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The catalytic promiscuity of a microbial 7α-hydroxysteroid dehydrogenase. Reduction of non-steroidal carbonyl compounds

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ABSTRACT

A thermostable 7α-hydroxysteroid dehydrogenase from *Bacteroides fragilis* ATCC 25285 was found to catalyze the reduction of various benzaldehyde analogues to their corresponding benzyl alcohols. The enzyme activity was dependent upon the substituent on the benzene ring of the substrates. Benzaldehydes with electron-withdrawing substituent usually showed higher activity than those with electron-donating groups. Furthermore, this enzyme was tolerant to some organic solvents. These results together with previous studies suggested that 7α-hydroxysteroid dehydrogenase from *B. fragilis* might play multiple functional roles in biosynthesis and metabolism of bile acids, and in the detoxification of xenobiotics containing carbonyl groups in the large intestine. In addition, its broad substrate spectrum offers great potential for finding applications not only in the synthesis of steroidal compounds of pharmaceutical importance, but also for the production of other high-value fine chemicals.

1. Introduction

Hydroxysteroid dehydrogenases, which catalyze the reversible oxidoreduction at different positions of the natural substrates steroid nucleus, are pyridine nucleotide-dependent oxidoreductases, belonging to aldo-keto reductases or the short-chain dehydrogenases/reductases [1,2]. This group of enzymes play critical roles in the biosynthesis and inactivation of all steroid hormones. Besides being involved in steroidogenesis and steroid metabolism, hydroxysteroid dehydrogenases have also exhibited activity toward the reduction of some nonsteroidal carbonyl compounds [3]. For example, 3α-hydroxysteroid dehydrogenase (3α-HSDH) from *Comamonas testosteroni* mediates the oxidoreduction at position 3 of a great variety of C_{19-27} steroids [4,5]. It also accepts as substrates a wide spectrum of non-steroidal carbonyl compounds, such as *p*-nitrobenzaldehyde, *p*-nitroacetophenone, metyrapone and the metyrapone-based insecticide NKI 42255 [5]. 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSDH1) physiologically catalyzes the interconversion of receptor-active 11-hydroxy glucocorticoids (cortisol) to their receptor-inactive 11-oxo metabolites (cortisone) [6,7]. Meanwhile, it is capable of playing the role of carbonyl reductase in the detoxification of aldehydes, ketones and quinones, for example, *p*-nitrobenzaldehyde, *p*-nitroacetophenone, metyrapone, oracin, ketoprofen and the tobacco-specific nitrosamine NNK [6–11]. 17β-Hydroxysteroid dehydrogenase (17β-HSDH) from the filamentous fungus *Cochliobolus lunatus* reduces 3-keto groups of 4,5-dihydro steroids, 20-keto groups, and most efficiently, 17-keto groups of steroidal substrates. It is also active toward quinones, menadione, *p*-nitrobenzaldehyde, etc. [12]. However, to the best of our knowledge, there is up to now no report about the alternative funtion of 7α-hydroxysteroid dehydrogenase for the reduction of nonsteroidal carbonyl compounds except our recent study [13].

The catalytic promiscuous property of hydroxysteroid dehydrogenases [14], together with their primary function of catalyzing the interconversion between the oxo group and hydroxyl counterpart of sterically bulky steroids, suggests that they might be a group of potentially useful enzymes for applications both in the synthesis of steroidal compounds of pharmaceutical importance, and for the production of other high-value fine chemicals. However, their use in organic synthesis as a biocatalyst has been largely unexplored. They have mainly been employed for the selective modification of bile acids and other steroid derivatives [15–19]. A preparative-scale regio- and stereo-specific oxidation of hydroxy groups and reduction of keto functions at C(3) of several bile acids, catalyzed by 3α-hydroxysteroid dehydrogenase (3α-HSDH) which was isolated from the cells of *Pseudomonas pauctmobilis* as a crude enzyme, has been reported [20]. Recently, Monti and co-workers reported the hydroxysteroid dehydrogenases(HSDHs)-catalyzed one-pot enzymatic synthesis of 12-ketoursodeoxycholic acid (3α,7β-dihydroxy-12-oxo-5β-cholanoic acid), a key intermediate for the synthesis of ursodeoxycholic acid, from cholic

