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Short communication

Solar-to-chemical conversion platform by Robust Cytochrome P450-P(3HB) complex

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ABSTRACT

Cytochrome P450 monooxygenases which catalyze a remarkable variety of oxidative transformation are of exceptional interest for the synthesis of fine chemicals. However, due to the instability and requirement of expensive cofactor, P450s have yet to be extensively used for industry. Here, we developed a new platform that combines a solar-driven cofactor regeneration and immobilization of P450 on biopolymer. Through photochemical cofactor regeneration using eosin Y as a photosensitizer, P450-P(3HB) complex successfully catalyzed a *O*-dealkylation reaction. Furthermore, using the P450-P(3HB) complex and a solar-tracking module, we achieved a P450-catalyzed reaction under natural sunlight for four consecutive days in a preparative scale.

Introduction

Cytochrome P450 monooxygenases (CYPs or P450s) have been given steady attention in the fields of industrial chemical reaction, pharmaceutical and metabolite synthesis because of its powerful and attractive biocatalytic ability for the regio- and stereospecific oxidation of non-activated hydrocarbons. Over the past decade, P450s mutants have been developed with specific biocatalytic oxidation activity on sp^3 hybridized C atoms, epoxidation of C=C double bonds, aromatic hydroxylation, *N*-oxidation, deamination and dehalogenation by rational and evolutionary engineering approaches [1–3]. However, despite the high potential of P450s, their practical applications is hindered by costly nicotinamide cofactors (e.g., NAD(P)H), which serve as a redox equivalent in P450-catalyzed reaction.[4–6]. Thus, numerous efforts have been devoted to achieve cofactor regeneration for sustainable cofactor supply [7–9]. However, the conventional cofactor regeneration methods by using secondary enzymes and chemical electrodes have intrinsic drawbacks, such as by-product formation, usage of secondary enzymes, low specific activity, and high overpotential, which hampered practical application of these methods [10–12]. Recently, the photochemical cofactor regeneration has come into the spotlight for the in situ supply of NAD(P)H, which enable to regenerate cofactor in a cost-effective and environmentally friendly manner by using abundant solar energy [13,14]. For the efficient photochemical regeneration of NAD(P)H, photosensitizer can successfully harvest sunlight and transfer photoexcited electrons to $NAD(P)^+$ [15,16]. An eosin Y (EY) is a promising photosensitizer, having high quantum yields in photocatalytic hydrogen evolution and dye-sensitized solar cells [17,18]. Especially, EY exhibits strong visible light absorption property and proper band edge positions for photochemical cofactor regeneration coupled with redox enzymatic reaction [19].

It is well known that the activity and stability of enzyme can be improved by immobilization on solid supports, and there are several successful results about the immobilization of P450 on various support including nanoparticles, polymers etc. [20–23]. In previous work, we also developed the new platform for the immobilization of P450 monooxygenase on biopolymer, poly(3-hydroxybutyrate) [P(3HB)] granules in *Escherichia coli* host [24]. The P450-P(3HB) complex clearly demonstrated higher stability and enzymatic activity in various harsh conditions

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compared to free P450. Here, we report on the development of robust P450 catalysis platform by the combination of solar-driven NADPH photo-regeneration and in situ P450 immobilization on P(3HB) granules. The photochemical NADPH regeneration system using the EY as a photosensitizer was coupled to the immobilized P450-catalyzed reactions, and P450-P(3HB) complex successfully performed visible light-driven P450-catalyzed *O*-dealkylation. Finally, with the solar tracking system, the natural sunlight was employed as a light source, and the P450-catalyzing reaction was successfully demonstrated in the preparative scale (0.5 L).

Experimental

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) in reagent grade purity and were used without further purification. The rhodium (III)-based electron mediator (**M** = $[Cp^*Rh(bpy)H_2O]^+$, $Cp^* = C_5Me_5$, bpy = 2,2′-bipyridine) was synthesized according to the method reported previously [25].

Bacterial strains and plasmids

E. coli XL1-Blue was used as a main host for P(3HB) synthesis, and protein expression. A low copy plasmid pMCS437ReAB containing the *Ralstonia eutropha* PHA biosynthesis genes [26] was used for the synthesis of PHA in *E. coli*. For the production of phasin-fused P450-BM3m2, pBM3_2P was used [24]. It contained the *Bacillus megaterium* P450 BM3 gene conjugated with two PhaP genes of *Aeromonas hydrophilia* 4AK4 (NCBI accession no. CP006579.1).

