members of the CHS superfamily [1]. CHS and STS are homodimers of identical subunits of 43 kDa and catalyze repetitive decarboxylative condensation of a *p*-coumaroyl residue with three $C_2$-units from malonyl-CoA. Although both enzymes share common starting materials with identical stoichiometry and apparently use the same condensation mechanism up to the common tetraketide intermediate, CHS and STS catalyze different ring closure reactions involving different atoms to give rise to different products, naringenin chalcone and resveratrol, respectively (Fig. 1). The amino acid homology between these two enzymes is more than 65%, and a phylogenetic analysis showed a higher homology between CHS and STS from related plants than among CHSs and STSs, suggesting that STSs have evolved from CHSs several times independently [2].

It has been demonstrated that, under certain in vitro conditions, CHS produces not only naringenin chalcone but also triacetic acid lactone (TAL) and styrylpyrone as early release (derailment) byproducts [3,4]. TAL was also detected from the reaction products by fatty acid synthase [5] and certain polyketide synthases [6,7]. On the other hand, such derailment products have not been observed in the reaction catalyzed by STS [8].

Using purified *Pueraria lobata* (kudzu) CHS and *Arachis hypogaea* (peanut) STS that was overexpressed in *Escherichia coli*, we have detected bisnoryangonin (BNY, the derailed lactone after two condensations) and *p*-coumaroyltriacetic acid lactone (CTAL, the derailed lactone after three condensations) from the reaction products produced by either CHS or STS. Furthermore, we found a cross-reaction between CHS and STS, i.e. resveratrol production by CHS and naringenin production by STS in small but detectable amounts.

## 2. Materials and methods

### 2.1. Cloning

Transcription of STS was induced by cutting peanut seedlings (Sakata, Japan) into slices (1 mm thick) that had been immersed in water for 3 days [9]. The slices were then left overnight in water at 25°C in darkness. Total RNA was prepared by the phenol method. cDNA was synthesized using an oligo dT-*Xba*I primer (5′-GTC GAC TCT AGA $(T)_{15}$-3′) and PCR-amplified with primers designed from the published *A. hypogaea* STS sequence [10]; the forward primer (5′-CAT *CCATG*G TGT CTG TGA GTG AAT TC-3′) and the reverse primer (5′-GTA TTA TAT GGC CAT GCT GCG GAG-3′). The forward primer carries an *Nco*I site suitable for cloning into the expression vector, pET-3d. The resulting PCR product was first blunt-ligated into the *Sma*I site of pBluescriptIISK(+) (MBI Fermentas), rescued by *Nco*I/*Bam*HI digestion, and ligated into the *Nco*I/*Bam*HI restricted vector pET-3d to give pET-3d/STS. The *Nco*I digestion was carried out in the presence of EtBr (40 μg/ml) to obtain the 1.2-kb full-length cDNA because of the presence of another *Nco*I site in the coding region. DNA sequencing was carried out on both strands with several forward and reverse primers using the Dye Terminator Cycle Sequencing kit from Applied Biosystems (CA, USA).

Construction of pET-3d/CHS using similar methods has been described [11].

### 2.2. Protein expression and purification

*E. coli* BL21(DE3)pLysE cells transformed with either pET-3d/CHS or pET-3d/STS were grown at 37°C in LB medium containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol and induced with 0.4 mM IPTG. After an induction period of 4 h at 37°C, the cells were harvested, washed and suspended in buffer A (10 mM potassium phosphate (KPi) buffer (pH 7.5), 2 mM DTT). Following sonication (10 s

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*Abbreviations:* CHS, chalcone synthase; STS, stilbene synthase; BNY, bisnoryangonin; CTAL, *p*-coumaroyltriacetic acid lactone; TAL, triacetic acid lactone; KPi, potassium phosphate; Trx-, thioredoxin-


