Short communication

Efficient bioreductive production of (S)-N-Boc-3-hydroxypiperidine using ketoreductase ChKRED03

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Ibrutinib is an anticancer drug targeting B-cell malignancies. The key chiral intermediate for ibrutinib synthesis is the alcohol (S)-N-Boc-3-hydroxypiperidine (S)-NBHP, which can be produced via ketoreductase (KRED)-catalyzed bioreduction. After screening a small inventory of 27 KREDs mined from the genome of Chrysobacterium sp. CA49, ChKRED03 was selected as the best performer, leading to the complete conversion of 200 g substrate/l within 10 h to yield (S)-NBHP with high enantiomeric excess (>99% ee). The enzyme was NADPH dependent, and the highest enzymatic activity was observed at 30 °C in potassium phosphate buffer (pH 7.0). At a substrate/catalyst ratio of 66.7 (w/w), ChKRED03 catalyzed the complete conversion of 200 g/L substrate within 3 h to yield (S)-NBHP with >99% ee, demonstrating great potential for industrial application.

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1. Introduction

Chiral alcohols have wide applications in the synthesis of bioactive compounds, chemical catalysts, and liquid crystals. A great deal of effort has been devoted toward the development of green catalytic methods for the chemo-, regio- and stereoselective reductions of prochiral ketones to afford enantiopure alcohols [1–4]. In recent decades, the application of biocatalytic asymmetric reduction using ketoreductases (KRED) has risen rapidly, largely due to the continuous development of new recombinant enzymes, both natural and engineered, as well as efficient cofactor regeneration systems, which have greatly promoted the industrial-scale bioreductive production of chiral alcohols [5–10].

(S)-N-Boc-3-hydroxypiperidine (S)-NBHP is the key chiral intermediate for the synthesis of ibrutinib, which, marketed with the name Imbruvica, is an anticancer drug targeting B-cell malignancies. It has been approved by the US Food and Drug Administration in 2013 for the treatment of mantle cell lymphoma, in 2014 for the treatment of chronic lymphocytic leukemia, and in January 2015 for the treatment of lymphoplasmacytic lymphoma [11–13]. (S)-NBHP as well as other enantiopure hydroxypiperidines are important synthetic synthons in the pharmaceutical industry [14]. They are mostly prepared via chemical approaches through multiple-step conversions from chiral starting materials [15–17]. Reddy et al. has also reported an asymmetric synthesis approach to (S)-NBHP via a 13-step conversion from achiral 4-methyl phenacyl bromide with ~35% yield [18].

To the best of our knowledge, the direct asymmetric reduction of the ketone precursor to produce chiral NBHP has not been reported by classic chemists despite of its atom economic nature. Biocatalytic approaches, on the other hand, have been reported in a few cases. The first bioreductive synthesis of (S)-NBHP was achieved together with other five hydroxypiperidines using the tissue of Daucus carota, resulting in moderate yield (73%) and good stereoselectivity (95% ee). However, the concentration of the substrate, N-Boc-piperidin-3-one (NBPO), was very low (3.3 mM) [19]. Recently, the first practical solution has been reported using a com-

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mercially available KRED, which involves adding substrate in two 50 g/L batches at a substrate/catalyst ratio of 20 (w/w), and delivers enantiopure (S)-NBHP (>99% ee) with 99.8% conversion after 24 h, corresponding to a space-time yield of 100 g/L/d [20].

In the present work, we screened a small inventory of recombinant KRESs mined from the genome of Chryseobacterium sp. CA49 [21] with the aim to achieve better productivity for the bioreduction of NBPO. The KRESs in this collection are quite diverse and generally share 20–30% identity at the protein level, and catalyze the reduction of a variety of ketones. One of the enzymes, designated as ChKRED03, was found to show much improved productivity, suitable for industrial scale asymmetric production of (S)-NBHP.

2. Materials and methods

2.1. Chemicals and analysis methods

The substrate NBPO was purchased from Alfa-Aesar (Tianjin, China). The racemic NBHP and (S)-NBHP were purchased from Sigma–Aldrich (St. Louis, MO, USA). Glucose dehydrogenase (GDH) was obtained from Sigma–Aldrich (St. Louis, USA). All other reagents were obtained from general commercial suppliers and used without further purification. The GenBank accession number of ChKRED03 is KC342003.

