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Two-step enzymatic synthesis of ursodeoxycholic acid with a new 7β-hydroxysteroid dehydrogenase from *Ruminococcus torques*

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ABSTRACT

7β-Hydroxysteroid dehydrogenase (7β-HSDH) is a key enzyme for the efficient biosynthesis of ursodeoxycholic acid (UDCA), an effective pharmaceutical for primary biliary cirrhosis and human cholesterol gallstones. In this work, a new 7β-HSDH from *Ruminococcus torques* ATCC 35915, designated as 7β-HSDH$_{Rt}$, was identified and heterologously overexpressed in *Escherichia coli* for the enzymatic synthesis of ursodeoxycholic acid from chenodeoxycholic acid (CDCA). 7β-HSDH$_{Rt}$ was firstly employed in one-pot mode together with 7α-HSDH$_{Ca}$, another NADPH-dependent 7α-HSDH from *Clostridium absonum*, to convert CDCA into UDCA without additional coenzyme regeneration. However, the final yield was limited to merely 73%, probably due to chemical equilibrium. Therefore, to enhance the UDCA yield, we alternatively adopted a two-step reaction strategy where the enzymes involved in the first reaction were simply heat-inactivated between the 1st-step reaction (dehydrogenation) and the 2nd-step reaction (hydrogenation), in order to prevent the undesired bioreduction of 7-oxo-LCA into CDCA in the 2nd step. Consequently, the analytic yield of UDCA was significantly improved up to above 98% at a substrate load of 10 mM (*ca.* 4 g L^{-1}), without any detectable intermediate (7-oxo-LCA) as observed in the case of one-pot reaction.

1. Introduction

As a traditional Chinese medicine, animal bile has a significant pharmaceutical function and value for unique clinical applications [1]. The conjugates and salts of bile acids are natural products and fundamental compositions of bile, playing an important role in the pathways of human fat metabolism [2]. Ursodeoxycholic acid (UDCA), a kind of endogenous bile acid, has various pharmaceutical applications due to its beneficial effects on solubilizing gallstone acid, improving liver engraftment and treating reflux gastritis, biliary pancreatitis, alcohol liver, primary biliary cirrhosis and drug-induced hepatitis diseases [3]. UDCA is usually prepared from its epimer chenodeoxycholic acid (CDCA) extracted largely from bovine and goose biles by chemical reactions involving 7-OH configuration inversion [4]. In this method, CDCA is completely converted to 7-oxo-LCA by *N*-bromosuccinimide or other weak oxidants [5], followed by the hydrogenation process where sodium borohydride or potassium borohydride is added under the protection of nitrogen gas to give a crude product of ursodeoxycholic acid. Finally UDCA was purified by adding chloroform [6]. Because of the complex reaction procedures and the low selectivity, the total yield of UDCA is so low that its industrial production is limited [7]. Compared to chemical epimerization, biosynthesis of UDCA from CDCA is mild and environmentally friendly.

In vivo, UDCA is synthesized by intestinal inhabitants *via* the action of hydroxysteroid dehydrogenases (HSDHs) belonging to the short-chain dehydrogenase/reductase (SDR) superfamily [8,9]. Therefore, the transformation of CDCA to UDCA by isolated intestinal bacteria has been well studied. *Clostridium limosum* [10], *Clostridium absonum* [11], *Clostridium baratii* [12] and *Xanthomonas maltophilia* [13] have been used to achieve the epimerization of CDCA to UDCA. The epimerization was also achieved using a mixed culture of *Eubacterium aerofaciens* and *Bacteroides fragilis* [14]. However, the growth of the cells is inhibited by CDCA at high concentration [11]. In addition, the reaction catalyzed by HSDH is reversible, thus the high conversion of the substrate is limited, which leads to difficulties in product recovery and purification. Furthermore, previous studies showed that the yield of the

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intermediate increased and UDCA decreased with the extension of incubation time [15,16]. 7α-HSDH (EC 1.1.1.159) and 7β-HSDH (EC 1.1.1.201) are two key enzymes for the efficient biotransformation of CDCA to UDCA. Genes encoding 7α-HSDH have been cloned from *Eubacterium* sp. [17], *Escherichia coli* [18], *Clostridium sordellii* [19], *B. fragilis* [20] and *C. absonum* [21]. Among them, the 7α-HSDH from *E. coli*, whose crystal structure has been determined, has the highest activity for converting CDCA into UDCA [22]. In contrast, much less information is available for 7β-HSDH. So far, only three genes encoding 7β-HSDHs have been cloned from *C. absonum* [21], *Collinsella aerofaciens* [23] and *Ruminococcus gnavus* [24]. Therefore, it is necessary to find more and better 7β-HSDHs for efficient synthesis of UDCA. Herein, we report a new and highly active 7β-HSDH whose gene was successfully cloned from *Ruminococcus torques* ATCC 35915 and heterogeneously expressed in *E. coli*. It was purified and its enzymatic properties were characterized.

Meanwhile, we describe the biotransformation of CDCA to UDCA using 7α-HSDH and 7β-HSDH *via* sequential two-step reactions in one pot (Fig. 2). In the oxidative step, CDCA was oxidized to 7-oxo-lithocholic acid (7-oxo-LCA) in the presence of 7α-HSDH, coupled by the regeneration of cofactor (NAD^+) with lactate dehydrogenase (LDH) and pyruvate. Similarly, 7β-HSDH catalyzed the reduction of 7-oxo-LCA to UDCA with glucose dehydrogenase (GDH) and glucose for the regeneration of cofactor (NADPH) in the reductive step. 7α-HSDH and LDH were inactivated by heating prior to the second step to avoid the reversible reaction in order to increase the yield of UDCA.

