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Note

Purification and Properties of a Carbonyl Reductase Involved in Stereoselective Reduction of Ethyl 4-Chloro-3-oxobutanoate from *Cylindrocarpon sclerotigenum* IFO 31855

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A NADPH-dependent carbonyl reductase (CSCR1) was purified to homogeneity from *Cylindrocarpon sclerotigenum* IFO 31855. The enzyme catalyzed the stereoselective reduction of ethyl 4-chloro-3-oxobutanoate to the corresponding (*S*)-alcohol with a >99% enantiomer excess. The relative molecular mass of the enzyme was estimated to be 68,000 by gel filtration chromatography and 24,800 on SDS polyacrylamide gel electrophoresis. The enzyme had an extremely narrow substrate specificity and it highly reduced conjugated diketone, 2,3-butanedion, in addition to ethyl 4-chloro-3-oxobutanoate. The enzyme activity was inhibited by $HgCl_2$ (100%), 5,5′-dithiobis(2-nitrobenzoic acid) (56%), dicoumarol (42%), and $CuSO_4$ (46%). The N-terminal amino acid sequence of the enzyme (P-Q-G-I-P-T-A-S-R-L) showed no apparent similarity with those of other oxidoreductases.

Key words: carbonyl reductase; *Cylindrocarpon sclerotigenum* IFO 31855; (*S*)-ethyl 4-chloro-3-hydroxybutanoate

Optically active ethyl 4-chloro-3-hydroxybutanoate (ECHB) could be used in the synthesis of biologically and pharmacologically important compounds such as L-carnitine[1] and hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitor.[2] Many studies have been reported on the asymmetric reduction of ethyl 4-chloro-3-oxobutanoate (ECAA) to chiral alcohols using microorganisms.[1,3–8] The several kinds of enzymes capable of reducing ECAA were isolated from yeasts; baker's yeast,[9,10] *Sporobolomyces salmonicolor*,[11] *Candida magnoliae*,[12] and *Kluyveromyces lactis*,[13] and characterized in detail. However, very few publications describe the isolation and characterization of ECAA-reducing enzymes in other organisms.[7,14] In our previous paper,[6] we reported that whole cells of *Cylindrocarpon sclerotigenum* IFO 31855 stereoselectively reduced ECAA to (*S*)-ECHB with a >99% enantiomer excess (*e.e.*) and that their cell-free extract also converted ECAA to (*S*)-ECHB in the presence of NADPH (*e.e.*; >99%). For the practical use of fungi for synthesis of chiral ECHB, more detailed information on the properties of the enzymes is needed. In this paper, we purified the ECAA-reducing enzyme from *C. sclerotigenum* IFO 31855 and characterized it briefly.

The culture and the preparation of a cell-free extract of *C. sclerotigenum* IFO 31855 were as described previously.[6] The enzyme was purified by four sequential column chromatographies at 0–4°C as follows. The standard buffer, 50 mM potassium phosphate buffer (KPB, pH 7.0) containing 20% glycerol and 0.5 mM DTT, was generally used throughout the enzyme purification. Step 1: The cell-free extract (250 ml) was put on a DEAE-Toyopearl 650M column (30 mm × 30 cm, Tosoh, Japan) equilibrated with the standard buffer. After washing of the column with the standard buffer, the enzyme was eluted with 700 ml of a linear gradient of NaCl (0 to 0.6 M) in the standard buffer. Two peaks showing NADPH-dependent ECAA reducing activities were observed on a DEAE-Toyopearl 650M column chromatography, one being non-adsorbed (CSCR1) and the other being eluted at 0.36 to 0.42 M NaCl (CSCR2). These two peaks gave (*S*)-ECHB preferentially (*e.e.*; >99%). On a native polyacrylamaide gel

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Abbreviations: ECAA, ethyl 4-chloro-3-oxobutanoate; ECHB, ethyl 4-chloro-3-hydroxybutanoate; *e.e.*, enantiomer excess; CSCR1, carbonyl reductase 1 from *Cylindrocarpon sclerotigenum*

Table 1. Purification of ECAA Reducing Enzyme (CSCR1)

Step	Total protein (mg)	Total activity (U*)	Specific activity (U/mg)	Overall yield (%)	Yield for CSCR1 (%)
Cell-free extract	737	1,360	1.85	100	—
DEAE-Toyopearl	87.4	903	10.3	66.4	100
Phenyl Toyopearl	9.0	666	74.0	49.0	73.8
Hydroxyapatite 1st	3.2	281	87.8	20.7	31.1
Hydroxyapatite 2nd	0.91	114	125.3	8.4	12.6

