# Chalcone and Stilbene Synthases Expressed in Eucaryotes Exhibit Reduced Cross-Reactivity *in Vitro*

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Received February 18, 2000; accepted March 24, 2000

**Chalcone synthase (CHS) and stilbene synthase (STS) catalyze different cyclization reactions of the common tetraketide to give different products, naringenin chalcone and resveratrol, respectively. We have previously observed *in vitro* cross-reaction of CHS and STS overexpressed in *Escherichia coli*, resveratrol production by CHS and chalcone production by STS. When expressed in eucaryotic cells, or in *E. coli* as thioredoxin-fusion proteins, CHS and STS exhibited reduced cross-reaction. STS refolded from inclusion bodies also showed reduced cross-reaction. While addition of bovine serum albumin and pH in the reaction were without noticeable effect, addition of glycerol decreased the cross-reaction of CHS likely due to its stabilizing effect on enzyme conformation. These results were interpreted to provide supporting evidence to our earlier proposition (Yamaguchi T. *et al.*, *FEBS Lett.*, 460, 457—461 (1999)) that the *in vitro* cross-reaction of CHS and STS is due to intrinsic capability of these enzymes to catalyze different types of cyclization, which, in turn, is endowed by conformational flexibility of their active sites.**

**Key words** Chalcone synthase; stilbene synthase; cross-reaction; heterologous expression

Flavonoids protect plants from UV irradiation and are responsible for the color in flowers.[1] The first committed reaction of flavonoid biosynthesis is catalyzed by chalcone synthase (CHS, E.C. 2.3.1.74).[2] In contrast, stilbene synthase (STS, E.C. 2.3.1.95), found in a limited number of plants, synthesizes the backbone of the stilbene phytoalexins which have antifungal properties and contribute to the plant defense against pathogens.[3,4] Belonging to the CHS superfamily,[5] CHS and STS share common starting materials with identical stoichiometry (a starter CoA ester from the phenylpropanoid pathway such as *p*-coumaroyl-CoA and three molecules of malonyl-CoA) and use the same decarboxylative condensation mechanism up to the common tetraketide intermediate (Fig. 1). However, they catalyze different ring closure reactions involving different atoms to give rise to different products, chalcones and stilbenes, respectively. CHS and STS are homodimers of 43 kDa subunits and the amino acid homology between these two enzymes is more than 65%.

It has been thought that the difference in products of CHS and STS is strictly controlled in nature. However, this distinction was not absolute under certain *in vitro* conditions, as we have previously reported *in vitro* cross-reaction of CHS and STS overexpressed in *Escherichia coli.*[6] Purified *Pueraria lobata* CHS overexpressed in *E. coli* produced resveratrol, the natural product of STS, up to 4% of its chalcone production, whereas purified *Arachis hypogaea* STS overexpressed in *E. coli* produced naringenin chalcone up to 2% of its resveratrol production. This cross-reaction probably does not occur in nature, but it was taken to reflect close semblance of the active sites of the two enzymes. It has been pro-

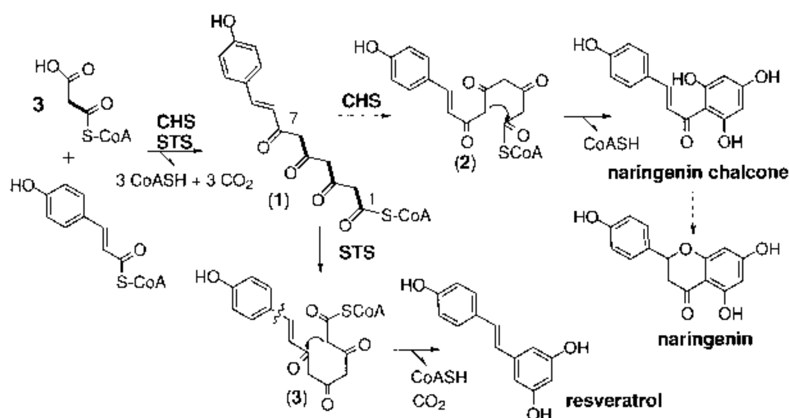


Fig. 1. Reactions Catalyzed by Chalcone Synthase (CHS) and Stilbene Synthase (STS)

