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Purification and Characterization of NADPH-Dependent Carbonyl Reductase, Involved in Stereoselective Reduction of Ethyl 4-Chloro-3-oxobutanoate, from *Candida magnoliae*

Masaru WADA,†† Michihiko KATAOKA, Hiroshi KAWABATA, Yoshihiko YASOHARA,* Noriyuki KIZAKI,* Junzo HASEGAWA,* and Sakayu SHIMIZU†

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan
**Fine Chemical Research Laboratories, Kaneka Corporation, 1-8 Miyamae-machi, Takasago-cho, Takasago 676, Japan*

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A NADPH-dependent carbonyl reductase was purified to homogeneity from *Candida magnoliae* AKU4643 through four steps, including Blue Sepharose affinity chromatography. The enzyme catalyzed the stereoselective reduction of ethyl 4-chloro-3-oxobutanoate to the corresponding (*S*)-alcohol with a 100% enantiomeric excess, which is a useful chiral building block for the chemical synthesis of pharmaceuticals. The relative molecular mass of the enzyme was estimated to be 76,000 on high performance gel filtration chromatography and 32,000 on SDS polyacrylamide gel electrophoresis. The enzyme reduced α-, β-keto esters and conjugated diketones in addition to ethyl 4-chloro-3-oxobutanoate. The enzyme activity was inhibited by quercetin and $HgCl_2$, but not by EDTA. The N-terminal amino acid sequence of the enzyme showed no apparent similarity with those of other oxidoreductases.

Key words: carbonyl reductase; stereoselective reduction; *Candida magnoliae*

Chiral β-hydroxy esters are versatile synthons in organic synthesis, especially in the preparation of natural and pharmaceutical products. The asymmetric reduction of ketoesters by baker's yeast has been widely used to obtain chiral alcohols because it is a cheap and readily available biocatalyst.[1] However, baker's yeast cannot always produce a β-hydroxy ester with the desired configuration with high optical purity. Some stereochemical control techniques for baker's yeast bioreduction have been developed to improve the optical purity.[2–5] Other microorganisms or enzymes that can catalyze the asymmetric reduction of ketoesters are also used to obtain optically pure β-hydroxy esters.[6–11] In previous papers, we reported the purification, crystallization, and characterization of a novel aldehyde reductase from *Sporobolomyces salmonicolor*.[12,13] It was also shown that this enzyme can catalyze the asymmetric reduction of 4-halo-3-oxobutanoate ester to the corresponding (*R*)-4-halo-3-hydroxybutanoate ester, which is a promising chiral building block in organic synthesis.[10]

During the course of a study on the microbial stereospecific reduction of 4-chloro-3-oxobutanoate ester, we investigated the distribution of β-ketoester reducing activity in various microorganisms, using ethyl 4-chloro-3-oxobutanoate (COBE) as a substrate, and found that *Candida magnoliae* AKU4643 produces a novel carbonyl reductase that reduces COBE to ethyl (*S*)-4-chloro-3-hydroxybutanoate ((*S*)-CHBE). The (*S*)-4-chloro-3-hydroxybutanoate ester is also a useful chiral building block for the chemical synthesis of pharmaceuticals.[7] We report here the purification, characterization, and N-terminal amino acid sequence of this enzyme. Comparison of the enzyme with COBE-reducing enzymes from other sources is also discussed.

Materials and Methods

Microorganisms and Cultivation. Microorganisms preserved in our laboratory (AKU Culture Collection, Faculty of Agriculture, Kyoto University) were screened. Air-dried cells were prepared as described previously.[14] *Candida magnoliae* AKU4643 was used for enzyme purification. The yeast was cultivated aerobically at 28°C for 40 h in a medium (pH 6.5) of 5% glucose, 0.5% peptone, 0.2% KH_2PO_4 0.1% K_2HPO_4, 0.1% yeast extract, and 0.02% $MgSO_4 \cdot 7H_2O$.

