

Molecular cloning and enzymological characterization of pyridoxal 5'-phosphate independent aspartate racemase from hyperthermophilic archaeon *Thermococcus litoralis* DSM 5473

Tsubasa Washio¹ · Shiro Kato² · Tadao Oikawa^{1,2}

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Abstract We succeeded in expressing the aspartate racemase homolog gene from *Thermococcus litoralis* DSM 5473 in *Escherichia coli* Rosetta (DE3) and found that the gene encodes aspartate racemase. The aspartate racemase gene consisted of 687 bp and encoded 228 amino acid residues. The purified enzyme showed aspartate racemase activity with a specific activity of 1590 U/mg. The enzyme was a homodimer with a molecular mass of 56 kDa and did not require pyridoxal 5'-phosphate as a coenzyme. The enzyme showed aspartate racemase activity even at 95 °C, and the activation energy of the enzyme was calculated to be 51.8 kJ/mol. The enzyme was highly thermostable, and approximately 50 % of its initial activity remained even after incubation at 90 °C for 11 h. The enzyme showed a maximum activity at a pH of 7.5 and was stable between pH 6.0 and 7.0. The enzyme acted on L-cysteic acid and L-cysteine sulfinic acid in addition to D- and L-aspartic acids, and was strongly inhibited by iodoacetic acid. The site-directed mutagenesis of the enzyme showed that the essential cysteine residues were conserved as Cys83 and Cys194. D-Forms of aspartic acid, serine, alanine, and valine were contained in *T. litoralis* DSM 5473 cells.

Keywords *Thermococcus litoralis* · Aspartate racemase · D-Aspartic acid · Hyperthermophilic archaeon · Molecular cloning

Abbreviations

AspR	Aspartate racemase
DTT	Dithiothreitol
KPB	Potassium phosphate buffer
LB	Luria-Bertani
PLP	Pyridoxal 5'-phosphate
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulfate

Introduction

Thermococcus litoralis DSM 5473 was isolated from a hot spring of a shallow sea in Naples, Italy, and was able to grow from 55 to 98 °C in a pH range of 4.0 to 8.0 and optimally at 88 °C and pH 6.0 (Neuner et al. 1990). *T. litoralis* DSM 5473 is one of the representative hyperthermophiles studied extensively so far, but little information is now available for its amino acid metabolism. For example, NADP⁺-specific L-glutamate dehydrogenase was purified to homogeneity from the cell-free extract of *T. litoralis* DSM 5473 to demonstrate that it was a major protein in the peptide-utilizing *T. litoralis* DSM 5473 (Ma et al. 1994). Two aromatic aminotransferases of pyridoxal 5'-phosphate enzymes were found in the soluble fraction of *T. litoralis* DSM 5473 and were thought to be involved in the catabolism of the L-form of aromatic amino acids in this organism (Andreotti et al. 1994). L-Aminoacylase of *T. litoralis* DSM 5473 was cloned and overexpressed in *Escherichia coli*, and its enzymological properties were examined (Toogood et al. 2002); the enzyme was specific

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✉ Tadao Oikawa
oikawa@kansai-u.ac.jp

- ¹ Department of Life Science and Biotechnology, Faculty of Chemistry, Materials and Bioengineering, Kansai University, 3-3-35 Yamate-Cho, Suita, Osaka-Fu 564-8680, Japan
- ² Kansai University High Technology Research Center, 3-3-35 Yamate-Cho, Suita, Osaka-Fu 564-8680, Japan

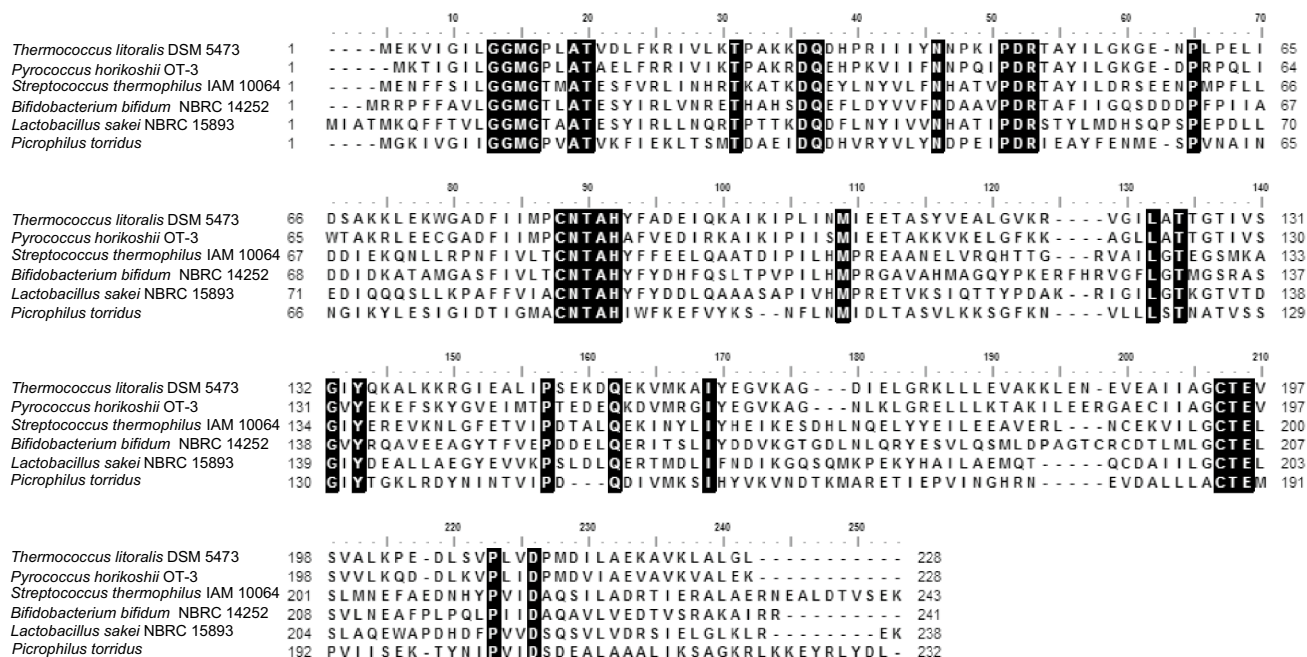


Fig. 1 Comparison of the primary structure of aspartate racemase from *Thermococcus litoralis* DSM 5473 with those from other microorganisms. *Pyrococcus horikoshii* OT-3 (accession no. PH0670);

