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Kyoto University
Preliminary

**Purification of Recombinant Type III Polyketide Synthases from *Pinus densiflora* and Its Crystallization**

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**Keywords:** type III polyketide synthases, chalcone synthase, stilbene synthase, crystallization, *Pinus densiflora*

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**Introduction**

Genus *Pinus* produces stilbenoids and flavonoids, especially in its heartwood. Among the heartwood extractives, pinosylvin is a naturally occurring stilbenoid phytoalexin showing antifungal and nematicidal activities, while pinocembrin is a precursor of the flavonoid showing antifungal and nematicidal activities. They were incubated 4°C for 2-4 weeks. The formed crystals were subjected to X-ray diffraction at Spring 8 of Harima Institute, RIKEN. Each stored cell (2-4 liter culture cells) was resuspended in 30 ml/L of a lysis buffer, which consisted of 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 40 mM imidazole (pH 8.0), 10% glycerol, and 1% (v/v) Tween-20. After the suspension was sonicated and centrifuged (18,000 rpm × 30 minute), each supernatant was passed through a Ni⁺⁺ NTA column (5 ml HiTrap™ Chelating HP; Pharmacia). The column was washed with 10 bed volumes of the lysis buffer without Tween-20. Each recombinant protein was then eluted with the lysis buffer without Tween-20, which contained 250 mM imidazole. Each purified recombinant protein was desalted and buffer-exchanged through two tandem-connected Sephadex G-25 superfine columns (5 ml HiTrap™ Desalting; Pharmacia). Incubation with Factor Xa (50 µg fusion protein/unit enzyme; Novagen) for 16 hours at 21°C in a Factor Xa cleavage buffer, which consisted of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM CaCl₂, removed the fused Tags. The protein was loaded on a Ni⁺⁺ NTA column and the flow-through depleted of Factor Xa using a Benzamidine-Sepharose column (1 ml HiTrap™ Benzamidine FF (high sub); Pharmacia). Purified each protein was stored at −85°C in 50 mM HEPES (pH 7.5) after buffer exchange. The Protein was quantified using a Coomassie protein assay reagent kit (Pierce, Rockford, USA) and bovine serum albumin as a standard.

Denatured electrophoresis was performed according to a standard procedure using 10% acrylamide running gels and 4.75% acrylamide stacking gels. After electrophoresis, separated proteins were stained with Coomassie Brilliant Blue (CBB) R-250 (Pierce, Rockford, USA).

**Experimental**

The cDNA clones and the vectors employed had described elsewhere. The *Escherichia coli* strains were Origami B (DE3) for PDCHSX and Origami B (DE3) plysS for PDSTS cells harboring the respective plasmids. They were cultured in the 1 liter of the media with the respective antibiotics in a 3-liter Erlenmeyer flask at 37°C for 16 hours at 210 rpm until the A₆₀₀ reached 1.0–1.2. After the culture was cooled on ice, isopropyl Thio-β-D-galactoside (IPTG) was added at 0.6 mM of the final concentration to induce protein expression. The culture was incubated further at 15°C on a shaker at 200 rpm for 20 hours. Cells were harvested by centrifugation (6,000 rpm for 5 min) and stored at −85°C until for use.

Each stored cell (2–4 liter culture cells) was resuspended in 30 ml/L of a lysis buffer, which consisted of 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 40 mM imidazole (pH 8.0), 10% glycerol, and 1% (v/v) Tween-20. After the suspension was sonicated and centrifuged (18,000 rpm × 30 minute), each supernatant was passed through a Ni⁺⁺ NTA column (5 ml HiTrap™ Chelating HP; Pharmacia). The column was washed with 10 bed volumes of the lysis buffer without Tween-20. Each recombinant protein was then eluted with the lysis buffer without Tween-20, which contained 250 mM imidazole. Each purified recombinant protein was desalted and buffer-exchanged through two tandem-connected Sephadex G-25 superfine columns (5 ml HiTrap™ Desalting; Pharmacia). Incubation with Factor Xa (50 µg fusion protein/unit enzyme; Novagen) for 16 hours at 21°C in a Factor Xa cleavage buffer, which consisted of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM CaCl₂, removed the fused Tags. The protein was loaded on a Ni⁺⁺ NTA column and the flow-through depleted of Factor Xa using a Benzamidine-Sepharose column (1 ml HiTrap™ Benzamidine FF (high sub); Pharmacia). Purified each protein was stored at −85°C in 50 mM HEPES (pH 7.5) after buffer exchange. The Protein was quantified using a Coomassie protein assay reagent kit (Pierce, Rockford, USA) and bovine serum albumin as a standard.

Denatured electrophoresis was performed according to a standard procedure using 10% acrylamide running gels and 4.75% acrylamide stacking gels. After electrophoresis, separated proteins were stained with Coomassie Brilliant Blue (CBB) R-250 (Pierce, Rockford, USA).

The PDCHSX and PDSTS crystals were grown by vapor diffusion in hanging drops of a protein buffer mixture (2 µl each). They were incubated 4°C for 2–4 weeks. The formed crystals were subjected to X-ray diffraction at Spring 8 of Harima Institute, RIKEN.

**Results and Discussion**

1. **Over-expression and purification of recombinant PDCHSX and PDSTS in *Escherichia coli***

The fusion protein encoded by PDCHSX cDNA with...
Trx-, His- and S-Tag was over-expressed in *Escherichia coli* cells (Origami B (DE3)). The other fusion protein encoded by PDSTS2 cDNA with the tags was over-expressed in *Escherichia coli* cells (Origami B (DE3) plysS). One-liter cultures of the PDCHSX and PDSTS2 yielded about 6.0 mg and 3.0 mg of the purified proteins, respectively. Their respective molecular sizes on SDS-PAGE were about 60 kDa. The found molecular sizes agreed with the predicted ones as follows. The 18 kDa of vector originated proteins (Trx-Tag, His-Tag and S-Tag) and 45 kDa of recombinant PDCHSX would produce 63 kDa fusion proteins. On the other hand, 18 kDa of vector originated proteins (thioredoxin, His-Tag and S-Tag) and 42 kDa of recombinant PDSTS2 would produce 60 kDa fusion protein. The tags were removed and purified to homogeneity by using an affinity chromatography.

### 2. Crystallization of PDCHSX and PDSTS2

The purified PDCHSX and PDSTS2 were screened for their crystals formed under various conditions. Out of about 250 conditions, only 0.05 M Bis-Tris propane (pH 6.3 at 4°C) and 1.0–1.05 M ammonium sulfate with 1.5–2.0 mg/ml of the protein was suitable for the PDCHSX crystallization. No PDSTS2 crystal was observed over 250 conditions tested. All the concentrations above described showed at final concentration.

X-ray diffraction for the PDCHS crystals showed 2.0 Å of the resolution, and revealed the unit cell dimensions being $a=b=120.75\,\text{Å}$, $c=213.05\,\text{Å}$ with $a=b=\gamma=90^\circ$ (a tetragonal system in a space group $P4_{22}$). According to the equation, this leaded the conclusion that the crystal had four monomers in the asymmetric unit. Further analyses will reveal the 3-D structure, for example, by using the MIR (multiple isomorphous replacement) method based on this data.

### References