Bioconjugate Chemistry

Communication

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New Platform for Cytochrome P450 Reaction Combining in Situ Immobilization on Biopolymer

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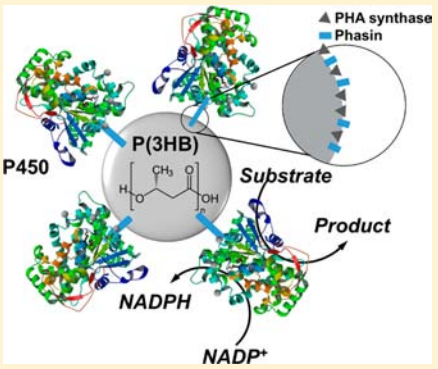
Supporting Information

ABSTRACT: We describe an efficienct chemical conversion platform with in situ immobilization of P450-BM3 on poly(3-hydroxybutyrate) granules. Through fusion with phasin, P450-BM3 is easily immobilized on poly(3-hydroxybutyrate) granules in *Escherichia coli*. In our work, the immobilized P450 exhibited higher stability and catalytic activity compared to free P450 against changes of pH, temperature, and concentrations of urea and ions. Through quick recovery of immobilized enzyme, the P450−P(3HB) complex successfully catalyzed an O-dealkylation reaction several times with maintained activity. Using the robust P450−P(3HB) complex, we performed a P450-catalyzed reaction on a preparative reactor scale (100 mL) and high-level production (12.3 μM) of 7-hydroxycoumarine from 7-ethoxycoumarin could be achieved.



INTRODUCTION

In the past 50 years, cytochrome P450 monooxygenases (CYPs or P450s) have been given significant attention for the synthesis of natural products (e.g., vitamins, steroids, lipids) and pharmaceuticals because of their unique and powerful catalytic abilities for regio- and stereospecific oxidation of nonactivated hydrocarbons.[1−6] Through intensive studies on P450 engineering using both rational mutagenesis and directed evolutionary approaches,[7−9] various kinds of P450 mutants have been created to have specific oxidation activities on sp^3-hybridized carbon atoms, epoxidation of C═C double bonds, aromatic hydroxylation, N-oxidation, deamination, and dehalogenation.[10−13] Despite their potential, however, critical issues exist for the practical applications of P450s. For the catalytic activity of P450s, costly nicotinamide cofactors such as NAD(P)H are critically required as reducing equivalents;[14−17] thus, in situ regeneration of NAD(P)H is essential to sustaining P450-catalyzed reactions.[18] Furthermore, poor stability of P450s has been considered as a hurdle, hampering industrial implementations of P450-catalyzed reactions.[19,20]

Herein we describe the development of a new P450 catalysis platform for in situ P450 immobilization on intracellular poly(3-hydroxybutyrate) [P(3HB)] granules. A P450-BM3 variant (Y51F/F87A, BM3m2) from flavocytochrome P450-(CYP102A1) of *Bacillus megaterium* was used as a model monooxygenase.[21] P(3HB) is an intracellular aliphatic carbon storage reserve that is produced as a granular inclusion body in many bacteria.[22] For the in situ immobilization of P450s on P(3HB) granules, we employed P(3HB)-associated protein (phasin or PhaP) that has a strong and specific binding ability to the surface of P(3HB) granules.[23−25] During cell cultivation, phasin-fused P450-BM3m2 and P(3HB) were coproduced in the cytoplasm of *Escherichia coli* to attach P450-BM3m2 to the surface of P(3HB) granules through the phasin tag (Figure 1A). After cell disruption, the complex of P450−P(3HB) could be easily recovered and used for chemical conversion reactions. The overall scheme for the preparation of the P450−P(3HB) complex is illustrated in Figure 1B.