NMR spectra were recorded on a Bruker-400 (400/100 MHz) spectrometer in CDE. All signals were expressed in ppm field from tetramethylsilane. Optical rotations were obtained on PerkinElmer 341 digital polarimeter. HPLC was conducted on a Shimadzu Prominence LC-20AD system connected to a PDA-detector. The conversion rates were determined on an SILO-100A column (4.6 × 250 mm, GL Sciences Inc., Japan) at 35 °C with a mobile phase of n-hexane/2-propanol (95/5, v/v) at a flow rate of 1.0 mL/min. The optical purities were determined on a CHI-RALPAK IC column (4.6 mm × 250 mm, Daicel, Japan) at 30 °C with n-hexane/2-propanol (92/8, v/v) as the mobile phase at a flow rate of 1.0 mL/min. The substrate and products were detected at 210 nm and the absolute configurations of the products were determined by comparing the retention times with the standard samples.

2.2. Preparation of crude ketoreductases extracts and purified ChKRED03

Each of the recombinant plasmids encoding one of the 27 KRESs of ChKRED01–ChKRED27 (GenBank accession numbers: KC342001 to KC342027) [21] was transformed into E. coli BL21 (DE3) cells for protein expression. Single colonies were grown overnight at 37 °C in Luria-Bertani (LB) medium containing 50 μg kanamycin/mL. Ten milliliters of overnight culture was then inoculated into 1 L of LB medium containing 50 μg kanamycin/mL. IPTG was added at a final concentration of 0.5 mM when the OD600 of the culture reached 0.6–0.8, and the cultivation was continued at 20 °C for 16 h. Cells were harvested by centrifugation, washed twice using 0.9% (w/v) aqueous sodium chloride solution, and resuspended in 100 mM sodium phosphate buffer. After disruption with a high-pressure homogenizer (ATSAH100B, ATS Engineering Inc., Canada), the cell debris was removed by centrifugation at 2 × 10^{10}g for 25 min at 4 °C. The resulting supernatant was used directly as crude enzyme extracts. The purification of the crude enzyme extracts followed the common practice of reported industrial-scale productions catalyzed with KRESs [1.5–7]. Briefly, a small fraction of the crude enzyme extracts was lyophilized and weighed. Then all references to enzyme loading (g/L) are based on the amount of lyophilized powder prepared from the crude enzyme extracts.

The crude enzyme extract of ChKRED03 was loaded onto a Ni^{2+}-nitritriacetic acid column (Qiagen, Valencia, CA, USA) equilibrated with buffer A (50 mM sodium phosphate buffer, pH 7.0, 300 mM NaCl and 10 mM imidazole), washed with buffer A containing 30 mM of imidazole, and eluted with buffer A containing 250 mM of imidazole at a flow of 1 mL/min. The fractions containing the target protein were collected and dialyzed against 100 mM potassium phosphate buffer (pH 7.0). Protein analysis was done with SDS-PAGE and the BCA Protein Assay Kit (Beyotime, China) with bovine serum albumin as a standard.

2.3. Recombinant enzyme screening

The first-round screening was carried out at 30 °C for 10 h in 1 mL potassium phosphate buffer (100 mM, pH 7.0) containing 10 mg substrate (50 mM), 1 mM NAD^+, 1 mM NADP^+, 250 mM glucose, and 3 mg of ketoreductase (crude enzyme extracts) and 30U GDH. The second-round screening was performed in a similar reaction system with the substrate and glucose concentrations increased to 100 g/L (500 mM) and 600 mM, respectively. The pH of the reaction mixture was monitored and maintained at 7.0–8.0 by the addition of NaOH (1 M). To test the ability of ChKRED3 to use isopropanol or ethanol as the ultimate reducing agent, the reaction was carried out at 30 °C for 10 h in 1 mL potassium phosphate buffer (100 mM, pH 7.0) containing 2 mg substrate (10 mM), 10 mM NADP^+, 3 mg ketoreductase (crude enzyme extracts), and 50–500 mM isopropanol or ethanol. The reaction was quenched by extraction with ethyl acetate. The organic phase was concentrated, analyzed with HPLC, and purified by silica gel column chromatography. Then the structure of the product was verified using 1H NMR analysis.

2.4. Measurement of enzyme activity

The standard reaction mixture (1 mL) contained 0.4 mM NADPH, 0.1 g/L of the crude extract of ChKRED03 and 5 mM substrate in potassium phosphate buffer (100 mM, pH 7.0). Then continuous spectrophotometric measurements were performed by monitoring the oxidation of NADPH at 340 nm for 1 min at 30 °C on a Shimadzu UV-1800 spectrophotometer. One unit of enzyme activity was defined as the amount of the enzyme catalyzing the oxidation of 1 μmol of NADPH per minute at 30 °C. The assay was generally performed in triplicate unless mentioned otherwise. Varied pH or temperature was applied when determining the reaction optimum.

