Tunnel Engineering for Modulating The Substrate Preference in Decarbonylase P450Bs\(\beta\)HI

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Research

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Abstract

An active site normally locates inside of enzymes, substrates should go through the tunnel to access the active site. Tunnel engineering is a powerful strategy for refining the catalytic properties of enzymes. Here, P450_{BsβHI} (Q85H/ V170I) derived from hydroxylase P450_{Bsβ} from *Bacillus subtilis* was chosen as study model, which is reported as a potential decarbonylase. However, this enzyme showed low decarboxylase activity towards long-chain fatty acids. Here, a tunnel engineering campaign was performed for modulating the substrate preference and improving the decarbonylase activity of P450_{BsβHI}. The finally obtained BsβHI-F79A variant had a 15.2-fold improved conversion for palmitic acid; BsβHI-F173V variant had a 3.9-fold improved conversion for pentadecanoic acid. The study demonstrates how the substrate preference can be modulated by tunnel engineering strategy.

1. Introduction

Enzymes are able to catalyze many specific reactions and are widely used in practical application. Previously, the enzyme functions were limited around their natural use; nowadays, the enzymes could be developed by engineering their activity and selectivity for meeting human demands (Bornscheuer et al., 2012; Damborsky and Brezovsky, 2014). Two common engineering strategies are the directed evolution based on Darwinian theory and rational design based on the structure-function relationship (Bornscheuer and Pohl, 2001). Rational design often focused on the substrate binding pocket which directly influence the enzymatic process (Bornscheuer and Pohl, 2001). However, the experiences of directed evolution told us the residues outside the active site also influence the enzyme properties (Kress et al., 2018). Researching on those “non-hotspot” rather than typical active site may be beneficial for expanding the understanding of proteins.

The urgent problem now confronting us is how to obtain the important “non-hotspot” domains beyond the enzyme active site. Tunnel engineering may be one of the answers. Enzymes spanning all of the six classes are found the exist of the tunnels (Kingsley and Lill, 2015). It was reported that more than 64% enzymes annotated in Catalytic Site Atlas library have the buried active site with the tunnels connecting the enzyme binding pocket and the environment (Pravda et al., 2014). The tunnels could support the transport of solvent, product and solvent between the enzyme active site and bulk solvent, which play important role in enzymatic reaction (Kokkonen et al., 2019; Zhou and McCammon, 2010). The behavior of substrate on the tunnels to the active site could affect the activity, stability and substrate selectivity (Kingsley and Lill, 2015; Lu et al., 2019; Yu et al., 2013). A typical example might be that the R47 and Y51 residues, two polar amino acids located at the end of access tunnel of P450 BM3, could regulate the entry of substrate, water and co-solvents (Whitehouse et al., 2012). Cheng et al. found that a single position located on the access tunnel of nitrile hydratase could invert the regio-selectivity towards aliphatic α,ω-dinitriles (Cheng et al., 2016). Tunnel engineering is becoming a promising strategy to optimize the enzyme property. Several scientists have developed many algorithms for determination of enzymatic tunnels, such as CAVER (Kozlikova et al., 2014), MOLE (Sehnal et al., 2013), AQUA-DUCT (Magdzier et al., 2017) and CCCPP (Benkaidali et al., 2014). However, compared with the prediction of tunnels, only a
few experimental tunnel engineering examples were reported (Kress et al., 2018). Here, we aim to develop the application of tunnel engineering for modulating the substrate preference.

α-alkenes are multifunctional compounds that perform an important industrial value and an extraordinary economic importance due to their flexible and active chemical performance. Particularly, long-chain α-alkenes can be used in the synthesis of high-value biofuel, lubrication and surfactant (Lee et al., 2008). However, α-alkenes mostly come from non-renewable petroleum cracking (Dutta et al., 2014). Energy-intensive process and harsh reaction conditions prompted researchers to focus on enzymatic synthetic strategy of α-alkenes (Schirmer et al., 2010). To date, three biotransformation strategies that convert fatty acids or its derivative to alkene were reported (Yi et al., 2014). A three-gene cluster from Micrococcus luteus represented a production of long-chain alkenes from a head-to-head condensation of fatty acids (Beller et al., 2010); a type I polyketide synthases from Synechococcus sp. was involved in a production of medium-chain α-alkenes via an elongation decarboxylation mechanism (Mendez-Perez et al., 2011); and some members from cytochrome P450 family are able to decarboxylate fatty acids and produce α-alkenes directly (Grant et al., 2015; Hsieh and Makris, 2016). Among those pathways, the decarboxylic reaction catalyzed by P450 is the simplest strategy. Free fatty acids could be used as substrate directly, which represent potential of producing α-alkenes on a large-scale based in engineered cell factory.

