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# Purification, characterization and amino acid sequence of a novel enzyme, D-*threo*-3-hydroxyaspartate dehydratase, from *Delftia* sp. HT23

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**D-*threo*-3-hydroxyaspartate dehydratase (D-THA DH) was purified from the cell-free extract of the soil-isolated bacterium *Delftia* sp. HT23. The enzyme exhibited dehydratase activity towards D-*threo*-3-hydroxyaspartate, L-*threo*-3-hydroxyaspartate, L-*erythro*-3-hydroxyaspartate and D-serine. Absorption of the purified enzyme at 412 nm suggests that it contains pyridoxal 5′-phosphate (PLP) as a cofactor. The $NH_2$-terminal and internal amino acid sequences showed significant similarity to hypothetical alanine racemase of genome-sequenced *Delftia acidovorans* SPH-1; however, the purified enzyme showed no alanine racemase activity. Using the sequence information of *D. acidovorans* SPH-1, the gene encoding D-THA DH was cloned. The deduced amino acid sequence, which belongs to the alanine racemase family, shows significant (26–36%) similarity to D-serine dehydratase of both *Saccharomyces cerevisiae* and chicken. In order to obtain purified D-THA DH efficiently, the gene was expressed in *Escherichia coli*. The recombinant enzyme was highly activated by divalent cations, such as $Mn^{2+}$, $Co^{2+}$ and $Ni^{2+}$. Site-directed mutagenesis experiment revealed that lysine 43 is an important residue involved in PLP binding and catalysis. This is the first reported enzyme that acts on D-THA. In addition, this enzyme is the first example of a prokaryotic dehydratase belonging to the fold-type III PLP-dependent enzyme family.**

*Keywords*: alanine racemase/*Delftia* sp. HT23/D-*threo*-3-hydroxyaspartate dehydratase/pyridoxal 5′-phosphate.

*Abbreviations*: DH, dehydratase; D-EHA, D-*erythro*-3-hydroxyaspartate; D-THA, D-*threo*-3-hydroxyaspartate; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria–Bertani; L-EHA, L-*erythro*-3-hydroxyaspartate; L-THA, L-*threo*-3-hydroxyaspartate; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NADH, nicotinamide adenine dinucleotide; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PLP, pyridoxal 5′-phosphate; SDS, sodium dodecyl sulphate; TLC, thin layer chromatography.

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 centres and their four stereoisomers, *i.e.* D-*threo*-3-hydroxyaspartate (D-THA), L-*threo*-3-hydroxyaspartate (L-THA), D-*erythro*-3-hydroxyaspartate (D-EHA) and L-*erythro*-3-hydroxyaspartate (L-EHA), have been difficult to synthesize (*3*).

The biochemical activity of 3-hydroxyaspartate has been investigated in considerable detail (*4*). Nevertheless, little is known about the enzymes that act on 3-hydroxyaspartate isomers, although two microbial enzymes, *erythro*-3-hydroxyaspartate aldolase (EC 4.1.3.14) (*5*, *6*) and *erythro*-3-hydroxyaspartate dehydratase (EC 4.3.1.20) (*7*), were identified many years ago. Recently, we reported that L-*threo*-3-hydroxyaspartate dehydratase (EC 4.3.1.16) isolated from the soil-isolated bacterium *Pseudomonas* sp. T62 exhibits dehydratase activity specifically towards L-THA and not towards other 3-hydroxyaspartate isomers (*8*, *9*). The amino acid sequence and detailed biochemical features, including side reactions, of this enzyme have also been reported (*9*). However, no enzyme acting on the D-*threo* form of 3-hydroxyaspartate has been reported. The enzyme degrading D-THA might be useful for enzymatic optical resolution of DL-THA to produce optically pure L-THA. Because there has been no report about enzyme acting on D-THA so far, in order to obtain the amino acid sequence information of D-THA converting enzyme, enzyme purification from microorganism that produces D-THA converting enzyme was necessary. Thus, we screened microorganisms that can utilize D-THA as a sole carbon source and found that a newly isolated bacterium, *Delftia* sp. HT23, which produces an enzyme that catalyses the dehydratase reaction of D-THA to oxaloacetate. We designated this enzyme as D-*threo*-3-hydroxyaspartate

dehydratase (D-THA DH; D-*threo*-3-hydroxyaspartate ammonia-lyase).

We report here the purification, partial characterization and amino acid sequence of this novel enzyme, D-THA DH, from *Delftia* sp. HT23. The comparison of amino acid sequence of this enzyme with known eukaryotic D-serine dehydratases is also discussed. This information may provide useful clues for understanding mechanisms of these PLP-dependent enzymes.

## Materials and Methods

### Materials

The L-THA was purchased from Tocris Cookson, Ltd (Bristol, UK); L-EHA, from Wako Pure Chemicals (Osaka, Japan); and DL-THA, from Tokyo Kasei Kogyo (Tokyo, Japan). D-THA was prepared from DL-THA by enzymatic resolution using L-THA dehydratase from *Saccharomyces cerevisiae* (*10*). Restriction endonucleases were obtained from Nippon Gene (Toyama, Japan). All other chemicals were of analytical grade and commercially available.

