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Purification and some properties of a novel racemase, which racemizes 2-oxothiazolidine-4-carboxylic acid and 5-oxoproline, from *Flectobacillus* sp. strain B-1

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Abstract

A novel racemase active toward 2-oxothiazolidine-4-carboxylic acid was purified 310-fold with 5% recovery to near homogeneity from a crude extract of *Flectobacillus* sp. B-1, which had been isolated as a bacterium being able to assimilate (*S*)-2-oxothiazolidine-4-carboxylic acid. The molecular weight was estimated to be 92000 by gel filtration. The purified preparation migrated as a single band of molecular weight 49000 upon SDS-polyacrylamide gel electrophoresis. The enzyme exhibited maximum activity at pH 8.0 and 45°C. The enzyme also racemized 5-oxoproline but did not act on proline and 4-hydroxyproline. The enzyme apparently had no coenzyme requirement. The enzyme activity was inhibited to 62–100% by SH-blocking reagents such as $HgCl_2$, $AgNO_3$, PCMB, iodoacetamide, *N*-ethylmaleimide and *N*-bromosuccinimide.

Key words: Racemase; 2-Oxothiazolidine-4-carboxylic acid; 5-Oxoproline

1. Introduction

Amino acid racemases are enzymes catalyzing interconversion between L-isomer and D-isomer of an amino acid. Since the discovery of alanine racemase, a variety of amino acid racemases and epimerase, for example, lysine racemase, arginine racemase and phenylalanine racemase, have been demonstrated in bacteria and actinomycestes [1]. But most of them are racemases which act on primary amino acids, and racemases toward imino acids are rare. Only two examples, proline racemase [2] and hydroxyproline 2-epimerase [3] were reported.

Amino acid racemases and epimerases play important role in the biological processes such as the synthesis of D-amino acids used in the construction of bacterial cell wall and the conversion of the nonmetabolizable isomer of a certain amino acid to the metabolizable one. These enzymes are also important in the field of enzyme engineering. The theoretical yield of the optical resolution is 50%, but it can be improved to 100% by the combination with racemase reaction. For instance, acylamino acid racemase, which recycles the D-acylamino acid produced in the optical resolution of DL-acylamino acid with the stereospecific L-aminoacylase [4], becomes a focus of attention.

Previously we reported a microbe which assimilated (*R*)-enantiomer of 2-oxothiazolidine-4-carboxylic acid (OTC) in preference to (*S*)-enantiomer, and preparation of (*S*)-enantiomer from racemic OTC using this microbe [5]. Subsequently we isolated a bacterium which was able to assimilate (*S*)-OTC in addition to (*R*)-OTC. In the course of the investigation of the catabolism of (*S*)-OTC, we found a new racemase which acted on imino acid, that is, a racemase active toward OTC. This racemase acted on 5-oxoproline in addition to OTC. As concerns the optical isomerization of 5-oxoproline, unidirectional isomerization of 5-oxo-D-proline to L-isomer by the crude enzyme preparation obtained from *Pseudomonas alcaligenes* was reported by Kawai et al. [6]. But the enzyme involved in this

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Abbreviations: OTC, 2-oxothiazolidine-4-carboxylic acid; Mes, 2-morpholinoethanesulfonic acid; Tricine, *N*-[Tris(hydroxymethyl)methyl] glycine; Tes, *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PCMB, *p*-chloromercuribenzoic acid.

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reaction has not been purified, and the nature of this reaction has not been revealed. From the viewpoint of applied enzymology, it is worth while examining the activity of this racemase toward acylamino acids because the chemical structure of 5-oxoproline contains acylated amino group. So we purified and characterized this racemase to study the enzymatic racemization of OTC and 5-oxoproline and to investigate its utility.

This report deals with the purification and some properties of a racemase racemizing OTC and 5-oxoproline from *Flectobacillus* sp. B-1.

