Bioscience, Biotechnology, and Biochemistry

ISSN: 0916-8451 (Print) 1347-6947 (Online) Journal homepage: http://www.tandfonline.com/loi/tbbb20

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To cite this article: Hitomi YAMAGUCHI, Nobuyoshi NAKAJIMA & Kohji ISHIHARA (2002) Purification and Characterization of Two α-Keto Ester Reductases from Streptomyces thermocyaneoviolaceus IFO 14271, Bioscience, Biotechnology, and Biochemistry, 66:3, 588-597, DOI: 10.1271/bbb.66.588

To link to this article: http://dx.doi.org/10.1271/bbb.66.588

Published online: 22 May 2014.

Biosci. Biotechnol. Biochem., **66** (3), 588–597, 2002

Purification and Characterization of Two α-Keto Ester Reductases from *Streptomyces thermocyaneoviolaceus* IFO 14271*

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Received October 1, 2001; Accepted November 9, 2001

Two NADPH-dependent α-keto ester reductases (*Streptomyces thermocyaneoviolaceus* keto ester reductase, STKER-II and -III) were purified from *S. thermocyaneoviolaceus* IFO 14271, one of thermophilic actinomycetes. The molecular masses of native STKER-II and -III were estimated to be 60 kDa and 70 kDa by gel filtration chromatography, respectively. These enzymes were both homodimers, with 29-kDa and 30-kDa subunit molecular masses based on SDS polyacrylamide gel electrophoresis. STKER-II and -III were stable from pH 7.0 to 10.0 and pH 5.5 to 9.0, respectively. Ethyl 3-methyl-2-oxobutanoate was reduced by both enzymes isolated to the corresponding (*R*)-hydroxy ester with excellent enantiomeric excess. STKER-III showed high stereoselectivity for the reduction of bulky substrates, while the selectivity of the STKER-II-catalyzed reduction was low except for ethyl 3-methyl-2-hydroxybutanoate. Both enzymes had small K_m values toward aliphatic keto esters having a long alkyl chain.

Key words: thermophilic actinomycete; reduction; reductase; enzyme purification; α-hydroxy ester producing enzyme

Biotransformations of exogenous substrates have been widely studied to synthesize chiral compounds.[1–6] The microbial reduction enables preparation of optically pure alcohols and helps to lessen the environmental impact of organic syntheses. For example, the asymmetric reduction of keto esters by bakers' yeast has been widely used to obtain chiral compounds, because the produced hydroxy esters are useful chiral building blocks for the synthesis of natural and bioactive compounds.[7–9] Furthermore, several yeast keto ester reductases (YKERs) were isolated from bakers' yeast and their enzymatic properties studied including the specific activity, stereoselectivity, and kinetic parameters.[10–14] Other microorganisms such as *Thermoanaerobactor brockii*,[15] *Geotrichum candidum*,[16,17] and *Klebsiella pneumoniae*[18] that can also catalyze the asymmetric reduction of keto esters are also used for the preparation of chiral hydroxy esters. However, little information is known from mechanistic studies of enzymatic reduction by their microorganisms.[13,14,19,20]

To date, we have studied the microbial reduction of some α- and β-keto esters with thermophilic *Bacillus* strains, actinomycetes, and micro algae and have reported that some of their strains could reduce keto esters with high conversion and excellent enantiomeric excess (e.e.).[21–25] In particular, we found that the stereoselectivity of α-keto ester reduction using a thermophilic actinomycete, *S. thermocyaneoviolaceus* IFO 14271, changed with alterations in the reaction conditions such as reaction temperature, substrate concentration, and introduction of additives.[23] For example, ethyl 3-methyl-2-oxobutanoate was reduced by *S. thermocyaneoviolaceus* to the corresponding (*R*)-hydroxy ester with high e.e. at 37°C, while the reduction at 55°C produced the corresponding (*S*)-hydroxy ester. In order to elucidate the reaction mechanism, we tried to isolate α-keto ester-reducing enzymes from a cell-free extract of *S. thermocyaneoviolaceus* IFO 14271; consequently, one enzyme (STKER-I) was purified and characterized. The enzyme reduced various α-keto esters to the corresponding (*S*)-hydroxy ester with excellent e.e.[26] However, (*R*)-hydroxy ester-producing enzyme(s) were still not isolated from the cells.

We would like to report here the purification and characterization of new two α-keto ester reductases from *S. thermocyaneoviolaceus* IFO 14271. We will also discuss the reaction mechanism in the actinomycete cells on the basis of enzyme properties.

Materials and Methods

Chemicals. *p*-ABSF (4-(2-aminoethyl)benzenesul-

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fonate fluoride) and ethyl pyruvate were purchased from Wako Pure Chemicals, Japan. Ethyl 3-methyl-2-oxobutanoate was obtained from Aldrich Chemicals, USA. Methyl benzoylformate and ethyl benzoylformate were purchased from Tokyo Kasei Kogyo, Japan. Other α-keto esters were synthesized according to methods from the literature.[27] DTT (dithiothreitol) and Bis-tris propane (1,3-bis[tris (hydroxymethyl)methylamino]propane) were obtained from Nacalai Tesque, Japan. NADPH and NADH were obtained from Kohjin, Japan. EDTA (ethylenediaminetetraacetic acid) and MES (2-(*N*-morpholino)ethanesulfonic acid) were purchased from Dojindo Laboratories, Japan. Extrelut was purchased from Merck, Germany. All other chemicals used in this study were of analytical grade and commercially available.

Microorganism and cultivation. *S. thermocyaneoviolaceus* IFO 14271 was purchased from the IFO (Institute for Fermentation, Osaka) culture collection, Japan. The actinomycete was cultivated aerobically at 45°C for 15 h. The cells were harvested by filtration *in vacuo* and washed with saline as described previously.[26]

Enzyme assay. The reducing activity of each reductase (STKER-II and -III) was measured spectrophotometrically using ethyl 2-oxoheptanoate as the substrate (final concentration was 4.0 mM and 2.0 mM, respectively). The standard assay mixture (1.0 ml) comprised 0.1 M potassium phosphate buffer (KPB) (pH 6.5), 0.15 mM NADPH, substrate solution, and enzyme solution. The consumption of reduced coenzyme was followed with a Beckman DU-640 spectrophotometer at 340 nm at 37°C. One unit of the enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADPH/min under the conditions specified.