### Screening of D-THA DH-producing microorganisms from soil

Basal agar medium contained 3 g of D-THA, 1 g of $(NH_4)_2SO_4$, 1 g of $KH_2PO_4$, 1 g of $K_2HPO_4$, 0.5 g of yeast extract, 0.2 g of $MgSO_4{\cdot}7H_2O$ and 2% agar in 1 l of tap water, pH 7.0. Each of the soil samples corrected from Sapporo city area was suspended in 0.85% NaCl solution, streaked onto the basal agar medium and incubated at 30°C for 48 h. Strains forming colonies were isolated and transferred to the same agar medium and cultured at 30°C until growth of the microorganisms was apparent. The isolated colonies were then transferred to liquid medium containing 10 g of glucose, 3 g of D-THA, 1 g of $KH_2PO_4$, 1 g of $K_2HPO_4$, 0.5 g of yeast extract, 0.2 g of $MgSO_4{\cdot}7H_2O$ in 1 l of tap water (pH 7.0) and cultivated 30°C for 16 h with shaking. The cells harvested by centrifugation were used for the reaction. Each reaction mixture contained 50 mM D-THA, 0.5 mM $MgSO_4{\cdot}7H_2O$ and 50 mM potassium phosphate buffer (pH 7.0). The reaction was carried out at 30°C for 16 h with shaking. Degradation of D-THA in the reaction mixture was monitored by thin layer chromatography (TLC) using the developing solvent ethanol/28% ammonia solution/water = 7/1/2 (v/v/v), and was visualized by ninhydrin. D-THA DH activity of the cell-free extract prepared from the strains showing high D-THA-degrading activity on TLC was measured as described below. Determination of 16S rDNA was done using a MicroSeq 500 16s rDNA Bacterial Identification Sequencing kit (Applied Biosystems, CA, USA).

### Microorganism and cultivation

*Delftia* sp. HT23, isolated from soil and identified in our laboratory was used. Bacteria were grown aerobically in medium containing 10 g of DL-THA, 10 g of glucose, 1 g of $KH_2PO_4$, 1 g of $K_2HPO_4$, 0.5 g of yeast extract and 0.2 g of $MgSO_4{\cdot}7H_2O$ in 1 l of tap water, pH 7.0. A loopful of *Delftia* sp. HT23 cells was inoculated into a test tube (16.5 × 165 mm) containing 5 ml of medium and was cultivated for 24 h at 30°C. The culture was transferred to a 2-l flask containing 400 ml of medium and grown for 48 h at 30°C, with shaking. *Escherichia coli* JM109 was used as the host cell for the cloning and expression of the D-THA DH gene *(dthadh)*. The *E. coli* cells were grown at 37°C in Luria-Bertani (LB) medium containing 1% polypeptone, 0.5% yeast extract and 1% NaCl (pH 7.0). When necessary, 100 μg/ml ampicillin were added to the medium.

### Enzyme assays

The activity of 3-hydroxyaspartate dehydratase was determined as described previously (*8*). One unit of the enzyme was defined as the amount capable of catalysing the oxidation of 1 μmol of NADH per min. Serine dehydratase activity (*11*) and alanine, serine and aspartate racemase activities (*12–14*) were measured as described earlier. Protein concentrations were determined by the dye-binding method of Bradford (*15*) with a Bio-Rad protein assay kit, using bovine serum albumin as the standard.

### 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, 0.1 mM $MnCl_2$ and 0.1 mM dithiothreitol, unless otherwise stated. *Delftia* sp. HT23 cells (30 g wet weight) obtained from a 2.4-l culture were disrupted with an ultrasonic oscillator. After centrifugation (8,000*g* for 40 min), the supernatant was fractionated with solid ammonium sulphate. The precipitate obtained at 40–60% saturation was collected, dialysed against 10 l of the buffer for 18 h and applied to a HiPrep Q FF 16/10 column (1.6 × 10 cm; GE healthcare, UK) equilibrated in the buffer. The enzyme was eluted with a linear gradient of 0–1.0 M NaCl in 160 ml of the buffer at a flow rate of 2.0 ml/min. The enzyme eluted at ~0.55 M NaCl.

The concentration of $(NH_4)_2SO_4$ was adjusted to 1 M by the addition of solid $(NH_4)_2SO_4$, and the enzyme solution was loaded onto a HiTrap Phenyl FF column (high sub, 1.0 × 10 cm; GE Healthcare), previously equilibrated with buffer containing 1 M $(NH_4)_2SO_4$, connected to a FPLC system (Pharmacia Biotech, Sweden). The enzyme was eluted with a linear gradient of 1 to 0 M $(NH_4)_2SO_4$ in 230 ml of buffer at a flow rate of 1 ml/min. The activity-containing fractions, which eluted with ~0.25 M $(NH_4)_2SO_4$, were pooled and dialysed against 3 l of buffer for 8 h.

