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

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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-

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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.1,8) 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 inserts10,11 were introduced into the NotI site of a yeast expression vector pNoteI12 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 methylotrophic yeast, C. boidinii TK6210 was performed by the modified lithium acetate method as described.13,14 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, 2% glucose). The cells were then transferred for induction to complete lysis. After centrifugation at 10000 g for 10 min, the precipitated cells were ruptured with a CRYO-PRESS and suspended in a two different solvent systems (methanol and hexane/ethyl acetate) and separated by French-press with a CR-YO-PRESS CP-100W (Microtech Nichion, Japan). The cell lysates were suspended in 20 ml of 0.1M potassium phosphate (pH 7.4) containing 100 g/ml of Triton X-100 in KPE buffer and twice with 2M urea in KPE buffer. The inclusion bodies were dissolved in denaturation buffer (8 M urea, 0.1 M β-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 mg/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-His-Tag-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 were carried out in the presence of 0.1 mM p-coumaryl-CoA and 16.8 μM [2-14C]malonyl-CoA (2.2 GBq/mmol, NEN) as substrates and analyzed by radio-TLC and an imaging plate analyzer (BAS2000, Fuji) as described previously.16) Specific enzyme activity was expressed in pmol of the product produced/s/mg (pkt/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 μg (yeasts) or 800 μg (insect cells) of proteins, 0.1 mM p-coumaryl-CoA and 16.8 μM [2-14C]malonyl-CoA (2.2 GBq/mmol, NEN). Both CHS and STS were incubated for 60 min at 20°C and disrupted by French-press with a CRYO-PRESS CP-100W (Microtech Nichion, Japan). The precipitated cells were disrupted by 0.4 mM IPTG. Following a freeze/thaw cycle, the cells suspended 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 : H2O : acetic acid = 50 : 50 : 1, v/v) and authentic naringenin (2 μg) and resveratrol (1.5 μg) were added as internal standards. HPLC was performed using an ODS-80TM column (TOSOH, 5 μm, 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 β-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 BamHI, and used for the transformation of C. boidinii TK62 (ura3). 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,45) 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.6) However, appreciable change in expression
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 Sf9 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 β-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. 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 mg/ml than at 7 mg/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. Specific activities of the refolded enzymes under the chosen conditions (I=0.1, pH 7.4, 4°C, 3.5 µM glucose, 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 Ni²⁺ chelation chromatography (Fig. 2C). When expressed as thioredoxin-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 products (resveratrol by CHS and naringenin by STS) were visible along with derailment products, bisnoryangonin (BNY) and p-coumaroyltriacetic acid lactone (CTAL).
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 $1.5 \times 10^5$ dpm/mmol (methanol), $1.3 \times 10^5$ (methanol), $1.3 \times 10^5$ (methanol), $1.4 \times 10^5$ (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 glycercol 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: 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. 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. Both CHS and STS are shown to be weakly membrane-associated in the plant cells.

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

| Enzyme | Yeasts | Expression hosts | E. coli
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>CHS</td>
<td>1.2%</td>
<td>1.4%</td>
<td>2.7—4.2%</td>
</tr>
<tr>
<td>STS</td>
<td>0.48%</td>
<td>0.36%</td>
<td>1.4—2.3%</td>
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\(a\) Values from ref. 6. Enzyme reaction was carried out in 0.1 m potassium phosphate, pH 7.5, containing 2 m DTT as described in Experimental.

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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 m potassium phosphate, pH 7.5, 2 n DTT</td>
</tr>
<tr>
<td>CHS expressed in E. coli</td>
<td>8.0±3.3 (b)</td>
</tr>
<tr>
<td>CHS expressed in yeasts</td>
<td>3.4±0.34 (b)</td>
</tr>
<tr>
<td>Trx-CHS (b)</td>
<td>6.0±2.4</td>
</tr>
<tr>
<td>STS expressed in E. coli</td>
<td>4.1±0.64 (b)</td>
</tr>
<tr>
<td>STS expressed in yeasts</td>
<td>1.2±0.13 (b)</td>
</tr>
<tr>
<td>Refolded STS (b)</td>
<td>2.6±0.49</td>
</tr>
<tr>
<td>Trx-STS (b)</td>
<td>2.7±1.6 (b)</td>
</tr>
</tbody>
</table>

Data are expressed as mean±S.D. \((n=3—6)\). \(a\) Purified enzymes overexpressed in E. coli BL21. \(b\) Purified thioredoxin-HisTag-fusion enzymes overexpressed in E. coli AD494. \(c\) Refolded enzyme from inclusion bodies isolated from E. coli BL21. 

References