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The *Sulfolobus tokodaii* gene ST1704 codes highly thermostable glucose dehydrogenase

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Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

NAD(P)-dependent glucose-1-dehydrogenase (GDH) has been used for glucose determination and NAD(P)H production in bioreactors. Thermostable glucose dehydrogenase exhibits potential advantage for its application in biological processes. The function of the putative GDH gene (ST1704, 360-encoding amino acids) annotated from the total genome analysis of a thermoacidophilic archeaon *Sulfolobus tokodaii* strain 7 was investigated to develop more effective application of GDH. The gene encoding *S. tokodaii* GDH was cloned and the activity was expressed in *Escherichia coli*, which did not originally possess GDH. This shows that the gene (ST1704) codes the sequence of GDH. The enzyme was effectively purified from the recombinant *E. coli* with three steps containing a heat treatment and two successive chromatographies. The native enzyme (molecular mass: 160 kDa) is composed of a tetrameric structure with a type of subunit (41 kDa). The enzyme utilized both NAD and NADP as the coenzyme. The maximum activity for glucose oxidation in the presence of NAD was observed around pH 9 and 75 °C in the presence of 20 mM Mg^{2+}. The enzyme showed broad substrate specificity: several monosaccarides such as 6-deoxy-D-glucose, 2-amino-2-deoxy-D-glucose and D-xylose were oxidized as well as D-glucose as the electron donor. D-Mannose, D-ribose and glucose-6-phosphate were inert as the donor. The enzyme showed high thermostability: remarkable loss of activity was not observed up to 80 °C by incubation for 15 min at pH 8.0. In addition, the enzyme was stable in a wide pH range of 5.0–10.5 by incubation at 37 °C. From the steady-state kinetic analysis, the enzyme reaction of D-glucose oxidation proceeds via a sequential ordered Bi–Bi mechanism: NAD and D-glucose bind to the enzyme in this order and then D-glucono-1,5-lactone and NADH are released from the enzyme in this order. The amino acid sequence alignment showed that *S. tokodaii* GDH exhibited high homology with the *Sulfolobus solfataricus* hypothetical glucose dehydrogenase and a *Thermoplasma acidophilum* one.

Keywords: *Sulfolobus tokodaii*; NAD(P)-dependent glucose-1-dehydrogenase; Thermoacidophilic archaeon; Modified Entner–Doudoroff pathway; Primary structure of glucose dehydrogenase

1. Introduction

Glucose dehydrogenase (β-D-glucose: NAD(P) 1-oxidoreductase EC 1.1.1.47), which catalyses the oxidation of D-glucose using NAD or NADP as a coenzyme.

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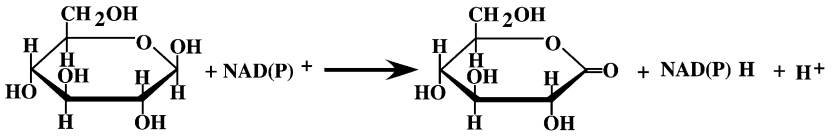

D-glucose D-glucono-1,5-lactone

GDH has been well used to supply NAD(P)H for the enzymatic production of useful chiral compounds with dehydrogenases and reductases [1,2]. The enzyme is also applicable to biochip and biosensor for the enzymatic determination of D-glucose [3,4]. Thus, a stable GDH may be more useful for applications. On the other hand, the presence of unique glucose metabolic pathways has been reported in thermophilic archaea [5]. Thermoacidophilic archaea such as *Sulfolobus solfataricus* and *Thermoplasma acidophilum*, are known to metabolize D-glucose via a modified Entner–Doudoroff pathway involving non-phosphorylated intermediates [6], and hyperthermophilic and neutrophilic arechaea such as *Pyrococcus furiosus* and *Thermococcus litoralis* to metabolize it via a modified Embden–Meyerhof pathway [7]. In the former pathway, GDH is a key enzyme which functions in the first step. We have recently found two putative glucose-1-dehydrogenase (GDH) genes (ST1704 and 2556) annotated from the total genome analysis of a thermoacidophilic archaeon *Sulfolobus tokodaii* strain 7 [8].

In this study, we have begun the functional analysis of gene ST1704 for clearer elucidation of the physiological role of GDH in the glucose metabolism of the thermoacidphilic crenarchaeon and for more abundant applications of the enzyme.