To investigate the thermostability of ChKRED3, the crude enzyme extract was incubated at 30 °C. Then the enzyme solution was withdrawn at intervals, cooled in ice, and the residual activity was assayed following the standard assay method. The solvent tolerance of ChKRED3 was measured in the presence of various organic solvents at concentrations of 5%, 10%, 15% (v/v) following the standard assay method.

The steady-state kinetic analysis was performed with triplicate assays in 1 mL reaction mixture contained 0.4 mM NADPH, 0.1 μM purified enzyme in potassium phosphate buffer (100 mM, pH 7.0) with varied substrate concentrations ranging from 0.1 to 4 mM. Then continuous spectrophotometric measurements were performed by monitoring the oxidation of NADPH at 340 nm for 1 min at 30 °C. The kinetic parameters K_m and k_cat were estimated using Michaelis– Menten equation and the program Graph-Pad Prism v5.0 (GraphPad Software, San Diego, CA, USA).

2.5. Time course and scale-up of the biotransformation

The reaction was performed with the addition of 3 mg crude enzyme extract of ChKRED03 to 1 mL potassium phosphate buffer (100 mM, pH 7.0) containing 1 mM NADP^+, the substrate (150–200 g/L), glucose (1.2 equiv.), GDH (30 U/mL), and 5% methanol (v/v), and incubated at 30 °C with shaking at 160 rpm. The
pH of the reaction mixture was monitored and maintained at 7.0-8.0 by the addition of NaOH (1 M). Samples were taken at intervals for analysis to monitor the time-course of the biotransformation.

The reaction was scaled up to 100 mL following the same conditions at a substrate concentration of 200 g/L (1 M). After 3 h, the reaction mixture was saturated with NaCl, and filtered through a Celite pad. After extraction with ethyl acetate, the two layers were separated, and the organic extracts were combined and dried over anhydrous Na₂SO₄. The solvents were then removed under reduced pressure to yield 19.26 g (95% isolated yield) (S)-NBHP with chemical purity of 95%. After the purification of silica gel column chromatography, 17.85 g (S)-NBHP (88% isolated yield) was achieved with >99% chemical purity. ¹H NMR (400 MHz, CDCl₃) δ 3.75 (d, J = 10.4 Hz, 2H, H₂, H₆), 3.60–3.49 (m, 1H, CHOH), 3.21–3.01 (m, 2H, H₂, H₆), 1.90 (dd, J = 12.4, 4.0 Hz, 2H, H₄, H₅). 13C NMR (100 MHz, CDCl₃) δ 155.2, 79.6, 66.0, 50.8, 43.8, 32.2, 28.4, 22.7. [α]D₂⁰ = +24.1 (c 0.63, EtOH) ([lit 17]. [α]D₂⁰ = +19.37 (c 0.65, EtOH) for 90% ee, (S)). >99% ee; retention time: tᵣ (S) 14.1 min, tᵣ (R) 15.2 min.

3. Results and discussion

3.1. Screening of the target enzyme

We have previously identified 27 ketoreductases from the genome of Chryseobacterium sp. CA49 [21]. This inventory of recombinant KREDs has been applied in the bioreduction of a spectrum of chiral alcohols with excellent stereoselectivity [22–25]. We first evaluated the ability of those KREDs to catalyze the bioreduction of N-Boc-piperidin-3-one to afford (S)-NBHP (Fig. 1A) at a lower substrate concentration of 10 g/L in the presence of the GDH/glucose system to recycle the cofactor. Four out of the KREDs showed excellent (S)-selectivity (>95% ee) as well as good activity (>90% conversion). ChKRED03 appeared to be the best owing to the highest conversion (>99%) and enantiomeric selectivity (>99% ee). The second-round of assay with increased substrate loading of 100 g/L (Fig. 1) confirmed that ChKRED03 was the best performer, leading to complete conversion and high enantiomeric excess (>99% ee) within 10 h (Fig. 1B). The ability of ChKRED03 to use isopropanol or ethanol as the ultimate reducing agent was very weak, as similar reactions in the absence of the GDH/glucose system resulted in a conversion of 59% and 8%, respectively within 10 h even at a low substrate concentration of 2 g/L (10 mM).