2. Materials and methods

2.1. Materials

The microbial strains used for genome mining were obtained from the American Type Culture Collection (ATCC), German Collection of Microorganisms and Cell Cultures (DSMZ), and China General Microbiological Culture Collection Center (CGMCC). *E. coli* DH 5α and *E. coli* BL21 (DE3) were used as the cloning and expression hosts and were grown in Luria–Bertani (LB) medium. NADH-dependent 7α-hydroxysteroid dehydrogenase (7α-HSDH, EC 1.1.1.159) from *E. coli* [18], NADPH-dependent 7α-hydroxysteroid dehydrogenase from *C. absonum* DSM 599 [21], lactate dehydrogenase (LDH, EC 1.1.1.27) from *Lactobacillus delbrueckii subsp. bulgaricus* DSM 20081 [25], and glucose dehydrogenase (GDH, EC 1.1.1.47) from *Bacillus megaterium* [26] were overexpressed in recombinant *E. coli* BL21(DE3). Chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid) and ursodeoxycholic acid (UDCA; 3α,7β-dihydroxy-5β-cholan-24-oic acid) were purchased from Shanghai Siyu Chemical Technology Co., Ltd. (Shanghai, China). 7-Oxo-lithocholic acid (7-oxo-LCA; 3α-hydroxy-7-oxo-5β-cholan-24-oic acid) was purchased from Mairuier Chemical Technology Co., Ltd. (Shanghai, China). All the other chemicals used in this work were obtained from commercial sources and were of reagent grade or better quality.

2.2. Cloning, expression and purification of 7β-HSDH$_{Rt}$

The genomic DNA of *R. torques* ATCC 35915 was extracted and purified using the TIANamp Bacteria DNA Kit from Tiangen (Shanghai, China). DNA fragments containing the 7β-HSDH$_{Rt}$ gene were amplified by polymerase chain reaction (PCR) using primers with *BamH* I and *Sal* I restriction sites. The resulting plasmid was transformed into *E. coli* DH5α for amplification. The plasmid was extracted using standard methods and then transformed into *E. coli* BL21 (DE3) cells.

The cells were cultivated in LB medium containing 50 μg mL^{-1} kanamycin at 37 °C. When the OD_{600} reached 0.6–0.8, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM. After cultivation at 16 °C for an extra 24 h, the cells were centrifuged at 8800 × *g* for 10 min and washed with normal saline. The cells were resuspended in buffer A (20 mM sodium phosphate buffer pH 7.4, 500 mM NaCl and 10 mM imidazole), and broken by sonication with an ultrasonic oscillator (JY92-II, Scientz Biotech Co.). The cell lysate was centrifuged at 12.000 × *g* for 20 min, and then the supernatant was filtered and loaded onto a Ni-NTA column (5 mL, GE Healthcare Co.) equilibrated with buffer A. After prewashing with buffer A, the column was eluted with a linear gradient from 10 to 500 mM of imidazole in buffer A at 5 mL min^{-1}. The purity of fractions was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing 7β-hydroxysteroid dehydrogenase (7β-HSDH) activity were collected and dialyzed extensively against sodium phosphate buffer to remove the high concentrations of imidazole and salt.

2.3. Protein assays

The enzyme activity was determined spectrophotometrically at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) and 30 °C by measuring the oxidation of NAD(P)H or reduction of $NAD(P)^+$.

The standard assay mixtures (1 mL) were: (a) For the NAD(P)H-dependent 7α-HSDH assay: 0.1 mM $NAD(P)^+$, 1 mM CDCA in 0.1 M phosphate buffer, pH 8.0; (b) For the 7β-HSDH$_{Rt}$ assay: 0.1 mM NADP(H), 1 mM 7-oxo-LCA (or UDCA) in 0.1 M phosphate buffer, pH 6.5 (or 8.0); (c) For the LDH assay: 0.1 mM NADH, 1 mM sodium pyruvate in 0.1 M phosphate buffer, pH 8.0; (d) For the GDH assay: 0.1 mM $NADP^+$, 1 mM glucose in 0.1 M phosphate buffer, pH 6.5, and an appropriate amount of enzyme. One unit of activity is defined as the amount of enzyme catalyzing the reduction (or oxidization) of 1 μmol NAD(P)(H) per min under the assay conditions used.

2.4. Characterization of purified 7β-HSDH$_{Rt}$

The effect of temperature was determined by assaying the 7β-HSDH$_{Rt}$ activity at different temperatures in the range of 20–50 °C in phosphate buffer (100 mM, pH 6.5). The optimum pH was determined using the standard activity assay at different pHs (5.0–9.0 for the forward reaction, 6.5–10.5 for the reverse reaction). The thermostability of 7β-HSDH$_{Rt}$ was investigated by incubating the purified enzymes (1.0 mg mL^{-1}) in the same phosphate buffer (100 mM, pH 6.5) at 30 °C, 40 °C and 45 °C, and measuring the residual activities at different times. The stability of 7β-HSDH$_{Rt}$ at different pH was investigated by incubating the purified enzymes (1.0 mg mL^{-1}) in the phosphate buffer (100 mM, pH 6.5), phosphate buffer (100 mM, pH 8.0) and Gly-NaOH (100 mM, pH 10.0) at 30 °C, and measuring the residual activities at different times.