2. Materials and methods

2.1. Materials

S. tokodaii strain 7 (JCM 10545) and *Escherichia coli* strain JM109 were obtained from Wako (Japan) and Toyobo (Japan), respectively. *E. coli* BL21(DE3)pLysS and plasmid DNA pET11a were purchased from Novagen (USA). All other reagents were of analytical grade.

2.2. Cloning and expression of S. tokodaii ST1704 gene in E. coli

The plasmid DNA containing ST1704 (pUC118-GDH, position 1,705,395-1,707,815 in the entire genome of *S. tokodaii* strain 7, has been inserted into the *Hin*cII site of pUC118) was obtained from the shotgun clone STLBA01109 prepared for genome analysis of *S. tokodaii* strain 7 (8). The following set of two oligonucleotide primers were used for amplification of ST1704 gene by PCR. The forward primer of 5′-GAGGCATATGAAAGCAATAGTTGTTACACC-TAAAAA-3′ was designed on the basis of the N-terminal amino acid sequence of *S. tokodaii* GDH, and a unique *Nde*I restriction site was introduced. The reverse primer of 5′-GAAAGGATCCTTAATCCCA-CACAATAACTGTTTTTATTTCTCC-3′ was designed on the basis of the C-terminal amino acid sequence and a unique *Bam*HI restriction site was introduced. The plasmid DNA pUC118-GDH was used as the template. The PCR products were size-fractionated by agarose gel electrophoresis. The 1.1 kb fragments were extracted from the gel and ligated with T vector using the Original TA cloning kit (Invitrogen, USA). Competent *E. coli* cells were transformed with the resulting hybrid plasmid (T vector-GDH). Positive *E. coli* cells were isolated and the DNA was purified by a DNA preparation kit (Qiagen, Japan). The nucleotide sequence was confirmed by the method of Sanger using a DSQ-600L(S)/-2000L DNA sequencer (Shimadzu, Japan). The DNA was digested with *Nde*I and *Bam*HI, and ligated into the expression vector of pET11a linearized with *Nde*I and *Bam*HI to generate pET11a-GDH. The *E. coli* BL21(DE3)pLysS was transformed with pET11a-GDH and *E. coli* pET11a-GDH was cultivated (up to $OD_{600} = 0.6$) at 37 °C in Luria–Bertani medium (1 l) containing 50 μg/ml of ampicillin and 34 μg/ml of chloramphenicol. The induction was carried out by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside, and the

cultivation was continued for 3 h and harvested by centrifugation (12,000 × *g* for 10 min).

2.3. Purification of GDH from E. coli

Washed *E. coli* cells (wet weight: 4.9 g) were suspended in 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA and incubated with 1 mg/ml lysozyme at room temperature for 1 h. The cells were disrupted by ultrasonication and centrifuged. The supernatant was obtained as the crude extract. The crude extract was heated at 70 °C for 10 min and denatured protein was removed by centrifugation. The supernatant was dialyzed against 10 mM potassium phosphate buffer (pH 7.0) containing 10% glycerol without EDTA. Solid ammonium sulfate was added to the solution until a final concentration of 20% saturation and the solution was loaded on a Butyl-Toyopearl column previously equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 20% ammonium sulfate and 10% glycerol. After washing with the same buffer, the enzyme was eluted with a linear reverse gradient of 20–0% ammonium sulfate (400 ml buffer). The active fractions were pooled and dialyzed against 10 mM Tris–HCl (pH 9.0) to remove ammonium sulfate and then concentrated by ultrafiltration (UP-20 membrane, ADVANTEC, Japan). The solution (30 ml) was loaded on an Uno-Q column (1.5 cm × 6.8 cm) (Bio-Rad, Japan) equilibrated with 10 mM Tris–HCl (pH 9.0) containing 10% glycerol. Tris–HCl buffer (pH 9.0) was used in this procedure because the isoelectric point of the enzyme is relatively high (pI = 7.1) (Database of Genomes Analyzed in National Institute of Technology and Evaluation (NITE)). After the column was washed with the equilibration buffer (30 ml), the enzyme was eluted with a linear salt gradient of buffer containing 0–0.5 M NaCl (100 ml). The active fractions were pooled, dialyzed against Tris–HCl (pH 8.0), and used as the purified enzyme. The purified enzyme was stored at 4 °C.