RESULTS AND DISCUSSION

For the synthesis of the P450−P(3HB) complex, we cultivated *E. coli* XL1-Blue harboring both pBM3_2P and pMCS437ReAB in LB medium. According to gas chromatographic analysis during cell culture, the content of P(3HB) in *E. coli* gradually increased and reached approximately 14.4% of cell dry weight after 10 h of cultivation (Supporting Information, Figure S1). The production of phasin-fused P450-BM3m2 was also induced by the addition of IPTG during the synthesis of P(3HB)

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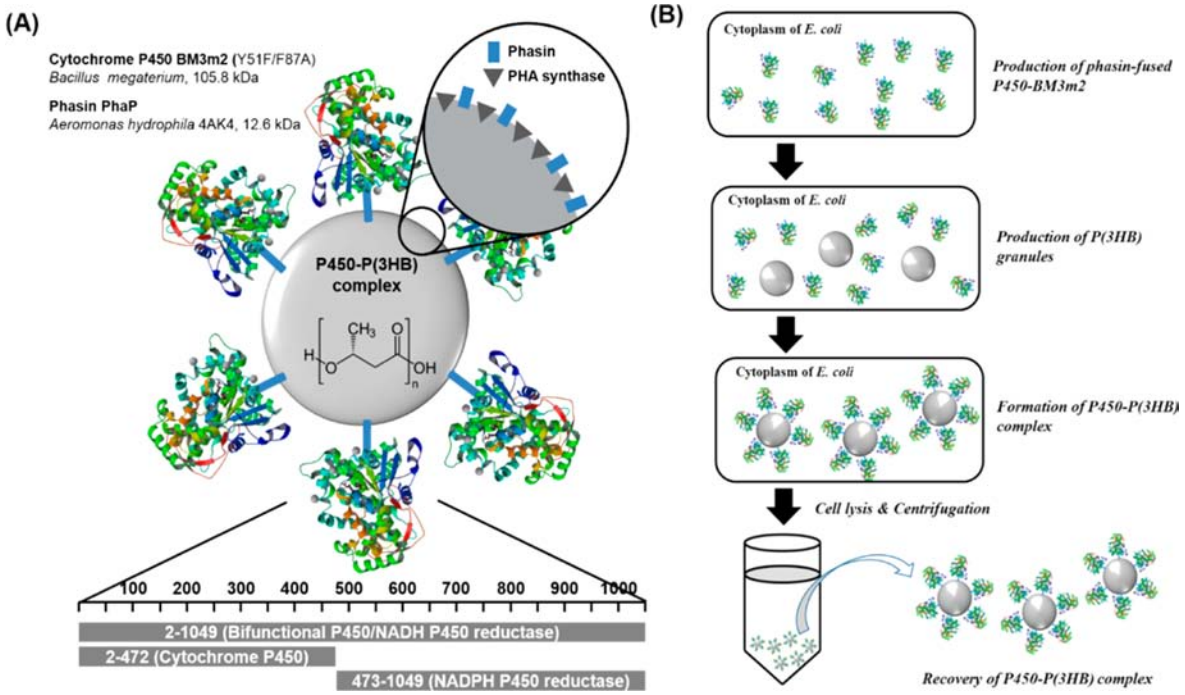

Figure 1. Synthesis of P450−P(3HB) complex in cytoplasm of *E. coli*. (A) P450-BM3m2 fused to two tandem repeats of phasin (PhaP) were produced in cytoplasm and immobilized on a P(3HB) granule via binding of phasin to the surface of P(3HB). P450-BM3m2 consists of two domains: heme domain (2−472 residues) and reductase domain (473−1049 residues). Two tandem repeats of phasin are fused to the C-terminus of the reductase domain. (B) The phasin-fused P450s and P(3HB) granules are coproduced in cytoplasm of *E. coli*, and P450s are immobilized on the surface of P(3HB) granules via the fused phasin tag. After cell lysis and centrifugation, the P450−P(3HB) complex can be recovered from the pellets with high purity and recovery yield.

granules in *E. coli*. Our SDS−PAGE analysis confirmed that most phasin-fused P450-BM3m2 (~130.1 kDa) were present in insoluble form unlike phasin-free P450-BM3m2 which was mostly in a soluble form (Figure 2). This result clearly indicates