The concentrated enzyme solution was applied to a Superdex-200 HR10/30 column (1.0 × 30 cm; GE Healthcare) equilibrated with buffer containing 0.15 M NaCl, and the enzyme was eluted with the same buffer.

The enzyme solution was then applied to a RESOURCE Q column (0.5 × 5 cm) previously equilibrated with the same buffer and was eluted by FPLC with a linear gradient of 0–0.8 M NaCl in 35 ml of buffer at a flow rate of 1 ml/min. The activity-containing fractions, eluting with ~0.3 M NaCl, were collected.

The $(NH_4)_2SO_4$ concentration of the enzyme solution was adjusted to 1 M by the addition of solid $(NH_4)_2SO_4$, and the enzyme solution was applied to a HiTrap Butyl FF column (1.0 × 10 cm; GE Healthcare) previously equilibrated with the buffer containing 1 M $(NH_4)_2SO_4$. The enzyme was eluted by FPLC with a linear gradient of 1–0 M $(NH_4)_2SO_4$ in 230 ml of buffer at a flow rate of 2 ml/min. The activity-containing fractions, which eluted with ~0.3 M $(NH_4)_2SO_4$, were pooled, dialysed against 3 l of the buffer for 8 h and used as the purified enzyme.

### Molecular weight determination

SDS–PAGE was performed using a 4.5% acrylamide stacking gel and 12.5% acrylamide separation gel. For molecular weight determination of the enzyme subunit by SDS–PAGE, the following molecular weight standards were used: phosphorylase *b* ($M_r$ = 97,400), bovine serum albumin (66,300), aldolase (42,400), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and lysozyme (14,400).

The molecular weight of the native protein was determined by gel-permeation liquid chromatography on TSKgel Super-SW3000 (Tosoh, 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 weight standards. The column was equilibrated and eluted with 100 mM potassium phosphate buffer (pH 6.7) containing 0.1 M $Na_2SO_4$, and 0.05% (w/v) $NaN_3$ at a flow rate of 0.35 ml/min.

The molecular weight of the recombinant enzyme was also estimated using a MALDI-TOF-MS (Voyager Biospectrometry, Applied Biosystems) in linear mode at 25-kV acceleration voltage, with sinapic acid as the matrix.

### Amino acid sequence analysis

The N-terminal and internal peptide sequences were determined as described earlier (*16*) with a modification. After lysyl endopeptidase digestion of the enzyme, the peptides were separated by HPLC (L-2000 system; Hitachi-Hitec Corp., Tokyo) on a CAPCELL PAK $C_{18}$ MGIII column (4.6 × 250 mm; SHISEIDO, Tokyo). Edman degradation was performed at the Center for Instrumental Analysis at Hokkaido University.

### Construction of the expression plasmid

Total DNA was isolated from *Delftia* sp. HT23 using Isoplant II (Nippon Gene, Toyama, Japan). For the expression of *dthadh* in *E. coli*, a DNA fragment containing the open reading frame of *dthadh* was prepared by PCR using *Delftia* sp. HT23 genomic

DNA as a template and oligonucleotide sense (5′-ATGCGGATCCATGCAAGACACACTTCTGAC-3′) and anti-sense primers (5′-ATATAAGCTTTCACCAGCCATGGAGCCGCT-3′) (the underlined sequences are *Bam*HI and *Hin*dIII sites, respectively). The PCR mixture (50 µl) contained 10 pmol of each primer, 0.2 mM of each dNTP and 1.25 U of PrimeSTAR HS DNA polymerase (Takara Bio, Ohtsu, Japan). The thermal cycler program was 98°C for 10 s, 55°C for 5 s and 72°C for 1.5 min. The unique amplified band corresponding to ~1,100 bp was digested with *Bam*HI and *Hin*dIII, and then ligated into the *Bam*HI and *Hin*dIII sites of pQE30 (Qiagen, Hilden, Germany) to obtain pQE30dthadh. The expression vector was introduced into *E. coli* JM109 cells and the nucleotide sequence of the insert was confirmed.

#### Expression and purification of recombinant D-THA DH

The transformed *E. coli* JM109 cells carrying pQE30dthadh were grown at 37°C in 50 ml of LB medium containing ampicillin. To induce gene expression, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture medium when the absorbance at 600 nm reached 0.3. After cultivation for another 8 h at 37°C, cells were harvested by centrifugation. All purification procedures were carried out at 4°C in 10 mM Tris–HCl buffer (pH 8.0) containing 0.01 mM PLP, 0.1 mM $MnCl_2$ and 0.1 mM dithiothreitol. The cells (0.62 g wet weight) obtained from a 50-ml culture were disrupted with an ultrasonic oscillator. After centrifugation (8,000*g* for 15 min), the supernatant was applied to a HisTrap HP column (0.7 × 2.5 cm; GE Healthcare, UK) equilibrated with buffer supplemented with 20 mM imidazole. The enzyme was eluted by FPLC with a step-wise gradient of 20–500 mM imidazole. Fractions showing activity, which eluted with ~150 mM imidazole, were collected, dialysed against the buffer and used as the enzyme for characterization.