PII: S0014-5793(99)01403-9

×10) on ice and centrifugation (12 000×*g*, 4°C, 15 min), soluble fractions were obtained. After $(NH_4)_2SO_4$ precipitation (30–80% saturation), CHS was purified to apparent homogeneity by successive chromatography procedures: (1) DEAE-Toyopearl (TOSOH, Tokyo, Japan) anion exchange chromatography using a linear gradient of NaCl (0–500 mM) in buffer A, (2) Bio-Gel hydroxyapatite (Bio-Rad) using a linear gradient of 10–300 mM KPi (pH 7.5) containing 2 mM DTT and (3) chromatofocusing on polybuffer exchanger (PBE94, Pharmacia) pre-equilibrated with 25 mM imidazole-HCl (pH 7.4), 2 mM DTT and eluted with Polybuffer74-HCl (pH 5.0), 2 mM DTT. STS was similarly purified after successive DEAE ion exchange and hydroapatite chromatography steps. Protein contents were determined with Bradford's dye method (Bio-Rad) [12], using bovine serum albumin as standard.

Expression and purification of *P. lobata* CHS and *A. hypogaea* STS as thioredoxin-HisTag fusion proteins (Trx-CHS and Trx-STS) used in the LC-MS analysis of cross-reaction will be described elsewhere.

### 2.3. Enzyme assay

For both CHS and STS, the reaction mixture (0.1 ml) contained appropriate amounts of protein (2–5 μg of purified enzyme), 0.1 mM *p*-coumaroyl-CoA and 16.8 μM [2-$^{14}$C]malonyl-CoA (2.2 GBq/mmol, NEN) in 0.1 M KPi buffer (pH 7.5) ($K_2HPO_4/KH_2PO_4$) containing 2 mM DTT. After incubation at 37°C for 30 min, 50 μl of 50% acetic acid was added and the reaction products were extracted with 200 μl of ethyl acetate. A portion (50 μl) of the extract was analyzed by TLC on RP18 plate (Merck) with methanol:$H_2O$:acetic acid (60:40:1) as solvent and the radioactive products were quantified with an Imaging Plate analyzer (BAS2000, Fuji). Authentic compounds, TAL ($R_f$ = 0.65, Tokyo Kasei), resveratrol ($R_f$ = 0.50, Sigma) and naringenin ($R_f$ = 0.32, Nacalai tesque) were used as internal standards to identify the products. The specific enzyme activity was expressed in pmol of the product produced/s/mg (pkat/mg).

### 2.4. Identification of bisnoryangonin and p-coumaroyltriacetic acid lactone

For identification of BNY, the ethyl acetate-extracted products produced by STS and CHS were first separated by HPLC. The column (5 μm, 4.6 mm I.D. ×150 mm, ODS80Ts, TOSOH) was washed at a flow rate of 0.6 ml/min at 40°C with 45% methanol in $H_2O$ containing 1% acetic acid for 10 min and then the concentration of methanol was increased to 55% during the next 30 min. Detection was with an UV monitor at 254 nm. The peak at $R_t$ = 16.3 min was collected and, after evaporation of the solvent, the residues were dissolved in methanol and reacted with trimethylsilyldiazomethane in the dark for 30 min at 22°C. The resulting sample was subjected to GC-MS (Hewlett Packard 5890 coupled with JEOL JMS-SX102A) analysis using a DB5 column (0.25 mm I.D. ×30 m, film thickness 0.25 μm, J and W) at 200°C to 250°C (10°C/min). The retention time and mass spectrum were found to be identical to those of authentic yangonin: GC, $R_t$ = 22 min; EIMS (70 eV, *m/z*), 258 ($M^+$), 230, 187.

Production of CTAL by CHS and STS was confirmed by LC-MS (see below). CTAL: Multi-stage LC-APCIMS, MS *m/z* 273.1 $[M+H]^+$, 271.1 $[M-H]^-$, MS/MS (precursor ion of *m/z* 271.1) 227.2 $[M-H-CO_2]^-$, MS/MS/MS (transitions *m/z* 271.1 > 227.2) 185.2 $[M-H-CO_2-CH_2CO]^-$.

