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Purification and characterization of new aldehyde reductases from *Sporobolomyces salmonicolor* AKU4429 [1]

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Abstract

New aldehyde reductases (AR), ARII and ARIII, which reduce ethyl 4-chloro-3-oxobutanoate (4-COBE) to ethyl 4-chloro-3-hydroxybutanoate (CHBE), with NADPH as a cofactor, were purified from *Sporobolomyces salmonicolor* AKU4429. The two enzymes were different from another aldehyde reductase (ARI) which had already been purified and characterized [Yamada et al., FEMS Microbiol. Lett., 70 (1990) 45; Kataoka et al., Biochim. Biophys. Acta, 1122 (1992) 57]. ARII catalyzed the stereospecific reduction of 4-COBE to (*S*)-CHBE (92.7% enantiomeric excess (e.e.)). In contrast, ARIII reduced 4-COBE to (*R*)-CHBE (38.4% e.e.). ARII was characterized further, and reduced aliphatic and aromatic aldehydes, as well as carbonyl compounds, such as camphorquinone, but did not accept aldose as a substrate. The enzyme is a monomer protein with a relative molecular mass of 34,000. Its isoelectric point is 5.0. The NH_2-terminal amino acid sequence of ARII is different from that of ARI, which catalyzes the stereospecific reduction of 4-COBE to (*R*)-CHBE (100% e.e.).

Keywords: Aldehyde reductase; *Sporobolomyces salmonicolor*; Asymmetric reduction

1. Introduction

Aldehyde reductase (EC 1.1.1.2) belongs to the aldo–keto reductase superfamily, which includes aldose reductase (EC 1.1.1.21) and carbonyl reductase (EC 1.1.1.184) [1]. The aldo–keto reductases metabolize a wide range of substrates, such as hydroxysteroids, prostaglandin F, chlordecone and 2,5-diketo gulonate, and are potential drug targets [2].

The asymmetric reduction of carbonyl compounds, with microorganisms or enzymes, is a useful method for the synthesis of optically active compounds. The NADPH-dependent aldehyde reductase (ARI) produced by *Sporobolomyces salmonicolor* AKU4429 was found to well catalyze the asymmetric reduction of 4-COBE to the corresponding (*R*)-isomer, which is a promising chiral compound for the chemical synthesis of L-carnitine [3,4]. Under preparative

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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scale reaction conditions with acetone-fractionated ARI and glucose dehydrogenase, (*R*)-CHBE (80% mol. yield, 86% e.e.) was produced [5]. In contrast, with untreated cells, the optical purities of the products were quite low [6]. These results suggested that *S. salmonicolor* AKU4429 cells might have an enzyme(s) which is able to reduce 4-COBE to (*S*)-CHBE, in addition to ARI. Recently, considerable attention has been paid to the microbial production of (*S*)-CHBE, which can be converted to (*S*)-1-benzyl-3-hydroxypyrrolidine [7].

In order to elucidate the catalytic mechanism underlying the stereospecific reduction and the physiological meaning of the presence of several kinds of aldehyde reductases which act on 4-COBE in *S. salmonicolor* AKU4429, we have separated the different aldehyde reductases from ARI and characterized the enzymes purified from the yeast.

2. Experimental

2.1. Materials

DEAE–Sephacel, phenyl–Sepharose CL-4B, Sephadex G-100, and Ampholine PAG plates were obtained from Pharmacia Biotech. Hydroxylapatite was obtained from nacalai tesque. The standard proteins for molecular mass calibration were products of Japan Bio-Rad Laboratories, Tokyo, Japan.

2.2. Microorganism and cultivation conditions

S. salmonicolor AKU4429 (Faculty of Agriculture, Kyoto University, Kyoto, Japan) was used. The organism was cultured in a 2-l Erlenmeyer flask containing 500 ml of a medium containing 1% yeast extract, 1% Bacto peptone, and 5% glucose. Cultivation was performed at 30°C for 48 h on a rotary shaker (100 rpm).

2.3. Enzyme assay

The enzyme activities of ARII and ARIII were determined photometrically using 4-COBE and *p*-nitrobenzaldehyde as the substrates as described previously [3]. The reaction mixture (1.0 ml) comprised 200 mM potassium phosphate buffer (KPB) (pH 7.0), 200 μM NADPH, and 200 μM substrate, and was incubated at 37°C. One unit of the enzyme was defined as the amount catalyzing the oxidation of 1 μmol NADPH/min.

