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Efficient synthesis of optically active halogenated aryl alcohols at high substrate load using a recombinant carbonyl reductase from *Gluconobacter oxydans*

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ABSTRACT

A carbonyl reductase from *Gluconobacter oxydans* (*Go*CR) exhibited good activity and strict enantioselectivity for the asymmetric reduction of a series of halogenated acetophenones, resulting in the corresponding *S*-alcohols. For efficient synthesis of alcohol products, an economical and satisfactory substrate-coupled cofactor regeneration system was constructed employing isopropanol as the co-substrate to regenerate NADH *in situ*. In the presence of 2.0 molar eq. of isopropanol, 500 mM *o*-chloroacetophenone was reduced to (*S*)-1-(2-chlorophenyl)-ethanol with >99% *ee* and a high conversion rate of 96% by recombinant *Escherichia coli* BL21 cells overexpressing *Go*CR. In this reaction system, a slight excess of isopropanol as hydride source could drive the reduction reaction to near completion due to the speculated forming of an intramolecular hydrogen bond between the Cl group and the new obtained alcohol moiety of (*S*)-1-(2-chlorophenyl)-ethanol. Furthermore, other tested halogenated acetophenones at 100 or 200 mM substrate load were also reduced at 71–96% conversion, affording *S*-configuration alcohols with >99% *ee*. Homology modeling and molecular dynamics simulation were performed to uncover the structural basis for the excellent enantioselectivity of *Go*CR toward halogenated acetophenones. These results imply the high potential of *Go*CR in the production of halogenated 1-phenylethanols, such as (*S*)-1-(2-chlorophenyl)-ethanol, an important chiral building block with wide application in pharmaceutical chemistry and fine chemicals.

1. Introduction

Optically active halogenated aryl alcohols are useful and valuable intermediates in medicinal chemistry [1–5]. A case in point is (*S*)-1-(2-chlorophenyl)-ethanol, which serves not only as a precursor of L-cloprenaline used for asthma treatment [6], but also as a chiral intermediate in the synthesis of thiophene-benzimidazole and imidazopyridine, which are promising PLK1 (serine-threonine kinase polo-like kinase 1) inhibitors for cancer therapy [7,8].

A popular route to obtaining enantiopure halogenated aryl alcohols is enantioselective reduction of the corresponding prochiral ketones using chemo-catalysts [9,10] or biocatalysts [2,11,12]. Except for very few successful examples, chemical processes employing heavy metals (iridium, rhodium, or ruthenium) as catalysts are commonly confronted with severe challenges, including low enantioselectivity, rigorous reaction conditions and poor environmental compatibility. In contrast, enzyme-catalyzed asymmetric reduction of ketones can perfectly solve these tough issues due to the inherent characteristics and advantages of biocatalysts [11,13,14], thus it may be an alternative to chemical methods.

Several groups have attempted to develop biocatalytic processes for the preparation of halogenated aryl alcohols from the corresponding ketones [4,7,8,15–19]. One typical research was performed by Regina et al., who employed a xylose reductase from *Candida tenuis* (*Ct*XR) for the asymmetric reduction of *o*-chloroacetophenone, using recombinant whole-cell catalysts co-expressing *Ct*XR and FDH (formate dehydrogenase) for the recycling of NADH. Through a series of process optimizations, the substrate loading was improved from 100 to 300 mM [8]. However, apart from a few reports [2,19], the often observed low tolerance of the enzymes for high substrate concentration and the low space-time yield of the biocatalytic reductions restrict the practical applications. It is still very important to search for new carbonyl reductases with higher catalytic efficiency and subsequently carry out biocatalytic reduction process at high substrate loading.

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Recently, we have isolated and identified a short-chain dehydrogenase, Gox2036 (denoted as *Go*CR in this study), from *Gluconobacter oxydans*, which could reduce a broad spectrum of prochiral ketones including α-diketones, hydroxy ketones, α-ketoesters, and β-ketoesters, as well as display preference for oxidation of secondary alcohols [20]. Here, we continue our studies toward the exploration of *Go*CR's application in asymmetric reduction of various halogenated acetophenones to produce the optically pure halogenated aryl alcohols at high substrate loading. To regenerate NADH *in situ*, a substrate-coupled system was constructed. Asymmetric reductions of *o*-chloroacetophenone and other halogenated acetophenones were subsequently investigated. Moreover, homology modeling and molecular dynamics simulation were performed to explain the strict enantioselectivity of *Go*CR from the point of view of tertiary structure.

2. Materials and methods

2.1. Chemicals

Halogenated acetophenones, isopropanol, NADH, NAD^+ and (*S*)-1-(o-chlorophenyl)-ethanol of high purity (>98%) were purchased from Roche (Switzerland), Aladdin (China), or Accela (China). Other chemicals involved were of analytical grade.

2.2. Synthesis of racemic alcohols catalyzed by sodium borohydride

Racemic alcohols were prepared through reduction of the corresponding prochiral ketones by sodium borohydride in an ethanol solvent. The reduction procedure was as follows: ketone (1 g) was added dropwise to 65 mL ethanol containing 5% (v/v) deionized water and 1 g sodium borohydride. The mixture was stirred continuously for 1 h at 4 °C, followed by the addition of 1 mL glacial acetic acid to terminate the reaction. After removal of the insoluble substances by filtration, the mixture was extracted with diethyl ether. The organic layer was then removed by rotary evaporation to obtain the final racemic alcohols.

