Characterization of an acid-resistant glucose 1-dehydrogenase from Bacillus cereus var. mycoides

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Abstract

A new glucose 1-dehydrogenase (BcGDH) from Bacillus cereus var. mycoides was isolated and characterized. This dehydrogenase was expressed in Escherichia coli and purified to homogeneity for biochemical and enzymatic analysis. The effect of pH and temperature assay showed that the optimal pH and temperature of BcGDH are 9.0 and 37 °C, respectively. Thermal stability assay showed that the stability of BcGDH is solvent dependent. At the presence of 2 M NaCl, BcGDH maintains its maximum activity even incubated at 65 °C for 1 h. pH stability assay showed that BcGDH is stable at a wide range of pH (4.4 to 8.5) indicates that BcGDH is an acid-resistant dehydrogenase. Substrate specificity results indicate that BcGDH has a highly substrate specificity toward D-glucose. Finally, the potential contribution of surface electrostatic effect and hydrophobic effect to the acid-resistant property of BcGDH was discussed.

Key words: glucose 1-dehydrogenase, Bacillus cereus, thermal stability, pH stability, substrate specificity, acid-resistance

Introduction

NAD(P)⁺-dependent dehydrogenase is an oxidoreductase that oxidizes a substrate by a reduction reaction in coupled with reducing of NAD(P)⁺ to NAD(P)H. Various of dehydrogenases with different substrate specificities, such as glucose dehydrogenase (GDH), formate dehydrogenase (FDH), aldehyde dehydrogenase (ADH), 1,5-anhydro-d-glucitol dehydrogenase (AGDH) and phosphite dehydrogenase (PTDH), have been isolated and characterized [1]. Some of these dehydrogenases have been used for medicinal and industrial applications, including blood glucose measurement, biotransformation and coenzyme regeneration system [2-4].

Two kinds of enzymes, glucose oxidase (GOD) and GDH, have been developed by some manufacturers for blood glucose measuring in diabetes care. Comparing to GOD, GDH has advantages of higher sensitivity and independent of oxygen for reaction on one hand, but has disadvantage of lower substrate specificity on the other hand [5-7]. A mass of efforts, including search of new GDHs from nature, directed evolution and structure-based rational design, have been ploughed into optimizing substrate specificity as well as other properties of GDHs [8-11]. However, new efforts still need to reach the ultimate goal.

Based on sequence similarity with other known GDH genes, a new GDH coding gene was identified in Bacillus cereus var. mycoides. The gene was cloned and expressed as recombinant protein in Escherichia coli. The characteristics, including dehydrogenation
activity, pH and thermal stability, steady-state kinetics and substrate specificity of the recombinant protein (BcGDH) were studied and compared with other GDH reported previously.

Materials and methods

Cloning of the glucose-1-dehydrogenase

Using *B. cereus* var. *mycoides* genomic DNA as template, GDH was amplified with the primer set of GDH_F (5'-ATGTATAGCGATTTAGAAGGG-3') and GDH_R (5'-TTACCCACGCCCCGCTTGAA-3'). PCR was performed with the following conditions: pre-heating at 94 °C for 4 min; 35 cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 55 °C and 1 min elongation at 72 °C. After the thermocycling, a step of 10 min extension was added before the reaction mixture was cooled to 4 °C. The product was separated by electrophoresis and visualized with UV light on a BioSpectrum® Imaging System (UVP LLC). The amplified fragment was recovered with the AxyPrep™ DNA Gel Extraction Kit (Axygen Biosciences) and inserted into pGEM-T Easy Vector (Promega). The positive plasmid was further confirmed by DNA sequencing and named as pGEM: BcGDH.

Multiple sequence alignment and phylogenetic analysis

Multiple-sequences alignment was performed by Clustal W 1.83 software [12] with default parameters. The Neighbor-joining (NJ) phylogenetic tree was constructed with MEGA 5.0 software with the suitable nucleotide substitution model, and mutation rate and pattern calculated by Model Selection provided by MEGA 5.0 [13]. The Bootstrap confidence limits were obtained by 1000 replicates.

Protein modeling and electrostatic surface potentials analysis

Structural models of GDHs were constructed by the SWISS-MODEL server [14] using BmGDH-IWG3 (PDB ID: 1GCO) as a template. The analysis and representation of the electrostatic surface potentials of GDHs were performed by using PyMOL 0.99 software [15].