Streptococcus thermophilus IAM 10064 (accession no. X61301); *Bifidobacterium bifidum* NBRC 14252 (accession no. AB179841); and *Lactobacillus sakei* NBRC 15893 (accession no. LC034559)

for *N*-benzoyl or *N*-chloroacetyl L-phenylalanine, L-methionine, and L-cysteine. Recently, a bifunctional proline racemase/hydroxyproline epimerase gene was identified in the genome of *T. litoralis* DSM 5473 (Watanabe et al. 2015), and to the best of our knowledge, its gene product is the first and only example of an enzyme that acts on the D-amino acid reported for this microorganism to date. Accordingly, we are interested in amino acid metabolism, especially D-amino acid metabolism, and the metabolizing enzymes of *T. litoralis* DSM 5473.

Recently, according to the development of an analytical technique (Gogami et al. 2011), D-amino acid has been widely found in nature and has shown several physiological functions in living organisms. In mammals, D-serine exists in the brain and acts as a coagonist of *N*-methyl-D-aspartic acid (NMDA) receptors (Fadda et al. 1988), while D-aspartic acid in the testes regulates the biosynthesis of testosterone (D'Aniello et al. 1996). In bacteria, D-alanine and D-glutamic acid generally exist as major components of peptidoglycan. In Archaea, D-aspartic acid was detected in the crude extract of *Desulfurococcus* sp. SY, *Pyrococcus* sp. O-II, or *Thermococcus* sp. KS-1, but its physiological function remains unknown (Matsumoto et al. 1999). Most D-amino acids in living organisms were synthesized by amino acid racemases. The two following types of amino acid racemases have been previously

reported: pyridoxal 5'-phosphate (PLP) dependent- and/or PLP-independent enzymes. Alanine racemase (AlaR: EC 5.1.1.1) (Oikawa et al. 2006), serine racemase (SerR: EC 5.1.1.18) (Gogami et al. 2009), and arginine racemase (ArgR: EC 5.1.1.9) (Matsui et al. 2009) require PLP as a coenzyme, but glutamate racemase (GluR: EC 5.1.1.3) (Choi et al. 1992) and proline racemase (ProR: EC 5.1.1.4) (Rudnick and Abeles 1975) do not. In the case of aspartate racemase (AspR: EC 5.1.1.13) (Fujii et al. 2015), both PLP-dependent and PLP-independent enzymes have been reported. Aspartate racemase from *Scapharca broughtonii* (bivalve mollusk) (Shibata et al. 2003) and *Thermoplasma acidophilum* (Long et al. 2001) require PLP as a coenzyme, but the enzyme from *Lactobacillus sakei* NBRC 15893 (Fujii et al. 2015), *Pyrococcus horikoshii* OT3 (Liu et al. 2002), *Picrophilus torridus* (Aihara et al. 2016), *Streptococcus thermophilus* (Okada et al. 1991), and *Bifidobacterium bifidum* NBRC 14252 (Yamashita et al. 2004) do not (Fig. 1). Interestingly, both types of aspartate racemases exist in Archaea, but their detailed enzymological properties remain unclear.

We describe the molecular cloning of the aspartate racemase homolog gene from the *T. litoralis* DSM 5473 genome, expression of this gene in *E. coli*, and the purification and enzymological characterization of PLP-independent archaeal aspartate racemase.

Materials and methods

Materials

L-Aspartic acid sodium salt, D-aspartic acid, and D, L-aspartic acid were purchased from Nacalai Tesuque (Tokyo, Japan). L-Cysteic acid and L-cysteine sulfinic acid were purchased from Wako Chemicals (Osaka, Japan). *Escherichia coli* NovaBlue, *Escherichia coli* Rosetta (DE3), pT7blue T-vector, and pET11b vector were purchased from Novagen (Madison, WI). Blend Taq polymerase, and KOD plus ver.2 polymerase were purchased from Toyobo (Osaka, Japan). The in vitro cloning kit and the DNA ligation kit, and restriction enzymes (BamHI and NdeI) were purchased from TaKaRa bioscience (Kyoto, Japan). Toyopearl DEAE-650M, Butyl-650M, and SuperQ-650M were purchased from Tosoh (Tokyo, Japan). All other reagents were of the best commercially available grade and were purchased from Wako Chemicals (Osaka, Japan), Kanto Chemical (Tokyo, Japan), Kishida Chemical (Tokyo, Japan), Nacalai Tesuque (Tokyo, Japan), Watanabe Chemical (Hiroshima, Japan), Invitrogen (Carlsbad, USA), Sigma Aldrich (St. Louis, USA) or Bio Rad (Hercules, USA) unless otherwise stated.

Cultivation of *Thermococcus litoralis* DSM 5473 and preparation of chromosomal DNA

The medium used for the cultivation of *Thermococcus litoralis* DSM 5473 throughout the course of this study was reported previously for *Thermococcus* sp. CKU-1 (Uchida et al. 2014). Cultivation (total volume: 0.5 L) was initiated by the addition of 100 mL of cell suspension of *T. litoralis* DSM 5473 in 0.5 % Na₂S medium. Cultivation was carried out at 82 °C for 16 h under anaerobic conditions with slow stirring and bubbling nitrogen gas. The culture of *T. litoralis* DSM 5473 (1 mL × 5) was centrifuged at 14,500×g for 1 min (Eppendorf, Hamburg, Germany), and the remained culture (75 mL × 3) was added separately to 25 mL of 20 mg/mL Na₂S solution in a vial with screw cap and was stored at 4 °C as a seed culture for next cultivation. The obtained cells were suspended in deionized water (460 µL) containing 1 mg/mL of lysozyme solution (60 µL), and the cell suspension was incubated at 37 °C. After approximately 30 min, 10 % sodium dodecyl sulfate (30 µL) and 20 mg/mL proteinase K solution (30 µL) were added to the mixture, and the cell lysate obtained was incubated at 65 °C. After approximately 18 h, 5 M NaCl (39 µL) was added to the mixture, and then the mixture was suspended in PCI solution (phenol:chloroform:isoamyl alcohol = 25:24:1) (700 µL) and was centrifuged at 5900×g for 5 min. The aqueous layer was collected in a microcentrifuge tube (volume: 1.5 mL); chloroform (700 µL) was added and mixed vigorously. The mixture was centrifuged again at 5900×g for 5 min. The aqueous

layer was collected in a microcentrifuge tube, and 0.6 volumes of 2-isopropanol was added. The precipitate obtained was washed with 70 % (v/v) ethanol solution (1 mL), resuspended in 0.04 mg/mL RNase solution (50 µL), and incubated at 37 °C for 30 min. The resulting chromosomal DNA of *T. litoralis* DSM 5473 obtained was stored at –20 °C.

