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Cloning, expression, and characterization of a novel diketoreductase from *Acinetobacter baylyi*

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Reductions of carbonyl groups catalyzed by oxidoreductases are involved in all biological processes and are often a class of important biocatalyst. In this article, we report a novel enzyme designated as diketoreductase (DKR) that was able to reduce two carbonyl groups in a diketo ester to corresponding dihydroxy ester with excellent stereoselectivity. The DKR was cloned from *Acinetobacter baylyi* by reverse genetic method, heterogeneously expressed in *Escherichia coli*, and purified to homogeneity by two chromatographic steps. This novel enzyme exhibited dual cofactor specificity, with a preference of NADH over NADPH. The dihydroxy ester product catalyzed by the DKR was only 3R,5S-stereoisomer with both diastereomeric excess and enantiomeric excess values more than 99.5%. In addition, some biochemical properties of the enzyme, such as the optimal pH and temperature, were also characterized. Furthermore, sequence analysis indicated that this new enzyme was homologous to bacterial 3-hydroxyacyl coenzyme-A dehydrogenase. More importantly, based on the unique catalytic activity and excellent stereoselectivity, the DKR could be utilized in the synthesis of valuable chiral drug intermediates, such as Lipitor®.

Keywords diketoreductase; oxidoreductase; stereoselectivity; chiral alcohols; statin

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Introduction

Ketoreductases, an abundant group of oxidoreductase, are present in various bacteria, yeast, and fungi. They commonly participate in many biological processes in all living organisms [1]. Due largely to the capability of producing chiral alcohols and high enantioselectivity, ketoreductases have been recognized and utilized as an important class of versatile biocatalysts in the chemical and pharmaceutical industries for the preparation of chiral intermediates [2–9].

Introduction of a chiral center in a substrate with single or multiple carbonyl groups can be easily accomplished by different ketoreductases with NADH or NADPH as a cofactor [10]. For example, *Lactobacillus brevis* converts the δ-keto group of a β, δ-diketo ester to its δ-hydroxy product [10,11]. However, the stereoselective reduction of two carbonyl groups in the same molecule to form a chiral β, δ-dihydroxy product by a single enzyme has not been evidently characterized. Previously, screening of microorganisms with ethyl-6-(benzyloxy)-3,5-dioxohexanoate (**1**) revealed that an *Acinetobacter* species was able to reduce both β and δ-carbonyl groups of the substrate to its 3R,5S-ethyl-6-(benzyloxy)-3,5-dihydroxyhexanoate (**2**) product with 93.2% enantiomeric excess ('ee') and 63.3% diastereomeric excess ('de'), and an enzyme possibly responsible for the conversion was isolated and preliminarily studied [12,13]. However, the identity and detailed characterization of such an interesting enzyme has not been further investigated.

In the present study, the gene encoding a diketoreductase (DKR), catalyzing double reduction of a β, δ-diketo ester as shown in **Fig. 1**, was cloned from *Acinetobacter baylyi* ATCC 33305 and expressed in *Escherichia coli* in a soluble form. After purification of the recombinant enzyme, the properties of the enzyme, in terms of the catalytic activity and stereoselectivity, were thoroughly characterized. It is expected that the unique reaction catalyzed by DKR with unprecedented stereoselectivity could be useful for the synthesis of chiral intermediates,

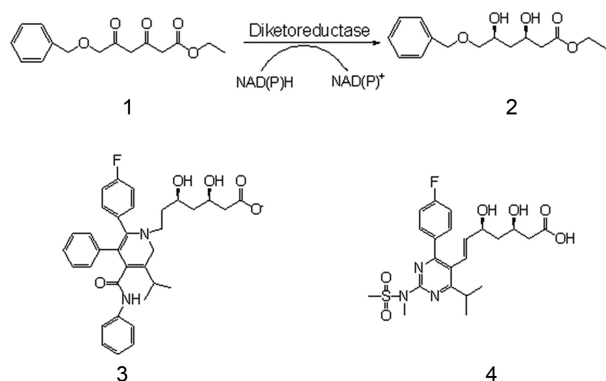

Fig. 1 Reaction catalyzed by DKR and structures of synthetic statins; **1**, ethyl 3, 5-diketo-6-benzyloxyhexanoate; **2**, ethyl 3R,5S-dihydroxy-6-benzyloxy hexanoate; **3**, Atorvastatin; **4**, Rosuvastatin.

particularly for the chiral dihydroxy hexanoate chains of synthetic statin drugs.

Materials and Methods

Strains and chemicals

Acinetobacter baylyi ATCC 33305 was obtained from American Type Culture Collection (ATCC). pET 22b (+) expression vector was purchased from Novagen (Gibbstown, USA). NADH, NADPH, NAD^+, and isopropyl β-*D*-1-thiogalactopyranoside (IPTG) were obtained from Sigma (St. Louis, USA). All chemicals and solvents were analytical grade. Ethyl 3,5-diketo-6-benzyloxy hexanoate (**1**), racemic ethyl 3,5-dihydroxy-6-benzyloxy hexanoate and ethyl 3R,5S-dihydroxy-6-benzyloxy hexanoate (**2**) were prepared according to the procedures described in the literature [13].

