# PURIFICATION AND PROPERTIES OF METHIONINASE FROM *PSEUDOMONAS OVALIS*

Hidehiko TANAKA*, Nobuyoshi ESAKI, Tatsuo YAMAMOTO and Kenji SODA**

*Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, Japan, and *Laboratory of Biochemistry, Kyoto College of Pharmacy, Kyoto 607, Japan*

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## 1. Introduction

Although the microbial metabolism of methionine has been extensively studied, the mechanism of the enzymatic α,γ-elimination of L-methionine has remained unsolved. The formation of methanethiol from methionine was first found using the dried cells of *E. coli* and *Proteus vulgaris* by Onitake [1]. The occurrence of methioninase (L-methionine-methanethiollyase (deaminating) (EC 4.4.1)) catalyzing the conversion of L-methionine into α-ketobutyrate, methanethiol and ammonia has been demonstrated in several bacterial strains; a soil bacterium [2], *E. coli* [3], *Pseudomonas sp.* [4,5], *Clostridium sporogenes* [6], rumen bacteria [7] and *Ps. putida* [8]. Little effort, however, has been devoted to the purification and the characterization of the enzyme. Recently, the enzyme was purified to homogeneity from *Cl. sporogenes* by Kreis et al. [9] to investigate its antitumor activity.

In this communication, we describe the purification of methioninase from *Pseudomonas ovalis* and some of its properties.

## 2. Materials and methods

*Ps. ovalis* IFO 3738 was grown in a medium containing 0.25% L-methionine, 0.1% urea, 0.1% glycerol, 0.1% $KH_2PO_4$, 0.1% $K_2HPO_4$, 0.01% $MgSO_4 \cdot 7H_2O$ and 0.025% yeast extract. The pH was adjusted to 7.2 with NaOH. Cultures were grown at 28°C for about 18 h under aeration. The cell harvested by centrifugation was washed twice with 0.85% NaCl solution, and subsequently with 0.01 M potassium phosphate buffer (pH 7.2) containing $10^{-5}$ M pyridoxal 5'-phosphate (pyridoxal-P) and 0.01% 2-mercaptoethanol. The yield of cells was approximately 4 g (wet weight) per liter of the medium.

α-Keto-γ-methiolbutyrate was prepared according to the method of Meister [10]. Amino acids were products of Ajinomoto Co., Tokyo, Japan. Methanethiol and ammonia were determined with *N,N*-dimethyl-*p*-phenylene diamine·HCl [11] and Nessler reagent [12], respectively.

### 2.1. *Enzyme assay*

Methioninase was routinely assayed by determining α-ketobutyrate formed. The standard reaction system contained 100 μmol of potassium phosphate buffer (pH 8.0), 100 μmol of L-methionine or other amino acids, 0.02 μmol of pyridoxal-P and enzyme in a final volume of 2.0 ml. Enzyme was replaced by water in a blank. Incubation was carried out at 30°C for 10 min, and the reaction was terminated by addition of 0.25 ml of 50% trichloroacetic acid. After centrifugation, α-ketobutyrate was determined with 3-methyl-2-benzothiazolinone hydrazone according to the procedure previously described [13].

One unit of enzyme is defined as the amount of enzyme that catalyzes the formation of 1.0 μmol of α-ketobutyrate per min. The specific activity is expressed as units per mg of protein. Protein was determined by the method of Lowry et al. [14] using crystalline egg albumin as a standard; with most column fractions, protein elution pattern was estimated by the 280 nm absorption.

** To whom correspondance should be addressed.

## 3. Results and discussion

### 3.1. *Purification of the enzyme*

All operations were performed at 0–5°C, unless otherwise stated and the buffers used contained 0.01% 2-mercaptoethanol.

Step 1: The washed cells (about 2.2 kg, wet weight) were suspended in 11 liters of 0.01 M potassium phosphate buffer (pH 7.2), containing $10^{-5}$ M pyridoxal-P, and disrupted continuously with a Dyno-Mill (Willy A, Switzerland) at a flow rate of about 2 liters per h followed by centrifugation. The supernatant solution was dialyzed for 24 h against 1000 volumes of the above-mentioned buffer. The precipitate formed during dialysis was discarded.

Step 2: The enzyme solution was applied to a DEAE-cellulose column (12 × 60 cm) equilibrated with the dialysis buffer. After the column was washed thoroughly with the buffer, and then with the buffer containing 0.12 M NaCl. The enzyme was eluted with the buffer supplemented with 0.15 M NaCl. The active fractions were combined and brought to 20% saturation with ammonium sulfate. The precipitate was removed by centrifugation and ammonium sulfate was added to the supernatant to 50% saturation. The resultant precipitate was dissolved in 0.1 M potassium phosphate buffer (pH 7.2) containing $10^{-4}$ M pyridoxal-P and 20% glycerol, and dialyzed against 10 000 volumes of 0.01 M potassium phosphate buffer, pH 7.2.