2. Materials and methods

Materials

Materials and their suppliers were as follows: (*R*)-OTC, 5-oxo-L-proline, 5-oxo-DL-proline, (*R*)-thiazolidine-4-carboxylic acid and (*R*)-5-oxotetrahydrofurane-2-carboxylic acid were from Aldrich Chemical Company, Wisconsin, USA. 5-oxo-D-proline was from Fluka Chemie, Buchs, Switzerland. D-proline, L-proline and 4-hydroxy-L-proline were from Nacalai Tesque, Kyoto, Japan. *N*-acetyl-L-alanine, *N*-acetyl-L-cysteine, *N*-acetyl-L-glutamic acid and *N*-acetyl-L-phenylalanine were from Sigma, Missouri, USA. Butyl-Toyopearl 650 M was from Tosoh, Tokyo, Japan. Fractogel EMD DEAE-650(S) and Hibar RT 100-8 Hydroxyapatite-MP column were from Kanto Chemical Co., Tokyo, Japan. DEAE MemSep 1000 chromatography cartridge was from Nihon Millipore, Tokyo, Japan. MCI GEL CRS10W column was from Mitsubishi Kasei, Tokyo, Japan. Crownpak CR(+) column was from Daicel Chemical Industries, Tokyo, Japan. UltraSpherogel SEC 3000 column was from Beckman Instruments, San Ramon, USA. (*RS*)-OTC and (*S*)-OTC were prepared from DL-and D-cysteine respectively by the method of Boettecher et al. [7] and DL-and D-cysteine were purchased from Sigma, St. Louis, MO, USA. (*R*)-2-thiothiazolidine-4-carboxylic acid was kindly provided by Dr. Yamazaki, National Institute of Bioscience and Human-Technology. All other chemicals were of analytical grade.

Bacterial strain and medium

Strain B-1 was isolated as a bacterium which was capable of assimilating (*S*)-OTC from a soil sample of the shores of the sea of Kashima. This organism was identified as a strain of *Flectobacillus* species and designated as *Flectobacillus* species B-1 strain [8].

The standard medium used for cultivation of *Flectobacillus* sp. strain B-1 contained (per liter) 5.0 g of (*RS*)-OTC, 40 g of glycerol, 20 g of NaCl, 3 g of K_2HPO_4, 3 g of KH_2PO_4, 1 g of yeast extract, 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.3 mg of H_3BO_3, 0.2 mg of $MnCl_2 \cdot 4H_2O$, 0.75 mg of $ZnCl_2$, 0.2 mg of $CuSO_4 \cdot 5H_2O$, 2.5 mg of $FeCl_3 \cdot 6H_2O$, 0.1 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.15 mg of $CoSO_4 \cdot 7H_2O$ (pH 7.0).

Purification of OTC racemase

The temperature was kept at 4°C throughout the purification.

Step 1. Preparation of crude enzyme. One liter of the standard medium was dispensed into ten 500-ml conical flasks. A 2-day-old culture of strain B-1 (5 ml) was inoculated into each flask, and cultivated aerobically for 24 h at 30°C on a rotary shaker. The cells were harvested by centrifugation and washed twice with 50 mM Mes buffer (pH 7.0). Then the cells were suspended in the same buffer, and disrupted with MSK cell homogenizer (B.Braun Melsungen AG, Germany). The cell lysate was centrifuged ($15000 \times g$, 60 min), and supernatant was used as a crude enzyme preparation.

Step 2. Ammonium sulfate fractionation. Solid ammnomium sulfate was added to the crude enzyme preparation to 60% saturation and the mixture was allowed to stand for 60 min on ice. The precipitate was collected by centrifugation ($20000 \times g$, 15 min), and dissolved in 1.2 M $(NH_4)_2SO_4$ – 50 mM Mes (pH 7.0).