Chemicals. (*R*, *S*)-CHBE was prepared by $NaBH_4$ reduction of COBE. (*S*)-CHBE was prepared as described previously.[15] All other chemicals used in this study were of analytical grade and commercially available.

Screening of COBE-reducing strains. Twenty milligrams of air-dried cells of each microorganism were incubated in a 1-ml reaction mixture of 100 μmol of potassium phosphate buffer (pH 6.0), 904 nmol of NAD^+, 784 nmol of $NADP^+$, 14.3 units of glucose dehydrogenase (Amano Pharmaceutical, Japan), 278 mmol of glucose, and 73.2 μmol of COBE. The reaction was done at 28°C for 18 h with shaking, and then the mixture was vigorously shaken with 1 ml of ethyl acetate. The ethyl acetate layer was analyzed for COBE

† Corresponding author.
†† Present address; *Department of Bioscience, Fukui Prefectural University, 4-1-1 Kenjyojima, Matsuoka-cho, Fukui 910-11, Japan.*
Abbreviations: COBE, ethyl 4-chloro-3-oxobutanoate; CHBE, ethyl 4-chloro-3-hydroxybutanoate; *e.e.*, enantiomeric excess.

and CHBE by gas chromatography as described previously.[10] The enatiomeric excess (*e.e.*) of the product was measured as described below.

Enzymatic preparation and analysis of CHBE. The enzymatic reduction of COBE was followed. A 3.3-ml reaction mixture of 830 μmol of potassium phosphate buffer (pH 7.0), 1.5 units of the enzyme, and 130 μmol of NADPH, was incubated at 37°C. At 2-min intervals for 60 min, 3.3 μmol of COBE was added. After another 2 h of incubation, the reaction mixture was extracted with 2 ml of ethyl acetate, and the ethyl acetate layer was concentrated and analyzed.

The optical purity of CHBE was measured by high performance liquid chromatography (HPLC) on a Chiralcel AS column (0.46×25 cm; Daicel, Japan) at room temperature. *n*-Hexane/2-propanol/ethanol/cyclohexanol=92/2.5/1.25/0.25 (by vol.) was used as the mobile phase, and the flow rate was 0.5 ml/min. The absorbance of the eluate was monitored at 217 nm. (*S*)-CHBE and (*R*)-CHBE were eluted at 23.7 and 25.3 min, respectively.

Enzyme assay. The standard assay mixture was, in 2.5 ml, 5 μmol of COBE (final concentraion: 2.0 mM), 0.80 μmol of NADPH, 500 μmol of potassium phosphate buffer, pH 7.0, and the enzyme. During the enzyme purification, substrate concentration of 0.2 mM was used for activity measurement. After 2 min of incubation without substrate at 37°C, the reaction was started by the addition of the substrate COBE, and then the decrease in absorbance at 340 nm was monitored at 37°C. One unit of the enzyme was defined as the amount catalyzing the oxidation of 1 μmol of NADPH per min. Protein concentrations were measured with a Bio-Rad Protein Assay kit using bovine serum albumin as the standard.[12]

N-terminal amino acid sequence analysis. The N-terminal amino acid sequence was analyzed on a model 476A pulsed liquid protein sequencer equipped with an on-line phenylthiohydantoin analyzer. The obtained N-terminal amino acid sequence was compared with those of proteins stored in SWISS-PROT (release: 34.0+/09-14, Sep. 97), PIR (release: 51.0, Dec. 96) and PRF (release: 97-07, Jul. 97) protein database.

Enzyme Purification. All purification procedures were done at 0–4°C in 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM dithiothreitol, unless otherwise specified.

Step 1. Preparation of a cell-free extract

The washed cells (500 g as wet weight) from 15 liters of culture broth were suspended in 1 liter of the buffer and then disrupted with 0.25×0.25 mm glass beads (Dyno-Mill KDL, Switzerland) for 15 min. The supernatant obtained on centrifugation was dialyzed against the same buffer.

Step 2. DEAE Sephacel chromatography

The resultant solution was put on a DEAE Sephacel column (3.5×36 cm), and washed with 1000 ml of the buffer. The unadsorbed active fractions were concentrated by ultrafiltration with a ultrafiltration module AIP-1010 (Asahi Kasei, Japan).

