Journal of Molecular Catalysis B: Enzymatic 67 (2010) 236–241

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Styrene monooxygenase from *Pseudomonas* sp. LQ26 catalyzes the asymmetric epoxidation of both conjugated and unconjugated alkenes

Hui Lin[a,b,1], Jing Qiao[a,b,1], Yan Liu[a], Zhong-Liu Wu[a,*]

[a] Chengdu Institute of Biology, Chinese Academy of Sciences, P.O. Box 416, Chengdu 610041, China
[b] Graduate University of Chinese Academy of Sciences, Beijing 100039, China

ARTICLE INFO

Article history:
Received 25 May 2010
Received in revised form 24 August 2010
Accepted 24 August 2010
Available online 18 September 2010

Keywords:
Epoxidation
Pseudomonas
Styrene monooxygenase
Unconjugated alkene
Biocatalysis

ABSTRACT

A novel styrene monooxygenase (SMO) was isolated from *Pseudomonas* sp. LQ26, a styrene degrader from activated sludge. Sequence alignment demonstrated that it was the most distant member of all SMOs originating from the genus of *Pseudomonas*. The substrate spectrum of this enzyme extended beyond typical SMO substrates to 1-allylbenzene analogues, previously reported as non-substrates for the SMO from *Pseudomonas fluorescens* ST. The results demonstrate for the first time the asymmetric epoxidation of both conjugated and unconjugated alkenes catalyzed by SMO and suggest that a much broader substrate spectrum is expected for SMOs.

1. Introduction

As a widely-used starting material for synthetic polymers, styrene is present in many industrial and domestic effluents. Over the last few decades, a large number of styrene-degrading microorganisms have been isolated [1,2]. The majority of reports indicate that the predominant catabolic pathway for styrene degradation is through the oxidation of vinyl side chain catalyzed by styrene monooxygenase (SMO). It is a two-component monooxygenase encoded by *styA* and *styB*, consisting of an oxygenase (StyA) and a NADH-flavin oxidoreductase (StyB) [3].

SMOs display excellent enantiomeric selectivity in styrene epoxidation, which has attracted many efforts to the synthesis of chiral epoxides [4–9], a group of extremely important building blocks in organic synthesis. SMOs from *Pseudomonas fluorescens* ST [10,11] and *Pseudomonas* sp. VLB120 [6], have been used to convert conjugated styrene derivatives as well as aromatic sulfides. A recent isolated SmoA from metagenome [12] and a self-sufficient SMO from *Rhodococcus opacus* [13] display similar substrate spectrum. Only one report has described the attempt to apply SMO in the epoxidation of unconjugated alkenes and the results suggest that 1-allylbenzene does not serve as a substrate [11].

Here, we present a novel SMO (designated as StyAB2) that can perform the asymmetric epoxidation of 1-allylbenzene and other unconjugated styrenes in addition to conventional conjugated styrene derivatives. StyAB2 was isolated from *Pseudomonas* sp. LQ26, a styrene degrader from activated sludge. Compared with all reported StyAB sequences from the genus of *Pseudomonas* at DNA level, SMO from *Pseudomonas* sp. LQ26 shows a maximum identity of 79%.

2. Materials and methods

2.1. Chemicals

Styrene, 4-bromostyrene, 3-chlorostyrene, 1,2-dihydronaphthalene, 2-vinylpyridine, 1*H*-indene and 1-allylbenzene were purchased from Alfa-Aesar (Tianjin, China) or Acros Organics (Geel, Belgium). 1-(2-Methylallyl)benzene, 1-allyl-2-hydroxybenzene, 1-allyl-3-methylbenzene and 1-allyl-4-methoxybenzene were synthesized using Wittig reaction [14]. Racemic and (*S*)-styrene epoxides were from Sigma–Aldrich (St. Louis, MO, USA). Other racemic mixtures of epoxides were synthesized according to the literatures [15,16].

2.2. Isolation of styAB2 from Pseudomonas sp. LQ26

The styrene degrading strain *Pseudomonas* sp. LQ26 was isolated from activated sludge collected from a municipal sewage treatment plant in Chengdu, China, using a modified indigo for-

* Corresponding author at: Chengdu Institute of Biology, Chinese Academy of Sciences, 9 South Renmin Road, 4th Section, Chengdu, Sichuan 610041, China. Tel.: +86 28 85238385; fax: +86 28 85238385.
E-mail address: wuzhl@cib.ac.cn (Z.-L. Wu).
[1] These authors contributed equally to the present work.