2.4. Enzyme assay and protein determination

GDH activity was assayed spectrophotometrically. The standard reaction mixture was composed of 200 mM glycine–NaOH buffer (pH 9.0), 10 mM glucose, 20 mM $MgCl_2 \cdot 6H_2O$, 1.25 mM NAD and enzyme in a final volume of 1.0 ml. The reaction mixture was incubated at 50 °C, and the change in absorbance of NADH was monitored at 340 nm ($\varepsilon = 6.2\,mM^{-1}\,cm^{-1}$). One unit of enzyme was defied as the amount catalyzing the formation of 1 μmol NADH/min at 50 °C in the D-glucose oxidation. Protein concentration was determined by the method of Bradford using the Bio-Rad Protein Assay kit. Bovine serum albumin was used as the standard.

2.5. Gel electrophoresis and molecular mass determination

Native gel electrophoresis and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were carried out by the procedures of Davis [9] and Laemmli [10], respectively. The protein band was stained with Coomassie brilliant blue R-250. The molecular mass of the GDH subunit was determined by SDS-PAGE using commercially available molecular marker proteins (NEW ENGLAND BioLabs, USA) as standard proteins. The molecular mass of native GDH was determined by gel filtration chromatography using a Superose^{TM} 6 column (Amersham Bioscience Corp., USA) equilibrated with potassium phosphate buffer (pH 7.0) containing 0.2 M NaCl. Filtration Calibration Kits (Amersham Biosciences Corp., USA) was used as molecular mass standards.

2.6. N-terminal amino acid sequencing

After SDS-PAGE of the purified GDH, the protein was electrophoretically transferred onto a PVDF membrane (Bio-Rad, Japan) and stained with Ponceau S (Aldrich, USA). The stained protein band was cut out and its N-terminal amino acid sequence was determined using protein sequencer PPSQ-10 (Shimadzu, Japan).

2.7. Effect of pH and temperature on enzyme activity and stability

The effect of pH on GDH activity was examined using 200 mM sodium acetate buffer (pH 3.0–5.5), bis-Tris–HCl (5.5–7.0), HEPES–NaOH (7.0–8.0), Tris–HCl (8.0–9.0) and sodium carbonate (9.0–11.0) buffers. The effect of pH on GDH stability was studied by incubating the enzyme in the buffers similar to those used for testing the effect of pH on the activity.

After incubation at 37 °C for 30 min, the activity of an aliquot obtained from each mixture was assayed. The effect of temperature on the enzyme stability was examined by incubating the enzyme at various temperatures. After incubation, the residual activity of an aliquot was assayed.

2.8. Effect of compounds on the activity and kinetic mechanism

The effect of a compound on GDH activity was evaluated by the measurement of the residual activity after addition of various compounds to the reaction mixture. The steady-state kinetic mechanism study was carried out by initial velocity and product inhibition analyses [11,12].

3. Results and discussion

3.1. Expression and purification of S. tokodaii GDH

We have found a gene ST1704 (1083 bp, a position of 1,705,465-1,706,547 on the entire genome) which may encode a putative GDH (a protein of 360 amino acids with a molecular weight of 40,282) in the complete sequence of the *S. tokodaii* gene. We performed cloning and expression of the ST1704 gene in *E. coli* BL21(DE3)pLysS and detected the activity on a transformant *E. coli*. *E. coli* has inherently no GDH activity. The transformant *E. coli* produced a high level of GDH activity and the enzyme was rapidly and simply purified by only three steps containing a heat treatment and two conventional chromatographies from the crude extract of transformant (Table 1). About 5.7 mg of the purified enzyme was effectively obtained with a 24% yield from the cells of 1 l culture (4.9 g wet weight.). The purified enzyme showed a single protein band by SDS-PAGE (Fig. 1A). The N-terminal sequence of the purified enzyme was determined to be M-K-A-I-V-V-T-P-K-K-S-G-V-E-V-K-D-I-P-M. The N-terminal amino acid sequence corresponded to that predicted from the ST1704 gene sequence. This shows that the ST1704 gene codes *S. tokodaii* GDH.

Table 1
Purification of GDH from recombinant *E. coli* cells

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	310	750	2.4	100
Heat treatment	54	670	12	89
Butyl-Toyopearl	18	300	17	40
Uno-Q	5.7	180	32	24

3.2. Molecular and catalytic properties of GDH

The molecular mass of GDH in the native form was estimated to be about 160 kDa by gel filtration (Fig. 1B). The molecular mass of a subunit was determined to be about 41 kDa by SDS-PAGE (Fig. 1A). This value is in fair agreement with the molecular weight (40,282) deduced from the gene sequence. This indicates that the native GDH is a homotetrameric protein. Two different kinds of subunit structure for bacterial glucose dehydrogenases have been reported: one is a homotetrameric structure for the enzymes from many microorganisms containing *Bacillus megaterium* [13,14], *S. solfataricus* [15] and *T. acidophilum* [16] and the other is the homodimeric one for the enzymes from *Haloferax mediterranei* [17] and *Thermoproteus tenax* [18]. The *S. tokodaii* GDH belongs to former group in the subunit structure.

