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Isolation and amino acid sequence of a dehydratase acting on D-*erythro*-3-hydroxyaspartate from *Pseudomonas* sp. N99, and its application in the production of optically active 3-hydroxyaspartate

Hiroyuki Nagano, Kana Shibano, Yu Matsumoto, Atsushi Yokota, and Masaru Wada*

Laboratory of Microbial Physiology, Research Faculty of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kita-ku, Sapporo, 060-8589, Japan

Running title: Novel D-*erythro*-3-hydroxyaspartate dehydratase

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*Corresponding author.

Email: wada@chem.agr.hokudai.ac.jp

Abbreviations:

DH, dehydratase; D-EHA, D-*erythro*-3-hydroxyaspartate; D-THA, D-*threo*-3-hydroxyaspartate; EAATs, excitatory amino-acid transporters; 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; NMDA, *N*-methyl-D-aspartate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PLP, pyridoxal 5'-phosphate; SDS, sodium dodecyl sulfate; TLC, thin layer chromatography.

Abstract

An enzyme catalyzing the ammonia-lyase reaction for the conversion of D-*erythro*-3-hydroxyaspartate to oxalacetate was purified from the cell-free extract of a soil-isolated bacterium *Pseudomonas* sp. N99. The enzyme exhibited ammonia-lyase activity toward L-*threo*-3-hydroxyaspartate and D-*erythro*-3-hydroxyaspartate, but not toward other 3-hydroxyaspartate isomers. The deduced amino acid sequence of the enzyme, which belongs to the serine/threonine dehydratase family, shows similarity to the sequence of L-*threo*-3-hydroxyaspartate ammonia-lyase (EC 4.3.1.16) from *Pseudomonas* sp. T62 (74%) and *Saccharomyces cerevisiae* (64%) and serine racemase from *Schizosaccharomyces pombe* (65%). These results suggest that the enzyme is similar to L-*threo*-3-hydroxyaspartate ammonia-lyase from *Pseudomonas* sp. T62, which does not act on D-*erythro*-3-hydroxyaspartate. We also then used the recombinant enzyme expressed in *Escherichia coli* to produce optically pure L-*erythro*-3-hydroxyaspartate and D-*threo*-3-hydroxyaspartate from the corresponding DL-racemic mixtures. The enzymatic resolution reported here is one of the simplest and the first enzymatic method that can be used for obtaining optically pure L-*erythro*-3-hydroxyaspartate.

Key words: 3-hydroxyaspartate, ammonia-lyase, enzymatic resolution, pyridoxal 5′-phosphate, *Pseudomonas* sp. N99

3-Hydroxyaspartate is a non-proteinogenic amino acid; however, it was first found in human cerebrospinal fluid in the 1960s[1)] and has been known as an inhibitor of aspartate aminotransferase[2)] or the intermediate of glycolate metabolism in some bacteria[3,4)] for a long time. More recently, its biological activities such as affinity to *N*-methyl-D-aspartate (NMDA) receptors,[5)] competitive blocking of human brain excitatory amino-acid transporters (EAATs),[6)] and inhibition of mammalian serine racemase[7)] have been reported. Thus, 3-hydroxyaspartate is an interesting molecule that plays an important role in the research of the mammalian central nervous system. However, 3-hydroxyaspartate has two chiral centers, and their four stereoisomers—D-*threo*-3-hydroxyaspartate (2*R*, 3*R*; D-THA), L-*threo*-3-hydroxyaspartate (2*S*, 3*S*; L-THA), D-*erythro*-3-hydroxyaspartate (2*R*, 3*S*; D-EHA), and L-*erythro*-3-hydroxyaspartate (2*S*, 3*R*; L-EHA)—are difficult to synthesize in an optically pure form. Various routes for the synthesis of optically active 3-hydroxyaspartate isomers have been reported, but most of them use tartrate derivatives as chiral templates and contain four or more steps to obtain optically pure isomers.[8)] In addition, the synthesis of L-THA from L-aspartate through a completely stereoselective cyclization to *trans*-oxazoline[9)] and a stereoselective synthetic approach for *erythro*-hydroxyaspartate through nucleophilic ring opening of racemic *trans*-aziridine-2,3-dicarboxylate also has been reported.[10)] These improved methods, however, still need chiral starting materials or special reagents. In contrast, a racemic mixture of DL-THA and DL-EHA can be easily synthesized from inexpensive *cis*- or *trans*-epoxysuccinate in a one-step reaction.[11,12)] Thus, enzymes that can distinguish the L-form or D-form of 3-hydroxyaspartate isomers are useful for the enzymatic resolution of DL-racemic 3-hydroxyaspartate mixtures.

We recently reported the identification of a novel enzyme, D-*threo*-3-hydroxyaspartate ammonia-lyase (dehydratase) (EC 4.3.1.27), from *Delftia* sp. HT23.[13)] The production of optically active L-THA from DL-THA by enzymatic resolution using this enzyme has also been reported.[12)] In

addition, the enzymatic syntheses of L-THA from L-aspartate or L-asparagine have also been reported.[14,15] However, no dehydratase acting on the D-*erythro* form of 3-hydroxyaspartate has been reported so far. Therefore, the enzymatic resolution of DL-EHA to produce L-EHA has not been achieved to date.

Threo-isomers have attracted more attention from biochemists, because these *threo*-isomers, especially L-THA, have more affinity to NMDA receptors[5] and more inhibitory activity towards EAATs in the mammalian central nervous system than *erythro*-isomers.[16] However, L-EHA has been identified as an inhibitor of mammalian serine racemase, producing D-serine, which is a co-agonist of NMDA receptors.[7] L-EHA also has affinity to EAATs and NMDA receptors, even though the affinities are lower than those of *threo*-isomers.[17] Thus, in addition to L-THA, L-EHA is also an important molecule for biochemical studies, and an enzyme capable of degrading D-EHA might be useful for the enzymatic optical resolution of DL-EHA to produce optically pure L-EHA.

