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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Identification, cloning, heterologous expression, and characterization of a NADPH-dependent 7β-hydroxysteroid dehydrogenase from *Collinsella aerofaciens*

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Abstract A gene encoding an NADPH-dependent 7β-hydroxysteroid dehydrogenase (7β-HSDH) from *Collinsella aerofaciens* DSM 3979 (ATCC 25986, formerly *Eubacterium aerofaciens*) was identified and cloned in this study. Sequence comparison of the translated amino acid sequence suggests that the enzyme belongs to the short-chain dehydrogenase superfamily. This enzyme was expressed in *Escherichia coli* with a yield of 330 mg (5,828 U) per liter of culture. The enzyme catalyzes both the oxidation of ursodeoxycholic acid (UDA) forming 7-keto-lithocholic acid (KLA) and the reduction of KLA forming UDA acid in the presence of $NADP^+$ or NADPH, respectively. In the presence of NADPH, 7β-HSDH can also reduce dehydrocholic acid. SDS-PAGE and gel filtration of the expressed and purified enzyme revealed a dimeric nature of 7β-HSDH with a size of 30 kDa for each subunit. If used for the oxidation of UDA, its pH optimum is between 9 and 10 whereas for the reduction of KLA and dehydrocholic acid it shows an optimum between pH 4 to 6. Usage of the enzyme for the biotransformation of KLA in a 0.5-g scale showed that this 7β-HSDH is a useful biocatalyst for producing UDA from suitable precursors in a preparative scale.

Keywords Cholic acid · Hydroxysteroid dehydrogenase · Short-chain dehydrogenase · Ursodeoxycholic acid

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Introduction

Ursodeoxycholic acid (UDA) is an important therapeutic agent for the treatment of human cholesterol gallstones (Makino et al. 1975; Stiehl et al. 1978; Salen et al. 1980), improving liver function in cholestatic diseases (Colombo et al. 1990; Combes et al. 1995) and preventing colon cancer (Im and Martinez 2004; Khare et al. 2003). UDA is prepared from cholic acid by a seven-step chemical synthesis (Kanazawa et al. 1955; Hofmann 1963). In view of these lengthy protocols and low yield (lower than 30%), various attempts have been made to shorten the synthesis and increase the yield of UDA by substituting a series of synthetic steps by appropriate enzymes. Thus, UDA can be synthesized by epimerization of chenodeoxycholic acid (CDA) via the intermediate 7-keto-lithocholic acid using a

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combination of 7α- and 7β-hydroxysteroid dehydrogenases (HSDH) (Lepercq et al. 2004). UDA can also be synthesized from cholic acid by enzymes in combination with chemical steps. An enzymatic production of the intermediate 12-ketochenodeoxycholic acid using 12α-HSDH with cofactor regeneration system was reported (Fossati et al. 2006). Sutherland et al. (1982) described a combination of 12α-HSDH, enzymatic epimerization using 7α- and 7β-HSDH and Wolff–Kishner reduction in different sequences. Monti et al. (2009) reported the synthesis of UDA from cholic acid through an intermediate 12-keto-ursodoxycholic acid using 7β-HSDH isolated from *Clostridium absonum* combined with oxidation by 7α-HSDH and 12α-HSDH. However, these enzymes are derived from anaerobic bacteria and cannot be easily prepared at a scale useful in industry.

This first report on a gene encoding 7β-HSDH and the characterization of this recombinant enzyme is part of a campaign to make suitable enzymes available in sufficient qualities for the industrial preparation of UDA.

The human intestinal microbial flora counts about 10^{14} bacterial cells and plays an important role in health and disease (Tancrede 1992; Eckburg et al. 2005). In the human terminal ileum and proximal cecum, about 5% bile salts escape active absorption by enterohepatic circulation and are biotransformed by intestinal bacteria (Baron and Hylemon 2000). The major biotransformation of bile salts in the human intestine includes deconjugation, epimerization, and oxidation of hydroxy groups at C-3, C-7, and C-12.

Epimerization of the 7α-hydroxy group of CDA reduces toxicity because UDA is less hydrophobic than CDA and thus less harmful to cell membranes (Armstrong and Carey 1982). MacDonald et al. observed that *C. absonum* can grow on plates containing 1 mM UDA but not on plates containing 1 mM CDA (MacDonald et al. 1983). The epimerization of 7α-hydroxy bile acids is carried out by the combination of 7α-HSDH and 7β-HSDH produced by intestinal microorganism (Hirano and Masuda 1981; MacDonald et al. 1982). Intraspecies 7-epimerization by 7α-HSDH and 7β-HSDH has been demonstrated in *Clostridium limosum* (Sutherland and Williams 1985) and *C. absonum* (MacDonald and Roach 1981), which can express both 7α-HSDH and 7β-HSDH. Oxidation of hydroxy groups of bile acids by HSDHs may provide reducing equivalents for cell metabolism (Sherrod and Hylemon 1977) but may also depend on the redox potential of the intestinal environment (Sutherland and MacDonald 1982).

