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Short communication

Asymmetric bioreduction of keto groups of 4- and 5-Oxodecanoic acids/esters with a new carbonyl reductase

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ABSTRACT

A novel carbonyl reductase from *Serratia marcescens*, *Sm*CR, was successfully cloned and overexpressed in *Escherichia coli*. *Sm*CR could catalyze the asymmetric reduction of long-chain keto acids/esters containing remote carbonyl groups, such as 4-oxo- and 5-oxodecanoic acids, yielding chiral γ- and δ-decalactones with high enantiopurity (up to 99% *ee*). This is the first report of enzymatic synthesis of (*R*)-γ- and (*R*)-δ-decalactones starting from γ-, δ-keto acids using free enzymes.

1. Introduction

Optically pure aliphatic hydroxy acids and their derivatives have been extensively studied as versatile chiral building blocks for the preparation of many valuable bioactive compounds and natural products [1–4]. For instance (*R*)-(+)-γ- and (*R*)-(+)-δ-decalactones, one class of flavors and fragrances that are widely used in the food industry, can be readily synthesized by the direct cyclization of (*R*)-4-hydroxydecanoic acid and (*R*)-5-hydroxydecanoic acid, respectively [5,6]. Because of their high application potential, the production of chiral hydroxy acids has attracted increasing attention. For example, the hydrolysis of biopolymer polyhydroxyalkanoate (PHA) is one of the major biotechnological sources of chiral hydroxy acids, and a lot of achievements have been made in this area [7,8]. In terms of synthesis, the asymmetric reduction of keto acids has been demonstrated to be one of the most efficient and atom economic routes to chiral hydroxy acids. However, although many chemical catalysts have been developed in the last few decades, the highly enantioselective reduction of remote carbonyl groups in keto acids (or esters) is still challenging because the majority of the substrates that have been studied have been limited to α-/β-aromatic or short-chain aliphatic keto acids (esters) [9–15]. Recently, Lin and co-workers developed a chiral surfactant-type catalyst for the asymmetric transfer hydrogenation of long-chain aliphatic keto esters in water [16]. However, this method was not applied for keto acids and the enantioselectivity was not satisfactory. Furthermore, the transition metals employed are expensive and inappropriate for applications in the food industry.

In contrast to chemical catalysts, the asymmetric reduction of keto acids/esters through biocatalysis is considered to be environmentally friendly and highly stereoselective. In the early 1960s, the wild-type *Saccharomyces cerevisiae* was found to be capable of converting γ- and δ-keto acids into optically pure hydroxy acids [17,18]. Later, it was employed to produce chiral lactones that were confirmed to have the *R* configuration, which is the enantiomer that is used widely in the food industry [19–22]. However, attempts to clone and express the enzyme that was responsible for the conversion from the genome of *Saccharomyces cerevisiae* were not successful. Currently, nothing is known about the exact enzyme catalyzing the reduction of the remote carbonyl groups in long-chain alkyl substituted (*R*)-lactones, despite a huge number of carbonyl reductases reported in the literature [23–28]. Recently, a number of chemo- or chemoenzymatic methods for preparing enantioenriched γ- and δ-lactones were reported; however, the substrates were still limited to short-chain alkyl or aryl substituted keto acids/esters [29–34].

In this work, we report a novel carbonyl reductase with high enantioselectivity toward γ- and δ- long-chain keto acids/esters and an efficient enzymatic route for the synthesis of optically pure (*R*)-(+)-γ- and (*R*)-(+)-δ-decalactones (Scheme 1). The benefit of using a carbonyl reductase is that the molecular structure of the functional enzyme is known. Therefore, the activity and stereoselectivity can be further enhanced by protein engineering, which is impossible to achieve by whole-cell biocatalysis employing wild-type microorganisms.

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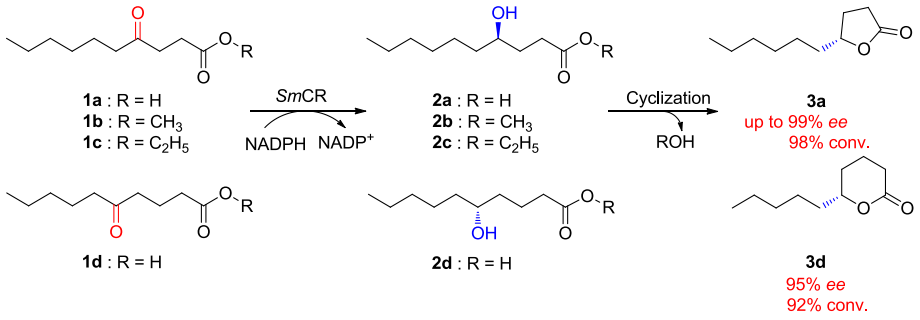

Scheme 1. Carbonyl reductase catalyzed asymmetric reduction of γ- and δ-keto acids/esters into chiral hydroxy acids/esters and lactones.

2. Experimental

2.1. Materials

4-Oxodecanoic acid, methyl 4-oxodecanoate, ethyl 4-oxodecanoate, 5-oxodecanoic acid, methyl 5-oxodecanoate and ethyl 5-oxodecanoate were obtained from Xiamen Bestally Biotechnology Co., Ltd. (Fujian, China). Racemic γ-decalactone and δ-decalactone were purchased from TCI (Shanghai, China). All other chemicals and reagents were obtained commercially. *E. coli* DH5α and *E. coli* BL21 (DE3) were used as the cloning and expression hosts, respectively. Plasmid pET-28a (+) was used for heterogeneous expression.