#### Site-directed mutagenesis

A mutant enzyme, K43A, was prepared according to the protocol for a PrimeSTAR Mutagenesis Basal Kit (Takara Bio). The nucleotide substitutions were confirmed by DNA sequencing. The mutant enzyme was produced in *E. coli* JM109 cells and purified by the same procedure as that used for the wild-type recombinant enzyme.

#### EDTA-treated enzyme

Recombinant D-THA DH was dialysed against 10 mM Tris–HCl buffer (pH 8.0) containing 0.01 mM PLP, 0.1 mM dithiothreitol and 5 mM EDTA for 12 h at 4°C. To remove EDTA, the enzyme solution was further dialysed against the same buffer without EDTA for 12 h at 4°C.

#### Nucleotide sequence accession number

The nucleotide sequence of the *dthadh* gene of *Delftia* sp. HT23 has been deposited in the DDBJ/EMBL/GenBank database under accession number AB433986.

#### Bioinformatic analysis

A homology search was performed with the FASTA program at DDBJ (http://www.ddbj.nig.ac.jp/search/fasta-j.html) (*17*). The amino acid sequence alignment was performed using ClustalW 1.83 and BOXSHADE 3.21 (*18*).

## Results

#### Result of screening

D-THA-utilizing microorganisms were screened and strains with high D-THA-degrading activity were isolated from soil. Four hundred and thirty-seven strains were isolated as D-THA-utilizing microorganisms, and 17 strains were selected by TLC as D-THA-degrading microorganisms. Most of these 17 strains showed 0.02–0.2 U/(mg protein) activity of D-THA DH which produces oxaloacetate from D-THA (*8*). Among these strains, strain HT23, which is rod-shaped, and gram-negative bacterium, was selected as the enzyme source, because HT23 showed the highest D-THA DH activity [~0.2 U/(mg protein)] among the strains tested.

The most variable region of the 16S rDNA sequence (460 bp) revealed 99.9% identity to *D. acidovorans* (*19*). These results indicate that strain HT23 belongs to the genus, *Delftia* sp. Thus, we designated this strain as *Delftia* sp. HT23.

#### Induction of the enzyme

When *Delftia* sp. HT23 was cultured in the medium described earlier, the cell-free extract showed an enzyme activity of ~0.2 U/(mg protein). However, when DL-THA in the medium was replaced by an equal amount of D-serine, D-threonine, D-aspartate or peptone, the cell-free extract showed no or only a trace [<0.02 U/(mg protein)] of enzyme activity. These results suggest that the enzyme was induced by 3-hydroxyaspartate in the medium.

#### Purification and molecular weight determination of D-threo-3-hydroxyaspartate dehydratase

With the purification procedures described earlier, the enzyme was purified ~116-fold to homogeneity with ~1.4% recovery (Table I). The purified enzyme preparation gave a single band on SDS–PAGE (Fig. 1). Furthermore, by high-performance gel-permeation liquid chromatography, the enzyme gave a single symmetrical protein peak.

Using a calibrated TSKgel Super-SW3000 column, the relative molecular weight of the enzyme was estimated to be 36,000. By SDS–PAGE the relative molecular weight of the subunit was estimated to be about 41,000, suggesting that the enzyme is a monomer.

#### Substrate specificity of the enzyme

The enzyme showed broad specificity towards 3-hydroxyaspartate isomers. Table II shows the substrate specificity and kinetic parameters of D-THA DH. In addition, D- and L-serine reacted as poor substrates for the enzyme. Normal hyperbolic kinetics was observed with all the substrates tested. The $K_m$, $k_{cat}$ and $k_{cat}/K_m$ values, calculated from Lineweaver-Burk plots, are shown in Table II.

Alanine, serine and aspartate racemase activities of the enzyme were investigated using a sensitive assay method. After a 12-h incubation of the enzyme with L-alanine, L-serine or L-aspartate, no D-alanine, D-serine or D-aspartate was observed by HPLC with fluorometric detection. Thus, any alanine, serine or aspartate racemase activity of D-THA DH is below the detection limit of $5.0 \times 10^{-2}$ pmol/h/(mg protein).

#### Effects of pH and temperature

The optimal pH and temperature of the enzyme were 8.5 and 50°C, respectively.

#### Absorption spectrum

The enzyme exhibited absorption maxima at 280 and 412 nm (Fig. 2). Solutions of the pure enzyme are distinctly yellow. These results suggest that the enzyme contains PLP as its prosthetic group.