Representative P450 decarboxylases belong to CYP152 subfamily, such as P450 Bsβ (CYP152A1) from Bacillus subtilis (Matsunaga et al., 1999), P450 Spα (CYP152B1) from Sphingomonas paucimobilis (Matsunaga et al., 2000), and P450 oleT (CYP152L1) from Jeotgalicoccus sp. (Yi et al., 2014). As the first member of CYP152 subfamily, P450 Bsβ was the research hotspot since it was discovered by Isamu et al. in 1999. However, wild-type P450 Bsβ showed higher hydroxylation activity rather than decarboxylation activity (Matsunaga, et al., 1999). In contrast, P450 oleT presented prominent decarboxylation property, which is able to convert medium- or long-chain fatty acids to corresponding carboxylic acids (Rude et al., 2011). Interestingly, some P450 Bsβ variants also exhibited satisfactory decarboxylation ability. Xu et al. reported a P450 BsβHI variant (Q85H/V170I) which displayed enhanced decarboxylation activity towards medium- or long-chain fatty acids (Xu et al., 2017). However, the yield of α-alkenes drops sharply as the length of the carbon chain of the substrate increases, which is the main limitation of the use of P450 BsβHI for long chain α-alkenes synthesis.

In the present work, we systematically analyzed the access tunnels in decarbonylase P450 BsβHI. In order to improve the substrate preference of P450 BsβHI to long chain fatty acids, two residues related to the access tunnels diameter were identified and mutated. In addition, the substrate selection mechanism controlled by tunnels of P450 BsβHI was briefly discussed.

2. Experimental

2.1. Strain, plasmid and chemicals
*Escherichia. coli* strain BL21(DE3) cells (used for gene expression) and TOP10 cells (used for molecular cloning) were purchased from TransGen Ltd. (China). The gene BsβHI-wt (NCBI Reference Sequence: WP_119898938.1) was synthesized by Inovogen Ltd. (China). Fast Mutagenesis kit was from Vazyme Biotech Co., Ltd (China). Plasmid extraction kits and gel extraction kits were obtained from Omega Bio-tek (USA). Capric acid, lauric acid, myristic acid, stearic acid, undecene and tridecene were purchased from BioRo Yee Ltd. (China). All chemicals used were of analytical grade.

### 2.2. Molecular modeling and simulation

The structure model of P450<sub> Bsβ </sub>Hl was constructed by PyMoL Molecular Graphics System (version 2.3.3) based using the crystal structure of P450<sub>Bsβ</sub> from *Bacillus subtilis* (PDB code: 1IZO with the resolution in 2.10 Å) as template (Lee et al., 2003). Molecular dynamics simulation was carried out in YASARA (version 17.8.15) using the built-in MD macro “md_run.mrc” with the AMBER03 force field (Hess et al., 2008). Structure were solvated into a 12 Å cube simulation cell of water molecules. The box was filled with 3857 water molecules. The simulations of the protein-water system was performed at 303 K, pH value of 8.0. Na<sup>+</sup> and Cl<sup>-</sup> were used to neutralize the systems. In all simulations, constant pressure periodic boundary conditions were used for 5 ns MD production. The simulation snapshots were capture every 100 ps from 2.5 ns to 5 ns (after RMSD stabilizes).

### 2.3. Tunnel analysis

Tunnel analyzed by MOLEonline (Pravda et al., 2018) with following parameters: Interior treshold 1.1 Å, Bottleneck Tolerance 3 Å, bottleneck radius 1.2 Å. Probe Radius 5 Å, Surface CoverRadius 10 Å. The starting point was the heme cofactor. Water molecules were not considered in channel analyses.