1381-1177/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2010.08.012

mation assay [17] by conventional enrichment approach. The strain was deposited at China Center for Type Culture Collection (Wuhan, China) under the acquisition number of CCTCC M 2010188. Partial segment of 16 s rDNA was amplified by PCR with two universal primers 5′-AGAGTTTGATCCTGGCTCAG-3′ (27f) and 5′-GGTTACCTTGTTACGACTT-3′ (1492r), cloned into pMD18-T vector (Takara, Dalian, China) and sequenced. The sequence has been deposited at GenBank under accession no. GU731675. Gene *styAB2* was amplified with primers 5′-ATGAAAAAGCGTATCGGTATTG-3′ and 5′-TTCGAAGGCATGAAGCATG-3′, designed according to the reported consensus sequence for *Pseudomonas* species and deposited at GenBank under accession no. GU593979.

2.3. Expression of StyAB2, StyA and StyB in E. coli

The DNA fragments encoding either *styAB2*, *styA* or *styB* were amplified with primers 5′-GAGGAGGTCATATGAAAAAGCGTATC-GGTATTGTTG-3′ (AB1F) and 5′-TGACAAGCTTTTAATTCAGGGGCA-GCGGATTG-3′ (AB2R), AB1F and 5′-TGACAAGCTTCAGGCTGCAA-TGGTCGGC-3′, 5′-GAGGAGGTCATATGACGCTAAAGACAGATGCGG-3′ and AB2R, respectively. They were each digested with *Nde* I and *Hind* III (New England Biolabs, Beverly, MA, USA), and inserted into pET-28a(+) vector (Novagen, Madison, WI). The resulting expression vectors, pETAB, pETA or pETB were transformed into *E. coli* BL21(DE3). Single colonies were grown overnight at 37 °C in LB media containing 50 μg kanamycin/ml. Two milliliter of overnight culture was then inoculated into 200 ml of Terrific Broth (TB medium) containing 50 μg kanamycin/ml in a 500 ml flask. For the expression of StyAB2, the cultures were incubated at 37 °C for 3 h before induction and the incubation continued at 20 °C for another 18 h with gyratory shaking at 220 rpm. For the expression of StyA or StyB, the induction was initiated with the addition of 0.05 mM IPTG at 20 °C. The incubation continued at 20 °C for another 24 h.

2.4. Purification of recombinant StyA and StyB

The entire procedure was performed at 4 °C. To prepare the soluble cell extract, 3 g recombinant *E. coli* cells (wet weight) treated with lysozyme were lysed by sonication in buffer A, which consisted of 0.1 M potassium phosphate (pH 7.0), 20% glycerol (v/v), 1 mM phenylmethyl sulfonylfluoride (PMSF), 0.5 M potassium chloride, 0.1 mM dithiothreitol and 5 mM imidazole. Insoluble cell debris was removed by centrifugation. The resulting supernatant was loaded onto a Ni^{2+}-NTA agarose column (Bio-Rad), which has been equilibrated with buffer A at a flow rate of 1 ml/min. The recombinant protein StyA or StyB was eluted at a flow rate of 1 ml/min with buffer A containing 250 mM imidazole (no PMSF). Fractions containing StyA or StyB were verified by SDS-PAGE, pooled, dialyzed and used freshly.

2.5. Determination of StyA activity in reconstituted system

The activity of StyA was determined by measuring the formation of (*S*)-styrene oxide by HPLC. The reaction mixture contained 8 μM of purified StyA, 20 μM of purified StyB, 20 μM formate dehydrogenase (from *Pichia pastoris* KM71), 0.16 M sodium formate, 0.2 mM NADH, 0.8 mM NAD^+, 0.03 mM FAD, and varying concentrations of styrene (from a 50-fold stock in DMSO), in 100 mM potassium phosphate buffer (pH 6.5). Reaction mixtures were incubated at 30 °C on a shaker at 280 rpm for 1 h. The organic phase was analyzed by reverse-phase HPLC on a Luna C18 (4.6 mm × 150 mm) column at a flow rate of 1 ml/min. The mobile phase consisted of a methanol–water mixture at a ratio of 80:20. Estimates of k_{cat} and K_m were obtained by non-linear regression with GradPad Prism program (Graphpad, San Diego, CA, USA).

2.6. Whole-cell bioconversion and product analysis

Recombinant *E. coli* BL21 cells with wet weight of 0.5–2.0 g were resuspended in 10 ml 100 mM potassium phosphate buffer (pH 6.5) containing 20% (v/v) bis-(2-ethylhexyl) phthalate (BEHP) and 10 mg substrate. The reaction was carried out at 30 °C for 24 h with gyratory shaking at 220 rpm and terminated by extraction with ether. The combined organic extracts were dried with anhydrous sodium sulfate, concentrated under reduced pressure and subjected to high performance liquid chromatography (HPLC) analysis performed on a Shimadzu Prominence LC-20AD system connected to a PDA-detector. Chemical yields were analyzed by reverse-phase HPLC on a Luna C18 column (4.6 mm × 150 mm, Phenomenex, Torrance, CA, USA). Optical purities were determined by chiral HPLC using Daicel Chiralcel OD-H (compound **4**) and OJ-H (compound **3**), or Chiralpak AD-H (compounds **1** and **2**) and AS-H (compounds **5**–**11**) columns.