The kinetic parameters of the 7β-HSDH$_{Rt}$ in both directions were determined by non-linear fitting using Microsoft-Excel 2010. The substrate, 7-oxo-LCA, at 0.008 mM, 0.01 mM, 0.015 mM, 0.02 mM, 0.025 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM and 0.8 mM, was used for the enzyme activity assay using the standard method. The concentrations of UDCA tested included 0.01 mM, 0.02 mM, 0.03 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM and 1.0 mM.

2.5. One-step cascade reaction in one-pot transformation of CDCA to UDCA using 7α-HSDH$_{Ca}$ and 7β-HSDH$_{Rt}$

7α-HSDH$_{Ca}$ from *C. absonum* DSM 599 is an NADPH-dependent enzyme. The 5-mL reaction starting from the CDCA consisted of 100 mM phosphate buffer (pH 8.0), 10 mM CDCA, 0.5 mM $NADP^+$, combined with 0.5 U mL^{-1}, 1.0 U mL^{-1}, or 2.0 U mL^{-1} 7α-HSDH$_{Ca}$ and 7β-HSDH$_{Rt}$ respectively. The 5-mL reaction starting from the

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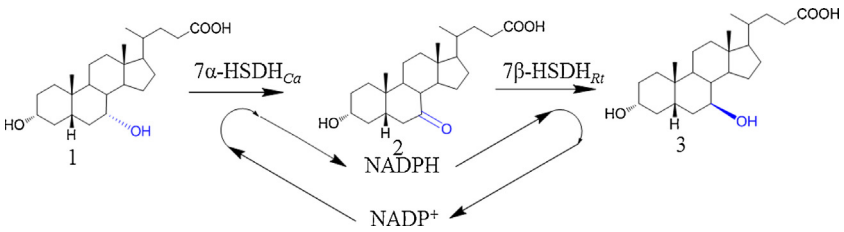

Fig. 1. One-step cascade reaction synthesis of UDCA (**3**) from CDCA (**1**).

UDCA was performed at 30 °C by incubating a mixture consisting of 100 mM phosphate buffer (pH 8.0), 10 mM UDCA, 0.5 mM $NADP^+$, combined with 0.5 U mL^{-1} or 1.0 U mL^{-1} 7α-$HSDH_{Ca}$ and 7β-$HSDH_{Rt}$ respectively. The reaction was stopped by adding 1 M HCl to lower the pH to 3 and the product was extracted with diethyl ether. The organic layer was evaporated, and then methanol was added for HPLC analysis.

2.6. Oxidation of CDCA to 7-oxo-LCA using *E. coli* 7α-HSDH and NAD^+ regeneration

CDCA was converted in a 1 mL solution containing 100 mM phosphate buffer, pH 8.0, 10 mM CDCA, 30 mM sodium pyruvate, 0.5 mM NAD^+, 5 U mL^{-1} or 15.0 U mL^{-1} LDH and 0.5 U mL^{-1}, 1.0 U mL^{-1} or 5.0 U mL^{-1} *E. coli* 7α-HSDH at 30 °C. The product was recovered as described above.

2.7. Reduction of 7-oxo-LCA to UDCA using 7β-$HSDH_{Rt}$ and NADPH regeneration

Biotransformations were carried out in a 1 mL solution containing 100 mM phosphate buffer, pH 6.5 or 8.0, 10 mM 7-oxo-LCA, 20 mM glucose, 0.5 mM $NADP^+$, 5 U mL^{-1} GDH and 0.1 U mL^{-1}, 0.5 U mL^{-1} or 2.0 U mL^{-1} 7β-$HSDH_{Rt}$ at 30 °C. The product was recovered as described above.

2.8. Two-step reaction in one-pot for more efficient enzymatic synthesis of UDCA

This reaction took place over two steps: oxidation of CDCA to 7-oxo-LCA and reduction of 7-oxo-LCA to UDCA. A 1-mL reaction containing 10 mM CDCA, 30 mM glucose, 0.5 mM NAD^+, 5 U mL^{-1} *E. coli* 7α-HSDH, 15 U mL^{-1} LDH and phosphate buffer (100 mM, pH 8.0) was carried out at 30 °C for 1 h. Then the reaction was stopped by boiling for 5 min. After cooling, 30 mM glucose, 0.5 mM $NADP^+$, 1 U mL^{-1} 7β-$HSDH_{Rt}$ and 5 U mL^{-1} GDH were added into the mixture and the reaction was continued at 30 °C. After an additional 1 h, the reaction was stopped by adding 1 M HCl to pH 3. The product was recovered as described above.

2.9. Analysis of bile acids

The bioconversion experiments were monitored *via* HPLC measurements. The sample was analyzed by UV detection at 210 nm using a mobile phase of methanol–water mixture (final ratio 80:20, pH 3.5 with phosphoric acid) using an Inertsil® ODS-4 column (250 mm × 4.6 mm ID; 5 μm, GL Sciences).