Sequence comparison using bioinformatic tools has revealed that HSDHs belong to the short-chain dehydrogenase superfamily (SDR) (Jörnvall et al. 1995). They catalyze the reversible oxidation and reduction of bile acids through a tyrosine residue in the active site, which is stabilized in the transition state by the side chain of an ionized lysine, acting as the proton donor and acceptor, respectively.

The regulation of expression of these HSDHs depends on the enzyme and the source organism. For instance, 7β-HSDH from *Collinsella aerofaciens* DSM 3979 is expressed constitutively and repressed by UDA (Hirano and Masuda 1982). CDA induces the expression of 7α- and 7β- HSDHs from *C. limosum* and *C. absonum*, while UDA represses the expression of both enzymes (Sutherland and Williams 1985; MacDonald and Roach 1981). 3α-HSDH from *Comamonas testosteroni* is not a constitutive enzyme either, which is induced by steroids (Möbus and Maser 1998).

C. aerofaciens has previously been reported to produce 7β-HSDH (Hirano and Masuda 1982). In our study, we have identified the gene encoding 7β-HSDH from *C. aerofaciens*. 7β-HSDH was cloned from this organism, heterologously overexpressed, purified, and functionally characterized. Moreover, a preparative scale biotransformation using recombinant 7β-HSDH was carried out to produce UDA from 7-keto-lithocholic acid.

Materials and methods

Enzymes and chemicals

The genomic DNA of *C. aerofaciens* DSM 3979 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). UDA and KLA were obtained from PharmaZell GmbH (Germany). All other chemicals were purchased from Sigma-Aldrich and Fluka (Germany). All the restriction endonucleases, T4 DNA ligase, *Taq* DNA polymerase, and isopropyl β-D-1-thiogalactopyranoside (IPTG) were obtained from Fermentas (Germany).

Bacterial strains and culture conditions

Escherichia coli strains DH5α (Novagen, Madison, WI, USA) was grown at 37 °C in LB medium containing appropriate antibiotics. *E. coli* strain BL21(DE3) (Novagen, Madison, WI, USA) was grown at 37 °C in LB medium containing appropriate antibiotics. At OD_{600} 0.8, it was induced with 0.5 mM IPTG. After that, it was incubated at 25 °C at 140 rpm for 15 h.

Cloning and creation of an expression construct

The gene (accession number ZP_01773061 of the GenBank database) encoding 7β-HSDH was amplified from genomic DNA of *C. aerofaciens* by PCR using the following primers: 5′-gggaattcCATATGAACCTGAGGGAGAAGTA-3′ and 5′-cccAAGCTTCTAGTCGCGGTAGAACGA-3′. The restriction endonuclease sites for *Nde*I and *Hin*dIII are underlined. The PCR product was purified and digested with *Nde*I and *Hin*dIII restriction endonucleases. The digested PCR product

was purified and cloned into the pET-28a(+) (Novagen, Madison, WI, USA) vector using T4 ligase to create an expression construct. The resulting expression construct was subsequently transformed into *E. coli* DH5α competent cells. The expected protein should include a pre-peptide consisting of 20 residues and a N-terminal 6xHis-tag as well as a thrombin cleavage site. The sequence of the insert DNA was sequenced for confirmation of the insertion and the absence of any mutations.

Overexpression and purification of 7β-HSDH

E. coli BL21(DE3) was transformed with the expression construct. *E. coli* BL21(DE3) containing the expression construct was grown in LB medium (2×400 ml in 2-l shake flask) containing 30 μg/ml kanamycin. The cells were harvested by centrifugation at 10,000×*g* for 15 min at 4 °C. The pellet was resuspended in 20 ml of 50 mM potassium phosphate buffer, pH 8, containing 0.1 mM PMSF. The cells were disrupted under constant cooling by sonication for 1 min with 40 W output, 40% work interval, and 1-min break using a sonicator-type Sonifier 250 (Branson, Germany). This process was repeated for three times. The cell extract was centrifuged at 22,000×*g* for 20 min at 4 °C. The supernatant was loaded onto a Talon column (Clontech, USA) equilibrated with loading buffer (50 mM potassium phosphate, 300 mM NaCl, pH 8). The process was performed at 24 °C. Unbound materials were removed by washing the column with loading buffer (three column volumes). The weakly bound proteins were removed by washing with washing buffer (20 mM imidazole in loading buffer; three column volumes). The His-tagged 7β-HSDH protein was eluted with elution buffer (200 mM imidazole in loading buffer). The eluate was dialysed in a dialysis tube with a molecular weight cutoff of 5 kDa (Sigma, USA) in 2 L potassium phosphate buffer (50 mM, pH 8) at 4 °C overnight. Finally, the sample was transferred into a new tube and stored at –20 °C for further analyses. Protein concentration was determined using the BCA assay kit (Thermo, USA) according to the manufacturer's instructions. Then, the sample was analyzed by 12.5% SDS-PAGE and visualized by Coomassie brilliant blue staining. The purity of the protein was determined densitometrically by Scion Image Beta 4.0.2 (Scion, USA).

