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Stereoselective Reduction of Carbonyl Compounds with Actinomycete: Purification and Characterization of Three α-Keto Ester Reductases from *Streptomyces avermitilis*

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We achieved the purification of three α-keto ester reductases (*Streptomyces avermitilis* keto ester reductase, SAKERs-I, -II, and -III) from *Streptomyces avermitilis* NBRC14893 whole cells. The molecular masses of the native SAKERs-I, -II, and -III were estimated to be 72, 38, and 36 kDa, respectively, by gel filtration chromatography. The subunit molecular masses of SAKERs-I, -II, and -III were also estimated to be 32, 32, and 34 kDa, respectively, by SDS-polyacrylamide gel electrophoresis. The purified SAKERs-II and -III showed a reducing activity for α-keto esters (in particular, for ethyl pyruvate). SAKER-I showed a high reducing activity not only toward the α- and β-keto esters, but also toward α-keto acid. The N-terminal region amino acid sequences of SAKERs-I, -II, and -III were identical to that of a putative oxidoreductase, SAV2750, a putative oxidoreductase, SAV1849, and a putative oxidoreductase, SAV4117, respectively, hypothetical proteins coded on the *S. avermitilis* genome.

Key words: enzyme purification; reductase; keto ester; α-hydroxy ester producing enzyme; *Streptomyces*

Optically active alcohols with proper functional groups are versatile building blocks in organic synthetic chemistry, especially for the preparation of enantiomerically pure pharmaceuticals, inhibitors, and pheromones.[1–6] Stereoselective reduction of carbonyl compounds with microorganisms is widely used to obtain such chiral alcohols. For example, chiral hydroxy acids and their esters are predominantly prepared on a laboratory scale by the reduction of the corresponding carbonyl compounds by microbes such as yeast, fungi, and bacteria.[7–13]

To date, several keto ester reductases have been isolated from microorganisms.[14–19] Recently, we investigated the reducing activity for keto esters in actinomycetes[20–22] and purified four α-keto ester reductases from *Streptomyces thermocyaneoviolaceus* (STKER-I, II, and -III)[23,24] and *S. coelicolor* A3(2) (SCKER).[25] The similarity of each amino acid sequence in the isolated enzymes with those of other proteins was examined by a computer search of the protein sequence databases.[26,27] In the N-terminal region amino acid sequences, between STKER-I and a putative oxidoreductase (SAV2750, one of hypothetical proteins) of *Streptomyces avermitilis*,[28] a high homology (100%) was observed. This result suggests that the putative oxidoreductase possesses a reducing ability toward α-keto esters. In other words, there is a possibility that the actinomycete (*S. avermitilis*) catalyzes the reduction of α-keto esters. Hence we investigated the reducing activity of *S. avermitilis* for α-keto esters and the enzymatic properties of the reductase in the actinomycete cells.

This paper describes the purification and characterization of three α-keto ester reductases from *S. avermitilis*.

Materials and Methods

Instruments. Gas chromatography was performed using GL Sciences Inc. (Tokyo, Japan) GC-353 (DB-WAX, Agilent Technologies, Palo Alto, CA, 0.25 mm × 30 m; CP-Chirasil-DEX CB, Chrompack, Netherlands, 0.25 mm × 25 m; GAMMA DEX™ 225, Supelco, Bellefonte, PA, 0.25 mm × 30 m) gas chromatographs.

Chemicals. Ethyl 3-methyl-2-oxobutanoate was obtained from Aldrich Chemical, St. Louis, MO. Ethyl

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benzoylformate was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. Ethyl pyruvate, ethyl 4-chloroacetoacetate, diatomaceous earth (granular), and *p*-ABSF (4-(2-aminoethyl)benzenesulfonate fluoride) were purchased from Wako Pure Chemicals, Osaka, Japan. Pyruvic acid, DTT (dithiothreitol), NADPH, NADH, $NADP^+$, and NAD^+ were obtained from Nacalai Tesque, Kyoto, Japan. The other α-keto and α-hydroxy esters were synthesized according to the literature methods.[29] All other chemicals used in this study were of analytical grade and are commercially available.

Microorganism and cultivation. Streptomyces avermitilis NBRC 14893 was obtained from the National Institute of Technology and Evaluation, Biological Resource Center, Tokyo, Japan. The actinomycete was aerobically cultivated at 28 °C for 64 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 collected by filtration on filter paper *in vacuo* and washed with saline (0.85% NaCl aq.).

