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Gene cloning and expression of pyridoxal 5’-phosphate-dependent L-*threo*-3-hydroxyaspartate dehydratase from *Pseudomonas* sp. T62, and characterization of the recombinant enzyme

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Running title: L-*threo*-3-hydroxyaspartate dehydratase from *Pseudomonas*

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Abbreviations:

ADP, adenosine 5’-diphosphate; AMP, adenosine 5’-monophosphate; ATP, adenosine 5’-triphosphate; DH, dehydratase; EDTA, ethylenediaminetetraacetic acid; GDP, guanosine 5’-diphosphate; LB, Luria–Bertani; L-THA, L-*threo*-3-hydroxyaspartate; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NADH, nicotinamide adenine dinucleotide ; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PLP, pyridoxal 5’-phosphate; SDS, sodium dodecyl sulfate; tdcB, the gene encoding biodegradative threonine dehydratase.

SUMMARY

L-*threo*-3-Hydroxyaspartate dehydratase (L-THA DH, EC 4.3.1.16), which catalyzes the cleavage of L-*threo*-3-hydroxyaspartate (L-THA) to oxalacetate and ammonia, has been purified from the soil bacterium *Pseudomonas* sp. T62. In this report, the gene encoding L-THA DH was cloned and expressed in *Escherichia coli*, and the gene product was purified and characterized in detail. A 957 bp nucleotide fragment was confirmed to be the gene encoding L-THA DH, based on the agreement of internal amino acid sequences. The deduced amino acid sequence, which belongs to the serine/threonine dehydratase family, shows similarity to YKL218c from *Saccharomyces cerevisiae* (64%), serine racemase from *Schizosaccharomyces pombe* (64%), and *Mus musculus* (36%), and biodegradative threonine dehydratase from *E. coli* (38%). Site-directed mutagenesis experiments revealed that lysine at position 53 is an important residue for enzymatic activity. This enzyme exhibited dehydratase activity specific only to L-THA ($K_m = 0.54$ mM, $V_{max} = 39.0$ μmol min^{-1} [mg protein]$^{-1}$), but not to other 3-hydroxyaspartate isomers, and exhibited no detectable serine/aspartate racemase activity. This is the first report of an amino acid sequence of the bacterial enzyme that acts on L-THA.

Key words: L-*threo*-3-hydroxyaspartate dehydratase, serine racemase, pyridoxal 5'-phosphate, *Pseudomonas* sp. T62, serine/threonine dehydratase

L-*threo*-3-Hydroxyaspartate (L-THA) is a non-proteinous amino acid with two chiral centers, in which the four stereoisomers are difficult to synthesize. In nature, polypeptide antibiotics, such as cinnamycin (Ro 90-0198), syringomycins, and cormycinA, contain 3-hydroxyaspartate (*1*, *2*, *3*). 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 (*4*). They should therefore serve as useful tools for elucidating the physiological roles of the glutamate transporter.

While the biochemical activity of 3-hydroxyaspartate has been investigated in considerable detail (*4*), little is known about the enzymes that act on 3-hydroxyaspartate isomers. Only two microbial enzymes, *erythro*-3-hydroxyaspartate aldolase (EC 4.1.3.14) (*5*) and *erythro*-3-hydroxyaspartate dehydratase (EC 4.2.1.38) (*6*) have been described. More recently, both eukaryotic and prokaryotic enzymes, L-*threo*-3-hydroxyaspartate dehydratase (L-THA DH; EC 4.3.1.16) from *Pseudomonas* sp. T62 and the YKL218c gene product (YKL218cp) of *Saccharomyces cerevisiae*, have been reported (*7*, *8*). In addition, L-THA β-elimination activity of serine racemase from *Mus musculus* (*9*), and aspartate racemase from *Scapharca broughtonii* (*10*) have been reported. These enzymes belong to the serine/threonine dehydratase family and relate to the fold-type II group of pyridoxal 5'-phosphate (PLP)-dependent enzymes (*11*).

There are several examples of PLP-dependent enzymes that display broad reaction specificities (*12*). For example, serine racemase from *M. musculus* exhibits stronger serine dehydratase activity than serine racemase activity (*13*). Cystathionine γ-synthase from *Salmonella typhimurium* can catalyze the various elimination and replacement reactions of β- and γ-substituted amino acids (*14*). In our previous report, this broad reaction specificity was absent in L-THA DH from *Pseudomonas* sp. T62 (*7*); however, the amino acid sequence and the detailed biochemical features, including serine or aspartate racemase activity, have not yet been investigated. Further characterization and elucidation of the structure-function relationship of this enzyme, and the comparison with eukaryotic serine and aspartate racemase in detail may provide useful clues for understanding mechanisms of the fold-type II group of PLP-dependent enzymes. To obtain enough amount of the enzyme for detailed characterization, we have done the cloning and characterization of the full-length DNA encoding L-THA DH from *Pseudomonas* sp. T62, together with expression of the recombinant enzyme in *E. coli*. This is the first report of an amino acid sequence of the bacterial enzyme that acts on L-THA. Using the constructed expression system, a large amount of the recombinant enzyme was prepared in purified form and characterized in detail.

MATERIALS AND METHODS

Materials - L-THA was purchased from Tocris Cookson, Ltd. (Bristol, UK). L-*erythro*-3-Hydroxyaspartate and phenylpyruvic acid were purchased from Wako Pure Chemicals (Osaka, Japan). DL-*threo*-3-Hydroxyaspartate was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). L-Aspartic acid, L-serine (ultra pure grade), and *N*-acetyl-L-cysteine were purchased from Sigma (St. Louis, MO, USA). 2-Keto-*n*-butyric acid was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical grade and commercially available. Restriction endonucleases were obtained from Nippon Gene (Toyama, Japan).