Therefore, in this study, we screened microorganisms that can utilize D-EHA as the sole carbon source. A newly isolated bacterium, *Pseudomonas* sp. N99, was found to produce an enzyme that catalyzes the dehydratase reaction for the conversion of D-EHA to oxaloacetate (Fig. 1). We report here the purification and amino acid sequence of this novel enzyme, D-EHA dehydratase (D-EHA DH). An efficient expression system for this enzyme was also established using *Escherichia coli*. Moreover, we also applied this enzyme for the production of optically pure L-EHA and D-THA by enzymatic optical resolution. The findings of this study provide a new approach for obtaining optically pure L-EHA.

Materials and methods

92 *Materials.* DL-EHA was synthesized by ammonolysis of (±)-*trans*-epoxysuccinic acid (Tokyo
93 Chemical Industry, Tokyo, Japan) at 60°C as described previously.[12] The synthesis was confirmed by
94 silica gel thin layer chromatography (TLC) using 7:1:2 (v/v/v) of ethanol/aqueous ammonia
95 (28%)/distilled water as a developing solvent. L-THA, L-EHA, and DL-THA were purchased from
96 Tocris Cookson, Ltd. (Bristol, UK), Wako Pure Chemical Industries (Osaka, Japan), and Tokyo
97 Chemical Industry, respectively. D-THA was prepared from DL-THA by enzymatic resolution using L-
98 THA dehydratase (L-THA DH) from *Saccharomyces cerevisiae*.[18] D-EHA was prepared from DL-
99 EHA by enzymatic resolution using D-THA dehydratase (D-THA DH) from *Delftia* sp. HT23.[12]
100 Restriction endonucleases were obtained from TaKaRa Bio Inc. (Kusatsu, Japan). All other chemicals
101 were of analytical grade and commercially available.
102
103 *Screening of D-EHA-degrading microorganisms from soil*. Screening of D-EHA-degrading
104 microorganisms was done as described previously.[13] The nitrogen source for the screening medium
105 used in previous studies, D-THA, was replaced by D-EHA. Identification of the isolated bacterium
106 was done by determining the most variable region of the 16S rDNA sequence (460 bp) using the
107 primer set of V1 (5'-AGAGTTTGATCCTGGCTCAG-3') and V3 (5'-ACCGCGGGKGCTGGC-3',
108 K=G/T).
109
110 *Microorganism and cultivation. Pseudomonas* sp. N99 isolated from soil and identified in our
111 laboratory was used as the enzyme source. *Pseudomonas* sp. N99 has been deposited in the AHU
112 Culture Collection of Hokkaido University under the accession number AHU2141. *Pseudomonas* sp.
113 N99 was cultivated as described previously.[19] *E. coli* DH5α (TaKaRa Bio Inc.) and *E. coli* BL21

(DE3) (Merck Millipore, Darmstadt, Germany) were used as the host cells for cloning and expression of the gene *(dehadh*), respectively, and were grown as described previously.[13)]

Enzyme assays. The activity of 3-hydroxyaspartate dehydratase was determined as described previously.[12)] The enzyme activity was coupled to malate dehydrogenase, which reduces oxaloacetate formed by the dehydratase to malate in the presence of NADH. One unit of the enzyme was defined as the amount capable of catalyzing the oxidation of 1 μmol of NADH per min. Serine racemase and serine dehydratase activities were determined as described previously.[13,19)]

Enzyme purification. Enzyme purification procedures were performed as described previously.[13)] The final Mono Q step was performed as follows: The partially purified enzyme after Superdex-200 chromatography was applied to a Mono Q 5/50 GL column (0.5 × 5 cm, GE Healthcare UK Ltd, Little Chalfont, UK) previously equilibrated with the buffer and was eluted using the ÄKTA system (GE Healthcare UK Ltd) with a linear gradient of 0–0.65 M NaCl in 20 mL of buffer at a flow rate of 2.0 mL/min. The activity-containing fractions, eluted with approximately 0.3 M NaCl, were collected, dialyzed against 3 L of the buffer for 16 h, and used as the purified enzyme.

Amino acid sequence analysis. The internal peptide sequences were determined using the In-Gel Tryptic Digestion kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). After trypsine digestion of the enzyme, the peptides were separated by HPLC (L-2000 system; Hitachi High-Tech Science Corp., Tokyo, Japan) on an Inertsil WP300 C8 column (4.6 × 250 mm; GL Science, Tokyo, Japan). Edman degradation was performed at the Global Facility Center, Hokkaido University.

Isolation of DNA encoding D-EHA DH (dehadh). Degenerate PCR was performed as described previously.[19] Oligonucleotide primer pools were designed based on the amino acid sequences of the N-terminal (MQIILPT) and internal (EIVHIDT) peptides. The alignment of the primers was 5'-ATGCARATHATHYTICCNAC-3' (sense strand) 5'-GGNGTRTCDATRTGIACDATYTC-3' (antisense strand) (D=A/G/T, H = A/C/T, I = inosinic acid, N = A/C/T/G, R = A/G, Y = C/T). The chromosomal DNA of *Pseudomonas* sp. N99 was used as the template. The chromosomal DNA of *Pseudomonas* sp. N99 was digested with *Eco*RI at 37°C overnight and purified by ethanol precipitation. The DNA fragments were then circularized with Ligation Solution I and II in the TaKaRa LA PCR in vitro cloning kit (Takara Bio Inc.) at 16°C for 30 min and then used as a template for inverse PCR. Inverse PCR was performed using the TaKaRa LA PCR in vitro cloning kit with a sense primer (5'-AGCGAAAGTCTGGAGTTGTAGCGTTCT-3') and an antisense primer (5'-TCATACGATCATCCGGATGTCTTGTCA-3'). The PCR was performed following with the manufacturer's protocol. The inverse PCR product was purified from the agarose gel and sequenced directly.