Step 3. Hydrophobic chromatography. The enzyme solution was applied to a Butyl-Toyopearl 650 M column (2.6×20 cm) previously equilibrated with 1.2 M $(NH_4)_2SO_4$ – 50 mM Mes (pH 7.0). The column was washed with 100 ml of the same buffer, then 200 ml of 0.9 M $(NH_4)_2SO_4$ – 50 mM Mes (pH 7.0) at a flow rate of 5.0 ml/min. The enzyme was eluted with a 150 ml negative linear gradient of 0.9 to 0.3 M $(NH_4)_2SO_4$ containing 50 mM Mes (pH 7.0) at the same flow rate. The active fractions, which were eluted at the elution volume from 115 to 130 ml, were pooled and dialyzed against 20 mM $(NH_4)_2SO_4$ – 10% (w/v) ethylene glycol – 10 mM Mes (pH 7.0).

Step 4. Ion exchanging chromatography using Fractogel EMD DEAE-650(S). The dialyzed enzyme solution was applied to a Fractogel EMD DEAE-650(S) column (1.6×10 cm) previously equilibrated with 10 mM $(NH_4)_2SO_4$ – 10% (w/v) ethylene glycol – 10 mM Mes (pH 7.0). The column was thoroughly washed with the same buffer and the enzyme was eluted with a 160 ml linear gradient of 10 to 240 mM $(NH_4)_2SO_4$ containing 10% (w/v) ethylene glycol and 10 mM Mes (pH 7.0) at a flow rate of 2.0 ml/min. The active fractions, which were eluted at the elution volume from 82 to 88 ml, were pooled and dialyzed against 20 mM $(NH_4)_2SO_4$ – 10% (w/v) ethylene glycol – 25 mM histidine (pH 7.0).

Step 5. Ion exchanging chromatography using DEAE MemSep 1000. The dialyzed enzyme solution was applied to a DEAE MemSep 1000 cartridge previously

equilibrated with 20 mM $(NH_4)_2SO_4$ – 10% (w/v) ethylene glycol – 25 mM histidine (pH 7.0) at a flow rate of 2.0 ml/min, and then the cartridge was washed thoroughly with the same buffer. The enzyme was eluted with a 40 ml linear gradient of 20 to 125 mM $(NH_4)_2SO_4$ containing 10% (w/v) ethylene glycol and 25 mM histidine (pH 7.0) at the same flow rate. The active fractions, which were eluted at the elution volume from 15 to 17 ml, were pooled.

Step 6. Hydroxyapatite column chromatography. The enzyme solution was applied to a Hibar RT 100-8 Hydroxyapatite-MP column (0.8 × 10 cm) previously equilibrated with 10 mM phosphate buffer (pH 7.0) containing 20 mM $(NH_4)_2SO_4$ and 10% (w/v) ethylene glycol. The column was washed with the same buffer at a flow rate of 1.0 ml/min, then the enzyme was eluted with a 60 ml linear gradient of 10 to 200 mM phosphate buffer (pH 7.0) containing 20 mM $(NH_4)_2SO_4$ and 10% (w/v) ethylene glycol at the same flow rate. The active fractions, which were eluted at the elution volume from 22 to 24 ml, were pooled.

Enzyme assay

Polarimetric assay. Racemase activity was routinely assayed at 37°C by measuring the rate of the decrease in the optical rotation at 585 nm with a JASCO polarimeter Model DIP-360. One ml of 125 mM Tricine buffer (pH 8.0) containing 37.5 mM (*R*)-OTC was mixed with 0.25 ml of appropriately diluted enzyme solution. The mixture was immediately transferred to a waterjacketed 1-dm polarimeter cell and the cell was placed in the chamber of JASCO instrument. Changing optical rotation was recorded as a function of time using an external recorder set. The cell was kept at 37°C by circulating warmed water through the waterjacket. One unit of enzyme activity was defined as the amount of enzyme which converted 1 micro-mole of (*R*)-OTC to (*S*)-OTC per min under the above conditions. This corresponds to a change in optical rotation of approximately 0.022° per min.

