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Purification and characterization of an aldehyde reductase from *Candida magnoliae* [1]

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Abstract

An NADPH-dependent aldehyde reductase was purified to homogeneity from *Candida magnoliae* AKU4643 through four steps, including Blue-Sepharose affinity chromatography. The relative molecular mass of the enzyme was estimated to be 33,000 on high performance gel-permeation chromatography and 35,000 on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The substrate specificity of the enzyme was broad and resembled those of other aldo–keto reductases. The partial amino acid sequences of the enzyme showed that it belongs to the aldo–keto reductase superfamily. The enzyme catalyzed the stereoselective reduction of ethyl 4-chloro-3-oxobutanoate to the corresponding (*R*)-alcohol, with a 100% enantiomeric excess. The enzyme was inhibited by 1 mM quercetin, $CuSO_4$, $ZnSO_4$ and $HgCl_2$. The thermostability of the enzyme was inferior to that of the (*S*)-CHBE-producing enzyme from the same strain.

Keywords: Aldehyde reductase; *Candida magnoliae*; Stereoselective reduction

1. Introduction

Recently, the aldo–keto reductase superfamily has attracted the attention of chemists and biochemists because of the interrelation to diseases such as diabetic complications. This superfamily includes not only a number of reductases, such as aldose reductase (EC 1.1.1.21), aldehyde reductase (EC 1.1.1.2), and prostaglandin F synthase [1], but also structural proteins, such as ε- and ρ-crystallins [2,3]. The aldo–keto reductases are monomeric and NADPH-dependent oxidoreductases that have common properties. These enzymes catalyze the reduction of a wide variety of carbonyl compounds to the corresponding alcohols to metabolize xenobiotics.

In a previous paper, we reported the purification and characterization of a novel carbonyl reductase from *Candida magnoliae* AKU4643 [4]. It was shown that this enzyme can catalyze

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the asymmetric reduction of ethyl 4-chloro-3-oxobutanoate (COBE) to the corresponding alcohol, ethyl (*S*)-4-chloro-3-hydroxybutanoate ((*S*)-CHBE), which is a useful chiral building block for chemical synthesis. During the purification of this enzyme, we found that *C. magnoliae* produces several COBE reducing enzymes and that one of these enzymes reduces COBE to (*R*)-CHBE.

We report here the purification, characterization and partial amino acid sequencing of this (*R*)-CHBE-producing enzyme. The properties of the enzyme, including its molecular form, cofactor requirement, substrate specificity and partial amino acid sequence, revealed that it is a kind of aldehyde reductase and belongs to the aldo–keto reductase superfamily. Comparison of the enzyme with the aldehyde reductase from *Sporobolomyces salmonicolor* [5–7] and the carbonyl reductase from *C. magnoliae* [4] is also described.

2. Materials and methods

2.1. Microorganisms and cultivation

C. magnoliae AKU4643 and *S. salmonicolor* AKU4429 were used. They were cultivated as described previously [4,5].

2.2. Chemicals

(*R*,*S*)-CHBE was prepared from COBE by $NaBH_4$ reduction. Both the (*S*)- and (*R*)-stereoisomers of CHBE were prepared enzymatically as described previously [4,8]. All other chemicals used in this study were of analytical grade and commercially available.

2.3. Enzymatic preparation and analysis of CHBE

The conditions for the preparation of CHBE from COBE with the *C. magnoliae* aldehyde reductase were essentially the same as those for that of (*S*)-CHBE with the carbonyl reductase from the same organism. Optical purity analysis was performed by high performance liquid chromatography (HPLC) on a Chiralcel AS column (Daicel, Japan), as described previously [4].

2.4. Enzyme assay

Each reductase activity was determined by measuring the COBE-dependent decrease of NADPH spectrophotometrically [4]. The standard assay mixture comprised, in 2.5 ml, 5 μmol of COBE (final concentration, 2.0 mM), 0.80 μmol of NADPH, 500 μmol of potassium phosphate buffer, pH 7.0, and the enzyme. During the enzyme purification, the substrate concentration of 0.2 mM was used for activity measurement.

