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Improved catalytic activities of a dye-decolorizing peroxidase (DyP) by overexpression of ALA and heme biosynthesis genes in *Escherichia coli*

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ABSTRACT

Hemoproteins are biotechnologically important heme-containing enzymes, and their production often requires optimal supply of precursors, such as 5-aminolevulinic acid (ALA) and hemin. In this work, endogenous ALA biosynthesis in *Escherichia coli* was increased as a strategy to improve catalytic activities of a recombinant dye-decolorizing peroxidase (DyP) from *Bacillus subtilis*. The positive effects of this expression system (pHemAL-DyP) are compared with individually expressed DyP strains grown with (pDyP + Hemin) and without (pDyP) the addition of hemin. The pHemAL-DyP plasmid increased intracellular ALA up to 117.5 mg/L, a 4.0-fold increased from control strain (pET22b, 29.7 mg/L of ALA). Soret peak in the UV–vis spectra was the highest for pHemAL-DyP strain with Reinheitszahl (A_{408}/A_{280}) value of 0.65 indicating higher heme content in the DyP produced compared to pDyP (0.39) and pDyP + Hemin (0.46). Peroxidase activity was increased up to 66.7 U/mg in the pHemAL-DyP strain compared to 39.0 and 43.4 U/mg for pDyP and pDyP + Hemin, respectively. Decolorization percentage of Reactive Blue 19 dye was the highest in the pHemAL-DyP strain with 84.7% as compared to the pDyP (69.9%) and pDyP + Hemin (72.8%) systems. In brief, enzymatic properties of recombinant DyP were successfully enhanced using this genetic engineering strategy thus eliminating the need for costly exogenous precursors.

1. Introduction

Hemoproteins comprise a broad group of heme-containing proteins that are essential for various biological processes. This class of proteins includes cytochromes, catalases, peroxidases and oxygen-carrying globin proteins [1]. Hemoproteins, such as horseradish peroxidase and hemoglobin, are important in medical and industrial biotechnology [2,3]. Genetic engineering approaches have been extensively used for the production of hemoproteins in recombinant microbial hosts, especially *Escherichia coli* [4,5].

Peroxidases are a family of enzymes that convert various compounds via oxidation reactions using hydrogen peroxide as an electron acceptor. The majority of peroxidases contain heme as a cofactor, and these heme peroxidases are generally classified in two superfamilies, animal and plant peroxidases [6]. A new family of heme peroxidase was recently discovered and classified as the dye-decolorizing peroxidase-type (DyP-type) family [7]. This group of enzymes degrades various dyes, including of xenobiotic and recalcitrant compounds, rendering them useful for biotechnological applications, particularly in waste treatments [8–10]. DyPs are naturally found in several fungal and bacterial species, and rapid progress has been made for recombinant DyP production using heterologous expression hosts [11,12].

Recent studies of recombinant hemoproteins indicate that the sufficient supply of the heme cofactor is an important aspect of soluble and functional hemoprotein production [13,14]. Incorporation of the heme prosthetic group into recombinant apoprotein is vital for the subsequent holoprotein solubility and activity [15]. Several strategies were developed to increase the solubility and stability of the hemoproteins, mainly by increasing the level of heme and its precursor, 5-aminolevulinic acid (ALA) in microbial culture media, so that efficient conjugation of newly synthesized protein and heme could occur efficiently [16,17]. Suboptimal levels of these cofactors in the production culture may lead to reduced enzymatic activity of the target hemoproteins [18,19].

Because of the importance of supplying sufficient amounts of heme cofactor and the cost involved for recombinant hemoprotein production, the objective of this study was to increase the catalytic activities of recombinant DyP by overexpressing the essential genes in the ALA and heme biosynthesis pathways in engineered

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E. coli. Therefore, this study reports the development of a bacterial expression system for recombinant dye-decolorizing peroxidase production, utilizing increased levels of intracellular ALA and heme as hemoprotein precursors.

2. Materials and methods

2.1. Strains and culture conditions

E. coli DH5α was used for molecular cloning and plasmid maintenance experiments. For protein expression experiments, *E. coli* BL21 (DE3) strain was selected for its T7 promoter-based expression system using isopropyl β-D-1-thiogalactopyranoside (IPTG) as an inducer. Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L sodium chloride) with ampicillin (50–100 μg/mL) was used for bacterial growth and plasmid propagation experiments. Bacterial growth was performed at 37 °C and 200 rpm. Glucose (0.5%) was added to the media when necessary to prevent leaky expression of the T7 promoter.