The effect of pH on activity was examined and the highest activity was observed around pH 9.0 (Fig. 2A). In addition, the enzyme exhibited maximal activity at 75 °C from the temperature profile (Fig. 2B). The enzyme was highly thermostable: it retains more than 90% activity up to 80 °C (Fig. 3) by incubation for 15 min at pH 8.0. At the optimum pH (pH 9.0) for activity, the thermostability was slightly low compared with that at pH 8.0: the activity was gradually lost above 60 °C and significantly lost above 80 °C by incubation for 15 min. The enzyme was stable in a wide range of pH (5.0–10.5) with incubation at 37 °C for 30 min, and half of the activity remained at pH 4.5 and 11.0.

In the case of GDHs from *S. solfataricus* and *H. mediterranei*, several divalent cations such as Mg^{2+} and Mn^{2+} have been reported to affect the activity [15,17]. The effect of various compounds on the activity was examined. The enzyme was activated by 1 mM $MgCl_2$ (the relative activity was 122% compared with that without the additive), but was inhibited by many other metal salts: 1 mM $ZnCl_2$ (relative

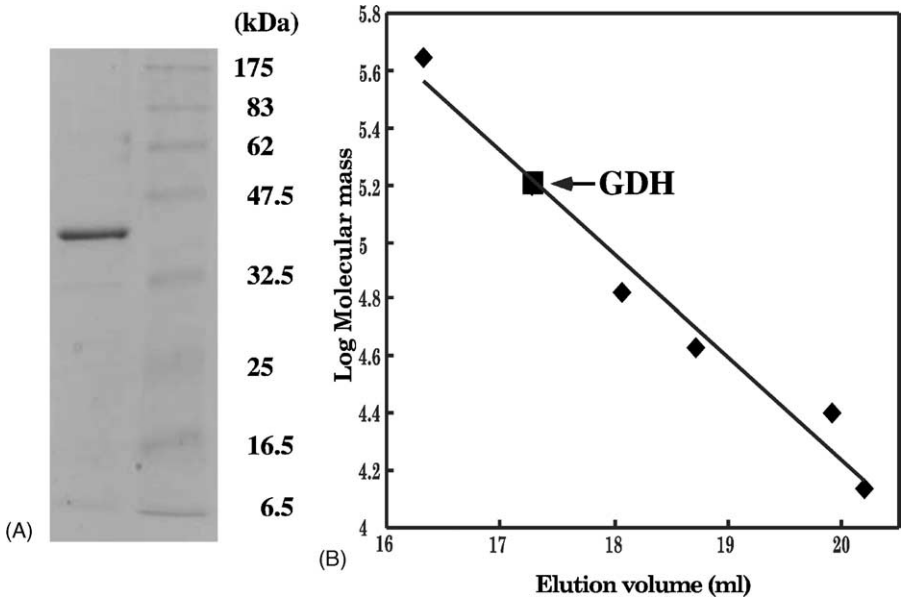

Fig. 1. SDS-PAGE of the purified enzyme (A) and a calibration curve for the determination of native molecular mass (B). The marker proteins used for SDS-PAGE are fusion of an *E. coli* maltose-binding protein and β-galactosidase (175 kDa), fusion of *E. coli* maltose-binding protein and paramysin (83 kDa), bovine glutamate dehydrogenase (62 kDa), rabbit muscle aldolase (47.5 kDa), rabbit muscle triosephosphate isomerase (32.5 kDa), bovine milk β-lactoglobulin A (25 kDa) and chicken eggwhite lysozyme (16.5 kDa), and used for gel filtration are horse spleen ferritin (440 kDa), rabbit muscle aldolase (158 kDa), bovine serum albumin (67 kDa), hen egg ovalbumin (43 kDa), bovine pancreas chymotrypsinogen (25 kDa) and bovine pancreas ribonuclease (13.7 kDa).