Enzyme assay. The reducing activity of the enzyme was determined spectrophotometrically. The reaction mixture, with a total volume of 1.0 ml, contained 5 mM of the substrate, 0.2 mM of the coenzyme in 0.1 M KPB (potassium phosphate buffer) (pH 6.5), and a limited amount of the enzyme solution. Consumption of the reduced coenzyme was followed using a Beckman DU-640 spectrophotometer (Fullerton, CA) at 340 nm and 37 °C. One unit (U) of the enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol NAD(P)H per min under the specified conditions.

Enzyme purification. All purification procedures were done below 4 °C, unless otherwise specified. Buffer-A was 10 mM KPB containing 1 mM DTT and 10% glycerol (pH 7.0). Buffer-B was 50 mM KPB containing 1 mM DTT, 10% glycerol, and 2.0 M ammonium sulfate (pH 7.0). Buffer-C was 0.1 M KPB containing 1 mM DTT, 10% glycerol, and 0.2 M KCl (pH 7.0).

Purification of SAKER-I.

Preparation of the cell-free extract. Eighty grams of the *S. avermitilis* cells was suspended in 250 ml of 50 mM KPB (pH 7.0) containing 1 mM DTT and 10% glycerol. 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, Tokyo, Japan) fitted with a micro tip at a power setting of 70 W. At the first interval, 0.5 mM of *p*-ABSF was added as a protease inhibitor. The cell debris in the homogenate was removed by centrifugation at 10,000 × *g* for 40 min at 4 °C, and the supernatant then served as the crude cell-free extract.

Fractionation with 30% saturated ammonium sulfate. Solid ammonium sulfate was added to the cell-free extract for making the solution 30% saturated. After stirring for 1 h and standing for 18 h at 2 °C, the precipitate was removed by centrifugation at 12,000 × *g* for 30 min at 4 °C. The supernatant (285 ml) was concentrated (50 ml) by a Stirred Ultrafiltration Cells Model 8200 (Millipore, Billerica, MA) equipped with an ultrafiltration membrane YM 10 (cut off MW 10,000, Millipore) and then dialyzed overnight against Buffer-A.

Step 1. TOYOPEARL® DEAE-650M chromatography. The dialyzed crude solution (87 ml) was applied to a TOYOPEARL® DEAE-650M (Tosoh, Tokyo, Japan) column (ϕ7.0 × 8.0 cm) equilibrated with Buffer-A. The column was washed with 400 ml of Buffer-A, then the proteins were eluted with a 0 to 1.0 M increasing linear gradient of KCl (200 ml). The major active fractions (128 ml) were collected and concentrated to 50 ml, and then dialyzed overnight against Buffer-A.

Step 2. Mono Q® HR 10/10 column chromatography. The dialyzed solution (78 ml) was applied to a Mono Q® HR10/10 column (GE Healthcare Science, Boston, MA) equilibrated with Buffer-A (flow rate was 3.0 ml/min). The protein was eluted with a 0.1 to 0.5 M increasing linear gradient of KCl in Buffer-A. The active fraction (eluted at about 0.15 M) was then collected (53 ml).

Step 3. Phenyl Superose® HR10/10 column chromatography. To the above enzyme solution was added solid $(NH_4)_2SO_4$ (final concentration was 50% saturated), and then the mixture was applied to a Phenyl Superose® HR10/10 column (GE Healthcare Science) equilibrated with Buffer-B. The column was washed with Buffer-B, and the protein was eluted with a 2.0 to 0 M decreasing linear gradient of $(NH_4)_2SO_4$. The active fractions (16 ml) were collected and concentrated to 1.5 ml by a Centricon® YM-10 (Millipore).

Step 4. Superdex™ 200 HR10/30 column chromatography. The enzyme solution was applied to a Superdex™ 200 HR10/30 (GE Healthcare Science) gel filtration column 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 SAKER-II.

Preparation of the cell-free extract. One hundred grams of the *S. avermitilis* cells was suspended in 300 ml of 50 mM KPB (pH 7.0) containing 1 mM DTT, 10% glycerol, and 0.5 mM *p*-ABSF. The suspension was cooled below 0 °C and subsequently sonicated with 10 pulses of 120 s each with 300-s cooling intervals. The cell debris in the homogenate was removed by centrifugation at 10,000 × *g* for 40 min at 4 °C, and the supernatant then served as the crude cell-free extract.