**Table I. Purification of D-THA DH from *Delftia* sp. HT23.**

| Step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Cell extract | 1,390 | 255 | 0.18 | 1.0 | 100 |
| Ammonium sulphate | 346 | 129 | 0.37 | 2.0 | 50.5 |
| Hi-Prep Q FF | 73.5 | 109 | 1.5 | 8.0 | 42.6 |
| Hi-Trap phenyl | 7.92 | 58.0 | 7.3 | 39.8 | 22.8 |
| Superdex-200 | 2.24 | 33.3 | 14.8 | 80.6 | 13.1 |
| Resource Q | 0.72 | 14.9 | 20.7 | 112.4 | 5.8 |
| Hi-Trap butyl | 0.17 | 3.6 | 21.3 | 115.8 | 1.4 |

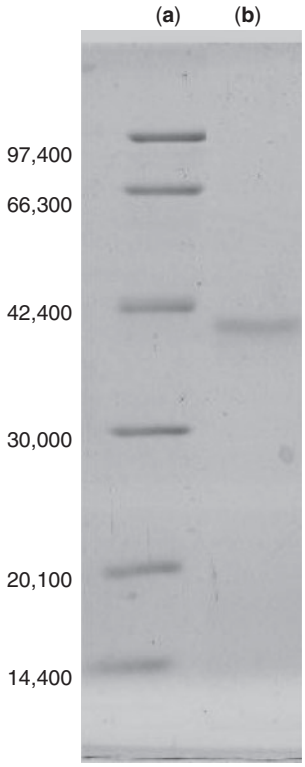


**Fig. 1 Molecular weight analysis of D-THA DH from *Delftia* sp. HT23.** SDS–PAGE analysis of D-THA DH. Lane a, molecular weight markers; lane b, purified D-THA DH.

**Table II. Kinetic parameters of D-THA DH.**

| Substrate | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ |
|---|---|---|---|
| D-THA | 10.93 | 0.42 | 25.96 |
| L-THA | 3.03 | 6.16 | 0.49 |
| D-EHA | N.D. | – | – |
| L-EHA | 8.68 | 0.16 | 54.25 |
| D-Serine | 0.89 | 0.15 | 5.91 |
| L-Serine | 0.18 | 38.70 | 0.0047 |

N.D., below the detection limit, *i.e.* <0.01 μmol/min/(mg protein).

### *Effects of chemicals*

Hydroxylamine or EDTA, each at a final concentration of 1 mM, was added to the standard reaction mixture and enzyme activity was measured. The enzyme was strongly inhibited by hydroxylamine (91.2% inhibition), suggesting that PLP participates in the enzyme reaction, as in the L-*threo*-3-hydroxyaspartate dehydratase reaction (*8–10*). The enzyme was also modestly inhibited by EDTA (27% inhibition), suggesting that the metal ions are involved in the enzyme reaction.

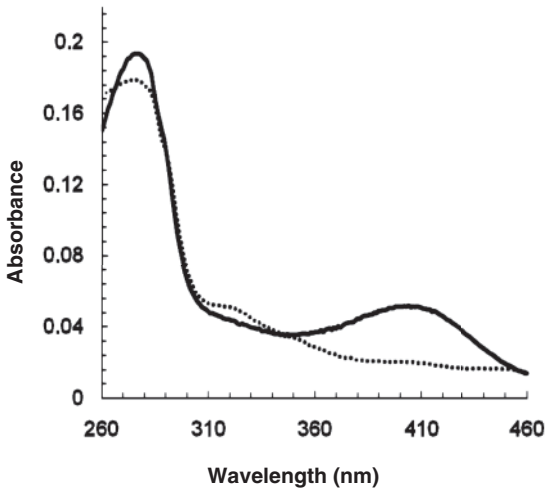


**Fig. 2 Absorption spectrum of the recombinant enzyme.** Absorption spectra were obtained with a Beckman DU-800 spectrophotometer. The recombinant enzyme (0.2 mg/ml) was dissolved in 10 mM Tris–HCl buffer (pH 8.0) containing 0.01 mM PLP, 0.1 mM $MnCl_2$ and 0.1 mM dithiothreitol. Solid line, wild-type enzyme; dotted line, K43A mutant enzyme.

### *N-Terminal and internal amino acid sequence analysis*

Automated Edman degradation of the enzyme protein was performed with a pulsed liquid phase sequencer giving an $NH_2$-terminal amino acid sequence of MQDTLLTLDTPAAVIDLDRMQXNIA, where X is an unidentified amino acid. The enzyme was digested with lysyl endopeptidase and the peptides were separated by HPLC. One peptide was isolated and the amino acid sequence was found to be RDRGTARQK. The two sequences were compared with sequences in a protein sequence database (nr-aa) using the sequence similarity search programme FASTA (*17*). Both sequences were almost perfectly matched to the partial amino acid sequences of the putative alanine racemase of *D. acidovorans* SPH-1 (accession no. ABX33617), whose whole genome, has been sequenced (http://genome.jgi-psf.org/delac/delac.home.html). These results strongly suggest that the enzyme purified from *Delftia* sp. HT23 is identical to or very closely related to the putative alanine racemase from *D. acidovorans* SPH-1.

### *Nucleotide sequence of the D-THA DH gene from* Delftia *sp. HT23*

Based on the sequence of the gene encoding *D. acidovorans* SPH-1 putative alanine racemase, a pair of specific PCR primers was synthesized. The PCR product was ligated into the vector pQE30 and the nucleotide sequence was determined.