2.4. Protein determination

Protein was assayed by measuring the absorbance at 280 nm or with a protein assay kit (Japan Bio-Rad Labs.), with bovine serum albumin as the standard [8].

2.5. Purification of aldehyde reductases from S. salmonicolor AKU4429

2.5.1. Purification of ARII

All procedures were carried out at 4°C. Throughout the enzyme purification procedure, 10 mM Tris–HCl buffer (pH 7.4) containing 0.1 mM dithiothreitol (DTT) was generally used.

Step 1. Wet *S. salmonicolor* AKU4429 cells (257 g) obtained from 25 l of culture broth were suspended in the buffer (430 ml) and then disrupted with 0.45-mm-diameter glass beads (Bee Braun Japan) at 4°C.

Step 2. The supernatant obtained on centrifugation at 12,000 × *g* for 60 min was applied to a DEAE–Sephacel column (8 × 20 cm) equilibrated with the buffer. After washing of the column with the buffer, the enzyme was eluted with a linear gradient of NaCl (0–0.4 M in the buffer, 5000 ml). The fractions containing the enzyme activity were combined and dialyzed against the buffer, and then concentrated with a membrane filter apparatus equipped with an Ultrafilter UK-10 (Toyo Roshi, Japan).

Step 3. The concentrated solution was applied to a DEAE–sephacel column (4 × 16 cm) equilibrated with the buffer. After washing of the column with the buffer, the enzyme was eluted with a linear gradient of NaCl (0–0.4 M in the buffer, 1200 ml). The fractions containing the enzyme activity (0.03–0.10 M NaCl) were combined.

Step 4. After adjustment of the NaCl concentration to 4 M with solid NaCl, the enzyme solution was placed on a phenyl–Sepharose CL-4B column (2.5 × 12 cm) equilibrated with the buffer containing 4 M NaCl. After the column had been washed with the same buffer, the enzyme was eluted with a linear decrease in the ionic strength of NaCl from 4 to 0 M, and a simultaneous linear increase in ethyleneglycol from 0 to 50% in the buffer. The active fractions were collected and dialyzed against 10 mM KPB (pH 7.4).

Step 5. The dialyzed enzyme solution was applied to a hydroxylapatite column (2.5 × 3 cm) equilibrated with 10 mM KPB (pH 7.4). The column was washed with 10 mM KPB (pH 7.4) and then eluted with a linear gradient of KPB (10–200 mM, 90 ml). The enzyme activity recovered in the washing solution was dialyzed against 10 mM KPB (pH 6.2) and then applied to a hydroxylapatite column.

Step 6. The dialyzed enzyme solution was applied to a hydroxylapatite column (2.5 × 3 cm) equilibrated with 10 mM KPB (pH 6.2). The column was washed with 10 mM KPB (pH 6.2), and then the enzyme was eluted with a linear gradient of KPB (10–200 mM, 90 ml). The active fractions were collected and dialyzed against 50 mM Tris–HCl buffer (pH 7.4). The dialyzed enzyme solution was concentrated with Centriprep (Amicon, Denver, MA) and then used for characterization of the enzyme.

2.5.2. Purification of ARIII

Step 1. The active fractions eluted at 0.10–0.18 M NaCl from DEAE–Sephacel column chromatography (Section 2.5.1, step 3), were combined. After adjustment of the NaCl concentration to 4 M with solid NaCl, the enzyme solution was placed on a phenyl–Sepharose CL-4B column (2.5 × 16 cm) equilibrated with the buffer containing 4 M NaCl. After the column had been washed with the same buffer, the enzyme was eluted with a linear decrease in the ionic strength of NaCl from 4 to 0 M, and a simultaneous linear increase in ethyleneglycol from 0 to 50% in the buffer. The active fractions were collected and dialyzed against 10 mM KPB (pH 7.4)

Step 2. The dialyzed enzyme solution was applied to a hydroxylapatite column (1.0 × 10 cm) equilibrated with 10 mM KPB (pH 7.4). The column was washed with 10 mM KPB (pH 7.4) and then eluted with a linear gradient of KPB (10–200 mM, 160 ml). The enzyme activity was dialyzed against 10 mM Tris–HCl (pH 7.4) containing 0.1 mM DTT, and then applied to a DEAE–Sephacel column.

