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Journal of Molecular Catalysis B: Enzymatic 26 (2003) 231–240
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Thermostable glutamate dehydrogenase from a commensal thermophile, *Symbiobacterium toebii*; overproduction, characterization, and application

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Received 4 April 2003; received in revised form 23 June 2003; accepted 27 June 2003

Abstract

A gene encoding glutamate dehydrogenase (GDH) was found in the genome sequence of a commensal thermophile, *Symbiobacterium toebii*. The amino acid sequence deduced from the *gdh I* of *S. toebii* was well conserved with other thermostable GDHs. The *gdh I* which encodes GDH consisting of 409 amino acids was cloned and expressed in *E. coli* DH5α under the control of a highly constitutive expression (HCE) promoter in a pHCE system. The recombinant GDH was expressed without addition of any inducers in a soluble form. The molecular mass of the GDH was estimated to be 263 kDa by Superose 6 HR gel filtration chromatography and 44 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicating that the GDH was composed of hexameric form. The optimal temperature and pH of the purified enzyme were 60 °C and 9.0, respectively, and the purified GDH retained more than 75% of its original activity after an incubation at 70 °C for 30 min. Although NADP(H) was the preferred cofactor, *S. toebii* GDH was able to utilize either NADP(H) or NAD(H) as coenzyme.

Keywords: Glutamate dehydrogenase; Thermostability; *Symbiobacterium toebii*; Overproduction; Regeneration system

1. Introduction

Glutamate dehydrogenases (GDHs, EC 1.4.1.2-4) found in almost every organism catalyze the reversible oxidative deamination of glutamate to α-ketoglutarate and ammonia, using either NAD or NADP as coenzyme [1]. GDHs have been identified as two types; one is tetrameric NAD-dependent enzyme consisting four identical subunits of 115 kDa, and the other, mainly functioning in ammonia assimilation, is hexameric enzyme formed by six subunits of around 48 kDa [2]. Comparison of GDHs from bacteria and archaea showed that microbial GDHs could be

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doi:10.1016/j.molcatb.2003.06.008

classified as three types according to the coenzyme used; NADP-dependent (EC 1.4.1.4) [3–5], NAD-dependent (EC 1.4.1.2) [6–8], and dual-coenzyme specific (EC 1.4.1.3) [9–11]. The GDH was also classified as two types according to their deduced amino acid sequence in which most GDH from thermophiles such as *Thermococcus profundus* and *Pyrococcus* sp. [12–14].

GDHs have been extensively studied due to both their metabolic importance and their interesting allosteric properties. GDH also plays an important role in nitrogen and carbon metabolisms [15]. Consalvi and Ohshima suggested that its primary role is biosynthesis but Robb and Klump showed that it was also responsible for glutamate catabolism in hyperthermophiles [16–18]. It was proposed that GDH is acting as an alternative to the glutamine synthetase/glutamate synthase cycle under the condition of high ammonium concentration, stress, or senescence [19–21]. L-Glutamate, the product of ammonia assimilation by the action of GDH is the major excitatory neurotransmitter released by the synaptic terminals in the mammalian brain and L-glutamate taken up into astrocytes is converted to glutamine and released into the synaptic cleft for uptake by neurons to be used for the resynthesis of neurotransmitter glutamate or utilized for energy [22,23].

In our previous study, we designed a multi-enzyme system composed of four enzyme reactions catalyzed by glutamate racemase (GluRa), D-amino acid aminotransferase (D-AAT), formate dehydrogenase (FDH) and GDH for producing D-amino acids such as D-phenylalanine, D-tyrosine and D-alanine which are valuable raw materials in the pharmaceutical applications for the synthesis of antibiotics, bioactive peptides and other physiologically active compounds [24] in which GDH plays an important role to regenerate L-glutamate from α-ketoglutarate with coupled reaction of FDH. And we found that after 10 h incubation at 37 °C, only GDH other than three enzymes lost more than 50% of its activity. So we tried to find a stable GDH for constructing more efficient multi-enzyme system and found a thermostable GDH from a commensal thermophile, *Symbiobacterium toebii* through the analysis of its genome information. In this study, we have reported the molecular cloning, expression, and characteristics of a thermostable GDH from *Symbiobacterium toebii*.

2. Experimental

2.1. Chemicals and enzymes

Restriction enzymes for gene manipulation were purchased from Roche Applied Science (Mannheim, Germany), *Taq* DNA polymerase was from TaKaRa Bio Co. (Otsu. Shiga, Japan), and reagents for genetic engineering experiments and enzyme characterization were from Sigma (St. Louis, MO, USA). PCR amplification was performed using a Thermal cycler from TaKaRa. Bio Co. and DNA sequences were confirmed by ABI PRISM 310 PE Applied BioSystem (Foster City, CA, USA).