Construction of recombinant plasmid

Using pGEM: BcGDH as template, the *gdh* gene was amplified with the primer set of BcGDH_F (5'-GGAATTCATATG TATAGCGATTTAGAAGGG-3', underline sequence is *Nde*I restriction site) and BcGDH_R (5'-GGAATTCCATATGTTACCCACGCCCCGCTTGAA-3', underline sequence is *EcoR*I restriction site). After purified by AxyPrep™ PCR Cleanup Kit (Axygen Biosciences), the PCR product was digested with *Nde*I and *EcoR*I and inserted into pET-28a (+) vector digested with the same restriction enzymes. The pET-28a (+) construct containing *gdh* gene (pET: BcGDH) was confirmed by sequencing to avoid any mutations which might be introduced during the PCR.

Protein expression and purification

Protein was expressed in *E. coli* BL21 (DE3) harboring pET: BcGDH at 16 °C over night at the presence of 0.5 mM IPTG. N-terminal 6 × histidines tagged BcGDH was purified in a native, non-denatured form by Ni²⁺-NTA agarose (Novagen) and gel filtration as described earlier [16]. The purified protein was concentrated into desired concentration with Amicon Ultra-15 Centrifugal Filter Units (Millipore). The final protein was eluted in TN buffer (10 mM Tris-HCl, 10 mM NaCl, pH 7.5) and stored at -80 °C after liquid nitrogen immersion. Protein concentration was determined using the Bio-Rad protein assay kit I (Bio-rad) as instructed.

Enzyme assay

Unless otherwise noted, the glucose 1-dehydrogenase activity was assayed by measuring the absorbance of NADH at 340 nm in 100 mM sodium phosphate (pH 8.0) at 25 °C with 200 mM D-glucose and 1 mM NAD⁺. The concentration of NADH was calculated...
based on the extinction coefficient of 6220 M\(^{-1}\) cm\(^{-1}\) [17]. One unit of enzyme activity was defined as the amount of GDH that catalyzing the formation of 1 μM NADH in one minute. All these reactions were conducted in triplicate to avoid artificial errors.

For steady-state kinetic study, 1 μL purified BcGDH was incubated with various concentrations of D-glucose (25 to 500 mM) in 100 mM phosphate buffer (pH 8.0) supplied with 1 mM NAD\(^+\) at 25 °C for 10 min. The kinetic constants of the enzyme were determined using a nonlinear fitting of the Michaelis-Menten equation: \(v = \frac{V_{\text{max}}[S]}{K_m+[S]}\), where \([S]\) is the concentration of D-glucose, \(K_m\) is the Michaelis constants for D-glucose. The turnover number \(k_{\text{cat}}\) was calculated by the equation: \(V_{\text{max}} = k_{\text{cat}}[E]\), where \(E\) is the enzyme [18].

**Effect of pH on activity and stability of BcGDH**

In order to estimate the effect of pH on BcGDH activity, the same amount of purified BcGDH was incubated with 200 mM D-glucose and 1 mM NAD\(^+\) in 100 mM sodium citrate (pH varied from 4.0 to 6.0 with 0.4 intervals), sodium phosphate (pH varied from 6.0 to 8.0 with 0.5 intervals), Tris-HCl (pH varied from pH 7.5 to 9.0 with 0.5 intervals) and glycine-NaOH (pH varied from pH 8.5 to 10.5 with 0.4 intervals) at 25 °C for 5 min. After incubation, the absorbance of NADH at 340 nm was recorded, and relative activities were calculated.

The pH stability of BcGDH was determined by comparing the residual dehydrogenation activity after incubating of the enzyme in different pH buffers at room temperature for 1 h with the initial dehydrogenation activity in the same pH buffer.

**Effect of temperature on activity and stability of BcGDH**

To illustrate the effect of temperature on activity of GDH, the purified GDH protein was incubated with 200 mM D-glucose and 1 mM NAD\(^+\) in 100 mM phosphate buffer (pH 8.0) at various temperatures from 25 to 60 °C with 5 °C intervals. After incubation, the concentration of NADH was measured for the dehydrogenation activity calculation.

To investigate the thermal stability of GDH, purified BcGDH proteins were treated for 1 h at different temperature from 25 to 60 °C with 5 °C intervals. The proteins were then mixed with 200 mM D-glucose and 1 mM NAD\(^+\) containing 100 mM phosphate buffer (pH 8.0) and incubated at room temperature for 10 min. After the incubation, the residual GDH activities of every temperature treatment were calculated based on the absorbance of NADH at 340 nm.

**Activity of BcGDH on different substrates**

Substrate specificity of BcGDH was tested with ten different substrates, including D-glucose, D-sucrose, D-maltose, D-galactose, D-fructose, D-xylose, D-arabinose, D-mannose, D-lactose and D-mannitol. The reactions were performed in 100 mM phosphate buffer (pH 8.0) containing 200 mM substrate and 1 mM NAD\(^+\) at 25 °C. Ten minutes after incubation, catalysis activity was calculated based on the absorbance of NADH at 340 nm.

