A Bacterial Enzyme Catalyzing Double Reduction of a β,δ-Diketo Ester with Unprecedented Stereoselectivity

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Various ketoreductases exclusively participate in all common biological events, and they are a class of important biocatalysts for the production of chiral alcohols. While many types of ketoreductase have been extensively studied and their functions, properties and utilities have been well known, the capability of stereoselectively reducing two carbonyl groups in the same diketohexanoate ester molecule to form a dihydroxy product by a single ketoreductase has not been evidently characterized. Here we show that a unique and novel enzyme, diketoreductase, was cloned from *Acinetobacter baylyi*, heterogeneously expressed in *Escherichia coli* and purified to homogeneity. The diketoreductase is up to 78% homologous to bacterial 3-hydroxyacyl coenzyme-A reductases. However, recombinant diketoreductase does not reduce HMG-CoA, showing that the inference of function of enzymes like the diketoreductase based on sequence homology may be in error. The enzyme directly converts a β,δ-diketo ester to the corresponding dihydroxy ester. More remarkably, our results demonstrate that the recombinant enzyme possesses unprecedented stereoselectivity with both diastereomeric and enantiomeric excesses of greater than 99%. This new enzyme is of immediate value in developing a practical biocatalytic route to the side chains of statin drugs, such as Lipitor®

Ketoreductases are among the most ubiquitous of microbial enzymes[1,2]. Microbial ketoreductases have been extensively studied and utilized as biocatalysts for the asymmetric synthesis of a variety of chiral alcohols owing to their high stereoselectivity[3-6]. While ketoreductases capable of reducing a single carbonyl group in a given substrate to form a chiral hydroxyl product is quite common, double reduction of a diketo ester by a single enzyme has not yet been well established. Reductions of β,δ-diketo esters could theoretically yield four stereoisomers from a diketo ester substrate. Stereoselective reductions of a β,δ-diketo-hexanoate ester have values in synthetic organic chemistry[1,7-8], particularly in the preparation of diols, such as those found in the side chains of statins. Diastereo- and enantioselective reductions of β,δ-diketo esters remains challenging in synthetic chemistry. Because of their intrinsic advantages of highly selective substrate binding leading to exceptional stereoselectivity under mild reaction conditions[7-9], enzymatic transformations have emerged as versatile tools for organic synthesis. Given that optically pure β,δ-dihydroxy-hexanoate esters are important building blocks for statins -a class of effective cholesterol lowering therapeutics currently with the largest market in the world[9], developing practical and more economic routes to β,δ-dihydroxy-hexanoate esters has attracted widespread attention [7-9].

In previous reports[10,11], multiple ketoreductases were purified in an *Acinetobacter* species. One isolated enzyme was suggested to have the potential to reduce diketo esters. However, this enzyme appears to be different than any identified ketoreductases with known sequences. Unfortunately, such a unique reaction has not been clearly characterized biochemically or genetically.

In order to ascertain the existence of and exploit the unique characteristics of this type of enzymatic transformation, we screened a number of *Acinetobacter* sp. for the presence of diketoreductases using ethyl 3,5-diketo-6-benzyloxy-hexanoate (**1**) as substrate (Fig. 1A). Screening revealed that growing cultures of *Acinetobacter baylyi* ATCC 33305 could stereoselectively catalyze the reduction of both ketone functional groups of the substrate. It is possible for such a reaction to occur by the action of either a single diketoreductase, or by the actions of more than one ketoreductase enzyme. To identify the real function and utility of the enzyme, we used a reverse genetic approach based upon reported peptide sequences of a ketoreductase from *Acinetobacter* sp. in the literature[10] for the cloning of the enzyme. cDNA probes were prepared for hybridization with a genomic DNA library prepared from *A. baylyi* ATCC 33305 to clone the gene encoding diketoreductase (DKR). After the hybridization, a gene containing an open reading frame encoding 283 amino acid residues (Fig. 1B, GenBank access number EU273886) was obtained. Sequence analysis showed a putative protein with calculated molecular mass of 30,237.4 Daltons showing up to 78% homology to 3-hydroxyacyl coenzyme-A reductase from various sources[12-14]. Recombinant DKR (rDKR) was heterogeneously expressed in *Escherichia coli* by constructing the gene with a pET22b(+) expression vector without changing the original sequence. The expression level reached a maximum at 12 hours after the induction (Supplementary Fig. 1). After evaluation of different conditions, the protein was successfully expressed in soluble form by induction with 50 μM IPTG at 15 ˚C (Fig. 1C). In the presence of NADH, the crude cell extract prepared from expressed *E. coli* cells directly reduced the 3,5-diketo ester (**1**) to corresponding dihydroxy ester (**4**). rDKR was purified to homogeneity (Fig. 1C) by