HPLC assay. Polarimetric assay could not be used for the kinetic studies because of the low sensitivity of polarimeter. The initial velocity for the racemase was determined by measuring the resulting antipode by HPLC with chiral column. Tes was used as a buffer in the HPLC assay because Tricine interfered with the HPLC analysis. Two ml of 125 mM Tes (pH 8.0) containing 0.2 to 0.5 mM of substrate was mixed with 0.5 ml of appropriately diluted enzyme solution. The mixture was incubated at 37°C. Aliquot was taken and mixed with one-tenth volume of 1 M HCl for the reaction to be stopped. When the substrate was OTC, each enantiomer of OTC was directly determined by HPLC. The conditions of the HPLC were as follows: column, MCI GEL CRS10W (4.6 × 50 mm), three of these columns being set in tandem; eluent, 2 mM $CuSO_4$ containing 15% (v/v) methanol; flow rate, 0.3 ml/min; detection, absorbance at 254 nm. When the substrate was 5-oxo-proline, 5-oxo-proline was converted to glutamate by aqueous alkali treatment [9] before HPLC analysis. After the stop of enzyme reaction, the mixture was added a quarter volume of 4 M NaOH, and allowed to stand at room temperature for 2 days, then added equal volume of 1 M HCl to be neutralized. The each enantiomer of glutamate was determined by HPLC. The conditions were as follows: column, Crownpak CR(+) (4 × 150 mm); eluent, aqueous solution of $HClO_4$ (pH 1.0); flow rate, 0.4 ml/min; column temperature, 10°C; detection, post-column labeling with *o*-phthalaldehyde [10].

Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out by using a premade gel system (ACI Japan Co., Kanagawa, Japan) according to the supplier's instructions. Phosphorylase *b* (M_r = 97400), bovine serum albumin (M_r = 67000), ovalbumin (M_r = 43000), carbonic anhydrase (M_r = 31000) and trypsin inhibitor (M_r = 21500) were used as molecular weight standards. Protein bands were visualized by silver staining with a Silver Stain II kit Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Molecular weight estimation of native enzyme

To estimate the molecular weight of the enzyme, the enzyme sample was subjected to HPLC using a Ultra-Spherogel SEC3000 column (7.5 × 300 mm) at a flow rate of 0.5 ml/min with an eluent buffer consisting of 100 mM KH_2PO_4 and 100 mM Na_2SO_4. A calibration curve was made with the following proteins: catalase (M_r = 232000), aldolase (M_r = 158000), bovine serum albumin (M_r = 67000) and ovalbumin (M_r = 43000). The void volume of the column was measured with Blue Dextran 2000 (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Isoelectric focusing

Isoelectric focusing was carried out by using a Rotofor IEF system (Bio-Rad Laboratories, Richmond, VA, USA) according to the supplier's instructions. Bio-Lyte 3/10 was used as carrier ampholite and glycerol was added to the enzyme-ampholite mixture to the final concentration of 10% (w/v) as stabilizer.

Protein determination

Protein concentration was determined by using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, USA). Bovine serum albumin was used as the standard.