Cloning of aspartate racemase homolog gene from *Thermococcus litoralis* DSM 5473 into the pET11b vector

At the beginning of this study, the genome sequence of *T. litoralis* DSM 5473 was under sequencing and was not available for designing the PCR primer for amplification of aspartate racemase homolog gene from *T. litoralis* DSM 5473 (*Tl-aspr*). Accordingly, we used genome-walking PCR to obtain the whole sequence of *Tl-aspr*. The forward and reverse primers (aspr-N-1-int, aspr-N-2-int, aspr-C-1-int, and aspr-C-2-int; Table 1) that were used were designed based on the conserved sequences of *aspr* homolog genes from *Thermococcus kodakarensis* KOD1, *Thermococcus onnurineus* NA1, *Thermococcus sibiricus* MM739, and *Thermococcus gammatolerans* EJ3 to determine the partial DNA sequence of *Tl-aspr*. These *Thermococcus* species are neighbors to *T. litoralis* DSM 5473 in 16S rDNA classification, and their genome sequences have already been sequenced. PCR was carried out with 20 pmol of each primer and 1 µg of chromosomal DNA from *T. litoralis* DSM 5473. The thermal program consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. The DNA fragments obtained (250 and 370 bp) were separately ligated to pT7Blue T-vector (Novagen), and then, the plasmids were transformed into *E. coli* NovaBlue to amplify the inserted DNA. The DNA sequences of 250 and 370 bp fragments were determined using an SQ550E DNA sequencer (Hitachi High-Technologies Corp., Tokyo, Japan) with a Thermo Sequenase primer cycle sequence kit (Amersham Bioscience, NJ, USA). To determine the upstream or downstream DNA sequence of 250 or 370 bp fragments, four primers (aspr-250S1, aspr-250S2, aspr-370S1, and aspr-370S2; Table 1) were designed based on the internal sequences obtained (250 and 370 bp). The genome-walking PCR was performed with an LA PCR in vitro cloning kit according to the manufacturer's instructions. The chromosomal DNA extracted from *T. litoralis* DSM 5473 cells was digested with HindIII, EcoRI, or PstI and was ligated to Hind III-, EcoRI-, or PstI-cassettes. The obtained DNA fragments were used as a template for PCR. PCR and DNA sequencing were carried out under the same conditions described above, and the whole sequence of *Tl-aspr* was determined. *Tl-aspr* amplification was carried out by PCR against the *T. litoralis* DSM 5473 genome with primers (aspr-exp-N, aspr-exp-C; Table 1) using KOD-plus DNA polymerase ver.2 (Toyobo,

Table 1 Primers and host strains used in this study

Primer	Sequence
aspr-N-1-int	AT(C/T)GG(A/C)AT(A/C)CT(A/C)GG(C/T)GG(A/C/G)ATGG
aspr-N-2-int	ACTT(C/T)ATAATAATGCC(A/C)TG(C/T)AA(C/T)AC(C/G)GCC
aspr-C-1-int	C(C/G)GT(A/G)TT(A/G)CA(G/T)GGCATTATTAT(A/G)AAGT
aspr-C-2-int	AC(A/G)CT(G/T)AC(C/T)TC(A/C)GT(A/G)CA(A/G)CC(A/G/T)GCTAT(C/T)A
aspr-250-S1	GCTGTCAATGAGTTCTGGAAGTGGATTTTC
aspr-250-S2	GAGAAATATAAGCAGTTCGATCGGGAATCTTG
aspr-370-S1	CTCAAAAAGAGAGGAATTGAAGCGCTCATTC
aspr-370-S2	CATGAAAGCTATCTATGAAGGAGTAAAGGCAGGTG
aspr-exp-N	TTACATATGGAGAAGGTCATAGGGA
aspr-exp-C	AGGATCCAAGGATGGTTATAAACCA
C83A-f	ATAATCATGCCCGCCAATACCGCTCATTACTTTTG
C83A-r	ATGAGCGGTATTGGCGGGCATGATTATAAAATCTGC
C194A-f	AATAGCCGGAGCCACGGAAGTAAGTGTAGCTTTAAAGC
C194A-r	TTACTTCCGTGGCTCCGGCTATTATAGCTTCAACTTC
Strain	Genotype
<i>Escherichia coli</i> NovaBlue	<i>endA1 hsdR17(r_k⁺m_k⁺)supE44 thi-1 recA1 gryA96 relA1 lac F'[proA⁺B⁺ lacI^q ZΔM15::Tn10] (Tet^R)</i>
<i>Escherichia coli</i> Rosetta (DE3)	<i>F⁻ ompT hsdS_b(r_b⁻m_b⁻) gal dcm (DE3) pRARE (Cam^R)</i>

Osaka, Japan). The PCR product of approximately 680 bp was treated with a 10× A-attachment mix (Toyobo, Osaka, Japan) and was purified with an UltraClean™ 15 DNA purification kit (MO Bio Laboratory, Carlsbad, CA USA). The purified product was ligated into the pT7Blue T-vector with a DNA ligation kit (TaKaRa biochemical, Kyoto, Japan). The plasmid that was constructed (pT7Blue-*Tl-aspr*) was transformed into *Escherichia coli* NovaBlue for amplification. After confirmation of the DNA sequence, *Tl-aspr* was cut out from pT7Blue-*Tl-aspr* with NdeI and BamHI and was ligated into the same restriction enzyme sites of the pET11b vector. As a result, pET11b-*Tl-aspr* was obtained and subsequently transformed into *Escherichia coli* Rosetta (DE3).

