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Efficient synthesis of an ε-hydroxy ester in a space–time yield of 1580 g L^{-1} d^{-1} by a newly identified reductase *Rh*CR

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ABSTRACT

A new NADH-dependent carbonyl reductase *Rh*CR capable of efficiently reducing the ε-ketoester ethyl 8-chloro-6-oxooctanoate (ECOO) to give ethyl (*S*)-8-chloro-6-hydroxyoctanoate [(*S*)-ECHO], an important chiral precursor for the synthesis of (*R*)-α-lipoic acid, was identified from *Rhodococcus* sp. ECU1014. Using recombinant *Escherichia coli* cells expressing *Rh*CR and glucose dehydrogenase used for the regeneration of cofactor, 440 g L^{-1} (2 M) of ECOO were stoichiometrically converted to (*S*)-ECHO in a space–time yield of 1580 g L^{-1} d^{-1} without the external addition of any expensive cofactor.

1. Introduction

(*R*)-α-Lipoic acid is a powerful antioxidant and important biologically active molecule, which is widely used in food supplement preparations, skin conditioners, and pharmaceuticals for treating diabetic neuropathy, chronic liver disease, and Alzheimer's disease.[1–5] As a result, a wide range of methods have been developed for the preparation of (*R*)-α-lipoic acid, including the kinetic resolution of the racemate or its intermediates using optically active chemical agents[6,7] and lipases,[8–10] and asymmetric synthesis using chiral auxiliaries.[11] (*R*)-α-Lipoic acid is produced on an industrial scale *via* the chemical resolution of racemates synthesized by chemical processes using the racemic 8-chloro-6-hydroxyoctanoic acid esters as intermediates.[12,13] However at most only 50% of the racemic intermediates are utilized to obtain enantiomerically pure α-lipoic acid, making this process appear economically unrewarding.

Asymmetric reduction of keto esters using keto reductases or alcohol dehydrogenases is an ideal process to produce enantiomerically pure hydroxy esters due to its inherent advantages, such as being able to provide up to 100% theoretical yield, excellent stereoselectivity, superior atom economy, and eco-friendliness.[14–18] A few dehydrogenases and microorganism cells that could reduce 8-chloro-6-oxooctanoic acid esters into the corresponding enantiomerically pure hydroxy esters have been reported in the literature.[19–22] However, the development of more efficient and practical biocatalysts to synthesize (*S*)-8-chloro-6-hydroxyoctanoic acid ester is still highly desirable promising and versatile reductases enantioselectivity towards or a variety of physiologically active compounds.

Herein our interests focused on exploring a novel reductase capable of efficiently reducing the ε-ketoester ethyl 8-chloro-6-oxooctanoate (ECOO) and developing an efficient synthesis of ECHO with high volumetric productivity.

2. Results and discussion

2.1. Enzyme screening

To begin with, several carbonyl reductases newly mined and stocked in our laboratory were screened. As shown in Table 1, most reductases exhibited low activity toward ECOO, which is probably because the substrate is an ε-ketoester, which is hard to be accepted by the majority of reductases, versus the readily reduced α-, β-, and γ-keto esters due to the long side chain of ECOO. Fortunately, a new reductase *Rh*CR from *Rhodococcus* sp. ECU1014 can efficiently covert ECOO into (*S*)-ECHO with >99% conversion and good enantioselectivity. After being purified to homogeneity by single step affinity chromatography, the *Rh*CR showed an extremely high activity in the reduction of ECOO (90.5 U mg^{-1} protein), for which the specificity constant (k_{cat}/K_m) was determined as 256 mM^{-1} s^{-1}.

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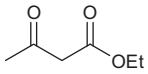
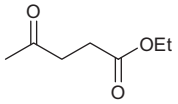
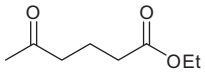
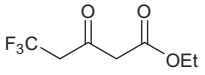
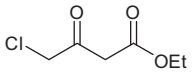
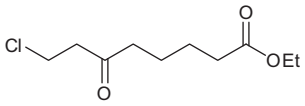
Table 1
Screening of reductases using ECOO as a substrate[a]

Entry	Enzyme	Specific activity (U mg^{-1} protein)	Conversion[b] (%)	ee[b] (%)/config
1	*Rh*CR	40	99	93 (*S*)
2	*Lbu*CR	30	99	79 (*S*)
3	*Cg*KR1	<1	8	79 (*R*)
4	*Lb*CR	3	90	11 (*R*)
5	*Cg*KR2	<1	<1	n.a.[c]
6	FabG	<1	<1	n.a.
7	YtbE	<1	<1	n.a.
8	YueD	<1	<1	n.a.
9	*Sc*CR	<1	<1	n.a.
10	*Kt*CR	<1	<1	n.a.
11	*Dn*CR	<1	<1	n.a.
12	*Cl*CR	<1	<1	n.a.
13	*Mp*CR	<1	<1	n.a.
14	*Ap*CR	<1	<1	n.a.