Step 3: The enzyme was re-chromatographed with a DEAE-cellulose column (6 × 60 cm) in the same manner as described above. The active fractions were combined and concentrated by addition of ammonium sulfate (50% saturation). The precipitate was dissolved and dialyzed as described in Step 2.

Step 4: The enzyme was concentrated by ultrafiltration through a Diaflow membrane (Amicon, Cambridge, Mass., USA) to approx. 100 mg of protein per ml, and the resulting solution was supplemented with pyridoxal-P (final concentration, $10^{-4}$ M). To the enzyme solution was added 1 M potassium phosphate buffer (pH 7.2) under stirring to bring its final concentration to 0.1 M. After heating at 60°C for 10 min, cooled rapidly and centrifuged to remove the precipitate.

Step 5: The supernatant solution was applied to a hydroxyapatite column (6 × 60 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.2) containing $10^{-5}$ M pyridoxal-P. The enzyme was eluted with 0.03 M potassium phosphate buffer (pH 7.2) containing $10^{-5}$ M pyridoxal-P. The active fractions were collected and concentrated by ultrafiltration.

Step 6: The enzyme solution was dialyzed for 10 h against 1000 volumes of 0.02 M potassium pyrophosphate buffer (pH 8.3). The enzyme was

Table 1
Purification of methioninase

| Step | Fraction | Total protein (mg) | Total units | Specific activity | Yield (%) |
|---|---|---|---|---|---|
| 1. | Crude extract | 132 000 | 5600 | 0.040 | 100 |
| 2. | First DEAE-cellulose chromatography | 17 700 | 3800 | 0.220 | 68 |
| 3. | Second DEAE-cellulose chromatography | 3500 | 1300 | 0.380 | 24 |
| 4. | Heat treatment | 1060 | 1300 | 0.800 | 24 |
| 5. | Hydroxyapatite chromatography | 550 | 900 | 1.640 | 17 |
| 6. | DEAE-Sephadex A-50 chromatography | 360 | 650 | 1.810 | 12 |
| 7. | Sephadex G-200 chromatography | 230 | 410 | 1.800 | 7 |

placed on a DEAE-Sephadex A-50 column (1.5 X 25 cm) equilibrated with the dialysis buffer containing 0.1 M KCl and the column was washed with the same buffer. Elution was carried out with a linear gradient between 60 ml of the buffer and 60 ml of the buffer supplemented with 0.3 M KCl. The active fractions were combined and quickly concentrated by ultrafiltration.

Step 7: The enzyme was applied to a Sephadex G-200 column (1.4 X 120 cm) buffered with 0.01 M potassium phosphate buffer (pH 7.2) containing $10^{-5}$ M pyridoxal-P and 0.3 M KCl. The column was developed with the same buffer. The active fractions were pooled and concentrated. A summary of the purification is given in table 1.

### 3.2. *Properties of the enzyme*

The purified enzyme was shown to be homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis (fig.1). The sedimentation coefficient ($S^0_{20}$, w) of the enzyme is 8.5 S. The mol. wt. was determined to be about 180 000 by the Sephadex G-200 gel filtration method of Andrews [15], with ovalbumin (43 000), bovine serum albumin (dimer, 136 000), bovine heart lactate dehydrogenase (140 000), bovine liver catalase (240 000) and bovine liver glutamate dehydrogenase (332 000) as standard proteins. The enzyme exhibits absorption maxima at 278 ($\epsilon$, 118 000) and 420 nm ($\epsilon$, 28 000) (fig. 2). No appreciable spectral shifts occurred on varying the pH (6.0–10.0). Incubation of

Fig.1(A)

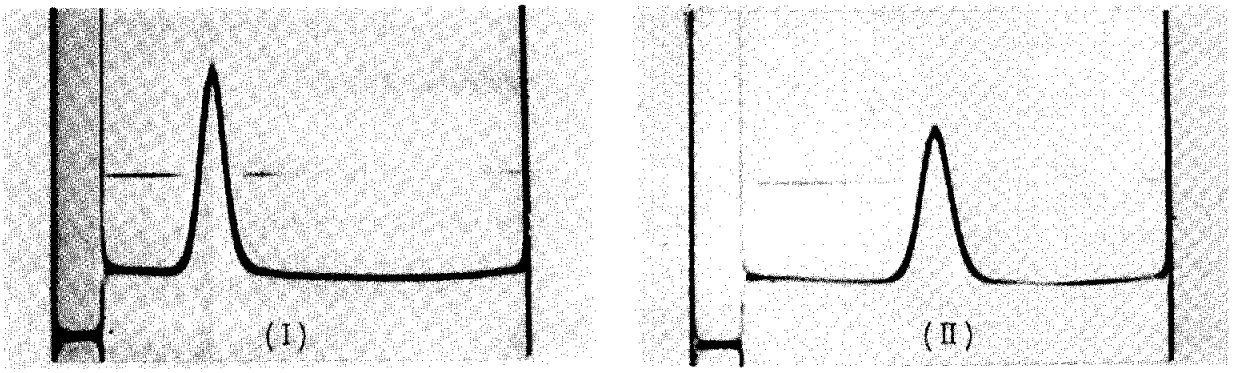


Fig.1(B)