Gel filtration

Gel filtration was carried out on a Pharmacia ÄKTA Protein Purifier system to determine the molecular mass of 7β-HSDH. The purified enzyme was applied to a Sephadex G-200 column, previously equilibrated with 50 mM Tris–HCl (pH 8) containing 200 mM sodium chloride, and eluted with the same buffer at a flow rate of 1 ml/min. The molecular weight of 7β-HSDH was determined by comparing its elution volume with that of the standard proteins, albumin serum (66 kDa), α-amylase from *Aspergillus oryzae* (52 kDa), trypsin from porcine pancreas (24 kDa), and lysozyme from chicken egg (14.4 kDa).

Enzyme assay and kinetic analysis

The enzyme assay mixture contained, in a total volume of 1 ml, 50 μmol potassium phosphate (pH 8), 0.1 μmol NAD(P)H or $NAD(P)^+$, 0.1 μmol UDA, KLA, or dehydrocholic acid, and protein in cuvettes with a path length of 1 cm. The 7β-HSDH activity was determined by recording the change of NAD(P)H concentration in absorbance at 340 nm with a spectrophotometer (Ultraspec 3,000, Pharmacia Biotech, UK). Enzyme activities were calculated as enzyme units (U, i.e., $\mu mol \times min^{-1}$) by using a molar extinction coefficient of 6.22 $mM^{-1} \times cm^{-1}$ at 25 °C. Different measurements with respect to substrate, coenzyme, concentration, pH value, buffer, and incubation temperature were performed. The kinetic constants for UDA, KLA, and dehydrocholic acid were determined in a range of concentration from 0.5 to 25 μM of UDA, KLA, and dehydrocholic acid with 0.1 mM appropriate cofactor. The kinetic constants for NADPH or $NADP^+$ were determined in a range of concentration from 1 μM to 50 μM of NADPH or $NADP^+$ with 25 μM KLA or UDA, respectively. The kinetic constants were calculated by using the standard method with Microsoft Excel 2003.

Biotransformation of KLA by 7β-HSDH

A conversion of KLA by 7β-HSDH was carried out to verify the biochemical function of 7β-HSDH. A total of 0.4 g of KLA was suspended in 10 ml potassium phosphate buffer (50 mM, pH 8) and the pH value was adjusted to pH 8 by adding 2 M sodium hydroxide. In addition, 0.2 ml 2-propanol, 100 U 7β-HSDH, 80 U alcohol dehydrogenase (ADH-TE) from *Thermoanaerobacter ethanolicus* (kind gift from Dr. K. Momoi), and 1 μmol $NADP^+$ were added. The same buffer was added to 20 ml of the total reaction volume. The reaction mixture was incubated at 24 °C and stirred for 24 h. The ADH regenerated NADPH via oxidation of 2-propanol. The product was acidified with 1 ml 2 M hydrochloric acid and extracted with 5×5 ml ethyl acetate. The organic phase was subsequently removed by distillation.

Chromatographic determination of product

HPLC analysis was performed on a column Purospher® STAR RP-18 (Hitbar® RT 125-4 Pre-Packed Column, Purospher® STAR RP-18 endcapped, Merck, Germany) protected by a guard column LiChroCART® STAR RP18 (endcapped, Merck, Germany) on a HPLC system LC20AD

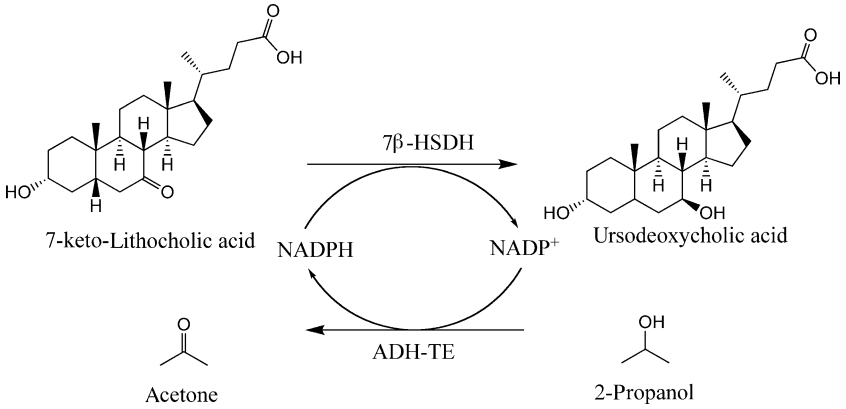


Fig. 1 Reduction of KLA by 7β-HSDH. The 7-carbonyl group of KLA was specifically reduced to 7β-hydroxy group (UDA). The cofactor NADPH was regenerated by ADH-TE