Step 3. Phenyl Sepharose CL-4B chromatography

The concentration of NaCl was adjusted to 4 M by the addition of NaCl to the enzyme solution. The enzyme solution was put on a Phenyl Sepharose CL-4B column (3.2×23 cm) equilibrated with the buffer containing 4 M NaCl. The enzyme was eluted with a 720-ml cross-linear gradient of NaCl and ethyleneglycol (from 4 M NaCl and 0% ethyleneglycol to 0 M and 50% (w/v), respectively, in the buffer). The combined active fractions were dialyzed against the buffer.

Step 4. Blue Sepharose CL-6B chromatography

The enzyme solution was put on a Blue Sepharose CL-6B column (1.8×16 cm) equilibrated with the buffer. The enzyme was eluted with a 240-ml linear gradient of NaCl (0–0.6 M in the buffer).

Step 5. Superdex 200 HR gel filtration chromatography

The concentrated enzyme solution was put on a Superdex 200 HR gel filtration column (1.0×30 cm) connected to a FPLC system (Pharmacia, Sweden), equilibrated with the buffer supplemented with 0.2 M NaCl. The enzyme was eluted with 30 ml of the buffer containing 0.2 M NaCl. The active fraction was used as the purified enzyme for characterization.

Results

Screening of COBE-reducing strains

COBE-reducing activity was widely distributed in various microorganisms, especially in yeasts. As shown in Table I, yeasts belonging to genus *Candida* catalyzed the stereoselective reduction of COBE. All *Candida* strains produced (*S*)-CHBE as the major reduction product. Among these strains, *C. magnoliae* AKU4643 was selected as the best producer of the enzyme that catalyzes the stereoselective reduction of COBE to (*S*)-CHBE.

Purification of the enzyme

The purification of the enzyme is summarized in Table II. The enzyme was purified 20-fold to homogeneity with an overall recovery of 0.9%. The purified enzyme gave a single band on SDS-polyacrylamide gel electrophoresis (Fig. 1). As shown in Table II, *e.e.* of CHBE prepared by a cell-free extract of *C. magnoliae* was low, suggesting that the cell-free extract of *C. magnoliae* contained an enzyme that reduces COBE to (*R*)-CHBE in addition to the enzyme purified here. This (*R*)-selective enzyme activity was completely removed on the Blue Sepharose step.

Molecular mass and subunit structure

The molecular weight of the enzyme was found to be 76,000 by gel filtration on TSK-G3000SW (Tosoh, Japan). The relative molecular mass of the subunit was estimated to be about 32,000 on SDS-polyacrylamide gel electrophoresis (Fig. 1). These results suggest that the enzyme is a dimer.

Table I. Reduction of COBE by Air-dried Cells of Several Yeasts[a]

Strain	CHBE Yield (%)	*e.e.* for (*S*)-CHBE (%)
Baker's yeast AKU4040	51	62
Pichia farinosa AKU4251	66	52
Pichia farinosa AKU4262	81	77
Debaryomyces castellii AKU4360	69	−24
Lipomyces lipofer AKU4420	66	80
Sporobolomyces salmonicolor AKU4429	41	−62
Candida lipolytica AKU4582	95	83
Candida lipolytica AKU4598	92	73
Candida lipolytica AKU4600	53	90
Candida magnoliae AKU4643	82	90
Candida apis AKU4645	72	90

[a] The assay conditions are given under Materials and Methods.

Table II. Purification of Carbonyl Reductase from *Candida magnoliae*[a]

Step	Total Act. (units)	Total Protein (mg)	Specific Act. (units/mg)	Yield (%)	*e.e.* for (*S*)-CHBE (%)
Cell-free extract	1,740	2,540	0.68	100	84
DEAE Sephacel	632	820	0.77	36	90
Phenyl Sepharose	198	55	3.6	11	95
Blue Sepharose	36	4.0	9.0	2.1	100
Superdex 200	14.9	1.1	13.5	0.85	100

[a] The enzyme activity was measured with 0.2 mM COBE.