Fractionation with 30% saturated ammonium sulfate. Solid ammonium sulfate was added to the extract for making the solution 30% saturated. After stirring for 1 h and standing for 16 h at 2 °C, the precipitate was removed by centrifugation at 12,000 × *g* for 30 min at 4 °C. The supernatant (323 ml) was concentrated (60 ml)

by a Stirred Ultrafiltration Cells Model 8200 equipped with an ultrafiltration membrane YM 10, and then dialyzed overnight against Buffer-A.

Step 1. TOYOPEARL® DEAE-650M chromatography. The dialyzed crude solution (76 ml) was applied to a TOYOPEARL® DEAE-650M column (ϕ7.0 × 7.0 cm) equilibrated with Buffer-A. The column was washed with 400 ml of Buffer-A, then the proteins were eluted with a 0 to 1.0 M increasing linear gradient of KCl (200 ml). The major active fractions (140 ml) were collected and concentrated to 50 ml, and then dialyzed overnight against Buffer-A.

Step 2. Mono Q® HR 10/10 column chromatography. The dialyzed solution (62 ml) was applied to a Mono Q® HR10/10 column equilibrated with Buffer-A (flow rate was 2.5 ml/min). The protein was eluted with a 0 to 0.5 M increasing linear gradient of KCl in Buffer-A. The active fraction (eluted at about 0.3 M) was then collected. The active fraction (23 ml) was concentrated (16.5 ml) by a Stirred Ultrafiltration Cells Model 8200 equipped with an ultrafiltration membrane YM 10, and then dialyzed overnight against Buffer-A.

Step 3. Phenyl Superose® HR10/10 column chromatography. To the above enzyme solution was added solid $(NH_4)_2SO_4$ (final concentration was 50% saturated), and the mixture was applied to a Phenyl Superose® HR10/10 column equilibrated with Buffer-B. The column was washed with Buffer-B, and the protein was eluted with a 2.0 to 0 M decreasing linear gradient of $(NH_4)_2SO_4$. The active fractions (10 ml) were collected and concentrated to 1.0 ml by a Centricon® YM-10.

Step 4. MonoQ® PC1.6/5 column chromatography (Smart system). The enzyme solution was applied to a MonoQ® PC1.6/5 (GE Healthcare Science) column equilibrated with Buffer-A (flow rate was 0.1 ml/min). The protein was eluted with a 0 to 0.5 M increasing linear gradient of KCl in Buffer-A. The active fraction was collected and concentrated to 0.12 ml by an Ultrafree-MC® (10,000 NMWL, Millipore).

Step 5. Superdex™ 200 PC1.6/30 column chromatography (Smart system). The enzyme solution was applied to a Superdex™ 200 PC1.6/30 (GE Healthcare Science) gel filtration column equilibrated with Buffer-C (flow rate was 50 µl/min). The active fraction was pooled and used as the purified enzyme for characterization.

Purification of SAKER-III.

Preparation of the cell-free extract. Forty grams of the *S. avermitilis* cells was suspended in 150 ml of 50 mM KPB (pH 7.0) containing 1 mM DTT, 10% glycerol, and 0.5 mM *p*-ABSF. This suspension was cooled below 0 °C and subsequently sonicated with 12 pulses of 120 s each with 300-s cooling intervals. The cell debris in the homogenate was removed by centrifugation at 10,000 × *g* for 40 min at 4 °C, and then the supernatant served as the crude cell-free extract. The supernatant (165 ml) was concentrated (50 ml) by a Stirred Ultrafiltration Cells Model 8200 equipped with an ultrafiltration membrane YM 10, and then dialyzed overnight against Buffer-A.

Step 1. TOYOPEARL® DEAE-650M chromatography. The dialyzed crude solution was applied to a TOYOPEARL® DEAE-650M column (ϕ7.0 × 5.5 cm) equilibrated with Buffer-A. The column was washed with 400 ml of Buffer-A, and then the proteins were eluted with a 0 to 1.0 M increasing linear gradient of KCl (200 ml). The major active fractions (98 ml) were collected and concentrated to 30 ml, and then dialyzed overnight against Buffer-A.

Step 2. Phenyl Superose® HR10/10 column chromatography. To the above enzyme solution was added solid $(NH_4)_2SO_4$ (final concentration was 50% saturated), and the mixture was then applied to a Phenyl Superose® HR10/10 column equilibrated with Buffer-B. The column was washed with Buffer-B, and the protein was eluted with a 2.0 to 0 M decreasing linear gradient of $(NH_4)_2SO_4$. The active fractions (16 ml) were collected and dialyzed overnight against Buffer-A, and then concentrated to 1.8 ml by a Centricon® YM-10.