(Shimadzu, Japan) at a flow rate of 1 ml/min. The mobile phase consisted of two eluents. Eluent A was HPLC–acetonitrile and eluent B was distillated water (pH 2.6 adjusted with orthophosphoric acid 85%). The gradient was: eluent A 35% (8 min)—35%–43% (1% min^{-1})—43%–70% (1% min^{-1})—70% (5 min)—70%–35% (17.5% min^{-1})—35% (5 min); eluent A 65% (8 min)—65%–57% (1% min^{-1})—57%–30% (1% min^{-1})—30% (5 min)—30%–65% (17.5% min^{-1})—65% (5 min). Totally, 20 μl samples at 1 mg/ml were analyzed. Authentic UDA, KLA, and CDA at the same concentration were used as references. The system was monitored by UV detector at 200 nm. The identity of the produced UDA was also verified by ^{1}H NMR (deuterated dimethylsulfoxide, 500 MHz, selected data): δ=3.25 (2 H, m, H-3α, H-7β) and ^{13}C NMR (deuterated dimethylsulfoxide, 125 MHz, selected data): δ=69.69 (CH, 3-C); δ=69.42 (CH, 7-C).

Sequence alignment and phylogenetic analysis

Multiple sequence alignments were created by using the Clustal X software (Thompson et al. 1997) and modified using the Jalview software (Clamp et al. 2004). The phylogenetic tree was drawn using program TreeView 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

Results

Cloning, expression, identification of the 7β-HSDH, and preparative scale biotransformation

Several 7β-HSDH were already reported from *C. absonum* (MacDonald and Roach 1981), *C. limosum* (Sutherland and Williams 1985), *Peptostreptococcus productus*, and *C. aerofaciens* (Hirano and Masuda 1982). However, no sequences annotating 7β-HSDH have been reported. Nevertheless, due to the rapidly increasing number of sequences, we have searched the genomic data in GenBank of NCBI (www.ncbi.nlm.nih.gov/Genbank) for putative 7β-HSDH genes. Strain *C. aerofaciens*, which was isolated from human feces, was reported to have 7β-HSDH activity (Hirano and Masuda 1982). From *C. aerofaciens*, nine candidate genes were annotated as short-chain dehydrogenase (accession number in GenBank: AAVN00000000).