2.2. Bacterial strains and plasmids

The commensal thermophile, *Symbiobacterium toebii* isolated from Korea compost [25,26] was used as a source of the GDH gene, and *E. coli* JM 109 and *E. coli* DH5α were used as hosts for cloning, sequencing and expression of GDH. Plasmids, pGEM-T easy (Promega Co., Madison, WI, USA) and pHCE(II)B (TaKaRa. Bio Co.) [27] were used for cloning and sequencing, and expression of GDH, respectively.

2.3. Cloning and overexpression of the glutamate dehydrogenase gene (gdh I) of S. toebii

Genomic DNA of *S. toebii* was extracted based on the method described by Saito and Miura [28] and the *gdh I* gene was amplified by PCR using genomic DNA as a template. The primer sequences for amplifying the *gdh I* were determined by analyzing the genome information of *S. toebii* and the sequence was followed; the forward primer, 5′-GGG*GAATTC*ACTGGCCAGGAGTTCTTCCGA-3′ and the reverse primer, 5′-CCCC*TGCAGT*CATGCCTGGTGGCGAGCCGG-3′. The forward primer contained *Eco*R I recognition sites, GAATTC and the reverse primer contained a *Pst* I recognition site, TGCAGT for convenient sub-cloning into pHCE(II)B vectors. PCR amplification was performed in 0.05 ml of a mixture containing 0.2 mM of each dNTP, 100 pmol of forward and reverse primers, 20 ng of template DNA, 5 μl of 10× reaction buffer, and 1 U of LA *Taq* DNA polymerase. The PCR product was ligated into

pGEM-T easy vector and the ligates were transformed into *E. coli* JM109 by electroporation. The DNA sequence from positive clones was confirmed on an ABI PRISM 310 using a Big Dye Terminator Kit. The *gdh I* gene was then subcloned into expression vector pHCE(II)B at *Eco*R I and *Pst* I sites, and transferred into *E. coli* DH5α. To overexpress the GDH, *E. coli* DH5α containing the pHCE(II)GDH harbored with the *gdh I* gene was cultured in LB medium containing 100 μg/ml of ampicillin at 37 °C for overnight.

2.4. Purification of recombinant glutamate dehydrogenase from E. coli transformant

The recombinant GDH was purified from the transformant *E. coli* DH5α harboring the pHCE(II)GDH. The recombinant cells overexpressing recombinant GDH genes were collected by centrifugation (6000 × *g*, 10 min, 4 °C), washed with 0.5% NaCl, and resuspended in 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA, and 7 mM β-mercaptoethanol. The suspended pellets were ultrasonicated for 5 min with sonication flush 5 s on/off. The cell debris was removed by centrifugation (24,000 × *g*, 10 min, 4 °C) and the cell lysate was incubated at 55 °C for 30 min to remove thermolabile *E. coli* proteins. Denatured protein aggregates were removed by centrifugation at 24,000 × *g*, 4 °C for 10 min. This crude enzyme solution was loaded onto a Mono Q HR anion-exchange column (0.5 cm × 5 cm; Pharmacia, Uppsala, Sweden) equilibrated with buffer A (20 mM Tris–HCl [pH 8.5] containing 1 mM EDTA and 7 mM β-mercaptoethanol) and the enzyme was eluted with a linear gradient of 0 to 0.25 M of NaCl. For further purification of GDH, the active fractions were collected and loaded onto a Superose 6 HR column (1.0 cm × 30.0 cm; Pharmacia, Uppsala, Sweden) and the enzyme was eluted with buffer B (50 mM Tris–HCl (pH 7.2) containing 1 mM EDTA and 7 mM β-mercaptoethanol) at a flow rate of 0.5 ml/min. All chromatographic procedures were conducted at room temperature.

2.5. Protein analysis

Protein was quantified by the Bradford method [29] using bovine serum albumin (BSA) as a standard (Bio-Rad, Hercules, CA, USA). Molecular weight of the denatured monomer of GDH was estimated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [30]. The molecular weight of native GDH was estimated by gel permeation chromatography with a Superose 6 HR column connected to a FPLC system (Pharmacia, Uppsala, Sweden). The purified enzyme solution was applied to a column and eluted with buffer B at a flow rate of 0.5 ml/min. To calculate the molecular mass of native GDH, the column was calibrated with thyroglobulin (670 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) as reference proteins.