The open reading frame was 1,143 bp long and encoded a protein of 380 amino acid residues with a predicted molecular weight of 40,300. A FASTA search revealed that the deduced amino acid sequence

was 99% identical with the putative alanine racemase from *D. acidovorans* SPH-1, described earlier. The amino acid sequence showed high identity with similar enzymes from various Gram-negative bacteria, including the putative alanine racemases from *Ralstonia solanacearum* (71%), *Burkholderia cenocepacia* (71%) and *Pseudomonas syringae* (68%), and the putative metal-activated pyridoxal enzyme from *Bordetella pertussis* (75%). The amino acid sequence of D-THA DH had relatively low, but significant similarity to two eukaryotic D-serine dehydratases those from *Gallus gallus* (chicken, 36%) (*20*) and *S. cerevisiae* (26%) (*21*), as shown in the alignment in Fig. 3. The PROSITE database (*22*) (http://au.expasy.org/prosite/) could not predict the PLP-binding site of D-THA DH; however, the three enzymes share a common motif, R(P/A)HVKT, in their N-terminal regions. In D-serine dehydratase from *S. cerevisiae*, the lysine residue in this motif binds PLP (*21*). Thus, Lys43 of D-THA DH is probably a PLP-binding residue.

### *Characterization of the recombinant enzyme*

We purified recombinant His-tagged D-THA DH from *E. coli* cells and characterized its enzymatic properties. The molecular weight determined by SDS–PAGE analysis (41,000) and that determined by MALDI-TOF-MASS analysis (41,600) were in agreement with that calculated from the deduced amino acid sequence of the recombinant enzyme (40,900). The first 15 N-terminal amino acid residues sequenced in recombinant His-tagged enzyme perfectly matched the deduced amino acid sequence. Moreover, the purified recombinant enzyme showed high activity, with specific activity of ~20 U/(mg protein) towards D-THA. From these results, we concluded that this protein is recombinant D-THA DH.

### *Effect of metal ions on the recombinant enzyme*

To investigate the role of metal ions in more detail, EDTA-treated recombinant enzyme was prepared as described in the 'Materials and Methods' section. The EDTA-treated enzyme showed ~0.1 U/(mg protein) activity towards D-THA, which was ~0.5% activity of the non-EDTA-treated enzyme. However, when $CoCl_2$, $MnCl_2$, $NiCl_2$, $ZnCl_2$, $CaCl_2$ or $FeCl_2$ was added to the enzyme solution at various concentrations, the relative activity was restored or even activated as shown in Fig. 4. No activity was detected when $SnCl_2$ or $CuCl_2$ was added to the enzyme solution, suggesting that $Sn^{2+}$ and $Cu^{2+}$ are inhibitors of this enzyme. These results indicate that divalent cations such as $Co^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Ca^{2+}$, $Zn^{2+}$ and $Fe^{2+}$ are activators of this enzyme.

### *Identification of PLP-binding lysine residue*

To identify the PLP-interacting lysine residue of the enzyme, a mutant enzyme, K43A, was constructed and purified as described in the 'Materials and Methods' section. The K43A mutant enzyme, in which Lys43 was replaced by alanine, showed no detectable activity (<0.01% of the activity towards D-THA) and lacked a large absorption maximum at 412 nm (Fig. 2). Thus, we concluded that Lys43 is most likely involved in both PLP binding and catalysis.

## Discussion

In this report, an enzyme that acts on D-*threo*-3-hydroxyaspartate was isolated and purified to homogeneity for the first time. The microbial metabolism of 3-hydroxyaspartate was described by Kornberg and Morris in the 1960s (*6*). They reported two enzymes in *Micrococcus denitrificans* that act on 3-hydroxyaspartate: *erythro*-3-hydroxyaspartate aldolase (EC 4.1.3.14) (*5*, *6*) and *erythro*-3-hydroxyaspartate dehydratase (EC 4.2.1.38) (*7*). However, these enzymes have not been purified to homogeneity or well characterized. More recently, the gene encoding 3-hydroxyaspartate aldolase was cloned and expressed in *E. coli* (*23*). In addition to these enzymes, L-THA DH, which acts only on L-THA, has been identified and characterized by our group (*8*–*10*), but enzymes acting on D-THA have not yet been reported. To our knowledge, the present study is the first to report an enzyme that catalyses the deamination of D-THA. Therefore, we designated this enzyme D-*threo*-3-hydroxyaspartate dehydratase (D-THA DH), although it also has some activity towards L-EHA. Based on this substrate specificity, the enzyme is clearly distinct from the *erythro*-3-hydroxyaspartate dehydratase (EC 4.2.1.38) reported by Gibbs and Morris (*7*), which acts only on L-EHA and not on D-THA.

D-THA DH had ~30% amino acid sequence similarity to two eukaryotic D-serine dehydratases, from chicken (*20*) and *S. cerevisiae* (*21*) (Fig. 3). Although D-THA DH acts on D-serine, the relative activity towards D-serine was only ~23% of the activity towards D-THA. Furthermore, the production of D-THA DH in *Delftia* sp. HT23 cells was not induced by D-serine, but was induced by D-THA in the medium. From these results, we concluded that this enzyme is a D-*threo*-3-hydroxyaspartate dehydratase, rather than a D-serine dehydratase.