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acid [21]. With this in mind, in our continuing project of searching for synthetically useful enzymes for asymmetic ketone reduction, a thermostable 7α-hydroxysteroid dehydrogenase from *Bacteroides fragilis* ATCC 25285 has drawn our attention [13]. This enzyme reduces sterically demanding native substrate 7-keto-lithocholic acid to cheno-deoxycholic acid in gastrointestinal tract and it is relatively thermostable, retaining 95% of initial activity after 1 h at 65 °C [22]. Therefore, this 7α-hydroxysteroid dehydrogease was produced as recombinant protein by over-expression in *E. coli*. Its substrate specificity and stereoselectivity toward reduction of various non-steroidal carbonyl compounds have been examined. In our previous paper we reported that 7α-hydroxysteroid dehydrogenase from *B. fragilis* catalyzed the reduction of aromatic and sterically demanding aliphatic α-ketoesters to the corresponding α-hydroxyesters in essentially optically pure form, and also demonstrated its synthetic application in the preparation of ethyl (*R*)-2-hydroxy-3-methylbutyrate, ethyl (*R*)-2-hydroxy-3,3-dimethylbutyrate and ethyl (*R*)-2-hydroxy-2-(3,5-diflurophenyl)acetate [13]. Herein, we would like to present that the microbial 7α-hydroxysteroid dehydrogenase can also reduce some aldehydes to their corresponding alcohols, further revealing its multi-functional properties, i.e. catalytic promiscuity [14].

2. Materials and methods

2.1. Materials

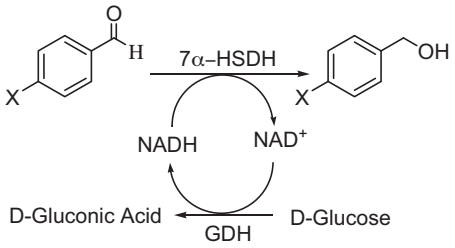
The HPLC analysis was performed on an Agilent 1200 high-performance liquid chromatography system with a Eclipse XDB-C18 column (4.6 × 150 mm). A mixture of water and methanol (v/v, 40/60) was used as eluent. All the benzaldehydes were obtained from commercial sources. The benzyl alcohols were either purchased from commercial sources or prepared by the reduction of the corresponding benzaldehydes using sodium borohydride in methanol. 7α-Hydroxysteroid dehydrogenase and D-glucose dehydrogenase were prepared as previously reported. A unit of activity (U) was defined as the enzyme that converts 1 micromolar of NADH in 1 min. The specific activity for methyl benzoylformate was 0.32 U/mg [13].

2.2. Reduction of various benzaldehydes catalyzed by 7α-hydroxysteroid dehydrogenase

The reaction procedure was as follows: glucose (18 mg), glucose dehydrogenase (0.35 U), NAD (2 mg), 7α-hydroxysteroid dehydrogenase (1.1 U) and benzaldehyde (0.02 mmol, dissolved in 10 μL of DMSO) were mixed in 1 mL of potassium phosphate buffer (100 mM, pH 7.0). The mixture was shaken for 12 h at 30 °C, and was then extracted with methyl *tert*-butyl ether (800 μL). The organic extract was dried over anhydrous sodium sulfate and subjected to HPLC analysis to measure the conversion. The products were identified by comparison with authentic samples in HPLC analysis.

