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Activity improvement of a *Kluyveromyces lactis* aldo-keto reductase KlAKR via rational design

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ABSTRACT

Optically pure *t*-butyl 6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate ((*R*)-**1b**) is the key chiral precursor for atorvastatin calcium, the most widely used cholesterol-lowering drug. Wild-type aldo-keto reductase KlAKR from *Kluyveromyces lactis* has ideal diastereoselectivity toward *t*-butyl 6-cyano-(5*R*)-hydroxy-3-oxohexanoate (**1a**, de_p >99.5%) but poor activity. A rational engineering was used to improve the KlAKR activity. Based on homology modeling and molecular docking, two amino acid residues (295 and 296) were selected as mutation sites, and two rounds of site-saturation mutagenesis were performed. Among the mutants, KlAKR-Y295W/W296L exhibited the highest catalytic efficiency (k_{cat}/K_m) toward **1a** up to 12.37 s^{-1} mM^{-1}, which was 11.25-fold higher than that of wild-type KlAKR. Moreover, the majority of mutations have no negative impact on stereoselectivity. Using KlAKR-Y295W/W296L coupled with *Exiguobacterium sibiricum* glucose dehydrogenase (EsGDH) for cofactor regeneration, (*R*)-**1b** was accumulated up to 162.7 mM with de_p value above 99.5%. KlAKR-Y295W/W296L represents a robust tool for (*R*)-**1b** synthesis.

1. Introduction

Chiral alcohols are remarkably versatile intermediates, which are widespread applied in the introduction of chiral center into pharmaceuticals, agricultural chemicals, foods and special materials (Chen et al., 2012; Clouthier and Pelletier 2012; Itoh 2014; Wang et al., 2011). Due to its mild reaction conditions, exquisite stereoselectivity, environmentally friendly and energy-effective operations, biocatalyzed asymmetric ketone reduction is identified as a reliable, scalable and straightforward route to optically active alcohols (Choudhury et al., 2014; He et al., 2014; Ni and Xu 2012). Therefore, asymmetric bioreduction gained extensive attention both in academy and industry. However, the shortcomings of nature enzymes such as low stability, low catalytic efficiency and low specificity hampered their industrial applications (Choi et al., 2015). Fortunately, with recent developments in computational techniques and bioinformatics, protein engineering using directed evolution or rational design has been developed, and acts as a powerful tool for tailoring enzymes (Lutz and Bornscheuer 2009; Zheng and Xu 2011).

The rapid development of genetic technologies has supplied convenience for redesigning enzyme properties by gene manipulation (Illanes et al., 2012). Combination of sequence-based information with computational modeling tools, promising target sites were selected by computational prediction. Using this strategy, the mutation library size is dramatically reduced. Therefore, the efficiency of biocatalyst tailoring, including stereoselectivity, substrate specificity and stability etc can be significantly increased (Illanes et al., 2012; Lutz 2010). Rational protein engineering was mainly applied to alter substrate specificity (Huang et al., 2014) and selectivity (Xue et al., 2015), and to enhance the activity (Jakoblinnert et al., 2013; Jeong et al., 2014), stability (Guo et al., 2015) and organic solvent tolerance (Dabirmanesh et al., 2015) of enzyme by changing amino acids in the substrate binding pocket.

In our previous work, a NADH-dependent aldo-keto reductase KlAKR has been discovered from *Kluyveromyces lactis*, which catalyzes the asymmetric reduction of *t*-butyl 6-cyano-(5*R*)-hydroxy-3-oxohexanoate (**1a**) to *t*-butyl 6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate ((*R*)-**1b**), the key chiral building block of atorvastatin calcium (Luo et al., 2015). Herein, a rational design

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based on the combination of molecular docking with two rounds site-saturation mutagenesis was applied to modify KlAKR, yielding a positive mutant Y295W/W296L with significantly enhanced activity.

2. Materials and methods

2.1. Materials

Optically pure **1a** (approximately 75 wt% content, *ee* > 99.5%) was generously presented by Neo-Dankong Pharmaceutical Co., Ltd (Taizhou, China). (*R*)-**1b** was purchased from Toronto Research Chemicals Co. Ltd. (Toronto, Canada). NADH was obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Restriction enzymes and T4 DNA ligase were purchased from Thermo Fisher Scientific Co., Ltd (Beijing, China). Prime Star HS DNA Polymerase was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Oligonucleotides synthesis and DNA sequencing were conducted at Shanghai Sunny Biotechnology Co., Ltd. (Shanghai, China). AxyPrep DNA Gel Extraction Kit and Plasmid Mini Kit were purchased from Axygen Scientific Inc. (Hangzhou, China). The *Escherichia coli* BL21 (DE3) and pET-28b (+) used for recombinant protein expression were purchased from Novagen (Shanghai, China). The *E. coli* BL21 (DE3)/pET-28b (+)-EsGDH was stored in our laboratory. Unless otherwise specified, all other reagents and chemicals used in this study were obtained from general commercial suppliers and used without further purification.