### 2.5. Cross-reaction

The enzyme reaction was carried out under the same conditions as described above except that the reaction volume was 1 ml and about 50 μg of enzyme (10–40 pkat/mg) was used. Radioactive products thus obtained were redissolved in 500 μl of 50% aqueous methanol containing 1% acetic acid in which authentic resveratrol (1.5 μg) and naringenin (2 μg) were added. Using a Hitachi 655 HPLC system and ODS-80Ts column (TOSOH), the radioactive products were separated with isocratic elution with 50% aqueous methanol containing 1% acetic acid for 30 min. Radioactivity of each 0.5 ml fraction was measured with a β-scintillation counter and the fractions corresponding to main product (naringenin for CHS and resveratrol for STS) and cross-reaction product (resveratrol for CHS and naringenin for STS) were collected. For carrier dilution analysis, recrystallization of the radioactive products with carrier compounds was performed at least four times in two different solvent systems (methanol and ethyl acetate/hexane) and specific radioactivity was measured after each recrystallization step.

The identity of the cross-reaction products was further confirmed by GC-MS and LC-MS. First, the CHS and STS reaction products were methylated with trimethylsilyldiazomethane and analyzed by GC-MS as described above. From the methylated CHS reaction products, a peak of *m/z* = 256 ($R_t$ = 12.99 min) corresponding to dimethylresveratrol was detected with mono- (*m/z* 286), di- (*m/z* 300) and trimethylnaringenin (*m/z* 314). Likewise, a peak of *m/z* = 300 ($R_t$ = 15.35 min) corresponding to dimethylnaringenin was detected from the methylated STS reaction mixture together with di- (*m/z* 256) and trimethylresveratrol (*m/z* 270). For LC-MS analysis, Trx-CHS (11 mg, 250 pkat) or Trx-STS (9.5 mg, 160 pkat) was incubated with 0.1 mM *p*-coumaroyl-CoA and 0.3 mM malonyl-CoA in 50 ml of 0.1 M KPi buffer (pH 7.4) containing 1 mM DTT for 40 min at 37°C. At the end of incubation, the pH was raised to 9 with 1 N NaOH, and the reaction mixture was further incubated at 37°C for another 10 min to complete chemical transformation of naringenin chalcone to naringenin [13]. The ethyl acetate extract was dried over $Na_2SO_4$ and the solvent was removed under vacuum at 30°C. The remaining products were analyzed by LC-MS (Thermoquest LCQ) as previously described [14].

Resveratrol: LC-APCIMS (positive), 229.1 $[M+H]^+$, MS/MS (precursor ion at 229) *m/z* (rel. %) 211 (65), 135 (100), 119 (12), 107 (11). Naringenin: LC-APCIMS (positive), 273.2 $[M+H]^+$, MS/MS (precursor ion at 273) *m/z* (rel. %) 171 (18), 153 (80), 147 (100).

## 3. Results

### 3.1. Sequence of A. hypogaea STS

When the deduced amino acid sequence of Japanese peanut STS cloned in this study (Accession number: AB027606) was compared to the published peanut STS sequence (Swiss-Prot P20178) [10], 8 out of 389 amino acids were found to be different. Those are Ser-46 corresponding to Gly-46 in Ref. [10], Ser-195 to Ala-195, Ser-202 to Asn-202, Leu-236 to Ile-236, Asp-281 to Gly-281, Pro-304 to Leu-304, Glu-315 to Gln-315 and Ala-356 to Thr-356. This difference in amino acid sequence probably reflects different origins of the plant materials used.

