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Efficient synthesis of (*R*)-2-chloro-1-phenylethol using a yeast carbonyl reductase with broad substrate spectrum and 2-propanol as cosubstrate

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ABSTRACT

In the screening of six *Escherichia coli* strains for overexpressing recombinant short-chain dehydrogenases/reductases from *Yarrowia lipolytica* ACA-DC 50109, an NADPH-dependent carbonyl reductase (YlCR) was identified with capability of producing chiral alcohols. The protein YlCR was over-expressed in *E. coli* BL21 (DE3), purified to homogeneity, and characterized of biocatalytic properties. The purified enzyme exhibited the highest activity at 35 °C and optimal pH at 7.0. The kinetic parameters K_m and K_{cat} of YlCR were 7.59 mM and 3.9 S^{-1} for α-chloroacetophenone, 366.1 mM and 2.94 S^{-1} for 2-propanol. YlCR showed a broad substrate spectrum toward aldehydes, ketones, α- and β-keto esters. Among them, α-chloroacetophenone was found to be efficiently converted to (*R*)-2-chloro-1-phenylethol, the precursor for the synthesis of anti-depressants and α- or β-adrenergic drugs. Using 2-propanol as the hydrogen donor, α-chloroacetophenone (50 mM) was reduced with the recombinant *E. coli*, and the obtained conversation and enantiomeric excess (e.e) were both 99%. When the α-chloroacetophenone concentration was up to 200 mM, the conversion achieved 63%, and the e.e was always 99%. The present study serves as a valuable guidance for the future applications of this versatile biocatalyst.

1. Introduction

The asymmetric reduction of carbonyl compounds is one of the most important reactions for the production of chiral alcohols, which can be transformed into various functions for many industrial chemicals such as pharmaceuticals, agrochemical and natural products [1,2]. For example, (*R*)- or (*S*)-2-choro-1-phenylethanol is a precursor of enantiopure intermediates in the production of anti-depressants and α- or β-adrenergic drugs, such as fluoxetine, tomoxetine and nisoxetine [3,4].

The coenzyme NADPH/NADH regenerating system is essential in efficient bioreduction processes to produce chiral alcohols from ketones [5]. However, the cofactor is so expensive that become a challenge of its application [6], and NADPH/ NADH is very unstable [7]. Therefore, efficient and cost-effective cofactor regeneration systems, such as enzyme- and substrate-coupled system, had been developed. Three practical methods have been reported for regenerating NADH/NADPH. Formate dehydrogenase (FDH) [8] or glucose dehydrodgenase (GDH) [9] could be used as enzyme-coupled systems to recycle NAD^+ or $NADP^+$. 2-propanol could be used as a co-substrate, owing to not only the simultaneous recycling of cofactor and reduction of prochiral ketone by a single dehydrogenase/reductase, but also the increase of substrate solubility in the presence of 2-propanol [10].

Carbonyl compounds are a challenge for biocatalysis because substrate solubility is low in the aqueous phase in which the enzyme are usually present [11,12]. *Yarrowia lipolytica* is a non-conventional yeast which is often found in oil fields [13], and therefore its enzymes seem to well metabolize with both polar and non-polar substrates [14]. In previous work, *Y. lipolytica* has been efficiently employed in the reduction of various carbonyl compounds [15–17]. With the rapid development of genomic, proteomics and bioinformatics, the candidates of ideal biocatalysts could be discovered and characterized from *Y. lipolytica* [18], which prompted us to investigate enzymes from this organism.

Based on the homology driven "genome mining" from *Y. lipolytica*, an NADPH-dependent carbonyl reductase (YlCR) was cloned and expressed in *Escherichia coli* BL21 (DE3). The reduction of α-chloroacetophenone to optically pure (*R*)-2-chloro-1 phenylethol

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was examined using the recombinant cells with NADPH self regeneration in the presence of inexpensive co-substrate 2-propanol.

2. Materials and methods

2.1. Bacterial strains, plasmids and chemicals

Y. lipolytica ACA-DC 50109 [19] was kindly offered by Prof. George Aggelis from Department of Biology, University of Patras, Greece. *E. coli* BL21 (DE3) was an expression host. The pMD18-T (TaKaRa, Dalian, China) vector and the pET28a plasmid (Novagen, Shanghai, China) were used as clone vector and expression vector, respectively. Restriction enzymes *Bam*HI, *Xho*I, T4 ligase were obtained from TaKaRa (Dalian, China). The enzyme GDH was from our laboratory. All carbonyl compounds were purchased from Alfa Aesar or Sigma–Aldrich.