Step 3. TOYOPEARL® AF-Red-650ML HR 5/5 column chromatography. The dialyzed solution was applied to a TOYOPEARL® AF-Red-650ML (Tosoh) HR5/5 column equilibrated with Buffer-A (flow rate was 0.8 ml/min). The protein was eluted with a 0.1 to 0.5 M increasing linear gradient of KCl in Buffer-A. The active fraction (eluted at about 0.15 M) was collected (3 ml) and concentrated to 0.15 ml by an Ultrafree-MC® (10,000 NMWL).

Step 4. Superdex™ 200 PC1.6/30 column chromatography. The enzyme solution was applied to a Superdex™ 200 PC1.6/30 gel filtration column equilibrated with Buffer-C (flow rate was 50 µl/min). The active fraction was then pooled and used as the purified enzyme for characterization.

Protein assay. Protein content was measured by the method of Bradford,[30] and was calibrated with γ-lactoglobulin as the standard (Protein Assay Kit, Bio-Rad, Hercules, CA).

Determination of molecular mass of enzyme. The molecular masses of the native enzymes were estimated by a Superdex™ 200 PC1.6/30 column with a standard molecular marker (Oriental Yeast, Tokyo, Japan) and Buffer-C. The molecular mass of the subunit was estimated by SDS-polyacrylamide gel electrophoresis (12.5%) using the SDS–PAGE standard marker (low range) (Bio-Rad) as the standard.[31]

Enzymatic reduction of α-keto esters. In a polypropylene tube were placed the purified enzyme solution (0.07 unit), NAD(P)H (8 µmol), the substrate (8 µmol), and 0.1 M KPB (pH 6.5, total volume was 0.46 ml). The mixture was gently shaken at 37 °C. After 6 h, the mixture was filtered by a diatomaceous earth short column, extracted with diethyl ether, and then concen-

Table 1. Purification of SAKER-I from *Streptomyces avermitilis*[a]

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Cell-Free Extract	13000	713	0.0548	100	1
30% $(NH_4)_2SO_4$	1120	368	0.329	52	6
TOYOPEARL® DEAE-650M	527	254	0.482	36	9
Mono Q® HR10/10	22.6	20.1	0.889	3	16
Phenyl Superose® HR10/10	2.23	5.54	2.48	0.8	45
Superdex™ 200 HR10/30	0.228	2.97	13.0	0.4	237

[a]Purified from 80 g of wet cells.

Table 2. Purification of SAKER-II from *Streptomyces avermitilis*[a]

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Cell-Free Extract	7290	469	0.0680	100	1
30% $(NH_4)_2SO_4$	6550	572	0.0872	122	1.3
TOYOPEARL® DEAE-650M	5870	318	0.0542	68	0.8
Mono Q® HR10/10	232	10.5	0.453	22	7
Phenyl Superose® HR10/10	34.2	44.6	1.30	10	19
MonoQ® PC1.6/5	5.19	17.5	3.37	4	50
Superdex™ 200 PC1.6/30	1.39	7.09	5.10	2	75

[a]Purified from 100 g of wet cells.

Table 3. Purification of SAKER-III from *Streptomyces avermitilis*[a]

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Cell-Free Extract	2720	582	0.214	100	1
TOYOPEARL® DEAE-650M	1936	480	0.247	82	1.2
Mono Q® HR10/10	147	330	2.24	57	11
Phenyl Superose® HR10/10	41.2	116	2.82	20	13
TOYOPEARL AF-Red-650ML	3.92	51.0	13.0	8.8	61
Superdex™ 200 PC1.6/30	0.542	17.4	32.1	3.0	150

[a]Purified from 40 g of wet cells.

trated under reduced pressure. The conversion of the product was determined by GLC equipped with a capillary DB-WAX column.

Stereochemistry of the products. The enantiomeric excesses (e.e.) of the products were determined by GLC analysis (Chirasil-DEX CB and GAMMA DEX™ 225). The absolute configuration of the isomer was determined by comparing its retention time with those of authentic samples prepared according to the literature methods.[29)]

Measurement of kinetic parameters. The kinetic parameters (K_m and k_{cat} values) of the purified enzymes toward ethyl pyruvate were calculated from the initial rates of the reaction in the appropriate range of the substrate concentration using at least five points by Lineweaver-Burk plots.[32)]

Measurement of the effects of pH. 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; and 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.

