Full Length Research Paper

Purification and characterization of recombinant glucose dehydrogenase isolated from a hyperthermophilic *Sulfolobus*-like bacterium

Liang-Jung Chien*, Yu-Ting Duan and Ni-Yin Yu

Graduate School of Biochemical Engineering, Ming Chi University of Technology, No. 84 Gongzhuan Rd., Taishan District, New Taipei City 243, Taiwan.

This study aimed to clone and characterize a thermal resistant glucose dehydrogenase (GDH) and investigate its clinical potential. A *Sulfolobus*-like thermophilic microbe was first isolated from a hot spring in Taitung, Chihpen County, Taiwan. The gene encoding GDH was cloned from the bacterium and expressed in *Escherichia coli*. The molecular weight of the enzyme was found to be approximately 39,000 kDa. The enzyme is stable over pH 4.0 to 11.0 and has an optimum pH of 8.0. The thermostability range of the enzyme correlated well with that of the natural environment for *Sulfolobus*. The GDH showed high substrate specificity for glucose. GDH could be useful in biotechnological applications because of its higher thermostability and substrate specificity when compared with that of other glucose-degrading enzymes.

Key words: Glucose dehydrogenase, sequencing, glucose test strip, blood glucose meter, diabetes mellitus.

INTRODUCTION

Glucose oxidoreductases are enzymes that catalyze glucose oxidation and have several important industrial applications. In particular, they are used as components of various enzyme sensors for clinical diagnosis of diabetes. On the basis of their electron acceptors, glucose oxidoreductases can be divided into glucose oxidases and glucose dehydrogenases (GDHs).

Glucose oxidases are used for measurement of blood glucose levels. However, this enzyme is said to have a problem, in that, glucose concentration measurement is affected by dissolved oxygen concentration because it may use molecular oxygen as an electron acceptor (Leskovac et al., 2005). Because GDHs are not affected by dissolved oxygen concentration, these enzymes have been mainly used for the measurement of blood glucose levels in recent years. GDHs include NAD-, pyrroloquinoline quinone (PQQ), and flavin-dependent GDHs. PQQ-dependent GDH has low substrate specificity, that is, it is as reactive towards maltose as it is toward glucose, while flavin-dependent GDH has stricter substrate specificity than PQQ-dependent GDH. However, the substrate specificity of flavin-dependent GDH is not necessarily sufficient because its reactivity toward xylose is approximately 9% as compared to that toward glucose, and it is stable only upto a temperature of approximately 50°C.

Among the known types of NAD-dependent GDHs, *Bacillus*-derived NAD-dependent GDH is well known. Although, the substrate specificity of this NAD-dependent GDH is relatively high, it is stable only up to approximately 50°C, which is not satisfactory (Hilt et al., 1991; Nagao et al., 1992).

Hyperthermophilic archaea are microbes that are systematically classified under Archaea and can grow at 90°C or more, with an optimum growth temperature of 70°C. Enzymes derived from hyperthermophilic archaea generally have high thermal stability. NAD-dependent GDH has been isolated from hyperthermophilic archaea, and its characteristics have been investigated. *Thermoplasma acidophilum*-, *Haloferax mediterranei*- and *Thermoproteus tenax*-derived GDHs were reported in 1989, 1996 and 1997, respectively (Bright et al., 1993; Bonete et al., 1996; Siebers et al., 1997). Although, these

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*Corresponding author. E-mail: ljchien@mail.mcut.edu.tw. Tel: +886-2-29089899. Ext: 4663.

enzymes have excellent thermal stability, their substrate specificity is poorer than that of enzymes derived from other bacteria. When NAD is used as a coenzyme, activity of *T. acidophilum*-derived GDH towards galactose is 70% as compared to that towards glucose. *T. acidophilum*-derived GDH is highly reactive towards xylose (Bright et al., 1993). Use of GDHs with low substrate specificity and high reactivity towards substances other than glucose results in inaccurate measurement of blood glucose levels, which is disadvantageous. However, NAD-dependent GDH with high thermal stability (stable at ≥70°C) and high specificity for glucose has not yet been isolated from hyperthermophilic archaea.

Therefore, we attempted to isolate a microbe from a hot spring in Taitung, Chihpen County, Taiwan, which expressed NAD-dependent GDH. We successfully isolated a *Sulfolobus*-like bacterium and expressed its NAD-dependent GDH-encoding gene in *Escherichia coli*. Our focus was to determine heat and pH sensitivities and substrate specificity of this enzyme for developing clinical assays. Our results strongly suggest that this NAD-dependent GDH may be very useful for developing next generation blood glucose meters.

