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Short communication

Efficient bioreductive production of (*S*)-*N*-Boc-3-hydroxypiperidine using ketoreductase *Ch*KRED03

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ABSTRACT

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 *Chryseobacterium* sp. CA49, *Ch*KRED03 was selected as the best performer, leading to the complete conversion of 100 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), *Ch*KRED03 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.

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 KREDs mined from the genome of *Chryseobacterium* sp. CA49 [21] with the aim to achieve better productivity for the bioreduction of NBPO. The KREDs 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 *Ch*KRED03, 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 *Ch*KRED03 is KC342003.

NMR spectra were recorded on a Bruker-400 (400/100 MHz) spectrometer in $CDCl_3$. All signals were expressed in ppm downfield 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 SIL-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 CHIRALPAK 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 Ch*KRED03*

Each of the recombinant plasmids encoding one of the 27 KREDs of *Ch*KRED01–*Ch*KRED27 (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 OD_{600} 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 $2 \times 10^4 g$ for 25 min at 4 °C. The resulting supernatant was used directly as crude enzyme extracts. The quantification of the crude enzyme extracts was following the common practice of reported industrial-scale productions catalyzed with KREDs [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 *Ch*KRED03 was loaded onto a Ni^{2+}-nitrilotriacetic 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 30 U 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 *Ch*KRED03 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 *Ch*KRED03 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 *Ch*KRED03, 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 *Ch*KRED03 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 *Ch*KRED03 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