### 3.2. Purification of CHS and STS

Purification of CHS and STS overexpressed in *E. coli* is summarized in Table 1. Typical experiments yielded 0.3–0.4

Table 1
Purification of *P. lobata* CHS and *A. hypogaea* STS overexpressed in *E. coli*

| | Stage | Total protein (mg) | Total activity (pkat) | Specific activity (pkat/mg) | Recovery (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| CHS | Crude extract | 29 | 36 | 1.2 | (100) | – |
| | DEAE ion exchange | 4.5 | 9.1 | 2.0 | 25 | 1.7 |
| | Hydroxyapatite | 1.7 | 6.5 | 3.8 | 18 | 3.2 |
| | Chromatofocusing | 0.42 | 4.2 | 10 | 12 | 8.3 |
| STS | Crude extract | 10 | 35 | 3.5 | (100) | – |
| | DEAE ion exchange | 1.3 | 14 | 10 | 39 | 2.9 |
| | Hydroxyapatite | 0.31 | 12 | 37 | 33 | 11 |

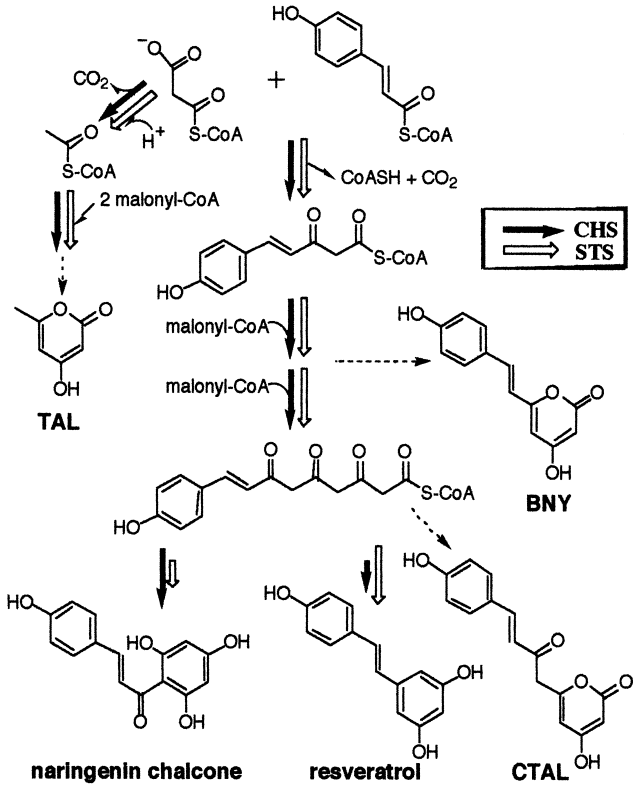


Fig. 1. Reaction steps catalyzed by CHS and STS and the products produced in vitro.

mg of apparently homogeneous enzymes from 300 ml bacteria culture (Fig. 2A). Recovery was 10–30% with about 10-fold enrichment.

### 3.3. Identification of BNY and p-coumaroyltriacetic acid lactone

Production of BNY and CTAL by STS as well as by CHS was clearly visualized by TLC (Fig. 2B). The TLC spots were identified with non-labeled carrier compounds (resveratrol and naringenin) and with parallel experiment using *p*-coumaroyltriacetic acid synthase which has been recently cloned from *Hydrangea macrophylla* and shown to produce CTAL and BNY as the main product and a major byproduct, respectively [14]. Further, the identity of BNY and CTAL was unambiguously determined by GC-MS and LC-MS. The production of these derailment products by CHS and STS were dependent on the presence of sulfhydryl reagents such as DTT in the reaction mixture, consistent with the earlier report with CHS [3]. It was thought that sulfhydryl reagent would act as an acceptor to the enzyme-bound triketide (or tetraketide) intermediate in a non-enzymatic *trans*-esterification to form unstable thiol adduct, which should be immediately converted to the corresponding pyrone [3]. In agreement with this notion, DTT showed apparently parallel effects on formation of BNY and CTAL. However, the requirement of the sulfhydryl reagent was not absolute, as these byproducts were still detectable when the enzyme reaction was carried out in the absence of DTT (data not shown). Acidification of the reaction mixture to pH $< 5$ greatly enhanced the amounts of BNY and CTAL detected on TLC. These results suggest that the majority of these compounds, both being pyrone derivatives, are present in the reaction mixture as free acids at neutral pH, thus poorly extractable by ethyl acetate, and that lactonization to form pyrone ring is non-enzymatic. Similar pH-dependence of extraction of pyrone derivatives was also reported recently by others [15]. Whereas CTAL was a major byproduct of the CHS reaction, BNY was a major byproduct of the STS reaction (Fig. 2B).