2.6. Enzyme assay

Enzyme activity was assayed spectrophotometrically using an Ultraspec 3000 spectrophotometer (Pharmacia Biotech., Cambridge, UK). The standard reaction mixture for oxidative deamination was composed of 100 mM of Glycine-KOH buffer, 10 mM of L-glutamate, 1.25 mM of NAD or NADP, and the enzyme in a final volume of 1.00 ml (pH 9.0). The increase of NADPH or NADH was monitored by measuring the absorbance at 340 nm and one unit of activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of product per min at 50 °C. For the reductive amination, enzyme activity was assayed by measuring the decrease in absorbance at 340 nm by depletion of NADH or NADPH. In this case, 100 mM of NH_4Cl, 10 mM of *a*-ketoglutarate, 0.20 mM of NADH or NADPH was used as substrates instead of L-glutamate and NAD or NADP.

2.7. Optimum pH and temperature

Optimum pH was estimated by oxidative deamination using 100 mM sodium acetate buffer (pH 5.0–7.0), 100 mM Tris–HCl buffer (pH 7.0–8.0), and 100 mM Glycin–KOH buffer (pH 8.0–10.0). The temperature optimum of purified GDH was determined by using various incubation temperatures in the pH 9.0.

2.8. Thermal stability

The thermal stability of GDH was analyzed by determining the residual activity after incubating at different temperatures in the range of 40–80 °C for 30 min. Also to determine the stability of GDH during

D-phenylalanine production, enzyme was incubated at 37 °C for 20 h and remaining activity was determined.

3. Results and discussion

3.1. Cloning of glutamate dehydrogenase gene

The DNA fragment containing the *gdh I* gene was amplified by PCR using the genomic DNA of *S. toebii* as a template. The 1239 bp amplified products were cloned into a pGEM-T easy vector and the plasmid with 1239 bp insert was designated pTGDH. After confirming the DNA sequence, 1.23 kb of *Eco*R I–*Pst* I fragment was cloned into pHCE(II)B and resulting plasmid was named as pHCE(II)GDH (Fig. 1). The two codon, GAA (Glu) and TTC (Phe), were inserted in the next of start codon (ATG) in the native sequence, because an *Eco*R I restriction enzyme site was imported for translational fusion in the N-terminal fusion region. The sequence of translational fusion region was verified by sequence analysis and the newly designed pHCE(II)GDH was transformed in *E. coli* DH5α to express the GDH (data not shown).