Step 3. The dialyzed enzyme solution was applied to a DEAE–Sephacel column (1.0 × 4 cm) equilibrated with 10 mM Tris–HCl (pH 7.4) containing 0.1 mM DTT. The column was washed with the buffer, and then the enzyme was eluted with a linear gradient of NaCl (0–0.4 M, 40 ml). The active fractions were collected and dialyzed against 50 mM Tris–HCl buffer (pH 7.4). The dialyzed enzyme solution was concentrated with Centriprep and then used for characterization of the enzyme.

2.6. Analyses

The amino acid sequence of the enzyme was determined by the Edman method with a Shimadzu PPSQ-10 protein analyzer. Polyacrylamide (10%) gel electrophoresis (PAGE) was performed at pH 9.5 according to the method of Davis [9]. Sodium dodecyl sulfate (SDS)-PAGE was performed by the method of Laemmli [10]. Isoelectrophocusing was performed at 10°C on an Ampholine PAG plate (pH 3.5–9.5) according to the manufacturer's instructions. The optical purity of 4-CHBE was determined as described elsewhere [3]. Western blotting (im-

munoblotting) was performed as described previously [11]. Ouchterlony double-immunodiffusion tests were conducted in 1.2% Noble agar (Difco) in 200 mM KPB (pH 7.0) [12].

3. Results and discussion

3.1. Purification of aldehyde reductases from S. salmonicolor AKU4429

Two NADPH-dependent *p*-nitrobenzaldehyde reductase activities were separated on the second DEAE–Sephacel column chromatography (Fig. 1). The activity, eluted at 0.03–0.10 M NaCl, was separated into two peaks on phenyl–Sepharose (Fig. 2), one being the aldehyde reductase (ARI) described previously [3,4] and the other, ARII, being a new enzyme. The peak eluted later was purified further as described under Section 2. The purification of ARII is summarized in Table 1. The enzyme was purified 31.2-fold, with a yield of 0.5%, from the cell-free extract. The purified ARII preparation gave a single band on both SDS-PAGE (Fig. 3A) and native-PAGE (Fig. 3B).

Another activity, ARIII, eluted from the first DEAE–Sephacel column at 0.10–0.18 M NaCl, was purified by sequential chromatography on phenyl–Sepharose, hydroxylapatite and DEAE–sephacel columns. The purified preparation, ARIII, was homogeneous on SDS-PAGE (data not shown). The amounts of ARII and ARIII in *S. salmonicolor* AKU4429 cells were not more than 1% of that of ARI.