**Results and discussion**

**Cloning and sequence analysis of the BcGDH gene from B. cereus**

Using the primers GDH_F and GDH_R, a fragment of about 800 bp containing the full coding region of BcGDH was amplified from *B. cereus* var. *mycoides*. The full coding region of BcGDH is 786 bp in length which encodes a putative 28.1 kDa peptide consisting of 261 amino acids. The nucleotide sequence of this gene was deposited in GenBank under the accession number of JQ266750. A phylogenetic tree was constructed based on the deduced amino acid sequence of BcGDH and other characterized GDHs [8-11, 19, 20]. In the phylogenetic tree (Figure 1), six out of the eleven GDHs clustered into two major groups (groups A and B). Whereas other GDHs were formed single isolate branches, except BcGDH and BtGDH were in the same branch, suggesting BcGDH may have similar enzymatic properties with that of BtGDH.
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**Figure 1.** Phylogenetic relationships among BcGDH and other characterized GDHs. The tree topology was generated by using the Neighbor-Joining method in MEGA 5 [13]. The number adjacent to a node indicates the percentage of 1000 bootstrap trees that contain the node. BmGDH, BmGDHIII, BmGDHIV and BmGDHI are from *B. megaterium* IAM1030 [8, 11], GDHA and GDHB are from *B. megaterium* M1286 [19], BmGDH-IWG3, BtGDH, BsGDH and LsGDH are from *B. megaterium* IWG3 [8], *B. thuringiensis* M15 [10], *B. subtilis* 168 [20] and *L. sphaericus* G10 [9], respectively.

**Expression and purification of BcGDH**

*BcGDH* was expressed in *E. coli* as a N-terminal histidines tagged recombinant protein. After Ni$^{2+}$-NTA affinity chromatography and gel filtration, a clear single band with molecular weight (MW) of about 28 kDa was detected in the SDS-PAGE assay (Figure 2), which is equal to the putative MW of BcGDH. The recombinant protein was expressed to about 35.7% of total intracellular proteins and about 82.5 mg purified protein was obtained from 1 L of *E. coli* culture. The MW of the recombinant BcGDH was calculated to be about 120 kDa by the gel filtration assay, indicating that BcGDH consisted of four identical subunits as well as other GDHs [21]. In the enzyme activity assay, 1 μL purified protein (0.26 μg) has a specific activity of 16.5 U (63.5 U/mg) when D-glucose and NAD$^+$ used as substrate and coenzyme, respectively. The kinetic constants of BcGDH were determined by using a nonlinear fitting plot. The $k_{cat}$ (s$^{-1}$) and $K_m$ of BcGDH are 56.2 and 49.2 mM, respectively. These results indicate that BcGDH has been expressed successfully.

**Figure 2.** SDS-PAGE analysis of total-cell lysate and purified protein. M, protein weight marker (Fermentas); 1, uninduced cell lysate sample; 2, IPTG induced cell lysate sample; 3, purified BcGDH.
Effects of pH on the activity and stability of BcGDH

In order to determine the effects of pH on the activity of BcGDH, purified BcGDH was incubated with D-glucose and NAD⁺ in a series of pH buffers, ranging from 4.0 to 10.5. As shown in Figure 3A, BcGDH exhibited dehydrogenation activity over a wide pH range from 4.0 to 10.5. The highest activity was observed at pH 9.0 in both Tris-HCl and Glycine-NaOH buffer systems. Furthermore, at the pH between 7.5 and 9.5, BcGDH exhibits more than 50 U/mg dehydrogenation activity (Figure 3A).

In the pH stability assay, BcGDH did not display obvious decrease of the activity from pH 4.4 to 8.5 on the whole (Figure 3B). However, BcGDH displayed varied stabilities in different buffer systems. Comparing to the BcGDH relative activity in Tris-HCl buffer, its relative activity decreased from 78% to 23% in sodium phosphate buffer from pH 7.5 to 8.5. Similarly, BcGDH showed higher relative activity in Tris-HCl than in Glycine-NaOH at the same pH.

Thermal stability of BcGDH

The activity of BcGDH increased as the temperature raised from 25 to 40 °C and reached its maximum activity at 40 °C. However, the activity of BcGDH decreased when the temperature continued increasing to 55 °C (Figure 3C). To understand the impact of temperature on the thermal stability, BcGDH was incubated at different temperatures for 1 h before NAD⁺ and D-glucose were added. Without the protective agents, the activity of BcGDH dwindled quickly as the temperature increased. However, the thermal stability was improved dramatically after the addition of 20% glycerol or 2 M NaCl. Especially, BcGDH

Figure 3. The effects of pH and temperature on the activity and stability of BcGDH. Effect of pH on the activity (A) and stability (B) of BcGDH. The activity was measured in sodium citrate, sodium phosphate, Tris–HCl, and glycine–NaOH buffer are indicated by hollowed square, diamond, triangle, and circle, respectively. B, Effect of pH on the stability of BcGDH. Effect of temperature on the activity (C) and stability (D) of BcGDH. For thermal stability assay, the enzyme was incubated at various temperatures for 30 min in different buffers, and the remaining activity was measured at 25 °C. The values are expressed as averages of triplicate and the error bars mean standard deviation.
remained 97% of its maximum activity after incubating at 65 °C for 1 h in the presence of 2 M NaCl (Figure 3D). Furthermore, the purified BcGDH did not show obvious loss of activity after incubating at 25 °C for 1 week in 2 M NaCl (data not shown).

