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Asymmetric synthesis of lipitor chiral intermediate using a robust carbonyl reductase at high substrate to catalyst ratio

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ABSTRACT

An NADPH-dependent carbonyl reductase (*Rp*CR) from *Rhodococcus pyridinivorans* was discovered by genome mining for the asymmetric reduction of ethyl 4-chloro-3-oxo-butanoate (COBE). *Rp*CR has been soluble expressed in *Escherichia coli* BL21(DE3). The highest activity is determined at pH 5.0 and 50 °C toward COBE. The apparent K_m and k_{cat}/K_m are 0.39 mM and 1747 s^{-1} mM^{-1}, endowing *Rp*CR with high catalytic efficiency in reduction of COBE. Employing merely 0.1 g recombinant *Rp*CR-GDH in a toluene-aqueous biphasic system, as much as 7.0 g COBE could be asymmetrically reduced into ethyl (*S*)-4-chloro-3-hydroxybutanoate [(*S*)-CHBE] (>99% *ee*) without addition of external cofactor, achieving molar isolation yield of 91%, substrate to biocatalyst ratio of 70 and space-time yield of 1480 g L^{-1} d^{-1}. Our results indicate the robust *Rp*CR could be potentially applied in the preparation of optically pure (*S*)-CHBE.

1. Introduction

Asymmetric reduction of ketones is one of the most important and practical approaches for the production of chiral secondary alcohols, which is essential for the synthesis of industrially important chemicals such as pharmaceuticals, agrochemicals and natural products [1–3]. As an alternative to chemical methodologies, bioreductive preparation of (*R*) or (*S*)-enantiomers of alcohols using isolated enzymes or whole-cell systems has been extensively investigated due to its high enantio-, regio- and chemoselectivities, mild conditions, reproducibility and easy operation [4,5]. However, two major challenges for the scale-up application of bioreduction systems are the lack of efficient biocatalysts and the necessity of expensive cofactors such as NAD(P)H/NAD(P)$^+$ [6,7]. The employment of robust biocatalysts with high cofactor utilization efficiency could reduce or even avoid the addition of external cofactors. Consequently, exploration of robust biocatalysts is of special interest to solve above issues [8,9].

Ethyl (*S*)-3-hydroxyl-4-chlorobutanoate [(*S*)-CHBE] is a versatile and important chiral intermediate for the production of chiral drugs, including the cholesterol lowering 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors (namely statins) [10,11], which rank the class I best-selling drugs due to their excellent therapeutic effect and low side effect. The asymmetric reduction of ethyl 3-oxo-4-chlorobutanoate (COBE) into (*S*)-CHBE is the most promising approach. In recent years, various microorganisms have been identified for the efficient synthesis of (*S*)-CHBE and well-reviewed by Ye et al. [11]. However the application of wild-type strains is often hindered by their complicated dehydrogenases/reductases systems with variable stereospecificity and low expression level of key reductases [11,12]. An effective solution is discovering novel reductases by genome mining and their heterogeneous overexpression [11,13]. A number of enantioselective carbonyl reductases have been identified with *S*-selectivity in the asymmetric reduction of COBE, including S1 from *Candida magnolia* [14], ARII from *Sporobolomyces salmonicolor* [15], CPE from *Candida parapsilosis* [16], PsCRI and PsCRII from *Pichia stipitis* [17,18], ScCR from *Streptomyces coelicolor* [19], SOU1 from *Candida albicans* [20] and *Dh*CR from *Debaryomyces hansenii* [21]. The highest catalytic efficiency (V_{max} = 349 μmol min^{-1} mg^{-1}) was reported for ARII, however at a high substrate concentration (K_m = 1.49), and the *ee* was relatively lower for application [15]. A carbonyl reductase CPE identified by Wang et al. displayed 99% *ee* and a low K_m (0.19 mM), while its V_{max} (200 μmol min^{-1} mg^{-1}) was not as high as ARII [19].

Besides high stability and enantioslelectivity, the lower K_m and higher V_{max} (or higher k_{cat}/K_m) is one of the crucial parameters of biocatalysts in large-scale application. Biocatalysts with high k_{cat}/K_m value could often reach the maximum velocity and maintain the activity for a longer time even at a low substrate concentration [22]. In current study, a novel carbonyl reductase from *Rhodococcus pyridinivorans* (*Rp*CR) was identified by genome

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mining, and soluble expressed in *Escherichia coli* BL21(DE3). *Rp*CR exhibited a high enantioselectivity, lower K_m than CPE and higher catalytic efficiency in the asymmetric reduction of COBE. Furthermore, the synthesis of (*S*)-CHBE employing *Rp*CR at high substrate loading was achieved in an organic solvent-aqueous biphasic system to evaluate its potential for industrial applications.

2. Experimental procedures

2.1. Cloning and expression of RpCR coding gene in E. coli BL21(DE3)

Genomic DNA was extracted from *R. pyridinivorans* using a TIANamp Bacteria DNA Kit from Tiangen (Shanghai). Primers with *Hin*dIII and *Xho*I restriction sites were designed according to the *Rp*CR coding gene (*rpcr*) sequence (GenBank accession No.: EHK80525.1). The PCR product of *rpcr* was double digested with *Hin*dIII and *Xho*I and then inserted into the expression vector pET28a. The resultant plasmid, pET28-*rpcr*, was transformed into *E. coli* BL21(DE3). The cells were cultivated at 37 °C in LB medium supplemented with 50 μg/mL kanamycin. When OD_{600} of the culture reached 0.6, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.2 mM, and the culture was further cultivated at 25 °C for 12 h.

