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Research paper

Enhancement of ethyl (*S*)-4-chloro-3-hydroxybutanoate production at high substrate concentration by in situ resin adsorption

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ABSTRACT

Asymmetric reduction of ethyl 4-chloro-3-oxobutyrate (COBE) by carbonyl reductases presents an efficient way to produce Ethyl (*S*)-4-chloro-3-hydroxybutanoate ((*S*)-CHBE), an important chiral intermediate for the synthesis of hydroxymethylglutaryl-CoA reductase inhibitors such as Lipitor®. In this study, an NADPH-dependent carbonyl reductase (SrCR) from *Synechocystis* sp. was characterized to demonstrate a broad substrate spectrum, and the highest activity (53.1 U/mg protein) with COBE. To regenerate the cofactor NADPH, *Bacillus subtilis* glucose dehydrogenase was successfully coexpressed with SrCR. Owing to the product inhibition, no more than 400 mM of COBE could be completely reduced to (*S*)-CHBE using the recombinant *Escherichia coli*/pET-SrCR-GDH. The macroporous adsorption resin HZ 814 was applied to adsorb (*S*)-CHBE in situ to alleviate the product inhibitio. Consequently, 3000 mM (494 g/L) of COBE was bioconverted within 8 h, resulting in a (*S*)-CHBE yield of 98.2%, with 99.4% *ee* and total turnover number of 15,000, revealed great industrial potential of (*S*)-CHBE production.

1. Introduction

Optically active ethyl 4-chloro-3-hydroxybutanoate (CHBE) is a key intermediate in the synthesis of pharmacologically active compounds (Asako et al., 2009; Forni et al., 2002). The (*R*)-enantiomer is a useful chiral building block for l-carnitine, whereas the (*S*)-enantiomer is a precursor of hydroxymethylglutaryl-CoA reductase inhibitors such as Lipitor®, which is the top-selling pharmaceutical product in the world (Ma et al., 2010; Yamamoto et al., 2004; Zhou et al., 1983). Asymmetric reduction of ethyl 4-chloro-3-oxobutyrate (COBE) by reductases is a more practical way to produce optically active CHBE, since it can be carried out under mild reaction conditions and results in a high yield and remarkable enantioselectivity (Goldberg et al., 2007; Mateo et al., 2007).

However, the biocatalysis of COBE to (*S*)- or (*R*)-CHBE by reductases often requires the presence of the expensive cofactor NADH or NADPH to donate electrons (Nakamura et al., 2003). Effective means for cofactor regeneration, such as through a substrate- and enzyme-coupled system, have attracted attention to reduce the high cofactor costs. Usually, 2-propanol is chosen as a cosubstrate for producing (*S*)-CHBE (Wang et al., 2011). However, the precondition is a robust reductase that can simultaneously transform the substrate and cosubstrate, and the cosubstrate must be applied in large excess to drive the equilibrium toward the desired direction (Cai et al., 2012). The enzyme-coupled system involves a second irreversible enzymatic reaction that frequently employs glucose dehydrogenase (GDH) or formate dehydrogenase for the required cofactor recycling (Liu and Wang, 2007). This method has the advantage of being irreversible, and has been successfully applied in many industrial-scale biocatalysis processes (Weckbecker et al., 2010). For example, a recombinant *Escherichia coli* strain, coexpressing an NADPH-dependent carbonyl reductase (PsCR II) from *Pichia stipitis* for the reduction reaction and a GDH from *Bacillus megaterium* for regeneration of the cofactor NADPH, was successfully used in the enantioselective reduction of COBE (Ye et al., 2010a).

Several reductases have already been cloned and used for the asymmetric synthesis of (*S*)-CHBE, such as SOU1 from *Candida albicans*, S1 from *Candida magnoliae*, and the carbonyl reductase ScCR from *Streptomyces coelicolor* (Cai et al., 2012; Kizaki et al., 2001; Wang et al., 2011). However, the inhibition of the reaction by the substrate or product is still a key limitation to the industrial application of these enzymes. Many attempts have been made to solve this problem. A water/toluene biphasic system was employed for the asymmetric reduction of COBE to (*S*)-CHBE to avoid substrate and product inhibition effects (Wang et al., 2011). Despite the pervasiveness and efficiency of such biphasic bioreaction systems, the high content of organic solvents in the reaction mixture can be quite toxic to the reductases, thereby inhibiting their activity. A robust system for

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alleviating the substrate or product inhibition effect and toxicity turns out to be necessary for large-scale applications.

In this study, an NADPH-dependent carbonyl reductase (SrCR) that showed good activity toward COBE was identified from *Synechocystis* sp. PCC 6803, and its biocatalytic properties were characterized. The GDH from *Bacillus subtilis* (BsGDH) was coexpressed with SrCR to achieve successful cofactor regeneration. Furthermore, a macroporous resin was introduced to alleviate the product inhibition effect, based on the resin's property of in situ adsorption of the product.

