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Asymmetric reduction of α-keto esters with thermophilic actinomycete: purification and characterization of α-keto ester reductase from *Streptomyces thermocyaneoviolaceus* IFO 14271☆

Kohji Ishihara [a,*], Hitomi Yamaguchi [b], Hiroki Hamada [c], Kaoru Nakamura [b], Nobuyoshi Nakajima [d]

[a] *Department of Chemistry, Kyoto University of Education, Fushimi-ku, Kyoto 612-8522, Japan*
[b] *Institute for Chemical Research, Kyoto University, Uji 611-0011, Japan*
[c] *Department of Applied Science, Okayama University of Science, Ridai-cho, Okayama 700-0005, Japan*
[d] *Department of Nutritional Science, Okayama Prefectural University, Soja, Okayama 719-1112, Japan*

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Abstract

An α-keto ester reductase was purified and characterized from *Streptomyces thermocyaneoviolaceus* IFO 14271, one of the thermophilic actinomycetes. The molecular mass of the native enzyme was estimated to be 64 kDa by gel filtration chromatography. The enzyme was a homodimer, with a 30-kDa subunit molecular mass estimated by SDS-polyacrylamide gel electrophoresis. The enzyme showed reducing activity toward aliphatic and aromatic α-keto esters exclusively and produced the corresponding (*S*)-alcohols with > 99% enantiomeric excess (ee). The kinetic constants (K_m values) for α-keto esters, $RCOCO_2Et$ (R = methyl, ethyl, *n*-propyl, *n*-butyl, *n*-pentyl, *n*-hexyl, and *iso*-propyl) were 0.079, 0.12, 1.6, 0.85, 0.70, 0.46, and 9.0 mM, respectively. The enzyme had high stability and was stable toward a variety of additives such as organic solvents and surfactants.

Keywords: Thermophilic actinomycete; Streptomyces; Stereoselective reduction; α-Keto ester; Enzyme purification

1. Introduction

The biological activities of chiral compounds such as agrochemicals, pharmaceuticals, and flavors often depend upon the configuration of their chiral center(s) [1,2]. Therefore, stereoselective synthesis is one of the important subjects in organic synthesis. Among them, the microbiological procedure has proved to be useful as an alternative method of chemical asymmetric synthesis [3,4]. Microbial reduction of carbonyl compounds is a convenient method of obtaining optically pure alcohols. In particular, because of their bifunctional properties, hydroxy esters are useful building blocks in organic chemistry and are employed as synthetic starting molecules for other chiral compounds [5–8]. For example, bakers' yeast (*Saccharomyces cerevisiae*) has

☆ Stereoselective reduction of α-keto esters with thermophilic actinomycete, Part 3. For the preceding paper in this series, see Ref. [30].

* Corresponding author. Tel.: +81-75-644-8265; fax: +81-75-645-1734.

E-mail address: kishi@wsml.kyokyo-u.ac.jp (K. Ishihara).

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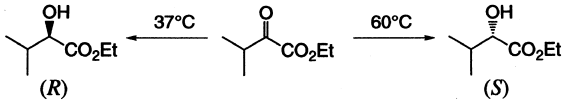
often been used for the reduction of keto esters to obtain optically active hydroxy esters [9–14]. Furthermore, seven yeast keto ester reductases (YKER I ~ VII) have been isolated from bakers' yeast and their enzymatic properties studied including the specific activity, stereoselectivity, and kinetic parameters [15].

Other microorganisms such as *Thermoanaerobactor brockii* [16–18], *Geotrichum candidum* [19–21], *Klebsiella pneumoniae* [22], *Bacillus stearothermophilus* [23], and *Candida magnoliae* [24] that can catalyze the asymmetric reduction of keto esters are also used for the preparation of chiral hydroxy esters. However, little information is known about a mechanistic study of the enzymatic reduction in their microorganisms [24–28].