2.3. Genes and strains

Genomic DNA of *G. oxydans* strain 621H (GenBank accession number: CP000009) was used as template for the PCR clone of *Go*CR (locus_tag: GOX2036). *Escherichia coli* BL21 (DE3) and pET-28a (Novagen) were used for overexpression of the recombinant *Go*CR. Molecular cloning, gene expression, and protein purification of *Go*CR were performed as described in our previous study [20].

2.4. Enzyme assay

Enzyme activities of *Go*CR toward halogenated acetophenones were determined spectrophotometrically by monitoring the change of NADH absorbance at 340 nm on a SpectraMax 190 instrument (Molecular devices, USA), and a molar extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$ for NADH was used for the calculation [21]. The reaction mixtures (0.2 mL) for enzyme assay consisted of 10 μg purified enzyme, 100 mM sodium phosphate buffer (PBS, pH 6.5), 0.2 mM NADH and 10 mM ketone substrates. All reactions were performed at 30 °C and initiated by adding NADH. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1.0 μmol NADH per minute. The concentration of protein was measured by the method of Bradford [22].

2.5. Asymmetric reduction of halogenated aryl ketones by whole-cell catalysts

2.5.1. Preparation of whole-cell catalysts

E. coli BL21 cells harboring pET28a-*gox2036* (gene encoding *Go*CR) were inoculated into a 200 mL LB medium (1% NaCl, 1% peptone, and 0.5% yeast extract) in a 500 mL shake flask. Isopropyl thio-b-D-galactoside (IPTG) was added at a final concentration of 0.25 mM when the cell density at OD_{600} reached 0.6. After cultivation at 30 °C for 10 h, the induced cells were harvested by centrifugation (10,000 × *g*, 10 min) at 4 °C. Wet cells were washed twice in 20 mM PBS (pH 7.4) before further use.

2.5.2. Reaction conditions

To perform reduction reactions, 0.1 g wet cells suspended in 100 mM PBS (pH 6.5) were added to the reaction mixture containing substrates and co-substrates, to a total volume of 1 mL. All reactions were performed as batch processes in 2 mL Eppendorf tubes, on a rotary shaker at 200 rpm for a period of time. Other reaction conditions including substrates (co-substrates) concentration, reaction temperature and reaction time were stated in the texts or footnotes of tables, respectively.

2.6. Analytical methods

After reaction completion, the mixture was extracted at least twice by *n*-hexane added in a 2:1 ratio. Centrifugation (12,396 × *g*, 10 min) was carried out to facilitate the phase separation. The combined organic layers were dried over Na_2SO_4. Supernatant was then directly subjected to GC (Agilent 6890N) or HPLC (Agilent 1100) for determination of yield and *ee* value. Chiral HPLC columns OD-H and OB-H were purchased from Daicel Chemicals (Japan). Capillary columns CP-ChiraSil-Dex CB and DB-5 were purchased from Varian (USA) and Agilent (USA), respectively. The absolute configuration of the products was identified by comparing the chiral chromatographic data with the authentic standards or literature reports [15,23]. The details of GC and HPLC analysis were shown in Table S1 and Figs. S3–S11.

The product of *o*-chloroacetophenone reduction was purified and subjected to GC–MS (Fig. S12) and NMR spectra assay (Fig. S13). Spectral data for (*S*)-1-(2-chlorophenyl)-ethanol were as follows: ^1H NMR (400 MHz, $CDCl_3$): δ = 7.59 (d, *J* = 7.8 Hz, 1H), 7.33–7.22 (m, 2H), 7.21–7.17 (m, 1H), 5.29 (m, *J* = 6.3 Hz, 1H), 2.20 (br, 1H), 1.49 (d, *J* = 6.3 Hz, 3H); GC–EI-MS *m*/*z* (M^+ 158, 156 for C_8H_9OCl) 141, 113, 77, 51, 43.

2.7. Molecular simulation experiments

The initial homology model of *Go*CR with NADH embedded in was constructed using the Modeller 9.12 package. The crystal structure of L-2,3-butanediol dehydrogenase (L-BDH, PDB ID: 3A28) was chosen as template. Molecular docking was performed with the Autodock 4.2 program [24] using *o*-chloroacetophenone as the model ligand and *Go*CR-NADH complex as the receptor. The *Go*CR substrate-binding pocket was examined by comparison with the corresponding X-ray structures of L-BDH. To encompass the entire substrate-binding pocket, the searching grid box was set to a size of 50 × 50 × 50 grid points with a spacing of 0.375 Å. The box center was set exactly at the OH atom of the catalytic residue Tyr155. Fifty possible docking conformations of ligands in receptors were obtained using the Lamakian genetic algorithm (LGA) with the maximum number of energy evaluation set to be 25,000,000. The resulting conformation of lowest binding energy in the largest cluster was chosen as the final solution. To obtain a more reasonable model, the docking solution was further subjected to energy minimization and molecular dynamics simulation (MD) using the