Bacterial strains, plasmids, and cultivation - *Pseudomonas* sp. T62 was isolated from soil (AKU882, Faculty of Agriculture, Kyoto University) and used as the DNA source. An expression plasmid of biodegradative threonine dehydratase (tdcBp) from *E. coli* was obtained from ASKA clone collection (NBRP, Japan), and named ASKA/JW3088 (*15*). *E. coli* JM109 was used as the host cell for the L-THA DH gene (*lthadh*) cloning and expression, and *E. coli* AG1 was used as the host cell for ASKA/JW3088. *Pseudomonas* sp. T62 and *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^{-1} ampicillin were added to the medium. *E. coli* AG1 cells were grown at 37°C in M9 minimal medium containing 2% casamino acid [M9C, reported previously (*8*)]. When necessary, 100 μg ml^{-1} chloramphenicol were added to the medium. The pGEM-T Easy Vector System (Promega, Madison, WI, USA) was used for TA cloning. The isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible vector pQE30 (Qiagen, Hilden, Germany) with the sequence encoding six consecutive histidine residues at the 5' end of the cloning sites was used for expression of *lthadh* in *E. coli* JM109.

Preparation of genomic and plasmid DNA - Isolation of total DNA from *Pseudomonas* sp. T62 was carried out using Isoplant II (Nippon Gene). The obtained DNA was further purified with phenol/chloroform (50/50, v/v) and phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v). Plasmid DNA was isolated using a Quantum Prep plasmid MiniPrep Kit (Bio-Rad, Hercules, CA, USA).

Isolation of DNA encoding lthadh - L-THA DH was purified from *Pseudomonas* sp. T62 as described previously (*7*). And then, N-terminal and internal peptide sequences were determined as described previously (*16*). Oligonucleotide primer pools were designed based on the amino acid sequences of N-terminal (YHDVIKA) and internal (EPEAGND) peptides. The alignment of the primers was 5'-TAYCAYGAYGTNATHAARGC-3' (sense strand) 5'-TCRTTNCCIGCYTCNGGYTC-3' (antisense strand) (Y = C/T, N = A/C/T/G, H = A/C/T, R = A/G, I = inosinic acid). The chromosomal DNA of *Pseudomonas* sp. T62 was used as the template. The PCR mixture (20 μl) contained 100 pmol each of the primers, 0.19 mM each of dNTP, and 0.05 U of Ex*Taq* DNA polymerase (Takara Bio, Ohtsu, Japan). The thermal cycler (GeneAmp 9700, Applied Biosystems, CA) was set at 94°C for 1 min, at 47°C for 30 sec, and at 72°C for 1 min. The PCR product, approximately 650 bp, was cloned into the pGEM-T Easy Vector for TA cloning and then sequenced. For inverse PCR, the chromosomal DNA of *Pseudomonas* sp. T62 was digested with *Pst*I at 37°C overnight and purified by ethanol precipitation. The DNA fragments were then circularized with Mighty Mix (Takara Bio) at 16°C overnight and used as a template for inverse PCR. Inverse PCR was done with TaKaRa LA*Taq* (Takara Bio) under the following conditions with a sense primer (5’-AAACCGGGAAAGCGCATTGAACGCACCGCG-3’) and an antisense primer (5’-ATGCTCTCCGGTACGGCATTGGCCACCCGT-3’). The PCR mixture (20 μl) contained 10 pmol each of the primers, 0.38 mM each of dNTP, and 0.05 U of LA*Taq* DNA polymerase (Takara Bio). The thermal cycler was set at 94°C for 1 min, at 65°C for 1 min, and at 72°C for 3 min. The inverse PCR product was purified from the agarose gel and sequenced directly.

Construction of expression plasmids - For the expression of *lthadh* in *E. coli*, a DNA fragment of the open reading frame of *lthadh* was prepared by PCR using genomic DNA of *Pseudomonas* sp. T62 as a template. The oligonucleotide sense primer 5’-ATATGGATCCATGCAACTGTCTTCGTACCA-3’ and an antisense primer 5’-TAGCAAGCTTTTGTTGGAGGTTTTAGCCCT-3’ (the underlined sequences represent either the *Bam*HI or *Hin*dIII site) were used. The PCR mixture (20 μl) contained 10 pmol each of the primers, 0.19 mM each of dNTP, and 0.025 U of PrimeSTAR HS DNA polymerase (Takara

Bio). The thermal cycler was set at 98°C for 10 sec, at 55°C for 5 sec, and at 72°C for 1 min. The unique amplified band corresponding to about 1,000 bp was digested with *Bam*HI and *Hin*dIII, and then ligated into the *Bam*HI and *Hin*dIII sites of pQE30. The plasmid obtained for the expression of *lthadh* was named pQE30lthadh and was introduced into *E. coli* JM109 cells. The nucleotide sequence of the insert DNA of pQE30lthadh was then confirmed.

Expression and purification of recombinant L*-THA DH* - The transformed *E. coli* JM109 cells carrying pQE30lthadh were grown at 37°C in 50 ml of LB medium containing ampicillin. For ASKA/JW3088 expression, the transformed *E. coli* AG1 cells were grown at 37°C in 50 ml of M9C medium containing chloramphenicol. In order to induce gene expression, 0.01 mM IPTG was added to the culture medium when the absorbance at 600 nm reached 0.3. After cultivation for another 16 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. *E. coli* JM109 and AG1 cells (0.62 g wet weight) obtained from a 50 ml culture were disrupted with an ultrasonic oscillator. After centrifugation (14,000 rpm for 15 min), the resulting supernatant was applied to a HisTrap HP column (0.7 × 2.5 cm; GE Healthcare, UK) connected to a fast protein liquid chromatography (FPLC) system (Amersham Biosciences, UK). The column was equilibrated with buffer supplemented with 20 mM imidazole. The enzyme was eluted with a 20–500 mM step-wise imidazole gradient. Active fractions were collected, dialyzed against the buffer, and used as the enzyme for characterization.

Enzyme assay - L-THA DH activity was determined spectrophotometrically as described previously (*7*). Serine and aspartate racemase activities were determined by fluorometric high-performance liquid chromatography (HPLC) methods as reported previously (*10*, *17*). In addition to these methods, threonine and phenylserine dehydratase activities were assayed by a colorimetric method based on the detection of α-keto acids using 2, 4-dinitrophenylhydrazine (*10*, *18*).