2.2. Purification of RpCR

Cells were harvested by centrifugation (8000 × *g*, 10 min), washed twice with saline and resuspended in buffer A (20 mM PBS, 500 mM NaCl, 10 mM imidazole, pH 7.4), followed by disruption by ultrasonication (400 W, work 3 s and stop 2 s for 15 min). The cell lysate was centrifuged (10,000 × *g*, 30 min) at 4 °C. Afterwards, the supernatant was loaded onto a Histrap column (1 mL, GE Corp.) pre-equilibrated with buffer A, and the proteins were eluted with an increasing imidazole gradient from 10 to 500 mM with buffer B (20 mM PBS, 500 mM NaCl, 500 mM imidazole, pH 7.4) at a flow rate of 1 mL/min. Then the collected eluents were desalted and concentrated at 4 °C. The purity of fractions was determined by SDS-PAGE. The purified *Rp*CR was stored at −80 °C with 20% glycerol for further use.

2.3. Enzyme activity assay protocol

Standard enzyme activity assay was performed spectrophotometrically by monitoring the changes in absorbance of NADPH at 340 nm and 30 °C. The reaction mixture for *Rp*CR consisted of 2 mM COBE, 0.5 mM NADPH in 190 μL PBS buffer (pH 7.0, 100 mM) and 10 μL enzyme solution with appropriated concentration. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol NADPH per minute under standard condition.

2.4. Characterization of purified RpCR

2.4.1. Optimum pH and temperature

The optimum temperature of *Rp*CR was determined under above mentioned standard condition at various temperatures (25–70 °C). The pH-profile of purified *Rp*CR was determined in the following buffers (final concentration 100 mM): sodium citrate (pH 3.0–6.0), sodium phosphate buffer (pH 6.0–8.0), Glycine-NaOH (pH 8.0–10.0). All the activities were assayed in triplicates.

2.4.2. Effect of metal ions and additives on RpCR activity

The effect of various metal ions and additives (including Mn^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Ag^{+}, Fe^{3+}, Ni^{2+}, Cu^{2+}, Al^{3+}, EDTA, DTT, imidazole, β-mercaptoethanol, SDS, Tris, Triton-X100 and Tween 20) on *Rp*CR activity was examined by adding each compound (final concentration of 1 mM) in the reaction mixture for 60 min at 30 °C, and the residual enzyme activity was measured under the standard condition. Control was performed in the absence of any test compound. All the activities were assayed in triplicates.

2.4.3. Effect of organic solvents on RpCR activity

The influence of organic solvents on *Rp*CR activity was performed by adding 50% *v*/*v* organic solvents (*n*-pentane, cyclohexane, *n*-hexane, *n*-heptane, *n*-decane, *n*-nonane, isooctane, toluene, ethyl lactate, ethyl acetate and glycerol), or 10% *m*/*v* PEG derivatives (PEG400, 600, 1000, 2000, and 4000), or 20% ionic liquid v/v (glycerol, choline chloride (ChCl), ChCl/glycerol, ChCl/urea, ChCl/formic acid, ChCl/acetic acid, ChCl/oxalic acid, ChCl/malonic acid and ChCl/citric acid) in the enzyme solutions (0.1 mg mL^{-1}), and incubated at 30 °C for 12 h. Then the mixtures were centrifuged at 8000 × *g* and 4 °C for 5 min. The activity of the enzyme aqueous phase was determined using standard activity protocol. Protein concentration was measured using Bradford method with BSA as standard protein.

2.4.4. Kinetic analysis

The kinetic parameters of the purified *Rp*CR were determined by determining the activity at different COBE concentrations (0.25–5.0 mM) and a fixed NADPH concentration of 1.0 mM. Similarly, the apparent K_m value for NADPH was determined at different NADPH concentrations (0.1–1 mM) and a fixed COBE concentration of 2 mM. All the activity was assayed in triplicates. The apparent K_m and V_{max} values of the purified *Rp*CR were calculated according to Lineweaver–Burk plot [23].

2.4.5. Substrate specificity

Substrate profile of purified *Rp*CR was determined using standard activity assay method, except different prochiral ketone substrates including diketone, aromatic ketone, α-ketoester and β-ketoester (final concentration, 2 mM) were used. All the activities were assayed in triplicates.

2.4.6. Enantioselectivity

Enantioselectivity of *Rp*CR in the asymmetric reduction of COBE was performed using chiral GC analysis as previously described [21].

2.5. Asymmetric reduction of COBE to (S)-CHBE employing RpCR

2.5.1. Construction of RpCR and GDH coexpression strain

Glucose dehydrogenase coding gene (*gdh*) was cloned from *Bacillus megaterium* and inserted into pACYDuet for expression as previously described [21]. The coexpression of *Rp*CR and glucose dehydrogenase (GDH) in *E. coli* was developed simply by transformation of pET28-*rpcr* and pACYDuet-*gdh* into *E. coli* BL21(DE3), and screening for double resistance against kanamycin and chloramphenicol. Recombinant strain *E. coli* BL21(DE3) harboring pET28-*rpcr* and pACYDuet-*gdh* (*Rp*CR-GDH) was cultivated in LB medium supplemented with 50 μg/mL kanamycin and chloramphenicol at 37 °C and 180 rpm. When OD_{600} reached 0.6, 0.5 mM IPTG was added and further cultivated at 25 °C and 180 rpm for 12 h. The cells were harvested by centrifugation followed by lyophilization. The dry cells of recombinant *E. coli* were stored at 4 °C and used as biocatalyst.

2.5.2. Optimization of (S)-CHBE production by RpCR

The effect of reaction pH on the performance of *Rp*CR was investigated with 1.65 g COBE, 3.0 g glucose and 0.05 g dry cells (*Rp*CR-GDH), in 5 mL PBS buffer (pH 6.0, 6.5, 7.0 and 7.5, 100 mM) and 5 mL toluene at 30 °C and 180 rpm for 6 h. Then the reaction