2.2. Construction of plasmids

pET-22b(+) plasmid (hereafter denoted as pET22b) from Novagen (San Diego, CA) was used as the expression vector. The *DyP* gene was amplified from the genomic DNA of *Bacillus subtilis* 168, including its signal sequence. For pDyP plasmid construction, the *DyP* gene was cloned into *NdeI* and *XhoI* restriction enzyme sites, replacing the original *pelB* peptide sequence. The primers used were DyP Fwd (5′-GCAAGACATATGAGCGATGAACAGAAAAAGCCAGAACAAATT-3′) and DyP Rev (5′-GCAAATCTCGAGTGATTCCAGCAAACGCTGGGCAAT-3′). To confer endogenous ALA overproduction in the recombinant *E. coli*, *hemA* and *hemL* genes were assembled as an operon and overexpressed together with the *DyP* gene to create the pHemAL-DyP plasmid. The *hemA* and *hemL* genes originated from *Salmonella typhimurium* LT2 and *E. coli BL21*, respectively, and were cloned from our laboratory plasmid by cutting the template plasmid via *BamHI* and *NotI* restriction enzyme sites to generate a DNA fragment containing the *hemA* and *hemL* genes flanked by a ribosomal binding site inserted in between the two genes. Enzymatic digestion of corresponding restriction sites in the pET-22b(+) vector led to the generation of pHemAL vector under the control of the strong T7 promoter. The *hemA* gene contained a site-directed mutation via the insertion of lysine codons at the third and fourth positions in accord to a previous report [20]. The *DyP* gene was cloned to the *hemAL*-containing plasmid via *XhoI* restriction enzyme site using primers AL-DyP Fwd (5′-GCATCTCGAGTTGTACACGGCCGCATAATCGAAATTAATACGACTCACT-3′) and AL-DyP Rev (5′-GCCTTCCTCGAGTGATTCCAGCAAACGCTG-GGCAATATA 3′). The restriction enzyme sites in the primers are underlined. All bacterial strains and plasmids used in this study are listed in Table 1.

2.3. Expression of recombinant proteins

To investigate the optimal expression systems for DyP production, recombinant strains carrying the pDyP plasmid were grown in LB broth containing 100 μg/mL ampicillin (LA medium) with and without the addition of hemin. The strain harboring the pHemAL-DyP plasmid construct was also grown under similar conditions to the pDyP strains except, for hemin addition. Specifically, each plasmid construct was initially transformed into the *E. coli* BL21 (DE3) strain and grown on LA agar plates with glucose (0.5%). For protein expression, the recombinant bacterial colonies were then grown as precultures in 10 mL LA broth with glucose (0.5%) for 8–12 h at 37 °C and 200 rpm. A volume of 1 mL of the overnight preculture was subsequently inoculated into 100 mL of LA medium in shake flasks and incubated at 37 °C and 200 rpm until the OD_{600} reached approximately 0.6. Then, 1 mM IPTG and 15 μM hemin, for the pDyP + Hemin culture only, were added to the culture medium and incubation was performed at 30 °C and 120 rpm for 24 h. For recombinant protein extraction, the cells were then harvested after centrifugation at 4000 rpm for 30 min. A volume of 5 mL sodium phosphate buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) was added to the cell pellets, and 0.5 mL lysozyme (10 mg/mL) was mixed with the sample solution. After 30 min, sonication was performed with 6 × 10 s pulses with a 10 s pause between pulses (VC 505 ultrasonic processor, 500 watt max; Sonics & Materials Inc., USA). Subsequently, the sample solution was centrifuged at 4000 rpm for 30 min to separate the soluble protein supernatant and cell debris. The supernatant was filtered using a 0.45 μM membrane filter and the resulting protein solution was used for further enzymatic analysis. All protein samples were placed on ice at all times and stored at −20 °C when necessary.

2.4. Analytical methods

UV–vis spectra, enzyme assays and ALA concentration were measured using a UV–vis spectrophotometer (Optizen POP, Mecasys Co., Ltd., South Korea). The protein concentration was measured via Bradford assay using a Bio-Rad protein assay kit (Bio-Rad, USA) with bovine serum albumin as the protein standard [21]. Peroxidase activity was analyzed using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the substrate in a reaction mixture consisting of 10 mM ABTS, 0.2 mM H_2O_2 and the appropriate dilution of protein sample in 100 mM sodium tartrate buffer (pH 5.0) at 25 °C. Measurement of the oxidation state of ABTS was performed at 420 nm (ε = 36,000 M^{-1} cm^{-1}). The specific enzyme unit (*U*) is defined as the amount of enzyme required to reduce 1 μmol ABTS per minute per mg of protein. Analysis of dye-decolorizing activity was performed at 25 °C in 100 mM sodium acetate buffer (pH 4.0) containing 0.1 mM Reactive Blue 19 (RB19) dye, 0.2 mM H_2O_2 and the appropriate dilution of protein sample. Decolorization of the RB19 dye was measured at 590 nm (ε = 10,620 M^{-1} cm^{-1}) after 15 min of incubation. The percentage of RB19 dye-decolorization was calculated using a method reported previously [22]. Intracellular ALA content was analyzed at 554 nm using the harvested sample according to the previously described method [23]. ALA, hemin, ABTS and RB19 dye were acquired from Sigma Aldrich (USA).

3. Results and discussion

3.1. Enhancing endogenous ALA biosynthesis in recombinant E. coli

The availability of heme and ALA precursor is integral in the biosynthesis of soluble and functional recombinant hemoproteins. In this study, we increased the endogenous ALA biosynthesis using a metabolic engineering strategy in lieu of relying on an exogenous supply of costly chemicals. To increase ALA biosynthesis, a mutated HemA (glutamyl t-RNA reductase) and glutamate 1-semialdehyde aminotransferase, HemL were overexpressed using the pET expression system. Intracellular ALA content was confirmed by measuring the ALA concentration in all recombinant strain samples accordingly.

As shown in Fig. 1, the recombinant strain expressing the *hemAL* operon and DyP exhibited the highest level of intracellular ALA. The strain harboring pHemAL-DyP produced 117.5 mg/L of ALA, which represents a 4.0-fold increase from the control strain