One unit of the enzyme was defined as the amount capable of catalyzing the oxidation of 1 μmol of NADH per min. Protein concentrations were determined by the dye-binding method of Bradford with a Bio-Rad protein assay kit (Bio-Rad) using bovine serine albumin as the standard (*19*).

Site-directed mutagenesis - The mutant enzyme, K53A, was prepared according to the protocol of the TaKaRa 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.

Nucleotide sequence accession number - The nucleotide sequence of the *lthadh* gene of *Pseudomonas* sp. T62 has been deposited in the DDBJ/EMBL/GenBank database under accession number AB297468.

Bioinformatic analysis - Amino acid sequences were obtained from Swiss-Prot from the ExPaSy proteomics server (http://kr.expasy.org/) of the Swiss Institute of Bioinformatics. A homology search was performed with the FASTA program at DDBJ (http://www.ddbj.nig.ac.jp/search/fasta-j.html) (*20*). The amino acid sequence alignment was performed with ClustalW 1.83 and BOXSHADE 3.21 (*21*). The phylogenetic tree was constructed with TreeView1.66 (*22*).

Molecular mass measurement - The molecular mass of the enzyme was estimated using a MALDI-TOF-MS (Voyager Biospectrometry, Applied Biosystem) using a 25-kV acceleration voltage. The samples were run in linear mode, and sinapic acid was used as the matrix.

RESULTS

Nucleotide sequence of the L-THA DH gene from Pseudomonas sp. T62 - We obtained N-terminal (YHDVIKA) and internal (EPEAGND) amino acid sequences of L-THA DH from *Pseudomonas* sp. T62 using Edman degradation. A pair of degenerate primers was then synthesized based on these sequences. Using these primers, a 615 bp DNA fragment was isolated using degenerate PCR. Based on the sequence information in this DNA fragment, a new pair of specific primers for inverse PCR was synthesized. The inverse PCR product was sequenced directly, and the obtained information was used to design a new set of primers for construction of the expression plasmid.

The open reading frame is 957 bp long and encodes a protein of 319 amino acid residues with a predicted molecular mass of 34.3 kDa. It contains a highly conserved pyridoxal-5'-phosphate binding motif (Prosite PS00165: [DESH]-x(4,5)-[STVG]-{EVKD}-[AS]-[FYI]-K-[DLIFSA]-[RLVMF]-[GA]-[LIVMGA]) that is also found in the serine/threonine dehydratase family proteins, consistent with the PLP dependence of the native enzyme (*7, 23*).

A FASTA search revealed that this deduced amino acid sequence showed 64% identity with YKL218cp from *S. cerevisiae,* which has L-THA DH activity (*8*). The amino acid sequence homology follows the eukaryotic serine/aspartate racemase and threonine dehydratase from various origins, such as serine racemase from *S. pombe* (64%), aspartate racemase from *S. broughtonii* (39%), serine racemase from *M. musculus* (36%), and tdcBp from *E. coli* (38%).

Fig. 1

Fig. 1

Figure 1 shows multiple alignments of these deduced amino acid sequences. In addition to the conserved sequence around the lysine residue at position 53 that binds PLP through a Schiff base, other residues that have been shown to interact with PLP in other serine/threonine dehydratase family proteins were also conserved; for example, the PLP-binding Lys62/Phe61/Gly241 that sandwiches the PLP ring, Ser315 whose side-chain is hydrogen-bonded to the pyridinium nitrogen of PLP, and Asn89, which stabilizes the 3' oxygen of PLP by a hydrogen bond in *E. coli* biosynthetic threonine dehydratase, corresponded to Lys53, Phe52, Gly232, Ser304, and Asn80 in L-THA DH, respectively (*24*). The glycine-rich group, which coordinates the phosphate sequence, part of PLP, comprises a tetraglycine loop (Gly179-182) in L-THA DH. This tetraglycine loop is also found in serine racemase of *M. musculus* and *S. pombe*, tdcBp of *E. coli*, and YKL218cp of *S. cerevisiae* (*24*).

The phylogenetic analysis presented in Fig. 2 suggests that L-THA DH from *Pseudomonas* sp. T62 is closely related to eukaryotic racemases, which also show L-THA DH activity, such as aspartate racemase from *S. broughtonii* (*25*) and serine racemase from *M. musculus* (*26*). However, tdcBp, which has some amino acid sequence homology with L-THA DH (38%), almost equal to those of aspartate racemase from *S. broughtonii* (39%) and serine racemase from *M. musculus* (36%), did not show L-THA DH activity (see below).

Fig. 2

Purification and relative molecular mass of the enzyme - We purified the recombinant L-THA DH from *E. coli* cells and characterized its enzymatic properties. The molecular mass of MALDI-TOF-MS analysis (36.0 kDa) was in agreement with the range obtained by the recombinant enzyme deduced from the amino acid sequence (35.7 kDa). This value was lower than that determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE;

Fig.3

Fig. 3

40 kDa, Fig. 3), and that of the enzyme purified from the original strain (approximately 39 kDa by SDS-PAGE) (*7*). The discrepancy may be due to the surface charge of the protein or some unknown factors such as the conformation of the enzyme.

Sequencing of the N-terminal amino acid of the recombinant His-tagged fusion enzyme up to the fifteenth amino acid residue perfectly matched the deduced amino acid sequence. From these results, we concluded that this protein was the recombinant L-THA DH.

Absorption spectrum and identification of the active site lysine residue - The recombinant enzyme had absorption maxima at 280 nm and 410 nm (Fig. 4), and this spectrum is hardly distinguishable from that of the native enzyme (*7*). Solutions of the pure enzyme were distinctly yellow in color. These results suggest that the enzyme contains PLP as the prosthetic group.

Fig. 4

To identify the PLP-interacting lysine residue of the enzyme, one mutant enzyme, K53A, was constructed and purified as described in the MATERIALS AND METHODS. The K53A mutant enzyme showed no detectable activity (<0.01% of the activity toward L-THA) and did not have a large absorption maximum at 410 nm (Fig. 4). The remaining small peak around 410 nm in K53A mutant suggests that this mutant can still retain PLP, presumably because all of the PLP-binding residues but Lys53 remain intact to form a Schiff base with a lysine residue other than Lys53. Nevertheless, Lys53 is the most plausible candidate that is involved in both PLP binding and catalysis, as its conversion to alanine abolished catalytic activity. Incidentally, enzyme activity of the K53A mutant was not observed even by the addition of 100 and 200 mM methylamine, implying that a chemical rescue does not work in the present system (*27, 28*).