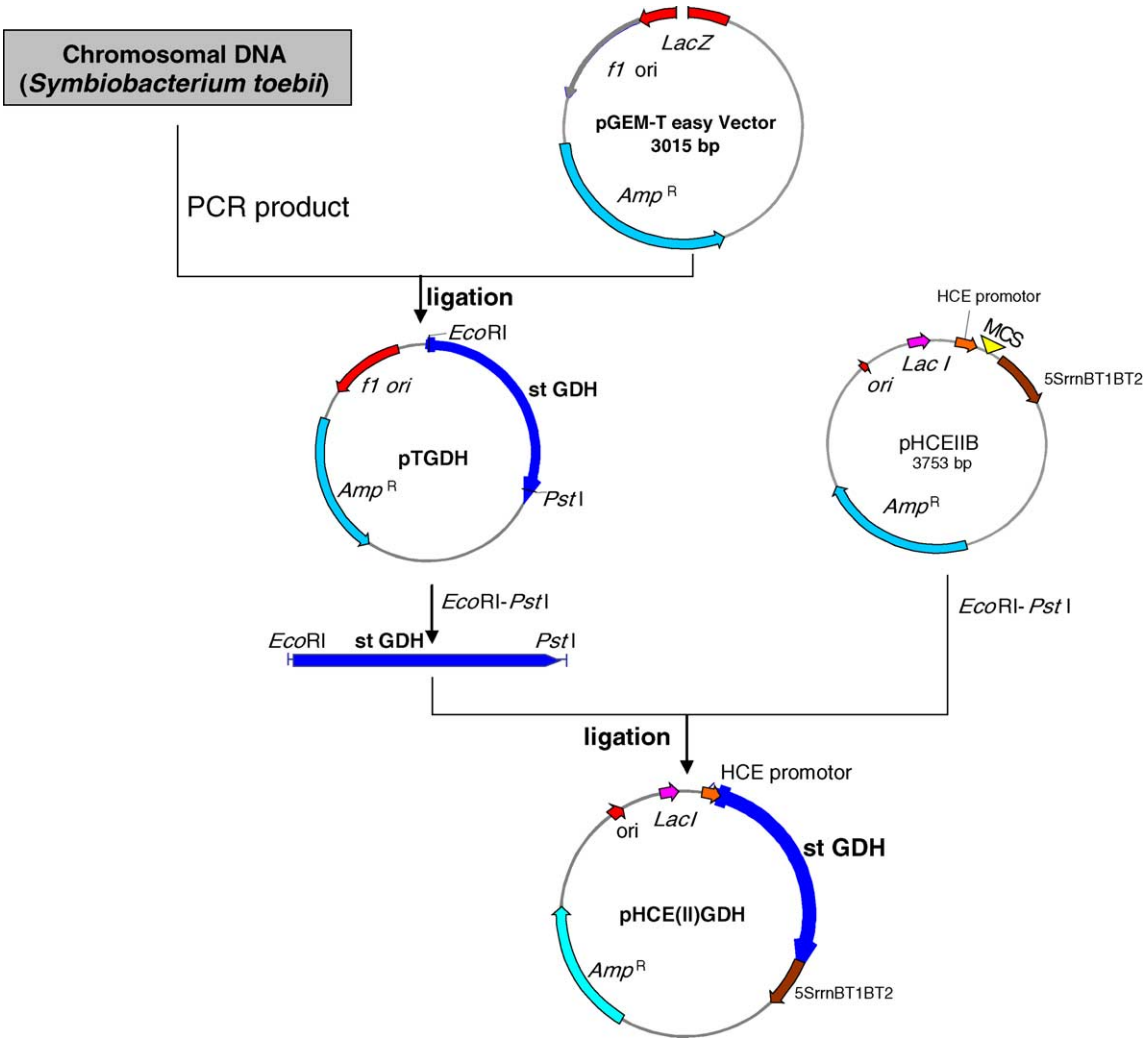


Fig. 1. Cloning of the glutamate dehydrogenase gene from *S. toebii*.

3.2. Comparison of the translated sequence with other glutamate dehydrogenases

Translation of the nucleotide sequence of *gdh I* gene led to a sequence of 411 amino acid length. When compared to SWISS PROT database, GDH of *S. toebii* exhibited the highest level of similarity to GDHs of thermophilic bacteria and gram (+) bacteria, such as *Thermoanaerobacter tengcongensis* (66.8%), *Thermoplasma acidophilum* (65.7%), *Clostridium difficile* (65.7%) and *Staphylococcus aureus* (65.6%). The amino acid sequence deduced from the *gdh I* of *S. toebii* was compared with those of enzymes from thermophilic bacteria (Fig. 2). As a result of sequence comparison, the GDH from *S. toebii* showed more than 60% similarity with the thermostable GDHs. Benachenhou-Lahfa et al. reported that most of GDH from various organisms was classified as two families according to their amino acid sequences and most of GDHs from thermophiles was clustered into family II