Microorganism and culture

The culture of *A. baylyi* ATCC 33305 is maintained in our laboratory as frozen vials at −70°C. Half milliliter of frozen *A. baylyi* ATCC 33305 culture was inoculated into 50 ml of LB medium consisting of 10 g peptone, 5 g yeast extract, 10 g NaCl per liter of water in a 250 ml flask and grown on a rotary shaker at 28°C, 220 rpm for 48 h. Ten milliliters of culture was used as an inoculum for the growth of cultures in a 1 l flask containing 200 ml of LB medium. The culture was grown at 28°C, 220 rpm for 24 h, and harvested by centrifugation at 13,500 *g* for 15 min.

Cloning of DKR

The genomic DNA was extracted from *A. baylyi* ATCC 33305 by a standard phenol–chloroform method. Degenerate PCR primers based on the partial amino acid sequences of the N-terminus TGITNVTV (Oligo No. 1, 5′-ACIGGIATIACIAAYGTIACIGTI-3′) and an internal peptide GELAPAK (Oligo No. 2, 5′-GGIGARCTRGCICCIGCIAAR-3′) of the previously purified ketoreductase from Acinetobacter species [13], where ‘R’ and ‘Y’ are A + G and C + T, were used to amplify the gene from *A. baylyi* ATCC 33305 genomic DNA. The amplification condition was as follows: 94°C for 4 min (1 cycle); 94°C for 1 min, 50°C for 1 min, 72°C for 2 min (30 cycles); 72°C for 7 min (1 cycle). The resulting approximately 350 bp PCR product was purified from agarose gel and ligated into cloning vector pTOPO-TA (Novagen) for sequencing. To obtain the gene with full length, the cDNA fragment was labeled with digoxigenin (Roche Applied Science, Basel, Switzerland). The labeled cDNA was then used as a probe to hybridize the genomic DNA library prepared with different endonuclease digestions.

Heterologous expression of DKR

To facilitate the ligation of the ‘DKR’ gene with expression vector pET22b (+) (Novagen), oligonucleotide primers were prepared to include 5′- and 3′-terminals of the gene along with compatible restriction cleavage sites:

Oligo 3 (5′-GATACGTGCCATATGACCGGCATCAC GAATGTCACCGTTCTC-3′; 5′-terminal of the gene, *Nde*I site underlined) and Oligo 4 (5′-GATACGTGCAGATAC GTGCAGGATCCTCAGTACCGGTAGAAGCCCTCGCC -3′; 3′-terminal of the gene; *Bam*HI site underlined). PCR reaction was performed with Oligo 3 and Oligo 4 as primers and the 5.2-kb *Kpn*I fragment as template under the same thermo cycling conditions as described above. Following agarose gel electrophoresis, an approximately 900 bp PCR fragment was amplified and purified using Axyprep DNA gel extraction kit (Shenzhen, China). The PCR product (2 μg) was digested with the restriction enzymes and 2 μg of the expression vector pET22b (+) was similarly cleaved with these enzymes in parallel. The digested DNA fragments were electrophoresed on a 1.0% TAE agarose gel for 1.5 h at 90 V and purified using Axyprep DNA gel extraction kit. Then, the digested PCR fragment and pET22b (+) were ligated at a 5:1 molar ratio using a Fast link kit (Axyprep) at room temperature. The constructed pET22b (+) vector with desired insert was confirmed by electrophoresis and DNA sequencing.

The pET22b (+)-DKR construct was transformed into *E. coli* BL21 (DE3) by $CaCl_2$. Transformed cells were selected on LB-ampicillin agar medium, and the

resulting transformants were cultured in 50 ml LB medium containing 100 μg/ml ampicillin at 37°C, 220 rpm for 20 h. The culture was diluted into fresh LB medium containing 100 μg/ml ampicillin with an OD_{600} of 0.30 and incubated under the same conditions with $OD_{600} = 0.9$. IPTG was added to final concentrations of 0.05, 0.2, and 1 mM from a sterile 1 M stock in H_2O at 15°C, 220 rpm to compare the expression levels. Samples of 1 ml each were taken at intervals of 2 h from 0 to 14 h after induction, and centrifuged at 13,500 *g* for 5 min. The cells were treated by SDS-loading buffer for SDS-PAGE analysis. For large scale expression, after 14 h of induction, the cells were pelleted by centrifugation (5000 *g*) for 15 min, resuspended in 0.5 volume of 0.1 M potassium phosphate buffer (pH 7.0), and centrifuged to collect cell pellets and store at −70°C for future uses.