Construction of the expression plasmid. For expression of *dehadh* in *E. coli*, a DNA fragment containing the open reading frame of *dehadh* was prepared by PCR using *Pseudomonas* sp. N99 genomic DNA as the template and the oligonucleotide sense (5′ -ATGCGGATCCGATGCAGATTATTTTGCCGACCTACAGCGAT-3′) and antisense primers (5′ -ATGCGGATCCTTAGGCTTGCAGCAAACGACCAAACTTATC-3′) (the underlined sequence are the *Bam*HI sites). The PCR was performed using KOD FX Neo (Toyobo Co., Ltd., Osaka, Japan). The thermal cycler program was set at 94°C for 2 min, followed by 35 cycles of 98°C for 10 s, and 68°C for 1 min. The unique amplified band corresponding to approximately 1,000 bp was digested with

160 *Bam*HI and then ligated into the *Bam*HI site of the pET15b expression vector (Merck, Darmstadt,
161 Germany) to obtain pET15b_*dehadh*. The expression vector was then introduced into *E. coli* BL21
162 (DE3) cells, and the nucleotide sequence of the insert was then confirmed.
163
164 *Expression and purification of recombinant D-EHA DH*. Expression and purification of recombinant
165 D-EHA DH was performed as described previously.[13] Briefly, the *E. coli* BL21 (DE3) carrying
166 pET15b_*dehadh* were grown at 37°C in LB medium containing ampicillin (100 μg/mL). In order to
167 induce gene expression, 0.1mM IPTG was added to the culture medium when the absorbance at 600
168 nm reached 0.5. After cultivation anther 8 h, cells were harvested by centrifugation. For the
169 recombinant enzyme purification, HisTrap HP column (1.6 x 2.5 cm; GE Healthcare) connected to the
170 ÄKTA system was used. The enzyme was eluted with a 20–500mM liner imidazole gradient. Active
171 fractions were collected, dialyzed, and used as the purified enzyme.
172
173 *Inhibitor experiments.* Hydroxylammonium chloride (Kanto Chemical Co. Inc., Tokyo, Japan) and *O*-
174 (carboxymethyl)hydroxylamine hemihydrochloride (Sigma-Aldorich Corp., St. Louis, USA) were
175 used as inhibitors. The inhibitors were added to the standard enzyme assay mixture minus PLP at the
176 concentration of 5 mM, and then the enzyme activities were measured.
177
178 *Optical resolutions using recombinant D-EHA DH.* Optical resolutions of DL-EHA and DL-THA
179 were carried out as described previously by modifying the reaction temperature (25°C).[12] Final
180 products were then identified with HPLC and NMR as described previously.[12] Other methods
181 including MALDI-TOF-MS analysis were done as described previously.[19]
182

Nucleotide sequence accession number. The nucleotide sequence of the *dehadh* gene of *Pseudomonas* sp. N99 has been deposited in the DDBJ/EMBL/GenBank database under the accession number AB771451.

Results

Screening. D-EHA-utilizing microorganisms were screened, and the strains with high D-EHA-degrading activity were isolated from soil. Three hundred and ninety strains were isolated as D-EHA-utilizing microorganisms, and 31 of them were selected by TLC as D-EHA-degrading microorganisms. Most of these 31 strains showed 0.02–0.2 unit (mg protein)$^{-1}$ activity for D-EHA dehydratase, which produces oxalacetate from D-EHA. Among these strains, strain N99, which is a rod-shaped gram-negative bacterium, was selected as the enzyme source, because it showed the highest D-EHA DH activity (around 0.2 unit (mg protein)$^{-1}$).

The most variable region of the 16S rDNA sequence (460 bp) revealed 98–99% identity to that of several *Pseudomonas* strains. These results indicated that strain N99 belongs to the genus *Pseudomonas*. Thus, we designated this strain as *Pseudomonas* sp. N99.

Induction of the enzyme activity. When *Pseudomonas* sp. N99 was cultured in medium containing DL-EHA or DL-THA as the sole nitrogen source, the cell-free extract showed an enzyme activity of approximately 0.2 unit (mg protein)$^{-1}$. However, when DL-EHA or 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 enzyme activity or only a trace [<0.02 unit (mg protein)$^{-1}$] of enzyme activity. These results suggested that the enzyme activity was induced by 3-hydroxyaspartate in the medium. It should be noted that this D-EHA DH activity was induced not only by DL-EHA but also by DL-THA.

Judging form the substrate specificity of this enzyme (see below), the actual inducer seems to be D-EHA and L-THA. However, the detail of enzyme induction including the actual inducer(s) remains unclear.

Purification and molecular weight determination of the enzyme. The enzyme was purified approximately 21-fold to almost homogeneity, with approximately 0.4% recovery (Table 1). The purified enzyme preparation produced two bands on SDS-PAGE (Fig. 2). Judging from the enzyme activity of each fraction from final Mono Q chromatography, the upper band with a relative molecular mass of 39,100 seemed to correspond to the enzyme having D-EHA DH activity.