4-Bromostyrene was used as the substrate to study the effects of reaction temperature and pH. Eighteen milligram of 4-bromostyrene was used as the substrate in 20 ml 100 mM potassium phosphate buffer containing 10% (v/v) cyclohexane with of 0.5 g wet weight of recombinant *E. coli* BL21 cells. The reactions were performed in duplicate assays at 30 °C with varied pH values for 18 h or at pH 6.5 with varied temperatures for 24 h. Substrate conversion was measured by reverse-phase HPLC on a Luna C18 column. The reactions were performed at 30 °C in potassium phosphate buffer with pH values ranging from 6.0 to 8.0, or in potassium phosphate buffer with a pH value of 6.5 at temperatures varying from 25 to 45 °C.

4-Chloroindole was used as the substrate to investigate the functional expression of SMO. The pelleted cells from 1 ml culture were resuspended in 1 ml 100 mM potassium phosphate buffer (pH 6.5) containing 0.5 mM 4-chloroindole in a 24-well plate and incubated at 37 °C with shaking at 220 rpm for 10 or 20 min. The formation of colored product was measured by determining the absorbance at 620 nm and the estimated concentrations were calculated based on the extinction coefficient of 4,4′-dichloroindigo [18]. 1U was defined as the activity which converts 1 μmol of 4-chloroindole per minute. The OD600 of one was corresponding to a dry cell weight (DCW) of 2.97 mg/ml.

2.7. Spectral data for epoxidation products

(*S*)-Styrene oxide (**1**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.27–7.35 (m, 5H, Ar–H), 3.85 (m, 1H, CH), 3.13 (m, 1H, CH_2), 2.79 (m, 1H, CH_2). $[\alpha]_D^{25}$ = +32.1 (*c* 1.02, $CHCl_3$) {lit. [19] $[\alpha]_D^{25}$ = +21.1 (83 mM, $CHCl_3$) for 99% ee, (*S*)}; >99% ee; retention times: t_R (*R*) 10.27 min, t_R (*S*) 10.67 min.

(*S*)-2-(4-bromophenyl)oxirane (**2**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.47 (d, 2H, Ar–H, *J* = 8.22 Hz), 7.15 (d, 2H, Ar–H, *J* = 8.22 Hz), 3.82 (m, 1H, CH), 3.14 (t, 1H, CH_2, *J* = 4.8 Hz), 2.74 (dd, 1H, CH_2, *J* = 2.4 Hz, *J* = 5.4 Hz). $[\alpha]_D^{25}$ = +14.8 (*c* 0.98, $CHCl_3$) {lit. [20] $[\alpha]_D^{24}$ = +20.5 (*c* 1.27, $CHCl_3$) for 99% ee, (*S*)}; >99% ee; retention times: t_R (*R*) 11.06 min, t_R (*S*) 11.64 min.

(*S*)-2-(3-chlorophenyl)oxirane (**3**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.09–7.19 (m, 4H, Ar–H), 3.75 (m, 1H, CH), 3.05 (m, 1H, CH_2), 2.67 (m, 1H, CH_2). $[\alpha]_D^{25}$ = +52 (*c* 0.52, $CHCl_3$) {lit. [19] $[\alpha]_D^{25}$ = +10.8 (65 mM, $CHCl_3$) for 99% ee, (*S*)}; >99% ee; retention times: t_R (*S*) 11.46 min, t_R (*R*) 11.70 min.

(*S*)-2-(oxiran-2-yl)pyridine (**4**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.57 (d, 1H, Ar–H, *J* = 4.86 Hz), 7.66–7.69 (m, 1H, Ar–H), 7.21–7.24 (m, 2H, Ar–H), 4.01 (dd, 1H, CH, *J* = 2.34 Hz, *J* = 4.26 Hz), 3.18 (dd, 1H, CH_2, *J* = 4.26 Hz, *J* = 5.88 Hz), 2.93 (dd, 1H, CH_2, *J* = 2.34 Hz, *J* = 5.88 Hz). $[\alpha]_D^{25}$ = +12 (*c* 0.2, $CHCl_3$) {lit. [21] $[\alpha]_D^{19}$ = +14 (*c* 0.56, $CHCl_3$) for 99% ee, (*S*)}; >99% ee; retention times: t_R (*R*) 15.05 min, t_R (*S*) 15.83 min.

(1*S*, 2*R*)-1,2-dihydronaphthalene oxide (**5**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.09–7.41 (m, 4H, Ar–H), 3.85 (m, 1H, CH), 3.73 (m, 1H, CH), 2.76–2.82 (m, 1H, CH_2), 2.53–2.57 (m, 1H, CH_2), 2.40–2.43 (m, 1H, CH_2), 1.74–1.80 (m, 1H, CH_2). $[\alpha]_D^{25} = -39$ (*c* 0.21, $CHCl_3$) {lit. [19] $[\alpha]_D^{20} = -134.5$ (68 mM, $CHCl_3$) for 99% ee, (1*S*, 2*R*)}; >99% ee; retention times: t_R (1*R*, 2*S*) 11.37 min, t_R (1*S*, 2*R*) 12.87 min.

