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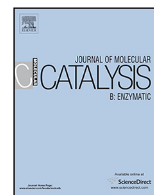

Genomic mining-based identification of novel stereospecific aldo-keto reductases toolbox from *Candida parapsilosis* for highly enantioselective reduction of carbonyl compounds

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ABSTRACT

Biocatalytic reduction of prochiral ketones offers significant potential in synthesis of optically active alcohols. However, so far the application of aldo-keto reductases (AKRs) in asymmetric reduction has been hampered due to limited availability of AKRs with high enantioselectivity and catalytic efficiency. Based on the genome sequence of *Candida parapsilosis*, a versatile bioresource for asymmetric reduction, eight open reading frames encoding putative AKRs were discovered and expressed, and the resulted enzymes (CPARs), comprising an AKR toolbox, were evaluated toward various carbonyl substrates. The CPARs were active to the selected substrates, especially 2-hydroxyacetophenone and ethyl 4-chloro-3-oxobutyrate. Additionally, most of them were obviously enantioselective to the substrates and gave alcohol products with optical purity up to 99%e.e. Of the enzymes, CPAR4 was outstanding with excellent enantioselectivity and broad substrate spectrum. All these positive features demonstrate that genomic mining is powerful in searching for novel and efficient biocatalysts of desired reactions for pharmaceuticals and fine chemicals synthesis.

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1. Introduction

Optically active alcohols are useful chiral intermediates for the synthesis of pharmaceuticals, agricultural chemicals, and specialty materials [1,2]. Compared with the conventional chemical process, biocatalytic asymmetric reduction is one of the efficient ways due to its high chemo-, enantio-, and regioselectivities [3–5]. For the production of chiral alcohols from the corresponding prochiral aldehydes and ketones, the aldo-keto reductases (AKRs) including aldehyde reductase (EC 1.1.1.21) and carbonyl reductase (EC 1.1.1.184) have the inherent advantages over other enzyme systems in terms of their effectiveness, pertinence, and diversity in catalyzing reduction [6,7].

The NAD(P)H-dependent AKRs with broad physiological roles have been found in vertebrates, invertebrates, plants, protozoa, fungi, eubacteria, and archaebacteria [8]. Several microbial enzymes have already been cloned and used for the asymmetric synthesis of chiral alcohols, such as ARI from *Sporobolomyces salmonicolor* [9], Conjugated polyketone reductase (CPR-C1 and CPR-C2) from *Candida parapsilosis* [10], and KER from *Penicillium citrinum* [11]. However, so far the resources of functional enzymes are yet not sufficient and the application of AKRs in the reduction of aldehydes or ketones has been hampered due to the limited availability [12]. Therefore, discovery and identification of AKRs with the application potential should be critical for bio-mediated asymmetric synthesis.

With the rapid development of genomics, proteomics, and bioinformatics, the candidates of ideal biocatalysts could be discovered and characterized significantly [13,14]. One typical example is that 18 key reductases from bakers' yeast were overexpressed and tested for their abilities of reducing α- or β-ketoesters after analysis of the yeast genome [15]. Within this area, the genus *Candida* spp. has been taken as an important source of oxidoreductases for biocatalytic redox reactions including enantioselective keto-reductions [16]. Of them, *C. parapsilosis* has been described as a highly efficient biocatalyst for kinds of asymmetric reductions [17–19], involving stereospecific alcohol dehydrogenases and

Abbreviations: AKR, aldo-keto reductase; ORF, open reading frame; IPTG, isopropyl-β-D-thiogalactopyranoside; TCEP, Tris (2-carboxyethyl) phosphine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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carbonyl reductases [20,21]. Besides the functional conjugated polyketone reductase [22], however, the available AKRs are yet limited for practical application and it would be necessary to discover and identify new enzymes systematically from the promising functional microorganism of *C. parapsilosis* [23].

The disclosure of microbial genome sequence allows scientists to search for novel enzymes with potential applications [24,25]. The genome sequence of *C. parapsilosis* (http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/) provides us a research avenue to dig biocatalytic resource of new enzymes from the microorganism by genomic mining [26]. In this report, by analyzing the genome sequence of *C. parapsilosis*, a biocatalytic toolbox was discovered, comprising eight open reading frames (ORFs) encoding putative AKRs. After expression of these ORFs, the encoded proteins were purified and functionalized as stereospecific AKRs catalyzing enantioselective reduction toward various carbonyl compounds including aryl ketones, aliphatic ketones, and ketoesters. This study would represent a systematic investigation on *C. parapsilosis* enzymes catalyzing stereoselective carbonyl reductions, which would serve as a useful guideline for future development of new AKRs and also the enzymatic processes for synthesis of optically pure alcohols.

2. Materials and methods

2.1. Materials

C. parapsilosis CCTCC M203011 was obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China). *Escherichia coli* strain XL-10 Gold was used for gene cloning. *E. coli* BL21 (DE3) was used for gene expression. The plasmid pET21c was obtained from Novagen (USA) served as expression vector. All enzymes used for DNA manipulations were obtained from TaKaRa Biotechnology Co., Ltd (Dalian, China). The cofactors including NAD(P)H and $NAD(P)^+$, the substrates including aryl ketones (acetophenone, 2-hydroxyacetophenone, *o*-chloroacetophenone *m*-chloroacetophenone, and *p*-chloroacetophenone), aliphatic ketones (2-octanone and 2-hexanone), and ketoesters (ethyl 4-trifluoro-3-oxobutyrate, methyl 3-oxobutyrate, ethyl 3-phenyl-3-oxopropionate, and ethyl 4-chloro-3-oxobutyrate), and the standard samples of chiral alcohol products corresponding to the above carbonyl substrates were purchased from the Sigma–Aldrich Chemical Co. (USA). All other used chemicals were of analytical grade and commercially available.

