Preliminary

Purification of Recombinant Type III Polyketide Synthases from *Pinus densiflora* and Its Crystallization*1

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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 thio-β-D-glucoside. They belong to polyketide super-gene family and classified into type III polyketide synthase. They are distributed in plant kingdom and bacteria, and probably originated from multi-enzyme complex of a fatty acid synthase. The type III enzyme is usually a single peptide, while the other type I and II enzymes form multi-enzyme complexes. The latter type enzymes are involved in macrolide-antibiotics biosyntheses such as erythromycin.

Recent structural studies on a type III enzyme revealed the active site and the reaction mechanism, leading to the enzyme molecular design partially possible. We have been studying STS and CHS in *Pinus densiflora*, and already isolated 3 STS clones (PDSTS1, PDSTS2 and PDSTS3) and one CHS clone (PDCHSX). After over-expressed in *Escherichia coli*, they have characterized. Although the three dimensional structure had reported, the reaction mechanism still remains ambiguous parts, especially in the type III enzyme from gymnosperm. Here, we have stepped into the first stage of X-ray crystallography, or the protein crystallization followed by X-ray diffraction.

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) pLyS for PDSTS2 cells harboring the respective plasmids. They were cultured in 1 liter of the media with the respective antibiotics in a 3-liter Erlenmeyer flask at 37°C for PDSTS2 cells harboring the respective plasmids. The suspension was sonicated and centrifuged (18,000 rpm for 5 min) and stored at -85°C until for use.

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) and bovine serum albumin as a standard.

The fusion protein encoded by PDCHSX cDNA with 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 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 Ni²⁺-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 reloaded on a Ni²⁺-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.

Results and Discussion

1. Over-expression and purification of recombinant PDCHSX and PDSTS2 in *Escherichia coli*

The fusion protein encoded by PDCHSX cDNA with
Trx-, His- and S-Tags 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) plosS. 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 condition. 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\(^3\)\(^-\)\(^5\). 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 \(\alpha=\beta=\gamma=90^\circ\) (a tetragonal system in a space group \(P4_22\)). According to the equation\(^6\), this led 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