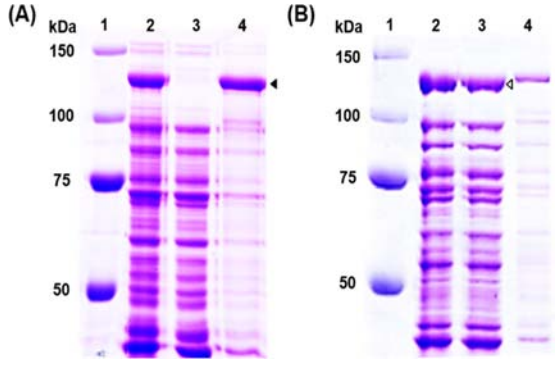

Figure 2. Analysis for production of P450-BM3m2 in *E. coli*. (A) *E. coli* (pBM3_2P and pMCS437ReAB) producing P(3HB) and phasin-fused P450-BM3m2 and (B) *E. coli* (pBM3) producing phasin-free P450-BM3m2 were cultivated, and protein production in each cell culture was analyzed by SDS−PAGE: lane 1, molecular weight markers (kDa); lane 2, total proteins; lane 3, soluble protein fraction; lane 4, insoluble protein fraction. Closed and open arrowheads indicate P450-BM3m2.

that most of phasin-fused P450-BM3m2 were successfully immobilized on P(3HB) granules with high efficiency. After cell cultivation, the P450−P(3HB) complex could be easily purified by simple cell disruption followed by centrifugation. From 1 L cultivation, the phasin-fused P450-BM3m2 could be purified as high as 1.28 g with high purity.

To validate the robustness of the P450−P(3HB) complex over free P450, we compared its activity and stability under different conditions of pH, temperature, urea, and ion concentrations. First, the activities of immobilized and nonimmobilized P450-BM3m2 were examined at different pH values (pH 5.0, 6.0, 7.0, and 8.0) (Figure 3A). The activity of free P450-BM3m2 decreased much faster than that of an immobilized one, indicating higher resistance of the P450−P(3HB) complex to pH change. Second, we observed much improved thermostability of P450-BM3m2 when it was immobilized on P(3HB) granules (Figure 3B). Furthermore, the P450−P(3HB) complex exhibited higher activity than did free ones in all examined concentrations of urea (up to 4 M) and phosphate ions (0, 25, 50, 75, and 100 mM) (Figure 3C and D). Taken together, we concluded that the P450−P(3HB) complex has strong resistance to environmental changes and P(3HB) granules can provide more stable platforms for applications of P450s.

Next, we applied the P450−P(3HB) complex to repetitive use through quick and easy recovery of enzyme complex. P(3HB) in P450−P(3HB) complex was used as an insoluble bead that can be simply separated by centrifugation (10 000 rpm, 4 °C, 10 min) after the reaction. The P450−P(3HB) complex used in NADPH dependent P450-catalyzed O-dealkylation reaction was reused more than 4 times with maintained activity after product saturation in 120 min (Figure 4A). The yield of 7-hydroxycoumarin by the immobilized P450 was kept to three cycles of reaction, and it showed just a gradual decreases of activities in the next two cycles. The rate of O-dealkylation reaction catalyzed by the P450−P(3HB) complex was almost the same in the all cycles. To examine the suitability of our system in scale-up operations, we increased the reaction