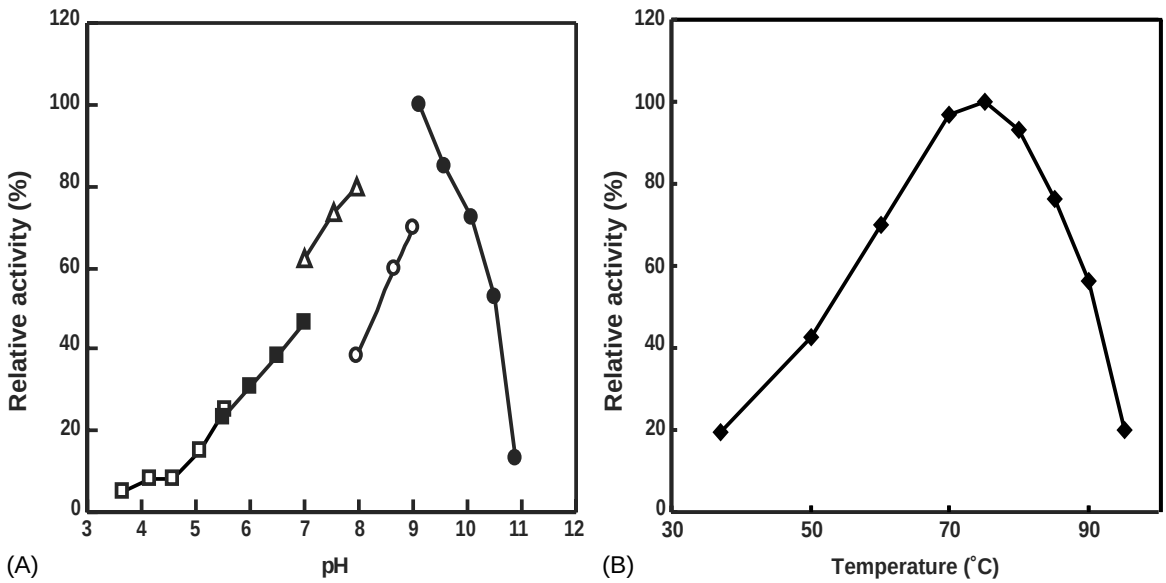

Fig. 2. Effect of pH (A) and temperature (B) on the enzyme activity.

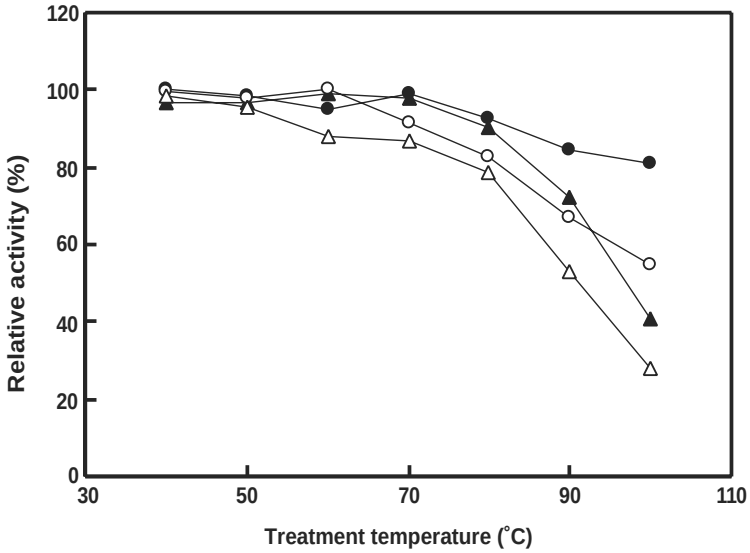

Fig. 3. Effect of temperature on enzyme stability. The enzyme was treated at various temperatures for 5 min (●) and 15 min (▲) at pH 8.0 and for 5 min (○) and 15 min (△) at pH 9.0.

activity: 75%), $CaCl_2$ (67%), $MnCl_2$ (59%), $CoCl_2$ (56%), $CuCl_2$ (17%), $NiCl_2$ (15%), $HgCl_2$ (15%) and *p*-chloromercuribenzoate (7%). EDTA (1 mM) also inhibited the activity (68%), and the addition of 20 mM $MgCl_2$ restored the full activity.

