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Purification and Characteristics of a NAD(P)-Dependent Gluconate 5-Dehydrogenase from Thermotoga maritima MSB8

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Abstract: NADP-dependent gluconate 5-dehydrogenase catalyzes the reversible oxidoreduction reaction between D-gluconate and 5-keto-D-gluconate. NADP-dependent gluconate 5-dehydrogenase from bacteria and fungi has been reported. The reported enzyme showed activity only for NADP as the cofactor. This study purified and characterized a NAD(P)-dependent gluconate 5-dehydrogenase (TmGNDH) from the archaea *Thermotoga maritima* MSB8. TmGNDH is composed of two identical subunits, and a single subunit has 255 amino acids with molecular mass of 28 kDa. TmGNDH is affiliated with the short-chain dehydrogenase/reductase family. Purified TmGNDH has a specific activity of 102.8 U/mg. The enzyme preferred NADP to NAD as its cofactor. TmGNDH exhibited a Km value of 121.3 ± 3.0 mM and k_{cat} value of 3181 ± 34.1 s^{-1} toward D-gluconate as a substrate. TmGNDH had lower activity with D-sorbitol, D-xylitol, D-glucose and D-xylose as substrates. The optimal pH and temperature of TmGNDH were observed at pH 9.0 and 60℃ with NADP as the cofactor. The thermostability of the enzyme suggests that it can be used as an industrially effective tool for synthesizing 5-keto-D-gluconate.

1. Introduction

5-keto-D-gluconate (5-KGA) can be converted to L-(+)-tartaric acid and L-ascorbic acid (by Gray's method) [1,2]. L-tartaric acid is used as an antioxidant in the food industry, an acidic reducing agent in the textile industry, and a chiral reagent in organic synthesis [1,3]. L-ascorbic acid (vitamin C) is a critical vitamin widely used as a nutrient or medicine in the pharmaceutical, cosmetics, and food industries [4].

5-KGA is produced by two types of gluconate dehydrogenases oxidizing D-gluconate. One type is Pyrroloquinoline quinone (PQQ)-dependent gluconate 5-dehydrogenase (EC 1.1.99.22) [5]. The other type is NADP-dependent gluconate 5-dehydrogenase (GNDH, EC 1.1.1.69) [6]. Both enzymes are involved in ketogluconate metabolism. They differ in terms of physiological function and biochemical properties.

PQQ-dependent gluconate 5-dehydrogenase is bound to cell membrane and may link to the

respiratory chain [7,8]. Therefore, the enzyme catalyzes the oxidation of D-gluconate to 5KGA irreversibly [8]. Only PQQ-dependent gluconate 5-dehydrogenase from *Gluconobacter suboxydans* has been reported [5,8,9]. The structure of the enzyme comprises three different subunits: dehydrogenase, cytochrome c, and a third subunit, whose function is unknown [8].

NADP-dependent gluconate 5-dehydrogenase is only present in the cytosol fraction [7]. The physiological role of the enzyme was found to reduce 5-KGA to D-gluconate [7,8]. The enzyme can catalyze the reversible oxidoreduction reaction between D-gluconate and 5-KGA in vitro [7,10]. Currently, several NADP-dependent gluconate 5-dehydrogenase from bacteria (*Gluconobacter liquefaciens* IFO 1238, *G. suboxydans*, and *Gluconobacter oxydans*) and fungi (*Aspergillus niger*) have been reported. The enzyme from *G. oxydans* and *A. niger* were reported to belong to the short-chain dehydrogenase/reductase family [10,11]. The reported enzyme have different subunit compositions. For example, the enzyme from *G. suboxydans* is composed of four identical subunits [7], while the enzyme from *G. oxydans* is a homodimer [10]. The reported enzyme had activity only for NADP as the cofactor [6,7,10,11]. The enzyme from *G. suboxydans* and *G. oxydans* had high specificity for the D-gluconate substrate [7,10]. D-glucose and D-sorbitol were not oxidized by the two enzyme [7,10]. Regarding stability, the enzyme from *G. liquefaciens* IFO 1238 and *G. suboxydans* lost all of activity and over 75% of activity respectively within a few days [6,7].

So far, the biochemical property of archaeal NADP-dependent gluconate 5-dehydrogenase has not been reported. In this study, we purified and characterized a thermostable NAD(P)-dependent gluconate 5-dehydrogenase (TmGNDH) from the archaea *Thermotoga maritima* MSB8. The crystal structure (PDB ID: 1VL8) of the enzyme has been resolved, but its biochemical properties have not been reported.

2. Materials and Methods

2.1 Chemicals

D-gluconate was purchased from Aladdin (Shanghai, China). NADP was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). *Escherichia coli* BL21 Star (DE3) was purchased from Tsingke Biotechnology Co., Ltd. (Beijing, China).