2.2. Synthesis and cloning of KlAKR gene

The codons of *klakr* (GenBank accession number: XM_455342.1) with a length of 927 bp was optimized regarding *E. coli* preference by Gene Designer (Villalobos et al., 2006), and synthesized by Shanghai Xuguan Biotechnological Development Co., Ltd. (Shanghai, China). The codon-optimized nucleotide sequence was deposited in the GenBank database under the accession number of KU145407, and cloned into the pET-28b (+) vector. Due to the absence of the termination codon (TAG) at the 3′ end of the nucleotide sequence, a 6 × his-tag was introduced into the C-terminus. Subsequently, the recombinant plasmid pET28b-*klakr*, was transformed into *E. coli* BL21 (DE3) competent cells using the heatshock method (Chung et al., 1989).

2.3. Homology modeling and docking

The three-dimension models of KlAKR and its mutants were constructed using the program Modeller 9.12, based on the crystal structure of the conjugated polyketone reductase C2 (CPR-C2) from *Candida parapsilosis* IFO 0708 (PDB ID: 3VXG) acting as template. The KlAKR showed 44% sequence identity with the CPR-C2. They share the catalytic tetrad (Asp-Tyr-Lys-His) of the aldo-keto reductase superfamily. The molecular docking procedure was performed according to the program AutoDock 4.0, and the images were created with PyMOL.

2.4. Site-saturation mutagenesis

The first round of site-saturation mutagenesis at the site Y295 was performed with pET28b-*klakr* as the template, and pairwise primers were designed as follows: Y295-F: 5′-TGCGTCTCNNSTGGACTGACGTGTACAGC-3′, Y295-R: 5′-GGCCGAGACGCAGAGNNSACCTGACTG-3′. The second round of site-saturation mutagenesis (combinatorial mutagenesis at the site of 295 and 296) at the site W296 was performed by using mutant Y295W as the template, and pairwise primers designed as follows: Y295W/W296-F: 5′-CGTCTCTGGNNSACTGACGTGTACAGCAAAT-3′, Y295W/W296-R: 5′-GCCGAGACGNNSAGAGACCGACTGACTGCA-3′. PCR was carried out with Prime Star HS DNA polymerase (Dalian, China) under the following conditions: initial denaturation at 95 °C for 5 min, and then 35 cycles (95 °C for 15 s, 56 °C for 15 s and 72 °C for 7 min), followed by a final extension at 72 °C for 10 min. The reactions were carried out in 25 μl Eppendorf tubes containing 1 × buffer with 1.0 mM $MgSO_4$, 0.2 mM dNTP, 0.2 mM both primers, 50 ng template and 1 U of Prime Star HS DNA polymerase. The PCR products were digested with Dpn I to remove parent plasmid, then transformed into *E. coli* BL21 (DE3) competent cells, and selected on Luria-Bertani (LB) agar plates containing 50 μg ml^{-1} kanamycin. The positive clones were verified by DNA sequencing.

2.5. Expression of KlAKR and glucose dehydrogenase

E. coli BL21 (DE3) harboring the pET28b(+) containing the gene *klakr* or *esgdh* was cultivated in LB medium in the presence of 50 μg ml^{-1} kanamycin for 10 h at 37 °C and 160 rpm. Then the preculture was transferred to fresh, sterilized LB medium containing 50 μg ml^{-1} kanamycin, and cultivated under the same conditions. When the optical density of the culture at 600 nm reached 0.8, cultivation temperature was altered to 28 °C, and lactose was added into the broth at a final concentration of 9.0 g l^{-1} to induce gene expression. After an additional incubation at 160 rpm for 12 h, cells were harvested by centrifugation at 12,000 rpm, 4 °C for 10 min, and then washed twice with sodium phosphate buffer (20 mM, pH 8.0).

2.6. Purification of KlAKR

The cell pellet of KlAKR (wild-type KlAKR or mutants) was suspended in buffer A (20 mM sodium phosphate buffer pH 8.0 containing 500 mM NaCl and 20 mM imidazole), and disrupted on ice by sonication. The supernatant of lysate was collected by centrifugation at 12,000 rpm, 4 °C for 20 min, and then loaded onto Ni^{2+}-nitrilotriacetic acid column (1.6 cm × 10 cm, Bio-Rad, USA) pre-equilibrated with buffer A. The column was first subjected to a thorough wash with 5 column volume buffer A to remove the unbound proteins, followed by elution with buffer B (20 mM sodium phosphate buffer pH 8.0 containing 500 mM NaCl and 500 mM imidazole) at a flow rate of 1.0 ml min^{-1}. The target protein fractions were collected, combined and dialyzed overnight against 20 mM sodium phosphate buffer (pH 8.0). All purification steps were conducted at 4 °C.

2.7. Enzyme assay and determination of kinetic parameters

Aldo-keto reductase activity was measured by a continuously monitoring the decrease in the absorbance of NADH at 340 nm using **1a** as the substrate. The standard assay mixture for the enzyme activity was composed of 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM NADH, 0.5 mM **1a** and appropriate amount of enzyme in a total volume of 200 μl. One unit of enzyme activity was defined as the amount of enzyme required for the oxidation of 1 μmol NADH per minute. The protein concentration was measured with bicinchoninic acid (BCA) protein assay kit (Nanjing KeyGen Biotech. Inc., China) (Smith et al., 1985). Since the majority of aldo-keto reductases obey the sequence reaction mechanism (Luo et al., 2015; Yuan 2012; Zuccotti et al., 2001), the kinetic analysis and the calculation of kinetic parameters were carried out as described previously (Luo et al., 2015).

2.8. Substrate specificity

To investigate the substrate spectrum, the specific activity of the KlAKRs toward various carbonyl compounds including **1a**,