Tunnels analyzed by Caver Analyst 2.0 BETA (Jurcik et al., 2018) with following procedures: After the MD simulation, 25 snapshots were introduced to Caver Analyst 2.0 BETA. Tunnel calculations were performed with the analytical parameters as follow: probe radius of 1.4 Å, shell depth of 4 Å, shell radius of 3 Å, clustering threshold of 3.5, and the starting point of surrounding the residues of R242, P243, and heme. After the tunnel calculations, the influence of the tunnel by related residues was extracted from the function of tunnel statistics and residue graph. Finally, amino acid residues with significant influence on the tunnel bottleneck were selected for further mutagenesis experiments.

### 2.4. Relative folding free energies (ΔΔGfold) analysis

The ΔΔGfold values were calculated using FoldX employing the YASARA plugin (version 19.12.4). The structure model of P450<sub>Bsβ </sub>Hl and its variants were constructed by PyMoL Molecular Graphics System (version 2.3.3) based using the crystal structure of P450<sub>Bsβ</sub> from *Bacillus subtilis* (PDB code: 1IZO with the resolution in 2.10 Å) as template. The initial structure of P450<sub>Bsβ </sub>Hl was constructed by the Automated Modeling Tool of Swiss Model Web Service (http://swissmodel.expasy.org/) using the crystal structure of P450Bsβ from Bacillus subtilis (PDB code: 1IZO with the resolution in 2.10 Å) as template. FoldX parameters was temperature 298 K, pH 8, and 0.05 M ionic strength.

### 2.5. Site-specific mutagenesis
The P450$_{B_{5}}$HI gene was inserted into plasmid pET22b(+) between the NdeI and HindIII restriction nuclease sites, with the His-tag encoding sequence at N-terminal. All of the variants were done by Fast Mutagenesis kit.

The variants with single mutation used P450$_{B_{5}}$HI as template, in which F79 and F173 site was dividually replaced with relatively small amino acids, including glycine, valine, alanine, serine, isoleucine, threonine, cysteine, leucine, and proline. Variants with double mutations used BsβHI-F173V variant as template.

### 2.6. Heterologous expression and purification

P450$_{B_{5}}$HI and its variants were transferred to E. coli BL21(DE3) for expression. Recombinant E. coli BL21 (DE3) cells were grown in LB medium (5 g/L yeast extract, 10 g/L tryptone, and 5 g/L NaCl) supplemented with ampicillin (100 µg/ml) at 37 °C until the OD600 reached about 0.6. All of genes were induced by the addition of 0.1 mM of isopropyl-β-d-thiogalactopyranoside (IPTG) at 18°C for 12 h. Then the cells were harvested and ultrasonic broken. Cell-free extractions were used for purification.

Enzymes were purified by His-tag affinity chromatography. Cell-free extractions was loaded onto a Ni-NTA column and equilibrated by Lysis buffer (50 mM of Tris-HCl, 10 mM of imidazole and 300 mM of NaCl, pH 7.8). Then the protein was sequential eluted by Wash buffer (50 mM of Tris-HCl, 30 mM of limidazole and 300 mM of NaCl, pH 7.8) and Elution buffer (50 mM of Tris-HCl, 300 mM of imidazole and 300 mM of NaCl, pH 7.8). Eluates were concentrated with an ultrafiltration (Millipore, Germany). The concentrations of purified proteins were determined by BCA kit (Solarbio, China).

### 2.7. Enzymatic assay

The bio-catalytic system contained 10 µM P450$_{B_{5}}$HI enzyme (or its variants), 500 µM fatty acid substrate (from a 100 mM stock solution in ethanol) and 1 mM H$_2$O$_2$ in a final volume of 1 mL of 100 mM potassium phosphate buffer (pH = 8.0). The reaction was carried out at 30°C for 2 h, then was quenched by additional 50 µL of 6 M HCl. The mixture was extracted by 800 µL hexane. Following extraction, the products were analyzed by gas chromatography-mass spectrometry (GC-MS-QP2020, Shimadzu, Japan) equipped with a Sh-Rxi-5Sil-Ms column (Shimadzu, Japan) using helium as carrier gas. The oven temperature was controlled initially at 50°C for 2 min, then increased at the rate of 10°C min$^{-1}$ to 280°C, and held for 10 min. The injecting temperature was 280°C. The peaks were identified by comparison of retention time and ion spectra with authentic references.