Specificity of the electron donor of GDH was examined. With NAD as the electron acceptor, D-glucose was the most preferable substrate: other monosaccharides, such as D-allose, D-gulose, 2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucose, were oxidized by the enzyme (Table 2). D-Mannose, D-ribose and glucose-6-phosphate were inert as the donor. This suggests that the *S. tokodaii* GDH enzyme can bind and oxidize substrate sugars which possess equatorial hydroxy groups at C-2, C-3 and C-4 in the pyranose-ring as reported in the case of the *S. solfataricus* enzyme [15]. The electron acceptor activity of NADP was 35% compared with that of NAD. This shows that the *S. tokodaii* GDH exhibits similar substrate specificity to that shown by *S. solfataricus* GDH. The reaction catalyzed by *S. tokodaii* GDH was irreversible: the enzymatic reduction of glucose-1,5-lactone in the presence of NADH and NADPH was not observed for the reverse reaction. On this point, the *S. tokodaii* GDH is different from the *H. salinarum* GDH, which catalyzes the reverse reaction [19].

The steady-state kinetic mechanism for the D-glucose dehydrogenase reaction was investigated to identify the reaction mechanism. From initial velocity analyses, the double reciprocal plots of initial velocity (v) against D-glucose concentrations in the presence of several fixed concentrations of NAD gave intersecting straight lines (Fig. 4). This pattern shows that the reaction proceeds via the formation of a ternary complex of the enzyme with NAD and D-glucose in

Table 2
Substrate specificity for electron donor

Electron donor (10 mM)	Electron acceptor	
	NAD	NADP
D-Glucose	100	35
D-Mannose	0	0
D-Allose	9	8
D-Galactose	0	6
D-Altorose	0	8
D-Gulose	6	7
2-Deoxy-D-glucose	7	6
2-Amino-2-deoxy-D-glucose	19	5
6-Deoxy-D-glucose	53	13
D-Xylose	11	10
D-Ribose	0	5
D-Glucose-6-phosphate	0	0

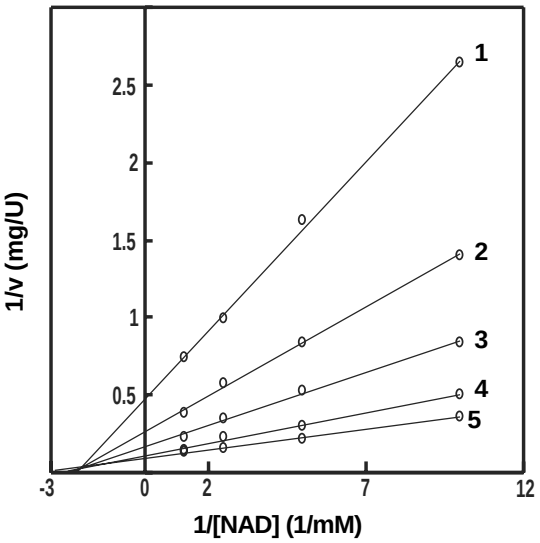

Fig. 4. Double reciprocal plots of initial velocity against NAD concentration at a series of fixed concentrations of D-glucose. The concentration of D-glucose used were 1: 0.625 mM; 2: 1.25 mM; 3: 2.5 mM; 4: 5 mM and 5: 10 mM.

the same manner as the *H. salinarum* enzyme [19]. K_m values for NAD and D-glucose were determined to be 0.46 and 4.2 mM, respectively, from replots of the reciprocal fixed substrate concentrations and vertical intersects. With NADP as the coenzyme, K_m values for NADP and D-glucose were calculated to be 0.015 and 0.17 mM, respectively. This shows that the affinity for NADP and D-glucose to the enzyme is much higher than that for NAD and D-glucose although the reaction rate in the case of NADP is rather low (one-third) compared with that in the case of NAD as the coenzyme.

Product inhibition patterns of D-glucose oxidation were performed to determine the order of substrate addition and product release. With NADH as an inhibitor, the double reciprocal plots of velocity (v) versus NAD concentrations at a constant concentration of D-glucose showed a typical competitive inhibition pattern (Fig. 5A). This indicates that NAD and NADH can bind to the free form of the enzyme. On the other hand, NADH showed non-competitive inhibition against D-glucose (Fig. 5B). This inhibition pattern by NADH against D-glucose indicates that D-glucose binds to the enzyme–NAD complex as the second substrate. In addition, another product,

