Note

Synthesis of Ethyl (R)-4-Chloro-3-hydroxybutanoate with Recombinant Escherichia coli Cells Expressing (S)-Specific Secondary Alcohol Dehydrogenase

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The synthesis of ethyl (R)-4-chloro-3-hydroxybutanoate ((R)-ECHB) from ethyl 4-chloroacetoacetate was studied using whole recombinant cells of Escherichia coli expressing a secondary alcohol dehydrogenase of Candida parapsilosis. Using 2-propanol as an energy source to regenerate NADH, the yield of (R)-ECHB reached 36.6 g/l (more than 99% ee, 95.2% conversion yield) without addition of NADH to the reaction mixture.

Key words: (S)-specific secondary alcohol dehydrogenase; ethyl (R)-4-chloro-3-hydroxybutanoate; NADH-regeneration; asymmetric reduction

Ethyl (R)-4-chloro-3-hydroxybutanoate ((R)-ECHB) is a chiral compound useful for the synthesis of biologically and pharmacologically important materials: (R)-carnitine,1,2 (R)-4-amino-3-hydroxybutyric acid,3,4 and (R)-4-hydroxy-2-pyrrolidone.5,6 Several microorganisms and enzymes have been found to catalyze the reduction of ethyl 4-chloroacetoacetate (ECAA) to (R)-ECHB.7-9 In particular, Kataoka et al.10 reported the production of (R)-ECHB, with a molar yield of 94% and an optical purity of 92% enantiomer excess (ee), using Escherichia coli cells co-expressing an aldehyde reductase11 from Sporobolomyces salmonicolor and a glucose dehydrogenase12 from Bacillus megaterium as catalyst, in an n-butyl acetate/water biphasic system.

In our previous report,13 we showed that an NAD+·dependent (S)-specific secondary alcohol dehydrogenase (CpSADH) produced by Candida parapsilosis IFO 1396 could catalyze the enantioselective oxidation of several secondary alcohols, such as (S)-1,3-butadiol, (S)-2-butanol, and (S)-1-phenylethanol, and also the oxidation of achiral 2-propanol, and the reduction of several ketones, such as 4-hydroxy-2-butanone, 2-butanone, acetone, and acetonophenone. Furthermore, we reported the cloning and expression of the gene coding for CpSADH.10 In this report, we describe the synthesis of (R)-ECHB by asymmetric reduction of ECAA without the addition of NADH, using whole E. coli cells overproducing CpSADH.

A high-expression plasmid for CpSADH, pSCECPA1, was constructed based on the pSE420 vector containing the trc promoter (Invitrogen Corporation, Carlsbad, California), as illustrated in Fig. 1. E. coli cells harboring pSCECPA1 were grown in 100 ml of 2

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Abbreviations: CpSADH, secondary alcohol dehydrogenase from Candida parapsilosis; ECAA, ethyl 4-chloroacetoacetate; ee, enantiomer excess; ECHB, ethyl 4-chloro-3-hydroxybutanoate

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Fig. 1. Construction of pSCECPA1.

The cloning vector pSE420 was digested with NcoI and BamHI, treated with the Klenow fragment, and self-ligated to give pSE420B. A 1,024-bp DNA fragment containing the entire CpSADH gene was amplified by PCR with the primers CPA-ATG2 (TTTGATCCAAACAGTCAATCCATCAAGC) and CPA-TAG2 (CCTCCTGAGTTACTATGGATTTAAAACCAAATC) from pKK-CPA1,10 and was inserted at the BamHI and XhoI sites of pSE420B to give the final construct pSCECPA1.
× YT medium (Bacto-Tryptone, 20 g/l; Bacto-Yeast extract, 10 g/l; NaCl, 10 g/l; pH 7.2) containing ampicillin (50 mg/l) in a 500-ml baffled shake flask at 30°C on a rotary shaker (140 rpm) to the optical density at 600 nm of 3-4; after addition of 2% lactose as an inducer, the culture medium was further shaken for 11 h. The cells harvested by centrifugation were suspended in 50 ml Tris-HCl (pH 9.0) and 0.02% 2-mercaptoethanol, and disrupted with a Bioruptor UCD-200 (Cosmo BIO Co., Ltd., Tokyo, Japan). The supernatant was obtained by centrifugation at 16,000 g for 10 min. CpSADH was assayed spectrophotometrically at 30°C. The standard assay mixture contained 50 μmol of (S)-1,3-butadiol, 2.5 μmol of NADH, 50 μmol of Tris-HCl buffer (pH 9), and enzyme in a final volume of 1 ml. One unit of the enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per min at 30°C.

_E. coli_ W3110 cells harboring pSE-CPA1 prepared by the method mentioned above showed a CpSADH activity of 43.2 U/ml-medium, which was 78-fold higher than that of _Candida parapsilosis_. ECA served as an about 3.4-times better substrate for CpSADH than 4-hydroxy-2-butaneone (212 U/mg-protein at pH 6.0 and 30°C).