(1*S*, 2*R*)-indene oxide (**6**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.20–7.52 (m, 4H, Ar–H), 4.28 (m, 1H, CH), 4.15 (m, 1H, CH), 3.24 (m, 1H, CH_2), 3.01 (m, 1H, CH_2). >99% ee; retention times: t_R (1*R*, 2*S*) 12.58 min, t_R (1*S*, 2*R*) 13.95 min.

(*S*)-2-benzyloxirane (**7**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.22–7.32 (m, 5H, Ar–H), 3.13–3.15 (m, 1H, CH), 2.91 (dd, 1H, CH_2, J = 5.64, J = 14.52), 2.91 (dd, 1H, CH_2, J = 5.22, J = 14.52), 2.81 (dd, 1H, CH_2, J = 4.44, J = 8.94), 2.91 (dd, 1H, CH_2, J = 5.64, J = 14.52). 36% ee; retention times: t_R (*R*) 6.12 min, t_R (*S*) 6.54 min.

(*S*)-2-benzyl-2-methyloxirane (**8**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.21–7.31 (m, 5H, Ar–H), 2.89 (d, 1H, CH_2, J = 14.16), 2.82 (d, 1H, CH_2, J = 14.16), 2.65 (d, 1H, CH_2, J = 4.98), 2.61 (d, 1H, CH_2, J = 4.98), 1.28 (s, 3H, CH_3). 13% ee; retention times: t_R (*R*) 9.41 min, t_R (*S*) 10.36 min.

(*S*)-2-(4-methoxybenzyl)oxirane (**9**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.16 (d, 2H, Ar–H, J = 8.58), δ 6.85 (d, 2H, Ar–H, J = 8.58), 3.79 (s, 3H, CH_3), 3.09–3.12 (m, 1H, CH), 2.86 (dd, 1H, CH_2, J = 5.52, J = 14.52), 2.74–2.78 (m, 2H, CH_2), 2.81 (dd, 1H, CH_2, J = 2.64, J = 4.94). 86% ee; retention times: t_R (*R*) 9.56 min, t_R (*S*) 9.90 min.

(*S*)-2-(oxiran-2-ylmethyl)phenol (**10**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.10–7.17 (m, 2H, Ar–H), 6.78–6.86 (m, 2H, Ar–H), 4.88–4.93 (m, 1H, CH), 3.85 (dd, 1H, CH_2, J = 3.36 Hz, J = 12.18 Hz), 3.75 (dd, 1H, CH_2, J = 6.36 Hz, J = 12.18 Hz), 3.25 (dd, 1H, CH_2, J = 9.54 Hz, J = 15.54 Hz), J = 12.18 Hz, 3.02 (dd, 1H, CH_2, J = 7.5 Hz, J = 15.54 Hz). 80% ee; retention times: t_R (*R*) 11.43 min, t_R (*S*) 10.47 min.

(*S*)-2-(3-methylbenzyl) oxirane (**11**): ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.19–7.21 (m, 1H, Ar–H), 7.04–7.06 (m, 3H, Ar–H), 3.12–3.15 (m, 1H, CH), 2.88 (dd, 1H, CH_2, J = 5.76 Hz, J = 14.46 Hz), 2.75–2.80 (m 2H, CH_2), 2.55 (dd, 1H, CH_2, J = 2.7 Hz, J = 5.04 Hz), 2.34 (s, 3H, CH_3). $[\alpha]_D^{25} = +18$ (*c* 0.05, $CHCl_3$); 29% ee; retention times: t_R (*R*) 5.6 min, t_R (*S*) 6.1 min.

3. Results and discussion

3.1. Isolation of styAB2 from Pseudomonas sp. LQ26

It is well-known that most of the experimentally characterized SMOs are from the genus of *Pseudomonas*. All the SMOs are two-component monooxygenases consisting of a FAD-dependent hydroxylase and a NADH-flavin oxidoreductase encoded by *styA* and *styB*. They are highly conserved with an overall identity of >94% for *styAB* at DNA level. Accordingly, we amplified the putative *styAB2* gene in *Pseudomonas* sp. LQ26 simply using primers complementary to the consensus sequence. The isolated *styAB2* gene showed a maximum of 79% sequence identity with other *styAB*s. It appeared as the most distant member of all SMOs originating from the genus of *Pseudomonas*, as shown in a rooted phylogenetic tree with all identified homological *styAB*s in *Pseudomonas* species aligned using the ClustalW method of DNAStar (Fig. 1).