2.5. Purification of reductases

The aldehyde reductase from *C. magnoliae* was purified by column chromatographies basically as reported previously [4]. In the Blue-Sepharose step, the COBE-reducing activity was separated into two peaks. The first peak, which was eluted at about 0.3 M NaCl, contained the (*S*)-CHBE-producing carbonyl reductase [4]. The activity in the second peak, which was eluted at about 0.45 M NaCl, was found to be due to the (*R*)-CHBE-producing enzyme (aldehyde reductase). The second peak was collected and applied to a Superdex 200 HR gel filtration column. 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.

The carbonyl reductase of *C. magnoliae* and the aldehyde reductase of *S. salmonicolor* were purified as described previously [4,5].

2.6. Lysyl endopeptidase digestion and isolation of the peptides

The purified enzyme was digested with lysyl endopeptidase (Wako Pure Chemicals, Japan)

under the conditions described previously [7]. The peptides were separated by reverse-phase HPLC on a μRPC C2/C18 column, connected to a Pharmacia Smart system, with a linear gradient of 0 to 80% of acetonitrile containing 0.1% trifluoroacetic acid.

2.7. Amino acid sequence analysis

The partial amino acid sequence was determined with an Applied Biosystems model 476A pulsed liquid protein sequencer as described previously [4].

2.8. Other methods

The molecular mass of the enzyme and protein concentrations were determined as described previously [4]. An antibody against the aldehyde reductase of *S. salmonicolor* was prepared as described previously [9]. Immunochemical characterization (Ouchterlony double-immunodiffusion experiment and Western blotting analysis) was performed as described previously [9].

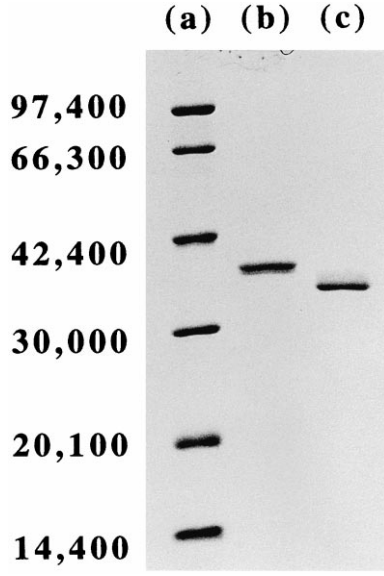

Fig. 1. SDS-polyacrylamide gel electrophoresis of the aldehyde reductases from *C. magnoliae* and *S. salmonicolor*. (a) Standards (from top): phosphorylase *b* ($M_r = 97{,}400$), bovine serum albumin (66,300), aldolase (42,400), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and lysozyme (14,400). (b) Purified aldehyde reductase from *S. salmonicolor* [5,6]. (c) Purified aldehyde reductase from *C. magnoliae*. The gel was stained for protein with Coomassie Brilliant Blue R-250 and destained in methanol/acetic acid/water (7:6:47).

3. Results

3.1. Purification of the enzyme

The purification of the enzyme is summarized in Table 1. The aldehyde reductase ((*R*)-CHBE-producing enzyme) was purified to homogeneity with a 19% recovery after the Blue-Sepharose step, and the apparent overall recovery was 0.9%. The purified enzyme gave a single band on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 1). The specific activity with 0.2 mM COBE of the purified aldehyde reductase (12.2 U mg^{-1}) was almost equal to that of the carbonyl reductase from the same strain (13.5 U mg^{-1}) [4].

Table 1
Purification of the aldehyde reductase from *C. magnoliae*[a]

	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Overall yield (%)	Yield for the aldehyde reductase (%)
Cell-free extract	1740	2540	0.68	100	–
DEAE-Sephacel	632	820	0.77	36	–
Phenyl-Sepharose	198	55	3.6	11	–
Blue-Sepharose	84	6.6	12.7	4.8	100
Superdex-200	16.1	1.32	12.2	0.93	19

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

3.2. Molecular mass and subunit structure

The molecular mass of the enzyme was determined to be 33,000 by gel filtration on TSK-G3000SW (Tosoh, Japan). The relative molecular mass of the subunit was estimated to be about 35,000 by SDS-polyacrylamide gel electrophoresis, while that of the aldehyde reductase purified from *S. salmonicolor* was 37,000 (Fig. 1). These results suggest that the enzyme is a monomer.

