Process Biochemistry 50 (2015) 104–110

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Enzymatic preparation of *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate by a whole-cell biocatalyst co-expressing carbonyl reductase and glucose dehydrogenase

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ARTICLE INFO

Article history:
Received 5 June 2014
Received in revised form
24 September 2014
Accepted 27 October 2014
Available online 13 November 2014

Keywords:
Carbonyl reductase
Glucose dehydrogenase
Nicotinamide cofactor
Whole-cell biocatalysis
Statin side chains

ABSTRACT

Statins are the most effective drugs for hyperlipidemia-related diseases by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase. Because of the difficulty and environmental concerns associated with chemical preparation of the chiral diols of statin side chains, different biocatalytic approaches have been explored and the two-step bio-reduction process for the introduction of two chiral hydroxyl groups has been industrialized. However, the high costs and poor stability of nicotinamide cofactors in the process was a major limiting factor. In the present study, a whole-cell biocatalyst simultaneously expressing carbonyl reductase and glucose dehydrogenase was constructed. This biocatalyst was then used to synthesize *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate *via* enzymatic reduction of *t*-butyl-6-cyano-(5*R*)-hydroxy-3-carboxylhexanoate, which involves in the self-recycling of endogenous cofactors. After systematic optimization, the bioconversion was complete with a productivity of 120 g l^{-1} day^{-1} without exogenous addition of cofactors after 7 h at 35 g/L substrate concentration. Thus, the present system has simplified the process and improved the overall efficiency for the preparation of statin side chains.

1. Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, were discovered from fungal secondary metabolites. The stereochemical similarity between the 3-hydroxy-3-methylglutaryl group of HMG-CoA and (3*R*, 5*S*/*R*)-dihydroxy ester in the side chain allows that statins competitively inhibit HMG-CoA reductase, making them the most effective drugs for the treatment of hyperlipidemia-related diseases as well as coronary heart disease [1–3]. Given the existence of two asymmetric hydroxyl groups in statin side chain, chemical synthesis of this chiral diol has resulted in poor atom-economy due to complicated synthetic route, large energy consumption and environmental pollution from harsh processing conditions [4–6]. In addition, it is difficult to meet the high standards on optical purity of chiral drugs by chemical synthesis [7]. Consequently, biocatalysis has emerged as a powerful approach for the synthesis of statin drugs owing to obvious benefits such as mild reaction condition, high catalytic efficiency, excellent stereoselectivity and environmental friendliness. This far, a number of biocatalytic approaches has been developed for the synthesis of statin side chains to replace or complement the existing chemical processes [8–11]. The employment of different biocatalysts including alcohol dehydrogenase, lipase, nitrilase, dehalogenase, aldolase and ketoreductase has resulted in various biosynthetic routes [12–17]. Nonetheless, very few of them have been applied in commercial practice, due mainly to the shortcomings of these biocatalytic approaches, including low substrate loading, high cost of cofactors and the complicated process for biocatalyst preparation.

Previously, a novel NADPH-dependent α-keto reductase from *Saccharomyces cerevisiae* (designated as CR1 in this study) was reported to exhibit strong activity on reducing aliphatic α-keto esters including *t*-butyl-6-cyano-(5*R*)-hydroxy-3-carboxylhexanoate (**1**) with enantiomeric excess (*ee*) and diastereomeric excess (*de*) values both greater than 99.5% [18,19]. The resulting product *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate (**2**) was then used as a key intermediate for the synthesis of atorvastatin. With a series of improvements, this biocatalytic approach has been the only industrialized process for the production of chiral side

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http://dx.doi.org/10.1016/j.procbio.2014.10.023
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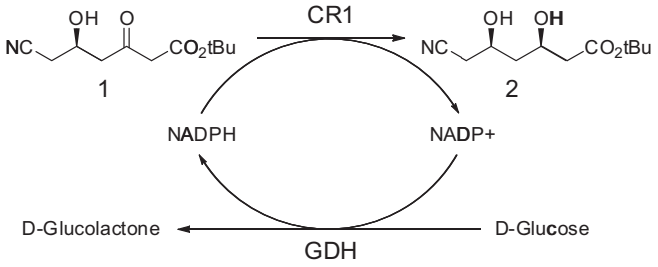

Scheme 1. Combination of CR1 and GDH for the biosynthesis of compound **2**. **1**: *t*-butyl-6-cyano-(5*R*)-hydroxy-3-carboxylhexanoate; **2**: *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate; CR1: carbonyl reductase from *Saccharomyces cerevisiae*; GDH: glucose dehydrogenase from *Bacillus megaterium*.

chain of atorvastatin using lyophilized CR1 and glucose dehydrogenase (Scheme 1). However, the requirements of exogenous addition of cofactors in the asymmetric reduction and complicated operation, such as cell disruption and enzyme lyophilization, have made this process economically inefficient [20]. Therefore, construction of a more economical and simpler biosynthetic process is a valuable and necessary improvement on the preparation of compound **2**.