Protein assay. The protein content was measured by the method of Bradford,[28] calibrated with γ-lactoglobulin as a standard (Bio-Rad Protein Assay Kit).

Enzyme purification. All purification procedures were done below 4°C unless otherwise specified. Buffer-A: 20 mM potassium phosphate buffer containing 1 mM DTT, 1 mM EDTA, and 10% glycerol (pH 7.2). Buffer-B: Buffer-A containing 1.3 M ammonium sulfate (pH 7.2). Buffer-C: Buffer-A containing 0.2 M KCl (pH 7.2).

Preparation of the cell-free extract. The cells were ground under cooled acetone (below −30°C) and filtered *in vacuo* (for preparing acetone-powder of the cells). Fifty-eight grams of the powdered cells were suspended in 550 ml of Buffer-A. This suspension was cooled at 0°C, then sonicated with 15 pulses of 120 sec each with 300-sec cooling intervals in a Sonicator (Ohtake Works, Japan), fitted with a microtip at a power setting of 70 W. At the first interval, *p*-ABSF was added as a protease inhibitor (the final concentration was 0.5 mM). Cell debris in the homogenate was removed by centrifugation at 10,000 × *g* for 30 min at 4°C, and the supernatant served as the crude cell-free extract.

Purification of STKER-II.

Step 1. DEAE-Toyopearl chromatography. The cell-free extract (176 ml) was applied to a DEAE-Toyopearl 650 M (TOSOH, Japan) column (φ 7.0 × 8.5 cm) previously equilibrated with Buffer-A. After the column was washed with Buffer-A (600 ml), the protein was eluted with a 0 to 0.3 M increasing linear gradient of KCl in Buffer-A (800 ml). The fractions containing the enzyme activity (eluted at about 0.2 M KCl) were pooled.

Step 2. AF-Red-Toyopearl column chromatography. The above enzyme solution (236 ml) without concentration or a desalting procedure was applied to a AF-Red-Toyopearl 650 ML (TOSOH, Japan) column (φ 3.2 × 3.0 cm) equilibrated with Buffer-A. After washing with Buffer-A, the protein was eluted with a 0 to 3.0 M increasing linear gradient of KCl in Buffer-A (200 ml). All fractions containing activity (eluted about 1.5 M) were collected (90 ml), concentrated to 21 ml using a Stirred Cells Model 8400 (Amicon Grace, Japan) with an ultrafiltration membrane YM 10 (Diaflo, cutoff MW 10,000), and dialyzed overnight against Buffer-A.

Step 3. POROS HS column chromatography. The dialyzate solution was applied to a POROS HS (Applied Biosystems, USA) column (φ 2.0 × 1.6 cm) equilibrated with Buffer-A. After the column was washed with Buffer-A, the protein was eluted with a 0.1 to 0.3 M increasing linear gradient of KCl in Buffer-A (150 ml). All fractions containing activity (eluted between 0.2 and 0.25 M) were collected (16 ml), concentrated to 9.8 ml by a Stirred Cells Model 8050 (Amicon Grace, Japan) equipped with an ultrafiltration membrane YM 10, and dialyzed overnight against Buffer-A.

Step 4. Mono Q HR10/10 column chromatography. The enzyme solution was applied to a Mono Q HR10/10 (Amersham Pharmacia Biotech, Sweden) column equilibrated with Buffer-A (flow rate was 1.5 ml/min). The protein was eluted with a 0.2 to 0.4 M increasing linear gradient of KCl in Buffer-A. The fractions containing the reducing activity (3.0 ml) were collected and concentrated to 0.5 ml by a Centricon-30 (cutoff MW 30,000, Amicon Grace, Japan).

Step 5. Asahipack GS 520 column chromatography. The concentrated enzyme solution was applied to an Asahipack GS 520 (Showa Denko, Japan) gel filtration column (ϕ 7.6×300 mm) equilibrated with Buffer-C (flow rate was 0.7 ml/min). The active fraction was pooled and used as the purified enzyme for characterization.

Purification of STKER-III.

Step 1. DEAE-Toyopearl chromatography. The cell-free extract (252 ml) was applied to a DEAE-Toyopearl 650 M column (ϕ 7.0×6.0 cm) previously equilibrated with Buffer-A. After the column was washed with Buffer-A (600 ml), the protein was eluted with a 0 to 0.2 M increasing linear gradient of KCl in Buffer-A (600 ml). All fractions containing the reducing activity (eluted at about 0.1 M KCl) were collected (168 ml) and concentrated to 53 ml by a Stirred Cells Model 8400 equipped with an ultrafiltration membrane YM 10.

Step 2. AF-Red-Toyopearl column chromatography. The concentrated solution was applied to a AF-Red-Toyopearl 650 ML column (ϕ 3.2×3.0 cm) equilibrated with Buffer-A, and another portion of Buffer-A was then allowed to flow into the column. The void fraction (not adsorbed) was collected and pooled (68 ml).

Step 3. Butyl-Toyopearl column chromatography. To the above enzyme solution was added 11.9 g of ammonium sulfate powder (adjusted pH 7.2 with NH_3 aq.), and then the mixture was stirred for 2 h at 0°C. The mixture was put on a Butyl-Toyopearl 650 M (ϕ 4.0×6.0 cm) column equilibrated with Buffer-B. The column was washed with Buffer-B, and the protein was eluted with a 1.3 to 0 M (240 ml) decreasing linear gradient of $(NH_4)_2SO_4$. The active fractions (eluted at about 0.3 M) were collected (43 ml), concentrated to 10 ml by a Stirred Cells Model 8050 equipped with an ultrafiltration membrane YM 10, and dialyzed overnight against Buffer-A.

Step 4. Mono Q HR10/10 column chromatography. The dialyzate solution was applied to a Mono Q HR10/10 column equilibrated with Buffer-A (flow rate was 2.0 ml/min). The protein was eluted with a 0 to 0.2 M increasing linear gradient of KCl in Buffer-A. The active fraction (eluted at about 0.13 M) was collected (5.2 ml) and concentrated to 0.6 ml by a Centricon-30.