In the preceding two papers, we reported that thermophilic actinomycetes can reduce various keto esters to the corresponding chiral alcohols in high enantiomeric excesses [29,30]. Especially, one of the thermophilic actinomycetes, *Streptomyces thermocyaneoviolaceus* IFO 14271, showed the reducing ability toward various α- and β-keto esters widely [30]. Furthermore, the stereochemistry of the alcohol produced was changed by the reaction temperature. For example, ethyl 3-methyl-2-oxobutanoate by *S. thermocyaneoviolaceus* was reduced to the corresponding (*R*)-alcohol in high ee at 37°C, while the reduction gave the corresponding (*S*)-alcohol at 60°C [30] (Scheme 1).

To elucidate the mechanistic interpretation and the stereoselectivity changing of the product, we carried out isolation of the enzyme relating to the reduction in the actinomycete cell. We report here the purification and characterization of one α-keto ester reductase from *S. thermocyaneoviolaceus* IFO 14271, and a comparison of the enzymatic properties of our purified enzyme with those of other keto ester reductases is also discussed.



Scheme 1.

2. Materials and methods

2.1. Chemicals

Ethyl pyruvate, ethyl acetoacetate, methyl acetoacetate, ethyl 2-methylacetoacetate, ethyl 2-chloroacetoacetate, ethyl 4-chloroacetoacetate, *p*-ABSF (4-(2-aminoethyl)benzenesulfonate fluoride), glucose 6-phosphate, and glucose 6-phosphate dehydrogenase (from yeast) were purchased from Wako Pure Chemicals, Japan. Ethyl 3-methyl-2-oxobutanoate, 1,2-cyclohexanedione, mandelic acid, and dihydro-4,4-dimethyl-2,3-furandione (ketopantolactone) were obtained from Aldrich Chemical, USA. Methyl benzoylformate, methyl pyruvate, ethyl benzoylformate, ethyl benzoylacetate, *tert*-butyl acetoacetate, benzyl acetoacetate, acetophenone, phenacyl chloride, α-trifluoroacetophenone, 1-acetoxy-2-propanone, and 1-methoxy-2-propanone were purchased from Tokyo Kasei Kogyo, Japan. Pyruvic acid, 2-oxobutanoic acid, poly(ethylene glycol) #20,000 (PEG), and DTT (dithiothreitol) were obtained from Nacalai Tesque, Japan. Ethyl 2-allylacetoacetate was prepared by the literature method [14]. Other α-keto esters were synthesized according to the literature method [31]. NADPH and NADH were obtained from Kohjin, Japan. EDTA (ethylenediaminetetraacetic acid) was purchased from Dojindo Laboratories, Japan. Bactopeptone and yeast extract were purchased from Difco Laboratories, USA. Meat extract (Ehlrich) was obtained from Kyokuto Pharmaceuticals, Japan. Extrelut® was purchased from Merck, Germany. Other chemicals used in this study were of analytical grade.

2.2. Microorganism and cultivation

S. thermocyaneoviolaceus IFO 14271 was purchased from IFO (Institution of Fermentation Osaka, Japan). The actinomycete was cultivated aerobically at 45°C for 15 h in a synthetic

medium (pH 7.2): bactopeptone (15 g/l), yeast extract (2 g/l), meat extract (2 g/l), glycerol (2 g/l), KH_2PO_4 (2 g/l), K_2HPO_4 (2 g/l), and $MgSO_4 \cdot 7H_2O$ (0.1 g/l). The cells were harvested by filtration in vacuo and washed with saline.

2.3. Enzyme assay

The standard assay mixture contained, in a total volume of 1.0 ml, 0.3 mM of ethyl pyruvate (substrate), 0.1 mM of NADPH (coenzyme) in 0.1 M potassium phosphate buffer (pH 6.5) and a limited amount of the enzyme solution. After 1 min of incubation without substrate at 37°C, the reaction was started by the addition of the substrate. The consumption of reduced coenzyme was followed in a Beckman DU-640 spectrophotometer at 340 nm at 37°C. One unit (U) of the enzyme activity was defined as the amount of enzyme which catalyzes the oxidation of 1 μmol of NADPH per minute under the conditions specified.