N-Terminal and internal amino acid sequence analysis. Automated Edman degradation of the enzyme was performed with a pulsed liquid phase sequencer and yielded an NH_2-terminal amino acid sequence of MQIILPTYSDVVDASQRLQGVANVTPPL. The enzyme was then digested by trypsin, and the resultant peptides were separated by HPLC. One peptide was isolated, and the amino acid sequence was found to be SFNAGEIVHIDTPDTIAD. The two sequences were compared with sequences in a protein sequence database (nr-aa) using the sequence similarity search program FASTA.[20] Both sequences showed relatively high identities to the partial amino acid sequences of putative serine/threonine dehydratases such as those from *Comamonas teststeroni, Pseudomonas fluorescens,* and *Acinetobacter baumannii* AB307-0294.

Nucleotide sequence of the D-EHA DH gene from Pseudomonas *sp. N99.* Based on the partial amino acid sequence, the full-length open reading frame of the gene encoding the enzyme was isolated through degenerate and inverse PCR approaches. The open reading frame was 966 bp long and

encoded a protein of 321 amino acid residues, with a predicted molecular weight of 34,240. The amino acid sequence showed high identity with similar enzymes from various gram-negative bacteria including putative *threo*-3-hydroxyaspartate ammonia-lyase from *Pseudomonas chlororaphis* subsp. *aureofaciens* 30-84 (87%) and putative catabolic threonine dehydratase from *Pseudomonas* sp. PAMC25886 (75%) and *Pseudomonas* sp. Ag1 (74%). In addition, the amino acid sequence showed high identity with L-THA DH (EC 4.3.1.16) from *Pseudomonas* sp. T62 (74%)[19] and *Saccharomyces cerevisiae* (64%)[18] and with serine racemase from *Schizosaccharomyces pombe* (65%),[21] whose functions have already been analyzed. Figure 3 shows multiple alignment of these deduced amino acid sequences. These results suggested that enzymes having similar amino acid sequence to D-EHA DH are probably distributed broadly in nature, not only in gram-negative soil bacteria but also in eukaryotic microorganisms. According to the PROSITE database (http://au.expasy.org/prosite/),[23] the pyridoxal-phosphate attachment site of serine/threonine dehydratase, 51-TGSFKFRGA-58, was found. This indicated that Lys55 of D-EHA DH seems to be a PLP-binding residue (Fig. 3).

Characterization of the recombinant enzyme. We purified recombinant His-tagged D-EHA DH from *E. coli* BL21 (DE3) cells and characterized its enzymatic properties. The molecular weight determined using SDS-PAGE (Fig. S1, 41,800) was not in agreement with that calculated from the deduced amino acid sequence of the recombinant enzyme including His-tag (36,900). However, the molecular weight determined using MALDI-TOF-MS analysis (36,847) showed good agreement with that calculated from the deduced amino acid sequence. This discrepancy was also observed in the case of L-THA DH from *Pseudomonas* sp. T62.[19] The first 25 N-terminal amino acid residues sequenced using Edman degradation in recombinant His-tagged enzyme perfectly matched the amino acid sequence deduced from the nucleotide sequence of pET15b_*dehadh*. Moreover, the purified

recombinant enzyme showed high activity, with specific activity of approximately 20 units (mg protein)$^{-1}$ toward D-EHA. From these results, we concluded that this protein is recombinant D-EHA DH.

Substrate specificity of the recombinant enzyme. Next, we attempted to determine the substrate specificity of D-EHA DH. Table 2 shows the substrate specificity and kinetic parameters of D-EHA DH. In addition to D-EHA, L-THA also acts as a good substrate of the enzyme (around 190% k_{cat}/K_m value compared to that of D-EHA). Other 3-hydroxyaspartate isomers such as D-THA, and L-EHA could not be used as substrates for the enzyme. In addition, the enzyme showed very low (around 0.73% k_{cat}/K_m value compared to that of D-EHA) dehydratase activity toward L-serine, but no activity toward D-serine, L-threonine, and D-threonine was observed. No serine racemase activity was detected using the sensitive fluorescence detection HPLC, as in the case of L-THA DH.[19] Normal hyperbolic kinetics were observed with D-EHA, L-THA, and L-serine. The K_m, k_{cat}, and k_{cat}/K_m values calculated from the Lineweaver-Burk plots are shown in Table 2.

Absorption spectrum and effect of inhibitors. The recombinant enzyme exhibited absorption maxima at 280 and 412 nm (Fig. S2). The solutions of the pure enzyme were distinctly yellow. In addition, both the inhibitors tested almost completely inhibited the enzyme activity at 5 mM (data not shown). These results suggested that the enzyme contains PLP as its prosthetic group.

Properties of the recombinant enzyme. The optimal pH and temperature of the recombinant enzyme were 8.5 and 45°C, respectively, under the standard assay conditions. The thermal stability of the recombinant enzyme was also determined. After 15 min of incubation at various temperatures, the

residual enzyme activity toward D-EHA was measured. The enzyme was stable below 25°C and lost its activity above 45°C.

L-EHA and D-THA production by enzymatic optical resolution. The enzyme showed remarkable activity towards D-EHA, while no dehydratase activity was observed toward L-EHA (Table 2). Thus, we conducted the optical resolution of DL-EHA to obtain optically pure L-EHA using the recombinant enzyme produced by the *E. coli* cells. After 14 h of reaction, no D-EHA was detected on the HPLC, and L-EHA remained with an optical purity of >99% *e.e.* (Fig. 4A, Fig. S3).

The chemical purity of the isolated product was then verified by NMR analysis (Fig. S4 and Fig. S5). L-EHA standard (purchased Wako Pure Chemical Industries): ^{1}H-NMR (500 MHz D_2O): δ＝4.03 (d1H *J* = 3.5Hz); 4.34 (d1H *J* = 3.5Hz); ^{13}C-NMR (125 MHz D_2O): 59.81; 73.58; 174.90; 179.04. The resolution product: ^{1}H-NMR (500 MHz D_2O): δ = 3.99 (d1H *J* = 3.5Hz); 4.32 (d1H *J* = 3.5Hz); ^{13}C-NMR (125 MHz D_2O): 60.55; 75.39; 179.48; 180.68. Finally, >99% chemically and optically pure L-EHA was obtained from DL-EHA with 35.1% yield in the isolated form.