Step 5. Superdex 200 HR10/30 column chromatography. The enzyme solution was applied to a Superdex 200 HR 10/30 (Amersham Pharmacia Biotech, Sweden) gel filtration column (ϕ 1.0×30 cm) equilibrated with Buffer-C (flow rate was 0.7 ml/min). The active fraction was pooled and used as the purified enzyme for characterization.

Determination of molecular mass of enzyme. The molecular masses of the native STKER-II and -III were estimated by high performance liquid column chromatography (HPLC) analysis with Asahipack GS 520 (ϕ 7.6×300 mm) and Superdex 200 HR 10/30 columns, respectively, with a standard molecular marker (Oriental Yeast, Japan) with Buffer-C. The molecular mass of the subunit was estimated by SDS-poly acrylamide gel erectrophoresis (12.5%) with SDS-PAGE marker (Bio-Rad, USA) as the standard.[29]

Measurement of kinetic parameters. The K_m and V_{max} of the purified enzymes toward various α-keto esters and NADPH were calculated from the initial rates of the reaction in an appropriate range of substrate concentrations using at least five points by Lineweaver-Burk plots.[30]

Measurement of the effects of pH. The optimum pH of the enzyme (relative activity) was measured at various pHs in the following buffers: pH 5.5 to 7.5, 0.1 M KPB; pH 7.5 to 9.0, 0.1 M Tris-HCl buffer; pH 5.5 to 7.0, 0.1 M Citrate-K_2HPO_4 and 0.1 M MES buffer; pH 6.0 to 7.5, 0.1 M Bis-tris propane buffer. The relative activity was calculated by arbitrarily setting the activity at pH 6.0 to be 100. The pH stability of the enzyme (residual activity) was measured at 37°C in 0.1 M KPB (pH 6.5) after incubation for 10 min in the following buffers: pH 5.0 to 7.5, 0.1 M KPB; pH 8.0 to 8.5, 0.1 M Tris-HCl buffer; pH 9.0 to 11.0, 0.1 M glycine-KOH buffer. The residual activity was calculated by setting the activity without incubation to be 100.

Effects of additives. The enzyme (residual) activity was measured in 0.1 M KPB (pH 6.5) at 37°C after a 5-min incubation in the buffer in the presence of each additive (metal ion, chelator, sulfhydryl reagent, or arginine-specific reagent). The residual activity was calculated by arbitrarily setting the activity in the absence of any additives to be 100.

Enzymatic reduction of α-keto esters. In a polypropylene tube were placed the purified enzyme solution (1.0 unit), NADPH (10 μmol), the substrate (10 μmol), and 0.1 M KPB (pH 7.0, 0.5 ml). The mixture was shaken gently at 37°C. After 2 h, the mixture was filtered by an Extrelut short column, extracted with ether, and then concentrated under reduced pressure.

Stereochemistry of the products. The enantiomeric excesses of ethyl lactate, ethyl 2-hydroxy butanoate, ethyl 2-hydroxy pentanoate, ethyl 2-hydroxy hexano-

Table 1. Purification of STKER-II from *S. thermocyaneoviolaceus* IFO 14271

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification fold (%)
Cell-free extract	9970	1920	0.192	100	1
DEAE-Toyopearl	1520	682	0.448	35.6	2.33
AF-Red-Toyopearl	69.4	577	8.31	30.1	43.2
POROS HS	10.3	531	51.6	27.7	268
Mono Q	0.465	326	701	17.0	3640
Asahipack GS520	0.137	158	1150	8.24	5980

Table 2. Purification of STKER-III form *S. thermocyaneoviolaceus* IFO 14271

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification fold (%)
Cell-free extract	14500	2660	0.184	100	1
DEAE-Toyopearl	897	933	1.04	35.1	5.66
AF-Red-Toyopearl	480	203	0.422	7.62	2.30
Butyl-Toyopearl	124	141	1.13	5.29	6.15
Mono Q	10.7	64.6	6.04	2.43	32.9
Superdex 200	0.178	37.0	208	1.39	1130

ate, ethyl 2-hydroxy heptanoate, ethyl mandelate, and methyl mandelate were determined by GLC (GL Science GC-353 gas chromatograph) analyses (Chirasil-DEX CB, Chrompack, Netherlands, 0.25 mm × 25 m, 85 to 130°C), and that of ethyl 2-hydroxy-3-methylbutanoate was also measured by GLC analysis (Chiraldex G-TA, Astec, USA, 0.25 mm × 25 m, 95°C). The absolute configuration of the isomer was identified by comparing its retention time with those of authentic samples prepared according to the method in the literature.[27]

N-Terminal amino acid sequence analysis. The N-terminal amino acid sequence was analyzed on a model 476A pulsed liquid protein sequencer (Applied Biosystems, USA) with an on-line phenylthiohydantoin amino acid analyzer.[31] The N-terminal sequences obtained were compared with those of proteins stored in protein sequence databases (GenBank, EMBL, PIR, and SWISS-PROT) using the sequence similarity search programs, BLAST and FASTA.[32,33]

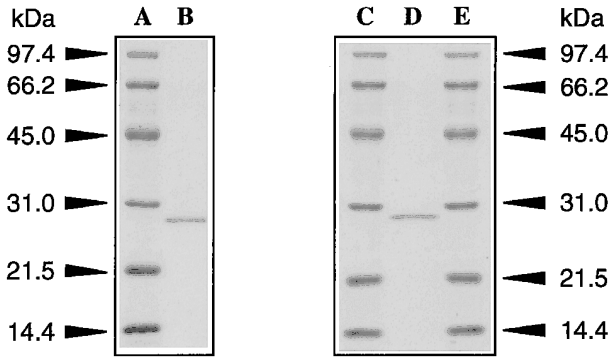


Fig. 1. SDS-PAGE of the Purified Enzymes.