2.4. Determination of protein content

For determination of the protein content, the method of Bradford was applied [32], calibrated with γ-lactoglobulin as a standard (Bio-Rad Protein Assay kit).

2.5. Enzyme purification

All purification procedures were done below 4°C unless otherwise specified. Buffer-A: 10 mM potassium phosphate buffer, 1 mM DTT, 1 mM EDTA, and 10% glycerol (pH 7.2, adjusted with 1 M KOH). Buffer-B: Buffer-A containing of 1.3 M ammonium sulfate (pH 7.2). Buffer-C: 0.1 M potassium phosphate buffer, 1 mM DTT, 1 mM EDTA, and 0.2 M KCl (pH 7.2).

2.5.1. Step 1: preparation of the cell-free extract

S. thermocyaneoviolaceus cell was ground under cooled acetone (below −30°C) and filtered in vacuo (preparing an acetone-powder cell). Thirty grams of the powder cells were suspended in 280 ml of 50 mM potassium phosphate buffer, 1 mM DTT, 1 mM EDTA, and 10% glycerol (pH 7.2). This suspension was cooled below 0°C and subsequently sonicated with 15 pulses of 120 s each with 300-s cooling intervals in a Sonicator® (Ohtake Works, Japan), fitted with a microtip at a power setting of 70 W. At the first interval, 0.5 mM of *p*-ABSF was added as a protease inhibitor. Cell debris in the homogenate was removed by centrifugation at $10{,}000 \times g$ for 30 min at 4°C, and the supernatant served as the crude cell-free extract.

2.5.2. Step 2: Butyl-Toyopearl column chromatography

Solid ammonium sulfate was added to the extract for making the solution 30% saturated. After stirring for an hour and standing for 2 h at 4°C, the precipitate was removed by centrifugation at $10{,}000 \times g$ for 30 min at 4°C. The supernatant (213 ml) was applied to a Butyl-Toyopearl 650M (TOSOH, Japan) column (4.0 × 12 cm) equilibrated with Buffer-B. The column was washed with 200 ml of Buffer-B, and then the proteins were eluted with a 1.3 to 0 M decreasing linear gradient of $(NH_4)_2SO_4$ (500 ml). All fractions containing activity (eluted between 0.5 to 0.4 M) were pooled and concentrated to 50 ml by dialysis against PEG at 4°C.

2.5.3. Step 3: Phenyl-Toyopearl column chromatography

To the above enzyme solution was added 12.5 g of $(NH_4)_2SO_4$, and the mixture was applied to a Phenyl-Toyopearl 650M (TOSOH, Japan) column (3.2 × 10.5 cm) equilibrated with Buffer-B. The column was washed with Buffer-A containing 1.0 M $(NH_4)_2SO_4$, and the protein was eluted with a 1.0 to 0 M decreasing linear gradient of $(NH_4)_2SO_4$ (500 ml). All fractions containing activity (eluted between 0.7 to 0.6 M) were pooled and concentrated to 127 ml by

dialysis against PEG and dialyzed overnight against Buffer-A at 4°C.

2.5.4. Step 4: DEAE-Toyopearl chromatography

The dialyzate solution was applied to a DEAE-Toyopearl 650M (TOSOH, Japan) column (3.2 × 9.5 cm) equilibrated with Buffer-A. The protein was eluted with a 0 to 0.5 M increasing linear gradient of KCl (400 ml). All fractions containing activity (eluted between 0.05 to 0.1 M) were pooled and dialyzed overnight against Buffer-A at 4°C.

2.5.5. Step 5: Red Sepharose CL-6B chromatography

The above enzyme solution was applied to a Red Sepharose CL-6B (Amersham Pharmacia Biotech, Sweden) column (1.6 × 4 cm) equilibrated with Buffer-A. The column was washed with Buffer-A containing of 0.5 M KCl, and the proteins were eluted with a 0.5 to 2.0 M increasing linear gradient of KCl (60 ml). All fractions containing activity (eluted between 1.7 to 1.8 M) were pooled and concentrated to 2 ml by a Stirred Cells Model 8050 (Amicon Grace, Japan) equipped with an ultrafiltration membrane YM 10 (Diaflo, cutoff MW 10,000).