Purification of aspartate racemase homolog from *Escherichia coli* Rosetta (DE3) harboring pET11b-*Tl-aspr*

Escherichia coli Rosetta (DE3) harboring pET11b-*Tl-aspr* was precultured on a reciprocal shaker (Personal-11, Taitec, Saitama, Japan; 125 rpm) in a test tube (φ18 × 180 mm) containing 5 mL of LB medium plus 100 μg/mL of ampicillin and 34 μg/mL of chloramphenicol. After cultivation at 37 °C for 16 h, the preculture was inoculated into a Sakaguchi flask (500 mL) containing 250 mL of the same medium. After cultivation at 37 °C for 16 h, cells were collected by centrifuging at 8266×g for 20 min. Then, the cells were suspended in 30 mL of a 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM 2-mercaptoethanol (buffer A) and were

disrupted by ultrasonication (UD-201, Tomy Seiko, Tokyo, Japan; output 6, duty cycle 30, 5 min × 7 times) with cooling in ice water. The cell homogenate that was obtained was centrifuged at 14,500×g for 20 min and was ultracentrifuged at 251,550×g for 1 h. The supernatant solution was collected and used as a cell-free extract. After heat treatment of a cell-free extract at 70 °C for 30 min, insoluble materials were removed by centrifugation at 18,900×g for 20 min.

The supernatant was collected and dialyzed against 3 L of buffer A at 4 °C for 16 h. Then, the dialysate was applied to a column of Toyopearl DEAE-650 M (φ25 × 150 mm) equilibrated with buffer A, and unabsorbed proteins were washed with the same buffer. The absorbed proteins were eluted by increasing the NaCl concentrations linearly in buffer A from 0 to 400 mM. The fractions (No. 40–61) containing aspartate racemase activity were collected and first dialyzed against a 50 mM potassium phosphate buffer (KPB) at a pH of 8.0 containing 1 mM 2-mercaptoethanol (buffer B). Subsequently, the fractions were dialyzed against buffer B containing 2.0 M (NH₄)₂SO₄ (buffer C). The enzyme solution was applied to a column of Toyopearl Butyl-650M (φ25 × 150 mm) that was equilibrated with buffer C, and unabsorbed proteins were washed with the same buffer. The absorbed proteins were eluted by decreasing the (NH₄)₂SO₄ concentrations linearly in buffer B from 2.0 to 0 M. The fractions (No. 65–96) containing aspartate racemase activity were collected and dialyzed against buffer A. The enzyme solution was applied to a column of Toyopearl SuperQ-650M (φ15 × 150 mm)

equilibrated with buffer A. The absorbed proteins were eluted by increasing the NaCl concentrations linearly in buffer A from 0 to 400 mM. The fractions (No. 42–62) containing aspartate racemase activity were collected and dialyzed against a 50 mM KPb (pH 8.0) containing 1 mM 2-mercaptoethanol and were stored at -80°C . The protein concentration was determined using a protein assay solution containing Coomassie brilliant blue (Nacalai Tesuque, Tokyo, Japan) by measuring the absorbance at 595 nm, which was the maximum for the Coomassie brilliant blue-protein complex. Bovine serum albumin was used as a standard. The purity of the enzyme was confirmed by SDS-polyacrylamide gel electrophoresis (13.5 % T, 4 % B).

Standard assay conditions

The aspartate racemase activity was measured at 90°C in the presence of D-aspartic acid as a substrate. The assay mixture (200 μL) contained 100 mM KPb (pH 8.0), 4 mM DTT, 50 mM D-aspartic acid, and enzyme solution. The enzyme reaction was started by the addition of an enzyme solution and was carried out at 90°C for 0, 2, 4, 6, 8, or 10 min. The enzyme reaction was stopped by the addition of 60 μL of 1 M trichloroacetic acid, and the mixture was neutralized with 140 μL of 0.5 M Na_2CO_3 . The L-aspartic acid produced was measured by HPLC with either a SUMICHIRAL OA-5000 column ($\phi 4.6 \times 150$ mm, Sumika Chemical Analysis Service, Osaka, Japan) or a Develosil ODS-UG-S column ($\phi 6.0 \times 250$ mm, Nomura Chemical, Aichi, Japan) after derivatization of the amino acid with *o*-phthalaldehyde and *N*-acetyl-L-cysteine (OPA/NAC method) according to methods previously described (Aswad 1984). One unit of aspartate racemase activity was defined as the amount of enzyme that produces 1 μmol of L-aspartic acid from D-aspartic acid under standard assay conditions.

Effects of temperature on enzyme activity and stability

The effects of temperature on the enzyme activity of aspartate racemase (*Tl*-AspR) purified from *Escherichia coli* Rosetta (DE3) harboring pET21b-*Tl*-*aspr* was examined by measuring the enzyme activity under standard assay conditions at various reaction temperatures between 25 and 95°C . The thermal stability of *Tl*-AspR was examined by measuring the residual activity in the enzyme solution (protein conc.: 0.5 mg/mL; solvent: 20 mM KPb, 1 mM 2-mercaptoethanol, pH 7.0) after heating at 90°C from 0 to 19 h.

Effects of pH on enzyme activity and stability

The effects of pH on the enzyme activity of *Tl*-AspR were examined by measuring the enzyme activity with the standard assay mixture at various reaction pH values between

6.0 and 8.5 at 50°C to reduce the influence of temperature on pH. The buffers used in this experiment were as follows: MES, pH 6.0–7.0; HEPES, pH 7.0–8.0; and Tricine, pH 7.5–8.5). The pH stability of *Tl*-AspR was examined by measuring the residual activity in the enzyme solution (protein conc.: 0.086 mg/mL) after treatment at various pH values at 4°C for 4 days. The buffers (final conc. 200 mM) used in this experiment were as follows: acetate, pH 4.0–5.5; MES, pH 5.5–7.0; HEPES, pH 7.0–8.0; TAPS, pH 8.0–9.0; and CHES: pH 9.0–10.0. The pH of each buffer solution was adjusted at room temperature.