* One unit of the enzyme was defined as the amount catalyzing the oxidation of 1 μmol of NADPH per min.

electrophoresis (PAGE), CSCR2 did not enter into the separation gel but was retained in the stacking gel. CSCR1 was further purified by sequential column chromatography as follows; Step 2: The CSCR1 solution was treated with ammonium sulfate at 80% saturation. The resultant precipitate was dissolved in 20 ml of the standard buffer containing 20% $(NH_4)_2SO_4$ (buffer A) and dialyzed against the same buffer. The enzyme solution was put on a phenyl Toyopearl 650M column (20 mm × 25 cm, Tosoh, Japan) equilibrated with buffer A. After the column was washed with buffer A, the enzyme was eluted with a linear decrease in the ionic strength (20 to 0% $(NH_4)_2SO_4$ in standard buffer, 400 ml). The fractions showing activities were collected, concentrated with a Centriprep (YM 10, Millipore), and dialyzed against 10 mM KPB (pH 7.0) containing 20% glycerol and 0.5 mM DTT (buffer B). Step 3: The dialyzed solution was put on a Bio Gel HTP hydroxyapatite column (16 mm × 13 cm, Bio-Rad, USA) equilibrated with buffer B. The enzyme was eluted with 200 ml of linear gradient of KPB (10 to 400 mM, pH 7.0). The fractions showing activities were concentrated with a Centriprep and dialyzed against buffer B. Step 4: The dialyzed enzyme solution was put on a 2nd hydroxyapatite column as described above. The enzyme solution was concentrated with a Centriprep and then used for characterization of the enzyme. The enzyme activity (standard assay) was measured at 35°C by measuring the rate of decrease in absorbance at 340 nm as described previously.[6] One unit of the enzyme was defined as the amount catalyzing the oxidation of 1 μmol NADPH per minute. Protein was measured by the protein-dye binding method[15] using bovine serum albumin as a standard. The amino acid sequence of the enzyme was analyzed by the Edman method with a Perkin Elmer Model 491-1 protein sequencer. Polyacrylamide (5 to 20%) gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE were done by the methods of Davis[16] and Laemmli,[17] respectively.

The purification of CSCR1 is summarized in Table 1. The purified CSCR1 gave a single band on both SDS-PAGE and native PAGE. The recovery of CSCR1 after the DEAE Toyopearl step and the apparent overall recovery of CSCR1 were 12.6% and 8.4% respectively. The ECHB formed by CSCR1 was the (*S*)-enantiomer with >99% *e.e.* in the presence of NADPH. The reversibility of the reaction was investigated with (*R*,*S*)-ECHB and $NADP^+$ using the standard assay. No increase of the absorbance at 340 nm was observed. Neither a decrease at 340 nm due to the reduction of ECAA nor ECHB formation were observed when NADPH was replaced by an equimolar concentration of NADH.

CSCR1 showed the maximum activity at 35°C and pH 6.5 for the reduction of ECAA in KPB.

The N-terminal amino acid sequence of CSCR1 was found to be P-Q-G-I-P-T-A-S-R-L. When this sequence was compared with those of proteins stored in NCBI nr, no apparent similarity was found with other oxidoreductases.

The molecular mass of CSCR1 was estimated to be 68,000 on a Superdex 75 HR 10/30 gel filtration column (Amersham Pharmacia Biotech.). The relative molecular mass of the denatured CSCR1 was approximately 24,800.

The substrate specificity of CSCR1 is shown in Table 2. CSCR1 showed restricted substrate specificity. The enzyme had high activities for ECAA but no activity for other β-keto esters or cyclohexanone. Low activities for ethyl pyruvate and hydroxyacetone were also observed. Interestingly, CSCR1 effectively reduced conjugated diketone, 2,3-butanedione and 2,3-pentanedione but 2,4-pentanedione was not reduced. The enzyme did not catalyze the reduction of typical substrates for aldo-keto reductase family enzyme, such as *p*-nitrobenzaldehyde, pyridine-3-aldehyde, or DL-glyceraldehyde. Aldoses, such as D(+)-glucose, D(+)-galactose, or D(+)-xylose which are good substrates for aldehyde- and aldose-reductases, were not also reduced by the enzyme.