2.2. Search for potential genes and sequence analysis

Discovery of potential AKRs was carried out by NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BLAST against the complete *C. parapsilosis* genome (http://www.sanger.ac.uk/sequencing/Candida/parapsilosis/) [26]. Multiple sequence alignment was performed using Clustal X software [27]. The phylogenetic trees were deduced from the alignment using the neighbor-joining method of MEGA [28].

2.3. Cloning and expression of genes encoding AKRs

The genes encoding the eight AKRs were amplified from *C. parapsilosis* genome using appropriate primer pairs with the restriction sites of *Nde*I and *Xho*I (Table 1). The purified fragments were digested with these restricted enzymes and ligated into pET21c expression vector. Then the verified recombinant plasmids were transformed into the *E. coli* BL21 (DE3) competent cells. The transformants were grown in Luria–Bertani (LB) medium (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl, 5 g L^{-1}) containing 100 μg mL^{-1} ampicillin at 37 °C.

Table 1
Oligonucleotide primers for amplification of the genes encoding CPARs from *C. parapsilosis* genome, involving restriction sites of *Nde*I and *Xho*I underlined.

Gene	Primer sequence
cpar1	F: 5′-CCCGCCCGCATATGACTCCACAACCAATTGAG-3′ R: 5′-GCCCGCTCGAGCTGAAACAATGAGCCTTCACTTTG-3′
cpar2	F: 5′-CCCGCCCGCATATGTCTACTACAATCAAGAAAGCC-3′ R: 5′-GCCCGCTCGAGATCAAATTCTTTACTCAAAGTGTCTC-3′
cpar3	F: 5′-CCCGCCCGCATATGACCCATCAGCGCAGTCTTC-3′ R: 5′-GCCCGCTCGAGCTCATCCTTATACAATGTTGGATC-3′
cpar4	F: 5′-CCCGCCCGCATATGTCAGCTCAATTGAAAGTAAAC-3′ R: 5′-GCCCGCTCGAGGTCATTGAAGTTGTTGAAGCCTG-3′
cpar5	F: 5′-CCCGCCCGCATATGTCATATAGACTAATTCAATTAAAC-3′ R: 5′-GCCCGCTCGAGTGGGGCATTGGTACATTCCC-3′
cpar6	F: 5′-CCCGCCCGCATATGAGCTCTCCTTTACCCTCAC-3′ R: 5′-GCCCGCTCGAGCAGGTGACCGCTTGGCCAC-3′
cpar7	F: 5′-CCCGCCCGCATATGACTCAAAGTAACTTACTACC-3′ R: 5′-GCCCGCTCGAGCAAATCTTTAAATTGCTCATGGAAG-3′
cpar8	F: 5′-CCCGCCCGCATATGTCAATTGAGCTCAAGTACAAT-3′ R: 5′-GCCCGCTCGAGAGAGAGGGACTGTTTGGGTTTA-3′

The recombinant cells were cultivated in 4 mL LB liquid medium containing 100 μg mL^{-1} ampicillin at 37 °C and 200 rpm for 12 h. Then the culture was inoculated into a 250-ml Erlenmeyer flask containing 50 ml fresh LB medium supplemented with 100 μg mL^{-1} ampicillin. When the culture turbidity (OD_{600nm}) increased to the level between 0.6 and 0.8, the expressions of target recombinant proteins were initiated with the optimization of the following conditions, where isopropyl-β-D-thiogalactopyranoside (IPTG) (0.1, 0.5, and 1.0 mM) or lactose (2%, 4%, and 6%) was added as inducer and the culture was incubated under different temperatures (17, 20, 25, 30, and 35 °C) at 200 rpm for additional 12 h. The yield of target protein was evaluated by calculating the amount of purified protein obtained from the corresponding culture broth.

2.4. Purification of recombinant enzymes

The cells were suspended in binding buffer (20 mM Tris-HCl, pH6.5, 0.3 M NaCl, 40 mM imidazole, 1× protease inhibitors, 1 mM Tris (2-carboxyethyl) phosphine (TCEP)) and disrupted on ice with an ultrasonic oscillator (VCX750, Sonic). The supernatant of the cell lysate was collected by centrifugation at 26,000 × *g* for 40 min at 4 °C and purified by an AKTAxpress system using HisTrap HP affinity column (GE Healthcare, USA). Elution was carried out with 300 mM imidazole in the same buffer at a flow rate of 2.0 mL min^{-1}. Then the purified fractions were exchanged into low salt buffer (10 mM Tris–HCl, pH 6.5, 0.1 M NaCl, 0.02% NaN_3, 5 mM D,L-dithiothreitol) using disposable PD-10 desalting columns (GE Healthcare, USA) [29]. The final recombinant enzymes were purified to homogeneity as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% polyacrylamide gels [29]. The amount of purified protein obtained from the corresponding culture broth was used to evaluate the yield of target protein. These final preparations of purified enzymes were used in all of the experiments in this study.

2.5. Enzyme activity assay

The enzyme activity of AKR was measured by a continuous spectrophotometric assay using the standard assay mixture containing 0.1 M potassium phosphate buffer (pH 6.5), 0.5 mM NAD(P)H, 5 mM substrate, and the appropriate enzyme in a total volume of 100 μL. The involved substrates of carbonyl compound included aryl ketones (acetophenone, 2-hydroxyacetophenone, *o*-chloroacetophenone *m*-chloroacetophenone, and *p*-chloroacetophenone), aliphatic ketones (2-octanone and 2-hexanone), and ketoesters (ethyl 4-trifluoro-3-oxobutyrate, methyl 3-oxobutyrate, ethyl

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