2.3. 7α-Hydroxysteroid dehydrogenase-catalyzed reduction of 4-cyanobenzaldehyde in reaction media with different co-solvents

4-Cyanobenzaldehyde was chosen as substrate for the reduction in reaction media with co-solvent such as dimethyl sulfoxide, methanol, isopropyl alcohol, methyl *tert*-butyl ether, ethyl acetate, butyl acetate and toluene. The reaction procedure was as follows: glucose (18 mg), glucose dehydrogenase (0.35 U), NAD (2 mg), 7α-hydroxysteroid dehydrogenase (1.1 U) and benzaldehyde (0.02 mmol, dissolved in 0.1 mL of organic solvent) were mixed in 0.9 mL of potassium phosphate buffer (100 mM, pH 7.0). The mixture was shaken for 12 h at 30 °C, and was then extracted with methyl *tert*-butyl ether (800 μL). The organic extract was dried over anhydrous sodium sulfate and subjected to HPLC analysis to measure the conversion. The products were identified by comparison with authentic samples in HPLC analysis.



Scheme 1. Reduction of benzaldehyde analogues catalyzed by 7α-hydroxysteroid dehydrogenase with a cofactor recyle system of GDH and D-glucose.

2.4. Preparative scale reduction of benzaldehyde derivatives catalyzed by 7α-hydroxysteroid dehydrogenase

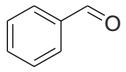
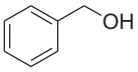
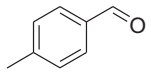
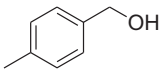
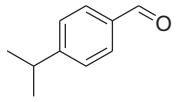
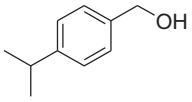
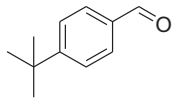
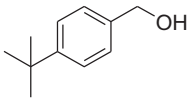
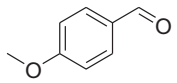
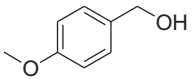
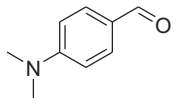
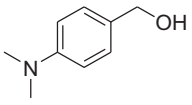
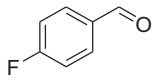
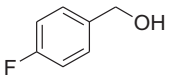
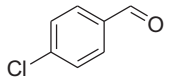
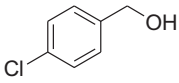
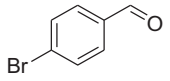
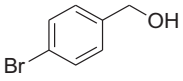
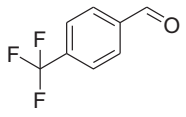
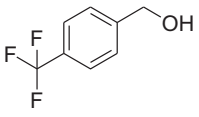
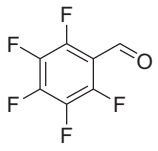
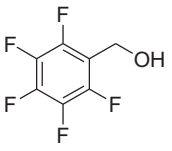
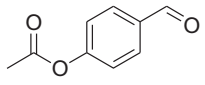
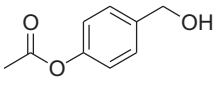
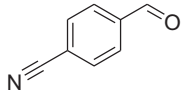
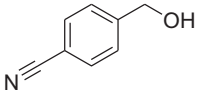
The preparative scale reactions were performed for 4-(trifluoromethyl)benzaldehyde, pentafluorobenzaldehyde, 4-acetoxybenzaldehyde and 4-cyanobenzaldehyde. The procedure was as follows (using 4-(trifluoromethyl)benzaldehyde as an example): 4-(trifluoromethyl)benzaldehyde (0.6 mmol in 300 μL DMSO), glucose (540 mg), glucose dehydrogenase (11 U), NAD (60 mg) and 7α-hydroxysteroid dehydrogenase (31 U) were mixed in 30 mL of potassium phosphate buffer (100 mM, pH 7.0). The reaction mixture was stirred at 30 °C. TLC was used to monitor the reaction. When the reduction was completed, the mixture was extracted with methyl *tert*-butyl ether (30 mL). The organic extract was dried over anhydrous sodium sulfate and the solvent was removed to give the product 4-(trifluoromethyl)benzyl alcohol (94 mg, yield 90%). The product was identified by comparing its ^{1}H NMR data with those reported in the literature [23]. Pentafluorobenzaldehyde (84 mg, yield 72%) [24]. 4-Acetoxybenzaldehyde (50 mg, yield 51%) [25]. 4-Cyanobenzaldehyde (66 mg, yield 85%) [26].

