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Purification and characterization of a novel enzyme, L-*threo*-3-hydroxyaspartate dehydratase, from *Pseudomonas* sp. T62

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Abstract

L-*threo*-3-Hydroxyaspartate dehydratase (L-*threo*-3-hydroxyaspartate hydro-lyase), which exhibited specificity for L-*threo*-3-hydroxyaspartate ($K_m = 0.74$ mM, $V_{max} = 37.5$ μmol min^{-1} (mg protein)$^{-1}$) but not for D-*threo* or D,L-*erythro*-3-hydroxyaspartate, was purified from a cell-free extract of *Pseudomonas* sp. T62. The activity of the enzyme was inhibited by hydroxylamine and EDTA, which suggests that pyridoxal 5′-phosphate and divalent cations participate in the enzyme reaction. The NH_2-terminal amino acid sequence showed significant similarity to the *Saccharomyces cerevisiae* YKL218c gene product, a hypothetical threonine dehydratase. However, the purified enzyme showed no threonine dehydratase activity.

Keywords: 3-Hydroxyaspartate dehydratase; Threonine dehydratase; Pyridoxal 5′-phosphate; Divalent cation

1. Introduction

3-Hydroxyaspartate and its derivatives have attracted the attention of biochemists because they are competitive blockers of the excitatory glutamate/aspartate transporters of the mammalian nervous system [1,2]. However, 3-hydroxyaspartate has two chiral centers and their four stereoisomers have been difficult to synthesize.

While the biochemical activity of 3-hydroxyaspartate has been investigated in considerable detail, little is known about enzymes which act on 3-hydroxyaspartate isomers. Only two microbial enzymes, *erythro*-3-hydroxyaspartate aldolase (EC 4.1.3.14) [3] and *erythro*-3-hydroxyaspartate dehydratase (EC 4.2.1.38) [4] are known and no enzyme which acts

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on the *threo* form of 3-hydroxyaspartate has been reported.

We found that an extract of a newly isolated bacterium, *Pseudomonas* sp. T62, exhibited dehydratase activity specific for L-*threo*-3-hydroxyaspartate but not for *erythro*-3-hydroxyaspartate. We report here the purification, partial characterization and NH_2-terminal amino acid sequence of this novel enzyme, designated as L-*threo*-3-hydroxyaspartate dehydratase (L-*threo*-3-hydroxyaspartate hydro-lyase) from *Pseudomonas* sp. T62.

2. Materials and methods

2.1. Materials

D,L-*threo*-3-Hydroxyaspartate was synthesized by the ring-opening reaction of *cis*-epoxysuccinic acid as has been described previously [5]. D,L-*erythro* and D- and L-*threo* isomers of 3-hydroxyaspartate were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Microorganism and cultivation

Pseudomonas sp. T62 isolated from soil and identified in our laboratory was used. This strain was deposited in the AKU culture collection (Faculty of Agriculture, Kyoto University) and numbered AKU882. The bacterium was grown aerobically in a medium containing 3 g D,L-*threo*-3-hydroxyaspartate, 10 g glucose, 1 g KH_2PO_4, 1 g K_2HPO_4, 1 g yeast extract and 0.2 g $MgSO_4 \cdot 7H_2O$ per 1 l of tap water, pH 7.0. A loopful of *Pseudomonas* sp. T62 cells was inoculated into a test tube (16.5×165 mm) containing 5 ml medium and then incubated for 16 h at 30°C. The culture was transferred to a 2-l shaking flask containing 500 ml medium and then grown for 35 h at 30°C.