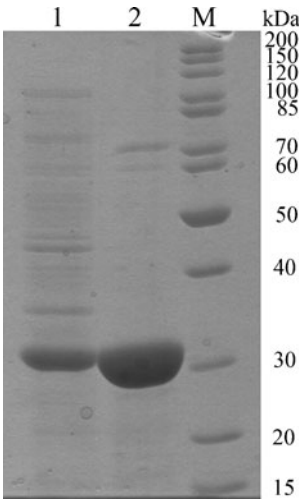
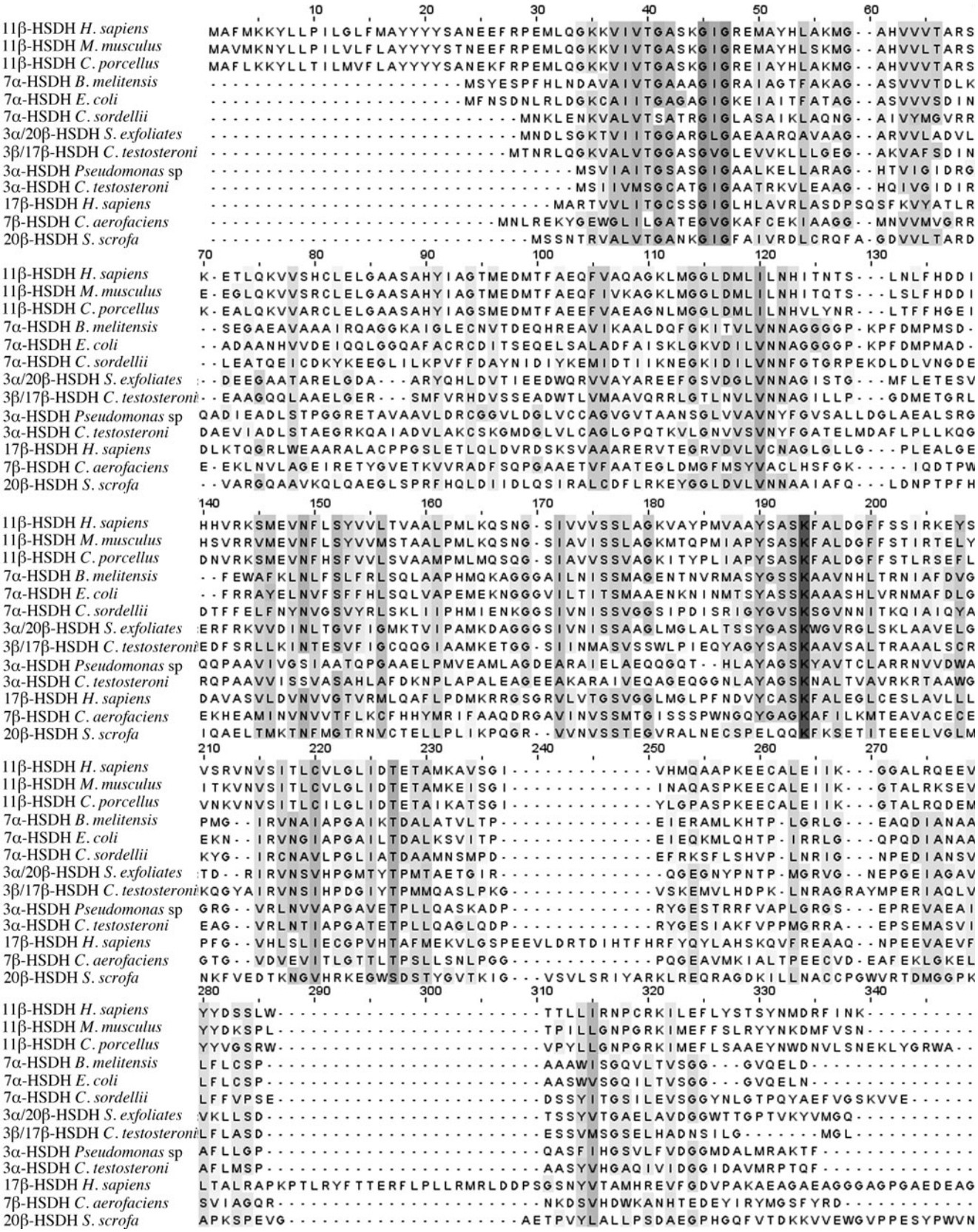


Fig. 2 *Lane 1*, crude cell extract; *lane 2*, purified protein; *lane M*, PageRuler™ Unstained Protein Ladder (Fermentas, Germany)

Fig. 3 Sequence alignment of 7β-HSDH from *C. aerofaciens* and selected HSDH proteins. The residues that are conserved in the sequences are *boxed*. The accession numbers are: 11β-HSDH from *H. sapiens*, GenBank NP_005516; 11β-HSDH from *M. musculus*, GenBank NP_001038216; 11β-HSDH from *C. porcellus*, GenBank AAS47491; 7α-HSDH from *B. melitensis*, GenBank NP_698608; 7α-HSDH from *E. coli*, GenBank NP_288055; 7α-HSDH from *C. sordellii*, GenBank P50200; 3α/20β-HSDH from *Streptomyces exfoliates*, Swiss-Port P19992; 3β/17β-HSDH *C. testosteroni* GenBank AAA25742; 3α-HSDH from *Pseudomonas* sp., GenBank BAA08861; 3α-HSDH from *C. testosteroni*, GenBank YP_003277364; 17β-HSDH from *H. sapiens*, GenBank NP_000404; 20β-HSDH from *S. scrofa*, GenBank NP_999238. The last 17 amino acids of 17β-HSDH from *H. sapiens* and the last one amino acid of 20β-HSDH from *S. scrofa* are not shown in this alignment because they are too long if compared to other HSDHs and no more alignment information is relevant within this range


11β-HSDH H. sapiens
11β-HSDH M. musculus
11β-HSDH C. porcellus
7α-HSDH B. melitensis
7α-HSDH E. coli
7α-HSDH C. sordellii
3α/20β-HSDH S. exfoliates
3β/17β-HSDH C. testosteroni
3α-HSDH Pseudomonas sp
3α-HSDH C. testosteroni
17β-HSDH H. sapiens
7β-HSDH C. aerofaciens
20β-HSDH S. scrofa

After the heterologous expression of all candidate genes in *E. coli*, only one was successfully found to demonstrate reversible activity towards UDA and KLA in the presence of $NADP^+$ or NADPH, respectively. To confirm the function of this enzyme, a 10-ml scale biotransformation of 0.4 g KLA was carried out using this enzyme combined with an ADH for regeneration of NADPH from 2-propanol. The reaction scheme is shown in Fig. 1.