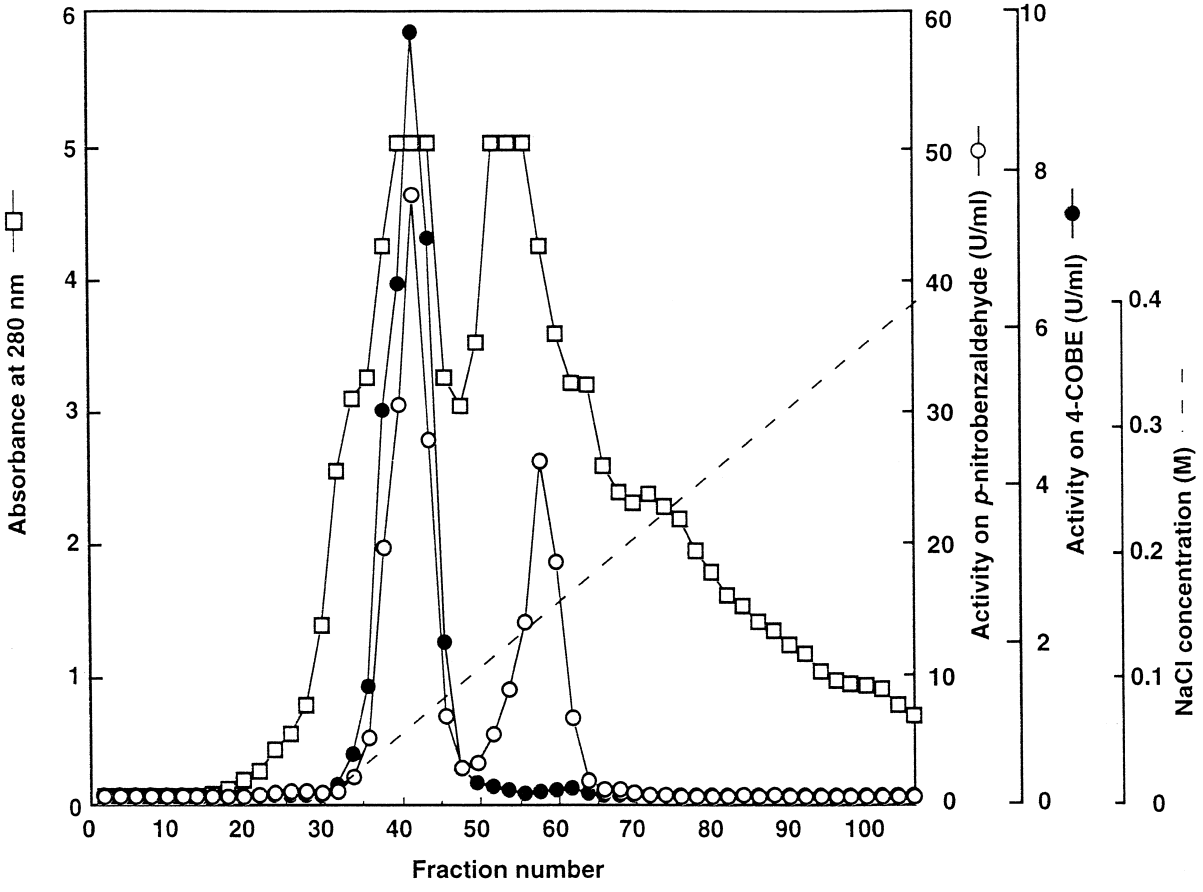

Fig. 1. Elution profile on DEAE–Sephacel column chromatography. Fractions of 6 ml were collected. Activities were measured as described in the text.