SDS-gel electrophoresis using 12.5% polyacrylamide was done in the presence of 0.1% SDS. (**A**, **C**, **E**) Standards (from top): phosphorylase b (M_r = 97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), carbonic anhydolase (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400). (**B**) STKER-II. (**D**) STKER-III. Proteins were stained with Coomassie Brilliant Blue R-250 and destained in 30% (v/v) methanol/10% (v/v) acetic acid/60% (v/v) water.

Results and Discussion

Purification of STKER-II and STKER-III

Two α-keto ester reductases, STKER-II and -III, were purified via five chromatographic steps from a cell-free extract of *S. thermocyaneoviolaceus* IFO 14271 to homogeneity with a 8.2% and 1.4% overall recovery, respectively. The purification of STKER-II and -III is summarized in Tables 1 and 2. Each of the purified enzymes gave a single band on SDS-PAGE (Fig. 1). The specific activity of STKER-II was 1150 units/mg with 4.0 mM ethyl 2-oxoheptanoate, and that of STKER-III was 208 units/mg with 2.0 mM ethyl 2-oxoheptanoate.

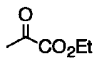
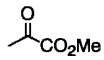
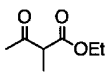
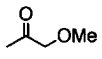
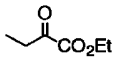
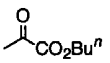
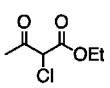
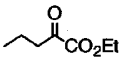
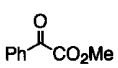
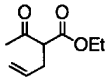
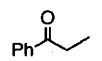
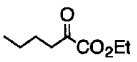
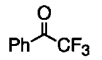
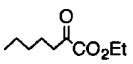
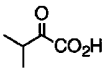
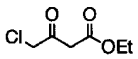
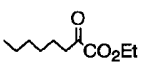
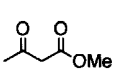
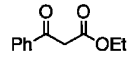
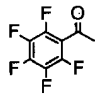
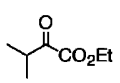
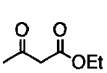
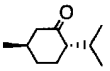
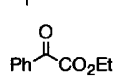
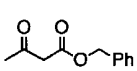
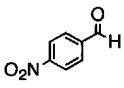
Molecular mass and subunit structure

The relative molecular masses of the native STKER-II and -III were estimated to be 60 kDa and 70 kDa, respectively, by gel filtration column chromatography. The molecular masses of the subunit were also estimated to be 29 kDa and 30 kDa, respectively, by SDS-PAGE (12.5%). Two α-keto ester reductases purified in this study had a similar size (molecular mass of the native and the subunit); furthermore, the size of the two enzymes was similar to that of STKER-I from the same actinomycete.[26]

Substrate specificity

The relative activities of the purified enzymes

Table 3. Substrate Specificity of STKER-II and STKER-III form *S. thermocyaneoviolaceus* IFO 14271[a]

Substrate	Rel. rate (%)[b]		Substrate	Rel. rate (%)[b]		Substrate	Rel. rate (%)[b]		Substrate	Rel. rate (%)[b]	
	STKER-II	-III		STKER-II	-III		STKER-II	-III		STKER-II	-III
	5	36		4	39		0	3		0	0
	20	60		25	71		—[c]	19		0	0
	42	100		7	73		0	7		0	0
	81	70		0	0					—[c]	29
	100	69		0	0		17	7			
	53	59		0	3		0	2		0	0
	59	34		0	4		8	—[c]		0	0
	7	43		0	0		0	0		0	0

[a] Concentration was 5 mM for α-keto esters and 20 mM for other substrates.
[b] Rel. rate = Relative rates were calculated by setting the activity to be 100 (STKER-II: ethyl 2-oxoheptanoate, STKER-III: ehyl 2-oxopentanoate).
[c] Not measured.

toward various keto esters and other carbonyl compounds were examined spectrophotometrically. These results are summarized in Table 3. STKER-II had greater activity toward aliphatic α-keto esters having a long alkyl chain such as 2-oxohexanoate and 2-oxoheptanoate, while the activity for smaller substrates such as ethyl pyruvate and methyl pyruvate was low. STKER-III showed high activity not only for aliphatic substrates but also for aromatic substrates. STKER-II had the reducing acitivity for ethyl 4-chloroacetoacetate in β-keto esters we tested. STKER-III also had activities toward some β-keto esters (ethyl 2-chloroacetoacetate, ethyl 2-allylacetoacetate, ethyl 4-chloroacetoacetate, methyl acetoacetate, ethyl acetoacetate, ethyl 2-methyl acetoacetate, and ethyl benzoylacetate) slightly. The reducing activities by STKER-II and -III for other substrates, such as benzyl acetoacetate, 1-acetoxy-2-propanone, 1-methoxy-2-propanone, acetophenone, propiophenone, 2′,3′,4′,5′,6′-pentafluoroacetophenone, and (-)-menthone were not observed. Both enzymes showed reducing activity toward neither α-keto acids such as pyruvic acid, 3-methyl-2-oxobutanoic acid, and mandelic acid nor *p*-nitrobenzaldehyde, a typical substrate for aldo-keto reductases.

These enzymes were highly specific for NADPH as a sole coenzyme. The oxidation activity for ethyl lactate and ethyl 2-hydroxy-3-methyl butanoate was not found for either enzyme.

Stereospecificity

The reduction of α-keto esters by the purified enzymes was carried out to clarify the stereochemistry of the products, as shown in Table 4. The enantioselectivity of the reduction by STKER-II was not as precise (from 46% e.e. (*S*) to 40% e.e. (*R*)) except for ethyl 3-methyl-2-oxobutanoate (>99% e.e. (*R*)) and ethyl 2-oxobutanoate (86% e.e. (*R*)). STKER-III reduced α-keto esters having a long alkyl chain, an isopropyl or a phenyl group to the corresponding (*R*)-hydroxy esters with excellent e.e. (such as ethyl 2-oxoheptanoate, ethyl 3-methyl-2-oxobutanoate, ethyl benzoylformate, and methyl benzoylformate), while the selectivity for ethyl pyruvate and 2-oxobutanoate was low (14% and 38% e.e., respectively) and gave the (*S*)-hydroxy esters.