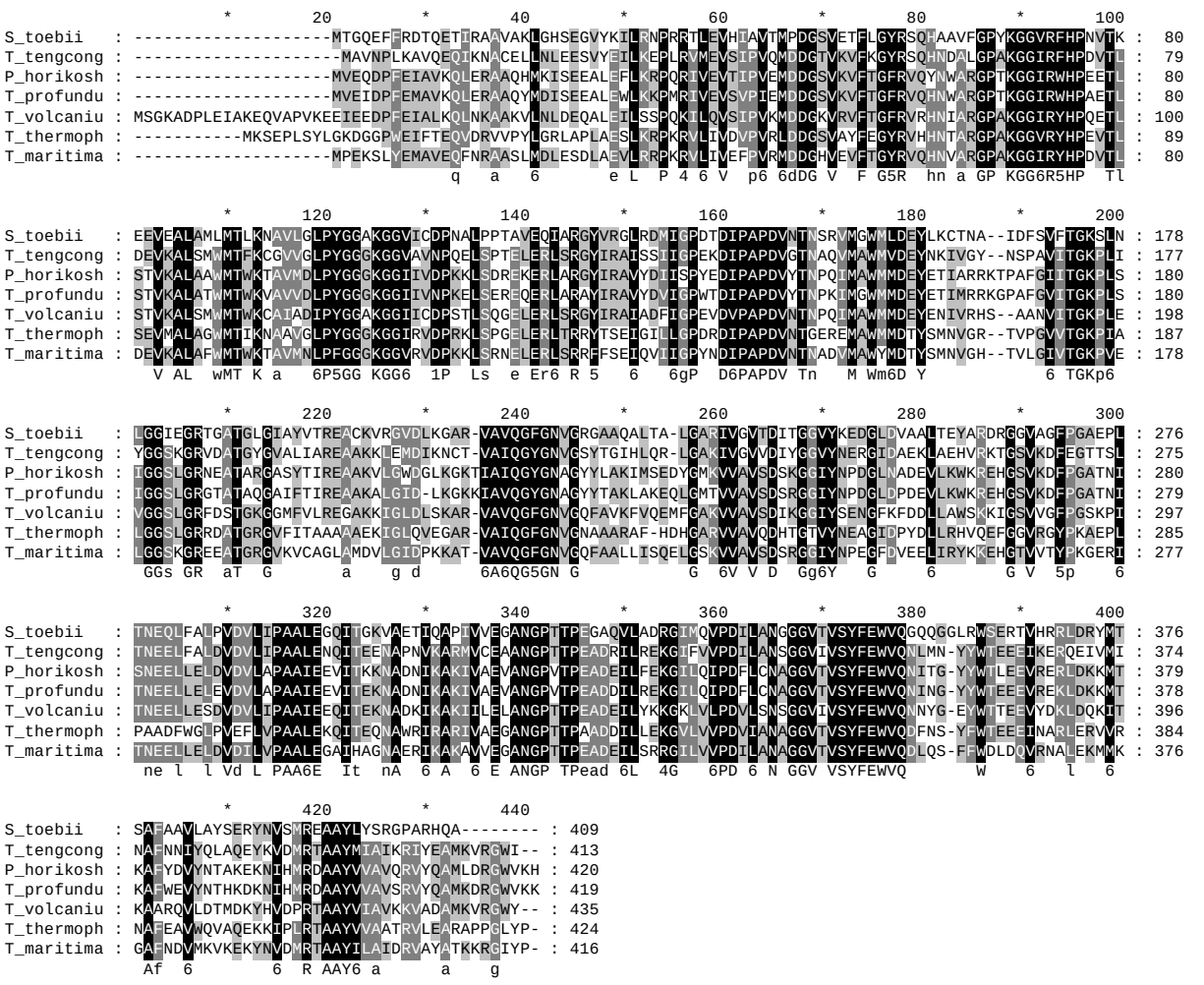
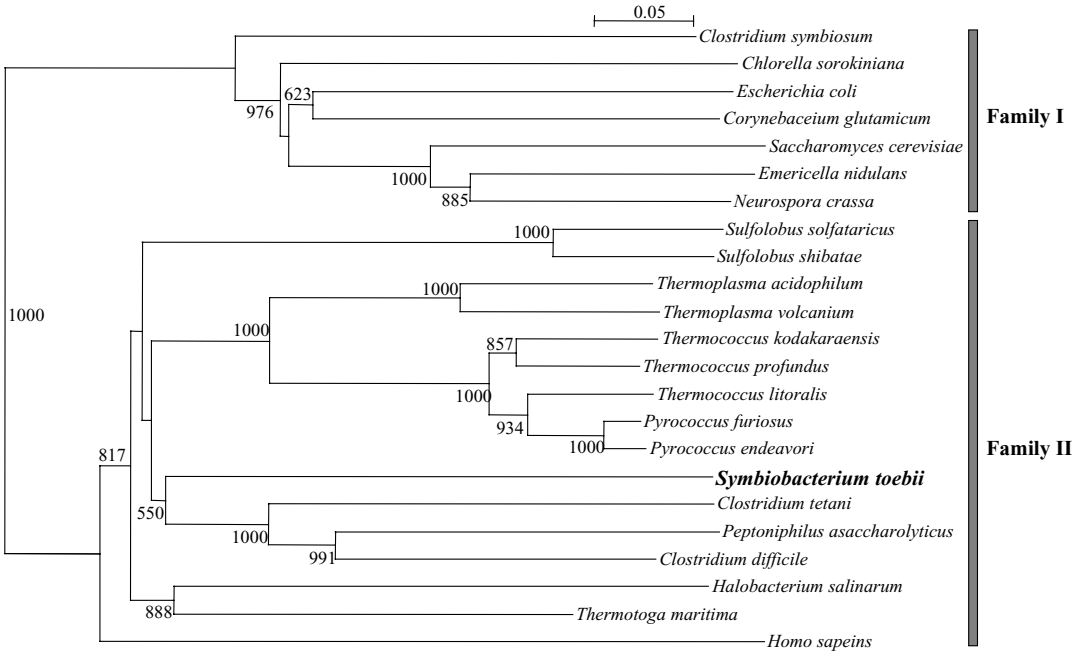
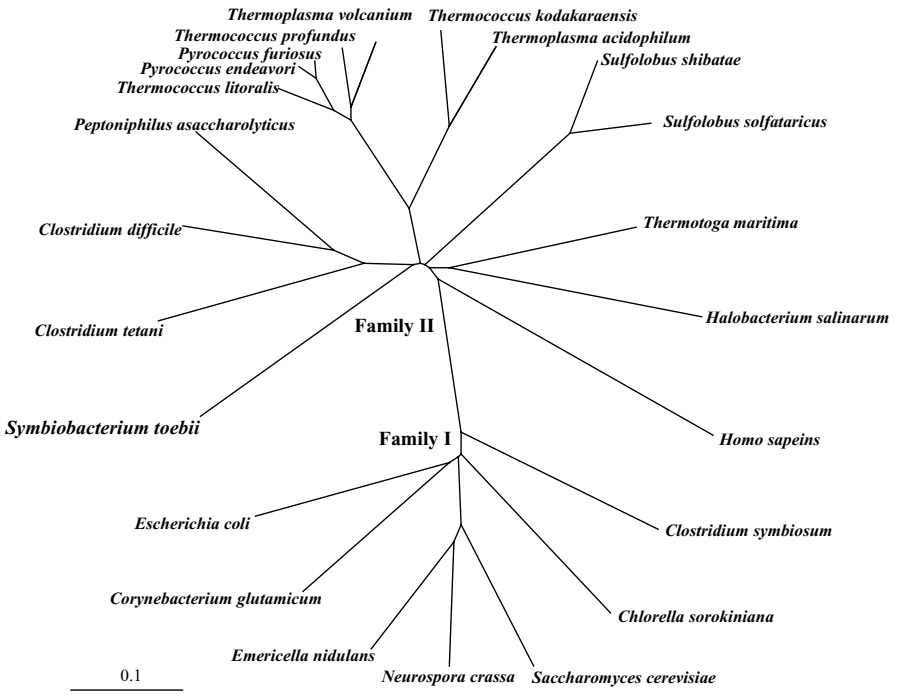