MATERIALS AND METHODS

Bacterial culture

The following bacterial strains were used as host cells: *E. coli* Eco 10B (Genotype: (F⁻) *endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 φ80lacZΔM15 araD139 Δ(ara, leu) 7697 mcrA Δ(mrr-hsdRMS-mcrBC)λ-*) and *E. coli.* BL21 (DE3) (Genotype: (F⁻) *ompT hsdSβ (rβ-mβ-) dcm gal λ-(DE3)*). Bacteria were cultured in either 3 ml of LB medium at 37°C with shaking (approximately 170 rpm) for 12 to 16 h, or on LB agar at 37°C for 12 to 16 h.

Construction of a NAD-GDH bacterial expression vector

Plasmids containing the glucose dehydrogenase gene (GenBank accession number: JN628989) were constructed (Figure 1) from *Sulfolobus*-like bacteria genomic DNA using PCR primers, *Nde*I (5′-CCGCATATGAAAGCGATTATCGTGAAACCGAGGAGA-3′) and *Xho*I (5′-CCGCTCGAGTTCCCACAGAATGCGGATTTTAATTTCGCC-3′).

The PCR product was digested with *Nde*I and *Xho*I, and the resulting fragment was cloned into the *Nde*I/*Xho*I site of pET-30b(+) and used to transform *E. coli* ECOS 10B. The colonies were counted, transferred to another solid medium, cultured at 37°C for 2 to 4 h, and used as template for a second round of PCR. The products were subjected to DNA electrophoresis analysis. The DNA sample with the brightest fluorescence was noted and the corresponding strain was cultured in the presence of 30 μg ml⁻¹ kanamycin for 12 h. Plasmid DNA was extracted from these cultures using the Mini Plus Plasmid DNA Extraction System and sequenced (Mission Biotech Inc., Taiwan). The plasmid was designated as pGD-75.

Protein expression

E. coli BL21 (DE3) was transformed with pGD-75 DNA and a single colony was grown in 40 ml of LB (plus antibiotic), incubated until mid-log phase (absorbance at 600 nm was 1.5), and NAD-GDH expression was induced with 0.1 mmol l⁻¹ isopropyl-beta-D-thiogalactopyranoside (IPTG) that was added into the culture and incubated for an additional 6 h at 30°C. In addition, *E. coli* BL21 (DE3) containing an expression plasmid encoding glucose dehydrogenase was subjected to fermenter culture.
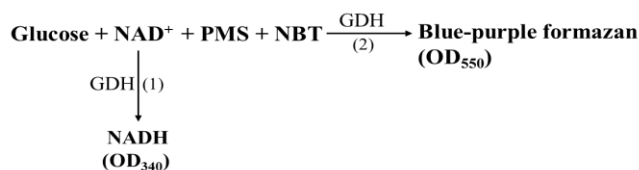
Protein purification

Histidine (His)-tagged protein was purified by immobilized metal ion affinity chromatography using a Ni^{2+} resin. The column was equilibrated with 50 mmol L⁻¹ Na_2HPO_4, 0.3 mol L⁻¹ NaCl and 30 mmol L⁻¹ imidazole (pH 8.0; start buffer). The homogenized and clarified sample was then applied to it. Several fractions were collected as a flow-through. The column was washed with start buffer and the enzyme was eluted with 50 mmol L⁻¹ Na_2HPO_4, 0.3 mol L⁻¹ NaCl and 250 mmol L⁻¹ imidazole (pH 8.0; elution buffer). Fractions were monitored using fluorescence spectrophotometry at an absorbance of 280 nm. Most bound proteins were eluted with 250 mmol l⁻¹ imidazole as determined in preliminary experiments. The eluate was collected and dialysed against deionized water and then with 1 mol L⁻¹ EDTA to remove nickel ions. Finally, the residual resin was washed away with 10 ml deionized water. The enzyme preparation was further dialysed to remove any residual imidazole, which could inhibit enzyme activity.

SDS-PAGE and western blot analysis

SDS-PAGE was performed by mixing the starting material or purification samples with 5× sample buffer (0.5 mol l⁻¹ Tris-HCl, pH 6.8, 10% glycerol, 5% SDS, 5% β-mercaptoethanol, 0.25% bromophenol blue), incubating at 95°C for 5 min, centrifuging at 16300 *g* for 10 min and loading onto a 12% acrylamide gel. Gels were stained with Coomassie Brilliant Blue R-250. Proteins were transferred from the gel to a PVDF membrane (Bio-Rad) for 60 min at 60 V using a Bio-Rad Mini-Trans Blot Cell and transfer buffer comprising 48 mmol L⁻¹ Tris, 39 mmol L⁻¹ glycine, 20% methanol, and pH 9.2. The PVDF membrane was probed with a 1:3000 dilution of rabbit polyclonal anti-His antibody and then incubated with a 1:5000 dilution of mouse anti-rabbit IgG conjugated to alkaline phosphatase. Immune complexes were detected with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT; Sigma) (Becht et al., 1988).