### 3. Results And Discussion

The result and discussion part is divided into three parts. In the first part, the P450$_{B_{5}}$HI access tunnels were analyzed and two amino acids were identified as beneficial key residues. In the second part, the variants based on the two key residues were characterized with lauric acid substrate then 5 best variants were selected. In the third part, the substrate preference of selected P450$_{B_{5}}$HI variants was investigated.
3.1. \( \text{P}450_{\text{Bs}\beta}\text{HI} \) access tunnel analysis and hot spots identification

Access tunnels are responsible for ligand transportation between active site and solvent environment in enzymes with buried active site (Kokkonen et al., 2019). Using MOLEonline serve (L. Pravda et al., 2018), two access tunnels were found in \( \text{P}450_{\text{Bs}\beta}\text{HI} \) (Fig. 1A, 1B and Table 1). The tunnels showed typical cytochrome features. The F and G helix define the most common tunnels among cytochrome P450s (Cojocaru et al., 2007). The peripheral flexible F-G loop can control the tunnel topology and influence substrate recognition. Representative tunnel in \( \text{P}450_{\text{Bs}\beta}\text{HI} \) is Tunnel 1, which goes through the A helix, B’ helix, B-B’ loop B’-C loop, and F-G loop. Tunnel 1 consists of a large number of non-polar residues, which facilitates the access of hydrophobic substrates (Table S1). Tunnel 2 threads through B-B’ loop and B’-C loop, which is also common among P450s. Compared with Tunnel 1, Tunnel 2 shows higher polarity and contains a hydrophilic area near the surface of \( \text{P}450_{\text{Bs}\beta}\text{HI} \) (Fig. 1C and Table 1). In addition to participating in substrate transport, this tunnel may be also involved in controlling water diffusion.

| Tunnel    | Length (Å) | Bottleneck radius (Å) | Polarity* |
|-----------|------------|-----------------------|-----------|
| Tunnel 1  | 29         | 1.4                   | 4.06      |
| Tunnel 2  | 37         | 1.2                   | 8.48      |

* Polarity is calculated as an average of amino acid polarities assigned according to the method of Zimmerman et al. (Zimmerman, Eliezer, & Simha, 1968). Polarity ranges from completely nonpolar amino acids (ALA, GLY = 0.00) through polar residues (e.g. SER = 1.67) towards charged residues (GLU = 49.90, ARG = 52.00).

In order to find potentially important residues, we set four criterions: 1.) Located in the tunnel bottleneck area; 2) being high influential among the entire tunnel; 3) Located in the loop area; 4) being not completely conserved in its homologous cytochromes. Caver Analyst 2.0 software was chosen for analyzing the residues influence, which provide an opportunity to explore the portion of the tunnel influenced by a particular amino acid (Jurcik et al., 2018). We analyzed the tunnel properties and bottleneck residues influence over 5 ns (Fig. 2). The results indicate that residues of F173 and V74 have high impact on Tunnel 1, and H85 and F79 are the main bottleneck residues in access Tunnel 2. Among high impact residues, H85 is the core residues for \( \text{P}450_{\text{Bs}\beta}\text{HI} \) decarboxylation activity (Xu et al., 2017). V74 is located in the middle of B’ helix, while the two phenylalanine, F79 and F173, are non-conservative (Figure S1) and located in the B’-C loop and F-G loop, respectively. Given that, F79 and F173 residues were chosen for further analysis to optimize the enzymatic property while avoid ruining tunnel architecture.
Theoretically, the transport ability of substrates between enzyme active site and solvent environment could be adjusted by gates located at the access tunnels (Gora et al., 2013). Aromatic amino acids in access tunnels bottleneck are often participated in the control of the putative gates (Pavlova et al., 2009). As show in Fig. 2, the huge aromatic side chains of F79 and F173 were involved in the tunnel bottleneck regions. Here, the two phenylalanine residues F79 and F173 are presumed to be the “gatekeeper”, i.e. control the access of substrate from solvent environment to P450 Bsβ HI active site. In addition, the F79 and F173 are also deemed to stabilize the fatty acid substrates via hydrophobic interactions (Lee et al., 2003). Based on the critical location of the two residues, it is assumed the large phenyl side chain of phenylalanine maintains substrate stability, but hinders the entry of long-chain fatty acid substrates. A more flexible substrate entrance may benefit to increase enzyme activity towards long-chain fatty acids substrate. To test the hypothesis, the F79 and F173 residues were replaced by relatively small amino acid including cysteine, isoleucine, leucine, alanine, glycine valine, serine, threonine and proline, in mutagenesis experiments, respectively. The variants stability was evaluated by analysis of the relative free energy of folding (ΔΔ\text{G}_{\text{fold}}) (Cui et al., 2020) and no unstable substitution was found (Figure S2). Variants were heterologously expressed in E. coli BL21(DE3).