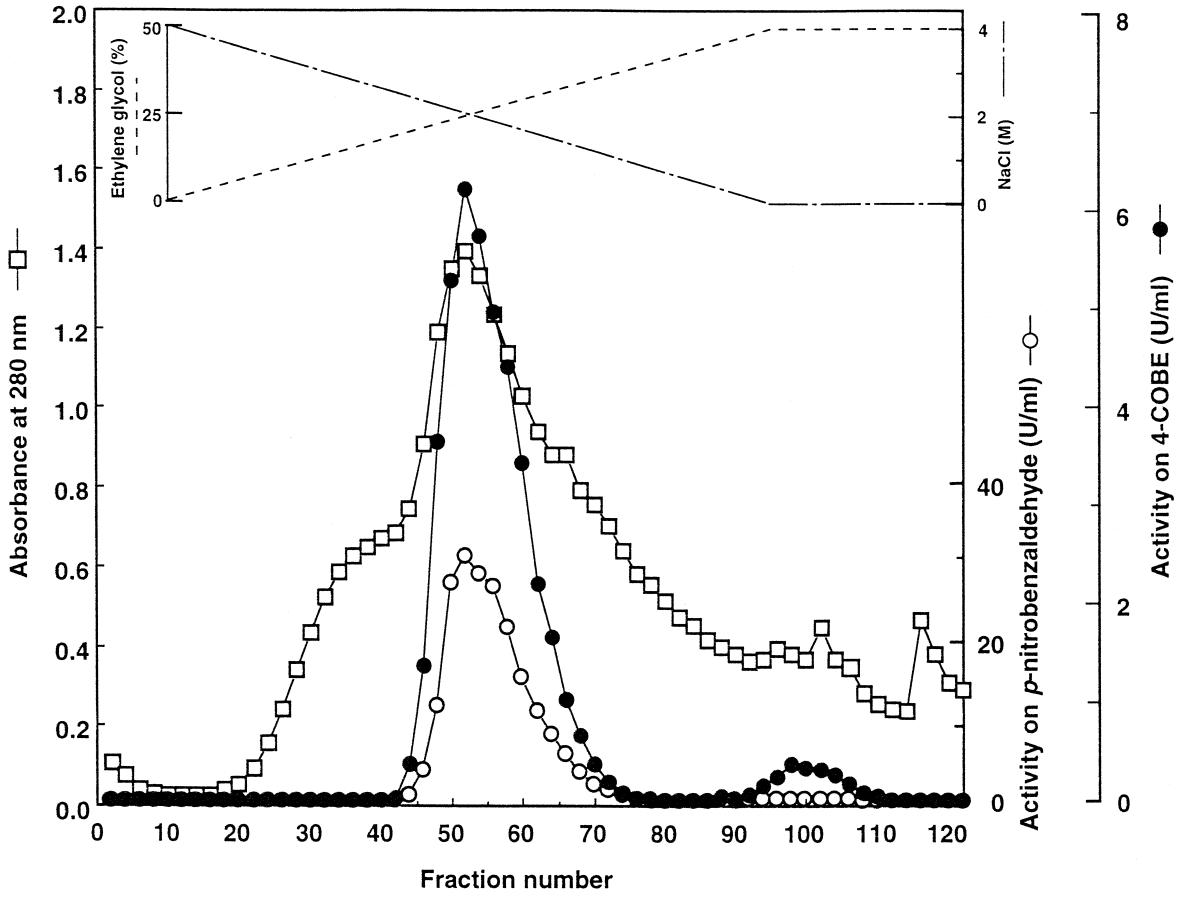

Fig. 2. Elution profile on phenyl–Sepharose column chromatography. Fractions of 5.5 ml were collected. Activities were measured as described in the text.

3.2. Properties of ARII

3.2.1. Molecular mass and subunit structure of ARII

The purified ARII was found to be homogeneous on PAGE and SDS-PAGE. The relative molecular mass of the denatured ARII was estimated to be 37,000 (Fig. 3A, lane 2). The molecular mass of ARII was estimated to be 34,000 on Sephadex G-100 (data not shown). ARII thus appears to be a monomer. Its isoelectric point was 5.0.

Table 1
Purification of ARII from *S. salmonicolor* AKU4429

Step	Total protein (mg)	Total activity[a] (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Cell-free extract	5020	1670	0.333	100	1
DEAE–Sephacel 1st	792	600	0.756	35.9	2.28
DEAE–Sephacel 2nd	88.2	402	4.56	24.1	13.7
Phenyl–Sepharose	10.8	13.8	1.28	0.826	3.84
Hydroxylapatite 1st	3.55	11.0	3.10	0.659	9.31
Hydroxylapatite 2nd	0.803	8.37	10.4	0.501	31.2

[a]4-COBE was used as the substrate.

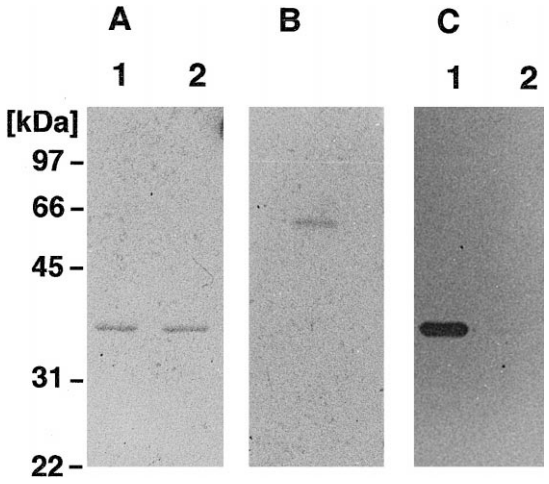

Fig. 3. Electrophoresis and Western blot analysis of the aldehyde reductases from *S. salmonicolor* AKU4429. (A) SDS-PAGE of ARI (lane 1) and ARII (lane 2). (B) Native-PAGE of ARII. (C) Western blot analysis with antibodies raised against ARI. Lane 1, ARI; lane 2, ARII.

3.2.2. NH_2-terminal amino acid sequence

The NH_2-terminal amino acid sequence of ARII was Ala-Lys-Ile-Asp-Asn-Ala-Val-Leu-Pro-Glu-Gly-Ser-Leu-Val-Leu-Val-Thr-Gly-Ala-Asn-Gly-Phe-Val-Ala. Comparison of its NH_2-terminal amino acid sequence with those of ARI and other members of aldo–keto reductase superfamily revealed no significant homology.

