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Design of a noncovalently linked bifunctional enzyme for whole-cell biotransformation

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ABSTRACT

Optical pure D-p-hydroxyphenylglycine (D-HPG) is a precursor for semi-synthetic antibiotics. It can be synthesized from D,L-hydroxyphenyl hydantoin (HPH) by a two-step reaction mediated by D-hydantoinase (HDT) and amidohydrolase (AHL). In this study, a bifunctional enzyme was originally created by in-frame fusion of AHL with HDT genes (AHL-HDT). However, the AHL-HDT fusion protein expressed in *Escherichia coli* was prone to aggregates, recognized as a frequently encountered problem for this conventional method. To address this issue, small interacting motifs, cohesin (Coh) and dockerin (Doc) domains of cellulosomes, were explored and illustrated to interact *in vivo*. Accordingly, Coh and Doc were fused with AHL and HDT, respectively. After co-expression in *E. coli*, Coh-tagged AHL and Doc-tagged HDT assembled into a soluble protein complex *via* the high-affinity interaction of Coh and Doc. Consequently, the protein assembly exhibited both AHL and HDT activities and a higher reaction rate than free counterparts. Whole cells expressing the protein assembly were more stable than ones with free proteins for D-HPG production, and they could be recycled six times with a conversion yield of D-HPG exceeding 90%.

1. Introduction

Amino acids of optical purity have a wide application range in industry, and they serve as building components for chemical syntheses of antibiotics, antifungal agents, pesticides, and sweeteners [1]. Of particular importance, D-p-hydroxyphenylglycine (D-HPG) is a precursor for the semi-synthetic antibiotics such as cephalosporin. Optical pure amino acids can be produced from racemic D,L-5-monosubstituted hydantoins by an enzymatic process mediated by stereoselective hydantoinase and amidohydrolase [2]. This production scheme has received attention from industry since a study first reported production of D-HPG from D,L-hydroxyphenyl hydantoin (HPH) by a biotransformation reaction based on *Agrobacterium radiobacter* NRRL B11291 [3]. This bacterial strain displays D-hydantoinase (HDT) and N-carbamoyl-D-amino acid amidohydrolase (AHL) activities. The former catalyzes the hydrolysis of HPH to N-carbamoyl-D-p-hydroxy phenylglycine (CpHPG) while the latter converts CpHPG to D-HPG. We show that the efficiency of this D-HPG transformation reaction is greatly improved using the surrogate strain *Escherichia coli* that expresses heterologous HDT and AHL [4].

In living cells, metabolic reactions interconnected in a complicated network proceed within the viscous cell cytoplasm that is highly crowed with proteins and metabolite molecules. To ensure effective metabolic activities, living cells have evolved to adopt specialized protein complexes for designed biological functions [5,6]. This multifunctional enzyme system is commonly conceived as an evolutional advantage for rapid turnover of substrates in a sequential reaction, driving intermediate metabolites away from competing pathways, and timely regulation of the enzyme assembly in response to the physiological status of cells [7]. Inspired by the potential of these nature's models, many efforts have been devoted toward artificial creation of bifunctional and multifunctional enzymes for various engineering purposes [8]. In general, the results of most cases are encouraging with respect to the kinetic efficiency of enzymes [9,10].

Creation of artificial bifunctional enzymes is not uncommon. This is frequently carried out by in-frame fusion of two distinct

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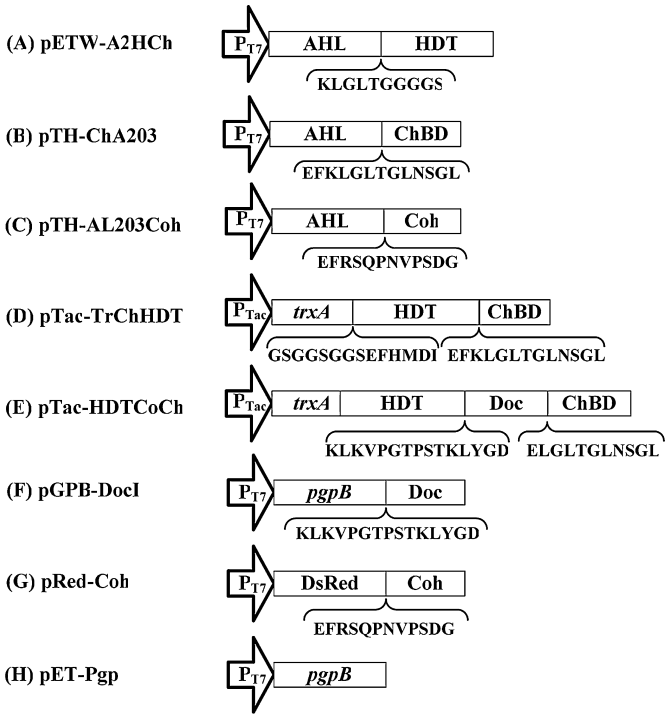