### 3.2. Decarboxylation activity of P450_{Bsβ HI} variants

As a hydrogen peroxide-dependent decarboxylase, P450_{Bsβ HI} relies on \text{H}_2\text{O}_2 as an electrons provider and oxygen supplier (Xu et al., 2017). To select promising variants of P450_{Bsβ HI}, we examined the enzyme activity with the substrate of lauric acid using \text{H}_2\text{O}_2 as the sole co-factor. Decarboxylation efficiency was characterized by \(\alpha\)-undecene yield. As shown in Fig. 3, 9 of 18 single-site variants showed improved yield of \(\alpha\)-undecene. Particularly, Bsβ HI-F79A, Bsβ HI-F79S, Bsβ HI-F79T, Bsβ HI-F79V, and Bsβ HI-F173V exhibited more than 1.5 fold increase of \(\alpha\)-undecene yield compared to P450_{Bsβ HI}. The optimal variant Bsβ HI-F79T and Bsβ HI-F79V showed the 3.1-fold improvement of \(\alpha\)-undecene yield. Then we focused on the alterations of identified tunnels in beneficial variants using MOLEonline serve (Table S3). The trends of decreased tunnel length and increased bottleneck radius were observed in the putative substrate tunnels (Tunnel 1) among variants, which is consistent with our previous assumptions. F79A variant showed the shortest substrate tunnel and the widest bottleneck radius, which were 2.8 Å decrease and 0.4 Å improvement, respectively. Interestingly, for both F79 or F173, we observed that the best decarboxylation performances were achieved by the valine substitution. The obvious feature of those two variants is the wider Tunnel 2. It is speculated that Tunnel 2 also undertakes the tunnel transfer task although this tunnel prefer polar water. Compared with Tunnel 1, Tunnel 2 has a longer length and a narrower bottleneck, which indicates that the Tunnel 2 may only serve as an auxiliary transportation for substrate. In short, the results indicated that the replacement of phenylalanine residue in positions 79 and 173 to relatively small amino acids (less bulky) had significant effect on the tunnels property and enzyme activity of P450_{Bsβ HI}.

To further investigate the influence of substitution combination on the catalytic performance of P450_{Bsβ HI}, we recombined the beneficial substitutions. Therefore, a series of double-site variants were constructed, which were Bsβ HI- F79A/F173V, Bsβ HI-F79S/F173V, Bsβ HI-F79T/F173V, and Bsβ HI-
F79V/F173V. However, all of the double-site variants show very low α-undecene production. One possibility is that the fatty acid substrates locate in the binding pocket lost the sufficient stability in the double site variants. The original F79 and F173 residues stabilize the substrate by hydrophobic interaction, so that substrate is structurally complementary to the P450_{ Bsβ HI} active site. Although the lauric acid substrate is more accessible to the binding pocket after double-site variants, the lack of strong hydrophobic interaction with F79 and F173 residues resulted in flexible and instable binding of substrate around heme. Hence, the single-site variants of BsβHI-F79A, BsβHI-F79S, BsβHI-F79T, BsβHI-F79V, and BsβHI-F173V were chosen for further test.