### 3.4. Cross-reaction

When the reaction products produced by purified recombinant enzyme were analyzed by HPLC, a small radioactive peak corresponding to resveratrol ($R_t = 10$ min) was detected in the CHS products and a peak corresponding to naringenin ($R_t = 20$ min) was detected in the STS products (Fig. 3). Cross-reaction was verified by carrier dilution analysis. Successive rounds of recrystallization of radioactive products in the presence of non-labeled compounds gave crystals of constant specific radioactivity. For resveratrol produced by CHS, the specific radioactivity was 8200 dpm/mmol (methanol), 7300 (methanol), 7000 (ethyl acetate/hexane) and 6700 (ethyl acetate/hexane), and for naringenin produced by STS, it was 8900 dpm/mmol (methanol), 7900 (methanol), 7500 (ethyl acetate/hexane) and 8300 (ethyl acetate/hexane). The cross-reaction was determined to be 2.7–4.2% (resveratrol/naringenin) for CHS and 1.4–2.3% (naringenin/resveratrol) for STS. The cross-reaction was further confirmed by GC-MS and LC-MS analyses. The retention time, molecular mass and major daughter ions in MS/MS were indistinguishable to those of authentic compounds and those found in the literature [16]. Trx fusion enzymes were indistinguishable from the native enzymes with regard to the pattern of byproduct production and kinetic parameters (data not shown).

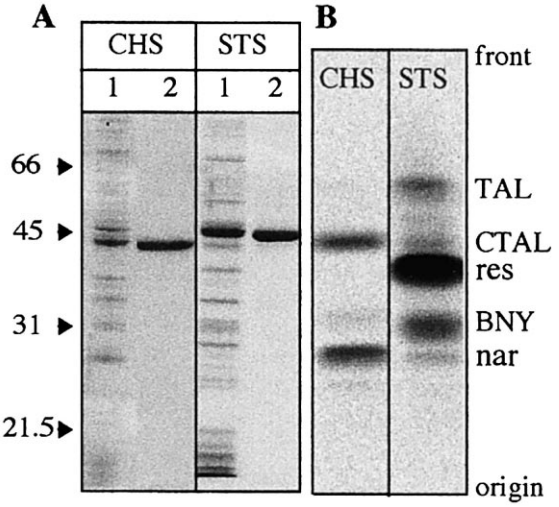


Fig. 2. Purification and enzyme assay of *P. lobata* CHS and *A. hypogaea* STS. A: SDS-PAGE was performed on a 12% acrylamide minislab gel under reducing conditions. For both CHS and STS, lane 1 contained 3 μg of proteins obtained after $(NH_4)_2SO_4$ fractionation and lane 2 contained 1 μg of purified enzyme. The proteins were stained with Coomassie blue R250 (Bio-Rad). The numbers on left indicate molecular weight in kDa of the $M_r$ marker proteins. B: Radio thin layer chromatogram of enzyme assays using purified recombinant CHS (5 μg) and STS (2 μg) and *p*-coumaroyl-CoA and [2-$^{14}$C]malonyl-CoA as substrates. The conditions for enzyme assay and TLC analysis are described in Section 2.

