

# Alanine Racemase from an Acidophilic Bacterium, *Acidiphilium organovorum*

Seow Teck Keong<sup>a)</sup>, Kenji Inagaki, Takashi Tamura  
and Hidehiko Tanaka

(Department of Bioresources Chemistry)

Alanine racemase (EC 5.1.1.1) was screened from several acidophilic bacteria. *Acidiphilium organovorum* 13H showed the highest activity and was chosen as the representative source to study alanine racemase from acidophilic bacteria. The enzyme was found to be localised in the cytoplasm of the bacteria. Relative molecular mass studies indicated that it had a dimeric native structure with identical subunits of about 34 kDa each. Maximum activity was observed between 50 and 60°C and at pH 9. There was no loss of enzyme activity even after incubation at 65°C. The loss of activity upon dialysis against pyridoxal 5'-phosphate-free buffer containing hydroxylamine, and its partial recovery upon subsequent dialysis against buffer containing pyridoxal 5'-phosphate suggested that the enzyme required pyridoxal 5'-phosphate as a co-factor for its catalytic activity.

**Key Words** : Alanine racemase, Acidophile, *Acidiphilium organovorum*, Pyridoxal 5'-phosphate

## Introduction

Alanine racemase (EC 5.1.1.1) is an enzyme that catalyses the racemisation of D- and L-alanine and is found widely in prokaryotes<sup>1,2)</sup>. This enzyme is essential for the metabolism of D-alanine in the synthesis of the peptidoglycan layer of the bacterial cell wall and has been studied as a target for various natural and synthetic antibiotics<sup>3)</sup>. All alanine racemases reported to date require pyridoxal 5'-phosphate for their activities through the formation of intermediary Schiff's bases with the substrates. Alanine racemase has been investigated in some detail from various bacteria such as *Bacillus stearothermophilus*<sup>4,5)</sup>, *Pseudomonas putida*<sup>6)</sup>, *P. fluorescens*<sup>7)</sup>, *Salmonella typhimurium*<sup>8)</sup> and *Escherichia coli*<sup>9)</sup>. Recently, the enzyme has also been isolated from an eukaryotic source, the fungus *Tolyposcladium niveum*<sup>10)</sup>.

In 1981, Harrison isolated an acidophilic bacterium from a culture of *Thiobacillus ferrooxidans* creating a new genus named *Acidiphilium* gen. nov.<sup>11)</sup> Since then many strains of the genus *Acidiphilium*, a group of gram-negative, non-spore forming and obligately acidophilic bacteria, have been isolated from soil, sewage and mine drainage and we have been studying the taxonomy, physiology, biochemistry and genetics of this genus<sup>12-15)</sup>.

As there have been no reports of the isolation of alanine racemases from acidophilic bacteria, this report represents the first attempt to study this enzyme from the genus *Acidiphilium*.

## Materials and Methods

*Materials* — All chemicals used were of ana-

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a) Graduate School of Natural Science and Technology

lytical grade. L-Alanine dehydrogenase (EC 1.4.1.1) was isolated from recombinant *E. coli* JM109 harbouring the plasmid pICR330 encoding the L-alanine dehydrogenase gene from *B. stearothermophilus*<sup>16</sup>.

*Cultivation of microorganisms* — Various strains of the genus *Acidiphilium* were cultured aerobically in an acidic (pH 3.0) medium containing 0.4% glucose, 0.05% yeast extract, 0.1% trypticase soy broth, 0.3% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% K<sub>2</sub>SO<sub>4</sub>, 0.01% K<sub>2</sub>HPO<sub>4</sub> and 0.05% MgSO<sub>4</sub>·7H<sub>2</sub>O at 30°C.

*Screening of alanine racemase* — The cells were harvested at the beginning of their stationary phases of growth by centrifugation, suspended in the standard buffer, 10 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol and 10 μM pyridoxal 5'-phosphate, and were disrupted by sonication at 4°C. After sonication, the suspension was centrifuged and the supernatant solution was dialysed against the standard buffer.

*Enzyme Assay* — Alanine racemase activity was assayed in the D- to L-alanine direction by a coupling reaction with L-alanine dehydrogenase<sup>4</sup>. The assay mixture contained 0.1 M CHES-NaOH buffer (pH 9.0), 2.5 mM NAD<sup>+</sup>, 30 mM D-alanine, 30 units of L-alanine dehydrogenase and an appropriate amount of enzyme solution, and the increase in absorbance at 340 nm was followed spectrophotometrically. One unit of alanine racemase activity was defined as the amount of enzyme catalysing the formation of 1 μmol of L-alanine per minute.