3.3. Stereoselectivity for COBE reduction

Using COBE as the substrate, the optical purity of the reduction product was analyzed by HPLC. The CHBE formed by the enzyme was confirmed to comprise only the (*R*)-enantiomer (100% enantiomeric excess (e.e.)).

3.4. Substrate specificity and catalytic properties

The substrate specificity of the enzyme is shown in Table 2. The enzyme catalyzed the reduction of typical substrates for aldo–keto reductases, such as *p*-nitrobenzaldehyde and pyridine-3-aldehyde, in addition to 4-chloro-3-oxobutanoate esters. Aldohexoses such as D-glucose, D-glucuronate, D-xylose and D-galactose were also reduced at high concentrations. The K_m and V_{max} values for COBE, calculated from Lineweaver–Burk plots, were 2.9 mM and 184 μmol min^{-1} mg protein^{-1}, respectively. The enzyme was highly specific for NADPH as a coenzyme; the K_m value for NADPH was 44.9 μM, and no decrease at 340 nm due to the reduction of COBE was observed when NADPH was replaced by an equimolar concentration of NADH.

3.5. Partial amino acid sequence analysis

Automated Edman degradation of the enzyme protein with a pulsed liquid phase sequencer was unsuccessful, and thus the N-terminal of the enzyme seemed to be blocked. The enzyme protein was digested with lysyl endopeptidase and the digest was separated with a Smart system. Five peptides (K-1–K-5) were isolated, and the amino acid sequences of these peptides were analyzed with a protein sequencer. K-1, Y-G-F-P-E-H-D-G-K; K-2, N-T-Q-P-A-N-V-V-L-S-W-G-V-A-R-K; K-3, L-W-D-Q-S-I-T-F-N-D-V-Y-A-L-M-F-K; K-4, N-A-Y-V-D-L-Y-L-M-H-W-P-F-A-V-D-E-N-K; and K-5, L-N-T-G-A-S-I-P-A-I-A-L-G-T-W-E-A-P-N-E-Q-V-A. When these sequences were compared with the amino acid sequence of the aldehyde reductase of *S. salmonicolor* [7], all the peptides obtained show some similarity. Moreover, as shown in Fig. 2, the sequence of K-5 showed high similarity to the N-terminal amino acid sequences of the aldehyde reductase

Table 2
Substrate specificity of the aldehyde reductase from *C. magnoliae*[a]

Substrate (2.0 mM)	Relative activity[b] (%)
p-Nitrobenzaldehyde	100
o-Nitrobenzaldehyde	180
m-Nitrobenzaldehyde	250
p-Chlorobenzaldehyde	58
o-Chlorobenzaldehyde	88
m-Chlorobenzaldehyde	460
Pyridine-3-aldehyde	330
Pyridine-4-aldehyde	260
Benzaldehyde	40
Glyoxal	29
Methylglyoxal	180
Diacetyl	40
Chloroacetaldehyde	170
Camphorquinone	85
Ethyl 4-Chloro-3-oxobutanoate	280
Ethyl 2-Chloro-3-oxobutanoate	430
Methyl 4-Chloro-3-oxobutanoate	72
Methyl 2-Chloro-3-oxobutanoate	270
Octyl 4-Chloro-3-oxobutanoate	150
D-Glyceraldehyde (100 mM)	56
D-Glucuronate (100 mM)	86
D-Glucose (100 mM)	2.0
D-Galactose (100 mM)	19
D-Xylose (100 mM)	20

[a] Enzyme activity was measured as described in Section 2.
[b] To calculate the relative activity, the activity with 2.0 mM *p*-nitrobenzaldehyde was taken as 100%.