3.2.3. Immunological properties

Ouchterlony double-immunodiffusion experiments performed with antibodies against *S. salmonicolor* ARI showed no coimmunoprecipitation with ARII. Western blot analysis showed that the purified ARII did not react with anti-ARI antibodies (Fig. 3C, lane 2). These results sug-

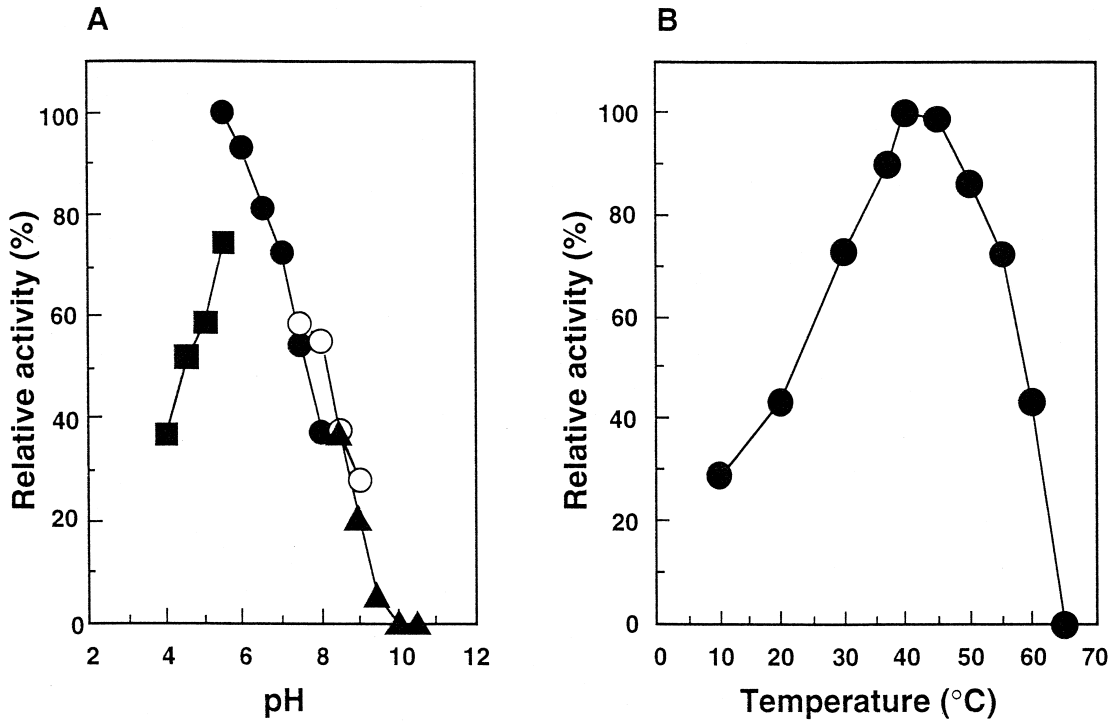

Fig. 4. Effects of pH and temperature on ARII activity. (A) Effect of pH. ARII activity was measured by using 4-COBE as substrate in the following buffer: ■, acetate buffer; ●, potassium phosphate buffer; ○, Tris–HCl buffer; ▲, glycine–NaOH buffer. (B) Effect of temperature. ARII activity was measured at various temperature in the reaction mixture described in the text.

gest that the immunochemical properties of ARI and ARII are quite different.

3.2.4. Effects of pH and temperature

The enzyme exhibited maximum activity at pH 5.5 (Fig. 4A) and 40°C (Fig. 4B). There was no loss of activity, when the enzyme was stored in the buffer with pH 5.0 to 9.0 at 4°C for 12 days (Fig. 5A). The enzyme activity was lost on incubation at 60°C for 10 min (Fig. 5B). As ARII is heat-labile, it is inactivated easily on heat treatment, when a cell-free extract of *S. salmonicolor* AKU4429 is used for the production of (*R*)-CHBE [6].

3.2.5. Inhibition studies

Of the compounds tested, quercetin, a non-specific inhibitor of mammalian oxidoreductases, and diphenylhydantoin, an inhibitor of aldehyde reductases, inhibited the enzyme (Table 2). Carbonyl reductase can be distinguished from aldehyde and aldose reductases by its partiality for quinone. These results suggested that ARII isolated from *S. salmonicolor* AKU4429 is a carbonyl reductase rather than an aldehyde reductase.