Both enzymes catalyze decarboxylative Claisen-type condensations between *p*-coumaroyl-CoA starter unit and three malonyl-CoA extender units leading to the enzyme-bound tetraketide intermediate (**1**). CHS catalyzes a cyclization reaction (acylation) involving C-1 and C-6 (6→1), producing naringenin chalcone, whereas STS catalyzes a cyclization involving C-2 and C-7 (2→7) accompanied by loss of carboxylate, producing resveratrol. Naringenin chalcone was chemically transformed to naringenin under the reaction conditions used.[6]

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posed that the cyclization reactions in CHS and STS are under pure topological control. Different spatial folding of the linear tetraketide ((**2**) and (**3**) in Fig. 1) governed by different surface geometry of the enzyme active sites would lead to different cyclization products.[7,8] Then, the observed *in vitro* cross-reaction of CHS and STS might be due to the intrinsic conformational flexibility of the enzyme active sites, which was exposed under non-optimal folding conditions during bacterial overexpression and/or under certain enzyme reaction conditions. Hence, to study the effects of expression hosts and reaction conditions on the cross-reaction, CHS and STS were expressed in eucaryotes, yeasts and insect cells, and also in *E. coli* as thioredoxin-fusion proteins, all of which are expected to provide better environments for adequate folding. In addition, active enzymes were refolded from inclusion bodies produced during overexpression in *E. coli.* The cross-reaction activities of these various enzyme preparations were compared under varying reaction conditions.

## Experimental

**Heterologous Expression of *P. lobata* CHS and *A. hypogaea* STS in *Candida boidinii*** The full-length CHS and STS inserts[6] were introduced into the *Not*I site of a yeast expression vector pNoteI[9] by blunt-end ligation, yielding pNoteI-CHS and pNoteI-STS, respectively. Direction of the inserted cDNA was confirmed by restriction enzyme mapping of the plasmids. Transformation of methylotropic yeast, *C. boidinii* TK62[10] was performed by the modified lithium acetate method as described.[11] Colonies of transformants were subcultured at 30 °C for 30 h in 3 ml of YNB medium (0.67% yeast nitrogen base, 2% glucose). The cells were then transferred for induction to 50 ml of YME medium (0.67% yeast nitrogen base, 0.5% yeast extract, 1.5% methanol) and allowed to grow aerobically at 28 °C for 40 h. The cells were harvested by centrifugation at 2000×***g*** for 10 min and disrupted by french-press with a CRYO-PRESS CP-100W (Microtech Nichion, Japan). The cell lysates were suspended in 20 ml of 0.1 M potassium phosphate (pH 7.5) containing 2 mM DTT and cell-free extracts were obtained after centrifugation at 10000×***g*** for 10 min. Colonies that allowed higher expression of CHS and STS were selected by SDS-PAGE analysis of the cell-free extracts. Insertion of the CHS and STS genes into the yeast DNA was further confirmed by PCR using appropriate primers designed from the sequences of 5′- and 3′-ends of the CHS and STS cDNAs. Total yeast DNA purified following a standard method[12] was used as template.

**Heterologous Expression of CHS and STS in *Spodoptera frugiperda* Sf9 Cells** The full-length *P. lobata* CHS and *A. hypogaea* STS cDNA were blunt-ligated into the *Sma*I site of the baculovirus expression vector pAcYM1[13] produced from *Autographa californica* nuclear polyhedrosis virus (AcNPV), yielding pAc-CHS and pAc-STS, respectively. Transfection of *Spodoptera fugiperda* (*Sf*9) cells with the derived recombinant baculovirus and insect cell culture were performed using the kit (BaculoGold, PharMingen) according to the manufacturer's protocol. The cells were harvested 72 h after infection by centrifugation at 2500×***g*** for 5 min. The precipitated cells were ruptured with a CRYO-PRESS and suspended in a proper volume ($4\times10^6$ cells/ml) of 0.1 M potassium phosphate (pH 7.5) containing 2 mM DTT. The resulting cell-free extracts (1 mg/ml) were analyzed by SDS-PAGE for enzyme expression and used as enzyme source without further purification.