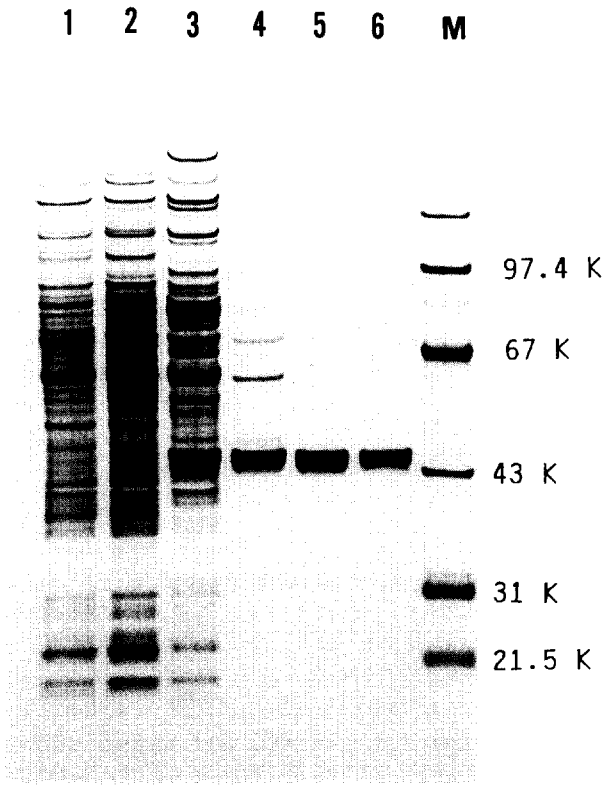

Fig. 1. SDS-PAGE of samples at various stages of purification on 10% gel. Lane 1, crude extract; lane 2, ammonium sulfate precipitate; lane 3, Butyl Toyopearl chromatography; lane 4, Fractogel EMD DEAE-650(S) chromatography; lane 5, DEAE MemSep chromatography; lane 6, hydroxyapatite chromatography; lane M, molecular weight standards.

3. Results

Production of racemase

Strain B-1 was aerobically cultivated in 100 ml of the standard medium in a 500-ml conical flask at 30°C. The maximum cell concentration was achieved after 24 h of incubation. The OTC racemase activity increased along with the cell growth and reached a maximum level at 24 h. The activity was detected in the cell extract but not in the culture broth. Substitution of (*RS*)-OTC with (*R*)- or (*S*)-OTC did not affect the racemase production. No activity was detected in the cells which had grown in the absence of (*R*)-, (*S*)- nor (*RS*)-OTC. The addition of 0.2% (w/v) $(NH_4)_2SO_4$ to the standard medium completely inhibited the racemase production. Therefore, the enzyme appeared to be formed inducibly.

Purification of the racemase

Table 1 summarizes the result of the purification procedure. The overall recovery from the crude extract was about 5%, with about 310-fold purification. Purification of the OTC racemase through the various steps was monitored by SDS-PAGE (Fig. 1). The final preparation eluted from the hydroxyapatite column gave a single protein band. In addition, this preparation gave a single protein peak accompanied with the enzymatic activity on both HPLC with a UltraSpherogel SEC 3000 column and isoelectric focusing. So the enzyme was judged to be homogeneous.

Cofactor requirement

Absorption spectrum of the enzyme from 190 to 600 nm showed no characteristic absorption except for a peak around 280 nm (data not shown). The enzyme activity was not stimulated by the addition of pyridoxal 5'-phosphate, flavin mononucleotide, FAD, NAD^+, $NADP^+$, NADH, NADPH nor ATP at a final concentration of 1 mM. So it seems that the enzyme has no cofactor requirement.

Molecular weight and isoelectric point

The molecular weight of the native enzyme was estimated to be approximately 92000 by gel filtration on HPLC (Fig. 2, A). The molecular weight estimated by SDS-PAGE was 49000 (Fig. 2, B). These results suggested that the enzyme was dimmer. The enzyme had an isoelectric point of pH 5.2.

Effect of temperature and pH

The effect of temperature on the racemase activity was tested at temperatures ranging form 35°C to 60°C. Other conditions were the same as those described in 'Materials and Methods'. Maximum activity was observed at 45°C. At 35°C the enzyme activity was 75% of maximum activity, and at 60°C it was 30%.

Table 1
Purification of OTC racemase

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
1. Crude extract	1185.00	1608.0	1.4	1	100
2. Ammonium sulfate precipitation	960.00	2395.2	2.5	1.8	149
3. Butyl Toyopearl chromatography	25.00	638.6	25.6	18.3	40
4. Fractogel EMD DEAE-650(S) chromatography	1.24	360.1	290.4	207.4	22
5. DEAE MemSep 1000 chromatography	0.45	190.1	422.4	301.7	12
6. Hydroxyapatite chromatography	0.18	79.8	443.6	316.8	5