3. Results

3.1. Screening of recombinant 7β-HSDHs and protein purification

Our initial goal was to identify a new 7β-HSDH with the ability to reduce 7-oxo-LCA to UDCA. After analysis of the 7β-HSDH gene from *C. absonum* DSM 599 [21], we adopted a data mining approach based on BLAST in the UniProt. During the screening process, a new enzyme from *R. torques* ATCC 35915 (Genbank accession no. CBL26204), designated as 7β-$HSDH_{Rt}$, was identified as being able to reduce 7-oxo-LCA to UDCA. A BLAST search revealed that 7β-$HSDH_{Rt}$ shares 47% identity with 7β-$HSDH_{Ca1}$ from *C. absonum* DSM 599, 76% identity with 7β-$HSDH_{Rg}$ from *R. gnavus* [24] and 7β-$HSDH_{Ca2}$ from *C. aerofaciens* [23]. Although the amino acid sequence of 7β-$HSDH_{Rt}$ had been compared with other 7β-HSDHs [21,24] (Fig. S1), the function of 7β-$HSDH_{Rt}$ has not yet been identified so far. In this work, the function of 7β-$HSDH_{Rt}$ was first confirmed by the product identification by UPLC-Q-tof-MS and NMR (*see* the supplementary material), and then its enzymatic properties were characterized after purification. Although CDCA and UDCA are epimers, their ^1H NMR results are not exactly the same because the chemical shifts of 7β-H and 3α-H are equal in UDCA (δ 3.5), but different in CDCA (δ 3.4 and δ 3.8 respectively) [36]. We found that the ^1H NMR of the product was consistent with that of the authentic UDCA (data not shown), but different from that of the authentic CDCA.

7β-$HSDH_{Rt}$ with an *N*-terminal His-tag was purified to electrophoretic homogeneity by immobilized metal affinity chromatography. The specific activity of the purified 7β-$HSDH_{Rt}$ toward 7-oxo-LCA was as high as 66 U mg^{-1} protein, indicating 6.3-fold purification from the crude extract. Analysis of the protein by SDS-PAGE (Fig. S2) clearly showed a protein band with a size of about 30 kDa, which is in a good agreement with that predicted from the gene sequence.

3.2. Characterization of purified 7β-$HSDH_{Rt}$

The optimum temperature of 7β-$HSDH_{Rt}$ activity was determined by measuring the enzyme activities at 20–50 °C (Fig. 3a). The enzyme showed a maximum activity at 40 °C. Since 7β-$HSDH_{Rt}$ has oxidation and reduction activities, their pH optima were determined in order to choose the most suitable pH for either the

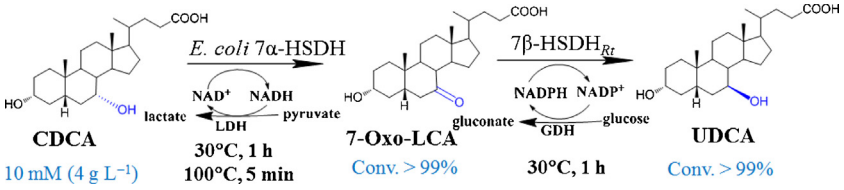

Fig. 2. Schematic representation of two-step in one-pot synthesis of UDCA.

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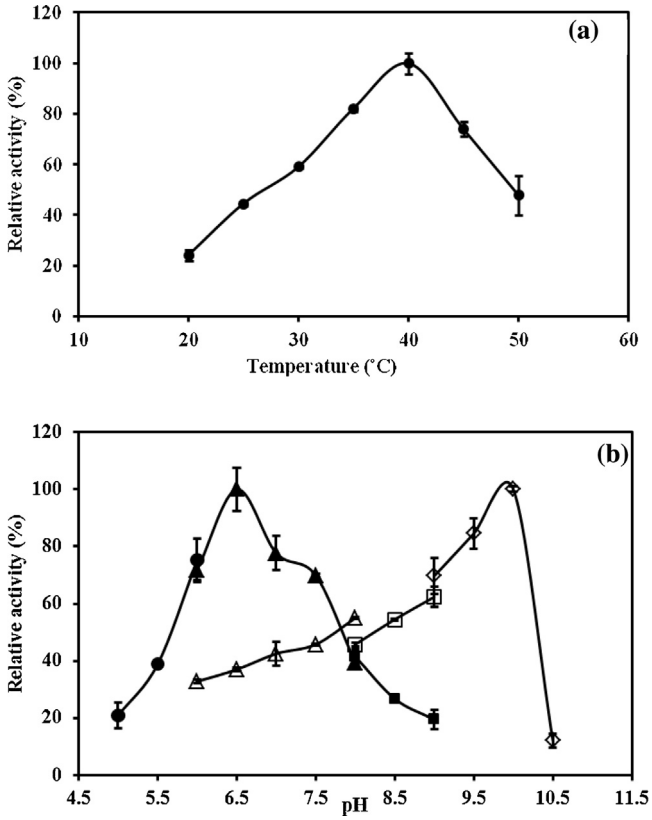

Fig. 3. Effects of temperature and pH on activity of the purified 7β-HSDH$_{Rt}$. (a) Temperature optima: the enzyme activity was measured at various temperatures (20–50 °C) in 100 mM sodium phosphate buffer (pH 6.5). (b) pH optima for 7-oxo-LCA (full symbols) or for UDCA (empty symbols). Enzyme assay was performed using standard assay procedure in the following buffers of 100 mM: (1) citrate (pH 5.0–6.0), (2) phosphate (pH 6.0–8.0), (3) Tris–HCl (pH 8.0–9.0), and (4) Gly-NaOH (pH 9.0–10.5).