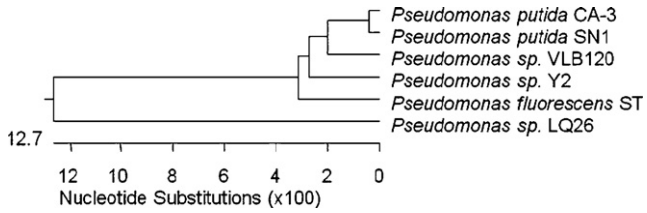

Fig. 1. Phylogenetic relationship of DNA sequences between *styAB2* from *Pseudomonas* sp. LQ26 and from other *Pseudomonas* strains retrieved from databases. Phylogenetic tree was generated with ClustalW method.

3.2. Overexpression of StyAB2 in recombinant E. coli

Functional expression of StyAB2 was achieved in the Novagen pET system and verified by both SDS-PAGE and whole-cell activity towards 4-chloroindole based on the known indigo-forming capacity of SMOs [12,22]. The substrate had been used in our previous work in the directed evolution of cytochrome P450 2A6, and proved a better substrate than indole for library screening because of enhanced color development [17]. Varied expression temperature (Fig. 2) and IPTG concentration were attempted. When 1 mM IPTG was used, expressions at 20 and 15 °C yielded the best results regarding both activity and specific activity per unit of dry cell weight. Enzymatic activity reached a maximum of ~5 U/mg DCW at around 21 h (Fig. 2). Lower incubation temperature (10 °C) significantly affected cell growth.

The functional expression of StyAB2 was further enhanced by reducing IPTG concentration to zero. The addition of IPTG had little effect on cell growth, but appeared unfavorable to StyAB2 expression. The maximum whole-cell specific activity was ~8 U/mg DCW in the absence of IPTG. Lower activities of 5–6 U/mg DCW was observed for cells induced with IPTG. The unintended induction of protein expression was most probably due to the leaky nature of the system, which has been reported previously [23].

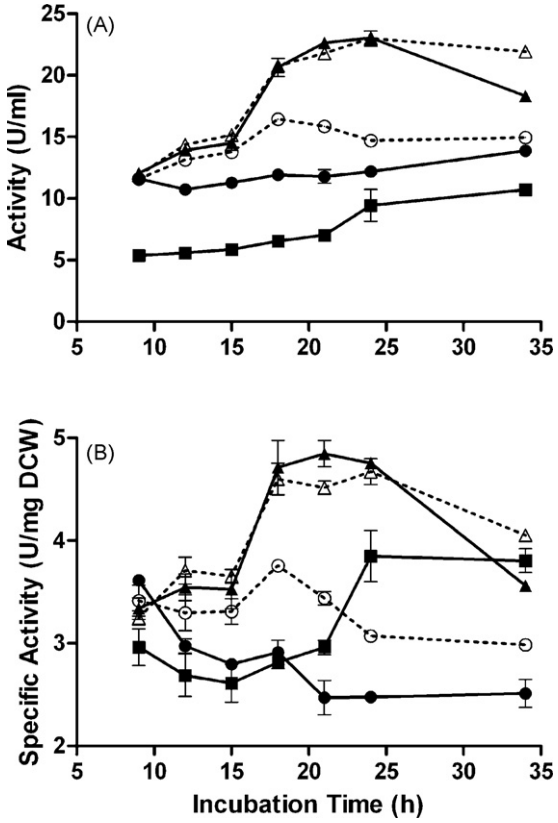

Fig. 2. Effect of temperature on the functional expression of StyAB2. Total activity (A) and specific activity (B) were measured in triplicate using whole *E. coli* cells expressing StyAB2. The expression was carried out at 10 °C (■), 15 °C (△), 20 °C (▲), 25 °C (○) and 30 °C (●) with the induction of 1 mM IPTG.

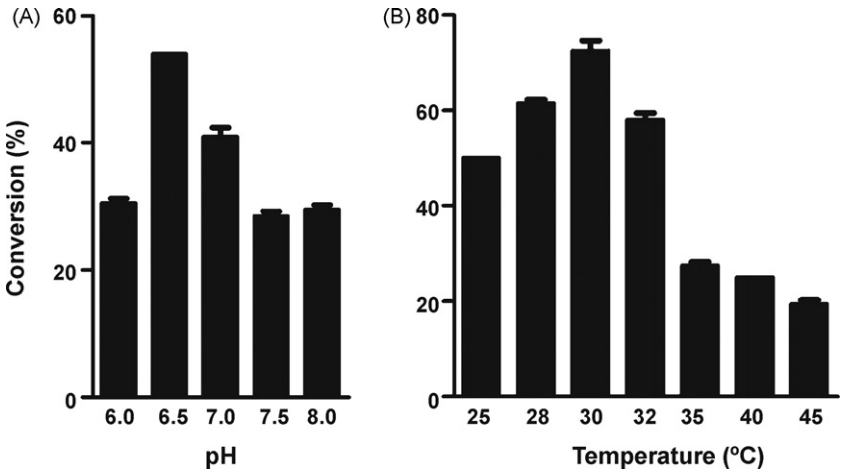

Fig. 3. pH (A) and temperature (B) dependence of activities of StyAB2. Whole *E. coli* cells expressing StyAB2 was used to convert 4-bromostyrene, and the yield of product was measured by reverse-phase HPLC.