The absorption spectrum of the purified enzyme revealed that D-THA DH contains PLP, as do other bacterial dehydratases, although the amino acid sequence of D-THA DH was not similar to that of L-THA DH, which catalyses the same reaction of L-THA to oxaloacetate (*9*, *10*). Despite catalysing the dehydratase reaction of D-THA, D-THA DH does not belong to the family of serine/threonine dehydratases, which contains most bacterial dehydratases (*24*). Based on their folding patterns, the PLP-dependent enzymes are classified into five groups, designated fold types I–V (*25*). The L-THA DH enzymes of *Pseudomonas* sp. T62 and *S. cerevisiae* belong to the fold type II group (*9*, *10*), whereas D-THA DH belongs to the fold type III group. Eukaryotic D-serine dehydratases, which are also fold-type III PLP-dependent enzymes, have been reported from chicken and from *S. cerevisiae* (*20*, *21*). However, D-THA DH is the first example of a prokaryotic dehydratase belonging to the fold type III PLP-dependent enzyme family.

The high amino acid sequence identities between D-THA DH and several putative alanine racemases

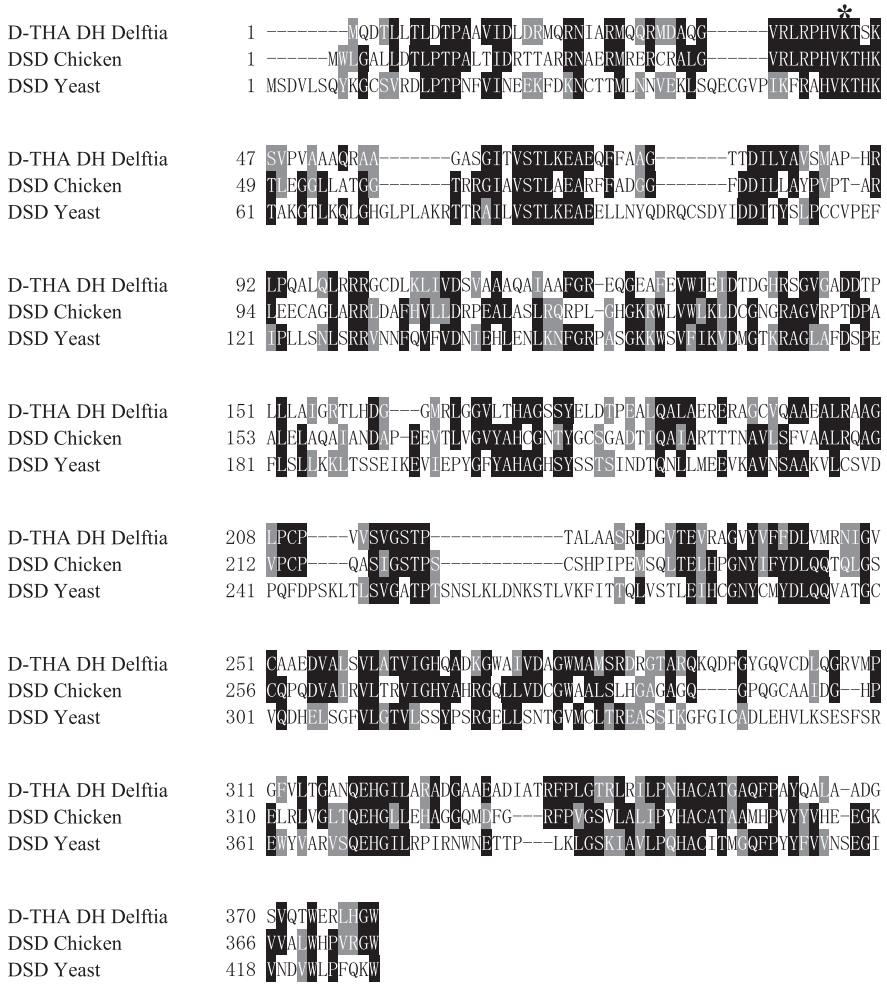


**Fig. 3 Multiple alignment of the amino acid sequences of D-THA DH from *Delftia* sp. HT23 (D-THA DH Delftia), D-serine dehydratase from chicken (DSD chicken) and D-serine dehydratase from *S. cerevisiae* (DSD Yeast).** The alignment was generated with Clustal W 1.83 and BOXSHADE 3.21. The numbers on the left are the residue numbers for each sequence. White letters on a black background indicate identical residues, and white letters on a grey background indicate similar residues. The asterisk indicates the PLP-binding residue.