3.2.6. Substrate specificity

A broad range of carbonyl compounds was used to investigate the substrate specificity of the enzyme (Table 3). Camphorquinone and 4-COBE were reduced rapidly. The reduction of 4-COBE was reflected by the corresponding (*S*)-CHBE (92.7% e.e.). The enzyme catalyzed the reduction of a number of aliphatic and aromatic aldehydes, and ketones. However, DL-glyceraldehyde or aldose, such as D-glucose, D-xylose or D-galactose, which are good sub-

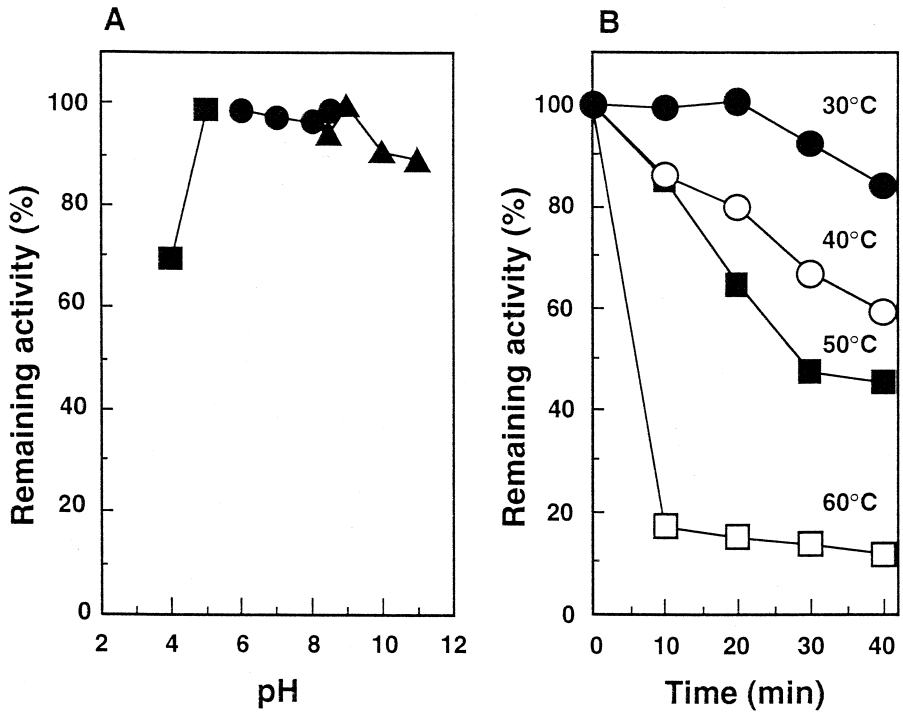

Fig. 5. Effects of pH and temperature on ARII stability. (A) Effect of pH. ARII was stored at 4°C for 12 days in the following buffer (100 mM): ■, acetate buffer; ●, potassium phosphate buffer; ▲, glycine–NaOH buffer. The remaining activity was measured as described in the text. (B) Effect of temperature. ARII was incubated in 200 mM KPB (pH 7.0), and then the remaining activity was measured as described in the text.

Table 2
Effects of various compounds on the activity of ARII

Compound	Concentration (mM)	Relative activity (%)
Quercetin	0.1	0
Dicoumarol	1	64.8
Barbital	1	101
Diphenylhydantoin	1	0
Citrate	1	107
DTNB	0.1	125
Iodoacetate	1	87
N-Ethylmaleimide	1	105
$HgCl_2$	0.1	65.3
PMSF	1	0
EDTA·2Na	1	101
8-Hydroxyquinoline	1	61.5
Phenylhydrazine	1	58.2
$ZnCl_2$	1	97.5
$MgCl_2$	1	97.9
$CoCl_2$	1	118
AgCl	1	58.4

4-COBE was used as the substrate.

strates for aldehyde and aldose reductases, did not serve as substrates. The enzyme can only reduce *o*-nitrobenzaldehyde and *o*-chlorobenzaldehyde, i.e., it cannot reduce a *p*- or *m*-substitution product. The K_m values and V_{max} values for *p*-nitrobenzaldehyde and 4-COBE were comparable to those of ARI [4].

The enzyme was highly specific for NADPH as a coenzyme; no decrease at 340 nm due to the reduction of 4-COBE was observed when NADPH was replaced by an equimolar concentration of NADH. The K_m values for NADPH (31.5 μM) was comparable to those of ARI [4].