3.3. Reaction conditions and kinetic parameters of SMO

The whole-cell biotransformation reactions using 4-bromostyrene as substrate were performed in a bi-phase system containing 20% (v/v) bis-(2-ethylhexyl) phthalate (BEHP) or 10% (v/v) cyclohexane, which had been demonstrated valuable to reduce the cytotoxicity effect of epoxide product [24]. The whole-cell bioconversion system performed fairly well at 25–32 °C with the maximum yield achieved at 30 °C. The yields dropped significantly when higher reaction temperature was applied (Fig. 3B). Fig. 3A showed that the optimum reaction pH was around 6.5. The product yield dropped from 54% to 41% and 31%, respectively, when the reaction pH was set to 7.0 and 6.0.

Based on these results, we measured the steady-state kinetics of styrene epoxidation at 30 °C, pH 6.5. The reactions were carried out in a reconstituted system with purified enzymes. Kinetic properties of StyA were investigated at varying concentrations of styrene (Fig. 4). The apparent K_m value determined by non-linear regression was 0.41 ± 0.14 mM, similar to that of StyAB for *Pseudomonas* sp. VLB120. Its turnover frequency k_{cat} was $1.5 \pm 0.1\ min^{-1}$, significantly lower than that of StyAB from *Pseudomonas* sp. VLB120 ($1.6\ s^{-1}$) [3].

3.4. Biotransformation of conjugated and unconjugated alkenes by StyAB2

The biotransformation of several alkenes with structure similar to styrene was attempted. Conjugated alkenes, such as styrene, 4-bromostyrene, 3-chlorostyrene, 1,2-dihydronaphthalene, 2-vinylpyridine, *1H*-indene or 1,2-dihydronaphthalene (Fig. 5A) afforded the corresponding (*S*)-epoxides with excellent enantiomeric excesses (>99%) at yields of up to 100%. Electron-withdrawing group appeared unfavorable for the reaction. Bromo- and chloro-substituted substrates afforded lower product yields. Compounds with electron-withdrawing group directly connected to the terminal alkene, such as (*E*)-4-phenylbut-3-en-2-one, (*E*)-1-(3-chloroprop-1-enyl)benzene and (*E*)-1-(2-nitrovinyl)benzene did not serve as substrates for StyAB2.

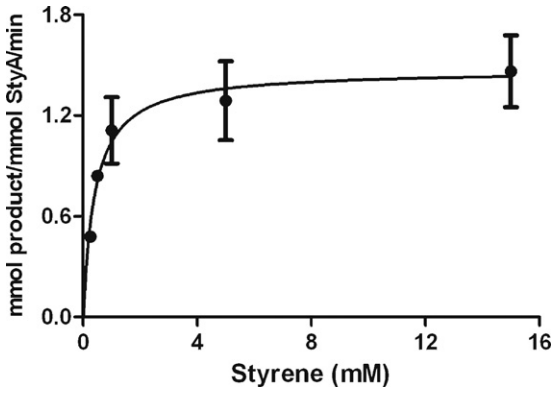

Fig. 4. Steady-state kinetics of styrene epoxidation catalyzed by StyAB2. Each point presented is the mean of triplicate assays in a reconstituted system.

The attempt to apply SMO in the epoxidation of unconjugated alkenes has been described in one report, and the results suggest that 1-allylbenzene does not serve as a substrate [11]. For the newly isolated StyAB2, we found that it was able to catalyze the epoxidation of 1-allylbenzene (Fig. 5B). The enantiomeric excess of the epoxide product was 36%, similar to a previous report on the same reaction catalyzed with chloroperoxidase (37% ee) [25]. Several unconjugated alkenes served as substrates as well, yielding the corresponding (*S*)-epoxides with low to moderate enantiomeric excesses (Fig. 5B). The highest enantioselectivity was found for the substrates 1-allyl-4-methoxybenzene (86%) and 1-allyl-2-hydroxybenzene (80%), which might be due to the advantage of electron-donating nature of the methoxy and hydroxy group.