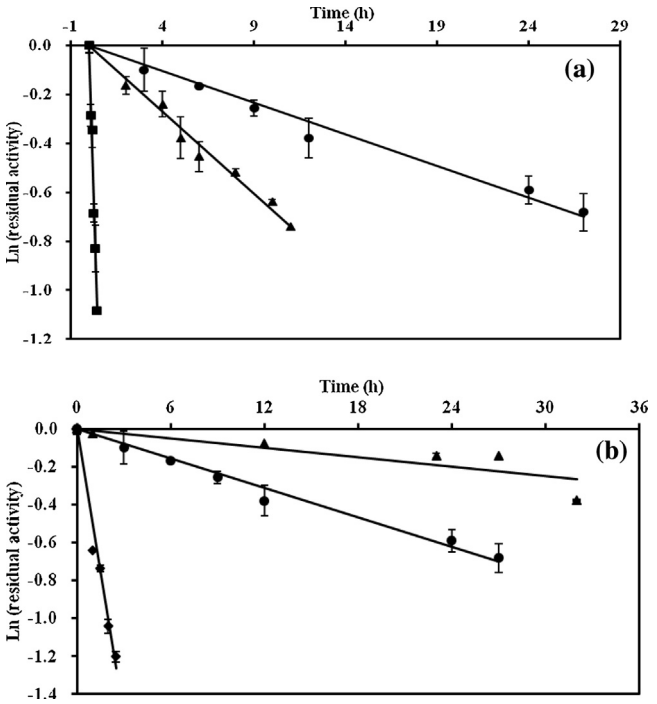

Fig. 4. Stability of temperature and pH on activity of the purified 7β-HSDH$_{Rt}$. (a) Thermostability of the purified 7β-HSDH$_{Rt}$ at different temperatures. The purified enzyme was incubated in phosphate buffer at different temperatures for a required period and the residual activity was measured using the standard assay protocol. *Circle*, 30 °C; *triangle*, 40 °C; *square*, 45 °C. (b) The stability of the purified 7β-HSDH$_{Rt}$ at different pH. The purified enzyme was incubated at 30 °C in different buffer for a required period and the residual activity was measured using the standard assay protocol. *Circle*, pH 6.5; *triangle*, pH 8.0; *rhombus*, pH 10.0.

oxidative or reductive reaction. As shown in Fig. 3b, the purified 7β-HSDH$_{Rt}$ showed a maximum activity at pH 6.5 for 7-oxo-LCA reduction and an optimum at pH 10.0 for UDCA dehydrogenation.

The thermostability of the purified 7β-HSDH$_{Rt}$ was examined at temperatures of 30 °C, 40 °C and 45 °C (Fig. 4a). The half-lives measured at 30 °C, 40 °C and 45 °C were 27 h, 10 h and 16 min, respectively. Incubation at higher temperatures resulted in a quick loss of activity, indicating that the thermal stability of this dehydrogenase requires further enhancement in the future. The stability of the purified 7β-HSDH$_{Rt}$ was also examined at pH 6.5, 8.0 and 10.0 (Fig. 4b). The half-lives measured at pH 6.5 and pH 10.0 were 27 h and 1.4 h, respectively. After 24 h, the residual activity of the purified enzyme at pH 8.0 was about 86%. Thus, 7β-HSDH$_{Rt}$ was most stable at pH 8.0.

The effects of various metal ions and EDTA on 7β-HSDH$_{Rt}$ activity are shown in Table S1. None of the metal ions significantly increased the enzyme activity. High concentrations of Fe^{3+} and Zn^{2+} inhibited the enzyme and 7β-HSDH$_{Rt}$ was very sensitive to Cu^{2+}. Other metal ions and EDTA did not show any obvious effects on 7β-HSDH$_{Rt}$ activity.

The kinetic parameters of 7β-HSDH$_{Rt}$ in both directions were determined by non-linear fitting using Microsoft-Excel 2010 at each optimal pH value. The kinetic parameters obtained are shown in Table 1, in comparison with other reported enzymes. 7β-HSDH$_{Rt}$ has a higher V_{max} than that of other 7β-HSDHs, except 7β-HSDH$_{Ca1}$ from *C. absonum* DSM 599 whose V_{max} is just apparent. The K_m value of 7β-HSDH$_{Rt}$ is lower than that of 7β-HSDH$_{Ca1}$ and 7β-HSDH$_{Rg}$. The low K_m value of the enzyme toward 7-oxo-LCA is advantageous for catalyzing the conversion of a low concentration of the dissolved substrate.

3.3. Cascade transformation of CDCA to UDCA in one-pot reaction using 7α-HSDH$_{Ca}$ and 7β-HSDH$_{Rt}$

The 7α-HSDH$_{Ca}$ from *C. absonum* DSM 599 and the 7β-HSDH$_{Rt}$ from *R. torques* ATCC 35915 are both NADPH-dependent enzymes. We attempted to employ these two enzymes for the one-pot synthesis of UDCA from CDCA without use of additional coupling enzymes for cofactor regeneration (Fig. 1). The results are shown in Fig. 5a. Because the enzymes have both oxidation and reduction activities, the reaction stopped at approximately 80% conversion, always accompanied by about 5% of the intermediate 7-oxo-LCA (data not shown), resulting in a UDCA yield of 73% after purification.