Substrate specificity

Substrate specificity of *Tl*-AspR was examined with L-aspartic acid, D-aspartic acid, D-glutamic acid, D-asparagine, D-glutamine, D-alanine, D-leucine, D-valine, D-proline, D-serine, D-threonine, D-arginine, L-cysteic acid or L-cysteine sulfinic acid as a substrate. Each substrate was dissolved in deionized water at a concentration of 250 mM to use as a stock solution and was added to the assay mixture at a final concentration of 50 mM. The concentration of D-aspartic acid and L-forms of glutamic acid, asparagine, cysteic acid, and cysteine sulfinic acid in the assay mixture were measured by the OPA/NAC method. The concentrations of the L-forms of aspartic acid, leucine, proline and valine in the assay mixture were measured by HPLC with a SUMICHIRAL OA-5000 column. The following two types of mobile phases were used to analyze aspartic acid, leucine, proline, and valine: for aspartic acid and leucine, 2 mM CuSO_4 in 2 % (v/v) 2-propanol aqueous solution; and for proline and valine, 2 mM CuSO_4 in water. In the cases of alanine, serine, threonine, glutamine, and arginine, a SUMICHIRAL OA-6100 column was used. For alanine, serine, and threonine, 1 mM CuSO_4 in water was used as the mobile phase, while for glutamic acid and arginine, 2 mM CuSO_4 in 2 % (v/v) acetonitrile aqueous solution was used. The activities except for L-forms of cysteic acid and cysteine sulfinic acid were measured based on the amount of L-counterpart produced under the standard assay conditions. For L-forms of cysteic acid and cysteine sulfinic acid, since D-forms of these compounds were not commercially available, we measured the enzyme activity based on the amount of substrate remained in assay mixture after enzyme reaction.

Effects of SH-reagents on enzyme activity

The purified enzyme (protein conc.: 0.125 mg/mL, 20 μL) was treated at 4°C with various SH-reagents (4980 μL), such as iodoacetate, *N*-ethylmaleimide, 5-5'-dithiobis (2-nitrobenzoate), and *p*-chloromercuribenzoate, dissolved in 50 mM KPb (pH 7.0) at a concentration of 1 mM. After

1 h, the enzyme solution was dialyzed against 50 mM KPBS (pH 7.0) containing 1 mM 2-mercaptoethanol. The residual activity was measured under the standard assay conditions.

Site-directed mutagenesis

Introduction of C83A, C194A, and C83A/C194A mutations to *Tl-aspr* was performed by quick-change mutagenesis using pET11b-*Tl-aspr* for C83A, C194A, and pET11b-*Tl-aspr* C83A for C83A/C194A as template (Fujii et al. 2016). Mutagenic primers were summarized in Table 1. After amplification of mutant plasmid using KOD plus ver.2 polymerase, the reaction product was treated with DpnI (10 U) for 1 h at 37 °C to digest the parental plasmid. The resultant plasmid, pET11b-*Tl-aspr* C83A, pET11b-*Tl-aspr* C194A, or pET11b-*Tl-aspr* C83A/C194A, was purified and concentrated by ethanol precipitation and was transformed into *Escherichia coli* NovaBlue by electroporation. The mutation in *Tl-aspr* gene was confirmed by DNA sequencing. Expression of pET11b-*Tl-aspr* C83A, C194A, or C83A/C194A in *Escherichia coli* Rosetta (DE3), and purification of each mutant enzyme were carried out by the same method for pET11b-*Tl-aspr* described above.

Effect of metal ions on enzyme activity

To determine the effect of metal ions on the aspartate racemase activity of *Tl-AspR*, we measured the enzyme activity in the presence of various metal ions (final concentration: 1 mM), such as CaCl₂, MgSO₄, FeSO₄, MnSO₄, CuSO₄, NiSO₄, and CoSO₄ and EDTA under standard assay conditions.

Kinetic analysis

The enzyme activities were measured under the standard assay conditions except pH (at 7.5) and K_m , k_{cat} , and V_{max} values for L- and D-aspartic acids were determined using a Lineweaver–Burk plot (Lineweaver and Burk 1934). The final substrate concentrations used in this experiment were 10, 20, 30, 40, or 50 mM.

Amino acid analysis of intracellular fractions of *Thermococcus litoralis* DSM 5473 cells

T. litoralis DSM 5473 was cultivated at 82 °C for 23 h, and the culture (200 mL) was centrifuged at 3000×g for 15 min. The cells obtained were suspended in 0.5 ml of 50 mM KPBS (pH 7.0) and were disrupted by ultrasonication (output 2, duty cycle 30 %, 1 min, once). The cell-free extract was treated with an equal amount of 10 % (w/v)

trichloroacetic acid, neutralized with 5 M NaOH, and subjected to amino acid analysis (OPA/NAC method) (Aswad 1984).

Accession number

The accession number of the aspartate racemase gene from *T. litoralis* DSM 5473, LC034559, was given by the Research Organization of Information and Systems, National Institute of Genetics, DNA Data Bank of Japan (DDBJ), Shizuoka, Japan.

Results

Expression of aspartate racemase homolog gene from *Thermococcus litoralis* DSM 5473 in *Escherichia coli* Rosetta (DE3)

DNA sequence analysis of *Tl-aspr* showed that an open reading frame of *Tl-aspr* consists of 687 bp and encodes 228 amino acid residues. The deduced amino acid sequence based on *Tl-aspr* was similar to that of various PLP-independent aspartate racemases from *Pyrococcus horikoshii* OT-3 (68.0 %) (Liu et al. 2002), *Bifidobacterium bifidum* NBRC 14252 (32.9 %) (Yamashita et al. 2004), *Streptococcus thermophilus* (39.2 %) (Okada et al. 1991), and *Lactobacillus sakei* NBRC 15893 (34.1 %) (Fujii et al. 2015). The important amino acid residues in the active center of these enzymes were well conserved in the primary structure of *Tl-AspR* as Arg49, Cys83, Lys165, and Cys194.