The background transformation was measured using the cell extract of E. coli BL21 carrying the empty vector as a control, which led to the same (S)-enantiomer with 7% conversion and 95% ee in 10 h (Fig. 1B). Using the standard spectrophotometric measurement, the activity of the control sample was not detectable within 10 min even with increased enzyme concentration, which corresponded to a specific activity of less than 10⁻⁵ of that of ChKRED03. Therefore, the background transformation was considered insignificant, and was not further monitored.

3.2. Reaction conditions and stability of ChKRED03

ChKRED03 is known to be NADPH-dependent [24]. For the substrate NBPO, the activity of ChKRED03 was determined to be 6.27 U/mg in the presence of NADPH, and 0.15 U/mg in the absence of NADH. Then the kinetic parameters for the purified ChKRED03 in the presence of NADPH were measured, and the estimated apparent Michaelis constant (Kₘ) and turnover frequency (kcat) were 0.56 ± 0.07 mM and 21.9 ± 0.9 s⁻¹, respectively.

Subsequent assays to determine the reaction conditions of ChKRED03 were also performed with NADPH as the cofactor. The pH dependence of activity of ChKRED03 was measured in the pH range from 5.5 to 9.5 in different buffer systems. The highest enzymatic activity was observed at pH 7.0 in potassium phosphate buffer (Fig. 2A). The optimal reaction temperature of ChKRED03 was 30 °C. Relative enzymatic activity at 20 °C and 35 °C were over 80% of the maximum activity (Fig. 2B).

To determine the thermal stability of ChKRED03, residual activities were measured after storage at 30 °C for different period of
time. The enzyme displayed moderate stability, maintaining 81% activity after 3 h and 53% activity after 11 h storage (Fig. 3A). In addition, organic solvents also had significant impact on the activity of ChKRED03 (Fig. 3B). Overall, the enzymatic activity dropped along with the increasing concentrations of organic solvents. The activity decreased sharply in the presence of N,N-dimethylformamide, 1,4-dioxane, ethyl acetate, tetrahydrofuran, and tert-butyl methyl ether, whereas methanol appeared to have less negative effect, affording 95% relative activity at concentration of 5% (v/v) (Fig. 3B). Therefore, methanol was selected as the co-solvent to increase the substrate solubility in aqueous system.

3.3. Production of (S)-NBHP using ChKRED03

The production of (S)-NBHP using ChKRED03 was performed in the presence of a cofactor regeneration system based on GDH-catalyzed glucose oxidation to ensure a sufficient supply of NADPH. Because the recycling system would result in the accumulation of stoichiometric gluconic acid, the pH of the reaction mixture was continuously monitored and maintained at 7.0–8.0 by the addition of 1 M NaOH.

At substrate concentrations of 150 and 200 g/L, the conversion rates reached >99% after 3 h with substrate/catalyst ratio of 66.7 (w/w) (Fig. 4). The product was achieved with excellent stereoselectivity (>99% ee) without the formation of any by-product. After scaling up to a 100 mL system with 20 g substrate, the reaction remained efficient, achieving excellent enantioselectivity (>99% ee) and high conversion (>99%) within 3 h. The isolated yield reached 88% after column chromatography.

Up until now, the only practical solution to the biocatalytic production of (S)-NBHP has been reported by Ju et al. [20]. It has the advantage of using NADH as the cofactor and isopropanol as the ultimate reducing agent, avoiding the use of a second enzyme. However, at a substrate concentration of 100 g/L, the system achieved a maximal conversion of 90.6% even after continuously removing acetone from the reactor, and no improvement was observed with prolonged reaction time. Only the fed-batch strategy of two 50 g/L batches afforded 97.7% conversion after 24 h [20]. Whereas, in our system, no significant substrate inhibition was observed for the tested concentration of 200 g/L, and the productivity was 16-fold of the previous process (Table 1). The use of the GDH/glucose system for the recycling of cofactor has the advantage of being irreversible, and has been successfully applied in several industrial settings [5,7,9]. Overall, the process parameters of our system have exceeded all the basic requirements for an economically feasible process for industrial-scale manufacture of chiral alcohols using KREDs [25] (Table 1), and are expected to be further improved by enhancing the activity and stability of ChKRED03.
via protein engineering, which is currently actively pursued in this laboratory [26].

4. Conclusions

An efficient biocatalytic process for the preparation of enantiopure (S)-NBHP (>99% ee) was realized by employing the ketoreductase ChKRED03 in the presence of a GDH-catalyzed cofactor-recycling system. This process resulted in the complete conversion of 200 g substrate/L within 3 h at a substrate/catalyst ratio of 66.7 (w/w), indicating great potential in the industrial scale production of (S)-NBHP.

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