Fig. 1. Schematic illustration of the organization of fusion genes in plasmids. The essential parts of plasmids (A–H) with the linkers (given as amino acid sequences) were shown. The size of fusion genes was not drawn to scale. *Abbreviations*: P_{T7}, the T7 promoter; P_{Tac}, the *tac* promoter.

structural genes separated by a short linker [11]. As expressed in cells, the fusion gene results in a hybrid protein that exhibits the dual activities of two individual enzymes. Although straightforward, this method becomes problematic to apply when two proteins with multimeric structures are fused together [12]. It leads to the fusion protein with a highly ordered and complex structure, which is poorly expressed and usually misfolds in host bacteria [13]. In this study, the conventional method was originally utilized to create a bifunctional enzyme by end-to-end fusion of AHL with the N-terminus of HDT (AHL-HDT). However, the resulting AHL-HDT fusion protein misfolded and aggregated after expression in *E. coli*. To address this issue, an alternative approach was implemented by exploration of cohesin (Coh) and dockerin (Doc) domains from cellulosomes. Coh and Doc were fused to AHL and HDT, respectively. Consequently, a soluble protein assembly exhibiting both AHL and HDT activities was obtained *in E. coli* by means of the high-affinity Coh–Doc interaction. Furthermore, the experiment illustrated that the protein complex gained a kinetic advantage over the free counterparts. For repeated production of D-HPG from HPH, the cells with the protein complex outperformed the ones with free counterpart proteins. Overall, the result indicates the feasibility of this approach in creating a bifunctional enzyme *in vivo*.

2. Materials and methods

2.1. Plasmid construction

The schematic structures of all plasmids applied in this study were summarized in Fig. 1. AHL-HDT was constructed as follows. The gene encoding AHL was first amplified from plasmid pChA203 [14] by polymerase chain reaction (PCR) with the designed primers (tttgtgatatcgctaccaccaccaccggtcaggccaagccaagcttg and taacttctagaaggagatatacatatgac). After digestion with *Eco*RV and *Xba*I, the PCR DNA was subcloned into the corresponding sites of plasmid pHDT [15] to produce plasmid pETW-A2HCh. The plasmid carried the AHL-HDT fusion gene under the control of the T7 promoter (P_{T7}).

By PCR, the DNA fragment containing the pSC101 replication origin and the kanamycin-resistant determinant was amplified from plasmid pTH18kr [16] with the designed primers (taaccagatctgattagaaaaactcatcg and agaacctgcagtcagatccttccgtatttagc). The PCR DNA and plasmid pChA203 were both cleaved by *Bgl*II and *Pst*I and then spliced together to produce plasmid pTH-ChA203. The resulting plasmid contained the chitin-binding domain (ChBD) of *Bacillus circulans* WL-12 fused to the C-terminus of AHL (AHL-ChBD).

The type I Coh domain was amplified from *Clostridium thermocellum* DSM1237 by PCR using the designed primers (atcatctcgagttatgcggccgcaagctttggtg and attagaattcgatctcagccaaatgttcc). After cleavage with *Eco*RI–*Xho*I, the PCR DNA was incorporated into plasmid pTH-ChA203 to give plasmid pTH-AL203Coh. The resulting plasmid carried C-terminal fusion of AHL with Coh (AHL-Coh) under the control of P_{T7}.