N-Terminal region amino acid sequence analysis. The N-terminal region amino acid sequences of the three purified enzymes were analyzed using a model 492A protein sequencer (Applied Biosystems, Foster, CA).[33)] The sequence obtained was compared to those of proteins stored in protein sequence databases (GenBank, EMBL, PIR, and SWISS-PROT) using the sequence similarity search programs BLAST and FASTA.[26,27)]

Results and Discussion

Purification of three SAKERs

Three α-keto ester reducing enzymes (SAKERs-I, -II, and -III) were purified from the cell-free extract of *S. avermitilis via* chromatographic procedures including TOYOPEARL® DEAE-650M, Mono Q®, Phenyl Superose®, TOYOPEARL® AF-Red-650ML, and Superdex™ 200. The activity and purity of these enzymes spectrophotometrically estimated by each step are shown in Tables 1–3. The final specific activity of

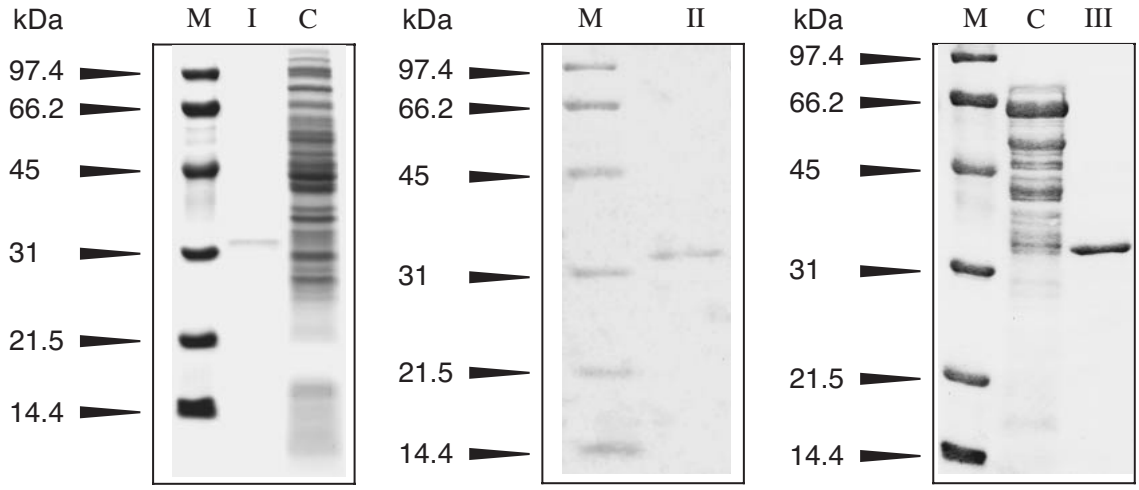


Fig. 1. SDS–PAGE of the Purified SAKERs.

SDS-gel electrophoresis using 12.5% polyacrylamide was performed in the presence of 0.1% SDS. M, 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). I, Purified SAKER-I. II, Purified SAKER-II. III, Purified SAKER-III. C, Cell-free extract. Proteins were visualized by Coomassie Brilliant Blue R-250 and destained in 30% (v/v) methanol/10% (v/v) acetic acid/water.

SAKERs-I, -II, -III were 13.0, 5.10, and 32.1 units/mg protein respectively, with 5 mM of ethyl pyruvate as the substrate and 0.2 mM of NADPH as the coenzyme. The three SAKERs gave a single band on SDS–PAGE (Fig. 1) and one peak on the gel filtration chromatograms.

Molecular mass and subunit structure

The molecular masses of the native SAKERs-I, -II, and -III were estimated to be 71.6, 38.1, and 35.9 kDa, respectively, by gel filtration chromatography (Superdex™ 200 PC1.6/30 column). Furthermore the subunit molecular masses of SAKERs-I, -II, and -III were 31.8, 32.3, and 33.7 kDa by SDS-polyacrylamide gel (12.5%) electrophoresis, respectively. These results suggest that SAKERs-I, -II, and -III had homodimer, monomer, and monomer structures, respectively.

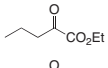
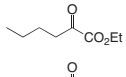
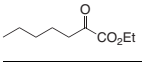
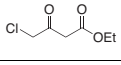
Substrate specificity

The substrate specificity of the SAKERs was investigated using α-keto esters, α-keto acid, and β-keto ester (Table 4). The three SAKERs showed the highest reducing activity toward ethyl pyruvate among the tested substrates. SAKER-I reduced not only aromatic α-keto esters, but also α-keto acid. Ethyl 4-chloroacetoacetate was slightly reduced by SAKERs-I and -II, however SAKER-III hardly reduced the substrate. Furthermore, the three SAKERs did not catalyze the oxidation of α-hydroxy ester with $NADP^+$.