Fig. 2. Multiple alignment of glutamate dehydrogenase amino acid sequence of *S. toebii* with the thermophilic bacteria. The sequences were aligned by using the ClustalX program. The *Thermoanaerobacter tengcongens*, *Pyrococcus horikoshii*, *Thermococcus profundus*, *Thermoplasma volcanium*, *Thermus thermophilus* and *Thermotoga maritima* sequences were obtained from the GeneBank database, using the accession numbers NP_622831, AAB99956, T44308, NP_111278, BAC21186, NP_228821 respectively. The residues common to all sequences indicated in shaded box.



(A)



(B)

Fig. 3. Schematic phylogenetic tree for glutamate dehydrogenase amino acid sequences showing the division of glutamate dehydrogenases into two families. Glutamate dehydrogenase of *S. toebii* was clustered into the middle of the GDH family II.

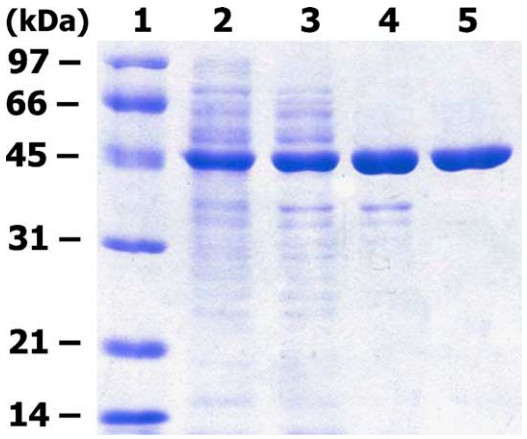


Fig. 4. SDS-PAGE analysis of purification. Lane 1: marker proteins; lane 2: crude extract; lane 3: supernatant after heat treatment; lane 4: Mono Q ion-exchange pool; lane 5: Superose 6 gel filtration pool.

[12]. As shown in Fig. 3A and B, analysis of evolutionary relationship by aligning amino acid sequence showed that the GDH of *S. toebii* was clustered into the middle of the GDH family II tree, suggesting that GDH of *S. toebii* is also a thermostable GDH.

3.3. Expression and purification of glutamate dehydrogenase in recombinant *E. coli*

E. coli DH5α containing the pHCE(II)GDH harboring the *gdh I* gene was cultured in LB medium containing 100 μg/ml of ampicillin at 37 °C. After overnight cultivation, the GDH was expressed well without addition of any inducer and specific activity of crude enzyme solution was 7.8 U/mg. The purified GDH revealed a molecular mass of about 44 kDa on 12% SDS-PAGE, which showed a good agreement with the value calculated from the deduced amino acid sequence (43,626 Da) (Fig. 4).