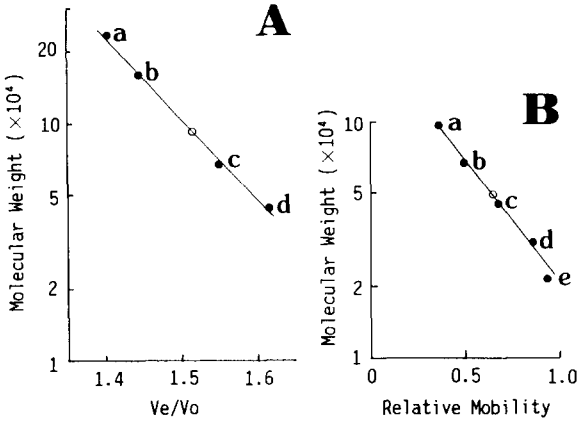

Fig. 2. Estimation of molecular weight of the OTC racemase. A: The molecular weight of the enzyme was estimated by gel filtration on HPLC. Ve/Vo was plotted against molecular weight in a semilogarithmic scale. The position of OTC racemase is shown by an open circle. The standard proteins used were: a, catalase; b, aldolase; c, bovine serum albumin; d, ovalbumin. B: The molecular weight of the subunit was estimated by SDS-PAGE. Relative mobility was plotted against subunit molecular weight in a semilogarithmic scale. The position of OTC racemase is shown by an open circle. The standard proteins used were: a, phosphorylase b; b, bovine serum albumin; c, ovalbumin; d, carbonic anhydrase; e, soybean trypsin inhibitor.

The effect of pH on the enzyme activity was determined in the buffers ranging from pH 6.5 to pH 9.0. The buffers used were as follows: from pH 6.5 to pH 7.5, Tes; from pH 7.5 to pH 9.0, Tricine. Other conditions were the same as those described in 'Materials and Methods'. The maximum activity was observed at pH 8.0. In the range from pH 7.5 to 9.0 the enzyme exhibited 85–100% of its maximum activity. It rapidly lost its activity below pH 7.5. No spontaneous racemization of OTC in the temperature and pH ranges tested was detected.

To examine the thermal stability, the enzyme was incubated in 10 mM phosphate buffer (pH 7.0) containing 20 mM $(NH_4)_2SO_4$ and 10% (w/v) ethylene glycol at various temperatures for 10 min and the remaining activity was measured. The enzyme retained all activity till 40°C. The residual activity was 80% at 50°C, but it was 0% at 60°C.

To examine the pH stability, the enzyme was incubated in the buffers of various pH values containing 20 mM $(NH_4)_2SO_4$ and 10% (w/v) ethylene glycol for 12 h at 4°C and the remaining activity was determined. The buffers used were as follows: from pH 4.0 to pH 5.5, acetate buffer; from pH 5.5 to pH 7.5, Mes. The enzyme was most stable at pH 5.5, and relatively stable in a pH range from 4.5 to 6.5. The activity was sharply declined below pH 4.5 and over pH 6.5 (100% inactivation at pH 4.0 and 80% inactivation at pH 7.5).

Effect of metal ions

Table 2 shows the effect of various metal ions on the racemase activity. None of the metal ion tested stimulated the enzyme activity. But $CuSO_4$ completely inhibited the enzyme activity, and $FeSO_4$ showed 70% inhibition.

Table 2
Effect of some metal ions on the activity of OTC racemase

Ion as:	Relative activity (%)
Control	100
LiCl	82
$MgCl_2$	111
$ZnCl_2$	102
$CoSO_4$	92
$MnCl_2$	112
$CaCl_2$	99
$PbCl_2$	86
$FeCl_3$	119
$FeSO_4$	30
$CuSO_4$	0

The enzyme activities were measured after the preincubation period of 10 min at 30°C with these substances. The concentration of the additive was 1 mM. The enzymatic activities are expressed as percentages of the activity measured in the absence of any additive.