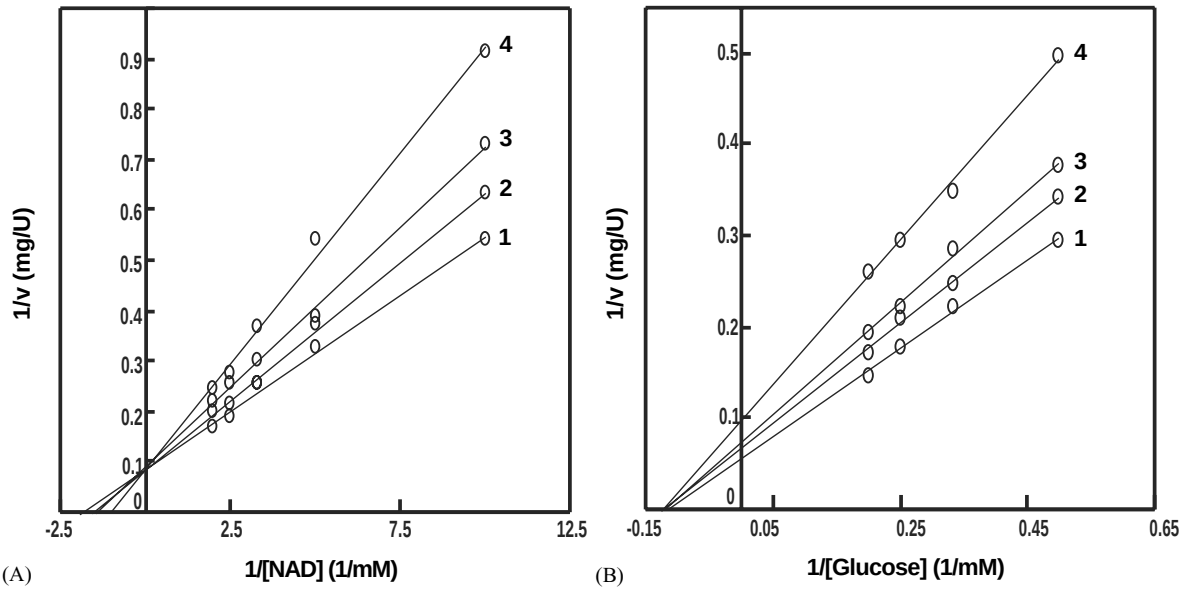

Fig. 5. Product inhibition patterns of NADH with the varied substrate concentrations of NAD (A) and D-glucose (B). The concentration of NADH used were 1: 0 mM; 2: 0.025 mM; 3: 0.05 mM and 4: 0.1 mM.

D-glucono-1,5-lactone, showed no inhibition against both D-glucose and NAD. Taking into consideration that the enzymatic reaction is irreversible, the reaction may proceed by a sequential ordered Bi–Bi mechanism in which NAD and D-glucose bind to the enzyme in this order, and D-glucono-1,5-lactone and NADH release from the enzyme in this order. The reaction of *H. salinarum* and *Schizosaccharomyces pombe* GDHs has been reported to proceed with a similar sequential ordered Bi–Bi mechanism [19,20]. In addition, in the case of *H. salinarum* enzyme the ternary abortive complexes of enzyme–NADP–glucono-1,5-lactone and enzyme–NADH–glucose are involved in the reaction scheme. Such ternary abortive complexes were not recognized for the *S. tokodaii* enzyme and that may be favorable for its application to D-glucose determination and a NAD(P)H production system.