Substrate and reaction specificity of the enzyme - The enzyme was highly specific toward L-THA. None of the other 3-hydroxy amino acids tested (i.e., DL-*erythro*- and

D-*threo*-3-hydroxyaspartate, D-threonine, L-threonine, DL-*allo*-threonine, D-serine, L-serine, and DL-phenylserine) was a substrate for this enzyme, either at 5 mM or 50 mM, as in the case of the enzyme from the original strain (*7*). With L-THA, normal hyperbolic kinetics were observed, and the K_m and V_{max} values, calculated from Eadie-Hofstee plots, were 0.54 mM and 39.0 μ mol min^{-1}(mg protein)$^{-1}$, respectively for L-THA, while the previously reported K_m and V_{max} values for the enzyme from the original strain were 0.74 mM and 37.5 μ mol min^{-1}(mg protein)$^{-1}$, respectively.

Serine and aspartate racemase activities of the enzyme were investigated using a sensitive assay method. After a 12-h incubation with L-serine or L-aspartate and the enzyme, no D-serine or D-aspartate was observed using HPLC with fluorometric detection. No serine and aspartate racemase activities were detected even under the most activated condition (in the presence of 10 mM adenosine 5'-monophosphate (AMP) at pH 9.0). Thus, we conclude that both serine and aspartate racemase activities of L-THA DH are below the detection limit, i.e., less than 5.0×10^{-2} pmol h^{-1} (mg protein)$^{-1}$.

Effects of amino acids - Addition of 5 mM L-*erythro*-3-hydroxyaspartate or 5 mM D-serine decreased the enzyme activity to 15% and 27%, respectively. L-*erythro*-3-hydroxyaspartate showed a strong competitive inhibition (K_i, 0.20 mM) against L-THA, and D-serine showed non-competitive inhibition (K_i, 22.8 mM) against L-THA. DL-Aspartate, L-serine, DL-threonine, DL-*allo*-threonine, DL-phenylserine, or malonic acid did not cause significant inhibition of the enzyme reaction at 5 mM.

Effects of metal ions - EDTA was added to the standard reaction mixture at a final concentration of 1 mM, and enzyme activity was then measured. The enzyme was strongly inhibited by EDTA

(69% inhibition), suggesting that metal ions were involved in the enzyme reaction. The effects of divalent cations were also measured using the enzyme as control dialyzed against the buffer minus $MnCl_2$, showing a decrease of the specific activity of the enzyme to approximately 36% of the initial activity after overnight dialysis. When 1 mM $MnCl_2$, $MgCl_2$, or $CaCl_2$ was added to the reaction mixture, the relative enzyme activity increased to 151%, 196%, and 159% of control activity, respectively. In contrast, $ZnCl_2$, $SnCl_2$, $CoCl_2$, or $CuCl_2$ caused inhibition of enzyme activity (56%, 70%, 81%, and 92% relative activity, respectively).

Effects of pH and temperature - Optimal pH and temperature of the recombinant enzyme were determined to be 9.0 and 35°C.

Effects of nucleotides - Addition of 10 mM AMP or adenosine 5'-diphosphate (ADP) increased the enzyme activity to 144% and 106% of the control, respectively, whereas 10 mM adenosine 5'-triphosphate (ATP) or guanosine 5'-diphosphate (GDP) decreased activity to 89% and 73%, respectively.

Side reaction of tdcBp - To assess whether tdcBp has L-THA DH activity, we purified the recombinant tdcBp from *E. coli* AG1 cells and characterized its enzymatic properties. The recombinant His-tagged fusion tdcBp was purified to give a single band, corresponding to a relative molecular mass of approximately 40 kDa, on SDS-PAGE (Fig. 3). Dehydratase activity toward L-threonine was 16.7 μmol min^{-1}(mg $protein)^{-1}$, whereas dehydratase activity toward L-THA could not be detected (<0.01% of dehydratase activity toward threonine). Serine and aspartate racemase activities of tdcBp were also below the detection limit [$<5.0 \times 10^{-2}$ pmol h^{-1}(mg $protein)^{-1}$].

DISCUSSION

Amino acid sequence analysis - The isolation and sequencing of the complete DNA coding for a PLP-dependent L-THA DH from *Pseudomonas* sp. T62, as well as the expression of the recombinant active enzyme, are reported here for the first time. The amino acid sequence deduced from the nucleotide sequence displays significant homology to the serine/threonine dehydratase family enzymes. The highest amino acid sequence identity to this enzyme family is exhibited by serine racemase from *S. pombe* (64%), for which the crystal structure has been solved [PDB code; 1V71]. The next highest identity is shown by *S. cerevisiae* YKL218cp (formally "serine racemase in yeast"), which represents the first eukaryotic L-THA DH (*8*). The identity is followed by aspartate racemase from *S. broughtonii* (39%), serine racemase from *M. musculus* (36%), and tdcBp (38%). The site-directed mutagenesis experiment revealed that Lys53 of the present enzyme is an important residue that can form a Schiff base with PLP. In addition to Lys53, the amino acid residues Phe52, Asn80, Gly232, Ser304, and Gly179-182, which interact with PLP in *S. pombe* and *M. musculus* serine racemase, *E. coli* tdcBp, and *S. cerevisiae* YKL218cp, are mostly conserved among the enzymes shown in Fig. 2 (*29, 30*).

A FASTA search revealed that the putative threonine dehydratase from Gram-negative bacteria such as *Delftia acidovorans* SPH-1 (74%), *Burkholderia cepacia* (72%), and *Pseudomonas aeruginosa* PAO1 (72%) have higher amino acid sequence identity than serine racemase from *S. pombe* (Fig. 2). This suggests that these enzymes, which share high identity with L-THA DH from *Pseudomonas* sp. T62, have L-THA DH activity. L-THA DH is most probably distributed broadly in nature, especially in Gram-negative soil bacteria.