DEAE-sepharose and Sephadex-100 chromatography, to give a protein with a specific activity of 9.5 μmoles/min/mg protein. After enzymatic conversion, the dihydroxy product was isolated and characterized by ^{1}H NMR (Supplementary Table 1) and mass spectrometry (Supplementary Fig. 2), and shown to be identical to reported data[10-11]. Thus, we named this 30 kDa enzyme as diketoreductase (DKR) based on its catalytic characteristics. Purified rDKR shows a relatively broad pH optimum centered at pH 6.0 (Supplementary Fig. 3) and broad temperature optimum centered at about 40 ˚C (Supplementary Fig. 4). rDKR retains more than 90% of activity after storage at 4 ˚C for two months. Through gel filtration, the natural form of the enzyme is a monomeric protein. The fairly good stability of the DKR is possibly due to this structural characteristic.

To examine the stereoselectivity of the rDKR and to compare it with wild type strain, we performed a biocatalytic conversion using the 3,5-diketo ester (**1**) as substrate with *A. baylyi* ATCC 33305 cells and purified rDKR with NADH. After 18 hours, the diastereomeric and enantiomeric excesses of the isolated products were evaluated by our chiral reverse phase high performance liquid chromatography (HPLC) method[15]. Compared to the HPLC chromatogram of the isomeric mixture of ethyl 3,5-dihydroxy-6-benzyloxy-hexanoate obtained by chemical reduction (Fig 2A), the product produced by whole cell reaction of *A. baylyi* ATCC 33305 showed a major peak for the (3R,5S)-stereoisomer along with peaks for （3S,5R)- and (3R,5R)- stereoisomers, indicating a *de* value of 66% and an *ee* value of 99%[10]. The dihydroxy ester produced by rDKR exhibited only a peak of (3R,5S)- stereoisomer (Fig. 2B). Thus, rDKR is an enzyme with

both excellent diastereo- and enantioselectivity, and reactions with wild type cells are likely catalyzed by mixtures of individual ketoreductases.

Since a common catalytic feature of ketoreductases is the requirement of a hydride donor[7,16-18], usually NADH or NADPH, preliminary steady state kinetic experiments were performed with rDKR in order to compare and assess the cofactor utilization and preference. rDKR shows dual cofactor specificity, NADH 50 – 80 times better than NADPH in the reduction of 3,5-diketo ester (Supplementary Table 2). We further compared cofactor reactivity by determining substrate and product concentrations for biocatalytic conversions. When NADH was used coupling with a formate: formate dehydrogenase regeneration system[19], the conversion of 3,5-diketo ester (**1**) to the dihydroxy ester (**4**) reached 93% within 3 hours (Fig. 3A). The reaction was much slower using NADPH and a regeneration system of glucose-6-phosphate: glucose-6-phosphate dehydrogenase[20] (Fig. 3B). During the course of the reaction, two monohydroxy esters with a hydroxyl groups at positions-3 (**2**) or 5 (**3**) are formed (Figures 3A, 3B), indicating that rDKR catalyzes a two-step reduction process. Using database analysis, we found that DKR is homologous to a variety of 3-hydroxyacyl dehydrogenases including 3-hydroxymethylacyl coenzyme-A reductase (HMG-CoA reductase) at both nucleic acid and amino acid levels[12-14]. Even though the sequence homology for DKR was as high as 78% for the enzyme from *Nocardia farcinica* IFM 10152 (GenBank access number YP_120052), all sequences in the databases with high homology (between 30 and 78%) are putative proteins without known function. Comparison of the DKR sequence with bacterial HMG-CoA reductases that have been expressed and functionally characterized (Supplementary Fig. 5) shows that the DKR sequence is neither homologous to HMG-

