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Highly efficient synthesis of ethyl (*S*)-4-chloro-3-hydroxybutanoate by a novel carbonyl reductase from *Yarrowia lipolytica* and using mannitol or sorbitol as cosubstrate

Qin Xu, Wei-Yi Tao, He Huang, Shuang Li*

College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 210009, China

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ABSTRACT

An NADPH-dependent carbonyl reductase (YlCR2) from *Yarrowia lipolytica* was discovered by genome mining, overexpressed in *Escherichia coli* BL21 and purified to homogeneity. To efficiently synthesize ethyl (*S*)-4-chloro-3-hydroxybutanoate ((*S*)-CHBE) (99%, e.e), the highly stereoselective bioreduction of ethyl 4-chloro-3-oxobutanoate (COBE) into (*S*)-CHBE with the recombinant *E. coli* BL21/pETYlCR2 was successfully demonstrated in an *n*-butyl acetate-water biphasic system (1:1, v/v) with NADPH self-regeneration by substrate-coupled system using sorbitol or mannitol as co-substrate. The optimum reaction condition for the biotransformation of COBE in the biphasic system were 3000 mM COBE, (1.2 or 1.3 mmol/mmol COBE) mannitol or sorbitol, 0.2 mM or 0.4 mM $NADP^+$, 0.12 or 0.14 g (wet weight)/ml cell dosage, pH 5.0, 30 °C; (*S*)-CHBE with yield of 90% and e.e of 99% was obtained after 10 h reaction. Furthermore, 3000 mM COBE could also be completely biotransformed after 20 h without addition of expensive cofactor $NADP^+$. Significantly, *E. coli* BL21/pETYlCR2 shows the high potential in the industrial production of (*S*)-CHBE.

1. Introduction

Ethyl (*S*)-4-chloro-3-hydroxybutanoate ((*S*)-CHBE), one of the optically active alcohols, is a key intermediate for the production of chiral drugs, including cholesterol-lowering HMG-CoA reductase inhibitors such as Lipitor [1,2]. Therefore, effective methods that can be practically used for synthesizing highly optically active (*S*)-CHBE (99% enantiomeric excess (e.e)) are of great interest.

In comparison with conventional chemical methods, asymmetric bioreduction of prochiral ketone using isolated enzymes or whole-cell system is more attractive because of the high enantioselectivity, mild and environmentally friendly reaction conditions [3,4]. However, carbonyl reductases for asymmetric biotransformation of ethyl 4-chloro-3-oxobutanoate (COBE) to (*S*)-CHBE often require expensive cofactor NADH or NADPH as an electron donor [5]. To overcome the problem, enzyme-coupled system and substrate-coupled system were developed for co-factor regeneration [6,7]. Substrate-coupled system, which simultaneously transforms substrate and co-substrate, involves a single enzyme and is simpler than enzyme-coupled system. However, the substrate-coupled system is commonly impeded by co-substrate inhibition of the enzyme [8]. Only a limited number of such systems have been reported because the enzyme in substrate-coupled systems should exhibit broad substrate specificity and high enantioselectivity toward chiral compounds [9–11]. Therefore, searching for new and efficient carbonyl reductases and improving their application performances are of great interest.

Yarrowia lipolytica is non-conventional yeast that is often found in oil fields. In previous work, *Y. lipolytica* has been efficiently employed in reduction of various carbonyl compounds [12,13]. In this work, we discovered an NADPH-dependent carbonyl reductase (YlCR2) from *Y. lipolytica* ACA-DC 50109 by genome data mining. The production of (*S*)-CHBE was performed using *Escherichia coli* cells expressing recombinant YlCR2 as the biocatalyst for both COBE reduction and sugar alcohols oxidation after optimization of the reaction parameters.

2. Materials and methods

2.1. Bacterial strains, vectors and chemicals

Y. lipolytica ACA-DC 50109 [14] was kindly offered by Prof. George Aggelis from Department of Biology, University of Patras, Greece. *E. coli* BL21 (DE3) and DH5α were purchased from Novagen

* Corresponding author at: College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, No.5 Xinmofan Road, Nanjing 210009, China. Fax: +86 25 83172094.
E-mail address: lishuang@njtech.edu.cn (S. Li).