Substrate and reaction specificity - From phylogenic analysis (Fig. 2), the eukaryotic racemases that act on L-THA, such as serine racemase from *M. musculus* (*26*) and aspartate racemase from *S. broughtonii* (*25*), are closely related to L-THA DH from *Pseudomonas* sp. T62. From this, it is possible that L-THA DH possesses racemase activity toward serine or aspartate as a side reaction. However, L-THA DH did not show any aspartate racemase or serine racemase activity. In addition to the eukaryotic racemases, tdcBp, which depends on PLP, also displays considerable sequence homology with L-THA DH (38%). Therefore, it is also suggested that L-THA DH and tdcBp may have overlapping substrate specificity. To confirm these hypotheses, we checked the L-THA DH activity of the recombinant that was obtained from NBRC. However, tdcBp did not exhibit detectable L-THA DH activity. In addition, L-THA DH did not show L-threonine dehydratase activity. These results indicate that L-THA DH from *Pseudomonas* sp. T62 can be clearly distinguished from aspartate racemase, serine racemase, and threonine dehydratase. From these results, we conclude that L-THA DH is an unique enzyme that is specific only to L-THA.

Effects of metals and inhibitors - L-THA DH requires divalent cations for activation, similar to YKL218cp from *S. cerevisiae* and serine racemase from *M. musculus*. The divalent cations, Mg^{2+}, Ca^{2+}, and Mn^{2+}, act as activators of L-THA DH (increased to 151%, 196%, and 159%), as in the case of serine racemase from *M. musculus* (increased to about 110%, 135%, and 120%) (*31*). Other PLP-dependent bacterial enzymes, such as D-threonine aldolase from *Arthrobacter* sp. DK-38, were also reported to be activated by divalent cations (*32*), however, the physiological relevance of those activations remains unclear.

As for the effect of nucleotides, aspartate racemase from *S. broughtonii* and tdcBp were dramatically activated by AMP (399% and ~1000% activation, respectively). These activations are thought to be associated with the energy metabolism (*33, 34*). In contrast to these dramatic activations, the effect of AMP on L-THA DH was limited (144%) as in the case of the effect of ATP on serine racemase from *M. musculus* (160%). These modest effects of the nucleotides suggest that L-THA DH is not directly involved in the energy metabolism in *Pseudomonas* sp. T62 cells. It is, however, difficult to find a correlation of the sensitivity of these enzymes to nucleotides from the amino acid sequence homology alone. The mechanism of activation and inhibition by nucleotides remains also to be clarified.

L-*erythro*-3-Hydroxyaspartate was a strong inhibitor of L-THA DH. The K_i value of L-*erythro*-3-hydroxyaspartate was 0.20 mM, which is similar to the K_i of serine racemase from *M. musculus* (0.049 mM) (*26*). In addition to the low K_i of L-*erythro*-3-hydroxyaspartate, high catalytic efficiency toward L-THA of serine racemase from *M. musculus* was also reported (*26*). The k_{cat}/K_m value for L-THA of serine racemase from *M. musculus* was reported to be 1800 min^{-1} mM^{-1}, while that of L-THA DH reported here was 2800 min^{-1} mM^{-1}. These results suggest that the active site structure of L-THA DH resembles that of the serine racemase from *M. musculus.* We are going to analyze the 3D structure of L-THA DH in a hope of unraveling factors underlying the stringent substrate/reaction specificity and the origin of susceptibility to regulation by metal ions and nucleotides in this enzyme at the molecular level.

Possible physiological function - Although the physiological function of L-THA DH in *Pseudomonas* sp. T62 remains unknown, there is one possible explanation. Several *Pseudomonas* bacterial strains produce antibiotics, such as syringomycins and cormycinA, which contain L-THA in their structure (*1*, *3*, *35*). It is possible that *Pseudomonas* sp. T62 itself or other bacteria in adjacent environments can produce antibiotics containing L-THA. If antibiotics containing L-THA are hydrolyzed by a peptidase or other hydrolyzing enzyme, free L-THA is released, and this is toxic to many bacteria (*36*). Thus, L-THA DH may play a role in detoxification of free L-THA in *Pseudomonas* sp. T62 cells. This hypothesis is also supported by the fact that the enzyme is inducible in *Pseudomonas* sp. T62 (*7*); however, the details still need to be elucidated.

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Figure legends

Fig. 1. Multiple alignment of the amino acid sequence of L-THA DH from *Pseudomonas* sp. T62 (PsLTHADH) with those of YKL218cp from *S. cerevisiae* (ScLTHADH), serine racemase from *M. musculus* (MsSR), serine racemase from *S. pombe* (SpSR), aspartate racemase from *S. broughtonii* (ScAspR), and biodegradative threonine dehydratase from *E. coli* (tdcBp).

The alignment was generated with Clustal W 1.83 and BOXSHADE 3.21. The numbers on the left side are the residue numbers of each amino acid sequence. White letters in a black background indicate identical residues, and white letters in a gray background indicate similar residues. Asterisk indicates the PLP-binding residues.