3.3. Investigation of substrate preference in P450_{ Bsβ HI} variants

Four additional fatty acids (capric acid, myristic acid, pentadecanoic acid, and palmitic acid) were investigated to probe the substrate profile of P450_{ Bsβ HI} variants and explain whether the decarboxylation activity of P450_{ Bsβ HI} variants are improved towards other medium- and long-chain fatty acids. In addition, given that the important industrial application of styrene, phenylpropionic acid was also added for testing. As shown in Fig. 4, the decarboxylation activities of BsβHI-F79T, BsβHI-F79V, and BsβHI-F173V are higher than P450_{ Bsβ HI} with the capric acid as substrate. While toward long-chain fatty acids substrate, as a general trend, all candidates of P450_{ Bsβ HI} variants exhibit higher catalytic efficiency than P450_{ Bsβ HI}. Intriguingly, optimal decarboxylation efficiency towards different substrates is exist in different variants. BsβHI-F173V possesses the highest activity towards myristic acid and pentadecanoic acid, while BsβHI-F79A is the best to palmitic acid. Both the two variants possessed broader access tunnels than the tunnels in P450_{ Bsβ HI} (Table S3). We reasoned that this observation may stem from the compatibility between the substrate and access tunnels. Alanine has the smallest side chain, so it provided the F79A variant the widest substrate tunnel, which is able to accommodate relatively larger fatty acid substrate, like palmitic acid. Meanwhile, hydrophobic interaction provided by nonpolar alanine or valine also reduces substrate flexibility to fatty acids substrate, leading to higher stability of enzyme-substrate complex during the reaction cycle. In addition, only trace styrene was detected when used the phenylpropionic acid as substrate, probably due to the strong preference of P450_{ Bsβ HI} towards fatty acid substrates (Xu et al., 2017).

4. Conclusions

Overall, an access tunnel engineering was carried out to understand the substrate preference as well as for improving the decarboxylation activity and of P450_{ Bsβ HI}. The structure indicates that two residues (F79 and F173) locate in the bottleneck of tunnels. A series of variants of P450_{ Bsβ HI} were generated and investigated. Significantly improved decarboxylation activity was observed in BsβHI-F79A and BsβHI-F173V variants towards long-chain fatty acids. The results reveal that the appropriate reduction of the amino acid size at the gate of tunnels improves the enzymatic activity towards larger substrates, like long-chain fatty acids. Furthermore, our study shows that identifying and engineering key residues lining
the access tunnels may be a valuable and efficiency strategy for improving the performance of enzymes with buried active sites.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this article and the supplementary information file.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors’ contributions

SQ-M performed the experiments and wrote this paper; RP-A and Prof. KL-N contributed to the calculation part; ZY-L analyzed the data; Prof. U-S, Dr. Y-J, Dr. MD-D helped to improve the paper; Prof. F-W, and Prof. M-Q and Dr. M-W were involved project administration, Prof. L-L designed the project and wrote the draft. All authors read and approved the final manuscript.

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Figures
Figure 1

The overall structure and substrate access tunnels information of P450BsβI. The structure model of P450BsβI was constructed by PyMoL based on the crystal structure of P450Bsβ (PDB: 1IZO). The access tunnel identification and hydrophobicity analysis were carried out with MOLEonline (Pravda, L, 2018). A and B: the structure of the P450BsβI enzyme. Access tunnel 1 (T1) and 2 (T2) were colored in yellow and green, respectively. The B-B' loop, B'-C loop and F-G loop were colored in orange, purple and cyan, respectively. C. The hydrophobicity property of identified tunnels. Hydrophobicity index accord to Hilda Cid et.al from the charged residues (Glu -1.14) to nonpolar residues (Ile 1.81) (Cid, Bunster, Canales, & Gazitúa, 1992).
Figure 2

Evolution of residues lining the access tunnels of P450BsβHI. The access tunnel analysis was carried out with Caver Analyst 2.0 BETA. The left part is bottleneck residues lining of tunnel 1 and 2. The upper right part shows graph representation plots the tunnel width along its centerline. Each line represents the tunnel in one timestep. The lower right part describes the list of all residues contributing to the tunnel. The horizontal position of the color in the line shows which part of the tunnel this residue influences. All tunnel structure information was from the 5 ns snapshot of MD simulation.
Figure 3

Evolution of the activity of P450BsβHI variants towards the decarboxylation of lauric acid. The α-undecene products were qualitatively determined by contrasting with standard sample, and quantified by external standard method. Results shown are mean ± SD of duplicated experiments.
Figure 4

Decarboxylation performance of P450BsβHI and its variants towards various organic acid. Capric acid, myristic acid, pentadecanoic acid, palmitic acid, and phenylpropionic acid were used as substrate. All of the α-alkene products were qualitatively determined by contrasting with standard sample, and quantified by external standard method. Results shown are mean ± SD of duplicated experiments.

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