Effect of inhibitors

The effects of various inhibitors on the racemase activity were examined at the concentration of 1 mM. The results are shown in Table 3. The inhibitory effect by chelating reagents such as EDTA, 2,2'-bipyridine and *o*-phenanthroline was negligible. Diisopropylfluorophosphate, D-penicillamine and hydroxylamine showed 26%, 17% and 26% inhibition, respectively. But the enzyme activity was inhibited to 62–100% by SH-blocking reagent such as $HgCl_2$, $AgNO_3$, PCMB, iodoacetamide, *N*-ethylmaleimide and *N*-bromosuccinimide. As mentioned in 'Molecular weight and Iso-

Table 3
Inhibition of OTC racemase

Inhibitor	Residual acitivity (%)
Control	100
$HgCl_2$	0
$AgNO_3$	0
PCMB (0.1 mM)	0
Iodoacetamide	29
Iodoacetamide (0.1 mM)	34
N-ethylmaleimide	38
N-ethylmaleimide (10 mM)	0
N-bromosuccinimide	0
EDTA	90
2,2'-bipyridine	100
O-phenanthroline	98
diisopropylfluorophosphate	74
D-penicillamine	83
$NH_2OH \cdot HCl$	74

The enzyme activities were measured after the preincubation period of 10 min at 30°C with these substances. The concentration of the additive was 1 mM unless otherwise mentioned. The enzymatic activities are expressed as percentages of the activity measured in the absence of any additive.

Table 4
Substrate specificity of OTC racemase

Substrate	Relative activity (%)
(*R*)-OTC	100
(*S*)-OTC	139
5-oxo-L-proline	56
5-oxo-D-proline	40
L-proline	0
D-proline	0
4-hydroxy-L-proline	0
(*R*)-2-thiothiazolidine-4-carboxylic acid	0
(*R*)-thiazolidine-4-carboxylic acid	0
(*R*)-5-oxotetrahydrofurane-2-carboxylic acid	0
N-Acetyl-L-alanine	0
N-Acetyl-L-cysteine	0
N-Acetyl-L-glutamic acid	0
N-Acetyl-L-phenylalanine	0

electric Point', the enzyme was suggested to be a dimmer. To examine whether the enzyme was a dimer linked by a disulfide bridge or not, the enzyme was denatured in the absence of 2-mercaptoethanol, and the migration on SDS-PAGE was compared with that of the enzyme denatured in the presence of 2-mercaptoethanol (the standard denaturation treatment for SDS-PAGE). The enzyme denatured in the absence of 2-mercaptoethanol migrated slightly faster than that denatured in the presence of 2-mercaptoethanol, and the position where its band emerged corresponded to the molecular weight of 43 000 (data not shown). So it seems likely that no disulfide bridge is involved in the dimerization of the enzyme. One explanation for the faster migration may be that the intramolecular disulfide bridge(s) make(s) the subunit molecule compact.

Substrate specificity and kinetic properties

The enzyme activity toward various compounds was examined at a concentration of 30 mM. As shown in Table 4 the enzyme acts only on 5-oxoproline other than OTC. The enzyme did not act on proline nor 4-hydroxyproline.

Kinetic studies were carried out to determine the Michaelis constants (Km) and maximum reaction velocities (Vmax) for OTC and 5-oxoproline from double-reciprocal plots. The initial velocity for racemase was measured by HPLC assay as mentioned in 'Materials and Methods'. The Km values for (*R*)- and (*S*)-OTC were 0.568 mM and 0.610 mM, respectively. The Vmax values for (*R*)-and (*S*)-OTC were 0.232 mmol/min/mg and 0.293 mmol/min/mg, respectively. The Km values for 5-oxo-L-proline and 5-oxo-D-proline were 0.909 mM and 0.470 mM, respectively. The Vmax values for 5-oxo-L-proline and 5-oxo-D-proline were 0.174 mmol/min/mg and 0.096 mmol/min/mg, respectively. When these values were used, the calculated Keq values for OTC racemization and 5-oxoproline racemization were 1.17 and 1.07, respectively, in good agreement with the theoretical value (1.0) for the chemical symmetric reaction.