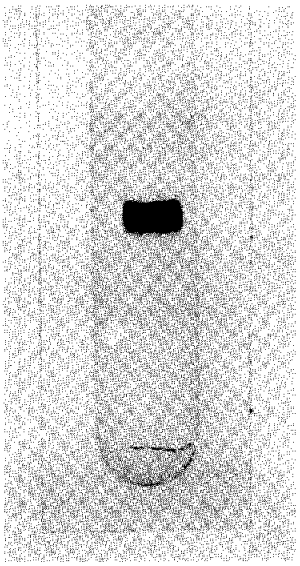

Fig.1. Sedimentation pattern (A) and disc gel electrophoresis (B) of methioninase. (A) Sedimentation pattern was obtained at 0.96% of protein concentration in 0.01 M potassium phosphate buffer (pH 7.2). Pictures were taken at a bar angle of 70°, 40 (I) and 60 (II) min after achieving top speed (59 780 rev/min). (B) A sample of the enzyme preparation (50 $\mu$g) was electrophoresed under the conditions of Davis [18].

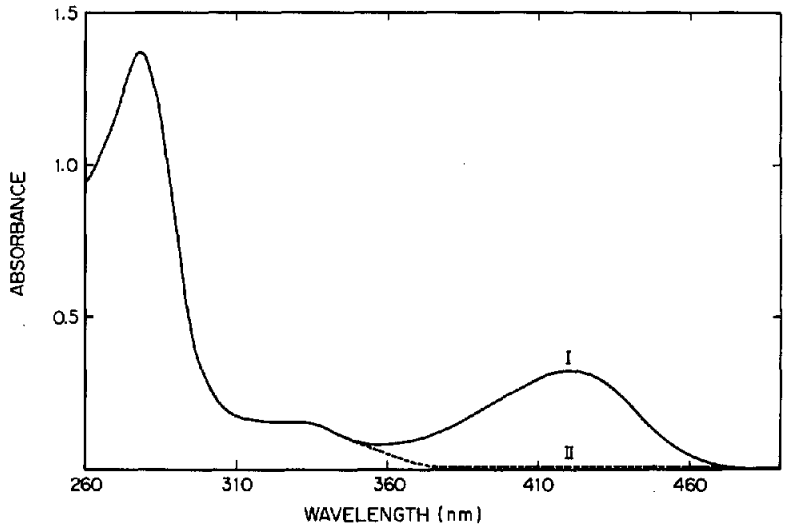


Fig.2. Absorption spectra of enzyme. Curve I, a 0.21% holoenzyme in 0.01 M potassium phosphate buffer (pH 7.2). Curve II, apoenzyme in the same buffer.

the enzyme with 1 mM hydroxylamine solution (pH 7.2), followed by dialysis against 0.01 M potassium phosphate buffer (pH 7.2) resulted in the formation of apoenzyme. The apoenzyme, which has no peak at 420 nm, can be fully reactivated by addition of pyridoxal-P.

L-Methionine was stoichiometrically converted by the enzyme into α-ketobutyrate, methanethiol and ammonia. In addition to L-methionine, which is the preferred substrate, several derivatives of L-methionine and L-cysteine, e.g., L-ethionine, DL-methionine sulfone, L-homocysteine and *S*-methyl-L-cysteine, serve as the effective substrates. *S*-Methyl-L-methionine and L-cysteine can be decomposed, though slowly, whereas D-methionine, D-cysteine and L-norleucine are inert. It has been reported that methioninase from soil bacterium [2], and also an enzyme from *Aspergillus* [16], which catalyzes the α,γ-elimination of D-methionine, but not the L-enantiomer, act on both α-keto and α-hydroxy analogues of methionine. α-Keto-γ-methiolbutyrate, however, is not susceptible to methioninase from *Ps. ovalis*, which is analogous to the enzyme of *Cl. sporogenes* [9] in this respect. These results provide evidence that the enzyme catalyzes both α,γ- and α,β-elimination reaction as noted previously [17].

$$RSCH_2CH_2CH(NH_2)COOH + H_2O \longrightarrow CH_3CH_2COCOOH + RSH + NH_3$$

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The enzyme has the maximum reactivity at about pH 8.0 for all the substrates. The $K_M$ values were calculated to be $1.33 \times 10^{-3}$ M for L-methionine, $0.77 \times 10^{-3}$ M for *S*-methyl-L-cysteine, $0.45 \times 10^{-3}$ M for L-ethionine and $0.43 \times 10^{-3}$ M for L-homocysteine. The detailed physicochemical properties and the reaction mechanism of the enzyme are currently under investigation.

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