CoA reductases from common pathogenic bacteria, such as *Staphylococcus aureus, Streptococous pneumoniae, or Enterococous faecalis*[21], nor to the organisms known for producing statins by secondary metabolism including *Pseudonomas citrinum, Pseudomonas mevalonii* and *Aspergirllus terreus*[12-14]. Thus, DKR does not appear to be a part of known systems involved in statin side-chain biosynthesis. To clarify the possible relationship of the DKR with HMG-CoA reductase, we tested HMG-CoA as a substrate with rDKR. No reduction occurred with varying amounts of HMG-CoA and rDKR with either NADH or NADPH. Further, the potent HMG-CoA reductase inhibitor calcium atorvastatin [22,23] showed no inhibition of rDKR activity in the reduction of 3,5-diketo ester at concentrations ranging from 0.01 to 0.5 mM. The experimental evidence indicates that DKR has no functional relationship with HMG-CoA reductase, and that DKR represents a novel class of oxidoreductase enzyme without any sequence homology to other known oxidoreductases.

Generally, *A. baylyi* ATCC 33305 has been regarded as a pathogenic organism, but little is known on the mechanism of its virulence[24-27]. So far, secondary metabolism in this organism also remains unexplored. From the present study, we speculate that DKR may play a role in the biosynthesis of secondary metabolites in this organism[28] since the double reduction of the carbonyl groups in a diketohexanoate ester molecule by a single enzyme is not involved in any primary metabolisms, which will be the subject of further study. Because of the same stereochemistry of the 3,5-dihydroxy hexanoate ester product and the chiral side chain of statin drugs, perhaps the enzyme functions in the biosynthesis of secondary metabolites with chiral hexanoate chains.

The biocatalytic synthesis of chiral side chain of statin drugs has been an extremely competitive area in which a number of approaches and routes have been reported[7-9]. A simple enzymatic process for the preparation of statin side chain can offer great potential in improving productivity of statins. To examine the utility of rDKR for such process, we tested 3,5-diketo ester at different concentrations with 5% ethanol as a co-solvent. As shown in Table 1, the conversion reached almost completion at 2 mg/ml substrate concentration although there is an obvious negative correlation between substrate concentration and conversion rate. At 10 mg/ml substrate level, increase of the enzyme amount, in terms of activity, by as much as 10 fold and extension of the reaction time for 3 times did not affect the product yield, indicating a typical product inhibition by the 3,5-dihydroxy product. Meanwhile, it should be noted that there were no detectable monohydroxy products after 18 hours in all cases, which ruled out the possibility of inhibition by the intermediate monohydroxy compounds. Since the substrate is poorly water soluble, co-solvent is necessary for the biocatalytic systems. We, therefore, evaluated different co-solvents and their concentrations. Five percent of ethanol gave the best results compared with other solvents, such as DMSO, DMF, and lower or higher concentrations of ethanol, which may be attributable to the combination of enzyme tolerance to a particular concentration of the organic solvent and the reasonable substrate solubility in the reaction system. Based on the availability of directed evolution for protein engineering[29-31], combining the potential of utilizing this unique enzyme for the preparation of chiral side chains of statin drugs, the issues of organic solvent tolerance and product inhibition with this enzyme and biocatlytic process would be feasibly solved[32-35]. With a variety of mutants that are being generated, we will be able to utilize

this enzyme to develop a practical and more economic route, compared to other biocatalytic approaches[9, 36-39], to make statin drugs with industrial utility.

METHODS SUMMARY

Acinetobacter baylyi ATCC 33305 was grown in LB medium at 28 °C and 220 rpm for 48 hours. Two-stage fermentation was performed to obtain cells for bioconversion.