**Expression in *E. coli*, Isolation of Inclusion Bodies and Refolding of Enzymes** Construction of expression plasmids pET3d-CHS and pET3d-STS coding *P. lobata* CHS and *A. hypogaea* STS, respectively, their expression in *E. coli* BL21(DE3)pLysE, and purification were previously described.[6]

Inclusion bodies were isolated from the induced *E. coli* cells according to the literature[14] with some modifications. The recombinant *E. coli* cells harboring pET3d-CHS or pET3d-STS were grown at 37 °C for 4 h after induction by 0.4 mM IPTG. Following a freeze/thaw cycle, the cells suspended in 1/25 culture volume of cold 40 mM potassium phosphate buffer ($I$=0.1, pH 7.4), 2 mM EDTA (KPE buffer) were subjected to bursts (3×5 s) of sonication to complete lysis. After centrifugation at 6000×***g*** for 10 min, the inclusion body pellet was sequentially washed with 1/10 culture volume of 0.1% Triton X-100 in KPE buffer and twice with 2 M urea in KPE buffer. The inclusion bodies were dissolved in denaturation buffer (8 M urea, 0.1 M $\beta$-mercaptoethanol in KPE buffer) to a concentration of 3 mg/ml. Refolding was performed by dialysis at 4 °C against KPE buffer containing 5 mM cystein with two changes of the buffer for >24 h. The volume and protein concentration of the dialysate were 1 ml and 3.5 to 5 $\mu$g/ml, respectively. The refolded enzymes were finally dialyzed against 0.1 M potassium phosphate (pH 7.4), 2 mM DTT prior to enzyme activity measurements. Protein concentration was determined by Bradford's dye method (BioRad)[15] using Trx-CHS as standard.

**Expression in *E. coli* and Purification of Thioredoxin-Fusion Proteins** Expression of CHS and STS as thioredoxin-HisTag-fusion proteins (Trx-CHS and Trx-STS) and purification by nickel chelation chromatography will be described elsewhere.

**Enzyme Assay and Quantification of Cross-reaction** Both CHS and STS reactions were carried out in the presence of 0.1 mM *p*-coumaroyl-CoA and 16.8 $\mu$M [2-$^{14}$C]malonyl-CoA (2.2 GBq/mmol, NEN) as substrates and analyzed by radio-TLC and an imaging plate analyzer (BAS2000, Fuji) as described previously.[6] The specific enzyme activity was expressed in pmol of the product produced/s/mg (pkat/mg).

To study cross-reaction of the enzymes expressed in yeasts and in insect cells, the enzyme reaction was carried out in a total volume of 1 ml containing 100 $\mu$g (yeasts) or 800 $\mu$g (insect cells) of proteins, 0.1 mM *p*-coumaroyl-CoA and 16.8 $\mu$M [2-$^{14}$C]malonyl-CoA (37 kBq) in 0.1 M potassium phosphate (pH 7.5) containing 2 mM DTT. After incubation at 37 °C for 15 to 30 min, the reaction was stopped by addition of 50% acetic acid (0.5 ml). The reaction products were then extracted with ethyl acetate (2×1 ml) and the solvent was removed using a spin-vac. The residue was dissolved in 0.5 ml of the HPLC mobile phase (methanol : $H_2O$ : acetic acid=50 : 50 : 1, v/v) and authentic naringenin (2 $\mu$g) and resveratrol (1.5 $\mu$g) were added as internal standards. HPLC was performed using a ODS-80TM column (TOSOH, 5 $\mu$m, 4.6×150 mm) at a flow rate of 1 ml/min; compounds were detected by their absorbance at 254 nm. Radioactivity of each 0.5 ml fraction was measured with a $\beta$-scintillation counter and the fractions containing the products were pooled. For carrier dilution analysis, recrystallization of the radioactive products with carrier compounds was performed four times in two different solvent systems (methanol and hexane/ethyl acetate) and specific radioactivity was measured after each recrystallization step.

