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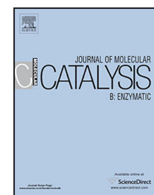

Characterization of a robust *anti*-Prelog short-chain dehydrogenase/reductase *Ch*KRED20 from *Chryseobacterium* sp. CA49

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ABSTRACT

*Ch*KRED20 is a short-chain dehydrogenase/reductase (SDR) cloned from *Chryseobacterium* sp. CA49 for the *anti*-Prelog bioreduction of 3,5-bis(trifluoromethyl)acetophenone to produce the chiral alcohol intermediate for aprepitant. Purified *Ch*KRED20 showed broad pH adaptability and stability with 91% of the maximal activity retained at pH 10.0. The temperature dependence of activity reached the maxima at 50 °C. Its half-lives of thermal inactivation were 163 and 9.8 h at 40 °C and 50 °C, respectively. The enzyme was resistant to a variety of metal ions, additives, and organic solvents. The enzymatic activity could be enhanced by the addition of particular metal ions or detergents to up to 168%. *Ch*KRED20 also displayed good activity and excellent *anti*-Prelog stereoselectivity toward a spectrum of acetophenone derivatives, providing chiral alcohols with >99% ee for the majority of the substrates.

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1. Introduction

Enantiopure alcohols are important building blocks applicable to the synthesis of numerous pharmaceuticals, agricultural chemicals and specialty materials. Despite the extensive development of transitional metal-catalyzed asymmetric reduction of ketones as the main approach to obtain chiral alcohols, the biocatalytic reduction has risen very rapidly, which often provides excellent activity, stereoselectivity and economic advantage under environment-friendly reaction conditions without the risk of metal contamination [1–7]. Both cell-free enzymes and whole microorganisms have been applied to the asymmetric reduction processes. Recent years, in particular, have seen the extensive application of isolated/crude recombinant enzymes coupled with efficient cofactor recycling systems being developed into industrial-scale production [8–13]. The use of recombinant enzymes greatly facilitates the scale-up with high volumetric productivity and the absence of side reactions [2,14–16].

The asymmetric reduction of 3,5-bis(trifluoromethyl) acetophenone (**1a**) using different biocatalysts can provide either isomer of 1-[3,5-bis(trifluoromethyl)-phenyl]ethanol (**1b**). Both of the isomers can serve as intermediates for pharmaceutical agents. (*S*)-**1b** is an intermediate for some antagonists currently under clinical evaluation [17], and (*R*)-**1b** is a key chiral intermediate in the synthesis route of Aprepitant, a neurokinin-1 (NK-1) receptor antagonist approved by the United States Food and Drug Administration and European Medicines Agency for the treatment of chemotherapy-induced emesis, depression and other potential symptoms [18]. The biocatalytic synthesis of (*S*)-**1b** has been established successfully in large scale using isolated alcohol dehydrogenase (ADH) from *Rhodococcus erythropolis* [17]. To produce the enantiomer, (*R*)-**1b**, whole cells of microorganisms following *anti*-Prelog's rule have been intensively exploited and quite a few can afford excellent enantioselectivity of >99% ee (enantiomeric excess) [19–22]. However, the productivity remains low with conversion of 31–95% achieved at a substrate concentration of ~5 g/l in the majority of cases. When higher substrate loading of ~50 g/l was applied, the best conversion reported was 91.8% after 30 h incubation with a high cell concentration of 300 g/l using the wet cells of *Leifsonia xyli* CCTCC M 2010241 [23]. The *anti*-Prelog enzymes LB-ADH and LK-ADH from *Leifsonia* species have been reported to catalyze the reduction of **1a** to (*R*)-**1b** at the expense of NADPH, however, the enantio-purity of the product is only 87% and 83% ee, respectively [17].

We have recently isolated a novel strain of *Chryseobacterium* sp. CA49 that is robust enough to tolerate 50 g/l ketone **1a** to produce

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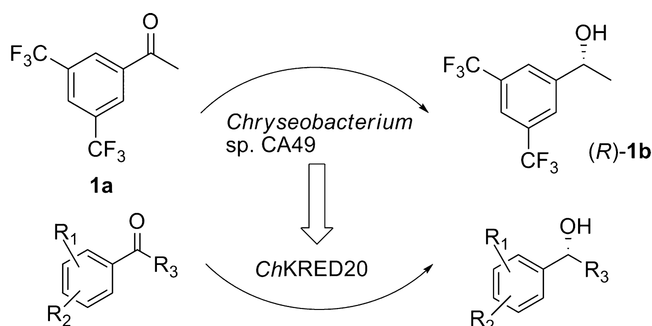

Fig. 1. Bioreduction of ketones catalyzed with *Ch*KRED20.

enantiopure (*R*)-**1b** with complete conversion within 24 h (Fig. 1). The identification of the key enzyme, *Ch*KRED20, has been accomplished through draft genome sequence analysis followed by heterologous expression, biotransformation and transcriptional analysis [24]. *Ch*KRED20 is a short-chain dehydrogenase/reductase (SDR) sharing the highest sequence identity with LSADH from *Leifsonia* sp. strain S749 [25]. It is able to use 2-propanol as a co-substrate to recycle the expensive cofactor, and the resulting reaction system catalyzed with the lyophilized powder of the crude enzyme is efficient enough to reduce 150 g/l substrate with >99% conversion and >99.9% enantiomeric excess within 24 h, which has met basic requirements of an economically feasible KRED-catalyzed process for industrial-scale manufacture of chiral alcohols.

In the present work, detailed characterization of *Ch*KRED20 was performed to further exploit the potential of this enzyme, which revealed unexpected robustness, good substrate adaptability (Fig. 1), and high tolerance of this enzyme toward a series of additives.