Enzyme assays

NAD-GDH activity can be analysed based on two mechanisms: 1) glucose matrix interaction with NAD⁺ catalyses the production of NADH by NAD-GDH. NADH absorbance can be measured at 340 nm; 2) glucose interaction with NAD⁺, PMS and NBT catalyses the production of NADH by NAD-GDH. The resulting formazan absorbance can be measured at 550 nm. We used the first method because we found that it produced a more rapid reaction. One unit of glucose dehydrogenase activity was the amount of the enzyme that produced 1 μmol of NADH per minute under the condition of the assay.

$$\text{Glucose} + \text{NAD}^+ + \text{PMS} + \text{NBT} \xrightarrow[(2)]{\text{GDH}} \text{Blue-purple formazan (OD}_{550})$$

$$\Bigg\downarrow \text{GDH}|(1)$$

$$\text{NADH (OD}_{340})$$

Enzyme analysis

Effects of pH on catalysis

The enzyme was added to 1 ml microcentrifuge tubes each containing 940 µl of buffer, 10 µl of 30 mmol l^{-1} glucose and 10 µl of 30 mmol l^{-1} NAD$^+$ with different pH values.

Effects of pH on stability

The enzyme was incubated in buffer solutions with different pH values at 4°C for 16 to 24 h and then added to a 1 ml microcentrifuge tube containing 940 µl of buffer, 10 µl of 30 mmol L^{-1} glucose and 10 µl of 30 mmol L^{-1} NAD$^+$ at pH 8.0. The enzyme activity was monitored by measuring absorbance at 340 nm.

Effects of temperature on catalysis

The enzyme was added to a 1 ml microcentrifuge tube containing 940 µl of buffer, 10 µl of 30 mmol l^{-1} glucose and 10 µl of 30 mmol L^{-1} NAD$^+$ at pH 7.0; absorbance was measured at 340 nm.

Thermostability analysis

The enzyme was incubated at pH 8.0 buffer solution at different temperatures for 15 min and then added to a 1 ml microcentrifuge tube containing 940 µl of buffer, 10 µl of 30 mmol L^{-1} glucose and 10 µl of 30 mmol L^{-1} NAD$^+$ at pH 8.0. The enzyme activity was determined by measuring absorbance at 340 nm.

Substrate specificity

Varying amounts of glucose was added to 1 ml microcentrifuge tube containing 940 µl of buffer, 10 µl of 30 mmol l^{-1} glucose and 10 µl of 30 mmol l^{-1} NAD$^+$ at pH 8.0 and incubated with the enzyme. NADH absorbance was measured at 340 nm. We also assayed enzyme activity in 30 mmol L^{-1} solutions of D-(+)-glucose, D-(+)-maltose, D-sorbitol, sucrose, D-(+)-lactose, D-(+)-galactose, D-(+)-xylose and mannitol.

RESULTS

The hot-spring water samples from Taitung, Chihpen County, Taiwan, were cultured in DSMZ medium 88 at 75°C to isolate hyperthermophilic strains. Four out of six hot-spring water samples yielded growth. We cultured the organisms on solid medium containing glucose and NAD to detect NAD-GDH activity. Two positive cultures were obtained. To confirm the protein sequence, the N-terminal protein sequencing analysis was performed. Their chromosomes were extracted and subjected to sequencing, which revealed that we had isolated *Sulfolobus*-like bacteria. This information made it possible to design PCR primers for isolating the gene encoding NAD-GDH. Figure 1A describes the construction of the NAD-GDH expression vector and the PCR colony screen for identifying transformed cells (15 out of 24). Figure 1B shows the results of colony screening for NAD-GDH expression.

Protein purification

Figure 2 shows the analysis of the purification procedure. SDS-PAGE revealed a single major band that migrated at the expected M$_r$ (Bright et al., 1993; Bonete et al., 1996). Figure 2 also shows the Western blot. The His-tag antibody detected a single band that comigrated with the major band observed in Figure 2.

Effects of pH on enzyme activity

We determined the activity of the purified enzyme at different pH values at room temperature (25°C). As shown in Figure 3A, enzyme activity was the highest at pH 7.97 (8.0) and >60% of activity was maintained at pH 7 to 9. The activity is lower than 50% at other pH values. Therefore, a pH of 7.97 was used in follow-up studies.

Effects of pH on enzyme stability

The activity of the purified enzyme was determined after 16 h at 4°C in the presence of buffers with varying pH values. NAD-GDH maintained >80% of activity for 20 h at 4°C and pH 3.97 to 11.

Effects of temperature on enzyme activity

Figure 4A shows that enzyme activity increased up to the highest temperature tested (85°C). In contrast, the activity of NAD-GDH isolated from *Haloferax mediterranei* started dropping at 70°C (Bonete et al., 1996).