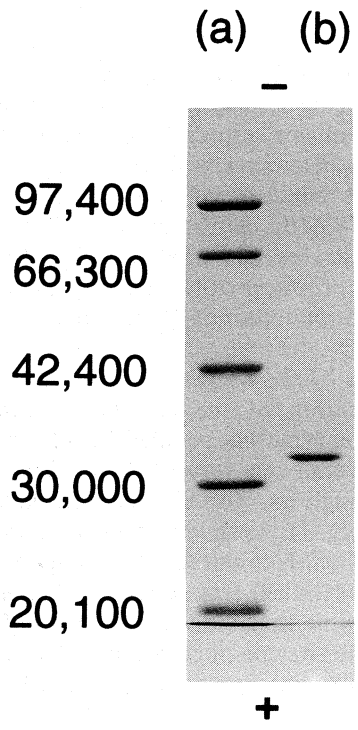

Fig. 1. SDS-Polyacrylamide Gel Electrophoresis of the Enzyme.
(a) Standards (from top): phosphorylase *b* (M_r=97,400), bovine serum albumin (66,300), aldolase (42,400), carbonic anhydrase (30,000), and trypsin inhibitor (20,100). (b) Purified enzyme. The gel was stained for protein with Coomassie Brilliant Blue R-250 and destained in methanol/acetic acid/water (7:6:47, by vol.).

Stereoselectivity for COBE reduction

Using COBE as a substrate, the optical purity of the reduction product was analyzed by HPLC. The CHBE formed was confirmed to only comprise the (*S*)-enantiomer.

Substrate specificity and catalytic properties

The substrate specificity of the enzyme is shown in Table III. The enzyme catalyzed the reduction of α- and β-ketoesters, and conjugated diketones, such as diacetyl, 2,3-pentanedione, and isatin, in addition to the 2- and 4-chloro-3-oxobutanoate esters. With these substrates, normal hyperbolic kinetics were observed, and the K_m and V_{max} for these substrates, calculated from Lineweaver-Burk plots, are also shown in Table III. Judging from the K_m and V_{max} values, COBE, ethyl 2-chloro-3-oxobutanoate and ketopantoyl lactone seems to be good substrates of this enzyme, however, the physiological substrate of this enzyme is still unknown.

The enzyme was highly specific for NADPH as a coenzyme; the K_m for NADPH was 16.7 μM. No decrease at 340 nm due to the reduction of COBE was observed when NADPH was replaced by an equimolar concentration of NADH.

The reversibility of the reaction was investigated with (*S*)-CHBE and $NADP^+$. The CHBE concentrations were varied from 2.0 to 16.0 mM with a fixed $NADP^+$ concentration of 320 μM, however, no formation of COBE and NADPH was observed at pH 6, 7, or 8.

Initial velocity studies on COBE reduction by the enzyme were done with respect to the concentration of COBE, with a fixed concentration of NADPH (20, 40, 80, and 160 μM). The intersecting lines in double reciprocal plots indicated a sequential mechanism for the enzymatic reaction (data not shown).

Table III. Substrate Specificity of the Carbonyl Reductase from *C. magnoliae*

Substrate	Concentration[a] (mM)	Relative Activity[b] (%)	K_m (mM)	V_{max} (μmol/mg protein/min)
Ethyl 4-chloro-3-oxobutanoate	2.0	100	4.6	270
Ethyl 2-chloro-3-oxobutanoate	2.0	85	4.1	190
Methyl 4-chloro-3-oxobutanoate	2.0	9.0	10	55
Methyl 2-chloro-3-oxobutanoate	2.0	9.0	39	140
Octyl 4-chloro-3-oxobutanoate	0.2	5.6	—	—
Ethyl 3-oxobutanoate	2.0	22	—	—
Methyl 3-oxobutanoate	2.0	0.32	—	—
Ethyl pyruvate	2.0	24	21	220
Ethyl 2-oxobutanoate	2.0	16	61	560
Ethyl 3-methyl-2-oxobutanoate	2.0	18	240	2000
Diacetyl	2.0	28	7.4	120
2,3-Pentanedione	2.0	11	33	140
Ketopantoyl lactone	2.0	86	10	410
Isatin	0.2	2.2	—	—