2.2. Cloning and expression of carbonyl reductase in E. coli

The genes of carbonyl reductases were amplified by PCR using the primers listed in Table S1 (see Supporting information). The amplified DNA were then inserted into expression plasmid pET-28a (+). The recombinant plasmids were then transformed into *E. coli* BL21 (DE3). The cells were cultivated at 37 °C in 100 mL LB medium containing 50 mg/L Kanamycin. The expression of encoding genes were induced by the addition of IPTG to a final concentration of 0.2 mM at OD_{600} of 0.6. After addition of IPTG, the cultures were grown at 16 °C for another 24 h.

2.3. Enzyme assays

The reductase activity was determined by monitoring the decrease in the absorbance of NADPH at 340 nm. The assay was performed in a 96-well half-area plate. Each reaction contained a final concentration of 0.1 mM NADPH, 0.2 mM substrate, 100 mM sodium phosphate buffer (pH 7.0) and an appropriate amount of enzyme. Absorbance reading were taken every 2 min at 30 °C for 60 min using a PowerWave XS2 spectrophotometer (BioTek, USA). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol NADPH per minute under above conditions.

2.4. Preparative procedures

For cell-catalyzed reaction, the reaction mixture was composed of 0.2 mmol substrate, 1 mmol glucose, 0.05 mmol $NADP^+$, 5 g wet cells of *E. coli/Sm*CR, 0.5 g lyophilized *E. coli/Bm*GDH and 100 mL of sodium phosphate buffer (100 mM, pH 6.0). For enzyme-catalyzed reaction, substrate (25–75 mM), glucose (5 equivalents of substrate), 0.5 mM $NADP^+$, 150 mg/mL lyophilized cell-free extracts of *E. coli/Sm*CR, and 10 mg/mL lyophilized *E. coli/Bm*GDH were mixed in a 500 mL sodium phosphate buffer (100 mM, pH 6.0). The reaction was performed at 30 °C and the pH was controlled at 6.0 with addition of 1.0 mol/L NaOH solution. GC was used to monitor the reaction progress. After 16 h, the mixture was acidified to pH 2.0 with 20% H_2SO_4 and heated at 90 °C for 2 h. The mixture was extracted with ethyl acetate, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The cured product was purified by column chromatography eluting with petroleum ether and ethyl acetate (10:1).

3. Results and discussion

3.1. Cloning and expression of carbonyl reductase

Previously, we discovered a carbonyl reductase (*Cp*AR2) that can efficiently reduce the ε-carbonyl group in ethyl 8-chloro-6-oxooctanoate [28]. This enzyme was initially tested to transform 4-oxodecanoic acid, but the product was confirmed to be (*S*)-4-hydroxydecanoic acid, which was not the desired enantiomer. Because no similar enzymes have been reported, we decided to search for new carbonyl reductases from microbes. Thousands of single bacterial colonies from environmental soil samples were screened, resulting in a bacterial strain, *Pseudomonas panipatensis*, which could convert 4-oxodecanoic acid into (*R*)-4-hydroxydecanoic acid with 97% *ee*. A novel carbonyl reductase was cloned from *Pseudomonas panipatensis*, designated as *Pp*CR, and expressed by *E. coli* BL21. *Pp*CR displayed a specific activity of 1.44 U/g toward 4-oxodecanoic acid with 99% *ee* (Table 1, entry 1).

To discover more enzymes, the gene sequence of *Pp*CR was used as the template to perform pBLAST searching in UniProt. On the basis of the results, we selected 16 bacterial strains stored in our lab and cloned the corresponding carbonyl reductases (SI, Table S1) that consist of a toolbox of 20 recombinant enzymes expressed in *E. coli* BL21 (DE3). These carbonyl reductases have moderate sequence identities (40–70%) compared with the template enzyme. Of them, nine reductases exhibited excellent stereoselectivity (> 99% *ee*) and various activities (from 0.196 to 3.32 U/g protein), as shown in the asymmetric reduction of 4-oxodecanoic acid (Table 1). *Sm*CR (from *Serratia marcescens*), which displayed the highest activity of 3.32 U/g and had a 47% amino acid identity to *Pp*CR, was chosen for further studies.

Table 1
Enzyme screening for the reduction of **1a**.

Entry	Enzyme	Amino acid identity (%)	Specific activity (U/g)[a]	*ee* (%)[b]
1	*Pp*CR	100	1.44	> 99 (*R*)
2	*Sm*CR	47	3.32	> 99 (*R*)
3	*Lg*CR	51	2.76	> 99 (*R*)
4	*Ar*CR	56	2.67	> 99 (*R*)
5	*Cn*CR	63	1.53	> 99 (*R*)
6	*Vp*CR	56	0.838	> 99 (*R*)
7	*Aa*CR	56	0.658	> 99 (*R*)
8	*Rp*CR	65	0.490	> 99 (*R*)
9	*Cb*CR	63	0.451	> 99 (*R*)
10	*Vb*CR	66	0.196	> 99 (*R*)

[a] Specific activity was determined spectrophotometrically in sodium phosphate buffer (100 mM, pH 7.0) containing 2 mM substrate **1a**, 0.1 mM NADPH, and a proper amount of purified enzymes at 30 °C.
[b] The *ee* values were determined by chiral GC analysis of the corresponding lactones.