Stereoselectivity

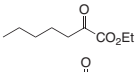
The stereoselectivity of the reduction by SAKERs was investigated by a gas chromatograph equipped with an optically active capillary column (Table 5). Each SAKER catalyzed the reduction of ethyl pyruvate to the corresponding (*S*)-hydroxy ester with a high enantioselectivity (>98% e.e.). In the case of the reduction of ethyl 2-oxoheptanoate and ethyl 3-methyl-2-oxobutyrate, SAKER-I preferentially produced the correspond-

Table 4. The Substrate Specificity of the Three Purified SAKERs

Substrate[a]	Rel. rate (%)[c]			Substrate[a]	Rel. rate (%)[c]		
	SAKER-I	-II	-III		SAKER-I	-II	-III
	100	100	100		29	5	2
	46	7	16		72[b]	24[b]	1[b]
	13	12	8		90	10	<1
	25	6	3		77	35	3
	33	10	2		36	37	<1

[a]The substrate concentration was 5 mM.
[b]The substrate concentration was 20 mM.
[c]Rel. rate = Relative rates were determined by setting the activity of ethyl pyruvate in the presence of NADPH to 100.

Table 5. The Stereoselectivity of the Enzymatic Reduction[a,b]

Substrate	SAKER-I		SAKER-II		SAKER-III	
	e.e. (%)	(*R*/*S*)	e.e. (%)	(*R*/*S*)	e.e. (%)	(*R*/*S*)
	98	*S*	98	*S*	99	*S*
	65	*R*	90	*S*	87	*S*
	87	*R*	85	*S*	82	*S*

[a]The purified enzyme solution (0.07 unit), NADPH (8 μmol), substrate (8 μmol), and 0.1 M potassium phosphate buffer (pH 6.5, 0.46 ml) were incubated for 6 h at 37 °C.
[b]Enantiomeric excesses and configuration were measured by GLC analysis.

Table 6. The Kinetic Parameters of the Three Purified SAKERs[a]

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m
SAKER-I	0.221	8.64	39.1
SAKER-II	1.93	214	110
SAKER-III	0.962	11.7	12.2

[a]The kinetic parameters for ethyl pyruvate were calculated by Lineweaver-Burk plots.

ing (*R*)-alcohol, however, SAKERs-II and -III produced the antipodal (*S*)-alcohol.

Kinetic parameters

The kinetic constants (K_m and k_{cat}) were calculated by Lineweaver-Burk plots as shown in Table 6. The K_m of SAKERs-I, -II, and -III for ethyl pyruvate were 0.221, 1.93, and 0.962 mM, respectively. SAKER-I showed the highest affinity for ethyl pyruvate, however, this enzyme also had the lowest k_{cat} value. On the contrary, SAKER-II had the highest K_m and k_{cat} values. These results suggest that SAKER-II mainly contributes to the reduction of ethyl pyruvate to give the corresponding ethyl (*S*)-lactate in the actinomycete cells.

pH stability

The effect of pH on the enzyme activity was examined as shown in Fig. 2. The three purified enzymes showed maximum activity at about pH 7.5 in potassium phosphate buffer. SAKERs-II and -III were stable in the pH 6.5 to 9.0 region and pH 5.5 to 8.5 region, respectively. While SAKER-I was unstable below pH 6.0 and stable under alkaline conditions, SAKER-III was stable in acidic conditions.

Thermostability

The thermostability of the three STKERs was investigated by measuring the residual activity after incubation at 37 °C and at 50 °C as shown in Fig. 3. The activities of SAKERs-I and -III were reduced to 40% after a 5-h incubation at 37 °C. The thermostability of SAKERs-I and -III were similar during incubation at 37 °C. After incubation at high temperature (50 °C), SAKER-II was deactivated, however, the enzyme retained over 70% of its activity after a 6-h incubation at 37 °C.