[a] Subtarate concentrations were near the saturating level.
[b] To calculate the relative activity, the activity with 2.0 mM of ethyl 4-chloro-3-oxobutanoate was taken as 100%. The K_m and V_{max} were estimated from Lineweaver-Burk plots. The following compounds were not reduced: acetaldehyde, chloroacetoaldehyde, pyruvate, 3-oxobutanoate, acetone, acetol, acetoin, menadione, glyoxal, benzaldehyde, *p*-nitrobenzaldehyde, *o*-nitrobenzaldehyde, *m*-nitrobenzaldehyde, nicotinaldehyde, isonicotinaldehyde, and pyridine-3-aldehyde.
—: not determined.

N-Terminal amino acid sequence analysis

Automated Edman degradation of the enzyme protein with a pulsed liquid phase sequencer showed that the N-terminal amino acid sequence is Ala-Lys-Asn-Phe-Ser-Asn-Val-Glu-Tyr-Pro-Ala-Pro-Pro-Pro-Ala-His-Thr-Lys-Asn-Glu-Ser-Leu-Gln-Val-Leu-Asp-Leu-Phe-Lys-Leu-Asn-Gly-Lys-Val-Ala-. When this sequence was compared with those of proteins stored in the protein-sequence database described under Materials and Methods, no apparent similarity was found with other oxidoreductases.

Spectral properties

The absorption spectrum of the enzyme had a maximum at 278 nm. No absorbance was detectable above 320 nm. Thus, the enzyme does not contain flavin, which is the coenzyme in most quinone reductases.[16]

Effects of pH and temperature on the enzyme activity

The optimum pH of the enzyme was 5.5. The effects of temperature were also examined and the optimum temperature for COBE reduction was found to be 55°C at pH 7.0.

Effects of chemicals

The various compounds and metal ions listed in Table IV were added to the standard reaction mixture, and then the relative activity was measured using COBE as a substrate. The enzyme was completely inhibited by quercetin, a nonspecific inhibitor of human brain carbonyl reductase[17] and aldose reductase.[18] But dicoumarol, which is a potent inhibitor of NAD(P)H dehydrogenase (quinone reductase)[19] and an inhibitor of the carbonyl reductase of human brain,[17] did not significantly affect the enzyme activity. 2,4-Dinitrophenol, which is an inhibitor of NADPH dehydrogenase (quinone)[20] was also not effective. The enzyme was also inhibited by $HgCl_2$ and $SnCl_2$. EDTA at 1 mM did not inhibit the enzyme activity, indicating that metal ions are not required for catalysis.

Table IV. Effects of Various Chemicals on the Enzyme Activity[a]

Compound	Concentration (mM)	Relative activity (%)
Quercetin	0.05	53
	0.1	0
Diphenylhydantoin	1	84
Dicoumarol	0.1	97
2,4-Dinitrophenol	0.1	86
DTNB	0.05	100
Iodoacetate	1	100
N-Ethylmaleimide	1	105
PMSF	1	93
p-CMB	1	88
EDTA	1	95
Phenylhydrazine	1	97
$SnCl_2$	1	77
$PbCl_2$	1	86
$CdCl_2$	1	91
$CuSO_4$	1	85
$HgCl_2$	0.1	49

[a] Enzyme activity was measured as described under Materials and Methods, except for the addition of the indicated compounds. COBE (2.0 mM) was used as the substrate and the activity without an inhibitor was taken as 100%. DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMSF, phenylmethylsulfonyl fluoride; *p*-CMB, sodium *p*-chloromercuribenzoate; EDTA, ethylenediaminetetraacetic acid.