## Results and Discussion

**Heterologous Expression in Yeasts and Insect Cells** Using a homologous recombination-based integrative transformation system, *P. lobata* CHS and *A. hypogaea* STS were functionally expressed in the methylotrophic yeast, *C. boidinii.* The constructed plasmids, pNoteI-CHS and pNoteI-STS, were linearized by *Bam*HI, and used for the transformation of *C. boidinii* TK62 (*ura*3). As the inserts were placed under the *C. boidinii* alcohol oxidase (*AOD1*) promoter, the CHS and STS proteins were produced at a high level when methanol was used as the carbon source. About 80% of the Ura$^+$ transformants selected (>100) showed the distinctive CHS or STS band on SDS-PAGE, and a single transformant with apparently higher expression was chosen for further study (Fig. 2A). Being non-glycoproteins, CHS and STS expressed in eucaryotes were indistinguishable on SDS-PAGE from the enzymes expressed in *E. coli.* The other 20% of the Ura$^+$ transformant colonies seemed not to contain the insert DNA, likely due to gene conversion or double crossing over within the *URA3* locus,[10] and this was confirmed by PCR. Total DNA from yeasts with or without enzyme (CHS or STS) activity was obtained and PCR-amplified using 5′- and 3′-flanking CHS or STS primer.[6] Only those DNA templates obtained from yeast colonies that exhibited enzyme activity showed the single DNA band corresponding to the full-length insert sequence (data not shown). It has been established that synonymous codons for any single amino acid are not randomly used and that this non-random usage is taxon specific.[16] However, no appreciable change in expression

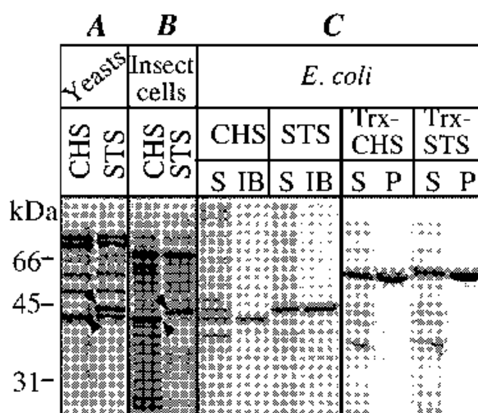


Fig. 2. SDS-PAGE of Various CHS (C) and STS (S) Preparations

A, B: Total cell-free extracts of transformed yeasts (A) and insect cells (B). C: Soluble fractions (*S*), isolated inclusion bodies (IB) of CHS and STS overexpressed in *E. coli*, and thioredoxin-HisTag-fusion enzymes (Trx-CHS and Trx-STS) purified by $Ni^{2+}$-chelation chromatography (*P*). SDS-PAGE was performed on 12% acrylamide minislab gels and the proteins were stained with Coomassie blue R250 (Bio-Rad). The protein bands corresponding to CHS and STS are indicated by arrowheads.

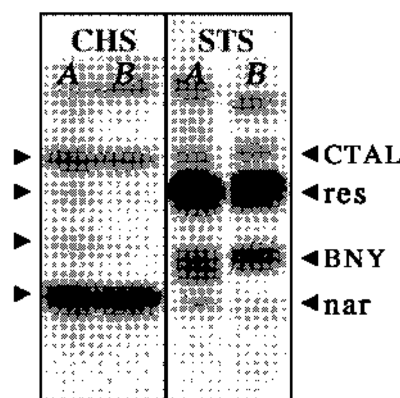


Fig. 3. Radio Thin-Layer Chromatogram of Enzyme Assays Using Trx-CHS and Trx-STS

Enzyme reaction was carried out in 0.1 M potassium phosphate buffer (pH 7.5) in the presence (A) or absence (B) of 2 mM DTT. Cross-reaction products (resveratrol by CHS and naringenin by STS) are visible along with derailment products, bisnoryangonin (BNY) and *p*-coumaroyltriacetic acid lactone (CTAL).[6)]