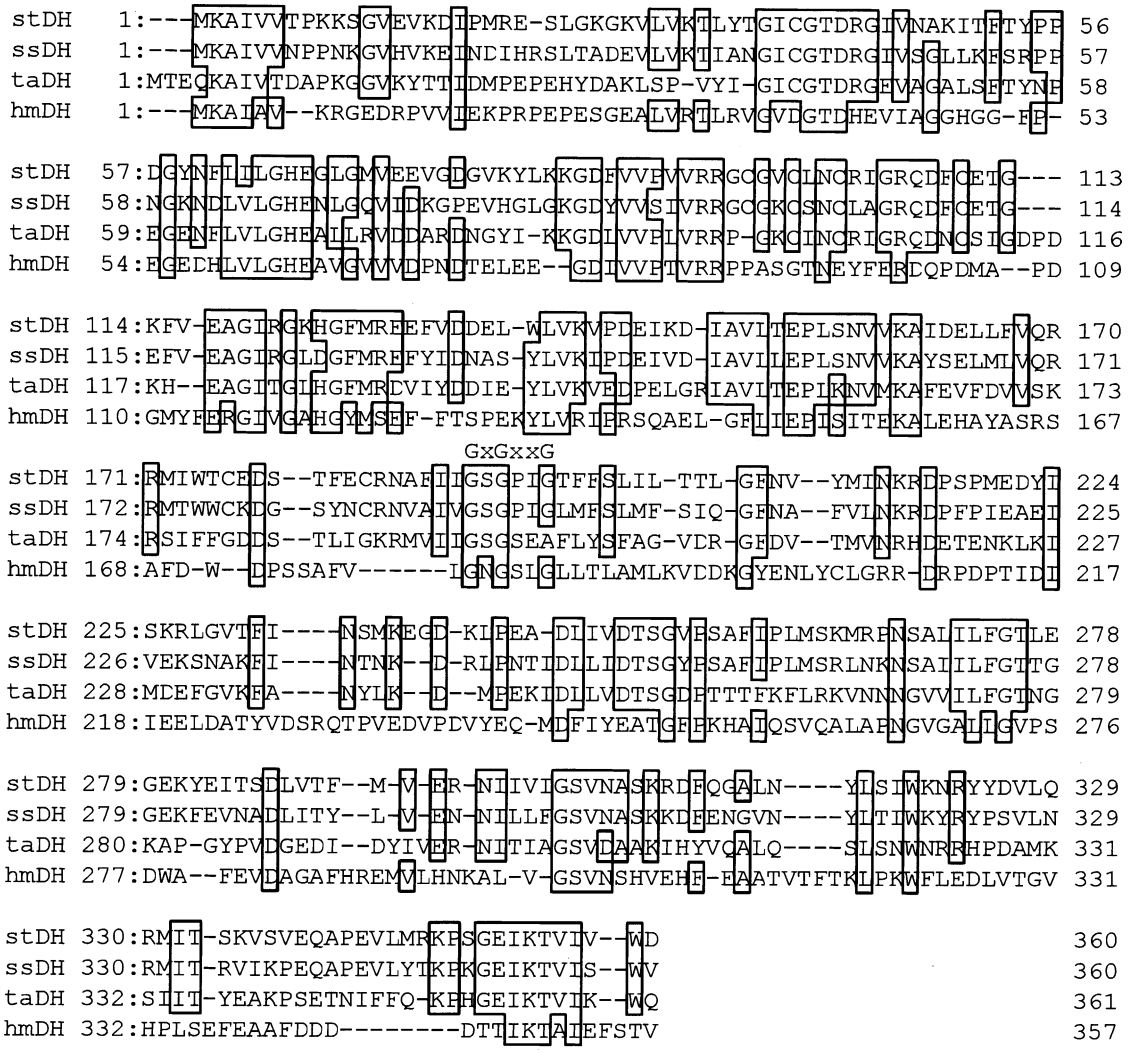

Fig. 6. Alignment of the amino acid sequences of GDHs from *S. tokodaii* (stDH), *S. solfataricus* (ssDH, putative enzyme), *T. acidophilum* (taDH) and *H. mediterranei* (hmDH). The conserved residues are boxed and GxGxxG above the sequence represents the motif recognized in the NAD(P)-binding domains of dehydrogenases and reductases.

3.3. Amino acid sequence analysis

The amino acid sequence of *S. tokodaii* GDH was aligned with those of the enzymes from other sources based on a database (Fig. 6). The *S. tokodaii* GDH exhibited high sequence homology with archaeal proteins from archaea such as the *S. solfataricus* hypothetical proteins (SSO3204: 57%, SSO3042: 42% and SSO3003: 39%), *T. acidophilum* enzyme (42%) and *H. mediterranei* enzyme (29%), but rather low homology with bacterial GDH from *B. megaterium* and *B. subtilis*. However, the value (about 57%) of the highest sequence homology of the *S. tokodaii* enzyme with the enzyme from the same genus *S. solfataricus* is fairly low compared with the values (more than 80%) observed among *Bacillus* species GDHs [21]. From the alignment, we identified some functionally important amino acid residues that are well conserved in the enzymes. In general, the GxGxxG or GxGxxA sequence motif has been recognized in the NAD(P)-binding domains of dehydrogenases and reductases [22,23]. In the case of *S. tokodaii* GDH, the similar sequence motif was recognized in the position from G190 to G195.

4. Concluding remarks

In this study, the function of *S. tokodaii* gene ST1704 was found to be GDH and the first primary structure as GDH from thermoacidophilic archaea *Sulfolobus* species was determined. In addition, we here achieved a simple and effective purification method of GDH from the transformant *E. coli* cells. The *S. tokodaii* enzyme is the most thermostable GDH found so far and may be more effectively applicable to biosensors and bioreactors than the enzyme from mesophiles such as *B. megaterium* and *B. subtilis*. The 3D structural analysis of the enzyme is now under investigation to obtain more detailed information about the relationships between structure and function.

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