Enzyme thermostability

To manufacture glucose test strips, the enzyme must be fixed on the strips and subjected to a drying process at a certain temperature for about 15 min. Therefore, in the present study, the purified enzyme was incubated at different temperatures at pH 7.97 for 15 min and then assayed for activity. NAD-GDH activity was slightly elevated at temperatures exceeding 45°C, reaching its highest rate between 55 and 70°C (Figure 4B). However, when the temperature exceeded 75°C, activity declined rapidly to approximately 60% and to 10% at 80°C. In contrast, high temperature can easily damage most of the enzymes or change their structures, while low temperature reduces enzyme activity. It is natural to speculate that because their strains originate from hot springs, the GDH enzyme is more stable than most enzymes at high temperature or has an excellent thermal stability. This characteristic is beneficial for the follow-up drying process of glucose test strips.

Enzyme kinetics

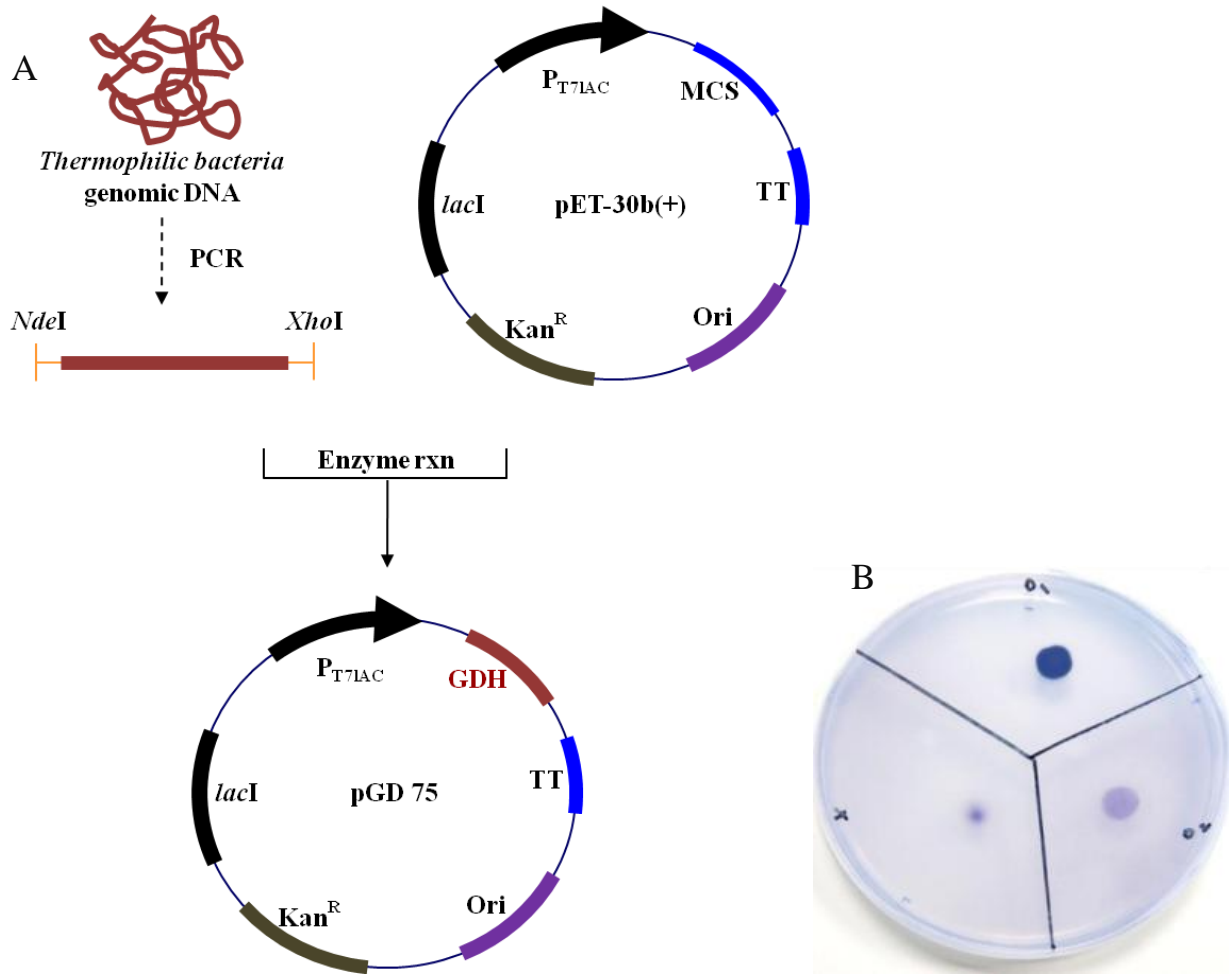Figure 5A shows that activity plateaued at 0.125 mol l^{-1}