3. Results and discussion

The 7α-hydroxysteroid dehydrogenase from *B. fragilis* ATCC 25285 was prepared as recombinant enzyme according to the method reported in the literature [13,22]. The obtained 7α-hydroxysteroid dehydrogenase was assayed for activity toward various benzaldehyde analogues. The reaction was carried at 1 mL scale and the co-factor NADH was regenerated with D-glucose dehydrogenase and D-glucose, as shown in Scheme 1. The conversion was measured by HPLC analysis, and the product was identified by comparison with authentic sample in HPLC analysis. The results are presented in Table 1.

From the results, it can be seen that 7α-hydroxysteroid dehydrogenase catalyzed the reduction of various benzaldehyde derivatives, affording the corresponding alcohols. For benzaldehyde and its derivatives with alkyl, methoxy and dimethylamino substituents, the coversions were low to medium, while the benzaldehyde analogues with electron-withdrawing substituents such as chloro, bromo, trifluoromethyl and cyano groups, were reduced to the corresponding benzyl alcohols in high conversions. This showed that the enzyme had higher activity toward benzaldehydes with electron-withdrawing group than those with electron-donating substituent. The stonger electron-donating group was,

Table 1
Reduction of various benzaldehydes catalyzed by 7α-hydroxysteroid dehydrogenase.

Entry	Benzaldehyde	Product	Conversion (%)[a]
1			57
2			49
3			52
4			46
5			38
6			1
7			55
8			79
9			85
10			94
11			87
12			99
13			100

[a] Determined by HPLC analysis.

the lower the conversion of the substrate was. With respect to benzaldehydes containing fluoro, chloro and bromo group, the conversion of 4-bromobenzaldehyde was the highest despite fluorine is the strongest electron-withdrawing group. This might be due to the lager size of bromine. Other benzaldehydes with relatively large electron-withdrawing substituents were also reduced with higher conversion. These results indicated that 7α-hydroxysteroid dehydrogenase showed higher activity toward benzaldehydes with electron-withdrawing group and when the substituent's abilities of withdrawing electron were about the same, steric factor determined the activity of this enzyme. The electronic factor might be due to the increased electron deficiency of the recipient carbonyl group undergoing reduction, which facilitates the H^- transfer from NADH to the carbonyl group.

Table 2
7α-HSDH-catalyzed reduction of 4-cyanobenzaldehyde in reaction media with different co-solvents.

Solvent in buffer[a]	Conversion (%)[b]
No solvent	93
DMSO	90
MeOH	94
i-PrOH	92
MTBE	94
Ethyl acetate	79
Butyl acetate	63
Toluene	85

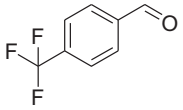
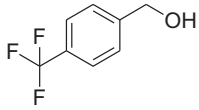
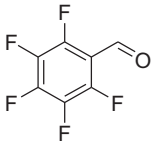
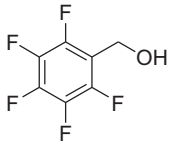
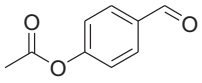
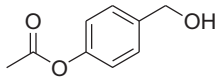
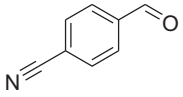
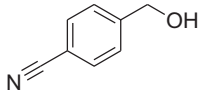
[a] Potassium phosphate buffer (100 mM, pH 7.0) containing 10% (v/v) of organic solvent except where indicated otherwise.
[b] Determined by HPLC analysis.