Purification of the GDH of *S. toebii* was summarized in Table 1. The activity of GDH remained in soluble fraction and exhibited more than 90% of the total activity of crude extract after incubation at 55 °C for 30 min. After heat treatment of crude extract, the GDH was purified to greater than 99% purity by using ion exchange column (Mono Q) and gel permeation column (Superose 6 HR) chromatography. Purified enzyme revealed a specific activity of 32.8 U/mg and it proved to be about 4.2-fold pure and a yield of 30%. The molecular mass of the native protein determined by Superose 6 HR gel filtration chromatography was about 260 kDa (data not shown) which indicated that native GDH of *S. toebii* could be composed of a hexamer with identical subunits like other GDHs [4–6,12–16,31].

3.4. Physical characteristics of GDH

The optimum pH of GDH for the oxidative deamination was 9.0 (Fig. 5A). But the activity of GDH was gradually decreased below and above this pH and GDH lost its activity completely below the pH 8.0. In case of reductive amination the optimum pH was 7.0 (data not shown). When the GDH was tested at various temperatures from 40 to 80 °C, the activity of the enzyme was increased with the change of temperature from 40 to 60 °C and it exhibited the maximum activity at 60 °C (Fig. 5B). This optimum temperature was related with the optimum growth temperature of *S. toebii* [25,26]. The enzyme retained its full activity after incubation at different temperature from 40 to 55 °C for 30 min but completely lost it after incubation at 80 °C for 30 min (Fig. 6).

To test the possibility of thermostable GDH as a biocatalyst, the enzyme was incubated at 37 °C at which the reaction for producing D-amino acid was carried out. The result showed that enzyme activity of GDH was retained after heating at 37 °C for 20 h

Table 1
Purification of recombinant *S. toebii* glutamate dehydrogenase

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	60.0	466.5	7.8	100	1
Heat treatment	30.0	428.4	14.3	92	1.8
Mono Q	5.1	147.4	29.1	32	3.7
Superose 6	4.3	141.5	32.8	30	4.2

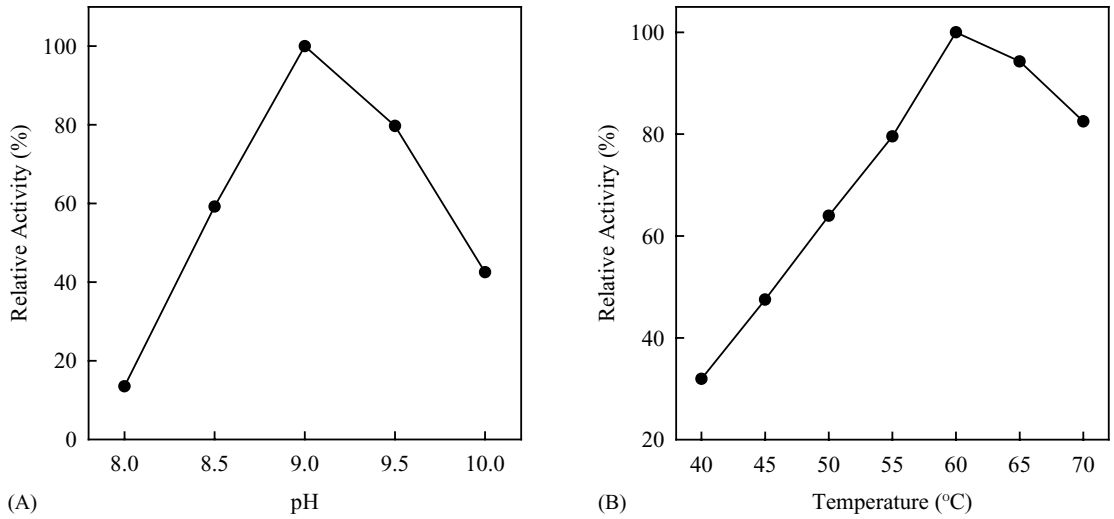


Fig. 5. Effect of pH (A) and temperature (B) on the activity of the glutamate dehydrogenase of *S. toebii*.

supports the possibility of the *S. toebii* GDH as an effective biocatalyst for the production of D-amino acid. In the previous study, multi-enzyme systems using the thermostable enzymes have been developed for the production of various D-amino acids by Bae et al. [24,32], in which aromatic D-amino acids, D-phenylalanine and D-tyrosine can be directly synthesized from phenylpyruvate and hydroxyphenylpyruvate, respectively. Three enzymes consisting the multi-enzyme system for the production of D-amino acid exhibited a high stability. However, only GDH from bovine liver (Boehringer Mannheim, Germany) used multi-enzyme system lost its activity up to 50%. We also reported that the instability of GDH was a critical point for the production of D-amino acid in a multi-enzyme system [32]. So above results show that *S. toebii* GDH has the possibility for using it as a biocatalyst to constitute a multi-enzyme system.