Kinetic parameters

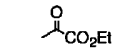
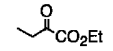
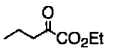
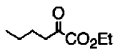
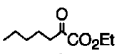
The kinetic constants (K_m and V_{max}) for aliphatic and aromatic α-keto esters were calculated by Lineweaver-Burk plots as shown in Table 5. The K_m of STKER-II toward ethyl pyruvate, ethyl 2-oxobutanoate, ethyl 2-oxopentanoate, ethyl 2-oxohexanoate, and ethyl 2-oxoheptanoate were 32.1, 21.6, 4.48, 2.45, and 0.831 mM, respectively. As the alkyl chain in the substrate molecule became longer, the corresponding K_m of STKER-II was decreased. On the contrary, the V_{max} tended to become larger with extension of the substituent. Thus, STKER-II prefers substrates having a long alkyl chain.

STKER-III showed strong reducing ability for aromatic α-keto esters. The V_{max}/K_m for ethyl benzoylformate was larger than those of STKER-I (1.41 μmol/min/mg/mM) and -II in the actinomycete cells. These results suggest that STKER-III contributes mainly to reduction of ethyl benzoylformate to give the corresponding (*R*)-α-hydroxy ester in actinomycete cells.

Optimum pH and pH stability

The effects of pH on the enzyme activity were examined as shown in Fig. 2. Both of the two purified enzymes showed maximum activity about pH 6.0–6.5

Table 4. Stereoselectivity of the Enzymatic Reduction[a,b]

Substrate	STKER-II		STKER-III	
	e.e. (%)	(*R*/*S*)	e.e. (%)	(*R*/*S*)
	40	*R*	14	*S*
	86	*R*	38	*S*
	37	*R*	81	*S*
	34	*S*	71	*R*
	46	*S*	>99	*R*
	>99	*R*	>99	*R*
	31	*R*	>99	*R*
	15	*S*	>99	*R*

[a] The purified enzyme solution (1.0 unit), NADPH (10 μmol), substrate (10 μmol), and 0.1 M potassium phosphate buffer (pH 7.0, 0.5 ml) were incubated for 2 h at 37°C.

[b] Enanitiomeric excesses and configuration were measured by GLC analysis with optically active capillary columns.

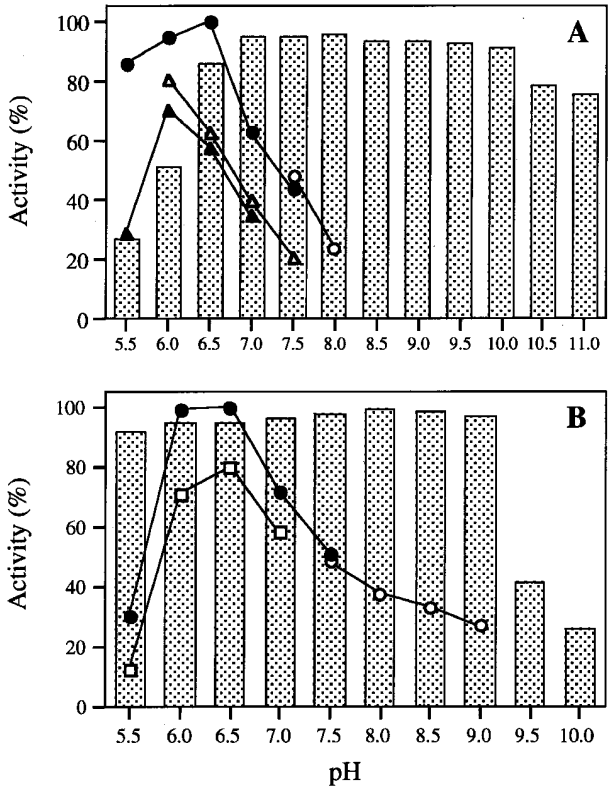
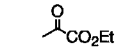
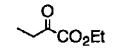
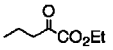
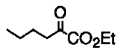
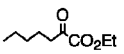


Fig. 2. The Optimum pH and pH Stability of the Enzyme.

The pH dependence (relative activity) was measured in 0.1 M buffer (pH 5.5 to 11.0) at 37°C (line graph). (●) Potassium phosphate buffer, (○) Tris-HCl buffer, (▲) Citrate-K_2HPO_4 buffer, (△) Bis-tris propane buffer, and (□) MES buffer were used. The relative activity was calculated by arbitrarily setting the activity at pH 6.5 to be 100. The pH stability (residual activity) was measured in 0.1 M potassium phosphate buffer after incubation for 10 min in 0.1 M buffer (pH 5.5 to 11.0) at 37°C (bar graph). The residual activity was calculated by setting the activity without incubation to be 100. **(A)** STKER-II. **(B)** STKER-III.

Table 5. Kinetic Parameters of STKER-II and STKER-III from *S. thermocyaneoviolaceus* IFO 14271

Substrate	STKER-II			STKER-III		
	K_m (mM)	V_{max} (μmol/min/mg)	V_{max}/K_m	K_m (mM)	V_{max} (μmol/min/mg)	V_{max}/K_m
	32.1	11.2	0.350	4.05	156	38.5
	21.6	1210	55.8	1.47	230	156
	4.48	1020	228	0.290	218	751
	2.45	1550	635	0.948	258	272
	0.831	1720	2070	0.402	250	622
	2.99	1170	383	8.12	312	38.4
	4.95	415	83.8	0.621	212	341
	—[a]	—[a]	—[a]	0.326	227	697

[a] Not measured.