The HPLC analysis indicates that UDA (retention time, RT, 15.5 min) was the only product from KLA (RT, 18.3 min) produced by this enzyme, with a conversion of 90% and a yield of 71%. The 7α-enantiomer, CDA (RT, 19.4 min), was not detected in the product. The result shows that the enzyme is an NADPH-dependent 7β-HSDH and able to selectively reduce the 7-carbonyl group of KLA into the 7β-hydroxy group of UDA.

Purification and gel filtration

The 7β-HSDH gene from *C. aerofaciens* was cloned into the pET28a(+) expression vector and the subsequent over-expression resulted in a N-terminal His-tagged fusion protein with a 7β-HSDH yield of 332.5 mg (5,828 U) per liter culture (OD_{600}=6). This His-tagged 7β-HSDH was purified in one step by an immobilized metal ion affinity chromatography (purity >90%, recovery 76%; Fig. 2). The main band in lanes 1 and 2 is the expected expression product at 30 kDa which corresponds to the molecular weight predicted from the amino acid sequence of its gene. However, the purified 7β-HSDH was eluted as a 56.1-kDa protein upon gel filtration. The result reveals the dimeric nature of 7β-HSDH from *C. aerofaciens*.

Sequence alignments

The amino acid sequence was compared to known HSDHs sequences (Fig. 3). Sequence similarity suggests that it belongs to the short-chain dehydrogenase (SDR) family. It is known that the SDRs share very low homology and sequence identity (Persson et al. 1991; Jörnvall et al. 1995). However, sequence alignment clearly showed conserved domains in the SDR primary structure. The N-terminal Gly-X-X-X-Gly-X-Gly sequence (Gly-41, Gly-45, and Gly-47, numbering according to the alignment) is the characteristic dinucleotide-binding motif of the SDR superfamily (Jörnvall et al. 1995). 7α-HSDH from *Clostridium sordellii* shows here an exception with the Gly-41 being replaced by Ser. Furthermore, there are three highly conserved residues, Ser-177, Thr-190, and Lys-194 (numbering according to the alignment), which constitute the catalytic triad of SDR enzymes (Jörnvall et al. 1995). However, the two 3α-HSDH from *Pseudomonas* sp. and *C. testosteroni* do not contain Ser at position 177, but Leu or Ile, respectively.

Phylogenetic analysis

The evolutionary tree based on the alignment in Fig. 3 is given in Fig. 4. The HSDHs are classified by function rather than species. 7α-HSDHs from *C. sordellii*, *Brucella melitensis*, and *E. coli* belong to the same subgroup. The two 3α-HSDHs show a much closer relationship than other HSDHs. Interestingly, this prokaryotic 7β-HSDH is related to the animal 11β-HSDH subgroup, which are from *Cavia porcellus*, *Homo sapiens*, and *Mus musculus*.

Kinetic constants

Steady-state kinetic analysis by Lineweaver–Burk plots was performed to determine the absolute values of V_{max} and K_M for UDA, KLA, dehydrocholic acid, $NADP^+$, and NADPH. The kinetic constants for all substrates and coenzymes obtained from substrate saturation curves and reciprocal plots are summarized in Table 1. The V_{max}, K_M, and K_{cat} values for all substrates and coenzymes are in the same range. The enzyme is NADPH dependent, and the kinetic constants for NAD^+ and NADH could not be determined due to very weak activity.

pH optimum

7β-HSDH activity towards several substrates as a function of pH was measured with purified enzyme (Fig. 5). The oxidation of UDA by 7β-HSDH demonstrated an optimal

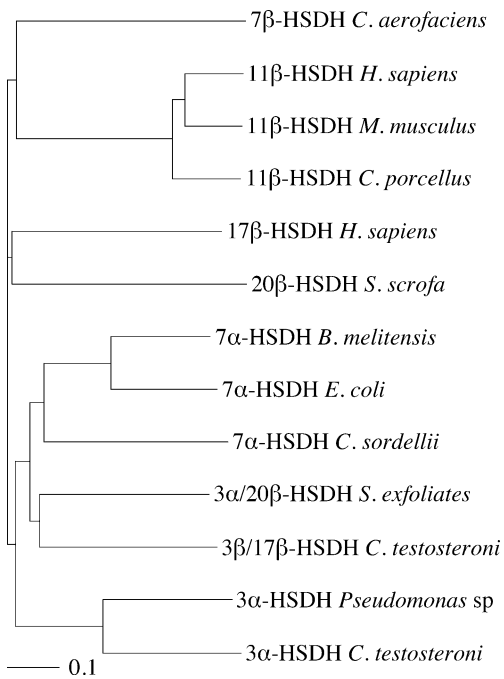


Fig. 4 Phylogenetic tree based on alignment of HSDH protein sequences, indicating the relationships between the selected HSDHs