2.3. Enzyme assays

3-Hydroxyaspartate dehydratase activity was determined by two different methods. In a spectrophotometric assay (malate dehydrogenase (MDH) method), the dehydratase was coupled to MDH, which in the presence of NADH reduces the oxalacetate formed to L-malate. The disappearance of NADH was measured at 340 nm. The standard assay mixture contained 5 mM D,L-*threo*-3-hydroxyaspartate, 10 μM pyridoxal 5′-phosphate (PLP), 1 mM $MnCl_2$, 1 U MDH (Oriental Yeast, Tokyo, Japan), 100 mM Tris-HCl buffer (pH 8.0) and an appropriate amount of enzyme, in a final volume of 2.5 ml. One unit of the enzyme was defined as the amount capable of catalyzing the oxidation of 1 μmol NADH per min.

In the chromatographic assay of pyruvate (high-performance liquid chromatography (HPLC) method), the oxalacetate formed after 30 min enzyme reaction was decarboxylated to pyruvate by heat treatment at 100°C for 3 min and the pyruvate formed was measured by ion-pair HPLC [6]. The standard assay mixture was basically the same as the MDH method, but PLP or $MnCl_2$ were omitted when chemicals and metal ions were added to the reaction mixture. The HPLC analysis of pyruvate was done as follows: column, Cosmosil $5C_{18}$-ARII 4.6×250 mm (Nacalai tesque, Kyoto, Japan); column temperature, 40°C; mobile phase, 10% acetonitrile/5 mM tetra-*n*-butylammonium phosphate/20 mM potassium phosphate buffer (pH 7.0); detection, 210 nm; flow rate, 0.5 ml min^{-1}.

Threonine, serine and phenylserine dehydratase activities were measured as described previously [7–9].

Protein concentrations were determined by the dye-binding method of Bradford [10] with a Bio-Rad Protein Assay kit using bovine serum albumin as the standard.

2.4. Purification of the enzyme

All purification procedures were carried out at 4°C in 10 mM Tris-HCl buffer (pH 8.0) containing 0.01 mM PLP, 1 mM $MnCl_2$, and 0.1 mM dithiothreitol unless otherwise stated. *Pseudomonas* sp. T62 cells (30 g wet weight) obtained from a 5-l culture were disrupted with an ultrasonic oscillator. After centrifugation ($8000 \times g$, 40 min), the resulting supernatant was fractionated with solid ammonium sulfate. The supernatant obtained on 20% saturation was collected, dialyzed against the buffer, and applied to a DEAE-Sepharose FF (Pharmacia Biotech, Uppsala, Sweden) column (3.0×20 cm) equilibrated with buffer. The enzyme was eluted with a 0–0.8 M linear

NaCl gradient. The dialyzed enzyme solution was applied to a MonoQ HR10/10 (Pharmacia Biotech, Uppsala, Sweden) column (1.0×10 cm) equilibrated with buffer connected to a FPLC system (Pharmacia Biotech, Uppsala, Sweden). The enzyme was eluted with a 0–1 M linear NaCl gradient. The concentrated enzyme solution was applied to a Superdex-200 HR10/30 (Pharmacia Biotech, Uppsala, Sweden) column (1.0×30 cm) equilibrated with buffer supplemented with 0.15 M NaCl. The enzyme was eluted with buffer supplemented with 0.15 M NaCl. The dialyzed enzyme solution was applied to a MonoQ HR5/5 column (0.5×5 cm) and eluted with a 0–0.6 M linear NaCl gradient. Active fractions were collected, dialyzed against the buffer, and used as the enzyme for characterization.

2.5. Molecular mass determination

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in gels containing 12.5% acrylamide for the separation gel and 4.5% acrylamide for the stacking gel. For molecular mass determination of the enzyme subunit by SDS-PAGE, the following molecular mass standards were used: phosphorylase *b* (relative molecular mass (M_r) = 97 400), bovine serum albumin (66 300), aldolase (42 400), carbonic anhydrase (30 000), trypsin inhibitor (20 100), and lysozyme (14 400).