UDCA was added as the initial substrate to validate this hypothesis that the limited conversion or yield is caused by the equilibrium. As expected, the final ratio of these three compounds from the revered reaction was indeed consistent with the previous result obtained from the forward reaction (Fig. 5b). This confirmed that CDCA cannot be transformed completely into UDCA using the one-pot method.

3.4. Oxidation of CDCA to 7-oxo-LCA using E. coli 7α-HSDH and NAD$^+$ regeneration

We next investigated a two-step method to increase the yield of UDCA (Fig. 2). Because 7α-HSDH from *E. coli* and LDH are both NADH-dependent enzymes; therefore we chose *E. coli* 7α-HSDH instead of NADPH-dependent 7α-HSDH$_{Ca}$ in the two-step reaction for cofactor regeneration. For the oxidative step, the amount of *E. coli* 7α-HSDH required to oxidize CDCA into 7-oxo-LCA was

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Table 1
Kinetic parameters of 7β-HSDHs purified from UDCA-producing bacteria.

Strains	Substrate	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})	Ref.
Clostridium absonum DSM 599^T	7-Oxo-LCA	2650	80.1[a]	3.8×10^7	14,500	[21]
	UDCA	3060	55.8[a]	2.6×10^7	8430	
Ruminococcus gnavus N53	7-Oxo-LCA	38.8	23.8	700	18	[24]
	UDCA	974	11.1	326	0.33	
Collinsella aerofaciens ATCC 25986^T	7-Oxo-LCA	5.20	30.8	951	183	[23]
	UDCA	6.23	38.2	1180	189	
Ruminococcus torques	7-Oxo-LCA	20	73.6	2136	107	This work
	UDCA	30	76.2	2214	74	

[a] In the case of *Clostridium absonum* DSM 599^T, the unit used in Table 1 for V_{max} and k_{cat} did not include the information of enzyme amount. Thus, these values are just apparent ones [21].

determined. As shown in Fig. 6, it is evident that when the amount of 7α-HSDH was 0.5 U mL^{-1}, the reaction was very slow. By adding 1.0 U mL^{-1} of 7α-HSDH, the reaction was increased and the transformation was complete after 2 h.

3.5. Reduction of 7-oxo-LCA to UDCA using 7β-HSDH$_{Rt}$ and NADPH regeneration

The reductive step was first performed at pH 6.5. When 1 U mL^{-1} of 7β-HSDH$_{Rt}$ was employed at pH 6.5, a conversion of merely 80% was achieved after 3 h (data not shown). Although the activity of 7β-HSDH$_{Rt}$ was the highest at pH 6.5, the solubility of substrate at this pH was very limited. Therefore, we attempted to increase the pH to improve the substrate solubility. Even though the relative activity of 7β-HSDH$_{Rt}$ at pH 8.0 was 50% compared with that at pH 6.5, the 7β-HSDH$_{Rt}$ was more stable at pH 8.0 than pH 6.5 (Fig. 4). As shown in Fig. 7, when the pH was increased up to 8.0, the addition of only 0.1 U mL^{-1} 7β-HSDH$_{Rt}$ resulted in an analytic yield of 98.3 ± 2.4% UDCA after 1.5 h. The complete conversion of 10 mM 7-oxo-LCA into UDCA was achieved within 0.5 h when the concentration of 7β-HSDH$_{Rt}$ was increased to 0.5–2.0 U mL^{-1}.

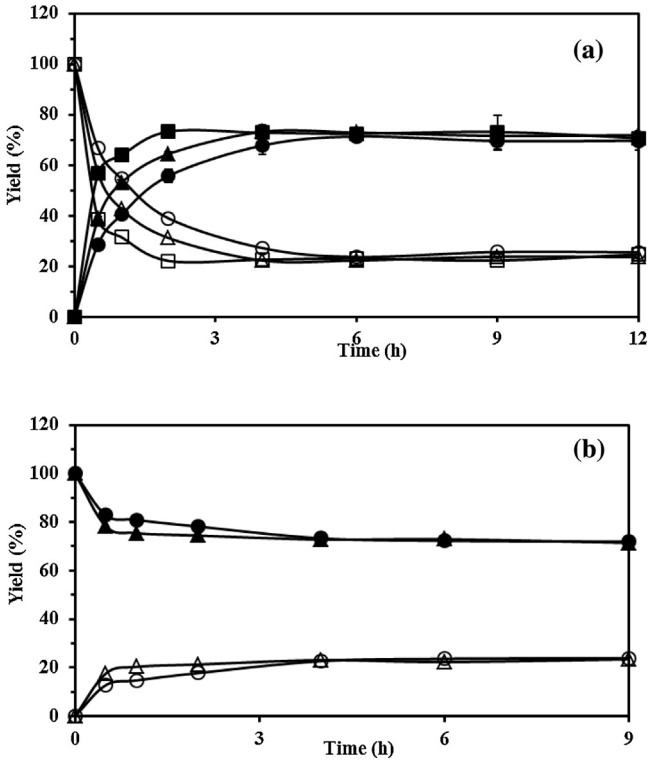

Fig. 5. Time courses of reaction by 7α-HSDH$_{Ca}$ and 7β-HSDH$_{Rt}$ with different doses. (a) The initial substrate was CDCA. (b) The initial substrate was UDCA. *Full symbol*, UDCA; *empty symbol*, CDCA; *Circle*, 0.5 U mL^{-1}; *triangle*, 1.0 U mL^{-1}; *square*, 2.0 U mL^{-1}. The yield was analyzed by HPLC.