Purification of aspartate racemase homolog of *Thermococcus litoralis* DSM 5473 from *Escherichia coli* Rosetta (DE3) harboring pET11b-*Tl-aspr*

Tl-AspR was expressed in a soluble fraction of *E. coli* Rosetta (DE3) harboring pET11b-*Tl-aspr* judging from its protein band of SDS–PAGE, and approximately 60 % of *Tl-AspR* was soluble. We succeeded in purifying the aspartate racemase homolog of *T. litoralis* DSM 5473 (Fig. 2). The purified aspartate racemase homolog of *T. litoralis* DSM 5473 showed aspartate racemase activity with a specific activity of 1590 U/mg (Table 2). The purification was carried out repeatedly three times. The specific activity of purified *Tl-AspR* was 1900 ± 350 U/mg. SDS–PAGE analysis showed that the molecular mass of a subunit in *Tl-AspR* was approximately 24 kDa, and the molecular mass of the native form of *Tl-AspR* was estimated to be approximately 56 kDa by gel-filtration chromatography with a Sephadex-200 10/300 GL column. Accordingly, *Tl-AspR* is a homodimer that is dissimilar to the *AspR* from *B.*

bifidum NBRC 14252 (monomer; molecular mass of subunit: 30 kDa, Yamashita et al. 2004), but it is similar to other PLP-independent AspRs from *P. horikoshii* OT-3 (homodimer; molecular mass of subunit: 25 kDa, Liu et al. 2002) and *S. thermophilus* (homodimer; molecular mass of subunit: 28 kDa, Okada et al. 1991).

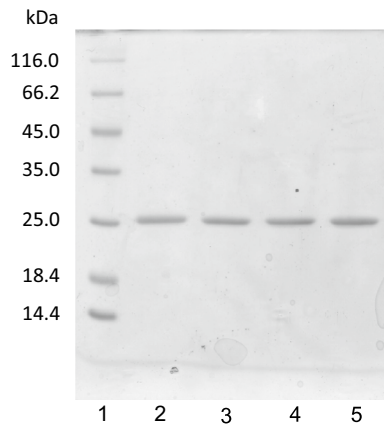
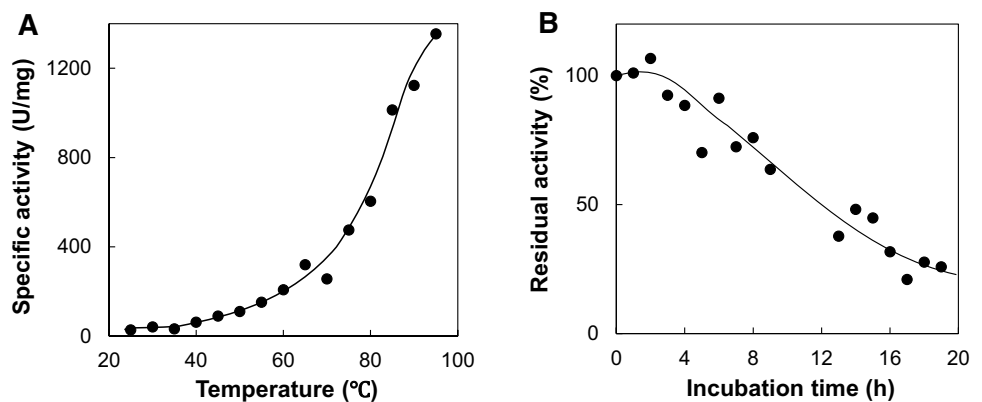


Fig. 2 SDS-polyacryl amide gel electrophoresis of purified aspartate racemase from *Thermococcus litoralis* DSM 5473. Lane 1 molecular mass marker (4 μ L)—14.4 kDa (lysozyme), 18.4 kDa (β -lactoglobulin), 25.0 kDa (REase Bsp981), 35.0 kDa (lactate dehydrogenase), 45.0 kDa (ovalbumin), 66.2 kDa (bovine serum albumin), and 116 kDa (β -galactosidase). Lane 2 purified *Tl*-AspR wild type (2.5 μ g). Lane 3 purified *Tl*-AspR C83A mutant (2.5 μ g). Lane 4 purified *Tl*-AspR C194A mutant (2.5 μ g). Lane 5 purified *Tl*-AspR C83A/C194A mutant (2.5 μ g)

Table 2 Purification of aspartate racemase from *Thermococcus litoralis* DSM 5473

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	273	422,000	1550	100
Heat treatment	106	334,000	3150	79.1
DEAE-Toyopearl	30.0	116,000	3870	27.5
Butyl-Toyopearl	19.8	29,000	1470	6.87
Super-Q-Toyopearl	18.3	29,100	1590	6.89

Fig. 3 Thermal profiles of aspartate racemase from *Thermococcus litoralis* DSM 5473. **a** Effect of temperature on enzyme activity. **b** Thermal stability



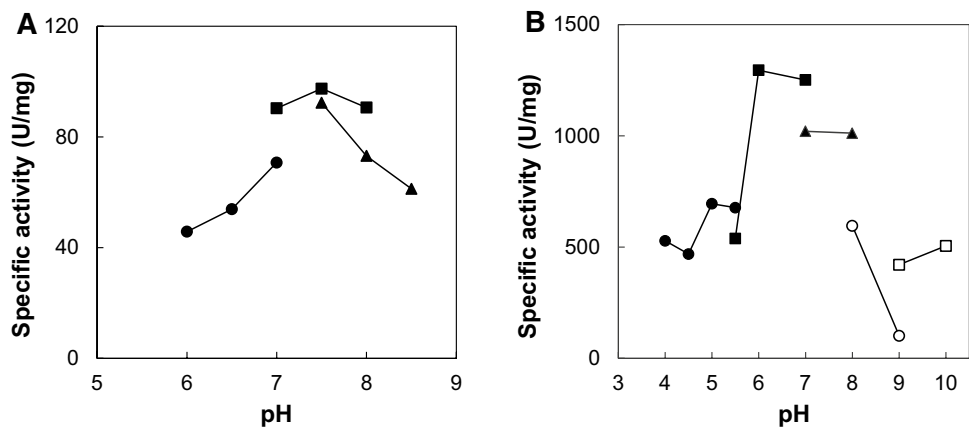
Effects of temperature on enzyme activity and stability