2.5.6. Step 6: Superdex® 200 HR gel filtration chromatography

The enzyme solution was applied to a Superdex® 200 HR 10/30 gel filtration column (Amersham Pharmacia Biotech) (1.0 × 30 cm) equilibrated with Buffer-C. The protein was eluted with Buffer-C. The active fractions were collected and concentrated to 1 ml by a Centricon-30 (cutoff MW 30,000, Amicon Grace).

2.6. Determination of the molecular mass

The molecular mass of the native enzyme was estimated by an HPLC gel filtration column, Asahipack® GS-520 (7.6 × 300 mm, GL-Sciences Inc., Japan) and Superdex® 200 HR10/30 with MW-Marker (Oriental Yeast, Japan) with Buffer-C. The molecular weight of the subunit was estimated by SDS-PAGE (12.5%) [33,34] with SDS-PAGE standards (low range) (Bio-Rad, USA) as the standard.

2.7. Determination of kinetic parameters

The K_m and V_{max} of the purified enzyme toward various α-keto esters and NAD(P)H were calculated from the initial rates of the reaction in an appropriate range of the substrate concentration using at least five points by [S]/v–[S] plots and Lineweaver and Burk plots [35].

2.8. Measurement of the effects of pH

The optimum pH of the enzyme (relative activity) was measured at 37°C in the following buffers: pH 4.5 to 6.5, 0.1 M potassium phosphate buffer; pH 6.5 to 9.0, 0.1 M bis-tris-propane buffer; pH 9.0 to 11.0, 0.1 M glycine-KOH buffer. The relative activity was determined by arbitrarily setting the activity at pH 6.0 to 100. The pH stability of the enzyme (residual activity) was measured at 37°C in 0.1 M potassium phosphate buffer (pH 6.5) after incubation for 10 min in the following buffers: pH 4.5 to 6.5, 0.1 M potassium phosphate buffer; pH 6.5 to 9.0, 0.1 M bis-tris-propane buffer; pH 9.0 to 11.0, 0.1 M glycine-KOH buffer at 37°C. The residual activity was determined by arbitrarily setting the activity at pH 6.5 to 100.

2.9. Effects of additives

The stability of the enzyme activity (residual activity) was measured in 0.1 M potassium phosphate buffer (pH 6.5) at 37°C after 7 days incubation in buffer-A in the presence of each additive. The residual activity was determined by arbitrarily setting the activity without incubation in the absence of additive to 100.

2.10. Enzymatic reduction of α-keto esters

In a polypropylene tube were placed the purified enzyme solution (10 units), NADPH (22

μmol), glucose 6-phosphate dehydrogenase (15 units), glucose 6-phosphate (Oriental Yeast) (1.0 mmol), substrate (0.5 mmol), and 0.1 M potassium phosphate buffer (pH 7.0, 3.0 ml). The reaction tube was shielded from light. The mixture was shaken gently at 37°C. After 24 h, the mixture was filtered by an Extrelut® short column, extracted with ether, and then concentrated under reduced pressure.

2.11. Stereochemistry of the products

The enantiomeric excesses of ethyl lactate, ethyl 2-hydroxy butanoate, ethyl 2-hydroxy pentanoate, ethyl 2-hydroxy hexanoate, ethyl 2-hydroxy heptanoate, and ethyl mandelate were determined by GLC analysis (Chirasil-DEX CB, Chrompack, Netherlands, 0.25 mm × 25 m, 70°C to 120°C), and that of ethyl 2-hydroxy-3-methylbutanoate was also determined by GLC analysis (Chiraldex G-TA, Astec, USA, 0.25 mm × 25 m, 90°C). The absolute configuration of the isomer was determined by comparing its retention time with those of authentic samples [9].