2. Materials and methods

2.1. Materials

Synechocystis sp. PCC 6803 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). *B. subtilus* CGMCC 1.1398 was obtained from the China General Microbiological Culture Collection Center (Beijing, China). *E. coli* BL21 (DE3) and the pET28a (+) plasmid were used for expressing the SrCR and BsGDH. The resins used were purchased from Shanghai Huazhen Science and Technology Co., Ltd. (Shanghai, China). Ketones and chiral alcohol standard samples were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

2.2. Cloning and expression of the SrCR gene in E. coli

The genomic DNA of *Synechocystis* sp. PCC 6803 was extracted and purified using the TIANamp Bacteria DNA Kit (Tiangen, Shanghai, China). Oligonucleotide primers (primer 1, 5′-AACGCGGATCCATGTTAAGTCTTGGTTTGGAAG-3′; and primer 2, 5′-AACCCAAGCTTAGGTGTGGTGGGCCCCATTT-3′; restriction sites underlined) with *Bam*HI and *Hin*dIII restriction sites were designed on the basis of the SrCR gene sequence (GenBank Accession No. **AKL80626**). The DNA fragment containing the SrCR gene was amplified by polymerase chain reaction (PCR), double-digested with *Bam*HI and *Hin*dIII, and then inserted into the expression vector pET-28a (+) to obtain plasmid pET-SrCR, which was subsequently transformed into *E. coli* BL21(DE3) cells. The cells were cultivated at 37 °C in Luria-Bertani medium containing 50 μg/mL of kanamycin. When the OD_{600} of the culture had reached 0.6–0.8, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.10 mM, and the induced cultures were further cultured at 20 °C for another 20 h. The cells were harvested by centrifugation (12,000 × *g*, 10 min) at 4 °C, and stored at −20 °C.

2.3. Purification of SrCR

Cells were harvested by centrifugation, washed twice with physiological saline, and then resuspended in buffer A (20 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl, 10 mM imidazole). Cell disruption was carried out by sonication with an ultrasonic oscillator (JY92-2D; Scientz Biotech. Co., Ltd., Ningbo City, China) and the cell lysate was removed by centrifugation (8000 × *g*, 20 min). Then, 10 mL of the supernatant was loaded onto a His-trap Ni-NTA Superflow column (Qiagen, Hilden, Germany) pre-equilibrated with buffer A, and the proteins were eluted with an increasing gradient (from 10 to 500 mM) of imidazole in buffer A at a flow rate of 1 mL/min. The eluted protein was desalted and concentrated by ultrafiltration using a 50-mLAmicon Ultra Centrifugal Filter Device with a molecular mass cutoff of 10 kDa (Millipore, Merck, Billerica, MA, USA). Finally, the sample was stored at −40 °C for further use. The crude extract and the pure enzyme were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined using the Bradford method, with bovine serum albumin as the standard. All purification steps were carried out at 4 °C.

2.4. Enzyme assays

The SrCR activity was assayed spectrophotometrically at 35 °C by monitoring the decrease in the absorbance of NADPH at 340 nm. The reaction mixture consisted of 10 mM of COBE, 0.2 mM of NADPH, 100 mM of sodium phosphate buffer (pH 6.8), 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 these assay conditions.

2.5. Effects of temperature and pH on the activity and stability of purified SrCR

The optimum pH for SrCR activity was determined in the following buffers (final concentration, 100 mM): sodium citrate-citric acid buffer (pH 5.2–6.4), sodium phosphate buffer (pH 6.4–8.0), and Tris–HCl buffer (pH 8.0–9.1). The optimum temperature was determined under the standard condition at various temperatures (25–55 °C). The thermal stability of the enzyme was determined by incubating the purified SrCR at different temperatures (30, 40, or 50 °C) for a required period of time and then assaying its residual activity.

2.6. Substrate specificity

The specific activity of the purified SrCR toward different prochiral ketones (10 mM) with structural diversity was measured under the standard condition by spectrophotometric assay. A control experiment without enzyme was performed for each ketone.

2.7. Effects of substrate and product on the reaction

10-mL reaction mixtures of sodium phosphate buffer (100 mM, pH 6.8), 20 mg/mL of dry cells and $NADP^+$ (0.2 mM) were firstly preincubated for 10 min at 35 °C. Different concentrations (100–800 mM) of COBE were added to the mixtures to measure the substrate inhibition. After 5 min of biotransformation, the reaction was terminated by centrifugation (14,000 × *g*, 5 min). The resulting supernatant was immediately extracted twice with equal volume of ethyl acetate for gas chromatography (GC) analysis to measure (*S*)-CHBE production. To measure the inhibition effects of product, different concentrations of (*S*)-CHBE (100–500 mM) were added to the mixtures, with a substrate concentration of 200 mM. The reaction was terminated by centrifugation to remove the cells after 5 min of biotransformation, and the supernatant was extracted twice with equal volume of ethyl acetate for GC analysis to measure the extent of COBE decrease.

The inactivation effects of substrate and product were also measured. 10-mL mixtures of sodium phosphate buffer (100 mM, pH 6.8), 20 mg/mL of dry cells and different concentrations of COBE (400–800 mM) or (*S*)-CHBE (200–400 mM) were shaken on an orbital shaker at 150 rpm for a certain time. Samples (1 mL) were taken at regular intervals, following by centrifugation and the cells were washed twice with physiological saline. The cells' residual activities for COBE were measured by spectrophotometric assay. The experiments were performed in triplicates.

2.8. Coexpression of the SrCR and BsGDH genes

To construct a coexpression plasmid, two synthetic primers (primer 3, 5′-CCCAAGCTTAAGGAGATATACATATGTATCCGGATTTA-3′; and primer 4, 5′-CCGCTCGAGTTAACCGCGGCCTGCCTG-3′; restriction sites underlined) were prepared for PCR using the genomic DNA of *B. subtilis*, which contains the *gdh* gene used as the template. The PCR-generated fragment was digested with *Hin*dIIIand *Xho*I, and the fragment was then ligated to the correspondingly digested plasmid pET-SrCR. The resultant plasmid pET-SrCR-GDH containing the genes of the two enzymes was transformed into *E. coli* BL21(DE3) cells (Fig. 1a). The expression