The activity of *Tl*-AspR increased gradually depending on the reaction temperature and showed a maximum activity at 95 °C (Fig. 3a). The value was higher than that of other PLP-independent AspRs reported to date, including 60 °C for *Picrophilus torridus* (Aihara et al. 2016), 45 °C for *B. bifidum* NBRC 14252 (Yamashita et al. 2004), and 37 °C for *S. thermophilus* (Okada et al. 1991). The activation energy of *Tl*-AspR was calculated to be 51.8 kJ/mol by Arrhenius plots. When 0.5 (mg/mL) of the enzyme was suspended in 1 mL of 20 mM KPB (pH 7.0) containing 1 mM 2-mercaptoethanol and incubated at 90 °C, approximately 50 or 25 % of the initial activity remained even after 11 or 19 h, respectively (Fig. 3b). The *Tl*-AspR was highly thermostable compared with other PLP-independent AspRs reported to date; for example, *B. bifidum* NBRC 14252 was thermostable at 60 °C for 30 min (Yamashita et al. 2004) and *S. thermophilus* was thermostable at 50 °C for 1 h (Okada et al. 1991).

Effects of pH on enzyme activity and stability

Tl-AspR showed maximum activity when a HEPES buffer (pH 7.5) was used in the assay mixture (Fig. 4a). *Tl*-AspR was highly stable between pH 6.0 and 7.0 in a MES–NaOH buffer (Fig. 4b). These pH profiles agreed well with those of other PLP-independent AspRs reported to date for *B. bifidum* NBRC 14252 (optimum pH, 7.0–7.5; pH stability, 5.5–7.5)

Fig. 4 pH profiles of aspartate racemase from *Thermococcus litoralis* DSM 5473. **a** Effect of pH on enzyme activity. The enzyme activity was measured at 50 °C. Filled circle MES–NaOH buffer, filled square HEPES–NaOH buffer, filled triangle Tricine–NaOH buffer. **b** pH stability. Filled circle acetate buffer, filled square MES–NaOH buffer, filled triangle HEPES–NaOH buffer, open circle TAPS–NaOH buffer, open square CHES–NaOH buffer



(Yamashita et al. 2004) and for *S. thermophilus* (optimum pH, pH 8.0; pH stability: 6.0–7.0) (Okada et al. 1991).

Substrate specificity

Tl-AspR acted on L-cysteic acid and L-cysteine sulfinic acid in addition to D- and L-aspartic acids. No activity was observed for other amino acids tested (Table 3). Similar substrate specificity was observed for the AspR from *S. thermophilus* (Okada et al. 1991).

Identification of essential amino acid residues for catalytic activity

The purified C83A-, C194A-, and C83A/C194A-*Tl*-AspR migrated as single band on SDS–PAGE (Fig. 2). C83A-, C194A-, and C83A/C194A-*Tl*-AspR showed no aspartate racemase activity under the standard assay conditions. These results suggest that both Cys83 and Cys194 residues are essential for catalytic activity of *Tl*-AspR.

Effects of SH-reagents and metal ions on enzyme activity

Tl-AspR was inhibited by treatment with various SH-reagents (Table 4). The enzyme was strongly inhibited by iodoacetic acid. The enzyme was slightly affected by several divalent metal ions (Table 4). EDTA did not affect enzyme activity.

Kinetic parameters

The k_{cat} , K_m , V_{max} , and k_{cat}/K_m values for D- and L-aspartic acids of *Tl*-AspR were determined using Lineweaver–Burk plots and were summarized in Table 5. *Tl*-AspR showed a higher K_m value for D-Asp rather than for L-Asp (K_m for

Table 3 Substrate specificity ($n = 2$)

Substrate	Relative activity (%)
D-Aspartic acid	100
L-Aspartic acid	56.4 ± 1.9
L-Cysteic acid	27.5 ± 4.2
L-Cysteine sulfinic acid	12.3 ± 3.0
D-Glutamic acid	0
D-Asparagine	0
D-Glutamine	0
D-Alanine	0
D-Leucine	0
D-Valine	0
D-Proline	0
D-Serine	0
D-Threonine	0
D-Arginine	0

L-Asp, 30.2 mM, K_m for D-Asp, 136 mM) in contrast to the PLP-independent AspRs from *B. bifidum* NBRC 14252 (K_m for L-Asp, 13.4 mM, K_m for D-Asp, 0.94 mM; Yamashita et al. 2004) and from *S. thermophilus* (K_m for L-Asp, 35 mM, K_m for D-Asp, 8.7 mM; Yamauchi et al. 1992). Similar profile was observed for K_m values of AspR from *P. horikoshii* OT-3 (AspR from *P. horikoshii*, K_m for L-Asp, 0.13 mM, K_m for D-Asp, 0.31 mM; Yoshida et al. 2006), but K_m value for D-Asp of *Tl*-AspR was about 439 times higher than that of *P. horikoshii* OT-3 AspR. However, the difference of reaction temperature (*Tl*-AspR, 90 °C; *P. horikoshii* OT-3 AspR, 70 °C) must be considered for further discussion. The ratio of the k_{cat}/K_m value for D-aspartic acid to that for L-aspartic acid was defined as K_{eq} (Lambert and Neuhaus 1972) and was calculated to be 1.15. Accordingly, *Tl*-AspR was regarded as an amino acid racemase that specifically acted on aspartic acid.

Amino acid analysis of cell-free extract

The amino acid analysis of the cell-free extract of *T. litoralis* DSM 5473 showed that the D-forms of aspartic acid, serine, alanine, and valine were contained in *T. litoralis* DSM 5473 cells (Table 6).

Discussion

We found that the aspartate racemase homolog gene of *T. litoralis* DSM 5473 encodes aspartate racemase. *Tl-AspR* was highly thermostable and showed the highest optimum temperature among the AspRs reported to date. Such characteristic thermal profiles of *Tl-AspR* most likely relate to the high optimum growth temperature of this organism. During purification, the specific activity of *Tl-AspR* decreased after Toyopearl Butyl-650M column chromatography. Probably, hydrophobic interaction between *Tl-AspR*

and Toyopearl Butyl-650M or presence of $(\text{NH}_4)_2\text{SO}_4$ caused inactivation of *Tl-AspR*.