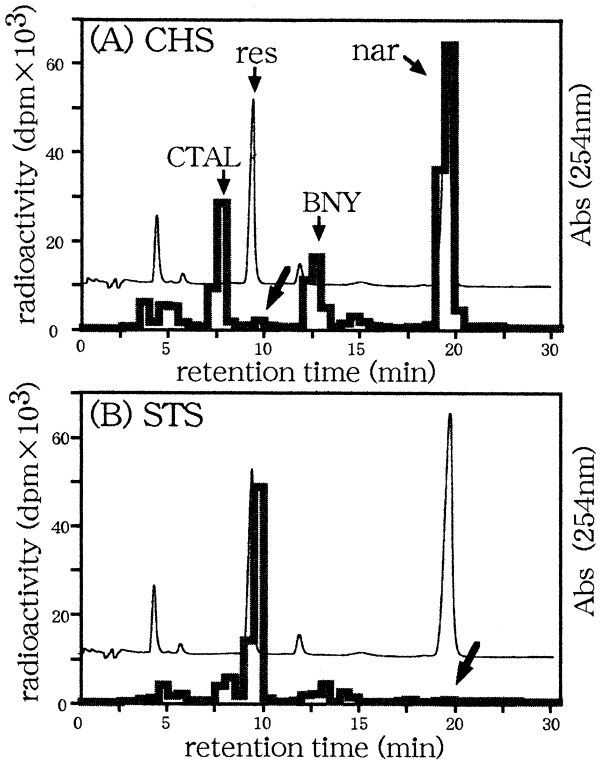


Fig. 3. HPLC analysis of reaction products produced by purified recombinant *P. lobata* CHS (A) and *A. hypogaea* STS (B). Authentic resveratrol and naringenin were added to the reaction products to aid detection by UV absorbance (thin line) and the resulting sample was injected onto a TOSOH ODS80-Ts column. The products were separated with an isocratic elution of 50% aqueous methanol containing 1% acetic acid. Radioactivity (thick line) of each fraction (0.5 ml) was measured and fractions corresponding to the cross-reaction products (resveratrol for CHS and naringenin for STS) were pooled and used for carrier dilution analysis.

## 4. Discussion

CHS purified from plants was shown to produce styrylpyrones and other minor byproducts in certain in vitro conditions [3,4]. In the present study, we showed that, like CHS, purified recombinant peanut STS produced not only BNY, a styrylpyrone, but also CTAL, the recently found derailment product at the stage of tetraketide intermediate [14]. This was not unexpected since it has been thought that CHS and STS catalyze identical condensation steps leading to the linear tetraketide intermediate prior to different cyclization steps [1]. Detection of BNY and CTAL from the STS reaction products provides an unequivocal evidence for this notion. Under the conditions employed, CHS produces more byproducts than STS (Figs. 2B and 3). This may reflect the more loosely structured active site of CHS compared with that of STS, in vitro, which allows to a greater extent premature thioester exchange with sulfhydryl reagent present in the reaction mixture or thioester hydrolysis by water to give derailment products. CHS is believed to be present in the cell as a part of multienzyme complex of flavonoid biosynthesis including polyketide reductase, chalcone isomerase and other modifying enzymes to achieve 'metabolite channeling' [1,17].

Cyclization steps in CHS and STS are different; Dieckmann condensation leading to naringenin chalcone in CHS and aldol condensation to stilbene in STS. Nonetheless, CHS and STS produce counterpart products in low amounts (2–4% of main products) which could not be detected by routine assays. The possibility that the cross-reaction products were formed during work-up by chemical transformation was thought to be unlikely based on the following. First, although the corresponding chemical transformations are known, they are normally carried out under harsh conditions unlike the work-up procedures used in this study. For example, the Dieckmann condensation of methyl ester of 7-phenyl-3,5,7-trioxoheptanoic acid to corresponding acylphloroglucinol required a prolonged incubation under strongly basic conditions, such as 2 M KOH [18]. Even though aldol condensation of triketo acids can proceed in weakly acidic (pH 5) aqueous buffer, the products are fairly stable β-resorcylic acids [18]. Decarboxylation of aromatic carboxylic acids such as conversion of 6-styryl-β-resorcylic acid to pinosylvin and of orsellinic acid to orcinol have been achieved either by heat (180°C) [18,19], by alcoholic KOH [20] or by a decarboxylase [21]. It should be noted that stilbene carboxylic acids, though found in nature [22], have not been detected from the STS reaction mixture. Secondly, neither naringenin nor resveratrol was detected after using an identical work-up procedure in the reaction mixture produced by *p*-coumaroyltriacetic acid synthase (CTAS), which catalyzes, like CHS and STS, three condensation reactions between *p*-coumaroyl-CoA and malonyl-CoA to triketo acid but does not catalyze any cyclization reaction [14]. This suggested that lactonization of the free triketo acid predominates during work-up to give CTAL as a major byproduct (Fig. 1) and, therefore, we concluded that the cross-reaction of CHS and STS was most likely enzymatic of nature.