[a] Reaction conditions: the reaction mixture consisted of the appropriate amount of reductase (cell lysate or lyophilized crude enzyme powders), 2 U of GDH from *Bacillus megaterium* (lyophilized crude enzyme powders), 10 mM of ECOO, 20 mM of glucose, 0.5 mM of NAD(P)$^+$ and 100 mM of sodium phosphate buffer (pH 6.0) in a total volume of 1 mL. The mixtures were incubated at 30 °C for 24 h.
[b] Conversion and ee were determined by GC.
[c] n.a.: not available.

Table 2
Substrate specificity of *Rh*CR

Entry	Substrate	Relative activity[a] (%)	ee[b] (%)
1		0.03	7.9
2		0.2	22
3		1.0	39
4		1.9	78
5		2.9	22
6		100	93

[a] The activity was determined by standard assay protocol. Activities were normalized to that for ECOO (100%).
[b] Determined by GC analysis.

2.2. Dependence of enzyme activity on pH and temperature

The effects of temperature and pH on the reductase activity were investigated over a temperature range of 25–70 °C (Fig. 1a), and a pH range of 3.0–10.0 (Fig. 1b). According to Figure 1a, the enzyme *Rh*CR exhibited maximum activity in the range of 30–45 °C and then the reaction rate decreased rapidly over 50 °C due to thermal inactivation. The activity–pH profile showed that the enzyme activity exhibited a pH optimum at 6.0. Therefore, the reductase *Rh*CR showed an optimum temperature of 30–45 °C and an optimum pH of 6.0.

The stability was examined by incubating the purified enzymes in the optimized buffer (pH 6.0) at 30, 40, and 50 °C for a varied period of time and the residual activities were determined. The reductase *Rh*CR gave half-lives of 84, 58, and 3.8 h at 30, 40, and 50 °C, respectively. Based on the above results, pH 6.0 and 30 °C were chosen as the optimal reaction conditions in the following work.

2.3. Substrate spectrum

The substrate specificity of *Rh*CR for various ketoesters was further explored (Table 2). We found that the reduction activity and stereoselectivity of *Rh*CR was significantly affected by the number of the insertion group (–CH_2–) between the carbonyl and ester bonds of the substrates, with a tendency of ε- > δ- > γ- > β-keto esters (entries 1–3 and 6). Furthermore, both the activity and stereoselectivity increased with the introduction of electron-withdrawing groups (entries 4–6). The highest activity and stereoselectivity were obtained during the reduction of ECOO (entry 6), an ε-ketoester with a strong electron-withdrawing group.

2.4. Bioconversion of ECOO into (*S*)-ECHO on an analytical scale

The newly discovered biocatalyst was utilized for the synthesis of (*S*)-ECHO to further demonstrate its potential in industry. It is well known that the requirement for expensive nicotinamide cofactors is a major hindrance in the practical application of reductases.[23,24] Therefore, a glucose dehydrogenase (GDH) from *Bacillus megaterium* was used for the regeneration of the oxidized cofactor (NAD$^+$) during the reduction of ECOO catalyzed by recombinant *E. coli* cells containing *Rh*CR. Without the external addition of any expensive cofactor, as much as 132 g L^{-1} of ECOO was completely converted within 6.5 h (Table 3, entry 1) in the cofactor-regenerated catalytic system. Even though the recombinant *E. coli* cell loading was decreased to 60 kU L^{-1} (ca. 5.0 g L^{-1}, an acceptable threshold in industry), the substrate was almost completely con-