3. Results and discussion

3.1. Purification of the enzyme

One of the α-keto ester reductases was purified from a cell-free extract of *S. thermocyaneoviolaceus* IFO 14271 by chromatographic methods, which included hydrophobic interaction, anion-exchange, affinity, and gel filtration chromatography. These results are summarized in Table 1. The overall 1043-fold purification was achieved from the crude cell-free extract with an overall yield of 16.3%. The purified enzyme gave a single band on SDS-polyacrylamide gel electrophoresis (Fig. 1) and only one peak on gel filtration and reverse-phase chromatography (data not shown).

3.2. Molecular mass and subunit size

The molecular mass of the purified enzyme was estimated to be 64.2 kDa by gel filtration chromatography on Asahipack® GS-520 and to be 63.8 kDa on Superdex® 200. The subunit size estimated by SDS-polyacrylamide gel electrophoresis (Fig. 1) was 30.1 kDa (single band). These results showed that the enzyme consisted of two identical subunits (homodimer structure).

3.3. Substrate specificity

The substrate specificity of the purified enzyme was examined spectrophotometrically toward various keto esters. These results are summarized in Table 2. The enzyme catalyzed the reduction of various aliphatic α-keto esters having variable chain length and had low reducing activities toward aromatic α-keto esters (alkyl benzoylformate) and a few β-keto esters (such as ethyl 2-allylacetoacetate and ethyl 2-chloro-

Table 1
Purification of α-keto ester reductase from *S. thermocyaneoviolaceus* IFO 14271

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Fold
Cell-free extract	10,900	876	0.0804	100	1
30% $(NH_4)_2SO_4$	10,600	818	0.0775	93.4	1.04
Butyl-Toyopearl	4270	767	0.180	87.6	2.24
Phenyl-Toyopearl	1600	634	0.395	72.4	4.91
DEAE-Toyopearl	72.4	510	7.05	58.2	87.7
Red-Sepharose CL-6B	10.2	352	34.5	40.2	429
Superdex® 200	1.70	143	83.9	16.3	1043

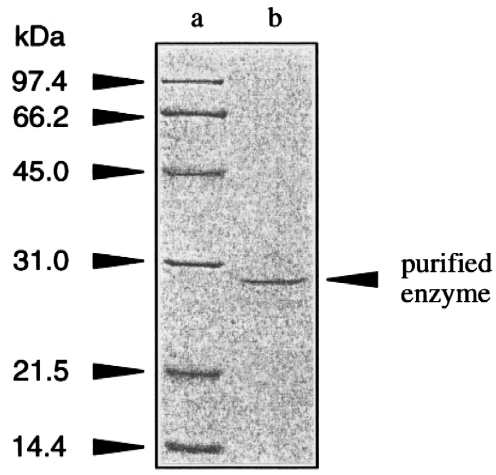


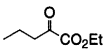
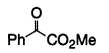
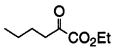
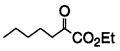
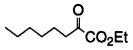
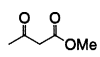
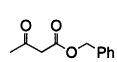
Fig. 1. SDS-polyacryl amide gel electrophoresis of the enzyme. SDS-gel electrophoresis using 12.5% polyacrylamide was performed in the presence of 0.1% SDS. (a) Standards (from top): phosphorylase b (M_r = 97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400). (b) Purified enzyme. Proteins were visualized by Coomassie Brilliant Blue R-250 and destained in a 30% (v/v) methanol/10% (v/v) acetic acid solution.

acetoacetate). Furthermore, the enzyme did not catalyze the reduction of α-keto acid, acetophenone (and its derivatives), ketopantolactone, and cyclic diketone. These results show that the enzyme is specific for the reduction of α-keto esters. A carbonyl group at the α-position and an acyclic ester structure would be necessary for the high reducing activity of the enzyme.

