Characterization of new type III polyketide synthase from *Dendrobium orchid*

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Abstract

Type III polyketide synthases (PKS) are a group of the enzymes that catalyze sequential condensations of malonyl-CoA and cyclization reaction to produce polyketide scaffold. Bibenzyl synthase (BBS), a type III PKS, is reportedly involved in the biosynthesis of dihydrostilbenes. In the previous study, we cloned a putative *bbs* gene (*DoBBS*) from *Dendrobium orchid*. To clariefy the DoBBS function, the *DoBBS* gene was expressed in *Pichia pastoris* and the DoBBS *in vitro* enzyme reaction products derived from dihydro-*p*-coumaroyl-CoA and *p*-coumaroyl-CoA with malonyl-CoA were analyzed by LC-MS. The constructed *Phichia* expression system gave DoBBS as soluble protein with high efficacy. However, the recombinant DoBBS did not produce any products from the substrates used in this study, suggesting that further investigation using the other substrate are required.

Keywords: *Dendrobium orchid*, dihydrostilbene, polyketide, protein expression.

1. Introduction

Polyketides are structurally diverse and pharmaceutically important natural products. They show a wide range of bioactivities such as antioxidant, anti-inflammatory, anticancer, antibacterial activities, and many polyketides reportedly recognize as bioactive constituents in the traditional medicines. Previous studies of the biosynthesis of the plant polyketides revealed that the remarkable structural diversity of the plant polyketides are principally derived from differences of the starter substrate specificity, the number of malonyl-CoA condensations, and the type of cyclization reaction of structurally simple type III polyketide synthases (PKS) (Austin & Noel, 2003; Abe & Morita, 2010). For example, chaclcone synthase (CHS), which is the pivotal enzyme in the biosynthesis of Flavonoid, produces naringenin chalcone from condensation of three molecules of malonyl-CoA with *p*-coumaroyl-CoA via the formation of the linear tetraketide intermediate and its C6-C1 Claisen type cyclization reaction (Figure 1). On the other hand, stilbene synthase (STS) generates resveratrol from the common linear tetraketide intermediate with CHS via an aldol type cyclization reaction. Nowadays, functionally distinct more than 40 type III PKSs have been identified from plants. Phylogentic analysis has demonstrated that STS evolves from CHS (Tropf et al., 1994). In addition, the site directed mutagensis studies of CHS converted its function to STS (Austin et al., 2004).

Among the reported type III PKSs, bibenzyl synthase (BBS), which is specifically involved in the biosynthesis of the *m*-hydrophenylpropionate-derived dihydrostilbene derivatives, has been obtained from the Orchidaceae plants such as *Epipactis palustris* (Gehlert & Kindle, 1991), *Bletilla striata* (Reinecke & Kindl, 1993), *Phalaenopsis* sp (Preisigmuller et al., 1995), and *Cymbidium hybrida* (Wang et al., 2014). The enzyme catalyzes the STS-like C2-C7 aldol type cyclization reaction of the linear tetraketide intermediate derived from three molecules of malonyl-CoAs and dihydro-*m*-coumaroyl-CoA to generate dihydrostilbene scaffold (Figure 1).