**Substrate specificity**

The substrate specificity of BcGDH was tested with ten different aldose sugars and disaccharides. Compare to the catalytic activity of BcGDH to D-glucose, only maltose offered catalytic activity more than 10 percentage (11.01%) (Figure 4). Furthermore, no catalytic activity was recorded when D-fructose, D-arabinose or D-mannitol was used as a substrate. These results indicate that BcGDH has a rigid substrate specificity.

![Figure 4 Substrate specificity of BcGDH. The activity was measured at 25 °C and pH 8.0. Reaction velocities for the different substrates were related to that of D-glucose. Error bars mean standard deviation.](image)

**Discussion**

pH stability is an important characteristic affects the application of GDH in blood glucose measurement as well as substrate specificity. The optimal pH in the present study is higher than that of GDHs from BtGDH [10], BsGDHs [22], and BmGDH I, BmGDH II and BmGDH III [8]. Comparing to BmGDH I, BmGDH II, BmGDH III, BmGDH IV, BtGDH, and BmGDH-IWG3, which were labile at a pH higher than 8.5 without addition of high concentration of NaCl [8, 11, 22-24], BcGDH could maintain its activity when the pH reached 9.0 in Tris-HCl buffer. Furthermore, BcGDH is stable within a wide range of pH from 4.4 to 8.5 (Figure 3B). Especially, BcGDH maintained its more than 90 percentages of maximum activity after incubated at pH 4.5 for 1h (Figure 3B). These results indicate that BcGDH is an acid-resistant dehydrogenase.
the weaker cation repulsion in the acidic pH solutions. At the P-axis interface (between subunit A and D), where hydrogen bonds are the major interactions [21], BcGDH and BmGDH-IWG3 are charged with negative charges, while LsGDH is almost neutral at this region (Figure 5C). The high alkali-resistance of LsGDH may owing to this difference. At the R-axis related interface (between subunit A and C), the loop structure at the C-terminal of subunit A interact with subunit C through hydrogen bonds [21]. Considering of the positive and negative charge distribution and the binding pattern of subunit A and C at the R-axis interface, BcGDH should has the strongest hydrogen bond interactions at this interface (Figure 5D). Thus, optimize the surface electrostatics of BcGDH through mutagenesis at the P-axis interface may improve the alkali-resistance of BcGDH.

The optimal temperature of BcGDH is about 37 °C, which is lower than other GDHs. This temperature is more ideal for blood glucose measurement since the reaction always conducts at room temperature. Thermal stability of BcGDH is lower than GDHs encoded by others[8, 11, 22-24]. However, this disadvantage can be overcome by the addition of protective agents, including glycerol and NaCl. Especially, BcGDH did not show any loss of activity at 65 °C when incubated in 2M NaCl. Consequently, BcGDH has an acceptable

Figure. 5. Comparison of the molecular electrostatic potential of association interfaces of BmGDH-IWG3, BcGDH and LsGDH. A, The cartoon representation of the tetramer of BmGDH-IWG3. The four subunits of BmGDH-IWG3 (A, B, C, D) are colored with red, yellow, purple and green, respectively. B-D, the surface electrostatics of Q, P and Q-axis interfaces, respectively. Positive charged surfaces are indicated in blue and negative charged surfaces are shown in red. Surface with different electrostatic potentials are indicated by green circles.
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thermal stability for practical applications.

Substrate specificity is an important characteristic affects the application of GDH in blood glucose measurement. Our results show that BcGHD has a rigid substrate specificity (Figure 4). Interestingly, BcGDH, the phylogenetic neighbor of BeGDH (Figure 1), also has a similar substrate specificity with that of BeGDH [10]. This result indicates that the phylogenetic branch which contains BcGDH and BtGDH may represents a subfamily of GDHs which possesses a relative narrow substrate specificity.

Conclusions

In conclusion, a new GDH from *B. cereus* var. *mycoides* had been successfully cloned, heterogenous expressed and well characterized. This GDH has a relative narrow substrate specificity and high acid-resistance, which make BcGDH a promising candidate for blood glucose measurements.

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