PLP-independent amino acid racemase contains two cysteine residues in its active sites (Liu et al. 2002). These two cysteine residues catalyze the deprotonation or protonation reaction of the $\text{C}_\alpha\text{-H}$ bond of aspartic acid to produce the racemic form of aspartic acid. Cys83 and Cys194 corresponding to these cysteine residues were found in the primary structure of *Tl-AspR*. C83A-, C194A-, and C83A/C194A-*Tl-AspR* showed no aspartate racemase activity. The enzyme activity of *Tl-AspR* was inhibited by several SH-reagents. The purified *Tl-AspR* did not show any absorption peaks at approximately 420 nm that attributed to the sciff base linkage between the ϵ -amino group of the Lys residue of the PLP-dependent amino acid racemase and PLP. Accordingly, *Tl-AspR* is thought to be a PLP-independent aspartate racemase, and both Cys83 and Cys194 are considered to be essential amino acid residues for *Tl-AspR* to show aspartate racemase activity.

After this study began, the genomic analysis of *T. litoralis* DSM 5473 was completed, which revealed that three types of aspartate racemase homolog genes [accession no.: OCC_13510 (length: 219 bp; 72 aa), OCC_13515 (length: 471 bp; 156 aa), and OCC_11152 (length: 678 bp; 225 aa)] exist in its genome. In comparing the primary structure of *Tl-AspR* with that of translated OCC_13510, OCC_13515, or OCC_11152, interestingly, the primary structure of gene products of both OCC_13510 and OCC_13515 overlapped partially with that of *Tl-AspR*, but the same coding DNA sequence of *Tl-AspR* was not found in the *T. litoralis* DSM 5473 genome. Judging from these data, we propose that the DNA sequences of both OCC_13510 and OCC_13513 were misread during the genome analysis of *T. litoralis* DSM 5473. Comparing the DNA sequence of this region in the *T. litoralis* DSM 5473 genome with that of

Table 4 Effect of SH-reagents and metal ions ($n = 2$)

Inhibitor	Relative activity (%)
None	100
<i>N</i> -Ethylmaleimide	38 ± 4.5
5,5'-Dithiobis(2-nitrobenzoic acid)	65 ± 5.2
<i>p</i> -Chloromercuribenzoic acid	64 ± 3.0
Iodoacetic acid	0
EDTA	93 ± 28
CaCl_2	120 ± 17
CuSO_4	85 ± 5.5
MnSO_4	82 ± 8.2
MgSO_4	52 ± 15
NiSO_4	69 ± 7.7
CoSO_4	64 ± 15
FeSO_4	57 ± 30

Table 5 Kinetic parameters ($n = 2$)

Substrate	K_m (mM)	V_{\max} ($\mu\text{mol}/\text{min}/\text{mg}$)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM)	K_{eq} (D/L)
D-Asp	136 ± 48	$(1.41 \pm 0.50) \times 10^4$	$(6.10 \pm 2.15) \times 10^5$	$(4.51 \pm 2.26) \times 10^4$	1.15 ± 0.47
L-Asp	30.2 ± 3.1	$(0.28 \pm 0.02) \times 10^4$	$(1.18 \pm 0.73) \times 10^5$	$(3.92 \pm 0.47) \times 10^4$	

Table 6 Amino acid analysis of cell-free extract ($n = 2$)

	Asp	Glu	Ser	Ala	Val
D-Form (nmol/mg wet cell)	2.18 ± 0.02	N.D.	0.28 ± 0.16	0.61 ± 0.03	0.16 ± 0.08
L-Form (nmol/mg wet cell)	2.11 ± 0.33	12.8 ± 0.61	N.D.	0.87 ± 0.00	0.38 ± 0.03
D/(D + L) %	50.8 ± 8.50	–	–	41.2 ± 1.36	30.0 ± 13.3

N.D. not detected

the *Tl*-AspR gene, the number of adenines was different (*Tl*-AspR gene 459–467, -GGAAAAGT-; genome sequence, -GGAAAAGT-), and this made the *Tl*-AspR gene that divides into two wrong genes, OCC_13510 and OCC_13513.

Two aspartate racemase homolog genes (accession no.: PH0670 and PH1733) were also found in the genome of *Pyrococcus horikoshii* OT-3. The X-ray crystallographic studies of these two homologs of *P. horikoshii* OT-3 have been completed already (Liu et al. 2002; Kita et al. 2008), but their enzymological properties remain unknown. The identity of the primary structure of *Tl*-AspR compared with that of PH0670 and PH1733 was calculated to be approximately 68.0 and 30.5 %, respectively. In addition, only one cysteine residue existed in the primary structure of PH1733. These results suggested that only PH0670 shows aspartate racemase activity between the two aspartate racemase homologs of *P. horikoshii* OT-3.

D-Aspartic acid was detected in an intracellular fraction of *T. litoralis* DSM 5473 (Table 6). Interestingly, a high concentration of D-aspartic acid existed in an intracellular fraction of *T. litoralis* DSM 5473. A similar observation was recorded for other hyperthermophilic archaea (Matsumoto et al. 1999) but not for bacteria; the molar ratio of D-aspartic acid to D- and L-aspartic acids was 43.0 % for *Thermococcus* sp. KS-1, 48.4 % for *Thermococcus* sp. KS-8, 45.2 % for *Pyrococcus* sp. OH, 49.1 % for *Pyrococcus* sp. GB-D, and 48.8 % for *Desulfurococcus* sp. SY. In addition, the K_m value of *Tl*-AspR for D-aspartic acid was fivefold higher than that for L-aspartic acid in vitro. Therefore, *Tl*-AspR is most likely important and is one of the key enzymes needed for *T. litoralis* DSM 5473 to produce D-aspartic acid from L-aspartic acid in vivo. In addition to D-aspartic acid, D-serine, D-alanine, and D-valine were also detected in an intracellular fraction of *T. litoralis* DSM 5473. Because *Tl*-AspR acted on aspartic acid, L-cysteic acid, and L-cysteine sulfinic acid and did not show any racemase activity for other amino acids, other amino acid racemases or enzymes acting on these D-amino acids might exist in *T. litoralis* DSM 5473 cells.

We are currently trying to study the physiological function of D-aspartic acid and other D-amino acids in *T. litoralis* DSM 5473 cells.

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