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Short communication

Efficient bioreductive production of (*R*)-*N*-Boc-3-hydroxypiperidine by a carbonyl reductase

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ABSTRACT

The 3-hydroxypiperidine moiety is a privileged scaffold encountered in many bioactive compounds. An NADPH-dependent reductase (YGL039W) from *Kluyveromyces marxianus* ATCC 748 was isolated to show excellent catalytic activity in (*R*)-*N*-Boc-3-hydroxypiperidine [(*R*)-NBHP] production. Using a GDH-catalyzed cofactor-recycling system to ensure a sufficient supply of NADPH, the effects of temperature, pH, metal ions, substrate concentration, biocatalyst dosage, and cofactors on the YGL039W-catalyzed bioreduction were investigated and optimized. Finally, an extremely high concentration of *N*-Boc-piperidin-3-one (NBPO, 400 g/L) could be completely reduced to (*R*)-NBHP (> 99% *ee*), with a total turnover number of 20,000. This process shows significant potential for the industrial production of (*R*)-NBHP.

1. Introduction

In the majority of natural and unnatural bioactive molecules, the so-called privileged skeletal fragments can be identified [1]. One of the most important classes of skeletal frameworks, which present in a diverse array of biologically active products, are chiral piperidines [2,3]. Benidipine, which is used as a calcium channel blocker, and cisapride, a gastroprokinetic agent, are both important biologically active compounds that possess piperidine moieties in their structures [4,5]. Other medicinally important compounds containing optically active piperidines in their skeletal framework include cassine, cholinotoxic agent, and nootropics or antiarrhythmic agents [6,7].

Addition of a hydroxyl group on the piperidine C3-position introduces a chiral carbon atom and may significantly affect the molecule's bioactivity [8]. Chiral hydroxypiperidines and their derivatives are thus important synthons in the pharmaceutical industry, which are mostly prepared via chemical approaches through multiple-step conversions from chiral starting materials [9,10]. Reddy et al. [11] reported an asymmetric synthesis approach to (*S*)-*N*-Boc-3-hydroxypiperidine [(*S*)-NBHP], via a 13-step conversion from achiral 4-methyl phenacyl bromide with 35% yield. However, this approach suffered from low yields and lengthy synthesis.

Recently, ketoreductase (KRED)-mediated biotransformation showed good performance in chiral piperidine synthesis. The first practical process in the biocatalytic production of (*S*)-NBHP was achieved by a commercially available KRED, which was batch-fed in two 50 g/L batches and shown to convert a total of 100 g substrate/L, with substrate/catalyst (enzyme powder) ratio of 20 (w/w) and high enantiomeric excess (> 99% *ee*), at a conversion rate of 97.7% after 24 h [12]. Recently, another robust KRED from *Chryseobacterium* sp. CA49 was reported to catalyze the conversion of 200 g/L NBPO to yield (*S*)-NBHP with substrate/catalyst (crude enzyme extract) ratio of 66.7 (w/w) and 99% *ee* within 10 h [13]. To date, the direct asymmetric reduction of the ketone precursor to produce (*R*)-NBHP has not yet been reported.

In the present study, we employed an NADPH-dependent carbonyl reductase (YGL039W) from *Kluyveromyces marxianus* ATCC 748 to explore the potential of biocatalytic reduction to synthesize (*R*)-NBHP (> 99% *ee*) from NBPO. Using glucose as a co-substrate for cofactor regeneration, highly efficient synthesis of (*R*)-NBHP (400 g/L) was achieved by an optimized methodology that shows significant potential for industrial application.

2. Experimental

2.1. Materials

All strains used for cloning carbonyl reductases were obtained from the China General Microbiological Culture Collection Center (CGMCC, Beijing, China), and American Type Culture Collection (ATCC,

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Manassas, VA, USA). The recombinant glucose dehydrogenase (GDH) strain was previously constructed in our laboratory [14]. *Escherichia coli* BL21 (DE3)/pET28a(+) was used for expressing the carbonyl reductase. Ketone and chiral alcohol standard samples were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

2.2. Cloning and expression of carbonyl reductase in E. coli

Molecular cloning procedures were performed according to standard laboratory techniques. Recombinant *E. coli* cells were cultivated in Luria-Bertani medium containing kanamycin at 37 °C. Isopropyl β-D-1-Thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM when the OD_{600} reached 0.6–0.8. The induced cultures were further incubated for 20 h at 20 °C.

2.3. Protein purification

Induced cells were harvested by centrifugation (10,000 × *g*, 5 min), washed twice with physiological saline, and resuspended in sodium phosphate buffer (100 mM, pH 7.0). Cell disruption was carried out by sonication, and the insoluble cell lysate was removed by centrifugation (10,000 × *g*, 15 min). The resulting crude extract was passed over a Ni-NTA Superflow column (1 mL, Qiagen) to purify the recombinant protein. All purification steps were carried out at 4 °C.

2.4. Enzyme assay

YGL039W activity was assayed spectrophotometrically at 35 °C by monitoring decrease in NADPH absorbance at 340 nm. The reaction mixture consisted of 10 mM NBPO, 0.2 mM NADPH, 100 mM Tris-HCl buffer (pH 8.0), and an appropriate amount of enzyme in a total volume of 1 mL. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADPH per minute under the specified conditions.