N-Terminal region amino acid sequences

The N-terminal region amino acid sequences of the three SAKERs were analyzed by an automated Edman degradation using a pulsed liquid phase protein sequencer. The sequences of SAKERs-I, -II, and -III were [1]Ala-Thr-His-Val-Ile-Thr-Gly-Ala-Gly-[10]Ser-Gly-Ile-Gly-Ala-Ala-, [1]Ser-Thr-Lys-Val-Pro-Pro-Ile-Thr-Leu-[10]Asn-Asn-Gly-Val-Glu-Met-, and [1]Thr-Ser-Leu-Arg-Lys-Leu-Gly-Pro-Ser-[10]Asp-Leu-Glu-Val-Phe-Pro-, respectively, as shown in Fig. 4 (the N-terminals of the three enzymes were not blocked). SAKER-I had a consensus sequence (-Gly-X-X-X-Gly-X-Gly-), the putative coenzyme (NAD(P)H) binding site, in the N-terminal region. The similarity of each sequence of the three enzymes with those of other proteins was examined by a computer search of the protein sequence database.[26,27] The sequences of SAKERs-I, -II, and -III were identical to those of SAV2750, SAV1849, and SAV4117, respectively. These identical proteins were putative oxidoreductases, hypothetical proteins, in the cells of *S. avermitilis*. In other words, these results suggest these hypothetical proteins are expressed as enzyme proteins in the *S. avermitilis* cells and the proteins have α-keto ester reducing activities. Furthermore, these three hypothetical proteins were not coded in the secondary metabolite clusters (*melC-1*, *tpc1*, *pks1*, *nrps1*, and so on) that are listed in the *S. avermitilis* genome database (http://avermitilis.ls.kitasato-u.ac.jp/metabolite/).

Comparison with other actinomycete keto ester reductases

The enzymological properties of the three STKERs (keto ester reductase from *S. thermocyaneoviolaceus*)[23,24] and the three SAKERs are listed in Table 7. While the STKERs and SAKER-I consist of a

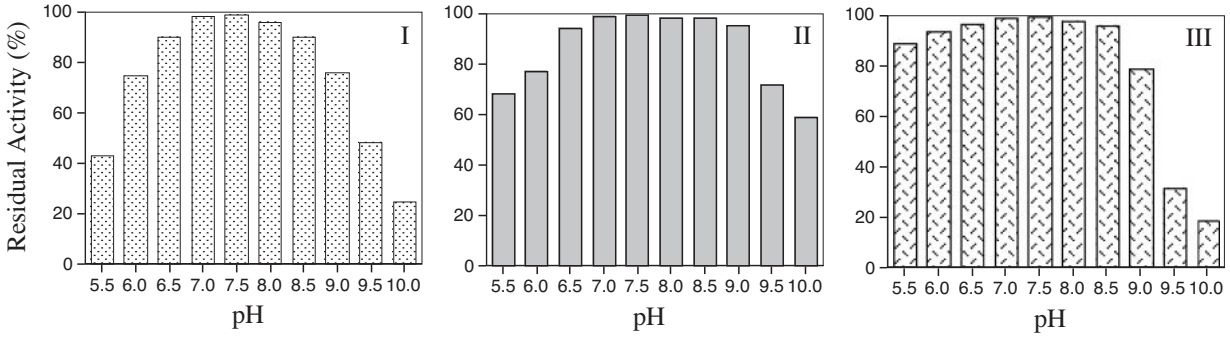


Fig. 2. The pH Stability of the Purified SAKERs.

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.

Table 7. Six α-Keto Ester Reductases from Actinomycetes

	STKER-I[23]	STKER-II[24]	STKER-III[24]	SAKER-I	SAKER-II	SAKER-III
Native M_r (gel filtration)	64 kDa	60 kDa	70 kDa	72 kDa	38 kDa	36 kDa
Subunit M_r (SDS–PAGE)	30 kDa	29 kDa	30 kDa	32 kDa	32 kDa	32 kDa
Subunit structure	dimer	dimer	dimer	dimer	monomer	monomer
Thermostability[a]	100% for 4 d	50% for 4 d	58% for 4 d	39% for 1 h	70% for 4 h	38% for 4 h
Favorite α-keto ester	CH_3COCO_2Et	n-$C_5H_{11}COCO_2Et$	$PhCOCO_2Me$	CH_3COCO_2Et	CH_3COCO_2Et	CH_3COCO_2Et
K_m for CH_3COCO_2Et (mM)	7.9×10^{-2}	3.2×10	4.1	2.1×10^{-1}	1.9	9.6×10^{-1}
k_{cat} for CH_3COCO_2Et (s^{-1})	5.7×10	5.3	7.9×10	8.6	2.1×10^2	1.2×10
k_{cat}/K_m for CH_3COCO_2Et	7.2×10^2	1.7×10^{-1}	2.0×10	3.9×10	1.1×10^2	1.2×10
E.e. (%) (*R*/*S*) for ethyl lactate	>99% (*S*)	40% (*R*)	14% (*S*)	98% (*S*)	98% (*S*)	99% (*S*)
Activity for pyruvic acid	−	−	−	+	−	−
Coenzyme	NADH/NADPH	NADPH	NADPH	NADPH	NADPH	NADPH
N-Terminal sequence	ATHVITGAGS-	TSVELPELSGK-	MKRLVTVVT-	ATHVITGAGS-	STKVPPITLN-	TSLRKLGPSD-

