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Characterization of two styrene monooxygenases from marine microbes

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ABSTRACT

Styrene monooxygenases (SMOs) are highly stereoselective enzymes that catalyze the formation of chiral epoxides as versatile building blocks. To expand the enzyme toolbox, two bacterial SMOs were identified from the genome of marine microbes *Paraglaciecola agarilytica* NO2 and *Marinobacterium litorale* DSM 23545, and heterologously expressed in *Escherichia coli* in soluble form. Both of the resulting whole-cell biocatalysts exhibited maximal activity at 30 °C and pH 8.0. They catalyzed the sulfoxidation reactions, and the epoxidation of both conjugated and unconjugated styrene derivatives with up to > 99%ee. *Mls*SMO displayed higher activity toward most substrates tested. Compared to an established SMO from *Pseudomonas* species (*Ps*SMO), *Mls*SMO achieved 3.0-, 3.4- and 2.6-fold conversions for substrates styrene, cinnamyl alcohol and 4-vinyl-2, 3-dihydrobenzofuran, respectively.

1. Introduction

Enantiopure epoxides bear a highly reactive oxirane function that can be opened by various nucleophiles or undergo elimination, reduction or rearrangements to a multitude of chiral intermediates, which makes them extremely versatile building blocks in organic synthesis and pharmaceutical industry [1–4]. Although chemo-catalyzed asymmetric epoxidations have been extensively developed in the last few decades, biocatalytic epoxidation remains a strong candidate for preparing enantiopure epoxides in an eco-friendly fashion [5–9]. Particularly, classical chemical methods often lack high stereoselectivity for nonfunctional terminal alkenes, such as styrene, while biocatalytic epoxidation can provide the corresponding epoxides with excellent enantiomeric excess.

Styrene monooxygenase (SMO) is a flavin-dependent monooxygenase that catalyzes the epoxidation of styrene to (*S*)-styrene oxide in the upper catabolic pathway of styrene degradation [10,11]. SMOs adopt oxygen as the oxidant, and often display absolute stereoselectivity to afford the (*S*)-styrene oxide with > 99%ee under mild reaction conditions. Their potential as a tool in organic synthesis has been demonstrated by the pilot-scale production of (*S*)-styrene oxide [12], the expanded substrate scope that includes a variety of conjugated and unconjugated styrene derivatives, heterocyclic analogues and aliphatic alkenes [13–16], and a series of cascade reactions based on the asymmetric epoxidation coupled with other enzymatic transformations [17–20].

Most SMOs reported so far are two component flavin-dependent monooxygenase composed of a FAD-dependent styrene epoxidase (StyA) and a NADH dependent flavin reductase (StyB), encoded by *styA* and *styB* genes [10]. They all originate from *Pseudomonas* [21–26] or *Rhodococcus* species [27,28] except one from metagenome [29]. A self-sufficient one-component SMO has been reported as well, which was isolated from *Rhodococcus opacus* 1CP [30]. In general, database analysis has revealed that SMOs represent only a small proportion of flavoprotein monooxygenases [29,31], and up to now the efforts devoted to the searching for new SMOs remain limited, which has hindered the extensive application of such a group enzymes of superior stereoselectivity and unique substrate preference that complements to classic chemical epoxidations.

The marine microbes are known to play many important roles in the ecosystem, and also have provided abundant resources for bioactive small molecules and proteins [32–34]. However, to the best of our knowledge, no SMO from such a source has been investigated. Here we report the functional characterization of two SMOs from marine microbes. The whole-cell systems expressing those two SMOs exhibited maximal activity on pH 8.0. They displayed similar substrate preference as the SMO from *Pseudomonas* species (*Ps*SMO), but one of them could yield significantly increased conversion toward styrene and derivatives with excellent stereoselectivity.