3.4. Stereospecificity

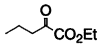
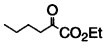
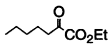
To clarify the stereochemistry of the products, the reduction of α-keto esters by the purified enzyme was carried out. As shown in Table 3, ethyl pyruvate, ethyl 2-oxobutanoate, ethyl 2-oxopentanoate, ethyl 2-oxohexanoate, ethyl 2-oxohepta-noate, ethyl 3-methyl-2-oxobutanoate, and ethyl benzoylformate were reduced to the corresponding (*S*)-hydroxy esters with excellent ee (> 99% ee). These results showed that both aliphatic and aromatic α-keto esters

Table 2
Substrate specificity of the enzyme from *S. thermocyaneoviolaceus* IFO 14271

Substrate[a]	Rel. rate[c] (%)	Substrate[a]	Rel. rate[c] (%)	Substrate[b]	Rel. rate[c] (%)	Substrate[b]	Rel. rate[c] (%)
	100		98		2		0
	96		114		2		0
	50		12[b]		8		0
	61		0		7		0
	82		0				0
	86		0[b]		0		0
	9		1[b]		1		
	2[b]		1[b]		0		0

[a] Concentration was 1 mM.
[b] Concentration was 20 mM.
[c] Rel. rate = Relative rates were determined as described in the Experimental section.

Table 3
Enzymatic reduction of α-keto esters[a]

Substrate	Yield (%)[b]	Ee (%)[c]	(*R*/*S*)[c]
	53	>99	*S*
	55	>99	*S*
	59	>99	*S*
	60	>99	*S*
	57	>99	*S*
	59	>99	*S*
	58	>99	*S*

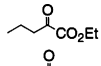
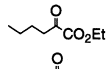
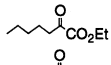
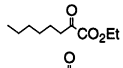
[a] The purified enzyme solution (10 units), NADPH (22 μmol), glucose 6-phosphate dehydrogenase (15 units), glucose 6-phosphate (1.0 mmol), substrate (0.5 mmol), and 0.1 M potassium phosphate buffer (pH 7.0, 3 ml) were incubated for 24 h at 37°C.
[b] Chemical yields were measured by GLC analysis.
[c] Enantiomeric excesses and configuration were measured by GLC analysis with an optically active capillary column.

were reduced stereospecifically by the purified enzyme.

3.5. Kinetic constants

The kinetic parameters (K_m and V_{max}) for aliphatic α-keto esters were calculated and are listed in Table 4. The K_m values increased as the side alkyl chain in substrates was changed from methyl to *n*-propyl group (corresponding from ethyl pyruvate to ethyl 2-oxopentanoate). However, the K_m value decreased with extension of the substituent from *n*-propyl to *n*-hexyl. These irregular changes in the K_m values reflect their relative activity (see Table 2). The K_m and V_{max} values of ethyl 3-methyl-2-oxobutanoate were 9.01 mM and 83.2 μmol/min · mg protein, respectively, which indicated that the enzyme had low affinity for the substrate. This would be due to the steric effects of the bulky isopropyl group (at the β-position site) in the substrate. The K_m values of NADPH and NADH were 0.023 and 0.53 mM, respectively. When the reduction of ethyl pyruvate by the purified enzyme was occurred, the reaction rate with NADPH as a coenzyme was faster than that with NADH. Although the enzyme is able to use both coenzymes, NADPH would be utilized mainly as a coenzyme in the cell.

Table 4
Kinetic parameters (K_m and V_{max}) of α-keto ester reductase from *S. thermocyaneoviolaceus* IFO 14271

Substrate		K_m (mM)	V_{max} (μmol/min•mg protein)	V_{max}/K_m
Ethyl pyruvate		0.079	113	1420
Ethyl 2-oxobutanoate		0.121	189	1560
Ethyl 2-oxopentanoate		1.55	105	67.9
Ethyl 2-oxohexanoate		0.853	103	121
Ethyl 2-oxoheptanoate		0.696	143	206
Ethyl 2-oxooctanoate		0.463	70.3	152
Ethyl 3-methyl-2-oxobutanoate		9.01	83.2	9.23