Table 1 Summary of kinetic constants for 7β-HSDH from *C. aerofaciens*

	K_M (μM)	V_{max} (U × mg^{-1} of protein)	K_{cat} (1 μmol× (μmol×min)$^{-1}$)
$NADP^+$	5.32	30.58	944.95
NADPH	4.50	33.44	1,033.44
UDA	6.23	38.17	1,179.39
KLA	5.20	30.77	950.77
Dehydrocholic acid	9.23	28.33	875.35

activity in the range of 9 to 10, with a gradual drop on the acidic side. On the contrary, the reduction of dehydrocholic acid and 7-keto-lithocholic acid by 7β-HSDH showed an optimal activity in the range of 4 to 6, with a sharp drop on the acidic and a gradual drop on the alkaline side. The use of different buffers showed only minor effects on the activity of 7β-HSDH at the same pH value.

Thermal stability

NADP-dependent 7β-HSDH is not very thermostable. In aqueous solution, the enzyme was completely inactivated at 50 °C within 5 min (Fig. 6, upper right) and at 40 °C within 400 min (Fig. 6). Due to thermodynamic activation, 7β-HSDH was about 18% more active at 30 °C as compared to 23 °C. As enzyme activity at 30 °C decreased much faster than at 23 °C, the residual activity after 400 min at 30 °C was about 30% lower than that at 23 °C. After 1,500 min, the enzyme was completely inactivated at 30 °C while at 23 °C 20% of the activity remained. No significant loss of activity was observed during storage at−20 °C in potassium phosphate buffer (50 mM, pH 8) for at least 3 months with freezing and thawing for more than 100 times.

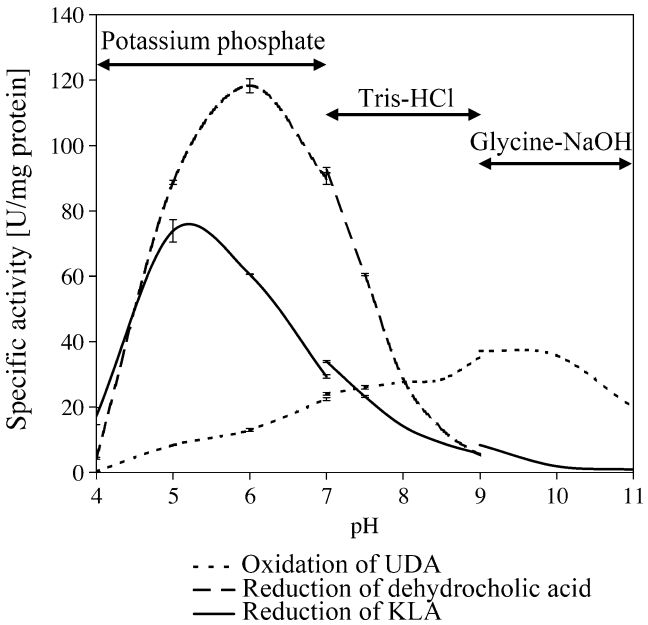


Fig. 5 Effect of pH on the activity of 7β-HSDH. The assay condition was: pH 4–7 in 50 mM potassium phosphate; pH 7–9 in 50 mM Tris–HCl; pH 9–11 in 50 mM Glycine–NaOH at 23 °C. The values for activity towards dehydrocholic acid and KLA at pH 9–11 in 50 mM Glycine–NaOH overlap. All measurements were repeated for three times. The standard deviations are shown; several values are too small to be recognized