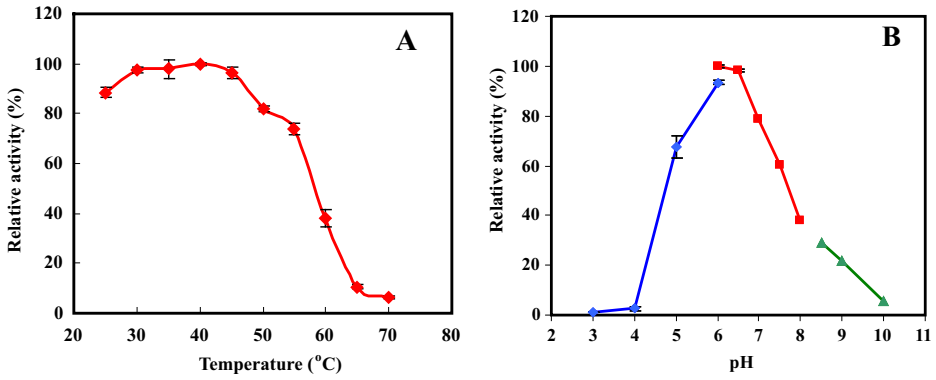


Figure 1. Effects of temperature and pH on activity of the purified *Rh*CR. (A) Activity–temperature profile was determined at various temperatures (25–70 °C) in a sodium phosphate buffer (100 mM, pH 6.0). (B) Activity–pH profile was determined using a standard assay in the following buffers (100 mM): (◆) sodium citrate (pH 3.0–6.0); (■) sodium phosphate (pH 6.0–8.0); (▲) Gly–NaOH (pH 8.5–10.0).

Table 3
Asymmetric reduction of ECOO with *E. coli* cells of *Rh*CR and GDH[a]

Entry	*Rh*CR (kU L^{-1})	ECOO (g L^{-1})	NAD^+ (mM)	Time (h)	ee[b] (%)	Conv.[b] (%)
1	120	132	0	6.5	93	>99
2	60	132	0	9	93	99
3	240	440	0	8	93	>99
4	360	660	0	24	93	95
5[c]	240	440	0	5	93	>99

[a] Reaction conditions: ECOO (0.6–3.0 M), D-glucose (1.5 equiv with respect to ECOO), lyophilized cells of *Rh*CR (60 kU are equals to approximately 5 g of lyophilized cells), lyophilized GDH powders (1 equiv activity with respect to *Rh*CR), 10 mL of sodium phosphate buffer (pH 6.0, 100 mM), 30 °C. pH was controlled at 6.0 with 2 M NaOH.
[b] Determined by GC.
[c] 100 mL of sodium phosphate buffer.

verted after 9 h (entry 2). Hence, this system was used for subsequent experiments.

Further optimization was accomplished toward higher substrate loading. Increasing the substrate loading to 440 g L^{-1} still gave >99% conversion within 8 h (entry 3). However, further improvement to 660 g L^{-1} resulted in incomplete conversion (95%) (entry 4). In all cases, the enantiomeric excess of the product was always kept at 93%.

2.5. Preparative scale synthesis of (*S*)-ECHO

Next, the bioreduction of ECOO was carried out at a 10-fold larger scale under the optimized reaction conditions to further confirm the feasibility of the process (entry 5). All of the substrate (ECOO, 44 g) was completely transformed within 5 h. After extraction and normal work-up, 34 g of (*S*)-ECHO (93% ee) was isolated. Although the ee of ECHO is not satisfactory, the enantiomeric purity of the final product (*R*)-α-lipoic acid could be improved by 99% by final product crystallization.

3. Conclusion

In conclusion, a more efficient and practical biocatalyst *Rh*CR for the synthesis of (*S*)-8-chloro-6-hydroxyoctanoic acid ester via asymmetric reduction was developed. The reductase *Rh*CR is the only one that can reduce a substrate of >200 g L^{-1} in a space–time yield as high as 1580 g L^{-1} d^{-1} (Table 4), which is greatly superior to the literature records, thus demonstrating its great potential for industrial application.

4. Experimental

4.1. General

Chemical reagents were purchased from Aladdin Chemicals Co. Ltd. (Shanghai, China) with >97% purity. Other chemicals were of reagent grade or better. Plasmid pMD18-T for the cloning of PCR products was obtained from TaKaRa (Dalian, China) and plasmid pET-28a (+) for the heterogeneous expression was obtained from Novagen (Shanghai, China). ^1H NMR and ^{13}C NMR were measured on a Bruker Avance 400 MHz and 500 MHz spectrometer with chemical shifts reported as ppm. The substrate conversion and product ee value were determined by GC analysis using a CP-Chirasil-DEX CB (Varian, USA).

4.2. Cloning and expression and purification

The reductase genes were amplified by polymerase chain reaction (PCR) from the genomic DNAs, which were extracted and purified from various bacteria using a TIANamp Bacteria DNA Kit (Tiangen, Shanghai). The amplified DNAs were digested with two restriction enzymes and ligated into expression plasmid pET-28a (+) or pET-43.1a (+) digested with the same enzymes. The constructs were ultimately transformed into *E. coli* BL21 (DE3) competent cells. Cells were inoculated in 100 ml LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl) at 37 °C and 200 rpm. When the OD_{600} reached 0.6–0.8, the enzyme expression was induced with 0.5 mM IPTG at 25 °C for another 12 h. Cells were harvested by centrifugation at 8800 rpm (4 °C for 10 min).