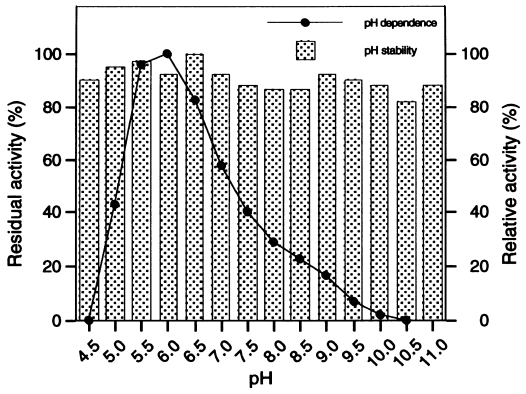


Fig. 2. The optimum pH and pH stability of the enzyme. The pH dependence (relative activity) was measured in 0.1 M buffer (pH 4.5 to 11.0) at 37°C. The relative activity was determined by arbitrarily setting the activity at pH 6.0 to 100. The pH stability (residual activity) was measured in 0.1 M potassium phosphate buffer after incubation for 10 min in the 0.1 M buffer (pH 4.5 to 11.0) at 37°C. The residual activity was determined by arbitrarily setting at pH 6.5 to 100.

3.6. Optimum pH and pH stability

The effect of pH on the enzyme activity was examined. The enzyme showed maximum activity between pH 5.5 and 6.0. The enzyme was quite stable over a wide region (pH 4.5 to 11.0) as shown in Fig. 2.

3.7. Inhibition study

The influence of a broad range of metal ions, sulfhydryl-protecting and -inhibiting reagents, chelators, and arginine-specific reagents on the activity of the purified enzyme was investigated (see Table 5).

Among sulfhydryl inhibitors tested, *p*-chloromercuribenzoate and iodoacetic acid inhibited enzyme activity. The enzyme was also inhibited by heavy metal ions, such as mercury, nickel, and zinc. This inhibition pattern indicates an essential sulfhydryl group. Metal chelating reagents such as *o*-phenanthroline, 2,2′-bipyridyl, and EDTA showed no inhibitory effect on the activity. This suggests that the enzyme does not contain an essential metal ion. Furthermore, arginine-residue-specific reagents such as phenylglyoxal, diacetyl, and 1,2-cyclohexanedione (1,2-CHDO) inhibited the enzyme activity. This result indicates an essential arginine residue.

3.8. Stability

The purified enzyme showed high stability at 37°C (Fig. 3). After 4 days, only 12% loss of activity was detected at 37°C. Storage at 4°C for 3 months caused no loss of activity. Furthermore, the enzyme retained above 60% of its initial activity after 12 h at 50°C. In particular, the activity in the presence of 30% dimethyl-

Table 5
Effects of additives on α-keto ester reductase from *S. thermocyaneoviolaceus* IFO 14271[a]

Additives	(Conc.)	Residual activity(%)
None		100[b]
Metal ions		
NaCl	(5 mM)	111
$MgCl_2$	(5 mM)	115
KCl	(5 mM)	105
$CaCl_2$	(5 mM)	109
$MnCl_2$	(5 mM)	100
$CoCl_2$	(5 mM)	81
$NiCl_2$	(5 mM)	48
$ZnCl_2$	(5 mM)	42
$HgCl_2$	(5 mM)	8
Chelators		
o-Phenanthroline	(1 mM)	97
2,2′-Bipyridyl	(1 mM)	95
EDTA	(1 mM)	102
Sulfhydryl-reagents		
p-CMB[c]	(1 mM)	52
ICH_2CO_2H	(1 mM)	56
Arginine-specific reagents		
Phenylglyoxal	(1 mM)	58[d]
Diacetyl	(15 mM)	60[d]
1,2-CHDO[e]	(15 mM)	70[d]

[a] The enzyme was incubated with additive for 5 min at 37°C before the reaction was started.
[b] The activity in the absence of inhibitor was determined by arbitrarily setting to 100.
[c] *p*-CMB = *p*-Chloromercuribenzoate.
[d] Measured after incubation for 15 min at 37°C.
[e] 1,2-CHDO = 1,2-Cyclohexanedione.