The optical resolution of DL-THA to obtain optically pure D-THA was also conducted in the same manner. Optically pure D-THA (>99% *e.e.*) was obtained with 37.4% yield in the isolated form (Fig. 4B, Fig. S6). NMR analysis also showed that the chemical purity of the isolated D-THA was >99% (Fig. S7 and Fig. S8). DL-THA standard (purchased from Tokyo Chemical Industry): ^{1}H-NMR (500 MHz D_2O): δ＝4.06 (d1H *J* = 2.0 Hz); 4.56 (d1H *J* = 1.5 Hz); ^{13}C-NMR (125 MHz D_2O): 60.68; 74.08; 176.46; 178.91. The resolution product: ^{1}H-NMR (500 MHz D_2O): δ = 3.82 (d1H *J* = 2.0 Hz); 4.46 (d1H *J* = 2.0 Hz); ^{13}C-NMR (125 MHz D_2O): 60.76; 74.32; 174.89; 179.04.

As shown in Fig. S3 and S6 (HPLC chromatograms), *e.e*. values of the substrate racemic mixture (DL-EHA and DL-THA) were not zero. The reason is unclear, however, *e.e.* value of laboratory-synthesized DL-EHA was around -18% for L-form, while that of commercially available DL-THA (Tokyo Chemical Industries) was around 3% for D-form. The reaction conditions of ring-opening ammonolysis may affect optical purity of the product. As shown in Fig. 4A, all the D-EHA in the reaction mixture were degraded within 12 h, even though *e.e*. value of the starting material were minus for desired L-EHA.

Discussion

In this study, we isolated an enzyme acting on D-EHA and purified it to almost homogeneity from the newly isolated soil bacterium, *Pseudomonas* sp. N99. Although 3-hydroxyaspartate dehydratases acting on L-THA or D-THA have been identified and characterized previously by our research group,[13,18,19,24] an enzyme acting on D-EHA has not yet been reported. To the best of our knowledge, the present study is the first to report an enzyme that catalyzes the deamination of D-EHA. Therefore, we designated this enzyme D-*erythro*-3-hydroxyaspartate dehydratase (D-EHA DH), although it also showed considerable activity toward L-THA.

The reason why substrate specificity of D-EHA DH in this study is different from L-THA DH of *Pseudomonas* sp. T62[19] in spite of the high primary structure identity (72%) is still unknown. However, according to the 3D structure of D-THA DH,[12] which catalyzes the very similar reaction (only the stereoselectivity is different) to this enzyme, very small change around the substrate-binding pocket may affect stereoselectivity of the enzyme reaction. The local environment around the substrate-binding pocket of these two enzymes, *i.e*., D-EHA DH and L-THA DH, may be different

320 from each other. The detailed substrate recognition mechanism, however, should be investigated in
321 further study such as 3D structure analysis.
322 Based on this substrate specificity, the enzyme was found to be clearly distinct from the
323 *erythro*-3-hydroxyaspartate dehydratase (EC 4.3.1.20) reported by Gibbs and Morris many years
324 ago,[25] which acts only on L-EHA but not on D-EHA. The D-EHA DH isolated and purified in this
325 study also showed weak activity toward L-serine in addition to D-EHA and L-THA. The L-serine
326 dehydratase activity of this enzyme, however, was less than 1% of the D-EHA DH activity (Table 2).
327 The production of D-EHA DH by *Pseudomonas* sp. N99 cells was not induced by L-serine, but by the
328 DL-EHA or DL-THA in the culture medium. These results strongly suggested that the physiological
329 substrates of this enzyme are 3-hydroxyaspartate isomers such as D-EHA and L-THA, but not L-serine.
330 Although the physiological function of D-EHA DH in *Pseudomonas* sp. N99 remains
331 unknown, one explanation is possible. 3-Hydroxyaspartate is a rare but naturally occurring amino acid.
332 It has been found in human cerebrospinal fluid[1], mammalian urine hydrolysate, [26] and some peptide
333 antibiotics such as cinnamycin,[27] and cormycin A.[28] Most 3-hydroxyaspartate found in peptides are
334 L-*threo* form. Thus, natural habitat of *Pseudomonas* sp. N99, *i.e.*, soil, may contain free L-THA,
335 which is toxic to many bacteria.[29] Thus, D-EHA DH may play a role in detoxification of free 3-
336 hydroxyaspartate in *Pseudomonas* sp. N99 cells. The facts that D-EHA DH is inducible in
337 *Pseudomonas* sp. N99 and D-EHA DH acts both on D-EHA and L-THA also support this hypothesis.
338 We also successfully isolated the gene encoding D-EHA DH using the inverse PCR
339 approach. The deduced amino acid sequence, which indicated that the enzyme belongs to
340 the serine/threonine dehydratase family, showed similarity to L-THA DH (EC 4.3.1.16) from
341 *Pseudomonas* sp. T62 (68%)[19] and *Saccharomyces cerevisiae* (64%) (Fig. 3).[18] In addition,
342 D-EHA DH also showed significant activity toward L-THA (Table 2). Together, these results

suggested that the enzyme is similar to L-THA DH from *Pseudomonas* sp. T62, but with broader substrate specificity, because the T62 enzyme does not act on D-EHA.[19] The primary structure of D-EHA DH also showed significant similarity to the structure of eukaryotic serine racemases (EC 5.1.1.18) from *Schizosaccharomyces pombe* (64%),[21] mouse (35%),[30] human (34%),[31] and *Caenorhabditis elegans* (28%).[32] Among these serine racemases, the enzymes from mouse[30] and *C. elegans*[32] have been shown to have activity toward L-THA. These results indicated that the enzymes having 3-hydroxyaspartate dehydratase activity are very broadly distributed in nature from bacteria to mammals. However, like L-THA DH from *Pseudomonas* sp. T62[19] and *S. cerevisiae*[18], D-EHA DH did not show any detectable serine racemase activity.