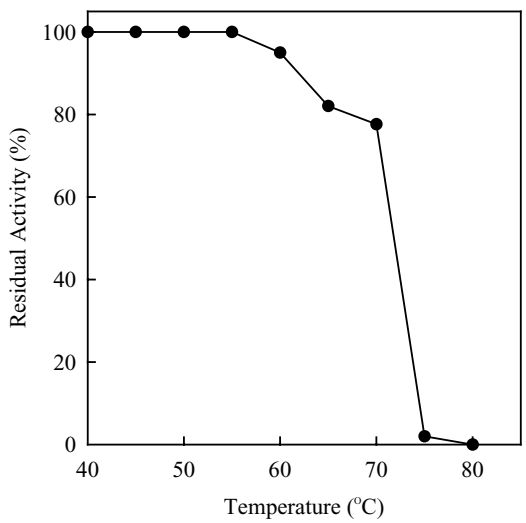


Fig. 6. Effect of temperature on the stability of the glutamate dehydrogenase of *S. toebii*.

3.5. Kinetic properties

S. toebii GDH was able to utilize either NADP or NAD as coenzyme for the oxidative deamination although NADP was the preferred cofactor. Not only the apparent K_m value for NADP (0.09 mM) was two order of magnitude smaller than that for NADH (3.6 mM), but also the k_{cat} value for NADP ($12.8 \times 10^4\ min^{-1}$) was three orders of magnitude higher than that of NADH ($0.01 \times 10^4\ min^{-1}$), indicating that NADP was a preferred coenzyme (Table 2).

Table 2 also revealed that NADPH showed a 4.3-fold higher k_{cat}/K_m value than NADP. These observations indicated that the reaction catalyzed by *S. toebii* GDH was biased towards glutamate formation, indicating that the enzyme has a biosynthetic role mainly. This result also observed in the case of NADH. The k_{cat}/K_m value for NADH was five order of magnitude higher than that of NAD. It indicated

Table 2
Kinetic parameters of recombinant *S. toebii* glutamate dehydrogenase

Substrate	K_m (mM)	k_{cat} ($\times 10^4$ min^{-1})	k_{cat}/K_m ($\times 10^4$ min^{-1} mM^{-1})
NADP	0.088	12.813	145.340
NAD	3.629	0.009	0.002
NADPH	0.280	171.875	614.876
NADH	0.368	36.864	100.168

that the reaction was also mainly biased towards glutamate synthesis from ammonia and 2-oxoglutarate, suggesting the possibility of GDH as a biocatalyst in the multi-enzyme system for synthesis of valuable aromatic D-amino acids.

4. Conclusions

We found a thermostable GDH from a commensal thermophile, *Symbiobacterium toebii* through the analysis of its genome information and, the GDH gene was successfully cloned and expressed in *E. coli* DH5α up to 50% of the total soluble protein under the control of constitutive expression promoter, HCE. The expressed GDH exhibited the thermostability after heat treatment at 55 °C for 30 min. The optimum temperature and pH of the GDH activity were 60 °C and 9.0, respectively, for the oxidative deamination, whereas for the reductive amination, it was 60 °C and 7.0, respectively. *S. toebii* GDH was able to utilize either NADP(H) or NAD(H) as coenzyme although NADP(H) was the preferred cofactor. In our previous study, it was concluded that of four enzymes consisting the multi-enzyme system for the production of D-phenylalanine exhibited a high stability at only GDH from bovine liver (Boehringer Mannheim, Germany) lost its activity up to 50% when these enzymes were incubated at 37 °C for 10 h [32]. We also reported that the thermostability of GDH was a critical point for the production of D-amino acid in a multi-enzyme system [32]. But GDH of *S. toebii* which was studied in this paper retained its activity more than 90% after incubation at 37 °C for 20 h. This result strongly supports the GDH of *S. toebii* could be a good enzyme for regenerating L-glutamate from α-ketoglutarate in a multi-enzyme system.

Acknowledgements

This work was supported by National Research Laboratory (NRL) program Grant M1-9911-00-0026 from the Ministry of Science and Technology of Korea.

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