3.3. Properties of ARIII

The purified ARIII was found to be homogeneous on SDS-PAGE. The relative molecular mass of the denatured ARIII was estimated to be 37,000 (data not shown). The substrate specificity of the enzyme was investigated with some carbonyl compounds. The enzyme catalyzed the reduction of 4-COBE, and *p*- and *o*-nitrobenzaldehyde, but slightly reduced 2-COBE or *m*-nitrobenzaldehyde. The relative activity on *o*-, *m*- and *p*-nitrobenzaldehyde (0.2 mM) were 265, 0 and 100, respectively. The reduction of 4-COBE was reflected by the corresponding (*R*)-CHBE (38.4% e.e.). Although further analysis could not be performed because of the small amount of the enzyme, these results suggested that ARIII must be distinct from ARI and ARII.

Besides ARI, two other kinds of aldehyde reductases, which reduce 4-COBE, were isolated from *S. salmonicolor* AKU4429 and characterized. Although molecular mass and subunit structure of ARII were quite similar to those of ARI, ARII showed the different stereoselectivity against 4-COBE from that of ARI. Another aldehyde reductase, ARIII, reduced 4-COBE to (*R*)-CHBE, but the optical purity of the product

Table 3
Substrate specificity, Michaelis constants and maximum velocities of ARII

Substrate (0.2 mM)	Relative activity (%)	K_m (mM)	V_{max} (μmol/min per mg)
p-Nitrobenzaldehyde	100	0.95	19
o-Nitrobenzaldehyde	3540	0.55	508
m-Nitrobenzaldehyde	38.8	–	–
p-Chlorobenzaldehyde	0	–	–
o-Chlorobenzaldehyde	597	0.51	13
m-Chlorobenzaldehyde	34.1	1.12	6
Nicotinaldehyde	14.2	27.7	19
Isonicotinaldehyde	34.1	1.82	9
Benzaldehyde	16.9	25.5	40
Glyoxal	0	–	–
Methylglyoxal	0	–	–
Diacetyl	229	20.4	698
Chloroacetaldehyde	0	–	–
Camphorquinone	20,700	0.16	448
Ethyl 4-chloro-3-oxobutanoate	1340	1.49	349
Ethyl 2-chloro-3-oxobutanoate	75.0	102	1597
Methyl 4-chloro-3-oxobutanoate	59.7	3.37	31
Methyl 2-chloro-3-oxobutanoate	278	0.75	17
Octyl 4-chloro-3-oxobutanoate	894	0.07	46
D-Glyceraldehyde[a]	0	–	–
DL-Glyceraldehyde[a]	0	–	–
D-Glucuronate[a]	0	–	–
D-Glucose[a]	0	–	–
D-Galactose[a]	0	–	–
D-Xylose[a]	0	–	–

[a] The substrate concentration was 100 mM.
–: Not determined.

was lower than that obtained by ARI. Presence of ARII and ARIII in cells of *S. salmonicolor* AKU4429 caused the low optical purities of (*R*)-CHBE, when the untreated cells were used as catalyst.

The NH_2-terminal amino acid sequence of ARII is not similar to that of ARI, which is a member of aldo–keto reductase superfamily [2,11]. Cloning and sequence analysis of the ARII and ARIII gene could clarify whether both enzymes belong to the aldo–keto reductase superfamily or not. The work along these lines is in progress. The physiological roles of the aldo–keto reductases produced by microorganisms have not been established so far. We suppose that each of these enzymes including the aldehyde reductases isolated from *S. salmonicolor* AKU4429 in this study, is involved in the respective secondary metabolism.

It remains unknown how ARI and ARII discriminate between CHBE-isomers. Three-dimensional structure analyses of the two enzymes is required to elucidate the mechanism underlying the stereospecific reduction of 4-COBE.

The asymmetric reduction of various carbonyl compounds using a stereospecific reductase is quite a significant method for the production of useful optically active alcohols. Recently, we developed a method for the asymmetric reduction of 4-COBE to (*R*)-CHBE involving *Escherichia coli* cells expressing the ARI gene as a catayst [13,14]. A recombinant *E. coli* strain expressing the ARII gene could also constitute an economical means for the production of (*S*)-CHBE.

Acknowledgements

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