Cell cultivation

The *E. coli* XL1-Blue harboring both pMCS437ReAB and pBM3_2P was inoculated in Luria-Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L). After overnight cultivation at 37 °C with shaking at 200 rpm, cells were transferred to a 1 L flask containing 200 mL of LB medium supplemented with 20 g/L glucose and 100 mg/L ampicillin (Ap) and 35 mg/L chloramphenicol (Cm), and cultivated in a shaking incubator at 30 °C with 200 rpm. At the optical density (OD_{600}) of 0.6, 0.4 mM isopropyl thiogalactopyranoside (IPTG, Sigma Chemical Co., St. Louis, MO, USA) was added for induction of gene expression. Simultaneously, 1 mM thiamine and 0.5 mM δ-aminolevulinic acid hydrochloride were added as the additives, and then cells were further cultivated for 22 h.

Protein purification

After flask cultivation, cells were harvested by centrifugation at 6000 rpm, 4 °C and for 10 min, and the pellets were resuspended in a 100 mM potassium phosphate buffer (pH 7.4). Cells were disrupted by a high pressure homogenizer (APV-2000, Invensys, Denmark) at 1000 bar for 10 min, then the soluble and insoluble protein fractions were separated by centrifugation (10,000 rpm, 4 °C, 10 min). The insoluble pellets were resuspended in the 100 mM potassium phosphate buffer (pH 7.4) and added to the reaction immediately. The total amount of proteins in cell lysates were quantified by the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) with BSA (Bovine Serum Albumin) as a standard.

Photochemical P450-catalyzed O-dealkylation

Eosin Y (20 μM), **M** (0.1 mM), $NADP^+$ (0.1 mM), and 7-ethoxycoumarin (1 mM) were dissolved in a phosphate buffer (100 mM, pH 8.0) containing triethanolamine (15% w/v). P450 BM3-P(3HB) complex solution was added into the reaction mixture, which was then exposed to the visible light from a white-light emitting plate (Viewone, EmbiTec, USA) or solar-tracking system (WDG 1500, Whilkor, Korea). The concentration of product (7-hydroxycoumarin) was measured by a multi-well plate fluorometer (VICTOR X3, Perkin-Elmer, USA) with excitation and emission wavelengths at 405 and 460 nm, respectively.

Results and discussion

A P(3HB) biopolymer, synthesized in *E. coli* host harboring P(3HB) biosynthesis genes (*phaCAB*), was used for the immobilization of P450 monooxygenase. As successfully demonstrated in

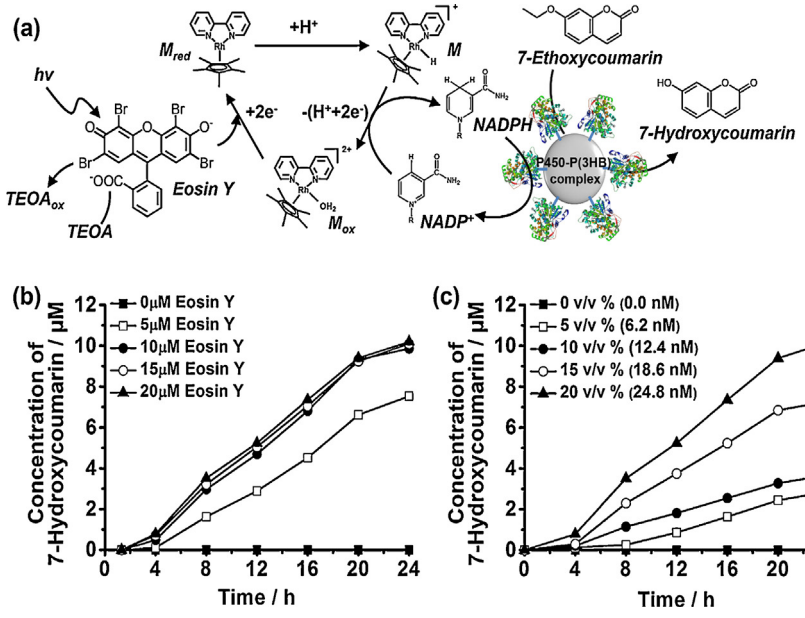

Fig. 1. (a) Schematic illustration of photochemical P450-catalyzed *O*-dealkylation by P450-P(3HB) complex. Photoenzymatic synthesis of 7-hydroxycoumarin with different concentrations of (b) Eosin Y (EY) and (c) P450-P(3HB) complex: For all experiments, EY (20 μM), M (0.1 mM), $NADP^+$ (0.1 mM), and 7-ethoxycoumarin (1 mM) were used in a phosphate buffer (100 mM, pH 8.0) containing triethanolamine (15% w/v). Reaction volume was 100 μL.