2.5. Analytical methods

The concentrations of NBPO and (*R*)-NBHP were determined on a Zorbax-C18 column (4.6 × 250 mm, Agilent, Shanghai) with a mobile phase of acetonitrile/water (30/70, v/v) at a flow rate of 1.0 mL/min, 25 °C column temperature and UV detection at 210 nm by a high-performance liquid chromatograph (model 1100, Agilent Technologies Co., Ltd.). The retention time for NBHP was 9.0 min, and the retention time for NBPO was 9.7 min.

Optical purities were determined on a Chiralcel OB-H column (4.6 × 250 mm, Daicel Chemicals, Japan) with *n*-hexane/2-propanol (98/2, v/v) as the mobile phase at a flow rate of 0.8 mL/min, 25 °C column temperature and UV detection at 210 nm, and the retention times for (*S*)-NBHP and (*R*)-NBHP was 15.0 and 16.8 min, respectively.

3. Results and discussion

3.1. Cloning and expression of carbonyl reductase

Screening of our internal collection of 10 wild-type enzymes revealed activity for a KRED (YGL039W, GenBank accession no. BAE46987) isolated from *Kluyveromyces marxianus* ATCC 748 that gave (*R*)-NBHP of > 99% *ee* and 100% conversion (Table S2). Based on the analysis of multiple sequence alignment (Fig. S2), YGL039W contained the distinct sequence motif of the short-chain dehydrogenase/reductase (SDR), the $G_8XXG_{11}XXA_{14}$ cofactor binding motif and the $S_{146}Y_{163}K_{167}$ triad for the catalytic activity of SDR proteins [15], thus it was classified to be a short-chain reductase. YGL039W was expressed as a soluble recombinant protein with great quantities, as shown in Fig. 1. The purified protein gave a single band approximately 38 kDa in size on SDS-PAGE, which was in accordance with the predicted molecular mass of 38.75 kDa. The purified protein showed a specific activity of 5.1 U/mg for NBPO when NADPH was used as the electron donor.

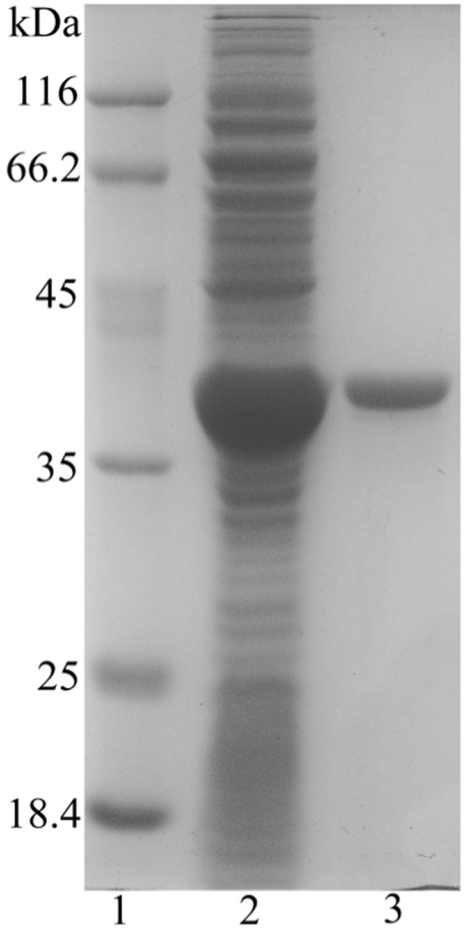

Fig. 1. SDS-PAGE analysis of enzyme expression. Lane 1, protein markers; Lane 2, soluble fraction of cell-free extract from *Escherichia coli* carrying pET-YGL039W; Lane 3, purified YGL039W.

3.2. Effects of temperature and pH on enzyme activity and stability

YGL039W showed maximal activity around 35 °C with more than half maximal activity detected at 25 and 45 °C (Fig. S3a). The optimum pH for activity was 7.0 (Fig. S3b). Fig. S3 shows that the enzyme retained > 50% of its initial activity after incubation at 45 °C for 54 h. The pH stability of YGL039W was also greatly influenced by storage buffer pH, as shown in Fig. S3. YGL039W displayed good thermal and pH stabilities, similar to those reductases from mesophilic bacterium, such as LCRIII from *Lactobacillus curieae* S1L19 [14]. Compared the reductases from mesophilic bacterium, the reductases from thermophilic bacterium usually demonstrate better stabilities due to their unique living environments, such as the reductase TkADH from *Thermococcus kodakarensis* KOD1 [16] and TADH from *Thermus* sp. ATN1 [17]. Thus, to obtain the enzyme with high stability, thermophilic strains would be better alternative resources.

3.3. Effect of metal ions and EDTA on enzyme activity

It has been established that metal ions could have remarkable effects on reductases. To test the effect of various metal ions and the metal chelating reagent ethylenediaminetetraacetic acid (EDTA) on YGL039W activity, Mn^{2+}, Ca^{2+}, Fe^{3+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Li^{+}, and EDTA (final concentration, 1 mM) were added into the reaction media, respectively. It was found that Mn^{2+} (2 mM) was the