Table 6. Effects of Additives on STKER-II and STKER-III from *S. thermocyaneoviolaceus* IFO 14271[a]

Additive	(Conc.)	Residual activity (%)		Additive	(Conc.)	Residual activity (%)	
		STKER-II	STKER-III			STKER-II	STKER-III
None		100[b]	100[b]	*Chelators*			
Metal ions				*o*-Phenanthroline	(1 mM)	95	101
NaCl	(5 mM)	96	110	2,2′-Bipyridyl	(1 mM)	96	100
$MgCl_2$	(5 mM)	88	107	EDTA	(1 mM)	100	103
KCl	(5 mM)	99	98	*Sulfhydryl reagents*			
$CaCl_2$	(5 mM)	94	104	*p*-CMB[c]	(1 mM)	71	62
$MnCl_2$	(5 mM)	103	0	Iodoacetic acid	(1 mM)	75	73
$CoCl_2$	(5 mM)	89	24	*Arginine-specific reagents*[d]			
$NiCl_2$	(5 mM)	82	16	Phenylglyoxal	(1 mM)	75	40
$ZnCl_2$	(5 mM)	55	0	1,2-CHDO[e]	(15 mM)	78	45
$HgCl_2$	(5 mM)	8	0				

[a] The enzyme was incubated with additive for 5 min at 37°C before the reaction was started.
[b] The activity of each enzyme without inhibitor was calculated by arbitrarily setting to be 100.
[c] *p*-CMB = *p*-Chloromercurybenzoate
[d] Measured after incubation for 15 min at 37°C.
[e] 1,2-CHDO = 1,2-Cyclohexanedione.

in potassium phosphate buffer. STKER-II and -III were stable in the pH 7.0 to 10.0 region and pH 5.5 to 9.0 region, respectively. While STKER-II was unstable below pH 6.0 and stable under alkaline conditions, STKER-III was stable in acidic regions and unstable above pH 9.5.

Thermostability

The thermostability of STKER-II and STKER-III was investigated by measuring the residual activity after incubation at 37°C and 45°C as shown in Fig. 3. Both enzymes retained 50–60% of their activity after 4 days of incubation at 37°C. The stability of STKER-II, STKER-III, and STKER-I were similar under incubation at 37°C. After incubation at high temperature (STKER-II and -III: 45°C, STKER-I: 50°C), STKER-II and STKER-III were deactivated for several hours. However, STKER-I did not lose activity at all for 4 hours at 50°C.[26]

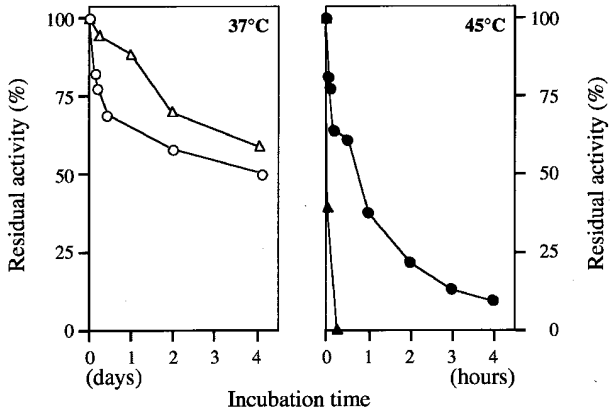


Fig. 3. Thermostability of the Purified Enzymes.
The stability of the enzyme activity (residual activity) was measured in 0.1 M potassium phosphate buffer (pH 6.5) at 37°C after incubation at 37°C or 45°C in Buffer-A. After the incubation, the enzyme activity was immediately assayed. The residual activities were the values expressed as percent relative to the activity without incubation. (○) STKER-II incubated at 37°C, (△) STKER-III incubated at 37°C, (●) STKER-II incubated at 45°C, (▲) STKER-III incubated at 45°C.

Inhibition study

The effects of a broad range of metal ions, chelators, sulfhydryl-protecting and -inhibiting reagents, and arginine-specific reagents on the activity of the two purified enzymes were investigated (see Table 6). Among the sulfhydryl inhibitors tested, *p*-chloromercurybenzoate and iodoacetic acid inhibited the reducing activity of both enzymes. STKER-II was inhibited by mercury and zinc ion. STKER-III was also inhibited by heavy metal ions, such as manganese, cobalt, nickel, zinc, and mercury. These inhibition patterns indicate an essential sulfhydryl group. Metal ion chelating reagents such as *o*-phenanthroline, 2,2′-bipyridyl, and EDTA showed no inhibitory effects on the acitivities. This suggests that both enzymes do not contain an essential metal ion. Furthermore, arginine-residue-specific reagents such as phenylglyoxal and 1,2-cyclohexanedione (1,2-CHDO) inhibited the activities of both enzymes. This result indicates an essential arginine residue for the catalytic activity.

N-Terminal amino acid sequences

The N-terminal amino acid sequences of three α-keto ester reductases, STKER-I (was purified again as described previously[26]), -II, and -III from *S. thermocyaneoviolaceus* were analyzed by an automated Edman degradation using a pulsed liquid phase protein sequencer. The sequences of the N-terminal amino acid of STKER-I, -II, and -III were ^{1}Ala-Thr-His-Val-Ile-Thr-Gly-Ala-Gly-^{10}Ser-Gly-Ile-Gly-Ala-Ala-, ^{1}Thr-Ser-Val-Glu-Leu-Pro-Glu-Leu-Ser-^{10}Gly-Lys-Val-Ala-Leu-Val-, and ^{1}Met-Lys-Arg-

Leu-Val-Thr-Val-Val-Thr-[10]Gly-Gly-Ser-Arg-Gly-Ile-, respectively as shown in Fig. 4 (the N-terminals of the three enzymes were not blocked). All the enzymes we purified had a consensus sequence (-Gly-X-X-X-Gly-X-Gly-), the putative coenzyme (NAD(P)H) binding site, in the N-terminal regions (underlined). The similarity of each sequence of the three enzymes with those of other proteins was examined by a computer search of the protein sequence databases. Among STKER-I and ProSc, STKER-II and PscoSc, STKER-III and PuoSc, a high homology (92, 92, and 73%, respectively) was observed. These homologous proteins were putative or probable oxidoreductases in the cells of *Streptomyces coelicolor* A3(2). These results suggest that STKER-I, -II, and -III belong to an oxidoreductase family.

Comparison with other microbial reductases

Several α-keto ester-reducing enzymes have been isolated from microorganisms such as *Saccharomyces cerevisiae* (bakers' yeast) and *Candida magnoliae*.[19] The enzymatic properties of these enzymes are compared with those of three enzymes in the cells of *S. thermocyaneoviolaceus* IFO 14271 as shown in Table 7. Although other enzymes including STKER-II and -III used NADPH as a sole coenzyme, STKER-I utilized both NADPH and NADH as the coenzyme. STKER-I had high affinity for ethyl pyruvate among the listed reductases. Furthermore, STKER-I reduced ethyl 3-methyl-2-oxobutanoate to the corresponding (*S*)-hydroxy ester in excellent e.e. (>99% e.e.). Therefore, STKER-I would be very useful for catalysis of the preparation of ethyl (*S*)-3-methyl-2-hydroxybutanoate.