Purification of recombinant DKR

Frozen *E. coli* cells (2.0 g) were suspended in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 5% glycerol. The cell suspensions were passed through a high-pressure cell press at 1000 psi for 5 min to disrupt the cells. Cell-free extract was obtained by centrifuging the mixture at 13,500 *g* for 30 min. The cell-free extract (10 ml) was applied onto a DEAE-Sepharose fast flow (GE Healthcare Biosciences, Wisconsin, USA) column (2.0 cm × 20 cm) equilibrated with 50 mM potassium phosphate buffer (pH 8.0) containing 1 mM DTT and 5% glycerol. After equilibration, the column was eluted with 200 ml of a linear gradient of 0−0.5 M NaCl. Fractions with 3 ml each were collected at a flow rate of 1.0 ml/min. Active fractions (30 ml) were pooled, concentrated, and desalted using an Amicon YM-10 membrane to a final volume of 2 ml. Next, approximately 2 ml of the concentrated enzyme solution was loaded to the Sephadex G-100 (GE Healthcare Biosciences) column (1.0 cm × 21 cm) and eluted with 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT, 0.1 M NaCl, and 5% glycerol at a flow rate of 0.5 ml/min. The active fractions were collected and assayed by spectrophotometric method. The purity of the enzyme was judged by SDS-PAGE, and protein concentration was determined by the Bradford method.

Enzyme assay

Spectrophotometric method was used to determine the DKR activity on a UV-1700 array spectrophotometer (Shimadzu, Kyoto, Japan) whose cell compartment was maintained at 40°C during the measurements of absorbance change at 340 nm for the oxidation of NADH or NADPH. Assay mixture contained 0.15 mM NADH or NADPH, 0.25 mM **1**, 5−50 μg DKR, and 0.1 M potassium phosphate buffer (pH 6.0) in a final volume of 1.0 ml. The decrease of absorbance at 340 nm was monitored by the addition of enzyme. The reaction was linear in 2 min. For all assays, enzyme activity was defined as one unit representing the oxidation of one μmole of NADH or NADPH per minute per milligram protein.

Determination of native molecular weight

The Sephadex G-100 column was employed to determine the molecular weight of DKR. The calibration curve of proteins with standard molecular weight was obtained by using gel filtration calibration kits from Sigma according to the manufacture's protocol.

Optimal pH and temperature

The optimal pH and temperature for the reduction of **1** catalyzed by DKR were determined by spectrophotometric method. For pH optima, assays were conducted at 30°C in a final volume of 1.0 ml using NADH as a cofactor. Sodium acetate buffer (0.1 M) was used for the pH range of pH 4.0−6.0, and 0.1 M potassium phosphate buffer for pH 6.0−8.0. For temperature optima, the reactions were conducted at pH 6.0 in a final volume of 1.0 ml at different temperatures ranging from 20°C to 50°C at intervals of 5°C. Specific activity of the enzyme was compared at different pH and temperatures to obtain the optimal reaction conditions.

Kinetic studies

To determine the kinetic constants for the reaction of **1** with NADH, reaction mixtures contained 0.15 mM NADH, various concentrations of **1**, 0.05 U DKR, and 0.1 M potassium phosphate buffer (pH 6.0) in a final volume of 1.0 ml. To determine the kinetic constants for the reaction of **1** with NADPH as a cofactor, assay mixtures contained 0.15 mM NADPH, various concentrations of **1**, 0.05 U DKR, and 0.1 M potassium phosphate buffer (pH 6.0) in a final volume of 1.0 ml. To determine the kinetic constants for NADH, reaction mixtures contained 0.25 mM **1**, various concentrations of NADH, 0.05 U DKR, and 0.1 M potassium phosphate buffer (pH 6.0) in a final volume of 1.0 ml. To determine the kinetic constants for NADPH, reaction mixtures contained 0.25 mM **1**, various concentrations of NADPH, 0.05 U DKR, and 0.1 M potassium phosphate buffer (pH 6.0) in a final volume of 1.0 ml.

speculate that DKR may play a role in the biosynthesis of secondary metabolites in this organism [31], which will be another subject of further study.

Statins, especially synthetic statins, such as Atorvastatin (**3**) and Rosuvastatin (**4**) (**Fig. 1**), are the top-selling cholesterol-lowering drugs in the world. However, because of the current complex and expensive route for synthesizing the chiral side chains, biocatalytic production of chiral side chain of statins has become a highly competitive area in which a number of approaches and routes have been reported [32–34]. Due to the exactly same stereochemistry of **2** compared with the chiral side chain of statins, the unique characteristics of DKR, in terms of the catalytic activity and the excellent stereoselectivity, make this enzyme as an attractive biocatalyst for the development of a practical, efficient, and economic process for the synthesis of statin drugs.

In conclusion, a novel oxidoreductase designated as DKR has been successfully cloned, expressed, and purified to homogeneity. Reduction of two carbonyl groups in the same molecule catalyzed by DKR and the stereoselectivity has also been investigated, indicating that DKR displays unique and rare characteristics. Because of the stereoselectivity and the reaction type, DKR could be utilized in the asymmetric synthesis of chiral drug intermediates, such as statin drugs. It is expected that exploration of DKR by detailed kinetics analyses and structure-function study may provide new insights on the novel catalytic mechanism.

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