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(Shanghai, China). The pMD-T vector, pET28a (+) plasmid, restriction enzymes and T4 ligase were obtained from Takara (Dalian, China). COBE, (*S*)-CHBE and (*R*)-CHBE were purchased from Aladdin Chemistry Co. Ltd., (Shanghai, China). All other chemical were also from local commercial sources and of analytical grade.

2.2. Cloning and expression of YlCR2 gene

Genomic DNA was extracted from *Y. lipolytica* ACA-DC 50109 using DNA extraction kit. Oligonucleotide primers with *Bam*HI and *Xho*I restriction sites were designed according to the putative carbonyl reductase sequence (GenBank accession No. XM_500963.1). The DNA fragment of the YlCR2 gene was amplified and double-digested with *Bam*HI and *Xho*I and then inserted into the expression vector pET28a (+). The resulting plasmid, pET28a-YlCR2, was transformed into *E. coli* BL21 (DE3) cells. The strain named recombinant *E. coli* BL21/pETYlCR2 was preserved in CCTCC (China Center for Type Culture Collection). Recombinant *E. coli* cells were cultivated at 37 °C in LB medium containing 50 μg/ml kanamycin. Protein expression was induced at 30 °C by the addition of IPTG (final concentration 1 mM) when the OD_{600} reached 0.8. After induction for 12 h, cells were harvested by centrifugation (5000 × *g*, 10 min) at 4 °C and then stored at −20 °C for further use.

2.3. Purification of the recombinant YlCR2

The cells were disrupted and the supernatant was collected by centrifugation at 12000 × *g* for 30 min at 4 °C. Protein purification was performed using an AKTA purifier 10 system with UNICORN 5 software. The YlCR2 was purified using HisTrapTM FF crude column (5 ml, GE Healthcare Corp, USA). The column was pre-equilibrated with 50 ml equilibrium buffer (100 mM sodium phosphate buffer, 500 mM NaCl, 20 mM imidazole, pH 7.4). The supernatant was loaded with a flow rate of 1.0 ml/min. After washing with 50 ml of the equilibrium buffer, the bounded target protein was eluted with elution buffer (100 mM sodium phosphate buffer, 500 mM NaCl, 300 mM imidazole, pH 7.4). The fractions containing target protein fraction were collected and dialyzed with 100 mM sodium phosphate buffer (pH 7.4) for desalting. The molecular weight of YlCR2 was determined on a SuperdexTM 200 column (GE Healthcare Life Science, NJ, USA). Purified enzymes were analyzed by SDS-PAGE and used for enzymatic assays. Protein estimations were carried out with a commercial BCA Protein Assay kit (Tiangen, China).

2.4. Enzyme assay

YlCR2 activity was assayed at 30 °C by monitoring the decrease of the absorbance of NADPH at 340 nm. The assay mixture (0.2 ml) consisted of 100 mM of sodium phosphate buffer (pH 7.0), 5 mM of COBE, 0.4 mM of NADPH and 10 μl of the purified enzyme YlCR2. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol of $NADP^+$ per minute under the described conditions.

2.5. Kinetic analysis

The YlCR2 kinetic parameters toward COBE were investigated using the standard activity assay. The apparent values of K_m and V_{max} were calculated by fitting the data into the Michaelis–Menten equation.

2.6. Effects of pH and temperature on the enzyme activity

In order to determine the optimum temperature and pH of YlCR2, the standard reduction enzyme assay was performed at different temperatures as well as different pH values. The temperatures were from 20 °C to 50 °C. The 100 mM acetate buffer (pH 4.5–6.0), 100 mM sodium phosphate buffer (pH 6.0–8.0) and 100 mM Tris–HCl buffer (pH 8.0–9.0) were used to determine the optimum reaction pH.