2.2 Bacterial strain and culture conditions

The gene (GenBank: AAD35526.1) was codon-optimized and synthesized by General Biol (Chuzhou, China). It was then inserted at *NdeI* in the pET-28a vector with an N-terminal 6-His tag. The *E. coli* BL21 Star (DE3) strain containing the gene was grown in culture medium (1.5% tryptone, 2.5% yeast extract, 1% sodium chloride, 0.2% glucose, 2% lactose, 250 mL) with 50 μg mL^{-1} kanamycin for 3 h at 37°C shaking at 200 rpm. Further, TmGNDH was expressed in the medium for 24 h at 16 ℃, and cells were harvested by centrifugation at 7,800 rpm and 4 ℃.

2.3 Purification of TmGNDH

Cells were lysed in 60 mL of wash buffer (25 mM Tris-HCl, 300 mM NaCl, pH 8.0) with sonication at 4°C. Lysates were then centrifuged for 25 min at 7,800 rpm. The supernatant was used as the cell-free extract. The extract was applied to a High Affinity Ni-Charged Resin FF Column (5 mL, GenScript) pre-equilibrated with wash buffer. Then, the column was washed with the same buffer and eluted with another buffer (25 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 8.0) at a flow rate of 2 mL/min.

The purity of the protein was monitored by sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) on 12% gel and stained with Coomassie brilliant blue R250. Protein concentrations were determined using the Bradford method [12].

2.4 Cofactor specificity

The enzyme activity of NAD and NADP was detected in 100 mM Tris-HCl buffer (pH 10.0) with 10 mM D-gluconate. The enzyme was pre-incubated at 60℃ for 20 min.

2.5 Kinetic parameters

Reactions were monitored at 45 ℃ using substrate (D-gluconate) concentration in the range of 50–100 mM in 100 mM Tris-HCl buffer (pH 10.0) and 1 mM NADP. The enzyme was pre-incubated at 60℃ for 20 min. The Michaelis constant (Km) was obtained by fitting the data to the Lineweaver–Burk double-reciprocal plot ($\frac{1}{v_0} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$), using the OriginPro 9.1 program.

2.6 Substrate specificity

Reactions were monitored at 45 ℃ using substrates (D-sorbitol, D-xylitol, D-glucose, and D-xylose) in the 100–200 mM concentration ranges with the same buffer and cofactor as mentioned in the above section. The enzyme was pre-incubated with the same condition mentioned in the above section. Reactions using substrate (D-arabinonate) were monitored in the concentration range of 0.5–5.0 mM. The substrate specificity of the enzyme was shown by Km values and turnover numbers (k_{cat}) (see above section for detail description of the Km value obtained).

2.7 Optimum pH

TmGNDH was pre-incubated at 60℃ for 20 min. The enzyme was then incubated with 1 mM NADP and 10 mM D-gluconate at pH 4.0–10.0. The used buffers were: 100 mM sodium acetate buffer (pH 4.0–5.0), 100 mM sodium phosphate buffer (pH 6.0–8.0) and 100 mM Tris-HCl buffer (pH 9.0–10.0).

2.8 Optimum temperature and stability

The enzyme was pre-incubated at a temperature of 30 ℃–90 ℃ for 20 min. The residual enzyme activity was then determined in 100 mM Tris-HCl buffer (pH 10.0) with 1 mM NADP and 10 mM D-gluconate. Stability experiments: TmGNDH was pre-incubated in the same buffer at 60℃ for 12 h. The residual TmGNDH activity was measured with the same conditions every 2 h.

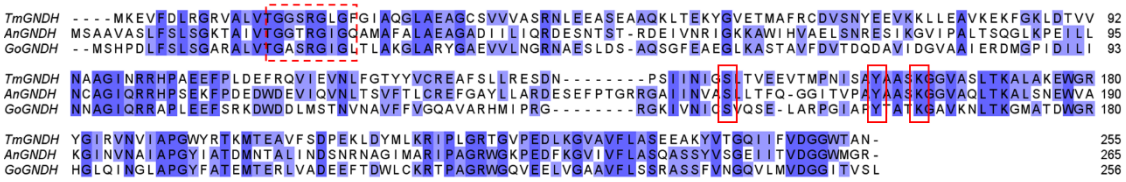
2.9 Enzyme assays

The enzyme reaction described above was conducted in 200 µl reaction volumes, using 96-well plates. The reaction was initiated by adding a coenzyme, and product formation was monitored at 340 nm UV. The amount of the product was determined using a molar extinction coefficient value of 6.22 $\mu M^{-1}cm^{-1}$. One unit (U) is defined as the amount of enzyme that reduces one micromole of cofactor per minute in the presence of a substrate. The reported results are the mean and standard deviation of at least three independent measurements.