2. Materials and methods

2.1. Chemicals

All substrates **1a**–**14a** were purchased from Alfa-Aesar (Tianjin, China). The racemic alcohols were prepared by reducing the ketones with sodium borohydride. Chiral alcohols (1*R*)- and (1*S*)-[3,5-bis(trifluoromethyl)phenyl]ethanol ((*R*)- and (*S*)-**1b**) were purchased from Sigma Aldrich (St. Louis, MO, USA). All other reagents were obtained from general commercial suppliers and used without further purification.

2.2. Expression and purification of ChKRED20 in Escherichia coli BL21(DE3)

The plasmid, pET-28a-*ch*KRED20, encoding *Ch*KRED20 [24] was transformed into *E. coli* BL21(DE3) (Novagen, Madison, WI, USA) competent cells and selected on Luria–Bertani (LB) agar plates containing 50 μg kanamycin/ml. Single colonies were grown overnight at 37 °C in LB medium containing 50 μg kanamycin/ml. The overnight culture was then inoculated into Terrific-broth (TB) medium containing 50 μg kanamycin/ml. IPTG was added to a final concentration of 1.0 mM when OD_{600} of the culture reached 0.6, and the cultivation was continued at 30 °C for 12 h. Cells were harvested by centrifugation at 6000 × *g* for 10 min at 4 °C, washed twice with distilled water and resuspended in buffer A (20 mM sodium phosphate buffer pH 8.0, 500 mM NaCl and 10 mM imidazole). After disruption with a homogenizer (Nano, ATS-AH100B, ATS Engineering Inc., Canada), the cell debris was removed by centrifugation at 20,000 × *g* for 25 min at 4 °C. The resulting supernatant was then loaded onto Ni^{2+}-nitrilotriacetic acid columns (Bio-Rad) equilibrated with buffer A. The enzyme was eluted with buffer A containing a gradient of imidazole from 10 to 500 mM at a flow rate of 1 ml/min, and five column volumes were used for the gradient elution. The fractions containing target protein were collected and dialyzed against 20 mM potassium phosphate buffer (pH 7.0).

Purified enzymes were analyzed by SDS-PAGE and used for enzymatic assays. Protein estimations were carried out with a commercial BCA Protein Assay kit with bovine serum albumin as a standard (Tiangen, Beijing, China).

2.3. Measurement of enzyme activity

All reactions were performed in triplicate. The reaction mixture comprised 100 mM potassium phosphate buffer (pH 6.0), 20 mM NADH and 10 mM substrate in a total volume of 1.0 ml. The reaction was carried out at 30 °C, and terminated by extraction with 1 ml ethyl acetate after 30 min. The resulting organic phase was subjected to gas chromatographic (GC) analysis to determine the conversion and ee. One unit of the enzyme activity was defined as the amount of enzyme that catalyzes the production of 1 μmol (*R*)-**1b** per minute.

To determine the pH optimum, standard assay method was applied except that different buffers were used for different pH ranges, which included sodium citrate (pH 4.0–6.0), potassium phosphate (pH 6.0–8.0), Tris–HCl (pH 8.0–9.5) and sodium carbonate (pH 9.5–11.5). To evaluate the pH stability, the enzyme was pre-incubated in buffers of different pH values (5.0–11.0) at 4 °C for 28 h.

The optimum temperature for *Ch*KRED20 was determined at various temperatures ranging from 20 °C to 75 °C. To investigate the thermostability of *Ch*KRED20, the enzyme was incubated at 40, 45 or 50 °C. The enzyme solution was withdrawn at intervals, cooled in ice, and the residual activity was assayed following the standard assay method.

The steady-state kinetic parameters toward the substrate **1a** were investigated using standard assay method except that 2.7 μM enzyme was applied with varied substrate concentrations ranging from 0.2 to 20 mM. Data were fitted to the Michaelis–Menten equation using Graph-Pad Prism v5.0 (GraphPad Software, San Diego, CA, USA) to generate estimates of K_m and k_{cat} values.

2.4. Biotransformation of various ketones and product analysis

Standard reaction conditions were followed to convert 10 mM substrates in the presence of 1% (v/v) isopropanol. Purified enzyme was applied at a concentration of 1 U/ml. After 1 h incubation at 30 °C, the reaction was terminated by extraction with ethyl acetate. The organic phase was dried with anhydrous sodium sulfate, concentrated under reduced pressure, and analyzed. Preparative biotransformation was carried out at 40 °C for 24 h to achieve a complete conversion of each substrate. The final product was purified with column chromatography and subjected to nuclear magnetic resonance (NMR) and optical rotation analysis to confirm the structure and purity. The absolute configuration was determined by comparison of the specific rotation data with those in the literatures.

The ee and conversion of the product were determined by chiral GC or HPLC analysis. Chiral GC analysis was performed on a Fuli 9790 II GC system connected to a flame ionization detector using a CP-Chirasil-DEX CB column (**1a**, **2a**, **4a**, **7a**–**14a**) (Varian, USA). The injector and detector were set at 260 °C and 280 °C, respectively. The column temperature was set at 115 °C (**1a**, **2a**, **8a**, **9a**, **10a**, **11a**), or 130 °C (**4a**), or 140 °C (**7a**), or 100 °C (**13a**), or 90 °C (**14a**). HPLC analysis was performed on a Shimadzu Prominence LC-20AD system connected to a PDA-detector using Chiralcel OJ-H column (4.6 × 250 mm, Daicel, Japan) for **3a** and **5a** or Chiralcel AS-H column (4.6 × 250 mm, Daicel, Japan) for **6a** with

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