The absorption spectrum of the purified recombinant enzyme and the inhibitor experiment revealed that D-EHA DH contains PLP, as do other bacterial dehydratases (Fig. S2).[33] Although it has not yet been identified, Lys55 is most probably the PLP-binding residue based on the sequence similarity to other related enzymes (Fig. 3).

There have previously been a few reports on the enzymatic production of L-THA. Mutated asparagine oxygenase (AsnO-D241N) from *Streptomyces coelicolor* A3(2) has been used for the direct hydroxylation of L -aspartate to produce L-THA.[14] This route of enzymatic synthesis is very concise and economical, but the mutant enzyme, AsnO-D241N, seems to be unstable, and the reaction can only be performed at 16°C. Hara et al. also reported whole cell catalysis from asparagine to L-THA using asparaginase-deficient- and wild-type AsnO-expressing *E. coli* cells.[15] However, the enzymatic production of L-EHA has not yet been reported. In this study, we successfully achieved the enzymatic resolution of DL-EHA and DL-THA to obtain optically pure L-EHA and D-THA, respectively, using the recombinant D-EHA DH produced by *E. coli* cells. This synthetic route is

one of the simplest methods that can be used to obtain optically pure L-EHA, because no hydroxylase yielding L-EHA as a reaction product has been reported so far. To the best of our knowledge, this is the first example of the enzymatic production or resolution of L-EHA. In addition, the concentration of L-EHA in the reaction mixture achieved in this study (100 mM) was much higher than previously reported values achieved using enzymatic L-THA production (about 10 mM using AsnO-D241N system,[14] and about 48 mM using wild-type AsnO-expressing *E. coli* system[15]). We also analyzed the final products using TLC, HPLC, and NMR spectroscopy and confirmed them to be chemically and optically pure L-EHA/D-THA. Pure compounds could be isolated by direct recrystallization from the reaction mixtures by taking advantage of the lower solubility of 3-hydroxyaspartate in acidic water (pH 2.0) compared to that of the other catalytic products of the enzyme.[12] Therefore, this enzymatic approach has an advantage for obtaining the pure products, as it does not require any chromatographic purification. It is also efficient and friendly to the environment because of minimal or no use of organic solvents.

In conclusion, in this study, we have discovered D-EHA DH for the first time, and established an enzymatic resolution process for obtaining optically pure L-EHA, which is an important compound for neurophysiological research. This process is also one of the simplest methods that can be used to obtain pure L-EHA, though the maximum yield of this process was theoretically 50% as a DL-racemic mixture of hydroxyaspartate used as substrates. Using this enzyme together with the already reported D-THA DH from *Delftia* sp. HT23 acting on D-THA and L-EHA, four isomers of 3-hydroxyaspartate can now be prepared from the inexpensive racemic mixture of DL-THA and DL-EHA. L-THA and D-EHA can be prepared from DL-THA and DL-EHA, respectively, using D-THA DH reported previously,[12] and D-THA and L-EHA can be prepared from DL-THA and DL-EHA, respectively, using the D-EHA DH reported here. These enzymatic resolutions may provide efficient and inexpensive

ways for obtaining optically pure 3-hydroxyaspartate isomers and thus contribute to the functional analysis of 3-hydroxyaspartate isomers in the future.

Author contribution

HN conducted the screening, enzyme purification, and gene cloning. KS characterized the recombinant enzyme and carried out the enzymatic resolution. YM prepared the substrates and carried out the enzymatic resolution. YA and MW conceived and designed the study and wrote the article.

Disclosure statement

No potential conflict of interest was reported by the authors.

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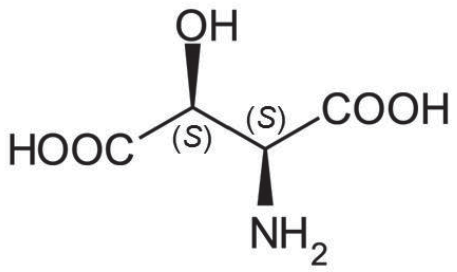
Figure legends

Fig. 1. Scheme of the ammonia-lyase reactions of D-*erythro*-3-hydroxyaspartate dehydratase (D-EHA DH).

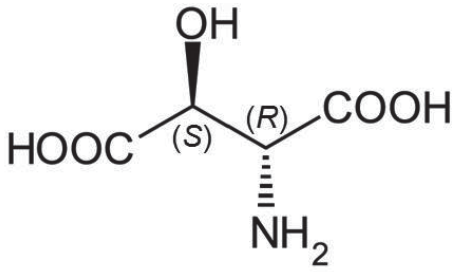
Fig. 2. SDS-PAGE analysis of D-EHA DH from *Pseudomonas* sp. N99. Lane (a), molecular weight markers; Lane (b), purified D-EHA DH after final Mono Q 5/50 GL chromatography.