The molecular mass of the native protein was determined by gel-permeation liquid chromatography (GPC) on Superdex-200 PC3.2/30 (Pharmacia Biotech, Uppsala, Sweden) and Cosmosil 5Diol-300 (Nacalai tesque, Kyoto, Japan) using glutamate dehydrogenase (M_r = 290 000), lactate dehydrogenase (142 000), enolase (67 000), myokinase (32 000), and cytochrome *c* (12 400) as molecular mass standards. The columns were equilibrated and eluted with 10 mM potassium phosphate buffer (pH 7.0) containing 0.2 M NaCl at a flow rate of 0.2 ml min^{-1}.

3. Results

3.1. Induction of the enzyme

When *Pseudomonas* sp. T62 was cultured in the medium described above, the cell-free extract showed approximately 0.12 U (mg protein)$^{-1}$ of the enzyme activity. However, when D,L-*threo*-3-hydroxyaspartate in the medium was replaced by an equal amount of L-threonine, L-aspartate, or peptone, the cell-free extract showed no or only a trace (< 0.02 U (mg protein)$^{-1}$) level of the enzyme activity. These results suggest that the enzyme was induced by the 3-hydroxyaspartate in the medium.

3.2. Purification of L-*threo*-3-hydroxyaspartate dehydratase and molecular mass

Through the procedures described above, the enzyme was purified approximately 68-fold to homogeneity, with approximately 2% recovery (Table 1).

The purified enzyme preparation gave a single band on SDS-PAGE. Further evidence for the purity of the enzyme was obtained by HPLC-GPC, which gave a symmetrical single protein peak.

The M_r of the enzyme was estimated to be 59 000 on a calibrated column of Superdex-200 PC3.2/30, and 57 000 on a Cosmosil 5Diol-300. The M_r of the subunit was estimated to be about 39 000 by SDS-PAGE, suggesting that the enzyme behaves in a non-ideal manner in HPLC-GPC and that the native enzyme may be either a monomer or dimer.

Table 1
Purification of L-*threo*-3-hydroxyaspartate dehydratase from *Pseudomonas* sp. T62

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Cell-free extract	2 700	319	0.118	1	100
Ammonium sulfate	1 840	269	0.146	1.2	84.3
DEAE-Sepharose	421	73.9	0.176	1.5	23.2
MonoQ (I)	12.4	33.1	2.67	22.6	10.4
Superdex-200	1.36	8.80	6.50	55	2.76
MonoQ (II)	0.84	6.8	8.07	68.2	2.13

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                    10                  20                  30
pHD:     M Q L S S Y H D V I K A A E R K E G F A N R T P V F T S R X L D ···
YKL218c: M I V P T Y G D V L D A S N R I K E Y V N K T P V L T S R M L N ···
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Fig. 1. Comparison of the NH_2-terminal amino acid sequence of pHD, the L-*threo*-3-hydroxyaspartate dehydratase from *Pseudomonas* sp. T62, and YKL218c, the YKL218c gene product of *S. cerevisiae*.

3.3. N-Terminal amino acid sequence analysis

Automated Edman degradation of the enzyme protein with a pulsed liquid phase sequencer revealed that the NH_2-terminal amino acid sequence is Met-Gln-Leu-Ser-Ser-Tyr-His-Asp-Val-Ile-Lys-Ala-Ala-Glu-Arg-Lys-Glu-Gly-Phe-Ala-Asn-Arg-Thr-Pro-Val-Phe-Thr-Ser-Arg-X-Leu-Asp- (X is an unidentified amino acid). When this sequence was compared with those stored in protein sequence databases (PRF, PIR, and SWISS-PROT) by means of the sequence similarity search programs BLAST [11] and Fasta [12], this sequence was found to be significantly similar to the partial amino acid sequences of the *Saccharomyces cerevisiae* YKL218c gene product, which is registered as a threonine dehydratase homologue. The sequence alignments are shown in Fig. 1.

3.4. Substrate specificity of the enzyme

The enzyme showed a high specificity towards L-*threo*-3-hydroxyaspartate. All other 3-hydroxyamino acids tested, i.e. D,L-*erythro*- and D-*threo*-3-hydroxyaspartate, D-threonine, L-threonine, D,L-*allo*-threonine, D,L-*threo*-3-phenylserine, D-serine, and L-serine, were not substrates for this enzyme either at 5 or 50 mM.