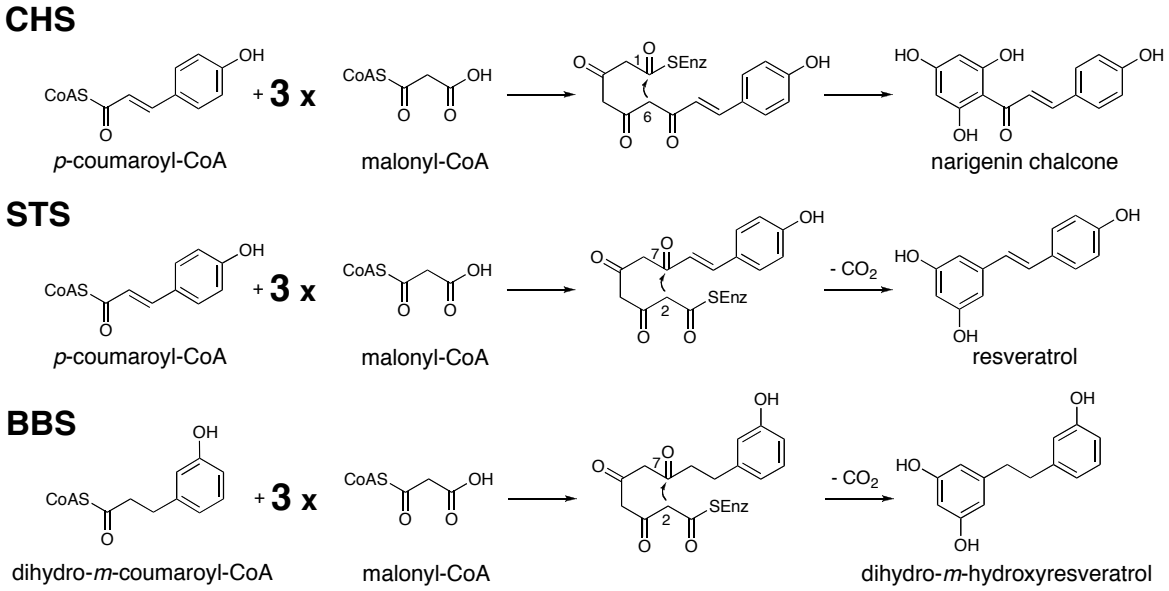

Fig. 1　The Biosynthesis of chalcone, stileben, and dihydrostilebe by type III PKSs

Bibenzyl derivatives such as Phenanthrenes and dihydrostilbenes have also been isolated from *Dendrobium* genus (Figure 2). However, the BBS enzymes in this genus have not be yet fully elucidated. We currently isolated putative *bbs* gene (*DoBBS*) from *D. orchid*. In this study, the putative *DoBBS* gene was expressed in *Pichia pastoris* and its function was evaluated by investigating *in vitro* reaction products of the recombinant DoBBS.

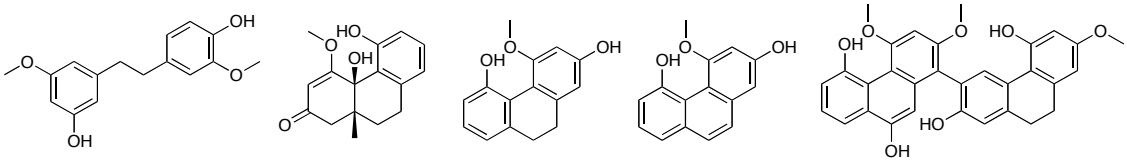

Fig. 2　Example of polyketides isolated from *Dendrobium* plants

2. Materials and Methods

2.1. Strains and culture conditions

The *E. coli* strain DH5α was used in subcloning. The cells were cultured in LB medium containing 25 μg/ml of Zeocin and incubated at 37 °C. The *P. pastoris* stain X33 and KM71H was used for expression of

recombinant DoBBS. Yeast strains were cultured in YPD medium (1% yeast extract, 2% peptone and 2% glucose) and incubated at 30 °C.

2.2. Recombinant enzyme expression

pPICZA with DoBBS cDNA was transformed into the *P. pastoris* stain KM71H and X33 by using LiCl method, and cultured on YPD plate containing 100 μg/ml and 500 μg/ml Zeocin, respectively. The transformants were confirmed with PCR. The KM71H and X33 cells harboring DoBBS cDNA were cultured in BMGH medium (100 mM potassium phosphate, pH 6.8, 1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 1% glycerol and 0.004% histidine) at 28 °C (200 rpm). After OD was reached to 2–6, the cells were harvested by centrifugation at 3,000 g for 10 min, and then the pellet were resuspended with BMMH medium (100 mM potassium phosphate, pH 6.8, 1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 1% methanol and 0.004% histidine) to OD_{600} = 1, and waa further incubated at 28 °C (200 rpm) for 4 days. During the culture of the cell in BNMH medium, the 1% methanol was added for everyday to maintain the protein induction.

2.3. Extraction and purification of recombinant protein

The induced cells were harvested by centrifugation at 3,000 g for 10 min (4 °C), and the pellet was resuspended with 3 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5% glycerol and 1 mM PMSF) per 1 g of cell. The cell suspension was mixed with 2 g acid-wash glass beads (sigma), and then vortex with high speed for 30 sec with interval for 30 sec on ice (≥ 7 times). The lyset was centrifuged at 2500 g for 3 min and the supernatant was centrifuged at 6,000 g for 10 min (4 °C) to prepare the crude enzyme solution. The crude enzyme solution was concentrated by 10K cutoff Macrosap Advance Centrifugal Device (Pall Life Sciences), and then subjected to Ni-NTA affinity column (GE healthcare). The recombinant DoBBS was eluted with 0.5 M imidazole. Fractions containing the DoBBS protein were determined on the basis of SDS-PAGE analysis, dialyzed, and concentrated. Protein concentration was measured by the Bradford method.