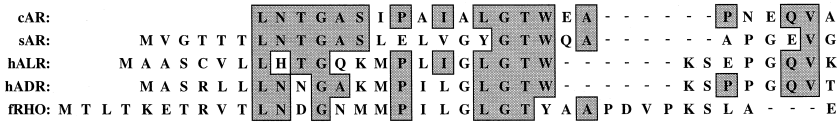

Fig. 2. Comparison of the amino acid sequences of internal peptide K-5 derived from the aldehyde reductase from *C. magnoliae* with the N-terminal amino acid sequences of other aldo–keto reductase superfamily enzymes. cAR, partial amino acid sequence of internal peptide K-5; sAR, aldehyde reductase of *S. salmonicolor* [7]; hALR, human liver aldehyde reductase [10]; hADR, human placenta aldose reductase [10]; fRHO, frog lens ρ-crystallin [2].

of *S. salmonicolor* [7] and other aldo–keto reductase superfamily enzymes [10].

3.6. Immunological properties

Ouchterlony double-immunodiffusion experiment performed with antibodies against *S. salmonicolor* aldehyde reductase showed no coimmunoprecipitation with the *C. magnoliae* aldehyde reductase. Western blotting analysis showed that the purified *C. magnoliae* aldehyde reductase did not react with anti-*S. salmonicolor* aldehyde reductase antibodies. These results suggest that the immunochemical properties of the two enzyme are quite different.

3.7. Spectral properties

The absorption spectrum of the enzyme showed 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 [11].

3.8. Effects of chemicals

Various compounds and metal ions were added to the standard reaction mixture to a final concentration of 1 mM, and then the relative activity was measured using COBE as the substrate. The enzyme was completely inhibited by quercetin, an inhibitor of human brain carbonyl reductase [12] and aldose reductase [13]. But, dicoumarol, which is a potent inhibitor of NAD(P)H dehydrogenase (quinone reductase) [14] and also an inhibitor of carbonyl reductase of human brain [12], did not significantly affect the enzyme activity. 2,4-Dinitrophenol, which is an inhibitor of NADPH dehydrogenase (quinone) [15], also inhibited the enzyme activity (51% inhibition). The enzyme was completely inhibited by 1 mM $CuSO_4$, $ZnSO_4$ and $HgCl_2$.

3.9. Effects of temperature on the enzyme activity and stability

The optimum temperature for COBE reduction was found to be 40°C at pH 7.0. As shown in Fig. 3, the enzyme was stable below 35°C for

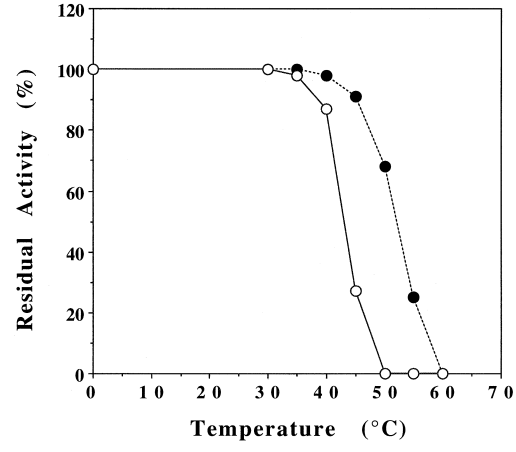

Fig. 3. Effects of temperature on the stabilities of the aldehyde reductase and carbonyl reductase from *C. magnoliae* [4]. Five micrograms each of the aldehyde reductase (○) and carbonyl reductase (●), in a total volume of 1.0 ml, was incubated in 10 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM dithiothreitol at various temperatures for 30 min. The residual activity was measured under the standard assay conditions. Relative residual activity is expressed as a percentage of the maximum residual activity attained under the experimental conditions.

30 min. At 50°C, 100% of the initial enzyme activity was lost, although the (*S*)-CHBE-producing carbonyl reductase from the same strain [4] retained 70% of its initial activity.

4. Discussion

In previous studies [5,6], we purified and characterized a COBE-reducing enzyme ((*R*)-CHBE-forming) from *S. salmonicolor* and concluded that it is an aldehyde reductase belonging to the aldo–keto reductase superfamily. The enzyme purified here also catalyzes the stereoselective reduction of COBE, i.e., the conversion of COBE to (*R*)-CHBE. Both enzymes are monomeric and require NADPH as a coenzyme.