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

Using DNA extraction kit, genomic DNA was extracted from *Y. lipolytica* ACA-DC 50109. Oligonucleotide primers with *Bam*HI and *Xho*I restriction sites were designed according to six putative carbonyl reductase sequences (GenBank accession No. XP_501554.1, XP_502306.1, XP_504895.1, XP_504870.1, XP_500246.1, XP_503594.1). The DNA fragments of six putative carbonyl reductase genes were amplified and double-digested with *Bam*HI and *Xho*I, and then inserted into the expression vector pET28a. The resulting plasmids, pET28a-CR, were transformed into *E. coli* BL21 (DE3) cells. The recombinant cells were cultivated at 37 °C in LB medium containing 50 μg/ml kanamycin. When the $OD_{600\,nm}$ of the culture reached 0.8, IPTG was added to a final concentration of 1 mM. Then the cultivation was continued at 30 °C for 10 h.

2.3. Purification of YlCR

Cells were harvested by centrifugation at 6000 × *g* for 10 min at 4 °C, washed twice with sodium phosphate buffer and resuspended in sodium phosphate buffer. After disruption with a high pressure homogenizer, the cell debris was removed by centrifugation at 12,000 × *g* for 30 min at 4 °C. Protein purification was performed using an AKTA purifier 10 system with UNICORN 5 software. The YlCR was purified using Ni-NTA HisTrap FF crude column chromatography (5 ml, GE Healthcare Corp). The column was pre-equilibrated with buffer A (100 mM sodium phosphate buffer, 0.5 M 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 B (100 mM sodium phosphate buffer, 0.5 M 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. Finally, the protein was concentrated and stored at −20 °C with 10% glycerol. Protein concentration was measured by BCA protein assay kit (Tiangen, Beijing, China).

2.4. Enzyme assay

YlCR activity was assayed at 35 °C by monitoring the decrease in 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 substrate, 0.4 mM of NADPH, and 10 μl of the purified enzyme YlCR. The oxidative reaction of YlCR was also measured at 340 nm in a 0.2 ml reaction mixture containing 100 mM of sodium phosphate buffer (pH 7.0), 5 mM of 2-propanol, 0.4 mM of $NADP^+$ and 10 μl of the purified enzyme YlCR. One unit of enzyme activity was defined as the amounts of enzyme that catalyzed the oxidation of 1 μmol of NADPH per minute or the reduction of 1 μmol of $NADP^+$ per minute.

2.5. Characterization of YlCR

2.5.1. Determination of pH optimum

The effects of pH on enzyme activity were examined in the range of pH 5.5–9.0. The buffers included 100 mM acetate buffer (pH 5.5–6.0), 100 mM sodium phosphate buffer (pH 6.0–8.0), and 100 mM Tris–HCl buffer (pH 8.0–9.0). The residual activity was measured under the standard assay condition.

2.5.2. Determination of temperature optimum and thermal stability

The optimum temperature for enzyme activity was determined at various temperatures in the range of 20–50 °C. For determining thermal stability, the enzyme was incubated at the desired temperatures (30 °C, 35 °C and 40 °C). The residual activity was tested under the standard assay condition.

2.5.3. Effects of metal ions, EDTA and organic solvents

The effects of various metal ions at 1 mM, EDTA at 1 mM and organic solvents at 10% v/v on the enzyme were tested by pre-incubating the enzyme in the reaction mixture for 20 min at 35 °C. The residual activity of the enzyme was measured under the standard assay condition. All experiments were conducted in triplicate.

2.6. Kinetic analysis

The YlCR kinetic parameters were performed by assaying the activity on the different reaction conditions. For α-chloroacetophenone, 0.4 mM of NADPH, and α-chloroacetophenone in the range of 2–30 mM were used for the activity assay. For NADPH, 5 mM of α-chloroacetophenone, and NADPH in the range of 0.04–0.24 mM were used for the activity assay. For 2-propanol, 0.5 mM of $NADP^+$, and 2-propanol in the range of 20–1500 mM were used for the activity assay. For $NADP^+$, 0.5 M of 2-propanol, and $NADP^+$ in the range of 0.05–0.7 mM were used for the activity assay. All data were fitted to the Michaelis–Menten equation using GraphPad Prism v5.0 (GraphPad Software, San Diego, CA, USA) to generate estimates of K_m and K_{cat} values.

2.7. Biotransformation of various carbonyl compounds

The enantioselectivity of YlCR in the reduction of carbonyl compound was determined using an NADPH regeneration system which contained 0.5 mM $NADP^+$, 20 mM carbonyl compound, 0.8 U of YlCR, 0.4 U of GDH, and 60 mM glucose in 1 ml of 100 mM sodium phosphate buffer. The reaction mixture was incubated at 35 °C with shaking for 12 h and extracted with ethyl acetate (1 ml). The organic extract was dried over anhydrous sodium sulfate and subjected to chiral GC or HPLC to determine the conversion and enantiomeric excess (e.e) as reported previously [20–22]. The absolute configurations of product alcohols were identified by comparing the retention time with that of the standard samples. A control experiment was performed under same conditions without addition the enzyme YlCR and no reduction product was observed. GC analyses were performed using a CP-Chirasil-DEX CB (Varian, USA). HPLC analyses were performed using Chiralcel OD-H column (Daicel Co., Japan).