*Purification of Enzyme* — All procedures were carried out at 4°C. The standard buffer was used throughout the purification procedures unless otherwise stated. The harvested cells (about 50 g) were suspended in 125 mL of the

standard buffer. After sonication, centrifugation and dialysis against the standard buffer, the resultant cell-free extract was brought to 50% saturation with ammonium sulphate. The resultant precipitate after centrifugation was dissolved in and dialysed against the standard buffer. The dialysed solution was loaded onto a 570 mL (ϕ 5 × 27 cm) DEAE-Toyopearl 650M column (Tosoh Co.) equilibrated with the standard buffer. The column was washed with the standard buffer containing 20 mM KCl and eluted with the same buffer containing 80 mM KCl. The active fractions were pooled, concentrated and dialysed against the standard buffer containing 2.0 M KCl. After dialysis, the enzyme solution was applied onto an 80 mL (ϕ 3.8 × 7 cm) Butyl-Toyopearl 650M column (Tosoh) equilibrated with the standard buffer containing 2.0 M KCl. After washing with the same buffer used for equilibration, the enzyme was eluted with the standard buffer containing 1.5 M KCl. The active fractions were pooled, concentrated and dialysed against the standard buffer containing 2.0 M KCl, and reloaded onto a 20 mL (ϕ 3.8 × 1.5 cm) Butyl-Toyopearl 650M column (Tosoh) equilibrated with the buffer used for the dialysis of the sample. The column was washed with the same buffer and eluted by a linear gradient from 2.0 M to 1.5 M KCl. After the active fractions were pooled, concentrated and dialysed against the standard buffer, the resultant solution was subjected to gel filtration on a Superdex 200 16/60 column (Pharmacia LKB Biotechnology Inc.) equilibrated with the standard buffer containing 0.2 M KCl and connected to a fast protein liquid chromatography (FPLC) system. Elution was carried out with the standard buffer containing 0.2 M KCl at a flow rate of 0.5 mL/min with a fraction size of 1 mL. Active fractions were collected, concentrated and dialysed against the standard buffer before being stored at -20°C until further use.

**Protein Determination** — Protein concentrations were determined by using the Bio-Rad Protein Assay kit based on the Bradford method<sup>17)</sup> with bovine serum albumin as a standard.

### Results

**Screening of alanine racemase activity** — We screened 6 strains of the genus *Acidiphilium* for alanine racemase activity and found that all of the strains exhibited alanine racemase activities with specific activities ranging from about 3 - 16 mU/mg (Table 1). From these 6 strains, *Acidiphilium organovorum* 13H demonstrated the highest specific activity (16.2 mU/mg). *A. organovorum* 13H was thus chosen for the purpose of studying alanine racemase from acidophilic bacteria.

**Enzyme production against cultivation time** — We harvested the *A. organovorum* 13H culture at various times, disrupted the cells to obtain the cell-free extracts as described in Materials and Methods and measured the alanine racemase activities of the extracts. As can be seen from Fig. 1, the highest activity was recorded from cells harvested after 30 hr of growth, corresponding to the beginning of the stationary phase with an optical density of about 2.0 at 540 nm. From these results, we decided to harvest the cells at about 30 hr after inoculation for the purification of alanine racemase from *A. organovorum* 13H.

**Localisation of enzyme in the cell** — We subjected the cells to lysis with lysozyme to obtain the periplasmic fraction and the resuspended debris was sonicated to obtain the cytoplasmic fraction, and we found that about 90% of the alanine racemase activity in *A. organovorum* 13H was localised in the cytoplasm of the cell (Table 2). Acidic phosphatase and  $\beta$ -galactosidase were used as marker enzymes for the periplasmic and cytoplasmic fractions, respectively. Sonication was thus chosen as the method to be used to disrupt the cells for the purpose of purifying alanine racemase from *A. organovorum* 13H.

**Purification of alanine racemase** — After fractionation by ammonium sulphate (0 - 50% saturation), ion-exchange by DEAE-Toyopearl 650M, hydrophobic interaction chromatography

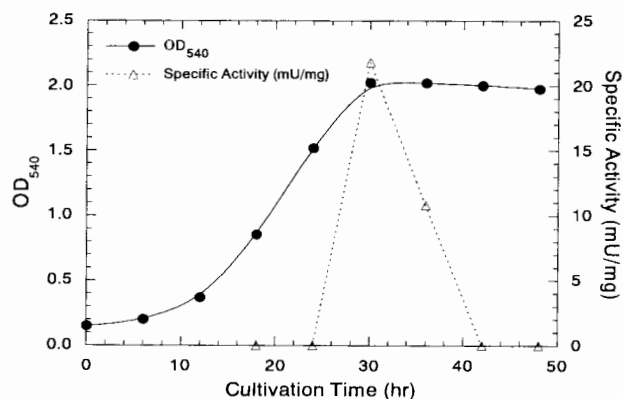


Fig. 1 Relationship between the growth of *A. organovorum* 13H and the production of alanine racemase.