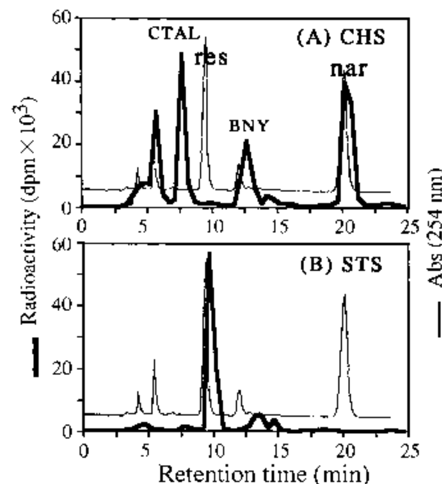


Fig. 4. HPLC Analysis of Reaction Products Produced by *P. lobata* Chalcone Synthase (A) and *A. hypogaea* Stilbene Synthase (B) Overexpressed in *S. frugiperda Sf*9 Cells

Authentic resveratrol and naringenin were added to the reaction products to aid detection by UV (thin line). 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) as well as the natural products were pooled and used for carrier dilution analysis.

level was observed when codons for a few N-end amino acids of CHS and STS (plant enzymes) were substituted with those preferred by yeast.

CHS and STS were also functionally expressed in *S. frugiperda Sf*9 cells using a baculovirus-based expression system. Both enzymes were overexpressed so as to give major bands on SDS-PAGE when total cell extracts were analyzed (Fig. 2B). Integration of the CHS and STS insert into the viral DNA was also confirmed by nested-PCR using primers derived from internal sequences of the enzymes and viral DNA as template.

**Purification of Inclusion Bodies and Refolding of CHS and STS** *E. coli* cells were disrupted with a combination of lysozyme (coded for by the pLysE plasmid), a freeze/thaw cycle and sonication. Inclusion bodies, sedimented with low speed centrifugation, were washed with detergent and urea for further purification (Fig. 2C). The inclusion bodies were solubilized in 8 M urea in the presence of $\beta$-mercaptoethanol as reducing agent, and refolding was performed by slowly removing the denaturing agent by dialysis. The yield of refolding depends on a number of variables including pH, temperature, protein concentration and additives.[14)] Therefore, refolding experiments were performed under varying conditions in order to find better refolding conditions. The yield of active enzyme was higher at pH 7.4 ($I=0.1$) than at pH 9.0 ($I=0.1$); at 4 °C than at 23 °C, and at a protein concentration of 3.5 $\mu$g/ml than at 7 $\mu$g/ml. The addition of free CoA at 0.5 mM in the dialysis buffer had little effect on the yield (data not shown). Cystein at 5 mM was used as redox agent and other redox agents were not tested. The recently solved 3-D structure of alfalfa CHS revealed no disulfide bond even though each CHS subunit contains seven cystein residues.[8)] Specific activities of the refolded enzymes under the chosen conditions ($I=0.1$, pH 7.4, 4 °C, 3.5 $\mu$g/ml, 5 mM Cys) were: refolded CHS, 2.7±1.8 pkat/mg for naringenin production, and refolded STS, 14±2.8 pkat/mg for resveratrol production. These values were substantially lower (6 to 19%) than those of Trx-fusion enzymes (see below). As a result, reliable cross-reaction data could not be obtained with refolded CHS due to the lower activity.

**Expression and Purification of Thioredoxin-Fusion Proteins** Trx-CHS and Trx-STS were overexpressed and purified to apparent homogeneity by a single step of $Ni^{2+}$ chelation chromatography (Fig. 2C). When expressed as thioredox-fusion proteins (60 kDa), more enzymes were recovered in soluble fractions allowing higher yields of purified enzymes (8 mg/100 ml culture). The specific activities of purified Trx-CHS and Trx-STS were 49±1.8 pkat/mg (for naringenin production) and 75±16 pkat/mg (for resveratrol production), respectively.

**Cross-Reaction** Cross-reaction was analyzed both by radio-TLC (Fig. 3) and HPLC (Fig. 4) followed by carrier dilution assay. In radio-TLC analysis, cross-reaction was calculated from the amounts of radioactive products quantitated with an imaging plate analyzer using standards of known specific radioactivity. In carrier dilution assay, four successive rounds of recrystallization of radioactive product in the

Table 1. Cross-Reaction Obtained from Carrier-Dilution Assays

| Enzyme | Expression hosts Yeasts | Insect cells | *E. coli*[a] |
|---|---|---|---|
| CHS | 1.2% | 1.4% | 2.7—4.2% |
| STS | 0.48% | 0.36% | 1.4—2.3% |

*a*) Values from ref. 6. Enzyme reaction was carried out in 0.1 M potassium phosphate, pH 7.5, containing 2 mM DTT as described in Experimental.