Heterogeneous expression of rDKR. The genomic DNA was extracted using a standard phenol-chloroform method. Degenerate PCR primers based on the peptide sequences were used to amplify the gene from *A. baylyi* ATCC 33305 genomic DNA. The PCR product was purified and labeled with digoxigenin to hybridize with the genomic DNA library prepared with different endonuclease digestions. The full length gene was cloned from a DNA fragment from *Kpn*I digestion. The dkr gene was amplified by PCR to introduce *Nde*I and *BamH*I sites to ligate with pET22b(+) vector for creating pET22b(+)-dkr construct. The plasmid was transformed into *E. coli* BL21(DE3) cells. After selection on LB-ampicillin agar, single colony was inoculated into LB medium. The culture was incubated at 37 °C, 250 rpm, for 20 hours, then diluted in fresh LB medium to an $OD_{600\ nm}$ of 0.30, and incubated under the same conditions until the OD_{600} was approximately 0.8-1.0. Optimal expression was achieved by adding 0.05 mM IPTG at 15 °C for an induction period of 12-14 hours.

Enzyme assay. Spectrophotometric method was used to assay the DKR activity at 40 °C by measuring the absorbance change at 340 nm for the oxidation of NADH or NADPH. The experiments for enzyme purification, temperature and pH optima, HMG-

CoA as substrate, inhibition by calcium atorvastatin and kinetic analyses were also conducted by the spectrophotometric assay.

Purification of rDKR. Frozen *E coli* cells were suspended in potassium phosphate buffer (pH 7.0) containing 1 mM DTT and 5% glycerol, and disrupted by passing through a high pressure cell press to yield cell-free extract. The cell-free extract was applied onto a DEAE-sepharose column equilibrated with a potassium phosphate buffer (pH 8.0). After washing, the column was eluted with a linear gradient of 0–0.5 M NaCl. After concentration and desalting of the active fractions, the concentrated enzyme was applied onto a Sephadex G-100 column and eluted with a potassium phosphate buffer (pH 7.0).

Determination of cofactor effects. The conversion of 3,5-diketo ester substrate using NADH as cofactor was performed using a mixture containing 3,5-diketo ester substrate**,** NAD^+, formate dehydrogenase, sodium formate, and DKR solution in potassium phosphate buffer (pH 6.0). To test the dependence with NADPH, reaction mixtures contained 3,5-diketone ester substrate, $NADP^+$, glucose-6-phosphate dehydrogenase, glucose-6-phosphate and DKR solution in potassium phosphate buffer (pH 6.0) Samples at different time points were taken for HPLC analyses.

Product identification. To isolate and identify the dihydroxy ester product, 50 ml of the reaction mixture was carried out with 0.26 μmoles of 3,5-diketo ester in the presence of the NADH regeneration system. After extraction and purification, the product was analyzed by ^{1}H NMR and ESI-MS.

Biocatalytic conversions. To compare different substrate concentrations and co-solvents, the reactions were carried out at 28 °C and 220 rpm for 18 hours. Reaction mixtures at 5 ml each contained 3,5-diketo ester dissolving in organic solvents**,** NAD^+,

formate dehydrogenase, sodium formate, and 0.5 U/ml rDKR in potassium phosphate buffer (pH 6.0), and the product was isolated and analyzed by HPLC methods.

HPLC analyses. The dihydroxy product was quantified by reverse phase HPLC with a gradient of 0.05% TFA in water and 0.05% TFA in acetonitrile on a C-18 column. OD-RH chiral column was used to determine diastereomeric and enantiomeric excesses of the 3,5-dihydroxy ester products.

Full methods and any associated references are available in the Supplementary information.

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Figure Legends

Fig. 1A. Reaction catalyzed by the diketoreductase.

Compound **1**: 3,5-diketo ester; Compound **2**: (3R)-momohydroxy ester; Compound **3**: (5S)-monohydroxy ester; Compound **4**: (3R,5S)-dihydroxy ester.

Fig. 1B. Deduced amino acid sequence of the diketoreductase from *Acinetobacter baylyi* ATCC 33305.

Fig. 1C. Heterogeneous expression of the diketoreductase.

SDS-PAGE was performed on a 12% gel under reduced condition. Lane 1, molecular weight markers. The respective molecular weights are indicated by arrows in the gel image. Lane 2, total proteins from *E. coli* cells containing pET22b(+) vector without addition of IPTG, 20 μg; Lane 3, total proteins from *E. coli* cells containing pET22b(+) vector with addition of 50 μM IPTG, 20 μg; Lane 4, total proteins from *E. coli* cells containing pET22b(+)-DKR construct without addition of IPTG, 20 μg; Lane 5, total proteins from *E. coli* cells containing pET22b(+)-DKR construct with addition of 50 μM IPTG, 20 μg; Lane 6, total soluble proteins from induced *E. coli* cells containing pET22b(+)-DKR construct with addition of 50 μM IPTG, 20 μg; Lane 7, purified recombinant diketoreductase, 3μg.