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2. Materials and methods

2.1. Chemicals

The substrates styrene (**1a**), cinnamyl alcohol (**2a**), thioanisole (**5a**), 4-vinyl-2, 3-dihydrobenzofuran (**6a**) were purchased from Alfa-Aesar (Tianjin, China) or Acros Organics (Geel, Belgium). The substrate 1-phenylprop-2-en-1-ol (**3a**) and 2-allylphenol (**4a**) were prepared from the corresponding aldehydes and Grignard reagent [14,35]. Racemic and (*S*)-styrene epoxides were from Sigma-Aldrich (St. Louis, MO, USA). Other racemic epoxides were prepared from the corresponding substrates using meta-chloroperoxybenzoic acid as the oxidant [36]. Methyl phenyl sulfone was purchased from Energy Chemical (Shanghai, China). Oligonucleotides were synthesized by Invitrogen (Shanghai, China). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA). Kanamycin and isopropyl-β-D-thiogalactoside (IPTG) were purchased from Amresco (Solon, OH, USA).

2.2. Construction of expression vectors

The DNA fragment encoding *Ps*SMO (GenBank accession no. GU593979.1) was previously isolated from *Pseudomonas* sp. LQ26, and annotated as *styAB2* [26]. It was constructed into pET28a (+) vector (Novagen, Madison, WI) for expression [26,37]. The amino acid sequence and the native DNA sequence of *Pa*SMO and *Ml*SMO were obtained from NCBI database (GenBank accession no.: NZ_BAEK01000054.1 and NZ_AUAZ01000037.1, respectively) using a genome mining approach that adopted the amino acid sequence of StyA from *Pseudomonas* sp. LQ26 as the reference sequence. The source strains were *Paraglaciecola agarilytica* NO2 (previously *Glaciecola agarilytica*) [38] and *Marinobacterium litorale* DSM 23545, respectively. Both DNA sequences were optimized for *E. coli* BL21 (DE3) expression using Optimum-Gene technology and synthesized by GenScript Biotech Co. (Nanjing, China). The DNA fragments were each digested with *Bam*H I and *Sac* I, and ligated into pET28a (+). The constructed plasmids were verified by DNA sequencing at Invitrogen Biotechnologies (Shanghai, China), and designated as pET-*Pa*SMO and pET-*Ml*SMO, respectively. The resulting plasmids were each introduced into *E. coli* BL21 (DE3) for expression.

2.3. Expression of SMOs in E. coli

Single colonies were grown overnight at 37 °C in Luria-Bertani (LB) media containing kanamycin (50 μg/mL) and D-glucose (5 g/L). Two milliliter of the overnight culture was then inoculated into 200 mL of Terrific Broth (TB medium) containing kanamycin (50 μg/mL) and D-glucose (5 g/L) in a 500 mL flask. The cultures were incubated at 37 °C for 3 h before the addition of 0.1 mM IPTG, and the incubation was continued at 20 °C for another 20 h with gyratory shaking at 200 rpm on an INFORS Multitron shaker. The cells were harvested by centrifugation, washed twice with potassium phosphate buffer (0.1 M, pH 7.0) and stored at 4 °C. Bacterial soluble fractions were prepared by high pressure homogenizer (ATS-AH100B, ATS Engineering Inc., Canada) followed by centrifugation at 4 °C, and the crude cell extracts were analyzed using SDS-PAGE.

2.4. Whole-cell biotransformation and product analysis

Harvested cells with a dry cell weight (DCW) of 0.2 g were re-suspended in 5 mL potassium phosphate buffer (pH 7.5, 100 mM) containing 5 mg substrate, and incubated at 30 °C for 1 h with gyratory shaking at 200 rpm. For the substrate styrene, a biphasic system [23] containing 10% (v/v) cyclohexane was applied instead. All the reactions were carried out in duplicate. The reactions were terminated by extraction with an equal volume of ethyl acetate for twice. The combined organic phases were dried with anhydrous sodium sulfate, concentrated under vacuum, and subjected to GC or HPLC analysis.

To determine the temperature optimum, the reactions were performed in 5 mL potassium phosphate buffer (pH 6.5, 100 mM) containing 10% (v/v) cyclohexane and 5 mg styrene for 4 h at temperatures varying from 20 to 45 °C. To determine the pH optimum, the reactions were performed in 5 mL potassium phosphate buffer (100 mM) or Tris-HCl buffer (50 mM) containing 10% (v/v) cyclohexane and 7.5 mg styrene for 2 h at 30 °C with pH values ranging from 5.5 to 9.0.