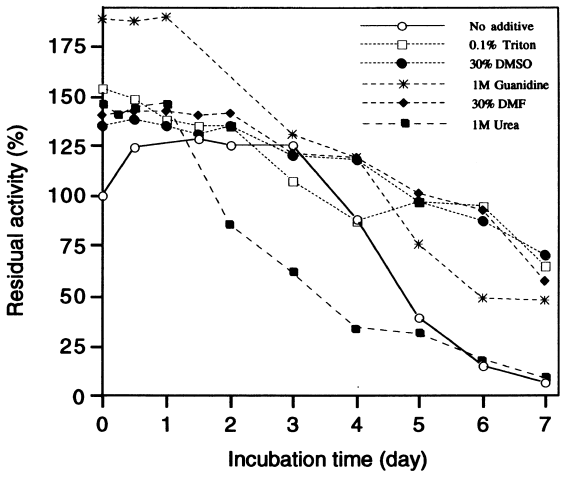


Fig. 3. Effects of additives on the stability of the enzyme. The stability of the enzyme activity (residual activity) was measured in 0.1 M potassium phosphate buffer (pH 6.5) at 37°C after incubation in Buffer-A in the presence of each additives. The residual activity was determined by arbitrarily setting the activity without incubation in the absence of additive to 100.

sulfoxide (DMSO), 30% dimethylformamide (DMF), 1 M guanidine, and 0.1% Triton X-100 (surfactant) showed high stability and loss of activity was not detected after 4-day incubation. The mechanism for the increase in the enzyme activity immediately after incubation is not clear. It might be caused by a conformational change in the enzyme to a more favorable form for the reduction.

In view point of the stability, the purified enzyme will serve mainly on the reduction of α-keto esters with *S. thermocyaneoviolaceus* cells at high temperature (60°C) [30].

3.9. Comparison with other microbial reductases

Several α-keto ester reducing enzymes have been isolated from microorganisms such as *Saccharomyces cerevisiae* (bakers' yeast) [15] and *C. magnoliae* [24]. The properties of these enzymes are compared with those of the purified enzyme in this study as shown in Table 6. Although other enzymes utilized NADPH as a sole coenzyme, our purified enzyme utilized both NADPH and NADH as the coenzyme. Our purified enzyme had high affinity for ethyl pyruvate and ethyl 3-methyl-2-oxobutanoate among the listed reductases and had high thermostability and resistibility toward organic solvents. Therefore, the enzyme would be very useful for catalysis of the reduction of α-keto esters to the corresponding (*S*)-hydroxy esters, in contrast to an (*R*)-hydroxy ester-producing

Table 6
Comparison of the characteristics of the reductases from various microorganisms

Source	Molecular mass						Coenzyme
	GFC[a] (kDa)	SDS-PAGE (kDa)		Ee (*R*/*S*)	K_m (mM)	Ee (*R*/*S*)	
S. thermocyaneoviolaceus[b]	64	30 (dimer)	0.079	>99 (*S*)	9.01	>99 (*S*)	NADPH / NADH
Candida magnoliae [24]	76	32 (dimer)	21	---[c]	240	---[c]	NADPH
Bakers' yeast (YKER-II) [15]	58	29 (dimer)	135	98 (*S*)	N.R.[d]	N.R.[d]	NADPH
Bakers' yeast (YKER-IV) [15]	39	31 (monomer)	0.434	>99 (*R*)	0.265	>99 (*R*)	NADPH
Bakers' yeast (YKER-V) [15]	83	41 (dimer)	5.06	94 (*S*)	79.4	77 (*S*)	NADPH

[a] GFC = Gel filtration chromatography.
[b] Purified in this study.
[c] ---: not determined.
[d] N.R. indicates no reaction.

enzyme, YKER-IV, which contributes mainly to the reduction by bakers' yeast at low substrate concentration.

Further detailed studies including purification of the (*R*)-hydroxy ester-producing enzyme, which contributes to the reduction system in the cells are now in progress in our laboratory.

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