Plasmid pTac-HDTDoCh was constructed in several steps. First, the internal *Nde*I site of plasmid pET32a (Novagen, USA) was eliminated by site-directed mutagenesis [17] to generate plasmid pET32-N using the designed primers (ggtgaataattttatcgctcatctgtatatctccttctagaggg and ccctctagaaggagatatacagatgagcgataaaattattcacc). The DNA bearing HDT-ChBD was then recovered from plasmid pET-TrHDTCh [15] by *Nde*I–*Xho*I and incorporated into the same sites of plasmid pET32-N to give plasmid pET-TrHDTCh. Secondly, the type I Doc was amplified from *C. thermocellum* DSM1237 by PCR with the designed primers (tgtgactcgagttagagctcgttcttgtacggcaatgtatc and gcatgaagcttaaagtacctggtactccttc). After cleavage by *Hind*III–*Xho*I, Doc was incorporated into plasmid pET-TrHDTCh to replace ChBD, resulting in plasmid pET-TrHDTDo. Moreover, the DNA containing ChBD was amplified from plasmid pET-TrHDTCh by PCR with the designed primers (agatgcttttctgtgactgg and agcatgagctcggcctgaccggtctgaac). The PCR DNA was digested by *Sac*I and subsequently spliced into the corresponding site of plasmid pET-TrHDTDo to give plasmid pET-HDTDoCh. The resulting plasmid carried the fusion of HDT-DocI-ChBD. Finally, the DNA containing the *tac* promoter (Ptac) was amplified from plasmid pJF-Trxfus [18] with the designed primers (gaatttctagacctgtgtgaaattgttatcc and gagtgagatatttatgccag). The PCR DNA was treated with *Apa*I–*Xba*I and incorporated into the same sites of plasmid pET32-N to produce pTac-N. By a *Pst*I–*Xba*I cut, HDT-Doc-ChBD was recovered from plasmid pET-HDTDoCh and ligated into the same sites of plasmid pTac-N. This construction gave rise to plasmid pTac-HDTDoCh, which created the C-terminal fusion of *trxA* (encoding thioredoxin) with HDT-Doc-ChBD (TrxA-HDT-Doc-ChBD).

The Ptac-containing DNA was removed from plasmid pTac-HDTDoCh by *Pst*I–*Xba*I. The DNA was then incorporated into the same sites of plasmid pET-TrHDTCh to replace P_{T7}, thus generating plasmid pTac-TrChHDT that carried the fusion of TrxA-HDT-ChBD.

In addition, PCR-amplification of Ds-Red was carried out from plasmid pDsRed-Express (Clontech, USA) with the designed primers (gaaacacatatgaccatgattacgccaag and gtcggaattcgtacaggaacaggtggtgg). The PCR DNA was digested by *Eco*RI–*Nde*I and then spliced into plasmid pTH-AL203Coh to obtain plasmid pRed-Coh. Meanwhile, the *pgpB* gene was amplified from strain BL21(DE3) (Novagene, USA) with the designed primers (gaatcatatgcgttcgattgccagacg and aacaaggtaccactttcttgttctcgttgcg). Restricted with *Kpn*I–*Nde*I, the PCR DNA was ligated into plasmid pET32a to give plasmid pET-Pgp. Doc was amplified from plasmid pET-HDTDoCh with the designed primers (caagtaagcttagccgagctcgttcttgtacg and agcaacccgggaaagtggacatgactc). The PCR DNA was cleaved with *Hind*III–*Sma*I and incorporated into plasmid pET-Pgp which was treated with *Eco*RV–*Hind*III. The construction resulted in plasmid pGPB-DocI, which created the C-terminal fusion of *pgpB* with Doc (PgpB-Doc).

2.2. Bacterial culturing and protein analysis

Bacterial growth was monitored using a spectrophotometer at 550 nm (OD_{550}). With the initial OD_{550} at 0.08, recombinant strains were cultured in shake flasks containing 20 mL Luria-Bertani (LB) medium [19] supplemented with ampicillin (30 μg/mL) and/or kanamycin (30 μg/mL) at 30 °C. To induce protein production, IPTG (100 μM) and/or L-arabinose (50 μM) were added to cell cultures upon OD_{550} reaching 0.3. Bacterial cultures were harvested by centrifugation after induction for 4 h. Followed by washing with 100 mM sodium phosphate buffer (PBS) at pH 7, the cells were resuspended in the same buffer solution to reach 20 at OD_{550}. The cells were then disrupted by sonication. After centrifugation, the supernatant was recovered as cell-free extract (CFX) and assayed for the protein content using Bio-Rad dye reagent. Each sample containing 20 μg proteins was resolved on dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The method for performing SDS-PAGE essentially followed our previous report [20]. In brief, the resolving gel (8%) was prepared and overlaid with a stacking gel (5%). The electric power (100 V followed by 200 V) was then applied to the gel which was mounted in the electrophoresis apparatus (Bio-Rad). The dismantled gel was finally stained with Coomassie blue for further analyses.

2.3. Analysis of bacteria by confocal microscopy

Bacterial cells were rinsed with PBS and fixed in 3.7% paraformaldehyde (Sigma, USA) for 30 min. Followed by washing three times with 100 mM PBS (pH 7.0), fixed cells were blocked with 3% BSA (Sigma, USA) in PBS. Bacterial cells were

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