Enantiomeric excesses or conversions were determined using chiral HPLC on a Shimadzu LC 20-AD (Shimadzu, Japan) connected to a photodiode array detector at 30 °C using a Daicel Chiralpak IC column (for **1a**, *n*-hexane/2-propanol 98:2, 0.6 mL/min, $t_R(R)$ 10.2 min, $t_R(S)$ 11.5 min; for **2a**, *n*-hexane/2-propanol 95:5, 0.5 mL/min, $t_R(R)$ 49.5 min, $t_R(S)$ 41.8 min; for **6a**, *n*-hexane/2-propanol 90:10, 0.5 mL/min, $t_R(R)$ 13.5 min, $t_R(S)$ 20.1 min), or Chiralpak AS-H column (for **3a**, *n*-hexane/2-propanol 90:10, 0.5 mL/min, t_{R1} 21.3 min, t_{R2} 25.3 min, t_{R3} 26.8 min, t_{R4} 30.3 min; for **4a**, *n*-hexane/2-propanol 90:10, 0.5 mL/min, $t_R(S)$ 17.4 min, $t_R(R)$ 19.0 min), or Chiralpak OD-H column (for **5a**, *n*-hexane/2-propanol 97:3, 0.5 mL/min, $t_R(R)$ 26.6 min, $t_R(S)$ 34.8 min).

3. Results and discussion

3.1. Sequence analysis of SMOs

The two subunits of both *Ml*SMO and *Pa*SMO are hypothesized monooxygenases (StyA) and flavin reductases (StyB) revealed by genome sequencing projects. The sequence of *Ml*SMO is mined from the genome of *Marinobacterium litorale* DSM 23545, a mesophile bacteria isolated from surface seawater, and that of *Pa*SMO is from the genome of *Paraglaciecola agarilytica* NO2, an agar-digesting marine bacterium isolated from marine sediment [39]. As far as we know, neither strain has been reported to degrade styrene or related compounds.

StyAs of *Ml*SMO and *Pa*SMO showed 65.8% identity to each other, and had similar identities to other two-component SMOs from *Pseudomonas* sp. LQ26 (69.2% and 65.5%, respectively) [26], *Rhodococcus* sp. ST-10 (57.9% and 55.6%, respectively) [27], or metagenome (50.0% and 48.8%, respectively) [29]. StyBs of *Ml*SMO and *Pa*SMO showed 43.3% identity to each other, and had similar identities to those of *Pseudomonas* sp. LQ26 (60.8% and 44.7%, respectively) [26] and *Rhodococcus* sp. ST-10 (41.6% and 33.2%, respectively) [27].

The amino acid sequences of *Ml*SMO and *Pa*SMO SMOs were aligned with other SMOs of known function, which showed the presence of conserved sequence motifs typical for a mechanistic involvement of flavins and nicotinamide adenine dinucleotides (Fig. 1a). Both SMOs have the functionally conserved residue Thr47 that is important to catalytic activity in StyA [40,41]. The FAD-binding fingerprints GXGXXG, GG and DX6G were found in both *Ml*SMO and *Pa*SMO (Fig. 1a). The GXGXXG sequence (residues 9–14) is a fingerprint sequence among FAD- and NAD(P)H-dependent oxidoreductases and play an important role in FAD-binding proteins in general [42–44]. The second FAD binding motif contains the GDX6P sequence (*Ml*SMO residues 294–302; *Pa*SMO residues 292–300), where the highly conserved Asp residue connects with the O3′ of the ribose moiety of FAD [45]. The DG sequence is highly conserved among all flavoprotein hydroxylases [43], but only the Gly is conserved in StyA (*Ml*SMO residue 171; *Pa*SMO residue 169).

3.2. Heterologous expression

The heterologous expression of both *Pa*SMO and *Ml*SMO were performed at 20 °C to achieve soluble proteins in *E. coli* BL21 (DE3) cells. SDS-PAGE analysis indicated that both SMOs showed a major band at around 45 kDa, which was in accordance with the size of the open reading frame of StyA (Fig. 2).

StyB was estimated to be around 19 kDa. Its over-expression was not