The effects of various chemicals on the activity of CSCR1 are shown in Table 3. Among the sulfhydoryl inhibitors tested, neither *N*-ethylmaleimide nor sodium *p*-chloromercuribenzoate inhibited the enzyme activity, but 5-5′-dithiobis(nitrobenzoate) inhibited the activity around 56%. $HgCl_2$ inhibited it completely but $CuSO_4$ and $ZnSO_4$ inhibited the activity around 46% and 0% respectively. These results indicate that CSCR1 resists SH-blocking reagents.[18]

Table 2. Substrate Specificity of CSCR1

Substrate	mM	Relative activity (%)[a]
Ethyl 4-chloro-3-oxobutanoate	33.3	100
Ethyl 3-oxobutanoate	33.3	5
Methyl 3-oxopentanoate	33.3	0
Ethyl 2-methyl-3-oxobutanoate	33.3	0
Cyclohexanone	33.3	0
Ethylpyruvate	33.3	14.6
Hydroxyacetone	33.3	12.5
2,3-Butanedione	33.3	106
2,3-Pentanedione	33.3	22.7
2,4-Pentanedione	33.3	0
o-Nitrobenzaldehyde	1	0
m-Nitrobenzaldehyde	1	0
p-Nitrobenzaldehyde	1	0
o-Chlorobenzaldehyde	1	0
m-Chlorobenzaldehyde	1	0
p-Chlorobenzaldehyde	1	0
Pyridine-3-aldehyde	33.3	0
DL-Glyceraldehyde	33.3	0
D-Xylose	33.3	3.2
D-Galactose	33.3	0
D-Glucose	33.3	0

[a] To calculate the relative activity, the activity with 33.3 mM ECAA was taken as 100%.

Table 3. Effects of Several Chemicals on the Activity of CSCR1[a]

Compound	(mM)	Relative activity (%)[b]
Quercetin	0.1	84.4
Diphenylhydantoin	1	100
Dicoumarol	0.3	57.6
2,4-Dinitrophenol	0.1	81.3
5,5′-Dithiobis(2-nitrobenzoic acid)	0.05	43.8
N-Ethylmaleimide	1	100
Sodium *p*-chloromercuribenzoate	0.05	100
Phenylmethylsulfonyl fluoride	0.5	100
Ethylenediaminetetraacetic acid	1	70.8
$ZnCl_2$	1	100
$MgCl_2$	1	100
$CdCl_2$	1	84
$HgCl_2$	1	0
$CuSO_4$	1	54.3
$ZnSO_4$	1	100

[a] The enzyme was icubated with the chemical for 3 min at 35°C before the reaction was started.
[b] ECAA (33.3 mM) was used as the substrate and the activity without an chemical was taken as 100%.

Dicoumarol, which is a potent inhibitor of NAD(P)H dehydrogenase (quinone reductase)[19] and is an inhibitor of the carbonyl reductase of human brain,[20] inhibited the enzyme by 42% at a concentration of 0.3 mM. The enzyme activity was not significantly affected by quercetin, a nonspecific inhibitor of mammalian oxidoreductases,[20,21] or 2,4-dinitrophenol, an inhibitor of NADPH dehydrogenase (quinone).[22]

Several enzymes that reduce ECAA to (*S*)-ECHB have been isolated.[7,9–14,23] Among them, two short-chain alcohol dehydrogenase/reductases (S1 and S4) from *Candida magnoliae* have been characterized.[12,23] The molecular masses of S1 and S4 are 77,000 and 86,000 as the native form, and 32,000 and 29,000 as the subunit form, respectively. These enzymes also have narrow substrate specificity, and do not reduce typical substrates for aldo-keto reductases, *i.e. p*-nitrobenzaldehyde, pyridine-3-aldehyde, or aldoses. The molecular mass of CSCR1 from *C. sclerotigenum* are 68,000 as the native form and 24,800 as the subunit form. CSCR1 seems to be a dimer or trimer. The properties of CSCR1, the molecular mass and narrow substrate specificity, resembled those of S1 and S4. CSCR1 may be a member of the short-chain alcohol dehydrogenase/reductase group. However, the N-terminal amino acid sequence of CSCR1 shows no similarity with those of S1, S4, and other oxidoreductases. Thus CSCR1 seems to be a novel NADPH-dependent carbonyl reductase. This work is thus the first to demonstrate the purification and properties of a carbonyl reductase from the genus of *Cylindrocarpon*. However, to find whether this enzyme can be used practically, further biochemical studies are needed.

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