We compared the protein sequences of StyAB2 and the SMO from *P. fluorescens* ST. They share 89% identity and 91% similarity, and none of the discrepant residues are apparently critical, either based on a model structure [26] or a recently released X-ray structure of SMO [27]. The alignment of the SMO from *P. fluorescens* ST with StyAB2 was shown in Fig. S1 of the Supplementary material. Consequently, no obvious differences have been observed for their active cavities modeled according to the X-ray structure (data not shown). It is hard to explain the different substrate spectrum between these two SMOs based on present information. Elucidation of the molecular basis for the novel activity of StyAB2 awaits further work on mutational analysis and sophisticated structure-relationship studies.

The novel activity presented here provides new insights into the substrate specificity of SMOs. The products from unconjugated alkenes represent a group of important synthetic building blocks and intermediates. It is also quite promising to harness the stereoselectivity of StyAB2 for this group of substrates with carefully designed structures and optimized reaction parameters. This work is currently under way in this laboratory.

Insofar as we know, this is the first report on the epoxidation of unconjugated alkenes catalyzed by SMO. Recently isolated SMOs,

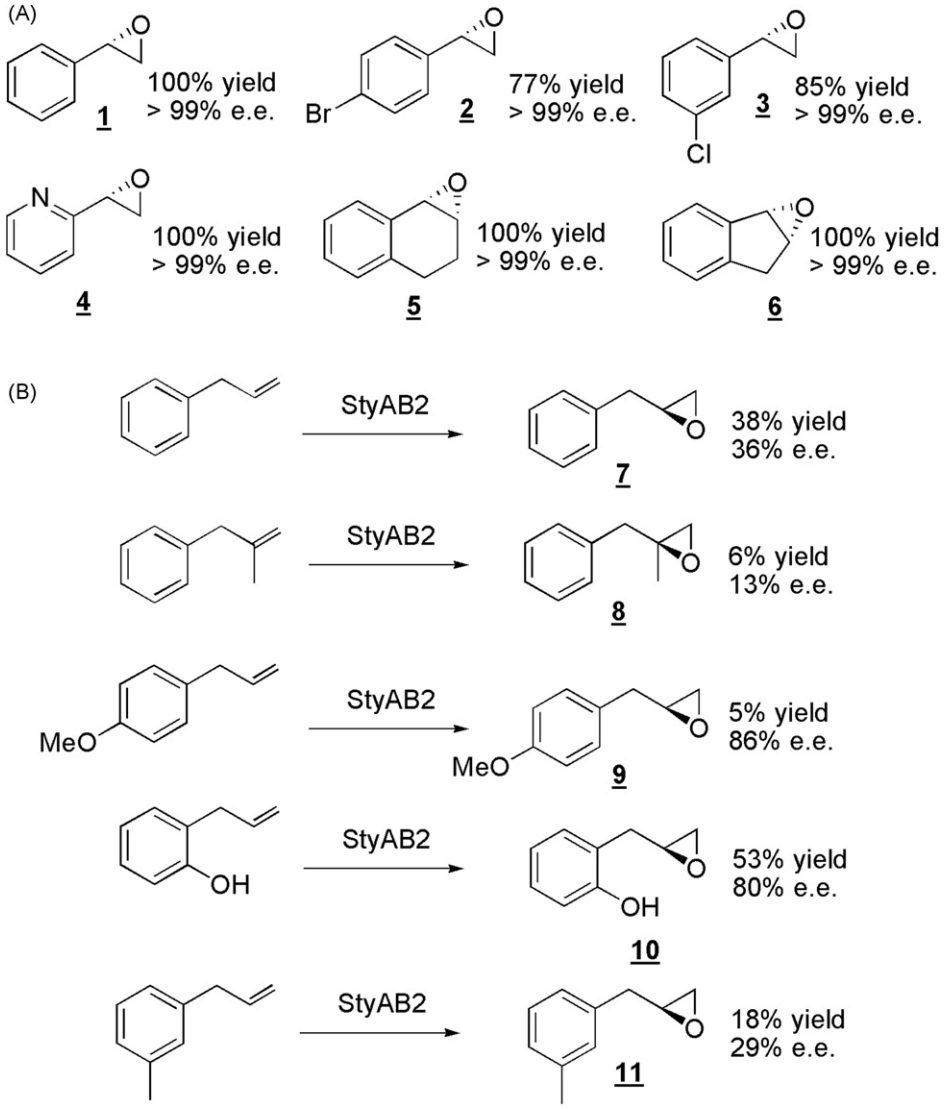

Fig. 5. Production of chiral epoxides by whole-cell catalyzed bioconversion of styrene derivatives (A) and unconjugated alkenes (B).

such as the self-sufficient SMO from *R. opacus* [13] and SmoA from metagenome [12], although both more diverse in their sequence spaces, have not been tested for unconjugated alkenes.

4. Conclusions

We have isolated a novel styrene monooxygenase StyAB2, and functionally expressed it in *E. coli*. The enzyme is able to catalyze the epoxidation of both unconjugated 1-allylbenzene analogues and conventional substrates of conjugated styrene analogues. Since only a very few SMOs have been experimentally assigned so far, the catalytic specificity of most SMO sequences from genomes are yet to be elucidated. Our results suggest that a much broader substrate spectrum is expected for SMOs and although known for decades, their applications in the generation of molecular diversity are yet to be explored.