Introduction of organic solvents, which improve the solubility of hydrophobic substrates, is increasingly used as a strategy to overcome the difficulty in the biocatalytic reaction of the poorly water-soluble substrates in aqueous media. Enzymes with ability to tolerate organic solvents is thus of great interest from both scientific and practical points of view. Thermostable enzymes usually are tolerant of organic solvent to some extent. The 7α-hydroxysteroid dehydrogenase from *B. fragilis* ATCC 25285 is a relatively highly thermostable enzyme. It would be interesting to test its tolerance of organic solvents. As such, 4-cyanobenzaldehyde was chosen as substrate to examine the activity of 7α-HSDH in potassium phosphate buffer with various organic solvents. The results are summarized in Table 2. From Table 2, it can be seen that toluene, ethyl acetate, especially, butyl acetate in reaction medium decreased the activity of 7α-HSDH, while dimethylsulfoxide, methanol, isopropanol and methyl *t*-butyl ether had little effect on the activity of the enzyme. The results showed that 7α-hydroxysteroid dehydrogenase from *B. fragilis* ATCC 25285 is highly tolerant of organic solvents.

The biocatalytic reduction of a few benzaldehyde derivatives was performed at preparative scale and the product benzyl alcohols were isolated and characterized by comparing their ^{1}H NMR data with those in the literature (Table 3). 4-Acetoxybenzylalcohol was obtained in 51% yield, while other benzyl alcohols, 4-trifloromethylbenzyl alcohol, 2,3,4,5,6-pentafluorobenzyl alcohol and 4-cyanobenzyl alcohol were prepared in high yields.

Therefore, it has been demonstrated that 7α-hydroxysteroid dehydrogenase from *B. fragilis* ATCC 25285 not only catalyze the interconversion of hydroxyl/oxo groups of bile acids, but also catalyze the reduction of nonsteroidal carbonyl compounds such as ketones, benzaldehydes and keto-esters [13]. Some hydroxysteroid dehydrogenases, e.g. 3α-hydroxysteroid dehydrogenase, 11β-hydroxysteroid dehydrogenase type 1 and 17β-hydroxysteroid dehydrogenase, have showed activity toward the reduction of some nonsteroidal carbonyl compounds. Thus it is commonly accepted that these enzymes play important roles in the detoxification of various xenobiotics that contain reactive carbonyl groups, besides their primary functions in steroidogenesis and steroid metabolism [6]. 7α-Hydroxysteroid dehydrogenase is a common class of bile acid hydroxysteroid dehydrogenases found in nature, having been detected in numerous genera of bacteria [27–35] and in mammalian liver [36]. The *Bacteroides* are the predominant genera in the large intestine [37]. Therefore, it is reasonable to believe that 7α-hydroxysteroid dehydrogenase also play multiple functional roles in biosynthesis and metabolism of bile acids, and in the detoxification of xenobiotics containing carbonyl groups.

Table 3
Preparative scale reduction of some benzaldehydes catalyzed by 7α-hydroxysteroid dehydrogenase.

Benzaldehyde	Product	Reaction time (h)	Isolated yield (%)
		42	90
		42	72
		21	51
		25	85

4. Conclusions

The present study has for the first time demonstrated that 7α-hydroxysteroid dehydrogenase from *B. fragilis* catalyzes the reduction of various benzaldehyde analogues to their corresponding benzyl alcohols. The enzyme activity is dependent upon the substituent on the benzene ring of the substrates. Usually, electron-withdrawing substituent increases the enzyme activity while the enzyme is less active toward substrates with electron-donating groups. Moreover, this enzyme shows good tolerance of organic solvents. These results together with previous studies [13,21,22] have demonstrated that 7α-hydroxysteroid dehydrogenase from *B. fragilis* not only reduces the native substrate 7-keto-lithocholic acid to cheno-deoxycholic acid, but also catalyze the reduction of nonsteroidal carbonyl compounds, suggesting the alternative functions of this enzyme in the detoxification of xenobiotics containing carbonyl groups in the large intestine. In addition, the broad substrate spectrum of 7α-hydroxysteroid dehydrogenase provides opportunity for finding applications in the construction of chiral alcohol building blocks for the production of high-value fine chemicals, as well as the synthesis of steroidal compounds of pharmaceutical importance.

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