Table 2. Cross-Reaction under Various Reaction Conditions Calculated from Radio-TLC

| Enzyme | Reaction conditions: 0.1 M potassium phosphate, pH 7.5, 2 mM DTT | 0.1 M potassium phosphate, pH 7.2, 10% glycerol, 1 mM DTT |
|---|---|---|
| CHS expressed in *E. coli*[a] | $8.0 \pm 3.3$[d] | |
| CHS expressed in yeasts | $3.4 \pm 0.34$[d] | |
| Trx-CHS[b] | $6.0 \pm 2.4$ | $4.3 \pm 0.94$ |
| STS expressed in *E. coli*[a] | $4.1 \pm 0.64$[d,e] | |
| STS expressed in yeasts | $1.2 \pm 0.13$[d] | |
| Refolded STS[c] | $2.6 \pm 0.49$[e] | |
| Trx-STS[b] | $2.7 \pm 1.6$[e] | $2.0 \pm 0.25$ |

Data are expressed as mean±S.D. ($n=3$—6). *a*) Purified enzymes overexpressed in *E. coli* BL21.[6] *b*) Purified thioredoxin-HisTag-fusion enzymes overexpressed in *E. coli* AD494. *c*) Refolded enzyme from inclusion bodies isolated from *E. coli* BL21. *d*, *e*) Significantly different at $p<0.01$,[d] $p<0.05$,[e] (Student's *t*-test).

presence of a non-labeled carrier compound yielded crystals of constant specific radioactivity. For example, for CHS expressed in yeasts, the specific radioactivity of naringenin produced was $1.2 \times 10^5$ dpm/mmol (methanol), $1.1 \times 10^5$ (methanol), $1.1 \times 10^5$ (methanol) and $1.0 \times 10^5$ (ethyl acetate/hexane), whereas that of resveratrol produced was $1.5 \times 10^3$ dpm/mmol (methanol), $1.3 \times 10^3$ (methanol), $1.3 \times 10^3$ (methanol) and $1.4 \times 10^3$ (ethyl acetate/hexane), yielding 1.2% of cross-reaction.

Cross-reactions of CHS and STS obtained using different expression systems were compared under otherwise identical reaction conditions. Both CHS and STS expressed in eucaryotic cells showed reduced cross-reaction as compared to the enzymes overexpressed in *E. coli* when analyzed by carrier dilution assay (Table 1) and radio-TLC (Table 2). The higher values obtained from radio-TLC were attributed to band tailing and high backgrounds. Similarly, the Trx-fusion enzymes and *in vitro* refolded STS also showed lower values of cross-reaction than those of purified *E. coli* overexpressed in intact enzymes (Table 2). We also studied cross-reaction by the Trx-fusion enzymes under varying reaction conditions, and the cross-reaction of CHS was found to be reaction condition-dependent. While protein concentration controlled by BSA and pH were without noticeable effect, addition of glycerol showed a tendency to decrease the cross-reaction, likely due to its stabilizing effect on enzyme conformation. These results were interpreted to provide supporting evidence to our earlier proposition[6]: that *in vitro* cross-reaction of CHS and STS are due to the intrinsic capability of these enzymes to catalyze different types of cyclization, which, in turn, is endowed by conformational flexibility of their active sites. The higher cross-reaction observed with the enzymes overexpressed in *E. coli* could thus be attributed to greater manifestation of this otherwise cryptic capability under a non-optimal folding environment during bacterial overexpression. This notion is completely compatible with the proposal that the cyclization reactions in CHS and STS are modulated by active site geometry.[7,8] Overexpression in *E. coli* often leads to misfolding especially for membrane-associated eucaryotic proteins, as bacteria lack intracellular membrane environments that may be necessary for efficient and accurate folding.[17] Both CHS and STS are shown to be weakly membrane-associated in the plant cells.[4,18,19]

**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 is a recipient of a fellowship (TBRF-98-10) from the Tokyo Biochemistry Research Foundation.

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