Cells were resuspended in buffer A (20 mM of Na_2HPO_4–NaH_2PO_4 buffer, pH 7.4, 500 mM of NaCl, 10 mM of imidazole) and sonicated. The cell lysate was centrifuged at 8800 rpm (4 °C for 20 min). The supernatant was transferred into an Ni-NTA column equilibrated with buffer A. Stepwise elution with imidazole was completed in 10 mM increments up to 500 mM. The purity of the fractions was analyzed by SDS–PAGE.

4.3. Enzyme assays

The reductase activity was analyzed spectrophotometrically at 30 °C by monitoring the decrease in the absorbance of NADH at 340 nm. The standard analysis mixture (1 mL) was composed of 100 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 6.0), 2.0 mM ketoesters, 0.1 mM NADH and an appropriate amount of enzyme. One unit was defined as the amount of enzyme leading to the consumption of 1 μmol of NADH per min.

4.4. Effect of pH and temperature on the activity

The effects of pH and temperature on the activity of *Rh*CR were examined within a pH range of 3.0–10.0 (sodium citrate 3.0–6.0; sodium phosphate 6.0–8.0; Gly–NaOH 8.5–10.0) and a temperature range of 25–70 °C. The stability was examined by incubating the purified enzymes in the optimized buffer (pH 6.0) at 30, 40 and 50 °C for a varied period of time and the residual activities were determined as described above.

4.5. Preparative scale enzymatic production of (*S*)-ECHO

A first, ECOO (44 g, 0.2 mol) was placed in 100 mL of sodium phosphate buffer (100 mM, pH 6.0) containing 24,000 U of lyophilized cells of *Rh*CR (2.0 g), 24,000 U of lyophilized GDH (2.0 g), and D-glucose (5.4 g, 0.3 mol) at 30 °C. The pH was adjusted to 6.0 by

Table 4
Comparison of *Rh*CR with other reported biocatalysts

Biocatalyst	Substrate (g L^{-1})	Cofactor (mM)	Time (h)	Conv. (%)	ee (%)/config	STY[a] (g L^{-1} d^{-1})	Refs.
Mr cell	5.0	0	24	66	92 (*S*)	3	19
Gc cell	5.0	0	24	62	88 (*R*)	3	19
LBADH	2.0	0.5	n.a.	>25	>65 (*S*)	n.a.	20
NGADH	45	4	24	>95	n.a.	<45	22
***Rh*CR[b]**	**440**	**0**	**5**	**>99**	**93 (*S*)**	**1580**	**This study**

[a] STY is an abbreviation of space–time yield.
[b] Reaction conditions ECOO (2 M), D-glucose (1.5 equiv with respect to ECOO), lyophilized cells of *RhCR* (24 kU), lyophilized GDH powders (24 kU), 100 mL of sodium phosphate buffer (pH 6.0, 100 mM), 30 °C. pH was controlled at 6.0 with 2 M NaOH.

adding 2 M NaOH. After 5 h, the reaction mixture was saturated with NaCl, and extracted with ethyl acetate. The collected organic phases were dried over Na_2SO_4, filtered, and evaporated under vacuum, to afford (*S*)-ECHO in 77% yield (34 g). $b_{0.5}$ 121 °C; ee 93%. The product was first acetylated according to the following procedure: A mixture of the appropriate amount of acetic anhydride, pyridine, and ECHO was maintained at 100 °C for 30 min, and the resultant product was then extract with ethyl acetate for chiral GC analysis.); $[\alpha]_D^{22}$ = +19.8 (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.13 (q, *J* = 7.1 Hz, 2H), 3.93–3.81 (m, 1H), 3.77–3.62 (m, 2H), 2.32 (t, *J* = 7.3 Hz, 2H), 1.97–1.80 (m, 2H), 1.77–1.58 (m, 3H), 1.55–1.45 (m, 3H), 1.44–1.35 (m, 1H), 1.26 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$, 400 MHz): δ 14.3, 24.7, 25.0, 34.2, 37.1, 39.7, 42.0, 60.4, 68.5, 173.8; HRMS (CI, N_2 as the reagent gas) *m*/*z* calcd for $C_{10}H_{20}O_3$-Cl(35) $(M+H)^+$ 223.1101, obsd 223.1105; $C_{10}H_{20}O_3Cl(37)$ $(M+H)^+$ 225.1071, obsd 225.1072.

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