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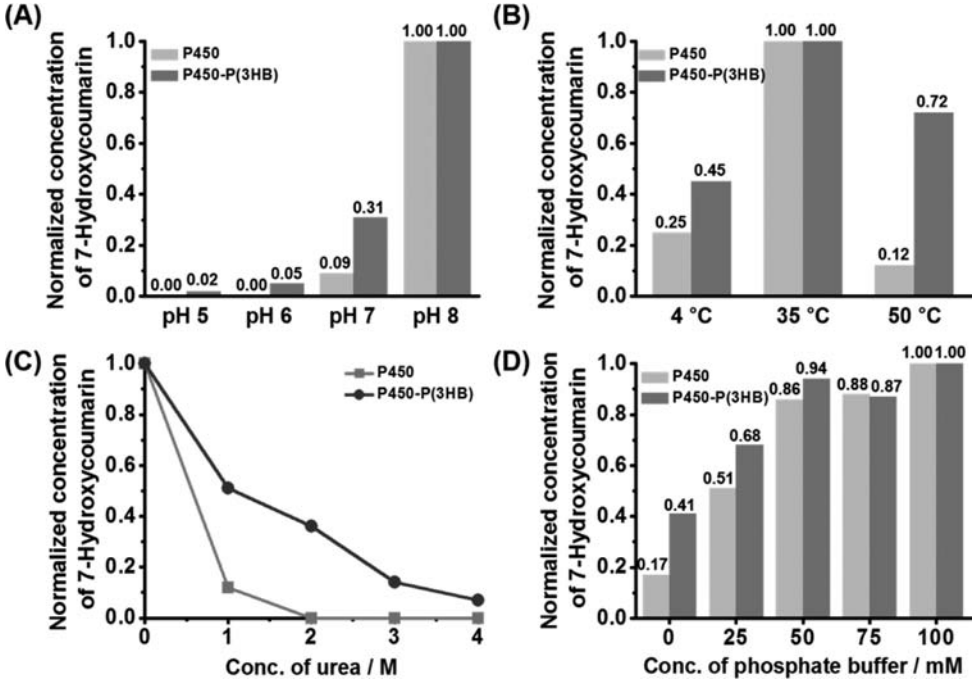

Figure 3. Property verification of nonimmobilized P450 and P450−P(3HB) complex. After concentration and purification, the stabilities and activities of P450 were compared at different conditions: (A) pH, (B) temperature, (C) urea concentration, and (D) phosphate concentration. In all experiments, nonimmobilized P450 and P450−P(3HB) complex (24.8 nM) were incubated for 24 h with NADPH (1 mM) and 7-ethoxycoumarin (1 mM) in a phosphate buffer. The turnover numbers for all enzyme reactions were calculated (Supporting Information, Table S3), and they were used for activity comparison. Reaction volume was 100 μL.

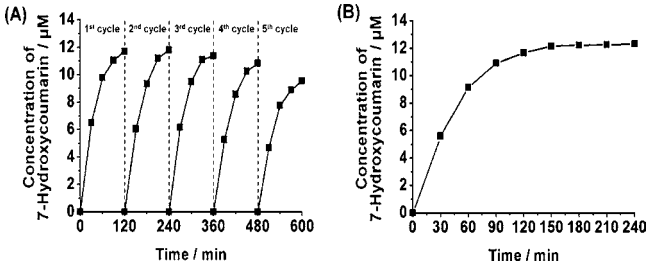

Figure 4. O-Dealkylation reaction by P450−P(3HB) complex in repetitive use (A) and in preparative scale (100 mL) (B). In all experiments, P450−P(3HB) complex (24.8 nM) was incubated with NADPH (1 mM) and 7-ethoxycoumarin (1 mM) in a phosphate buffer.

volume to 100 mL and carried out the same reaction for saturation point. We clearly observed that the concentration of 7-hydroxycoumarin did not change in the large scale reactor (Figure 4B). These results indicate that the P450−P(3HB) complex was stably maintained without loss of activity with repetitive use as well as in the preparative scale reactions.

CONCLUSION

We developed an economic and robust process of P450-catalyzed reactions by in situ P450 immobilization. P450 monooxygenase could be immobilized on P(3HB) biopolymer with high efficiency, which enabled simple purification from the *E. coli* host. We clearly demonstrated that the P450−P(3HB) complex exhibited a much higher enzymatic yield and stability than free P450 did against changes of pH, temperature, and concentrations of urea and ions. By use of the robust P450−P(3HB) complex, the P450-catalyzed reactions were successfully conducted in the repetitive use as well as in the preparative scale (100 mL). These results also proved the advantages of P450−P(3HB) complex compared to free P450 which is not suitable for repetitive use and large scale reaction because of the requirement of high cost and labor-intensive purification. We believe that our robust platform using simple immobilization and quick recovered method promises a significant breakthrough for the broad applications of cytochrome P450 monooxygenases.

ASSOCIATED CONTENT

Supporting Information

Details of the purification of P450−P(3HB) complex, gas chromatography analysis, and chemical P450-catalyzed O-dealkylation as well as supplemental tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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