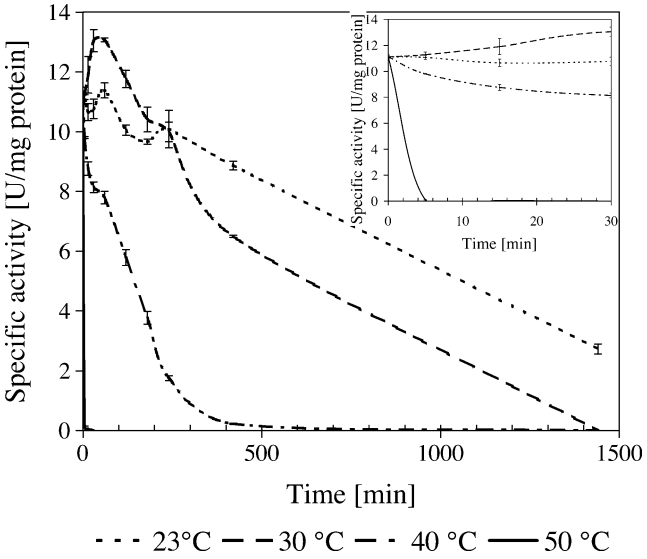


Fig. 6 Thermal inactivation of the NADP-dependent 7β-HSDH from *C. aerofaciens*. The enzyme was incubated at 23, 30, 40, or 50 °C for various periods of time up to 1,500 min (in 50 mM potassium phosphate, pH 8.0). The remaining enzymatic activity was measured under standard conditions. The thermal inactivation curve within the first 30 min is shown at the *upper right* of the diagram. All measurements were repeated for three times. The standard deviations are shown; several values are too small to be recognized

Discussion

7β-HSDH catalyzes the reversible, stereospecific oxidation/reduction of the 7β-hydroxy group of bile acids and plays a key role in 7-epimerization. Molecular cloning of genes encoding for 7β-HSDH enzymes will help to understand the genetic organization and regulation of metabolic pathways of bile salt-modifying bacteria and the phylogenetic evolution of SDRs. From a practical point of view, they may become useful enzymes for producing UDA, an important pharmaceutical agent.

In the present study, we have used information from GenBank (www.ncbi.nlm.nih.gov/Genbank) to identify, among nine putative gene sequences from *C. aerofaciens*, a gene encoding 7β-HSDH protein. This gene was overexpressed in *E. coli*, further purified and characterized. An analysis of the crystal structure of 7-oxo-GLCA substrate/enzyme complex revealed that the binding strength of bile

acid is independent of the presence of the conjugated part (Tanaka et al. 1996). Thus, only free bile acids were investigated in this study.

Whereas a native 7β-HSDH from *C. aerofaciens* has been previously reported (Hirano and Masuda 1982), the reductive activity of this enzyme was not observed in this earlier investigation. Furthermore, K_M and V_{max} for UDA obtained with the recombinant enzyme in the present study are 16-fold lower and 20-fold higher, respectively, than in the earlier study (Hirano and Masuda 1982). Since there was no sequence information available from the previous study, it is possible that the enzymes differ.

Interestingly, 7β-HSDH exhibits a pH optimum for reduction at low pH where the solubility of bile acids is low. The pH optimum for oxidation is in the alkaline range, where bile acid solubility is high. 7α-HSDH from *B. fragilis* also shows an alkaline pH optimum for oxidation (Hylemon and Sherrod 1975). Similar pH properties were observed by Braun et al. (1991) for 12α-HSDH from *Clostridium* group P strain C 48-50. It is quite probable, if unproven, that this observation is of functional relevance.

Upon gel filtration, the purified enzyme elutes as a 56.1-kDa protein, revealing the dimeric nature of 7β-HSDH from *C. aerofaciens*. It is comparable to the dimeric 3α-HSDH from *C. testosteroni* (Maser et al. 2000) but distinct from the 12α-HSDH from *Clostridium* group P strain C 48-50 with a tetrameric quaternary structure (Braun et al. 1991).

The sequence alignment shows that 7β-HSDH from *C. aerofaciens* belongs to the short-chain dehydrogenases superfamily. Although the short-chain dehydrogenases show only 15–30% residue identity, the conserved tertiary structures, the highly conserved N-terminal cofactor-binding motif Gly-X-X-X-Gly-X-Gly, and the Thr-X-X-X-Lys motif of short-chain dehydrogenases indicate that they have common origins and early divergent evolution (Jörnvall et al. 1995). Phylogenetic analysis clearly shows the functional relationship among HSDHs. In further studies, the new sequence of 7β-HSDH may be helpful to identify additional HSDH functions by sequence alignments.

Biotransformation experiments in a 0.5-g scale showed the capability of 7β-HSDH to produce UDA acid from KDA in the presence of a cofactor regeneration system, and the conversion achieved 90%. The reaction can, however, be further optimized, e.g., by in situ removal of acetone to shift the reaction equilibrium towards UDA formation (Schroer et al. 2007). As 7β-HSDH was observed to be able to reduce dehydrocholic acid in this study, a subsequent or concomitant reduction by 3α-HSDH, which is available in recombinant form from several sources, e. g., from *C. testosteroni* (Möbus and Maser 1998), leads to the important intermediate 12-keto-UDA. UDA can then be obtained by Wolff–Kishner reduction, a chemical reaction in strong alkali which, in the presence of hydrazine, turns a keto group first into a hydrazone, which eliminates nitrogen and forms the corresponding alkane (Huang 1949). This new synthetic pathway to effectively produce UDA is presently under investigation.

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