Table 1 Alanine racemase activity of different strains of the genus *Acidiphilium*

<i>Acidiphilium</i> spp. Strains	Volume of Extract (mL)	Enzyme Activity (mU/mL)	Protein Concentration (mg/mL)	Specific Activity (mU/mg)
<i>A. aminolytica</i> JCM8796	13.0	10.0	0.961	10.4
<i>A. facilis</i> ATCC35904	12.0	2.81	0.897	3.13
<i>A. facilis</i> 16R	14.0	66.8	5.58	12.0
<i>A. facilis</i> 22M	13.0	4.04	0.599	6.75
<i>A. facilis</i> 24R	12.0	3.58	0.526	6.81
<i>A. organovorum</i> 13H	17.0	104	6.42	16.2

by Butyl-Toyopearl 650M (twice) and gel filtration through Superdex 200, we were able to partially purify the alanine racemase from *A. organovorum* 13H by about 700-fold with a yield of 5% (Table 3). The enzyme was then stored at  $-20^{\circ}\text{C}$ .

#### Relative molecular mass determination

Using a Superdex 200 16/60 column (Pharmacia) that was calibrated with molecular mass markers (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; bovine serum albumin, 67 kDa;

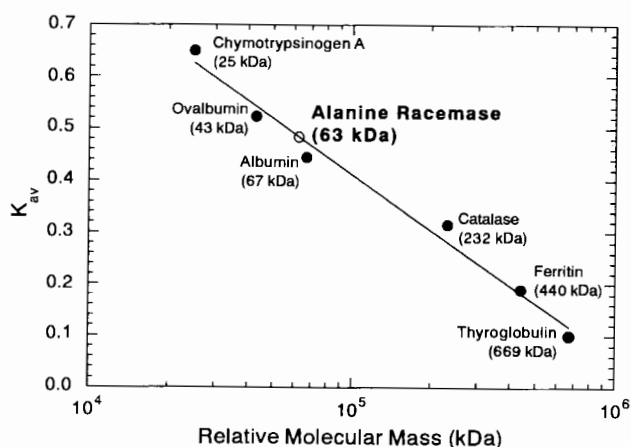


Fig. 2 Relative molecular mass of native alanine racemase determined by gel filtration through a Superdex 200 column.

ovalbumin, 43 kDa; chymotrypsinogen A, 25 kDa), we determined that the alanine racemase had a relative molecular mass of about 63 kDa (Fig. 2).

Results from SDS-PAGE showed that the denatured enzyme had a relative molecular mass of about 34 kDa when compared against the relative mobility of protein standards (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa) (Fig. 3).

The difference in the molecular mass values obtained from the native and denatured enzymes suggests that the alanine racemase from *A. or-*

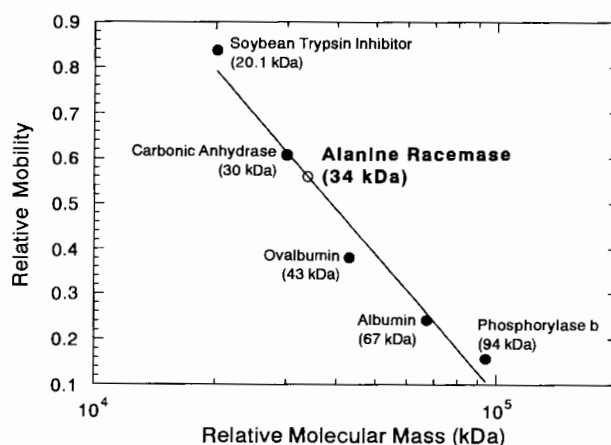


Fig. 3 Relative molecular mass of denatured alanine racemase determined by SDS-PAGE.

Table 2 Localisation of alanine racemase activity in *A. organovorum* 13H

Cellular Fractions	Total Protein (mg)	Total Activity (U)	Percentage of Activity (%)
Periplasmic fraction	18.0	0.58	9.4
Cytoplasmic fraction	315	5.6	90.6

Table 3 Purification of alanine racemase from *A. organovorum* 13H

Purification Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Purification Index (Fold)
Cell-Free Extract	733	46.4	0.0633	100	1.0
0.50% $(\text{NH}_4)_2\text{SO}_4$	575	50.8	0.0884	109	1.4
DEAE-Toyopearl 650M	60.4	46.7	0.774	101	12
1st Butyl-Toyopearl 650M	14.3	44.8	3.14	96.6	50
2nd Butyl-Toyopearl 650M	2.17	9.17	4.22	19.8	67
Superdex 200	0.0522	2.32	44.5	5.01	700

*ganovorum* 13H consists of two identical subunits.