Fig. 2A. Chiral HPLC chromatogram of stereoisomers of 3,5-dihydroxy ester reduced chemically.

Column: Chiralcel OD-RH; temperature: 25 ˚C; injection volume: 20 μl; mobile phase: 0.1% trifluroactetic acid in water (A) and 0.1% trifluroacetic acid in acetonitrile (B); elution: gradient of 25-30% B in 25 min and kept at 30% B for additional 5 min; flow rate: 0.5 ml/min; detection: UV 220 nm. Retention times for (3R,5S), (3S,5R), (3R,5R)

and (3S,5S)-dihydroxy ester stereoisomers were 23.5, 19.1, 14.4 and 17.3 min, respectively.

Fig. 2B Chiral HPLC chromatogram of stereoisomers of 3,5-dihydroxy ester produced by the diketoreductase.

Same conditions were used as Fig. 2A. The peak at 23.6 was the (3R,5S)-stereoisomer of the dihydroxy ester product.

Fig. 3A. Time course of reduction of β,δ-diketo ester by the diketoreductase in the presence of NADH.

The reaction was performed at a 3,5-diketo ester substrate concentration of 1 mg/ml with a formate:formate dehydrogenase regeneration system for NADH. The data represent the averages of 3 experiments. Closed diamond: (3R,5S)-dihydroxy ester; open diamond: 3,5-diketo ester substrate; closed triangle: (3R)-monohydroxy ester; open triangle: (5S)-monohydroxy ester.

Fig. 3B. Time course of reduction of β,δ-diketo ester by the diketoreductase in the presence of NADPH.

The reaction was performed at a 3,5-diketo ester substrate concentration of 1 mg/ml with a glucose-6-phosphate: glucose-6-phosphate dehydrogenase regeneration system for NADPH. The data represent the averages of 3 experiments. Closed diamond: (3R,5S)-dihydroxy ester; open diamond: 3,5-diketo ester substrate; closed triangle: (3R)-monohydroxy ester; open triangle: (5S)-monohydroxy ester.

Figure 1 A. Reaction catalyzed by the diketoreductase.

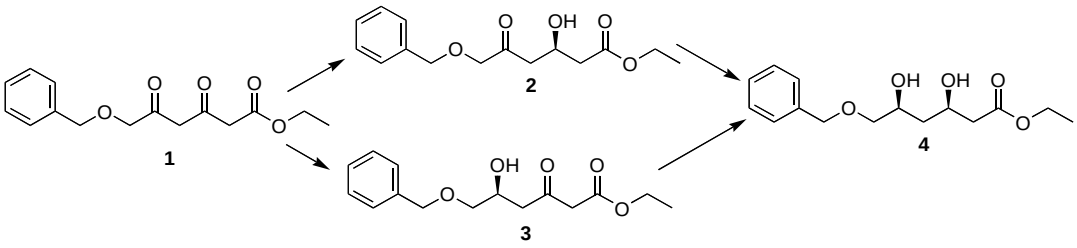

Figure 1B. Deduced amino acid sequence of the diketoreductase from *Acinetobacter baylyi* ATCC 33305.

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MTGITNVTVLGTGVLGSQIAFQTAFHGFAVTAYDINTDALDAAKKRFEGL     50
AAVYEKEVAGAADGAAQKALGGIRYSDDLAQAVKDADLVIEAVPESLDLK    100
RDIYTKLGELAPAKTIFATNSSTLLPSDLVGYTGRGDKFLALHFANHVWV    150
NNTAEVMGTTKTDPEVYQQVVEFASAIGMVPIELKKEKAGYVLNSLLVPL    200
LDAAAELLVDGIADPETIDKTWRIGTGAPKGPFEIFDIVGLTTAYNISSV    250
SGPKQREFAAYLKENYIDKGKLGLATGEGFYRY
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Figure 1 C. Heterogeneous expression of the diketoreductase in *E. coli*.