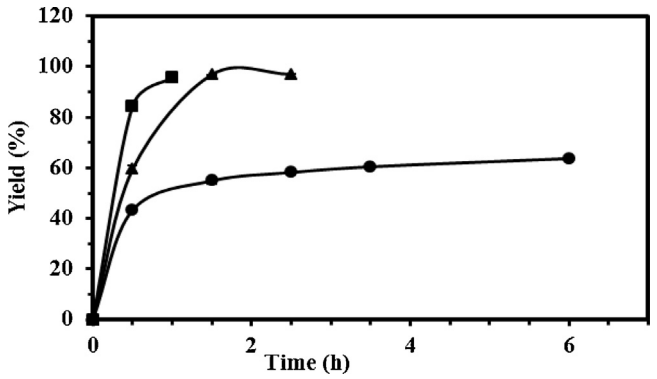

Fig. 6. Time course of chenodeoxycholic acid oxidization by *E. coli* 7α-HSDH with different concentration. *Circle*, 0.5 U/mL; *triangle*, 1.0 U/mL; *square*, 5.0 U/mL. Yield of 7-oxo-LCA is analyzed by HPLC.

3.6. Two-step reactions in one-pot for efficient enzymatic synthesis of UDCA

Subsequently, the above-mentioned two steps were combined together in a single pot. In the first step, 10 mM CDCA was transformed to 7-oxo-LCA by using *E. coli* 7α-HSDH, with LDH for cofactor regeneration. After complete conversion of 10 mM CDCA into 7-oxo-LCA, the solution was heated to inactivate 7α-HSDH and LDH. Then 7β-HSDH$_{Rt}$ and GDH were added into the mixture to transform 7-oxo-LCA to UDCA. As shown in Table 2, the space–time yield of UDCA in the two-step reaction was obviously higher than that in the other experiments.

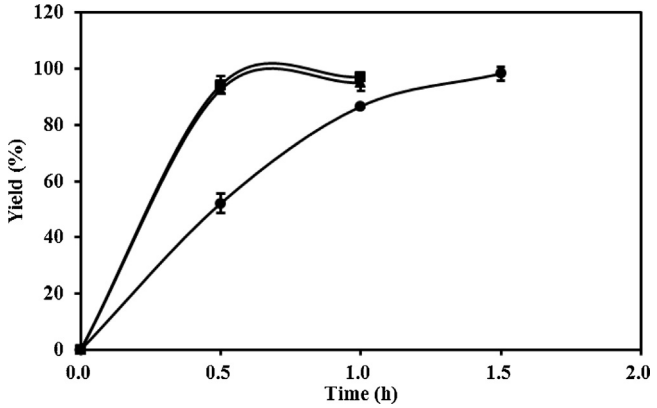

Fig. 7. Time course of 7-oxo-LCA reduction by 7β-HSDH$_{Rt}$ with different concentration. *Circle*, 0.1 U/mL; *triangle*, 0.5 U/mL; *square*, 2.0 U/mL. The yield of UDCA was analyzed by HPLC.

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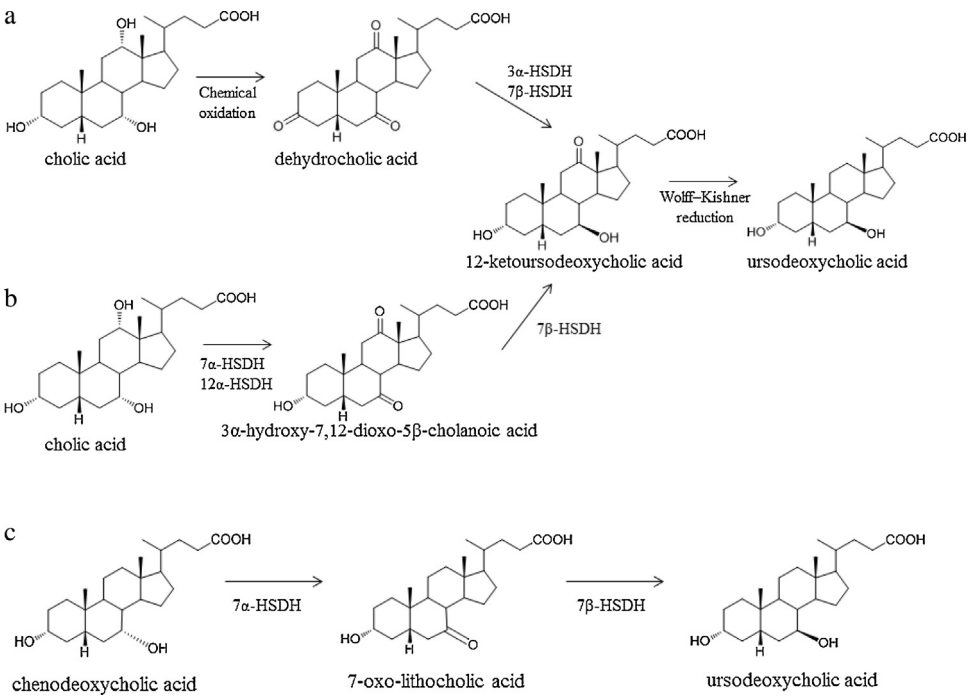

Fig. 8. Synthetic routes for the preparation of ursodeoxycholic acid (UDCA).