[a]Residual activity after incubation at 37 °C.

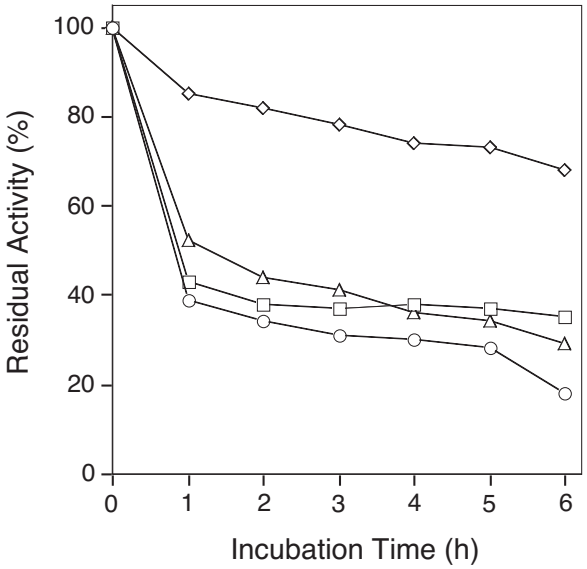


Fig. 3. Thermostability of the Purified SAKERs.

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 50 °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. ○, SAKER-I incubated at 37 °C; ◇, SAKER-II incubated at 37 °C; □, SAKER-II incubated at 50 °C; △, SAKER-III incubated at 37 °C.

```
SAKER-I    ATHVITGAGS GIGAAVTRRL HARGD
           :::::::::: :::::::::: :::::
SAV2750    ATHVITGAGS GIGAAVTRRL HARGD

SAKER-II   STKVPPITLN NGVEMPQLGF GVWQVPDDEA
           :::::::::: :::::::::: ::::::::::
SAV1849    STKVPPITLN NGVEMPQLGF GVWQVPDDEA

SAKER-III  TSLRKLGPSD LEVFPLSLGG NVFGWTADEA
           :::::::::::::::::::: ::::::::::
SAV4117    TSLRKLGPSD LEVFPLSLGG NVFGWTADEA
```

Fig. 4. Sequence Comparison of N-Terminal Region Amino Acid Sequences of SAKERs and Hypothetical Proteins in *S. avermitilis*.

SAV2750, Putative oxidoreductase from *S. avermitilis*. SAV1849, Putative oxidoreductase from *S. avermitilis*. SAV4117, Putative oxidoreductase from *S. avermitilis*. The underlined sequences are the putative coenzyme binding site.

homodimer, SAKERs-II and -III had a monomer structure. The three SAKERs had a low heat-resistance compared with that of the STKERs. SAKERs-I and -II catalyzed the reduction of the α-keto acid, however, the three STKERs and SAKER-III did not reduce the α-keto acid at all. The stereoselectivity of the reduction of ethyl pyruvate by the SAKERs was higher than those of STKERs-II and -III. SAKER-I had the same enzymological properties as those of STKER-I with regard to the N-terminal region of the amino acid sequence (residues 1 to 16), the favorite substrate, the subunit structure, and the reduction stereoselectivity of ethyl pyruvate, however, the heat-resistance and coenzyme specificity of SAKER-I differed from those of STKER-I.

It was found that the cells of *S. avermitilis* had α-keto ester reducing activity, and that SAKER-I was identical to SAV2750 (one of the hypothetical proteins in the actinomycete cells) as we predicted based on a bioinformatics approach during our initial study. Furthermore, we purified and characterized SAKERs-II and -III by an orthodox and traditional approach with the guidance of the catalytic function (α-keto ester reducing activity). The gene cloning and expression of STKER-I and SAKER-I and analysis of the structure-function relationship of the two enzymes are now in progress in our laboratory.

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