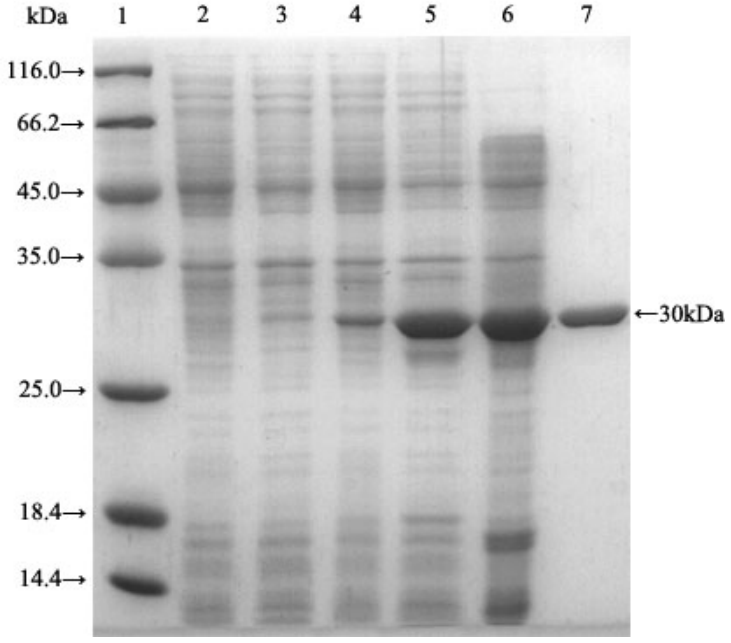

Figure 2A. Chiral HPLC chromatogram of stereoisomers of 3,5-dihydroxy ester reduced chemically.

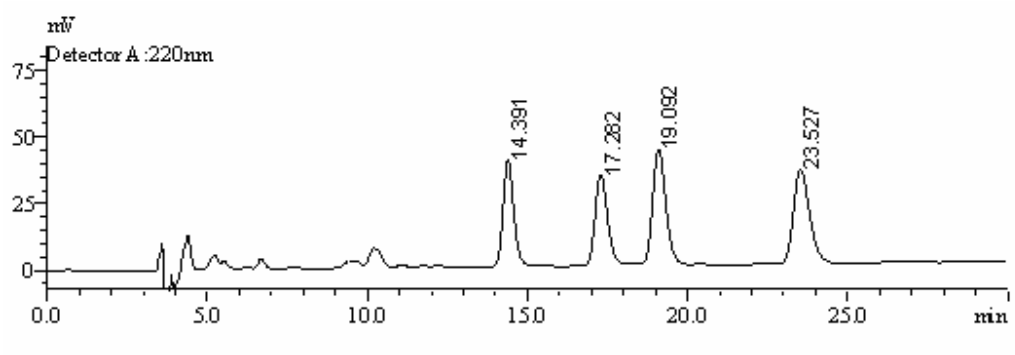

Figure 2B. Chiral HPLC chromatogram of stereoisomers of 3,5-dihydroxy ester produced by the diketoreductase.

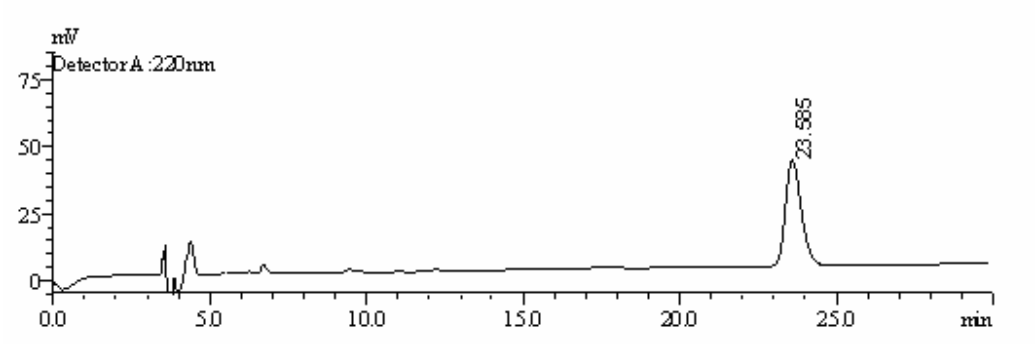

Figure 3 A. Time course for reduction of 3,5-diketo ester by the diketoreductase in the presence of NADH