The properties of the aldehyde reductases purified from *C. magnoliae* and *S. salmonicolor* are summarized in Table 3. Although the relative molecular mass of the *C. magnoliae* aldehyde reductase was smaller than that of the *S. salmonicolor* enzyme, the two enzymes showed similar substrate specificities; they can reduce *p*-nitrobenzaldehyde or pyridine-3-aldehyde, which are typical substrates for microbial aldehyde reductases [6,16,17], as well as COBE. However, the *C. magnoliae* enzyme preferred *m*-substituted benzaldehyde among nitrobenzaldehyde and chlorobenzaldehyde isomers, while the *S. salmonicolor* enzyme preferred *p*-substituted benzaldehyde [6]. For the reduction of 4-chloro-3-oxobutanoate esters, the *S. salmonicolor* enzyme favored the octyl ester (K_m, 0.1 mM; V_{max}, 242 μmol min^{-1} mg protein^{-1}) [6], however, the *C. magnoliae* enzyme showed high activity toward esters of shorter carbon length (i.e., methyl and ethyl esters). The K_m value for COBE of the *C. magnoliae* enzyme (2.9 mM) was quite different from that of the *S. salmonicolor* enzyme (0.36 mM). The sensitivity to chemical inhibitors was also different between them. Although the *S. salmonicolor* enzyme was inhibited by dicoumarol, the *C. magnoliae* enzyme was not affected by dicoumarol. The effects of metal ions on the enzyme activity also seemed to be different. The *S. salmonicolor* enzyme was inhibited only 20–30% by 1 mM $ZnCl_2$ and $CdCl_2$ [6], however, the *C. magnoliae* enzyme was completely inhibited by 1 mM $CuSO_4$ and $ZnSO_4$.

The partial amino acid sequence of the *C. magnoliae* enzyme showed similarity to those of other aldo–keto reductase superfamily proteins [10]. These results suggest that the *C. magnoliae* enzyme purified here is a kind of aldehyde reductase and belongs to the aldo–keto reductase superfamily. Besides the aldehyde reductases purified here and from *S. salmoni-*

Table 3
Properties of the aldehyde reductases from *C. magnoliae* and *S. salmonicolor* [5,6]

	C. magnoliae	*S. salmonicolor*
Molecular mass (SDS-PAGE)	35,000	37,000
Number of subunits	1	1
N-terminal sequence	Blocked	VGTTTLNTGASLELVGYGTWQAA
Thermal stability	$<$ 40°C (30 min)	$<$ 60°C (10 min)
Optimum pH	7.0	5.5
Optimum temperature	40°C	60°C
Stereoselectivity for COBE reduction	100% e.e. for (*R*)	100% e.e. for (*R*)
K_m for COBE	2.9 mM	0.36 mM
K_m for NADPH	44.9 μM	37.5 μM
V_{max} for COBE	184 μmol min^{-1} mg^{-1}	144 μmol min^{-1} mg^{-1}
Inhibitors	Quercetin, $CuSO_4$, $ZnSO_4$	Dicoumarol, Quercetin

color, several other enzymes which are thought to be members of the aldo–keto reductase superfamily have been purified from yeasts such as *Saccharomyces* [16,17], *Pichia* [18], *Pachsolen* [19] and *Candida* [20,21]. These results suggest that aldo–keto reductase family enzymes are widely distributed in yeasts.

The *C. magnoliae* enzyme showed no immunochemical reactivity with the anti-*S. salmonicolor* aldehyde reductase serum. This result agreed that high immunochemical reactivity with the anti-*S. salmonicolor* aldehyde reductase serum was only found in the yeasts of the genera *Sporobolomyces*, *Sporidiobolus* and *Rhodotorula* [9].

As reported previously, air-dried cells of *C. magnoliae* catalyzed the stereoselective reduction of COBE to (*S*)-CHBE (90% e.e.) [4], although the enzyme purified here produced (*R*)-CHBE (100% e.e.) from COBE. In air-dried cells, this (*R*)-CHBE-producing enzyme (i.e., aldehyde reductase) may be inactive, because this enzyme is less stable than the (*S*)-CHBE-producing enzyme (i.e., carbonyl reductase). Heat-treated cells of *C. magnoliae* may be useful as a catalyst for obtaining optically pure (*S*)-CHBE from COBE. The asymmetric reduction of COBE with *C. magnoliae* is currently in progress.

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