One reasonable interpretation for this seemingly unnatural cross-reaction is that parts of heterologously overexpressed enzymes are in unnatural conformational states and these misfolded, yet soluble enzymes are responsible for the observed cross-reaction. Overexpression in *E. coli* often leads to misfolding especially for membrane-associated eukaryotic proteins, as bacteria lack an intracellular membrane environment that may be necessary for efficient and accurate folding [23]. Both CHS and STS are shown to be weakly membrane-associated in the plant cells [24,25] and CHS is associated with other enzymes involved in the flavonoid biosynthesis pathway [17]. However, an attempt to resolve conformational heterogeneity using hydroxyapatite chromatography was unsuccessful, as cross-reaction activities were co-purified with the natural activities (data not shown). Another intriguing and probably more conceivable possibility is that CHS and STS possess intrinsic potential to catalyze cross-reaction possibly due to conformational flexibility of their active sites, and that this intrinsic, yet 'cryptic' capability of these enzymes to perform the cross-reaction was manifested under non-optimal folding conditions during bacterial overexpression. Our recent finding that cross-reaction of CHS and STS expressed in eukaryotic systems (yeasts and insect cells) apparently decreased as compared to the cross-reaction found in this study with bacterial expression system seems to support this idea (Sankawa, unpublished data). In line with this idea, it should be noted that cyclization steps in CHS and STS are proposed to be under pure topological control [8]. Indeed, the recently solved crystal structure of alfalfa (*Medicago sativa*) CHS by Ferrer et al. did not reveal apparent functional amino acids in the cyclization pocket that might participate in a specific

CHS-type cyclization reaction [26]. The authors, therefore, proposed that alterations in the surface topology of the cyclization pockets of CHS and STS may affect the stereochemistry of the cyclization reactions.

Based on phylogenetic analysis, Schröder et al. [2] proposed different STSs evolved from CHSs independently several times by a limited number of amino acid exchanges. However, consensus (signature) amino acid sequences characteristic to CHS or to STS have yet to be recognized. It may result from the insufficient number of STS sequences available (6 entries in Swiss-Prot). On the other hand, STS conceivably gained its new activity by changing active site geometry rather than by introducing a few new functional amino acids in the active site of STS. A closely related case can be found in chemical diversity of higher plant fatty acids, where independent, multiple evolution of oleate 12-hydroxylase from oleate 12-desaturase was proposed. Site-directed mutagenesis studies involving as few as seven amino acids suggested that catalytic plasticity in these two enzymes is achieved by changing active site geometry leading to different partitioning of a common intermediate into different reaction products [27]. Clearly, further studies with regard to the origin of the cross-reaction of CHS and STS overexpressed in *E. coli* are warranted.

*Acknowledgements:* The authors are grateful to the Ministry of Education, Science, Sports and Culture of Japan for Grants-in Aid for Scientific Research (B) (No. 09044212) and (C) (No. 10680564). D.-Y. Suh thanks the Tokyo Biochemistry Research Foundation for a fellowship (TBRF-98-10).

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