Figure 1. Schematic presentation of pGD-75 plasmid. (A) and colony screening for NAD-GDH expression (B).
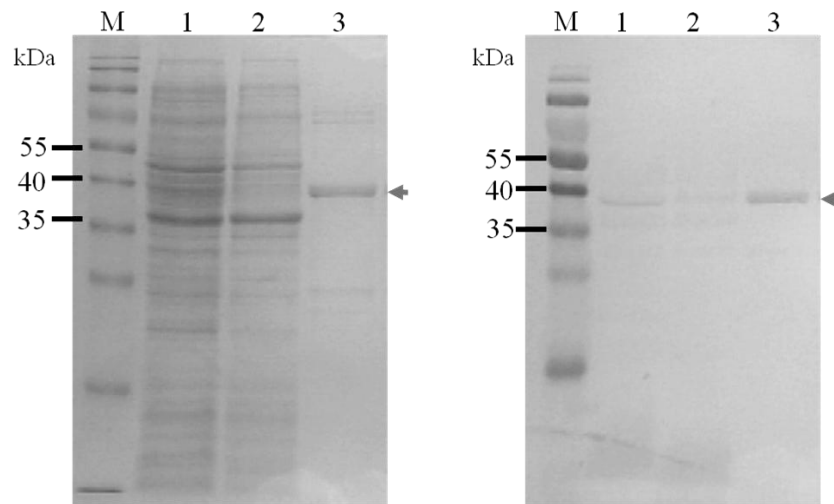

Figure 2. SDS-PAGE and Western blot analysis of the recombinant GDH before and after purification with IMAC. M, Markers; lanes 1, soluble protein; 2, insoluble proteins; 3, elution fraction. The arrow indicates the target protein glucose dehydrogenase, GDH.
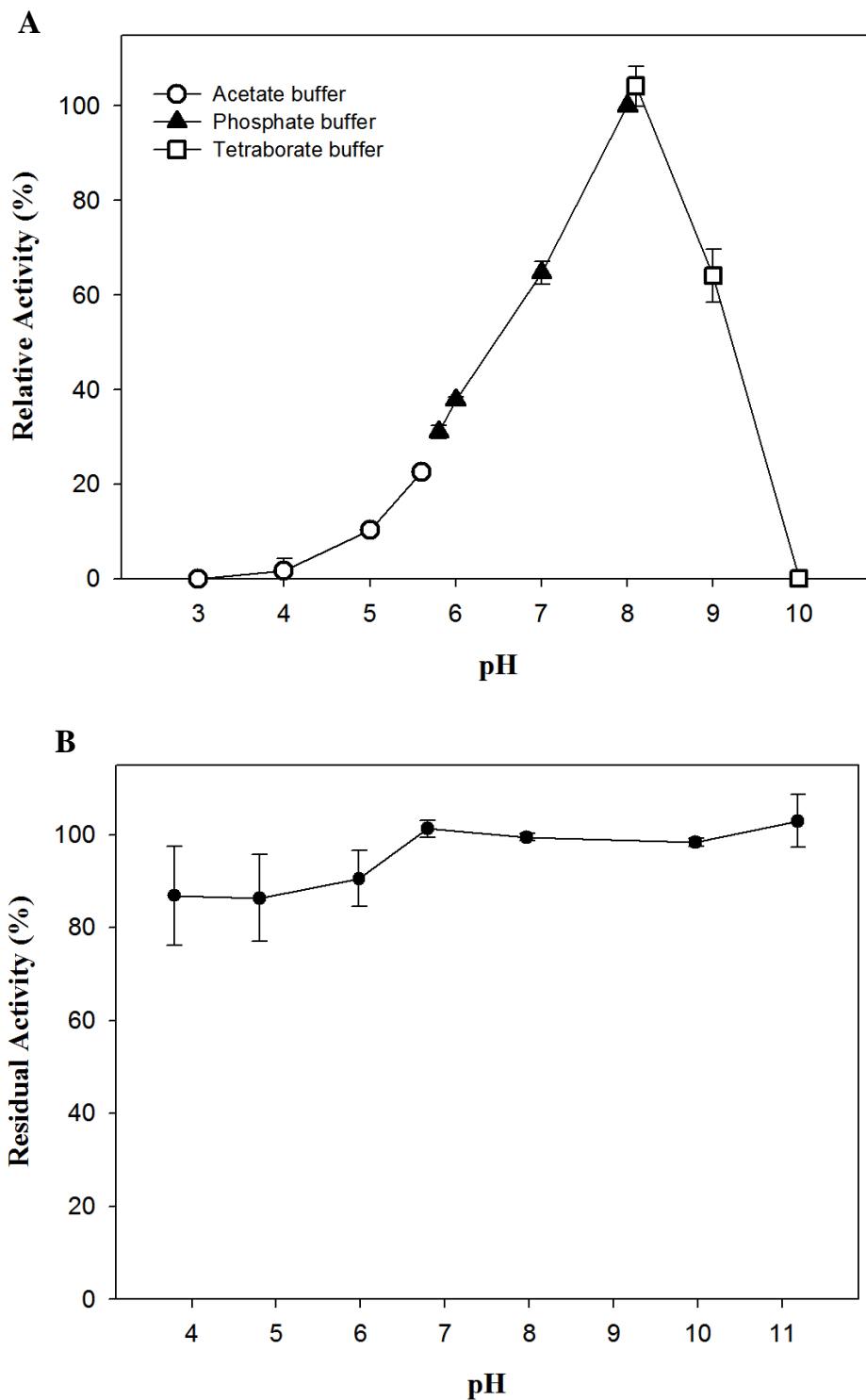
Figure 3. The GDH activity at different pH (A) and pH stability of the enzyme (B). The residual activity of the enzyme was assayed at different pH at 4°C for 16 h.