STKER-II and -III reduced ethyl 3-methyl-2-oxobutanoate to the corresponding (*R*)-hydroxy ester with excellent e.e. as did YKER-IV from bakers' yeast.

Finally, we examined the mechanistic interpretation about the stereoselectivity change in the reduction of ethyl 3-methyl-2-oxobutanoate by whole cells of *S. thermocyaneoviolaceus* IFO 14271. The reduction of ethyl 3-methyl-2-oxobutanoate by the whole cells at 37°C gave the corresponding (*R*)-hydroxy esters.[23] This result suggests that the substrate was reduced mainly by STKER-II (the lowest K_m and the highest V_{max} of the three reductases) in the actinomycete cells. However, the stereoselectivity of the actinomycete reduction was low at high temperature.[23] It is presumed that this stereoselectivity change may be attributed to differences in the thermostability of the reductases which contribute to the reduction in the cells, that is, because the thermostability of STKER-I is higher than that of STKER-II and -III, STKER-I contributes to the reduction at high temperature; consequently, the stereoselectivity sifted toward (*S*)-hydroxy ester production at 55°C. The stereoselectivity change in the reduction of ethyl pyruvate, ethyl benzoylformate, and methyl benzoylformate would be explicable in the same manner.

The isolation of α-keto ester reductase from *S. coe-*

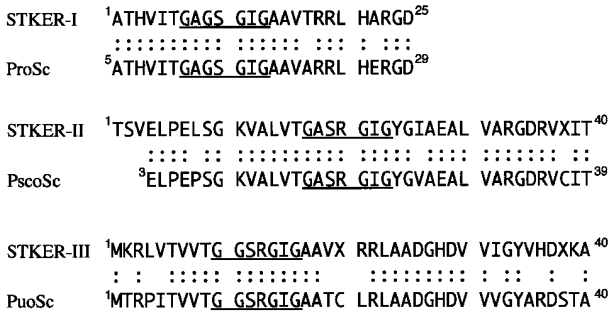


Fig. 4. Sequence Comparison of N-Terminal Amino Acid Sequences of α-Keto Ester Reductases from *S. thermocyaneoviolaceus* and Other Oxidoreductases.

ProSc: Probable oxidoreductase from *S. coelicolor* A3(2), accession number: T35808. PscoSc: Putative short chain oxidoreductase from *S. coelicolor* A3(2), accession number: AL359949, gene: 2SC2G61.27c. PuoSc: Putative oxidoreductase from *S. coelicolor* A3(2), accession number: AL359949, gene: 2SC2G61.17c. The underlined sequences are putative coenzyme biding sites.

Table 7. Comparison of the Characteristics of the Reductases from Various Microorganisms

Enzyme (Source)	Molecular mass						Coenzyme
	CFC[a] (kDa)	SDS-PAGE (kDa)	K_m (mM)	e.e. (*R*/*S*)	K_m (mM)	e.e. (*R*/*S*)	
STKER-I[26]	64	30 (dimer)	0.079	>99 (*S*)	9.01	>99 (*S*)	NADH/NADPH
STKER-II[b]	60	29 (dimer)	32.1	40 (*R*)	2.99	>99 (*R*)	NADPH
STKER-III[b]	70	30 (dimer)	4.05	14 (*S*)	8.12	>99 (*R*)	NADPH
YKER-II (Bakers' yeast)[14]	58	29 (dimer)	135	98 (*S*)	N.R.[c]	N.R.[c]	NADPH
YKER-IV (Bakers' yeast)[14]	31	39 (monomer)	0.434	>99 (*R*)	0.265	>99 (*R*)	NADPH
YKER-V (Bakers' yeast)[14]	83	41 (dimer)	5.06	94 (*S*)	79.4	77 (*S*)	NADPH
Carbonyl reductase (*Candida magnoliae*)[19]	76	32 (dimer)	21	—[d]	240	—[d]	NADPH

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

licolor A3(2) and the comparison of enzymatic properties including the N-terminal amino acid sequence of the reductase with those of STKER are now in progress in our laboratory.

Acknowledgments

The work was partially supported by the Ministry of Education, Science, Sport and Culture of Japan, through a Grant-in-aid for Scientific Research, Encouragement of Young Scientists, No. 13780452 (2001–2002). We wish to express our appreciation to Prof. N. Tokitoh, Institute for Chemical Research, Kyoto University, for his useful advice during the course of the work.