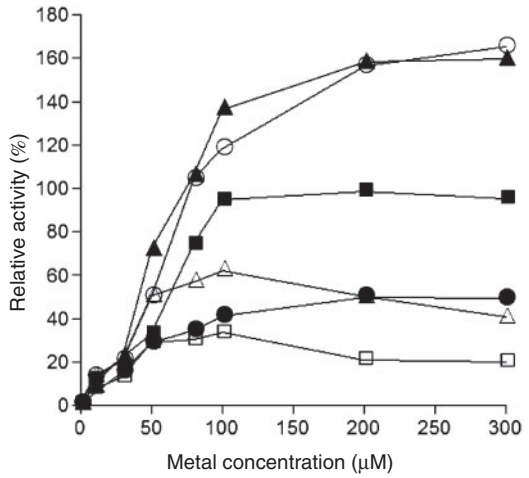


**Fig. 4 Effect of metal ions on EDTA-treated D-THA DH.** $MnCl_2$, $CaCl_2$, $ZnCl_2$, $CoCl_2$, $FeCl_2$, and $NiCl_2$ were added to the reaction mixture at various concentrations, and incubated for 10 min. The activity of non-EDTA-treated enzyme, which dialysed against 100 μM $MnCl_2$, was taken as 100%. Values are means of two independent experiments. Symbols: open circle, $Mn^{2+}$; filled circle, $Ca^{2+}$; open triangle, $Zn^{2+}$; filled triangle, $Co^{2+}$; open square, $Fe^{2+}$; filled square, $Ni^{2+}$.

and putative metal-activated pyridoxal enzymes from various Gram-negative bacteria, including enzymes from *Bordetella pertussis* (75%), *Ralstonia solanacearum* (71%), *Burkholderia cenocepacia* (71%) and *Pseudomonas syringae* (68%), suggest that these putative enzymes may have D-THA DH activity. D-THA DH is expected to be present in other organisms, especially the Gram-negative soil bacteria mentioned above.

Site-directed mutagenesis experiment revealed that lysine 43 is an important residue involved in PLP binding and catalysis (Fig. 2), however, the K43A mutant enzyme still has weak absorption peaks around 320–420 nm. The reason why K43A mutant enzyme has these absorption peaks remains unknown, however, non-covalently bound PLP may cause these absorption peaks as in the case of aspartate aminotransferase of *E. coli (26)*. Another explanation is also possible; Lys residue other than 43 (for example, Lys46) may bind PLP weakly, and gave catalytically inactive enzyme-PLP complex with absorption ~320 and 420 nm. But, further investigation is required to elucidate this phenomenon.

Like L-THA DH from *Pseudomonas* sp. T62, D-THA DH requires divalent cations for its activation (*8*, *9*), but the activation pattern differed between the two enzymes. Both $Co^{2+}$ and $Zn^{2+}$ act as inhibitors of L-THA DH, whereas these divalent cations are activators of D-THA DH. The pattern of D-THA DH activation by divalent cations was similar to that of D-threonine aldolase from *Arthrobacter* sp. DK-38 (*27*, *28*), which is also activated by $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Fe^{2+}$ and $Ca^{2+}$. D-Threonine aldolase from *Arthrobacter* sp. DK-38 also belongs to the fold-type III PLP-dependent enzymes (*28*) and shares around 30% amino acid identity to D-THA DH (data not shown). D-Threonine aldolase from *Arthrobacter* sp. DK-38 can bind 1 mol of $Mn^{2+}$ ion per mol of subunit (*28*), however, the amount of metal ion, which binds to D-THA DH has not yet been determined.

Although the reason why activity of the EDTA-treated enzyme added with 100 μM $MnCl_2$ is higher than that of the non-EDTA-treated enzyme dialysed against the same concentration of $MnCl_2$ is not clear (Fig. 4), the following explanation might be possible; the EDTA-treated enzyme is somewhat more stable than the non-EDTA-treated enzyme, *i.e.* the non-EDTA-treated enzyme (containing $Mn^{2+}$) might be partially inactivated during the dialysis, however, the EDTA-treated enzyme (containing no or very little amount of metal ions) may become more active than the non-EDTA-treated enzyme when $Mn^{2+}$ is added just before activity measurement. Effect of the metal ions on stability of the enzyme, however, still needs to be elucidated.

Although the physiological function of this enzyme remains to be clarified, the dehydratase reaction catalysed by D-THA DH may be one of the reactions enabling *Delftia* sp. HT23 to grow on medium containing D-THA as the sole carbon source. This hypothesis is also supported by the fact that D-THA DH is inducible in *Delftia* sp. HT23; however, the details have not been elucidated.

To analyse the detailed reaction mechanism and 3D structure of this enzyme, an efficient expression system is necessary. Unfortunately, the expression level of D-THA DH in *E. coli* was low. The specific activity of the cell-free extract of *E. coli* expressing the D-THA DH gene was ~0.2 U/(mg protein), which is almost equal to that of the cell-free extract of the original strain, *Delftia* sp. HT23. The reason for poor expression in *E. coli* is unknown; however, the high GC content of the gene (71.9%) is one possible explanation. Currently, we are trying to improve the expression level of D-THA DH in *E. coli*.

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**Conflict of interest**

None declared.

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