glucose, thus, only the value less than this was used in the subsequent kinetic calculations. Figure 5B shows that the maximum V_{max} was 0.305 U/ml and the K_m was 21.9 mmol L^{-1}. The K_m value was lower (higher affinity for glucose) when compared with that of glucose oxidase, pyrroloquinoline quinine (PQQ) and glucose dehydrogenase (33, 25 and 30 mmol L^{-1}) (Tranulis et al., 1994; Bankar et al., 2009).
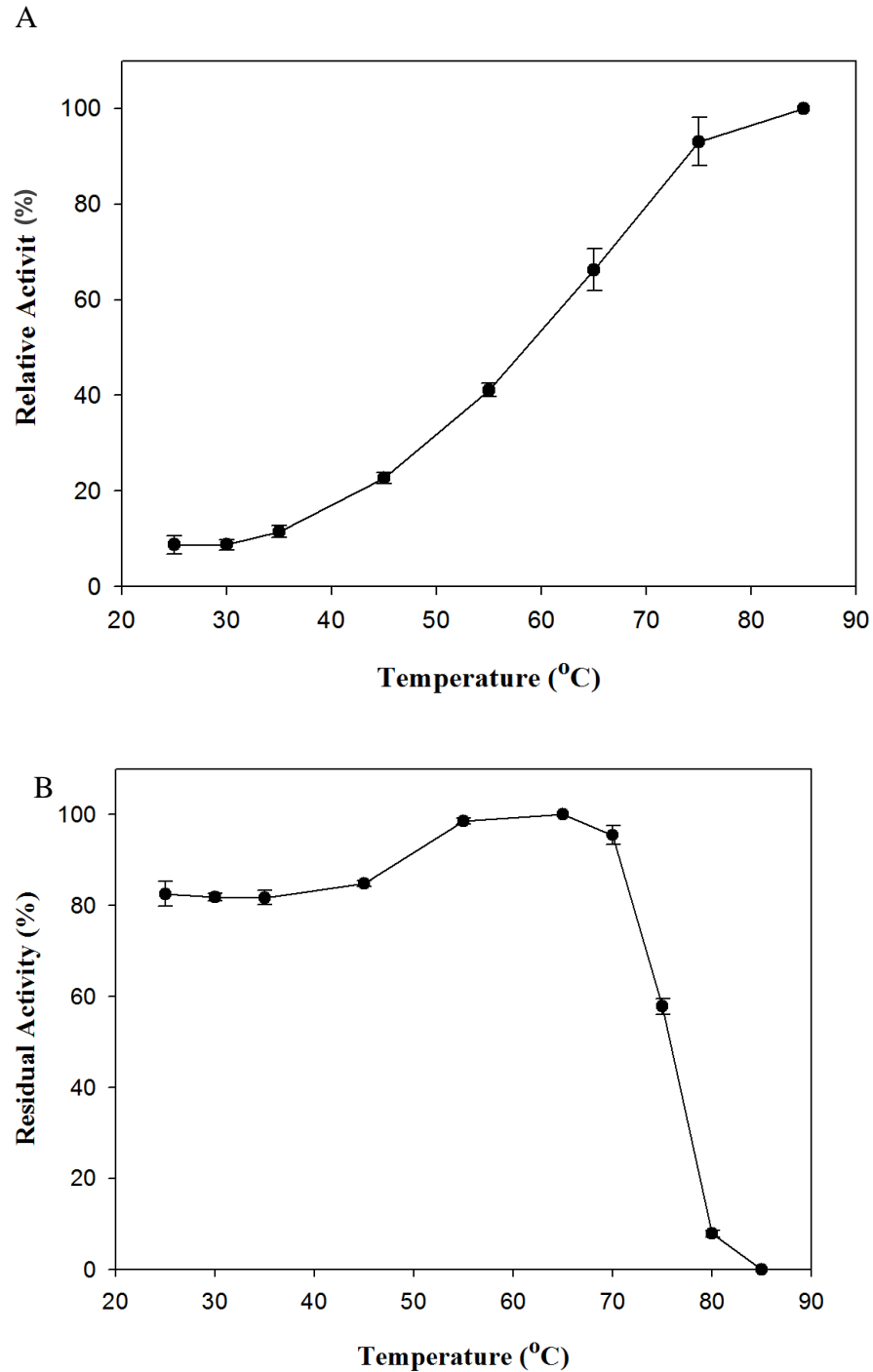
A