References

1) Nakamura, K., Kawai, Y., Kitayama, T., Miyai, T., Ogawa, M., Mikata, Y., Higaki, M., and Ohno, A., Asymmetric reduction of ketones with microbes. *Bull. Inst. Chem. Res., Kyoto Univ.*, **67**, 157–168 (1989).
2) Suga, T. and Hirata, T., Biotransformation of exogenous substrates by plant cell cultures. *Phytochemistry*, **29**, 2394–2406 (1990).
3) Cusk, R. and Glänzer, B. I., Baker's yeast mediated transformations in organic chemistry. *Chem. Rev.*, **91**, 49–97 (1991).
4) Wong, C. -H., and Whitesides, G. M., Enzymes in synthetic organic chemistry, Tetrahedron Organic Series Vol. 12, 1st ed., Pergamon, Elsevier, Amsterdam, pp. 41–194 (1994).
5) Roberts, S. M., Preparative biotransformations: the employment of enzymes and whole cells in synthetic organic chemistry. *J. Chem. Soc., Perkin I*, 157–169 (1998).
6) Sugai, T., Application of enzyme- and microorganism-catalyzed reaction to organic synthesis. *Curr. Org. Chem.*, **3**, 373–406 (1999).
7) Brooks, D. W. and Kellogg, R. P., Synthetic study of polyene macrolides, synthesis of a C29–37 fragment for amphotericin B and nystatin. *Tetrahedron Lett.*, **23**, 4991–4994 (1982).
8) Kikukawa, T., Imaida, M., and Tai, A., Synthesis of the sex-attractant of Pine Sawflies. *Bull. Chem. Soc. Jpn.*, **57**, 1954–1960 (1984).
9) Mori, K., Biochemical methods in enantioselective synthesis of bioactive natural products. *Synlett.*, 1097–1109 (1995).
10) Furuichi, A., Akita, H., Matsuzaka, H., Oishi, T., and Horikoshi, K., Purification and properties of an asymmetric reduction enzyme of 2-methyl-3-oxobutyrate in baker's yeast. *Agric. Biol. Chem.*, **49**, 2563–2570 (1985).
11) Shieh, W. -R., Gopalan, A. S., and Sih, C. J., Stereochemical control of yeast reductions. 5. Characterization of the oxidoreductases involved in the reduction of β-keto esters. *J. Am. Chem. Soc.*, **107**, 2993–2994 (1985).
12) Nakamura, K., Kawai, Y., Nakajima, N., and Ohno, A., Stereochemical control of microbial reduction. 17. Mechanism to control the enantioselectivity in the reduction with bakers' yeast. *J. Org. Chem.*, **56**, 4778–4783 (1991).
13) Nakamura, K., Kawai, Y., Miyai, T., Honda, S., Nakajima, N., and Ohno, A., Stereochemical control in microbial reduction. 18. Mechanism of stereochemical control in diastereoselective reduction with bakers' yeast. *Bull. Chem. Soc. Jpn.*, **64**, 1467–1470 (1991).
14) Nakamura, K., Kondo, S., Kawai, Y., Nakajima, N., and Ohno, A., Purification and characterization of α-keto ester reductases from bakers' yeast. *Biosci. Biotechnol. Biochem.*, **58**, 2236–2240 (1994).
15) Sonnleitner, B. and Fiechter, A., Application of immobilized cells of *Thermoanaerobium brockii* for stereoselective reductions of oxo-acid esters. *Appl. Microbiol. Biotechnol.*, **23**, 424–429 (1986).
16) Biosson, D., Azerad, R., Sanner, C., and Larcheveque, M., A study of the stereocontrolled reduction of aliphatic β-ketoesters by *Geotrichum candidum*. *Biocatalysis*, **5**, 249–265 (1992).
17) Nakamura, K., Highly stereoselective reduction of ketones by *Geotrichum candidum*. *J. Mol. Catal., B: Enzym.*, **5**, 129–132 (1998).
18) Miya, H., Kawada, M., and Sugiyama, Y., Stereoselective reduction of ethyl 2-methyl-3-oxobutanoate by bacteria. *Biosci. Biotechnol. Biochem.*, **60**, 95–98 (1996).
19) Wada, M., Kataoka, M., Kawabata, H., Yasohara, Y., Kizaki, N., Hasegawa, J., and Shimizu, S., Purification and characterization of NADPH-dependent carbonyl reductase, involved in stereoselective reduction of ethyl 4-chloro-3-oxobutanoate, from *Candida magnoliae*. *Biosci. Biotechnol. Biochem.*, **62**, 280–285 (1998).
20) Kita, K., Nakase, K., Yanase, H., Kataoka, M., and Shimizu, S., Purification and characterization of new aldehyde reductases from *Sporobolomyces salmonicolor* AKU4429. *J. Mol. Catal., B: Enzym.*, **6**, 305–313 (1999).
21) Ishihara, K., Iwai, K., Yamaguchi, H., Nakajima, N., Nakamura, K., and Ohshima, T., Stereoselective reduction of α- and β-keto esters with aerobic thermophiles, *Bacillus* strains. *Biosci. Biotechnol. Biochem.*, **60**, 1896–1898 (1996).
22) Ishihara, K., Nishitani, M., Yamaguchi, H., Nakajima, N., Ohshima, T., and Nakamura, N., Stereoselective reduction of α-keto esters using thermophilic actinomycetes. *J. Ferment. Bioeng.*, **84**, 268–270 (1997).
23) Ishihara, K., Yamaguchi, H., Hamada, H., Nakajima, N., and Nakamura, K., Stereocontrolled reduction of α-keto esters with thermophilic actinomycete, *Streptomyces thermocyaneoviolaceus* IFO 14271. *J. Mol. Catal., B: Enzym.*, **10**, 429–434 (2000).
24) Ishihara, K., Yamaguchi, H., Adachi, N., Hamada, H., and Nakajima, N., Stereocontrolled reduction of α- and β-keto esters with micro green algae, *Chlorella* strains. *Biosci. Biotechnol. Biochem.*, **64**, 2099–2103 (2000).
25) Ishihara, K., Nakajima, N., Yamaguchi, H.,

Hamada, H., and Uchimura, Y., Stereoselective reduction of keto esters with marine micro algae. *J. Mol. Catal., B: Enzym.*, **15**, 101–104 (2001).
26) Ishihara, K., Yamaguchi, H., Hamada, H., Nakamura, K., and Nakajima, N., Asymmetric reduction of α-keto esters with thermophilic actinomycete: purification and characterization of α-keto ester reductase from *Streptomyces thermocyaneoviolaceus* IFO 14271. *J. Mol. Catal., B: Enzym.*, **10**, 419–428 (2000).
27) Nakamura, K., Inoue, K., Ushio, K., Oka, S., and Ohno, A., Stereochemical control on yeast reduction of α-keto esters. Reduction by immobilized bakers' yeast in hexane. *J. Org. Chem.*, **53**, 2589–2593 (1988).
28) Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).
29) Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).
30) Lineweaver, H. and Burk, D. J., The determination of enzymes dissociation constants. *J. Am. Chem. Soc.*, **56**, 658–666 (1934).
31) Hewick, R. H., Hunkapilla, M. W., Hood, L. E., and Dreyer, W. J., A gas-liquid solid phase peptide and protein sequenator. *J. Biol. Chem.*, **356**, 7990–7997 (1981).
32) Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J., Basic local alignment search tool. *J. Mol. Biol.*, **215**, 403–410 (1990).
33) Person, W. R. and Lipman, D. J., Improvement tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA*, **85**, 2444–2448 (1988).