Fig. 2. Phylogenetic relationships among amino acid racemase and serine/threonine dehydratases from various organisms. The phylogenetic tree was created with the Clustal W 1.83 and TreeView 1.6.6 programs. The scale bar represents 0.1 amino acid substitution per site. The asterisks indicate putative enzymes. Swiss-prot number: Q8VBT2, L-serine dehydratase from *Mus musculus*; Q9GZT4, serine racemase from *Homo sapiens*; Q9QZX7, serine racemase from *Mus musculus*: Q9ZSS6, biosynthetic threonine dehydratase, chloroplastic from *Arabidopsis thaliana*; Q76EQ0, serine racemase from *Rattus norvegicus*; A4F2N8, L-THA DH from *Pseudomonas* sp. T62; Q9KVW1, threonine dehydratase from *Vibrio cholera*; Q2L695, aspartate racemase from *Scapharca broughtonii*; P20132, L-serine dehydratase from *Homo sapiens*; P04968, biosynthetic threonine dehydratase, from *Escherichia coli* (strain K12); P36007, YKL218cp from *Saccharomyces cerevisiae*; P0AGF6, biodegradative threonine dehydratase,

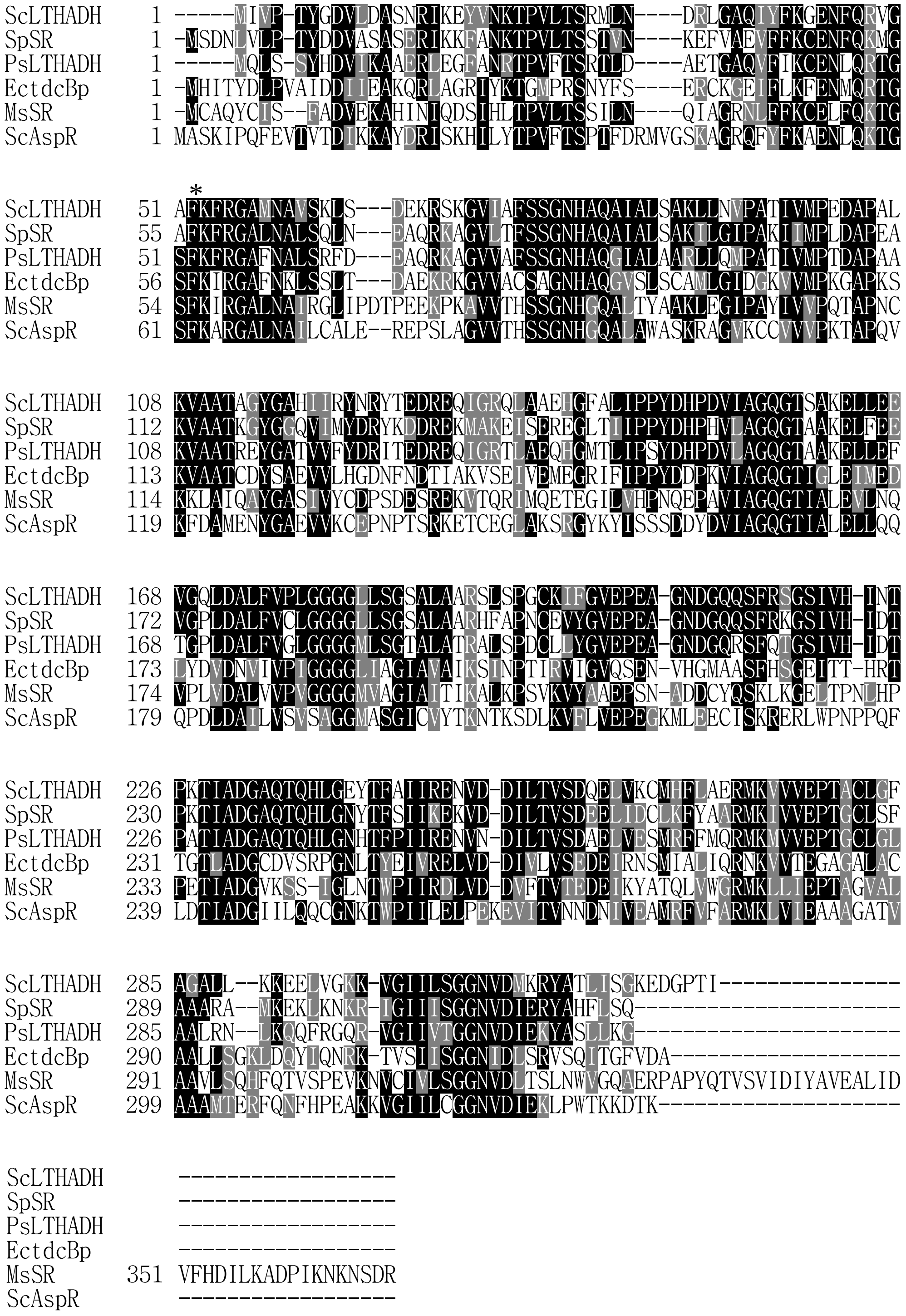
from *Escherichia coli* (strain K12); Q7A5L8, biodegradative threonine dehydratase, from *Staphylococcus aureus* (strain N315);Q9I0F5, threonine dehydratase from *Pseudomonas aeruginosa* PAO1; A9C0S1, threonine dehydratase from *Delftia acidovorans* SPH-1; Q0B8T7, threonine dehydratase from *Burkholderia cepacia* (strain ATCC 53795/AMMD); O59791, serine racemase from *Schizosaccharomyces pombe*.

Fig. 3. SDS-PAGE analysis of samples at the purification stages for recombinant L-THA DH and tdcBp from *E. coli* cells.

Lane 1, cell extract of *E. coli* JM109/pQE30lthadh; lane 2, purified L-THA DH; Lane 3, cell extract of *E. coli* AG1/ASKA/JW3088; lane 4, purified tdcBp; Lane M, molecular weight markers.

Fig. 4. Absorption spectrum of the recombinant enzyme.

Absorption spectra were obtained with a Beckman spectrophotometer DU-800. The recombinant enzyme (0.7 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, K53A mutant enzyme.