With L-*threo*-3-hydroxyaspartate, normal hyperbolic kinetics were observed and the K_m and V_{max} values, calculated from Eadie-Hofstee plots, were 0.74 mM and 37.5 μmol min^{-1} (mg protein)$^{-1}$, respectively.

3.5. Absorption spectrum

The enzyme exhibited absorption maxima at 280 and 412 nm. Solutions of the pure enzyme are distinctly yellow in color. These results suggested that the enzyme contains PLP as prosthetic group.

3.6. Effects of chemicals and metal ions

Hydroxylamine and EDTA were added to the standard reaction mixture each at a final concentration of 1 mM, and enzyme activity was then measured by the HPLC method. The enzyme was strongly inhibited by hydroxylamine (91.2% inhibition), suggesting that PLP participates in the enzyme reaction, as were *erythro*-3-hydroxyaspartate dehydratase and L-threonine dehydratase. The enzyme was also strongly inhibited by EDTA (77% inhibition), suggesting that metal ions were involved in the enzyme reaction.

The effects of divalent cations were also measured using the enzyme dialyzed against the buffer minus $MnCl_2$. The specific activity of the enzyme was decreased to approximately 0.5 U mg^{-1} after 20 h dialysis against the buffer minus $MnCl_2$. When 1 mM $MnCl_2$, $CoCl_2$, $FeCl_2$, $MgCl_2$, and $CaCl_2$ were added, the relative enzyme activity increased to 290, 200, 180, 160, and 160% of control activity, respectively. In contrast, $CuCl_2$ and $ZnCl_2$ caused inhibition (63 and 36% relative activity, respectively).

4. Discussion

The microbial metabolism of 3-hydroxyaspartate was described by Kornberg and Morris [13]. They reported two enzymes, *erythro*-3-hydroxyaspartate aldolase (EC 4.1.3.14) and *erythro*-3-hydroxyaspartate dehydratase (EC 4.2.1.38), in *Micrococcus denitrificans*, which act on 3-hydroxyaspartate. However, these enzymes have not been purified to homogeneity or well characterized. To the best of our knowledge, the present report reveals the first instance of an enzyme which catalyzes the deamination of *threo*-3-hydroxyaspartate. This enzyme does not act either on D,L-*erythro*-3-hydroxyaspartate or L-threonine, although the NH_2-terminal amino acid sequence showed a high similarity to the hypothetical threonine dehydratase of *S. cerevisiae*. Thus, the enzyme is distinct from *erythro*-3-hydroxyaspartate dehydratase (EC 4.2.1.38.) or L-threonine dehydratase (EC 4.2.1.16.).

The open reading frame YKL218c of *S. cerevisiae* is registered as threonine dehydratase homologue on the basis of its sequence similarity. However, the protein from the YLK218c gene has not been identified and characterized yet, and whether YLK218c encodes L-*threo*-3-hydroxyaspartate dehydratase activity or not is still unknown.

Divalent cation-activated or -dependent pyridoxal enzymes are rare. To our knowledge, only two, D-threonine aldolase from *Arthobacter* sp. DK-38 [14,15] and D-glucosaminate dehydratase from *Pseudomonas fluorescens* [16,17], have been purified to homogeneity and well characterized. *erythro*-3-Hydroxyaspartate dehydratase in *M. denitrificans* has also been reported to be divalent cation-dependent [4]. However, it has not been purified to homogeneity.

Although the physiological function of this enzyme remains to be clarified, an enzyme which recognizes the configurations of two asymmetric carbon atoms is unique. The dehydratase reaction catalyzed by this enzyme may be one of the reactions which enables *Pseudomonas* sp. T62 to grow on a medium containing D,L-*threo*-3-hydroxyaspartate as a sole carbon source.

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