**Physical characteristics** — The enzyme exhibited maximum activity between 50 and 60°C, and retained almost 100% of its activity even after being incubated at 65°C for 30 min. The enzyme, however, lost most of its activity upon incubation for the same duration of time at 70°C (Fig. 4). We also found that the optimum pH of the enzyme was at pH 9.

**Requirement of co-factor** — The enzyme retained all of its activity even after dialysis against pyridoxal 5'-phosphate-free standard buffer. However, all activity was lost upon dialysis against pyridoxal 5'-phosphate-free standard buffer containing 10 mM hydroxylamine. Subsequent dialysis against 10 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol and 100 µM pyridoxal 5'-phosphate restored the activity of the enzyme by about 18%, thereby suggesting that the enzyme required pyridoxal 5'-phosphate for its activity.

### Discussion

This is the first report of an alanine racemase from an acidophilic bacteria and from the genus *Acidiphilium*. We found that alanine racemase

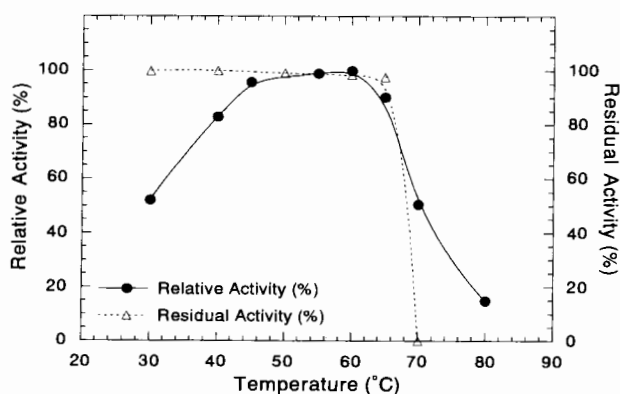


Fig. 4 Optimum temperature and thermal stability of alanine racemase from *A. organovorum* 13H.

activity was present in all of the 6 strains that were screened. This result concurs with the wide occurrence of the enzyme in bacteria.

We have found that the properties of the enzyme from *A. organovorum* 13H are very similar to alanine racemases from other sources. Gel filtration and SDS-PAGE results indicate that the enzyme comprises of a dimer of identical subunits with a relative molecular mass of about 34 kDa for each subunit. This is similar to other alanine racemases which are either monomers or dimers with molecular sizes of about 40 kDa<sup>18</sup>. The *A. organovorum* 13H enzyme is most active between 50 and 60°C and at pH 9, which is similar to the alanine racemase from *B. stearrowthermophilus*<sup>4</sup>, the most extensively studied of all reported alanine racemases<sup>19,20</sup>.

Although *A. organovorum* 13H thrives in an acidic environment, the pH of the cytoplasm of the cell is at around pH 7. Thus the optimum pH of the alanine racemase being in the alkaline region corresponds well with the results of the localisation study. That the alanine racemase occurs mainly in the neutral environment of the cytosol explains how the enzyme remains catalytically active even though the cell is cultivated at a pH 3-medium.

All known alanine racemases require pyridoxal 5'-phosphate as a co-factor for their catalytic activities<sup>2</sup>. Results from co-factor studies indicated that the enzyme from *A. organovorum* 13H may also be a pyridoxal 5'-phosphate dependent enzyme. However, further studies are necessary to confirm this, since there was no loss of activity seen after dialysis against pyridoxal 5'-phosphate-free buffer.

The alanine racemase from *A. organovorum* 13H is also relatively stable to heat and this makes it suitable for studies on the mechanism of its enzymatic reaction, which will be the basis of our further studies on this enzyme. Meanwhile, we are also investigating alanine racemases from other acidophilic microorganisms such as the

genus *Sulfolobus*, a hyperthermophilic acidophilic archaeon.

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## 好酸性細菌 *Acidiphilium organovorum* 由来の アラニンラセマーゼ

スィアオ テッキョン・稲垣 賢二・田村 隆・田中 英彦  
(生物資源開発学講座)

*Acidiphilium* 属好酸性細菌中のアラニンラセマーゼ活性を検索した。*Acidiphilium organovorum* 13H が最も高い活性を示したので、好酸性細菌由来の代表的なアラニンラセマーゼとして研究対象とした。本酵素の局在性を調べたところ、細胞質に存在することが明らかとなった。また本酵素は同一のサブユニット (約 34 kDa) からなる二量体構造をとることが示された。至適反応条件は 50-60°C, pH 9 であった。また 65°C で 30 分のインキュベーション後も活性が失われず、熱に対して比較的安定な酵素であった。Hydroxylamine による透析で活性が失われ、これを pyridoxal 5'-phosphate を含む緩衝液により透析をした結果、活性が部分的に回復したことから、本酵素は pyridoxal 5'-phosphate を補酵素として要求することが示唆された。