2.7. Establishment and optimization of COBE bioreduction

The effects of various parameters (e.g., organic solvent, co-substrate, co-substrate concentration, $NADP^+$ concentration and cell dosage) on the bioreduction reaction were studied. The whole-cell-catalyzed production of (*S*)-CHBE were performed in 50 ml flasks. The reaction mixture comprised 5 ml of sodium phosphate buffer (100 mM, pH 5.0), 5 ml of organic solvent, 3000 mM COBE, and a predetermined amount of organic solvent, co-substrate, $NADP^+$, and cell dosage. The reactions were carried out at 30 °C and 600 r/min with magnetic stirring. Samples were taken periodically and centrifuged, and the isolated organic phase was dried over anhydrous Na_2SO_4 and then assayed by GC. The conversion of COBE was calculated as: [1-(the residual COBE)/(the initial COBE)] × 100%. The yield of (*S*)-CHBE was calculated as: [the concentration of (*S*)-CHBE (mM)]/[the initial concentration of COBE (mM)] × 100%.

To investigate the effects of organic solvent on the reaction, the reaction mixture contained 0.14 g/ml wet cells, 0.5 mM $NADP^+$, 3000 mM COBE, 1.3 mmol mannitol/mmol COBE, organic solvent (hexane, toluene, *n*-butyl acetate, dibutyl phthalate and benzene).

To investigate the effects of co-substrate on the reaction, the reaction mixture contained 0.14 g/ml wet cells, 0.5 mM $NADP^+$, 3000 mM COBE, 1.3 mmol co-substrate/mmol COBE (isopropanol, glucose, sorbitol, mannitol and glycerol), 5 ml sodium phosphate buffer and 5 ml *n*-butyl acetate.

To investigate the effects of co-substrate concentration on the reaction, the reaction mixture contained 0.14 g/ml wet cells, 0.5 mM $NADP^+$, 3000 mM COBE, various concentration of mannitol or sorbitol (0.6–1.5 mmol/mmol COBE), 5 ml sodium phosphate buffer and 5 ml *n*-butyl acetate.

To investigate the effects of $NADP^+$ consumption on the reaction, the reaction mixture contained 0.14 g/ml wet cells, certain concentration of $NADP^+$ (0–0.5 mM), 3000 mM COBE, 1.2 mmol manitol or 1.3 mmol sorbitol/mmol COBE, 5 ml sodium phosphate buffer and 5 ml *n*-butyl acetate.

To investigate the effects of enzyme loading on the reaction, the reaction mixture contained wet cells (0.08–0.16 g/ml), 0.2 mM or 0.4 mM $NADP^+$, 3000 mM COBE, 1.2 mmol manitol or 1.3 mmol sorbitol/mmol COBE, 5 ml sodium phosphate buffer and 5 ml *n*-butyl acetate.

2.8. Instrumental analyses of the purified (S)-CHBE

The reaction mixture was extracted with ethyl acetate twice. The organic solution was combined, dried over anhydrous Na_2SO_4 and evaporated under vacuum. The product was purified by silica gel column chromatography (eluent: petroleum ether/ethyl acetate, 15:1, v/v). The production was characterized on GC, GC–MS, ^1H NMR. The absolute configuration of the product was determined by comparison of the specific rotation data with that in the literature.

GC analysis: CP-Chirasil-DEX CB column, 30 m × 0.25 mm × 0.25 μm, oven temperature 120 °C, injector temperature 260 °C, detector temperature 300 °C, carrier gas nitrogen (0.1 MPa), retention times: t_R (R) 6.73 min, t_R (*S*) 6.91 min. GC–EI–MS analysis: *m*/*z* (M^+ 168,167 for $C_6H_{11}O_3Cl$). ^1H NMR (400 MHz, $CDCl_3$), δ/ppm: 1.26–1.35 (*t*, 3H, —CH_3), 2.61–2.66 (d, 2H, —CH_2—CO—), 3.16–3.25 (m, 1H,—OH), 3.58–3.64 (d, 2H, —CH_2—Cl), 4.17–4.21 (d, 2H, —CH_2—O—), 4.26–4.28 (m, H, —CH—OH).