B

Figure 4. (A), The optimum enzyme reaction temperature. The residual activity of the enzyme was assayed at pH 7.97 under various temperatures. (B), The thermal stability of the enzyme; the residual activity of the enzyme after treatment at different temperatures for 15 min was determined.

Substrate specificity

When test strips with PQQ-GDH are used, maltose, galactose or xylose in blood are misinterpreted as glucose, potentially resulting in erroneously elevated serum glucose levels (Flore and Delanghe, 2009; Ng et al., 2010). Therefore, we investigated the effects of different carbohydrates on enzyme specificity. Figure 6
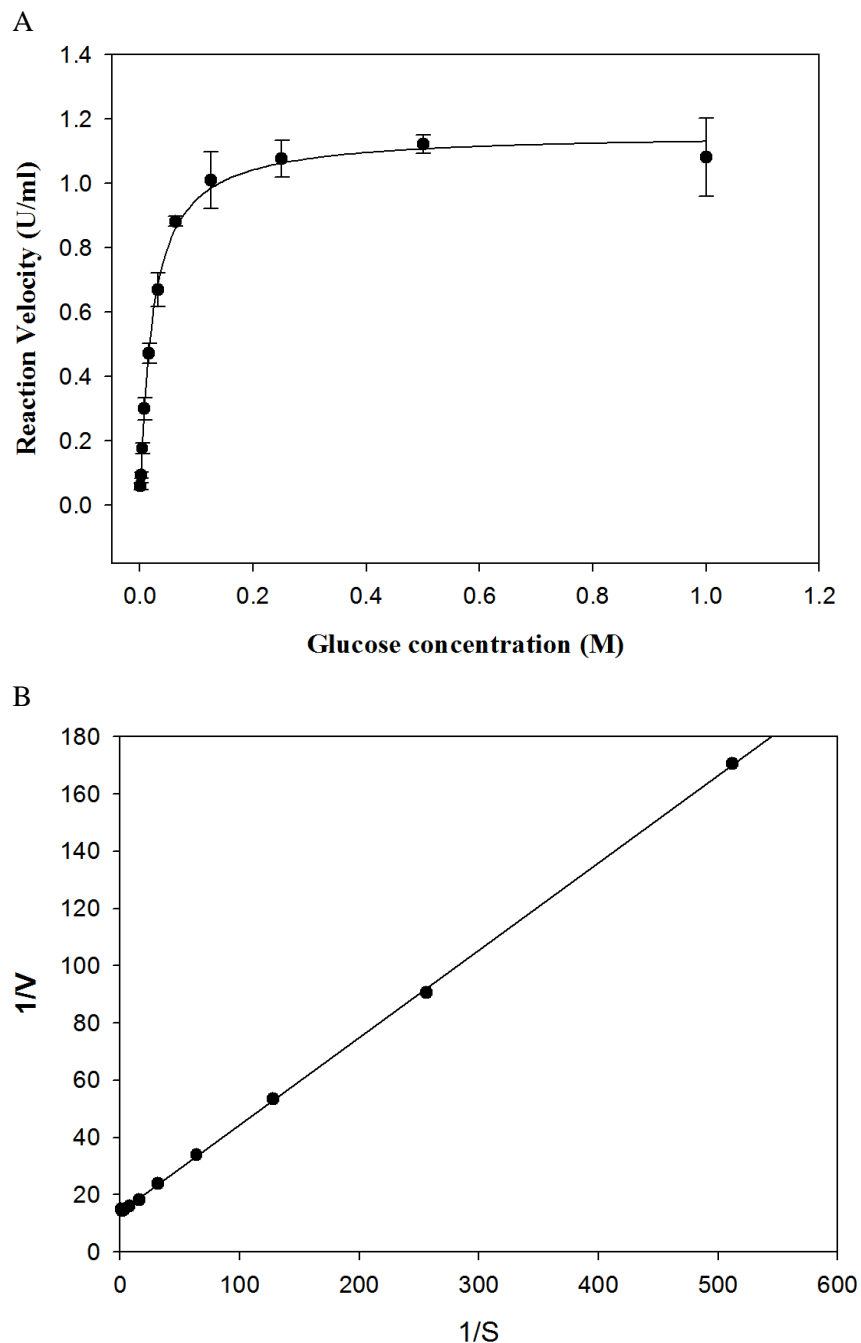
A