2.4. Enzyme assay

Crude enzyme (100 μg) or the purified DoBBS (20 μg) were incubated with 0.3 mM malonyl CoA, 0.2 mM *p*-coumaroyl CoA or 0.2 mM dihydro-*p*-coumaroyl CoA in 100 μl of 20 mM Tris-HCl buffer, pH 7.0, at 20°C and 30 °C for 12 h and 24 h, respectively. The reaction was stopped by adding 10 μl 20% HCl and the enzyme reaction products were extracted with 200 μl ethyl acetate (triplicate). After the extract was dried under reduced pressure, the residue was resuspended with 50 μl methanol and subjected to LC/MS using water (A) and acetonitrile (B) containing 0.1% (v/v) TFA as mobile phase to analyze the enzyme reaction products. The gradient solvent system was follows: 0-5 min, 10% B; 5-20 min, 20% B; 20-30 min, 60% B; 30-50 min, 100% B; 50-60 min, 10%B. The flow rate was 0.5 ml/min and chromatograms were acquired at 290 nm.

Results and Discussion

The recombinant DoBBS was heterologously expressed in *P. pastoris* strain KM71H and X33 as a

fusion protein with His_6-tag at the C-terminus under controlling AOX1 promoter and purified with Ni-NTA affinity column, respectively. The recombinant DoBBSs expressed in KM71H and X33 migrated as a band with a molecular weight of 44.7 kDa on SDS-PAGE (Figure 3), which agree well with the calculated value of 44.5 kDa, respectively. Furthermore, the SDS-PAGE analysis indicated that DoBBS was expressed in KM71H with 19 times higher efficacy over X33 (Table 1). Thus, the functional analysis of DoBBS was performed with the recombinant DoBBS expressed in KM71H.

Table 1. Summary of protein purification

	Strain	
	X33	KM71H
Total protein (mg)	34.90	10.00
Ni-NTA column (mg/ml)	0.15	0.83
Target soluble protein (%)	0.22	4.16

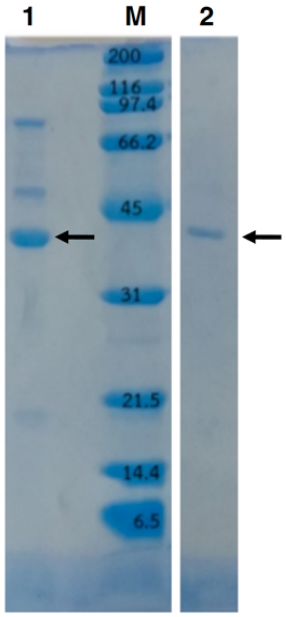

Fig. 3　Analysis of the DoBBS expression in *P. pastoris* strains KM71H and X33 by SDS-PAGE

The expression of DoBBS was evaluated after the recombinant DoBBS was purified by using Ni-NTA affinity column. M: Molecular weight marker, 1: DoBBS expressed by KM71H, 2: DoBBS expressed by X33. The recombinant BBS was indicated with arrows.

BBS has been reported to efficiently catalyze the iterative condensation of three molecules of malonyl-CoAs with dihydroxy-*m*-coumaroyl-CoA to generate dihydroresveratrol. Due to the lack of dihydroxy-*m*-coumaroyl-CoA, we utilized dihydro-*p*-coumaroyl-CoA as a substrate with malonyl-CoA in order to investigate the DoBBS reaction products. Since, functionally related STS has been known to utilize *p*-coumaroyl-CoA as a substrate with malonyl-CoA to produce resveratrol, we also carried out the DoBBS

enzyme reaction using *p*-coumaroyl-CoA and malonyl-CoA. Boiled DoBBS was used as the negative control. However, the recombinant DoBBS did not accept dihydro-*p*-coumaroyl-CoA and *p*-coumaroyl-CoA with malonyl-CoA to produce products. These observations suggested that either the recombinant DoBBS was expressed in *E. coli* as inactive enzyme or DoBBS accepts other CoA thioester substrate to produce compound.

Conclusion

DoBBS was expressed in *P. pastoris* strain KM71H and X33 as a fusion protein with His6-tag at the C-terminus and investigated the enzyme function. The KM71H strain efficiently expressed DoBBS as a soluble protein. However, the recombinant DoBBS did not produce any products. Thus, further functional analysis of DoBBS is needed.

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