Acknowledgments

This work was supported by National Natural Science Foundation of China (20802073/B020104), 100 Talents Program of the Chinese Academy of Sciences and Sichuan Province Science Foundation for Young Scholars 08ZQ026-023.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.08.012.

References

[1] A. Mooney, P.G. Ward, K.E. O'Connor, Appl. Microbiol. Biotechnol. 72 (2006) 1–10.
[2] N.D. O'Leary, K.E. O'Connor, A.D. Dobson, FEMS Microbiol. Rev. 26 (2002) 403–417.
[3] K. Otto, K. Hofstetter, M. Rothlisberger, B. Witholt, A. Schmid, J. Bacteriol. 186 (2004) 5292–5302.
[4] J.B. Park, B. Buhler, T. Habicher, B. Hauer, S. Panke, B. Witholt, A. Schmid, Biotechnol. Bioeng. 95 (2006) 501–512.
[5] S. Panke, M. Held, M.G. Wubbolts, B. Witholt, A. Schmid, Biotechnol. Bioeng. 80 (2002) 33–41.
[6] F. Hollmann, P.C. Lin, B. Witholt, A. Schmid, J. Am. Chem. Soc. 125 (2003) 8209–8217.
[7] K. Hofstetter, J. Lutz, I. Lang, B. Witholt, A. Schmid, Angew. Chem. Int. Ed. 43 (2004) 2163–2166.
[8] G. Sello, F. Orsini, S. Bernasconi, P. Di Gennaro, Tetrahedron: Asymmetry 17 (2006) 372–376.
[9] K.L. Tee, O. Dmytrenko, K. Otto, A. Schmid, U. Schwaneberg, J. Mol. Catal. B: Enzyme 50 (2008) 121–127.
[10] G. Bestetti, P. Di Gennaro, A. Colmegna, I. Ronco, E. Galli, G. Sello, Int. Biodeterior. Biodegrad. 54 (2004) 183–187.

[11] S. Bernasconi, F. Orsini, G. Sello, A. Colmegna, E. Galli, G. Bestetti, Tetrahedron Lett. 41 (2000) 9157–9161.
[12] E.W. van Hellemond, D.B. Janssen, M.W. Fraaije, Appl. Environ. Microbiol. 73 (2007) 5832–5839.
[13] D. Tischler, D. Eulberg, S. Lakner, S.R. Kaschabek, W.J.H. van Berkel, M. Schlomann, J. Bacteriol. 191 (2009) 4996–5009.
[14] G. Wittig, U. Schoellkopf, Org. Syn. Coll. 5 (1973) 751.
[15] R.P. Hanzlik, M. Edelman, W.J. Michaely, G. Scott, J. Am. Chem. Soc. 98 (1976) 1952–1955.
[16] M. Fieser, L.F. Fieser, Reagents for Organic Synthesis, Wiley-Interscience, New York, 1967.
[17] Z.-G. Zhang, Y. Liu, F.P. Guengerich, J.H. Matse, J. Chen, Z.-L. Wu, J. Biotechnol. 139 (2009) 12–18.
[18] Z.-L. Wu, L.M. Podust, F.P. Guengerich, J. Biol. Chem. 280 (2005) 41090–41100.
[19] A. Schmid, K. Hofstetter, H.-J. Feiten, F. Hollmann, B. Witholt, Adv. Synth. Catal. 343 (2001) 732–737.
[20] K. Matsumoto, T. Kubo, T. Katsuki, Chem. Eur. J. 15 (2009) 6573–6575.
[21] Y. Genzel, A. Archelas, Q.B. Broxterman, B. Schulze, R. Furstoss, J. Org. Chem. 66 (2000) 538–543.
[22] S. Panke, B. Witholt, A. Schmid, M.G. Wubbolts, Appl. Environ. Microbiol. 64 (1998) 2032–2043.
[23] F.W. Studier, Protein Expr. Purif. 41 (2005) 207–234.
[24] S. Panke, V. de Lorenzo, A. Kaiser, B. Witholt, M.G. Wubbolts, Appl. Environ. Microbiol. 65 (1999) 5619–5623.
[25] A.F. Dexter, F.J. Lakner, R.A. Campbell, L.P. Hager, J. Am. Chem. Soc. 117 (1995) 6412–6413.
[26] K.A. Feenstra, K. Hofstetter, R. Bosch, A. Schmid, J.N.M. Commandeur, N.P.E. Vermeulen, Biophys. J. 91 (2006) 3206–3216.
[27] U.E. Ukaegbu, A. Kantz, M. Beaton, G.T. Gassner, A.C. Rosenzweig, Biochemistry 49 (2010) 1678–1688.