To date, whole-cell biocatalysts with co-expression of an oxidoreductase and an NAD(P)H regeneration enzyme including formate dehydrogenase (FDH) or glucose dehydrogenase (GDH) or alcohol dehydrogenase have been utilized in various asymmetric bio-reductions to eliminate or to reduce the exogenous addition of cofactors [21]. Unfortunately, in most cases, the process still requires the addition of sufficient amount of expensive cofactors to initiate the enzymatic transformation and to achieve complete conversion of the substrate on large scales [17,22,23], which could be a result of lower catalytic efficiency of the biocatalyst or the incompatibility between a reductase and a cofactor regeneration system in the host cells. Previously, a close correlation between intracellular cofactor concentration and biocatalytic efficiency was observed when we coupled diketoreductase with GDH as a whole-cell biocatalyst for the preparation of a chiral diol (ethyl 3*R*, 5*S*-dihydroxy-6-benzyloxy hexanoate). Further analysis revealed that the order of genes cloned in the same vector under different promoters could still affect enzyme expression and enzymatic activity [24]. Therefore, due to sequence diversity of various genes and different properties of genes and promoter, the compatibility of co-expressed enzymes could be an issue to affect their functional expression, especially the order of genes in a co-expression vector, in order to identify more valuable whole-cell biocatalyst. In the present study, to completely eliminate the addition of exogenous NAD(P)(H) and simplify the operation for the preparation of **2**, we established a biocatalytic process with whole-cell biocatalyst coupling CR1 and GDH-cofactor regeneration system.

A recombinant *Escherichia coli* strain simultaneously overexpressing CR1 (GenBank no. NP_010159.1) from *S. cerevisiae* and glucose dehydrogenase (GDH) (GenBank no. YP_003563827.1) from *Bacillus megaterium* was constructed by co-expression vector pETDuet-1 with two independent T7 promoters. Subsequently, after comparing two *E. coli* strains expressed both enzymes in different orders and optimizing the biocatalysis conditions, an efficient *in situ* cofactor-regenerating system was established to improve the biocatalytic efficiency with a productivity of 120 g l^{-1} day^{-1}, and the complete elimination of cofactor addition significantly reduced the preparation costs for the chiral side chain of atorvastatin.

2. Materials and methods

2.1. Materials

Co-expression vector pETDuet-1 was obtained from Novagen, USA. All restriction endonuclease were obtained from TaKaRa Bio Inc., Japan. *t*-Butyl-6-cyano-(5*R*)-hydroxy-3-carboxylhexanoate and *t*-butyl-6-cyano-(3*R*, 5*R*)- dihydroxyhexanoate were purchased from J&K Scientific Ltd. China. Chromatographic grade acetonitrile used for HPLC was purchased from Tedia Company Inc., USA. Other biological and chemical reagents used in this study were of analytical grade.

2.2. HPLC analysis

Achiral HPLC method was performed with mobile phase A (0.25% acetic acid in water) and mobile phase B (acetonitrile) at 30 °C with UV detection at 220 nm. The analyses were achieved on a Thermo ODS-2 HYPERSIL column (5 μm, 250 mm × 4.6 mm) with an injection volume of 20 μL and a flow rate of 1 mL/min. The conversion catalyzed by CR1 was determined by an isocratic elution 25% B in 20 min. The retention times for **2** and **1** were 6.9 min and 10.2 min, respectively. Meanwhile, the *de* value of compound **2** was also analyzed according to reported method [20].

2.3. Constructions of *E. coli* (pETDuet-cr1-gdh) and *E. coli* (pETDuet-gdh-cr1)

DNAs with the nucleotide sequences of *cr1* and *gdh* were synthesized by Generay Biotech Ltd., China. The pETDuet-1 vector with two multiple cloning sites (MCS), each of which is preceded by a T7 promoter was selected to construct co-expression system for CR1 and GDH. The DNA fragment of *cr1* was then cloned into the MCS1 of pETDuet-1 vector between *Nco* I and *Bam*H I restriction sites while *gdh* fragment was cloned into the MCS2 of pETDuet-1 vector between *Nde* I and *Xho* I restriction sites. The constructed plasmid was then designated as pETDuet-*cr1-gdh*. With the same strategy, pETDuet-*gdh-cr1* was built accordingly. Constructed plasmids were transformed into *E. coli* BL21 (DE3) respectively to obtain recombinant *E. coli* containing pETDuet-*cr1-gdh* and *E. coli* containing pETDuet-*gdh-cr1*. After screening by PCR and DNA sequencing, the strains with correct plasmids were subsequently used and named as pCG and pGC, respectively.

2.4. Expression of CR1 and GDH and preparation of cell-free extract

Recombinant *E. coli* cells were grown in LB medium containing 100 μg/mL ampicillin at 37 °C on a rotary shaker (220 rpm). When OD_{600} value reached 0.8 ± 0.1, co-expression of CR1 and GDH was induced by addition of IPTG for 16 h. Temperatures from 15 to 35 °C were applied to examine the effects on CR1 and GDH expression at 0.2 mM IPTG. IPTG concentrations from 0.2 to 1.2 mM were employed for optimal induction. Cells were harvested *via* centrifugation at 5000 × *g* for 10 min and washed twice with 0.1 M potassium phosphate buffer (pH 7.0). The cells were resuspended in same buffer for high pressure cell disruption (35 kpsi). After centrifugation at 15,000 × *g* for 30 min, cell-free extract was obtained for SDS-PAGE and enzyme activity assay.

2.5. Enzyme activity assays of CR1 and GDH

Enzyme activity of CR1 and GDH in the cell-free extract was assayed *via* spectrophotometric method. Assay mixture for CR1 composed of 0.1 mM NADPH, 5 mM **1**, 0.1 M potassium phosphate buffer (pH 7.0) and 10 μL cell-free extract in a final volume of