Using the whole _E. coli_ W3110 cells harboring pSE-CPA1, the synthesis of _R_-ECHB was investigated. At first, the energy source for the microbial reduction was examined. The reduction of ECAA was done at 30°C for 17 h in the reaction mixture (25 ml) containing 200 mM potassium phosphate buffer (pH 6.5), 1% ECAA, cultured cells obtained from 25 ml of the medium, and 5% (w/v) of a compound as an energy source (either glucose or 2-propanol) with shaking at 140 strokes per min in a 500-ml Sakaguchi flask. These reactions were done at pH 6.5, as ECAA was unstable and was degraded at more than pH 7.0, and the optimal pH for asymmetric reduction of ECAA was 6.5 as a result of preliminary experiments. 2-Propanol was expected to function as a co-substrate for CpSADH to regenerate NADH, as it was a good substrate for oxidation by CpSADH and an inexpensive compound. The amounts of ECAA and ECHB in the reaction mixture were measured by gas chromatography (Shimadzu GC-14A, Kyoto, Japan) under the following conditions: column, ThermoSpin 3000 5% Chromosorb W (Shinwa Chemical Industries Ltd., Kyoto, Japan); column temperature, 150°C; carrier gas, N2; detection, flame ionization detector. The optical purity of ECHB was measured by chiral HPLC with a Chiralcel AS packed column (4.6 × 250 mm, Daicel Chemical Industries Ltd., Tokyo, Japan) at 80°C, eluted with n-hexane:ethanol:2-propanol:cyclohexane (9:2.5:1:2.5) at a flow rate of 1 ml/min, and detected at 220 nm. As shown in Table 1, the use of glucose as an energy source afforded only 1.40 g/l of _R_-ECHB (13.8% conversion) from 10 g/l of ECAA, while the use of 2-propanol afforded 9.83 g/l of ECHB (97.1% conversion), resulting in an almost quantitative conversion with very high optical selectivity (more than 99% ee). These findings suggested that NADH regeneration using glucose as an energy source was inefficient in _E. coli_, or alternatively CpSADH itself could regenerate its cofactor efficiently by oxidation of 2-propanol as a co-substrate as shown in Fig. 2.

Table 1. Effects of Energy Source on the Synthesis of _R_-ECHB Using Whole _E. coli_ Cells Harboring pSE-CPA1

<table>
<thead>
<tr>
<th>Energy source</th>
<th>ECAA (g/l)</th>
<th>(R)-ECHB (g/l)</th>
<th>ee (%)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Glucose</td>
<td>4.28</td>
<td>1.40</td>
<td>&gt;99</td>
<td>13.8%</td>
</tr>
<tr>
<td>5% 2-Propanol</td>
<td>ND</td>
<td>9.83</td>
<td>&gt;99</td>
<td>97.1%</td>
</tr>
</tbody>
</table>

The reaction mixture (25 ml) containing 200 mM potassium phosphate buffer (pH 6.5), 1% ECAA, cultured cells obtained from 25 ml of the medium, and 5% (w/v) of the indicated compound for energy source (glucose or 2-propanol) was shaken in a 500-ml Sakaguchi flask at 140 strokes per min and 30°C for 17 h. The amounts of ECAA and ECHB, and the optical purity of ECHB in the reaction mixture were measured by gas chromatography and chiral HPLC. ND, not detected.

Table 2. Effects of the Ratio of 2-Propanol to ECAA on the Synthesis of _R_-ECHB

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>ECAA (g/l)</th>
<th>(R)-ECHB (g/l)</th>
<th>ee (%)</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>4%</td>
<td>13.4</td>
<td>26.9</td>
<td>&gt;99</td>
<td>66.4%</td>
</tr>
<tr>
<td>2-propanol (243 mm)</td>
<td>10.6</td>
<td>29.1</td>
<td>&gt;99</td>
<td>71.9%</td>
</tr>
<tr>
<td>2-propanol (180 mm)</td>
<td>4.4</td>
<td>31.9</td>
<td>&gt;99</td>
<td>78.8%</td>
</tr>
</tbody>
</table>

Reaction conditions were the same as those in Table 1 except for the use of 4% ECAA and the amounts of 2-propanol added. Eq., molar excesses.

![Fig. 2. Enzymatic Reduction of ECAA and Regeneration of a Coenzyme, NADH, Using CpSADH.](image-url)
shown in Table 3, the reaction at 20°C gave the best result for the reaction with 3.8% ECAA and 2 molar excesses of 2-propanol over ECAA (36.6 g/l of (R)-ECHB; conversion yield, 95.2%). These findings suggested lower temperatures resulted in the increased stability of the substrate, ECAA, and less inhibition of CpSADH by ECAA.

In conclusion we have established a highly enantioselective and efficient method for the enzymatic synthesis of (R)-ECHB by asymmetric reduction using whole recombinant cells expressing CpSADH without addition of an expensive coenzyme, NADH. The microbial reduction for the synthesis of (R)-ECHB using the recombinant cells could be scaled up to a 30-l reactor scale in our laboratory. Surprisingly, CpSADH had little dehydrogenase activities for (R)-ECHB as well as (S)-ECHB, although it showed a high activity for ethyl (S)-3-hydroxybutanoate without chlorine substitution at the 4-position. Furthermore, ECAA was found to be a suicide substrate for CpSADH by kinetic analysis (unpublished results). Investigation concerning these unique properties of CpSADH is in progress.

References