Discussion

In previous studies,[12,13] we purified and characterized the COBE-reducing enzyme from *S. salmonicolor*, and concluded that this enzyme belongs to the aldo-keto reductase family. The enzyme purified here also catalyzed the stereoselective reduction of COBE. However, its stereoselectivity was opposite, i.e., this enzyme pro-

Table V. Comparison of the Characteristics of the Purified (*S*)-Selective COBE Reductases from Various Microorganisms

Source	M_r Gel-filtration	M_r SDS/PAGE	K_m for COBE (mM)	Optimum pH	Optimum Temperature (°C)	Coenzyme
Candida magnoliae[a]	76,000	32,000	4.6	5.5	55	NADPH
Candida macedoniensis[21]	45,000	42,000	8.7	6.5	45	NADPH
Geotrichum candidum[7]	950,000	—	0.003	—	—	NADPH
Baker's yeast[23]	2,400,000	—	1.8	—	—	NADPH
Baker's yeast[23]	38,000	—	1.0	—	—	NADPH
Baker's yeast[24]	25,000	—	0.6	8.5	50	NADPH
Baker's yeast[24]	1,600,000	—	4.5	6.5	40	NADPH

[a] This study; —: not determined.

duced (*S*)-CHBE from COBE, while the *Sporobolomyces* enzyme gave (*R*)-CHBE. Both enzymes required NADPH as a coenzyme, however, the substrate specificity of this new enzyme was quite different from that of the *Sporobolomyces* enzyme. This enzyme was not able to reduce *p*-nitrobenzaldehyde or pyridine-3-aldehyde, which are typical substrates for the microbial aldehyde reductase.[12,13,21]

Besides the *Sporobolomyces* enzyme, several COBE-reducing enzymes have been purified from microorganisms such as *Candida macedoniensis*,[22] *Candida parapsilosis*,[8] *Geotrichum candidum*,[7] *Rhodococcus erythropolis*,[9,23] and *Saccharomyces cerevisiae* (baker's yeast).[24-27] Among them, the enzymes from *C. macedoniensis*, *G. candidum*, and *S. cerevisiae* were reported to be (*S*)-selective. The properties of these (*S*)-selective enzymes are listed in Table V. Although most enzymes listed in Table V have not been well characterized, the molecular weight of the present enzyme was different from those of other enzymes.

We have also reported microbial NADPH-dependent reductases that show rather wide substrate specificities toward carbonyl compounds, i.e., conjugated polyketone reductases, from *Mucor ambiguus*[28] and *C. parapsilosis*,[29] and carbonyl reductase from *C. macedoniensis*.[22] The enzyme purified in this study also reduced several polyketones such as ketopantoyl lactone and isatin (Table III), which are good substrates for the conjugated polyketone reductases and carbonyl reductase described above. However, these enzymes cannot reduce α- or β-ketoesters, such as ethyl pyruvate or ethyl 3-oxobutanoate. The *C. macedoniensis* carbonyl reductase also catalyzed the stereoselective reduction of COBE to (*S*)-CHBE,[22] but this enzyme is monomeric and it reduces menadione, which does not serve as a substrate for the *C. magnoliae* enzyme.

EDTA did not affect the enzyme activity, suggesting that the enzyme is not a zinc-dependent one, such as alcohol dehydrogenases I, II, and III of *S. cerevisiae*.[30] The subunit M_r of the enzyme (32,000) indicated that it is not a member of the "iron-activated" alcohol dehydrogenase family, which generally contain approximately 385 amino acid residues.[30] Moreover, the NH_2-terminal amino acid sequence of the *C. magnoliae* enzyme shows no apparent similarity with those of other oxidoreductases, such as aldo-keto reductase,[31] rat quinone reductase,[32] or short chain alcohol dehydrogenase/reductase.[33]

From these results, the *C. magnoliae* enzyme introduced here seems to be a novel NADPH-dependent carbonyl reductase having an unique substrate specificity. The enzyme may be stable, because it was active even in air-dried cells. Although the physiological role of this enzyme remains to be clarified, it may be useful for the enzymatic synthesis of optically pure (*S*)-CHBE.

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