Fig. 3. Multiple alignment of the amino acid sequences of D-EHA DH from *Pseudomonas* sp. N99 (D-EHA DH N99), L-THA DH from *Pseudomonas* sp. T62 (L-THA DH T62), L-THA DH from *S. cerevisiae* (L-THA DH Yeast), and serine racemase from *Schizosaccharomyces pombe* (SR Pombe). The alignment was drawn using Clustal W and BOXSHADE.[22] The numbers on the left side indicate the residues of each amino acid sequence. White letters in the black box indicate the identical residues and white letters in the gray box indicate similar residues. The asterisk indicates a possible PLP-binding residue.

Fig. 4. Optical resolution of DL-EHA and DL-THA. (A) Biocatalytic preparation of L-EHA from DL-EHA using purified D-EHA DH in a reaction mixture containing 100 mM Tris-HCl (pH 8.5), 0.01 mM PLP, 0.1 mM $MnCl_2$, 200 mM DL-EHA, and 20.8 unit (for D-EHA) of the enzyme (6.05 mg) in a total volume of 100 mL. During incubation, the D-form and L-form of 3-hydroxyaspartate in the reaction mixture were monitored using HPLC. The enantiomeric excess (*e.e.*) was calculated from the peak area of the stereoisomers. (B) Biocatalytic preparation of D-THA from the DL-THA using purified D-EHA DH. The methods were the same as mentioned above, except for an enzyme amount (20.8 unit for L-THA, 1.72 mg). The curves shown in the figure are representative value of several experiments, which gave similar results.

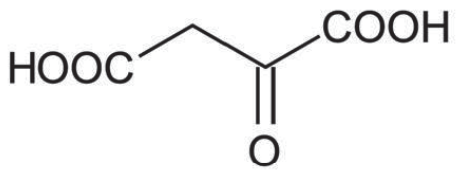


L-*threo*-3-hydroxyaspartate
(L-THA)



D-*erythro*-3-hydroxyaspartate
(D-EHA)

D-EHA DH



Oxaloacetate

+ NH_3

Fig. 1 Nagano et al.

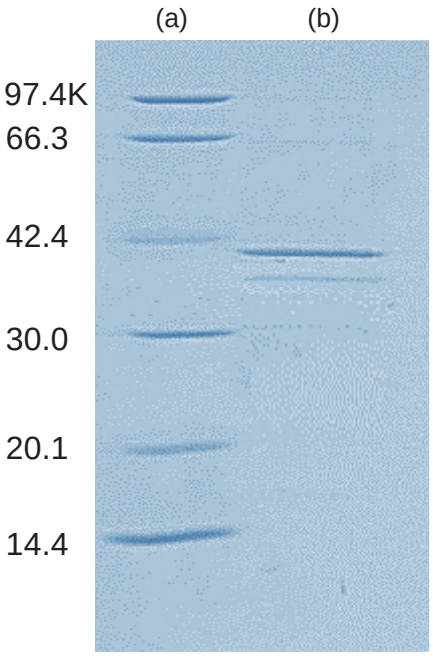


Fig. 2. Nagano et al.