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SpSR        1 -MSDNLVLP-TYDDVASASERIKKFANKTPVLTSSTVN----KEFVAEVFFKCENFQKMG
PsLTHADH    1 -----MQLS-SYHDVIKAAERLEGFANRTPVFTSRTLD----AETGAQVFIKCENLQRTG
EctdcBp     1 -MHITYDLPVAIDDIIEAKQRLAGRIYKTGMPRSNYFS----ERCKGEIFLKFENMQRTG
MsSR        1 -MCAQYCIS--FADVEKAHINIQDSIHLTPVLTSSILN----QIAGRNLFFKCELFQKTG
ScAspR      1 MASKIPQFEVTVTDIKKAYDRISKHILYTPVFTSPTFDRMVGSKAGRQFYFKAENLQKTG

               *
ScLTHADH   51 AFKFRGAMNAVSKLS---DEKRSKGVIAFSSGNHAQAIALSAKLLNVPATIVMPEDAPAL
SpSR       55 AFKFRGALNALSQLN---EAQRKAGVLTFSSGNHAQAIALSAKILGIPAKIIMPLDAPEA
PsLTHADH   51 SFKFRGAFNALSRFD---EAQRKAGVVAFSSGNHAQGIALAARLLQMPATIVMPTDAPAA
EctdcBp    56 SFKIRGAFNKLSSLT---DAEKRKGVVACSAGNHAQGVSLSCAMLGIDGKVVMPKGAPKS
MsSR       54 SFKIRGALNAIRGLIPDTPEEKPKAVVTHSSGNHGQALTYAAKLEGIPAYIVVPQTAPNC
ScAspR     61 SFKARGALNAILCALE--REPSLAGVVTHSSGNHGQALAWASKRAGVKCCVVVPKTAPQV

ScLTHADH  108 KVAATAGYGAHIIRYNRYTEDREQIGRQLAAEHGFALIPPYDHPDVIAGQGTSAKELLEE
SpSR      112 KVAATKGYGGQVIMYDRYKDDREKMAKEISEREGLTIIPPYDHPHVLAGQGTAAKELFEE
PsLTHADH  108 KVAATREYGATVVFYDRITEDREQIGRTLAEQHGMTLIPSYDHPDVLAGQGTAAKELLEF
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MsSR      114 KKLAIQAYGASIVYCDPSDESREKVTQRIMQETEGILVHPNQEPAVIAGQGTIALEVLNQ
ScAspR    119 KFDAMENYGAEVVKCEPNPTSRKETCEGLAKSRGYKYISSSDDYDVIAGQGTIALELLQQ

ScLTHADH  168 VGQLDALFVPLGGGGLLSGSALAARSLSPGCKIFGVEPEA-GNDGQQSFRSGSIVH-INT
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EctdcBp   173 LYDVDNVIVPIGGGGLIAGIAVAIKSINPTIRVIGVQSEN-VHGMAASFHSGEITT-HRT
MsSR      174 VPLVDALVVPVGGGGMVAGIAITIKALKPSVKVYAAEPSN-ADDCYQSKLKGELTPNLHP
ScAspR    179 QPDLDAILVSVSAGGMASGICVYTKNTKSDLKVFLVEPEGKMLEECISKRERLWPNPPQF

ScLTHADH  226 PKTIADGAQTQHLGEYTFAIIRENVD-DILTVSDQELVKCMHFLAERMKVVVEPTACLGF
SpSR      230 PKTIADGAQTQHLGNYTFSIIKEKVD-DILTVSDEELIDCLKFYAARMKIVVEPTGCLSF
PsLTHADH  226 PATIADGAQTQHLGNHTFPIIRENVN-DILTVSDAELVESMRFFMQRMKMVVEPTGCLGL
EctdcBp   231 TGTLADGCDVSRPGNLTYEIVRELVD-DIVLVSEDEIRNSMIALIQRNKVVTEGAGALAC
MsSR      233 PETIADGVKSS-IGLNTWPIIRDLVD-DVFTVTEDEIKYATQLVWGRMKLLIEPTAGVAL
ScAspR    239 LDTIADGIILQQCGNKTWPIILELPEKEVITVNNDNIVEAMRFVFARMKLVIEAAAGATV

ScLTHADH  285 AGALL--KKEELVGKK-VGIILSGGNVDMKRYATLISGKEDGPTI---------------
SpSR      289 AAARA--MKEKLKNKR-IGIIISGGNVDIERYAHFLSQ----------------------
PsLTHADH  285 AALRN--LKQQFRGQR-VGIIVTGGNVDIEKYASLLKG----------------------
EctdcBp   290 AALLSGKLDQYIQNRK-TVSIISGGNIDLSRVSQITGFVDA-------------------
MsSR      291 AAVLSQHFQTVSPEVKNVCIVLSGGNVDLTSLNWVGQAERPAPYQTVSVIDIYAVEALID
ScAspR    299 AAAMTERFQNFHPEAKKVGIILCGGNVDIEKLPWTKKDTK--------------------