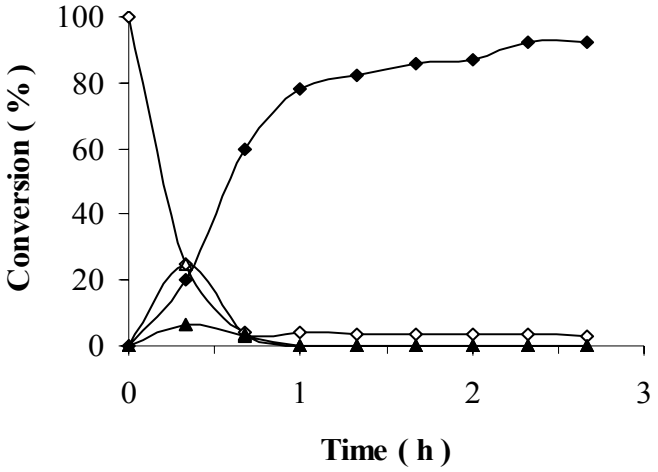

Figure 3 B. Time course for reduction of 3,5-diketo ester by the diketoreductase in the presence of NADPH

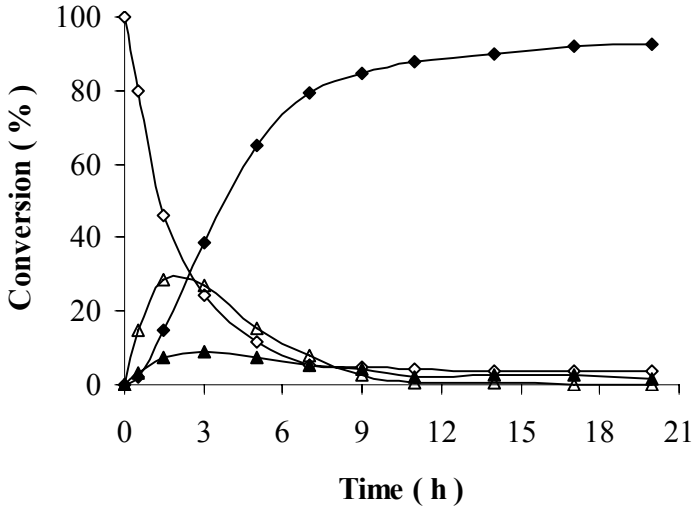

Table 1. Effect of 3,5-diketo ester concentration on the biocatalytic reduction by rDKR[a]

Substrate concentration (mg/ml)	Conversion[b] (%)	Product yield[c] (%)	*de*[d] (%)	*ee*[e] (%)
1	96.8	90.5	>99	>99
2	91.2	82.4	>99	>99
5	81.4	73.0	>99	>99
10	68.3	59.1	>99	>99

[a]The reactions were carried out at 28 °C and 220 rpm for 18 hours. Reaction mixtures at 5 ml each contained different amounts of 3,5-diketo ester**,** 5% ethanol**,** 0.5 mM NAD^+, 20 U of formate dehydrogenase, 200 mM sodium formate, and 0.5 U/ml rDKR in 0.1 M potassium phosphate buffer (pH 6.0).

[b]Conversion of **1** to **4** were determined by reverse phase HPLC with a ODS-A column. HPLC conditions: temperature: 40 °C; injection volume: 10 µl; mobile phase: 0.05% trifluroactetic acid in water (A) and 0.05% trifluroacetic acid in acetonitrile (B); elution: gradient of 20-90% B in 12 min; flow rate: 1 ml/min; detection: UV 220 nm. Retention times for 3,5-diketo ester and 3,5-dihydroxy ester were 9.7 and 7.0 min, respectively.

[c]The 3,5-dihydroxy product was purified and quantified by HPLC as described in the Supplementary information.

[d,e]The diastereomeric excess (*de*) and enantiomeric excess (*ee*) values were determined on Chiralcel OD-RH column using the same conditions as Fig. 2A .