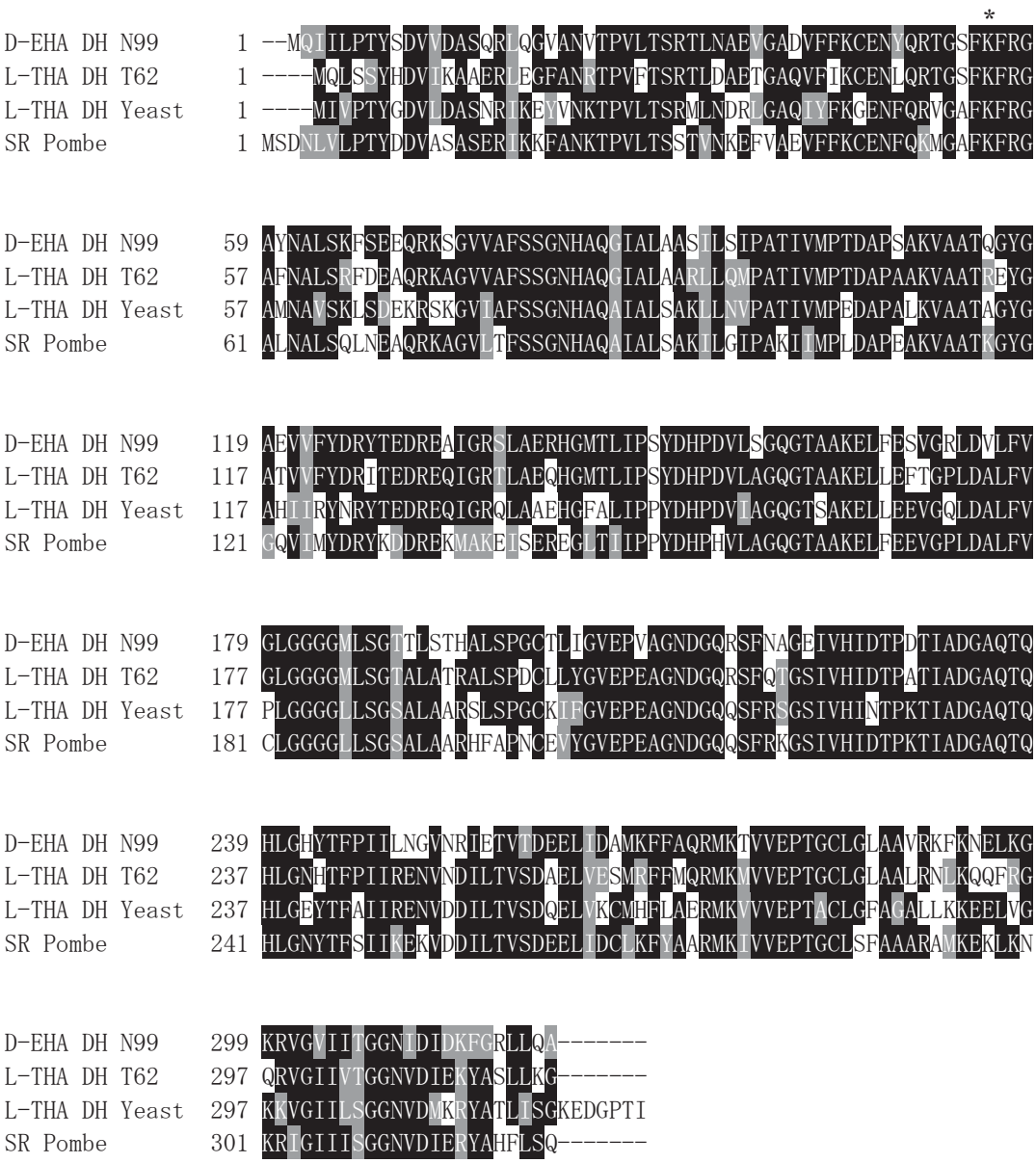


Fig. 3 Nagano et al.

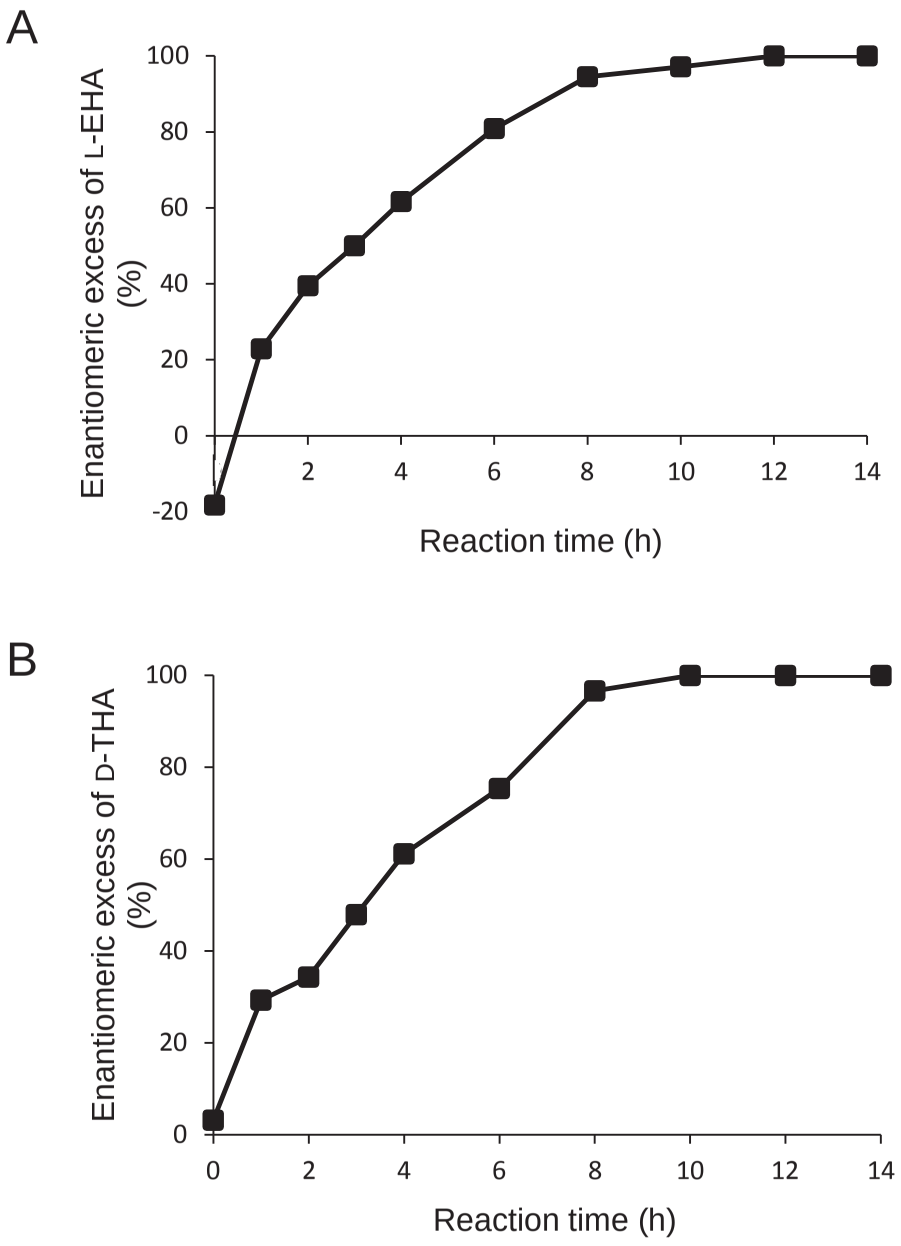


Fig. 4. Nagano et al.

Table 1. Purification of D-EHA DH from *Pseudomonas* sp. N99.

Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
Cell extract	903	108	0.12	1.00	100
Hi-Prep Q FF	78.6	22.8	0.29	2.41	21.1
Ammonium sulfate	72.2	11.6	0.16	1.33	10.7
Hi-Trap Butyl FF	9.68	7.45	0.77	6.42	6.90
Resource Q	2.21	2.19	0.99	8.25	2.03
Superdex-200	0.37	0.78	2.12	17.7	0.72
Mono Q 5/50 GL	0.17	0.43	2.54	21.2	0.40

Table 2. **Kinetic parameters of D-EHA DH.**

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m
D-THA	N.D.	-	-
L-THA	8.61	0.57	15.22
D-EHA	2.19	0.27	8.01
L-EHA	N.D.	-	-
L-serine	0.84	144.0	0.00584

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