Induction of the racemase by 5-oxoproline

The OTC racemase acted on 5-oxoproline besides OTC. In connection with the activity toward 5-oxoproline, the ability of 5-oxoproline to induce OTC racemase was examined. In this experiment (*RS*)-OTC contained in the standard medium was replaced with 5-oxo-DL-proline. OTC racemase activity was detected in the crude extract prepared from the cells grown on such medium. This racemase could be purified by the same procedure used in the purification of OTC racemase from the cells grown on the standard medium. Furthermore the racemase migrated to the same position as the racemase purified from the cells grown on the standard medium on SDS-PAGE (Fig. 3). In addition, both racemases eluted at the same position on the chromatographies used in the purification procedure. So the racemase induced by 5-oxoproline is considered to be identical with that induced by OTC.

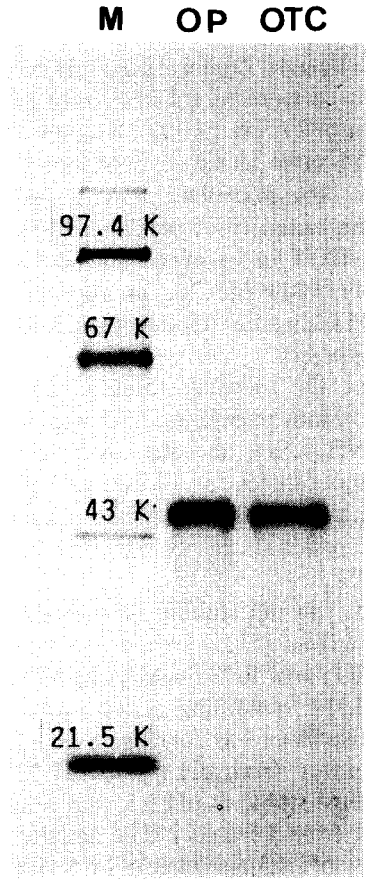

Fig. 3. SDS-PAGE of OTC racemase purified from the cells grown on the standard medium and 5-oxoproline containing medium on 10% gel. Lane M, molecular weight standards; lane OP, OTC racemase purified from the cells grown on 5-oxoproline containing medium; lane OTC, OTC racemase purified from the cells grown on the standard medium.

4. Discussion

Amino acid racemases/epimerases are classified into two groups with respect to cofactor requirement [1]. One group is comprised of racemases for primary amino acids; these require pyridoxal phosphate as a cofactor. The second group is represented by proline racemase and hydroxyproline 2-epimerase. These enzymes contain cysteine residue in the active site, do not require pyridoxal or any other known coenzyme. Recently pyridoxal independent diaminopimelate epimerase [11], glutamate racemase [12] and aspartate racemase [13] have been reported. Our enzyme may belong to the second group because it has no coenzyme requirement and it is markedly inhibited by SH-blocking reagent.

The racemases for primary amino acid such as alanine racemase and glutamate racemase are used for the synthesis of D-amino acids used in the construction of peptidoglycan layer of bacterial cell wall [14]. Proline racemase and hydroxyproline 2-epimerase are necessary for the catabolism of L-proline and 4-hydroxy-L-proline, respectively [15]. In *Clostridia*, most important route of proline degradation is reductive cleavage to 5-aminovaleric acid by a D-specific reductase. So preliminary racemase step is necessary for the utilization of L-proline. In *Pseudomonas* and some other bacteria, allohydroxy-D-proline is the proximate energy-yielding substrate for the degradation pathway of free 4-hydroxyproline and 4-hydroxy-L-proline can be utilized only after conversion to allohydroxy-D-proline. If OTC racemase functions as 5-oxoproline racemase in the bacterial cells, what is its role? Most plausible candidate is a catalyst involved in the catabolism of 5-oxoproline. Although some bacteria have been reported to be able to assimilate 5-oxoproline, and although enzymes which act on 5-oxoproline or which produce 5-oxoproline have been found in certain bacteria, the information about the metabolism of 5-oxoproline in bacteria is limited [16]. The role of OTC racemase remains to be resolved.

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