B

Figure 5. Enzyme kinetic analysis. A, the analysis of enzyme reaction velocity corresponding to glucose concentration. B, Reciprocal transformation of the corresponding values in A.

shows the relative activity with each substrate, where the activity with glucose was assumed to be 100. When NAD+ was used as a coenzyme, the specific activities with mannitol and maltose were about 3%, and the specific activities with galactose and xylose were about 1.5%, based on the specific activity with glucose. The results clearly demonstrate that NAD-GDH activity was extremely low when maltose, xylose and galactose were used as substrates, indicating that substrate specificity is appropriate for glucose tests.

DISCUSSION

To our knowledge, this is the first report concerning the cloning, purification and characterization of recombinant
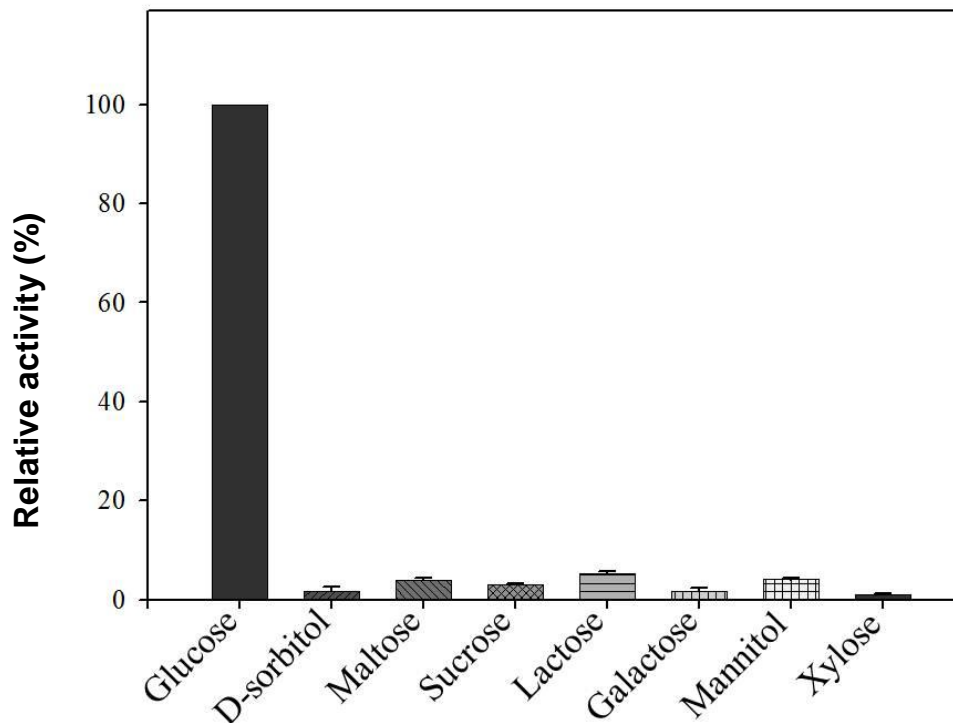
Figure 6. Substrate specificity of the GDH.

GDH from a hyperthermophile *Sulfolobus*-like bacterium. Both SDS-PAGE and Western blot analyses indicate that its molecular weight is approximately 39000 kDa, consistent with that of the naturally occurring enzyme. Biochemical characterization established its hyperthermostability, a feature consistent with its cellular physiology. Accordingly, this work provides a glucose dehydrogenase that is an extremely stable enzyme having a thermostability of 70°C. The recombinant GDH reported here also exhibited high specificity for glucose and was much more efficient when compared with other related glucose-degrading enzymes. It was found that the enzyme has an excellent substrate specificity when compared with known GDH derived from hyperthermophilic archaea (Bright et al., 1993; Bonete et al., 1996; Siebers et al., 1997). Therefore, we concluded that it could be a more useful reagent for biotechnological purposes such as in the manufacture of glucose test strips. At present, the physiological benefits *Sulfolobus*-like bacterium could accrue by retaining the glucose dehydrogenase NAD-GDH showing such disparate catalytic efficiency is not clear. As compared to first generation blood glucose tests employing glucose oxidase (GOD) which is susceptible to oxygen concentration, recombinant *Sulfolobus* NAD-GDH exhibited faster reaction kinetics and was not influenced by dissolved oxygen. Thus, NAD-GDH may be able to replace GOD and become the mainstream enzyme used in the future to manufacture non-fingertip blood glucose meters. Collectively, these results can be further applied in the manufacture of glucose test strip for the second generation non-fingertip blood glucose meters. in the manufacture of glucose test strip for the second generation non-fingertip blood glucose meters.

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