ScLTHADH      ------------------
SpSR          ------------------
PsLTHADH      ------------------
EctdcBp       ------------------
MsSR      351 VFHDILKADPIKNKNSDR
ScAspR        ------------------
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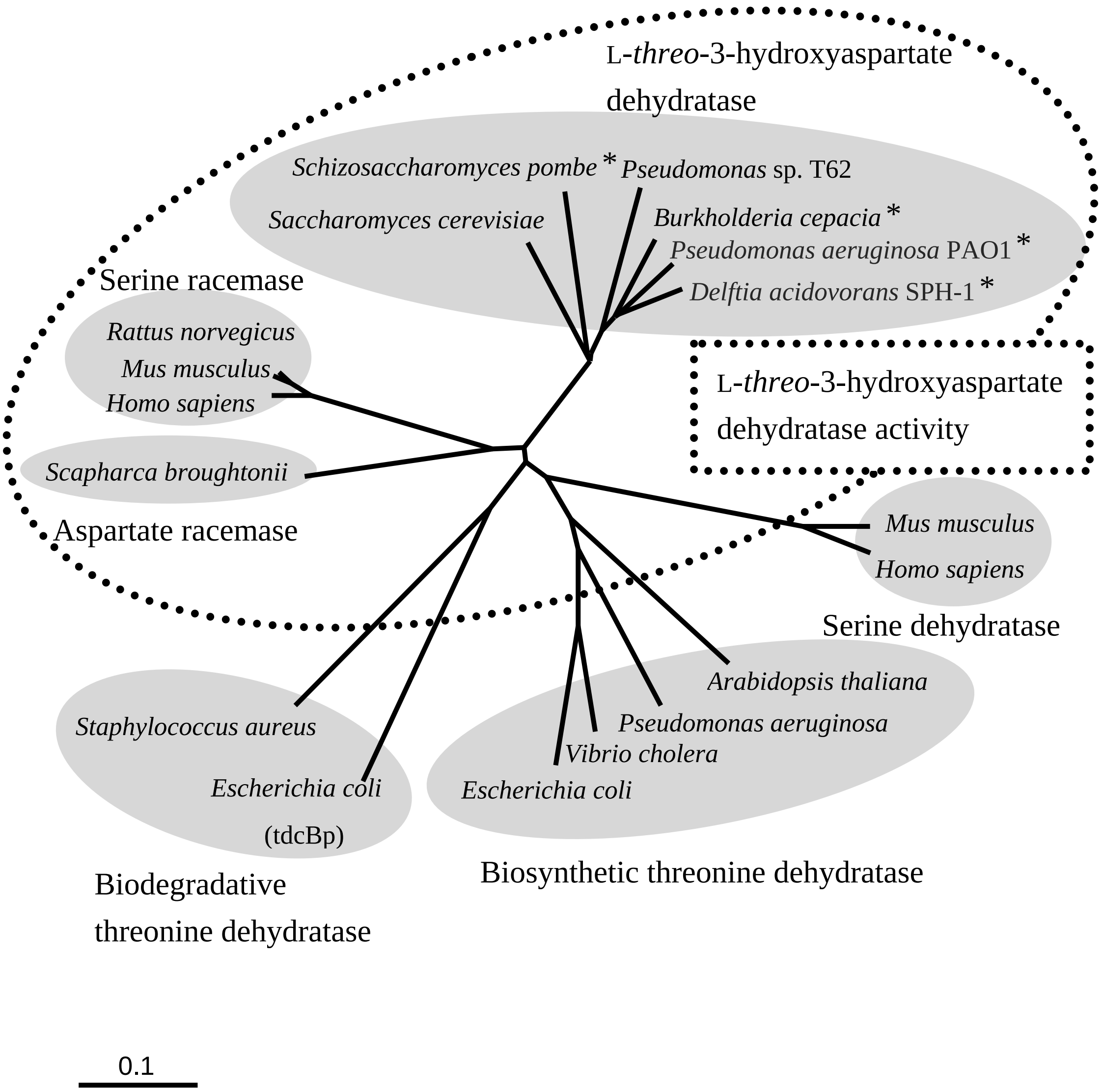
Fig. 1　Murakami et al.



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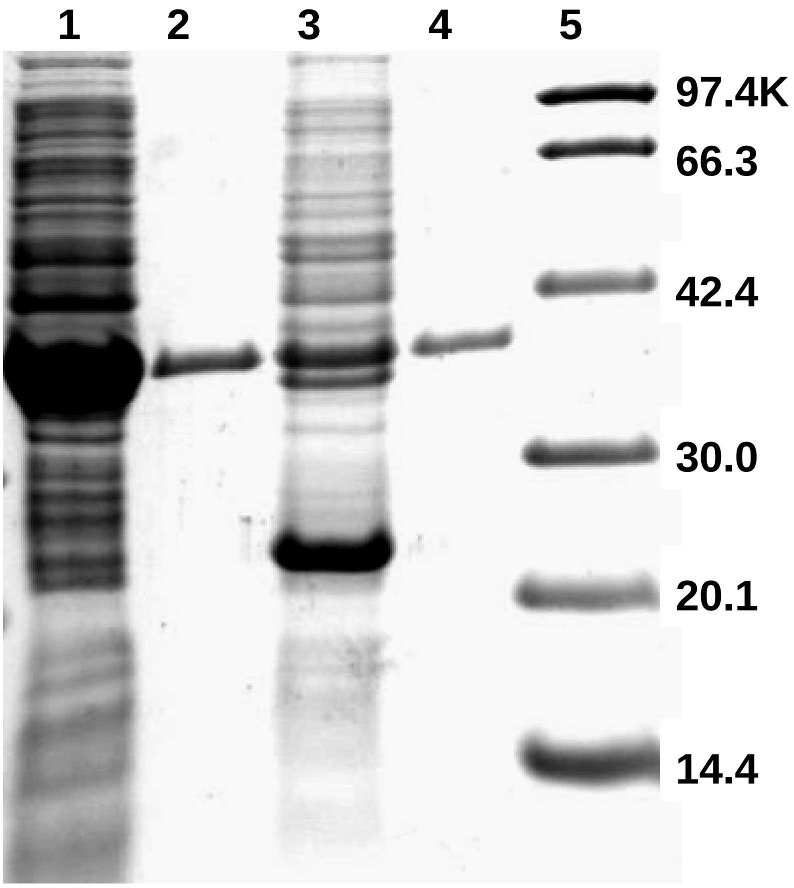

Fig. 3. Murakami et al.

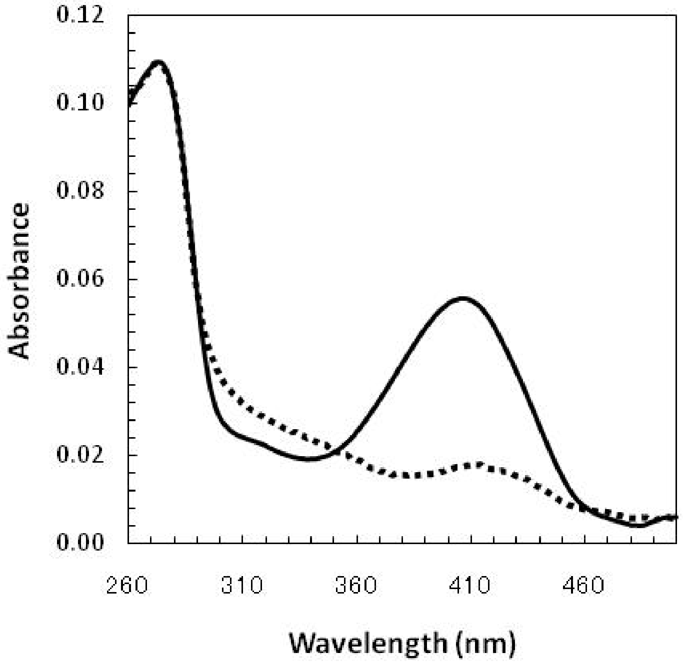

Fig. 4. Murakami et al.