4. Discussion

To the best of our knowledge, only three genes encoding 7β-HSDHs have been cloned so far [21,23,24]. In this work, a new 7β-HSDH was identified, and its enzymatic properties were characterized. 7β-HSDH$_{Rt}$ and other reported 7β-HSDHs contain three highly conserved residues, Ser-143, Tyr-156, and Lys-160 [24], which are the catalytic triad of SDR superfamily [29]. Furthermore, the Gly-X-X-X-Gly-X-Gly sequence (Gly-15, Gly-19, Gly-21) of *N*-terminal contains amino acidic residues to bind with the NAD(P)H cofactor of SDRs [29]. In addition, 7β-HSDH$_{Rt}$ could completely convert 10 mM 7-oxo-LCA into UDCA within 1.5 h at 0.1 U mL^{-1} (Fig. 7).

The overall yield of UDCA was too low through chemical preparation starting with cholic acid [4,7,30]. To improve the yield, three major routes have been investigated. In the first route, cholic acid was chemically oxidized to dehydrocholic acid, followed by enzymatic reduction to 12-ketoursodeoxycholic acid using 3α-HSDH and 7β-HSDH. Then UDCA was formed by chemical reduction of 12-ketoursodeoxycholic acid by Wolff–Kishner reduction [31–33] (Fig. 8a). In the second route, cholic acid was first oxidized enzymatically to 3α-hydroxy-7.12-dioxo-5β-cholanoic acid by 7α-HSDH and 12α-HSDH, then biochemically reduced to 12-ketoursodeoxycholic acid by 7β-HSDH, and finally the carbonyl group at C-12 was chemically reduced by Wolff–Kishner reduction (Fig. 8b). For example, Monti et al. [34] has reported the synthesis of 12-ketoursodeoxycholic acid from cholic acid *via* a one-pot reaction. The one-pot reaction was not successful because of the unspecific driving force of the GDH, so they used sequential synthesis of 12-ketoursodeoxycholic acid with compartmentalized enzymes. The third route begins with CDCA as a substrate (Fig. 8c). Previously, the transformation of UDCA from CDCA employed mostly isolated intestinal bacteria *in vivo* whose yield was low [10–13].

Firstly, we investigated the feasibility to directly biosynthesize UDCA from CDCA in a cascade one-pot reaction by combining 7α-HSDH and 7β-HSDH with self-recycling of cofactors without additionally coupled enzymes *in vitro* (Fig. 1). However, since the reaction catalyzed by HSDH is reversible, the complete conversion of substrate could not be achieved which is the main difficulty in biotransformation of CDCA to UDCA through such a one-pot method. In the present work, this shortage has been successfully overcome by a two-step reaction strategy (Fig. 2). The enzymes 7α-HSDH and LDH employed in the 1st-step reaction were simply inactivated by heating to avoid the reverse reaction. Furthermore, the yield was improved because the intermediate did not need to be extracted between the two steps.

Recently, Ertl et al. have filed a patent about biotransformation of UDCA from CDCA in one-pot [35]. It is worth noting that the high substrate load was achieved at the cost of a very long reaction time (about 72 h). In our study, we found that the poor solubility

Table 2
The biotransformation results of CDCA to UDCA.

No.	Substrate load (mM)	Yield (%)	Space–time yield (mmol L^{-1} d^{-1})	Ref.
1	0.10	52–76	0.03–0.04	[12]
2	0.20	90	0.2	[14]
3	0.58	75	0.2	[28]
4	1.0	55–60	0.3	[10]
5	0.50	80	0.4	[11]
6	12.5	86	5.4	[27]
7	25	27	6.8	[13,16]
8	250	>99	83	[35]
9	10	73	29	This study (one-step)
10	10	98	118	This study (two-step)

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of the substrate might lead to the long reaction time. Solubility is the second difficulty in this route. Cholic acid and dehydrocholic acid are well soluble in aqueous solution with a concentration at least up to 100 mM in their salt form (pH 8.0), but CDCA is poorly soluble with only about 10 mM concentration under the same condition (pH 8.0). In order to improve the bioconversion efficiency, we decreased the substrate load to 10 mM for a complete dissolution so that the biotransformation could complete after merely 2 h. Overall, in this work, the use of two-step-in-one-pot strategy permitted a higher space–time yield (Table 2).

5. Conclusions

In summary, a new 7β-HSDH from *R. torques* ATCC 35915 was discovered by genomic data mining and used for high-performance transformation of UDCA from CDCA. The one-step cascade reaction synthesis of UDCA from CDCA can be achieved by using 7α-HSDH$_{Ca}$ and 7β-HSDH$_{Rt}$ simultaneously without additional coupling enzymes, in spite of a relatively low UDCA yield of about 73% due to chemical equilibrium. The yield of UDCA was further improved up to 98% by using a two-step method, avoiding the reverse reaction by simply inactivating the enzymes 7α-HSDH and LDH used in the oxidative reaction of CDCA, which enables the complete reduction of the intermediate 7-oxo-LCA. More importantly, such a strategy allows the final product very easy to be isolated since either the initial substrate (CDCA) or the intermediate (7-oxo-LCA) has been almost completely converted into the desired product (UDCA).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2014.12.026.

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