3. Results and Discussion

3.1 Sequence analysis of TmGNDH

The TmGNDH coded by the gene (described in the Materials and Methods section) had 255 amino acids. The amino acid sequence of TmGNDH shows identity and similarity with GNDH from *A. niger* (40.45% identity, 60% similarity) and GNDH from *G. oxydans* (37.21% identity, 56.98% similarity). Aligning the amino acid sequences of the two GNDH and TmGNDH, we identified the catalytically conserved "Ser-Tyr-Lys triad," and a consensus sequence of NADP binding motif (GxxxGxG) stressed in Figure 1. These characteristics in the sequence alignment confirmed that TmGNDH is a member of the short-chain dehydrogenases/reductases family [13].



The amino acid sequences of TmGNDH, GNDH (GoGNDH) from G. oxydans, and GNDH (AnGNDH) from A. niger were aligned using the Clustal Omega program using default parameters. A solid red line shows the "Ser-Tyr-Lys triad" in three boxes. GxxxGxG is stressed in a box by a red dotted line.

Figure 1. Multiple sequence alignment of GNDH colored on Percentage Identity.

3.2 Expression and purification of TmGNDH

TmGNDH was expressed in *E. coli* BL21 Star (DE3) cells and purified as described in the Materials and Methods section. The protein was produced in soluble form. The activity of the purified enzyme increased 5.0-fold to a specific activity of 102.8 U/mg compared to the cell-free extract (Table 1). The molecular mass calculated from the amino acid sequence (about 28 kDa) agreed with the value obtained with SDS-PAGE (Figure 2). TmGNDH is composed of two identical subunits according to its crystal structure from the PDB database (RCSB, https://www.rcsb.org/).

Table 1: Summary of purification of TmGNDH. Reactions were monitored at 45°C in 100 mM Tris-HCl buffer (pH 10.0) with 1 mM NADP and 10 mM D-gluconate.

Step Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Cell-free extract	154.0	3169	20.6
Purified enzyme	16.7	1721	102.8

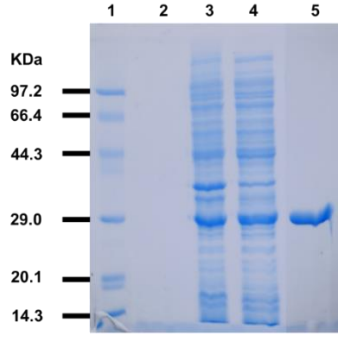


Figure 2: SDS-PAGE of crude and purified TmGNDH. Lane 1: Protein Marker; Lane 2: Blank; Lane 3: Insoluble fraction; Lane 4: Cell-free extract; Lane 5: Purified TmGNDH.

3.3 Coenzyme specificity

TmGNDH showed activity for both NAD and NADP. The enzyme preferred NADP to NAD as its coenzyme. About 56% of the activity was detected when NADP was replaced with NAD (Table 2). TmGNDH exhibited a difference from other reported GNDH in terms of coenzyme specificity (see the Introduction section for detail description of the coenzyme specificity of the other reported GNDH).

Table 2: Coenzyme specificity of TmGNDH.

Substrate	Relative activity (%)	
	NADP	NAD
D-Gluconate	100	55.64

3.4 Kinetic properties

The Km value of TmGNDH with D-gluconate as the substrate was 121.3 ± 3.0 mM (see the Materials and Methods section for detail description of the Km value obtained). TmGNDH showed the lowest Km value among reported GNDH with D-gluconate as the substrate (see Table 3 for Km values of the reported GNDH).

Table 3: Using D-gluconate as the substrate, the Km value of TmGNDH was compared with that reported for other GNDH.

Enzyme	Km (mM)
TmGNDH (this study)	121.3 ± 3.0
GNDH from *G. liquefaciens*[6]	18
GNDH from *G. suboxydans*[14]	20
GNDH from *G. oxydans*[10]	20.6
GNDH from *A. niger*[11]	8.4 ± 0.1

3.5 Substrate specificity

The substrate specificity of TmGNDH was detected against six substrates. The Km and k_{cat} values of TmGNDH with D-arabinose as the substrate were not obtained due to the slight solubility of this substrate. Therefore, the enzyme had kinetic parameters for only five substrates (D-gluconate, D-sorbitol, D-xylitol, D-glucose, and D-xylose) (Table 4). The Km values decreased in the order of D-glucose > D-gluconate > D-sorbitol > D-xylose > D-xylitol. The k_{cat} values decreased in the order of D-gluconate > D-xylitol > D-sorbitol > D-xylose > D-glucose. The substrate specificity of TmGNDH was different from GNDH from *G. suboxydans* and *G. oxydans* (see the Introduction section for detail description of substrate specificity of the two GNDH).

Table 4: Substrate specificity of TmGNDH.

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1}/mM)
D-gluconate	3181 ± 34.1	121.3 ± 3.0	26.2 ± 0.4
D-sorbitol	17.4 ± 0.2	142.8 ± 4.3	0.1
D-xylitol	95.7 ± 2.5	254.5 ± 5.1	0.4
D-glucose	9.2 ± 0.1	48.1 ± 0.8	0.2
D-xylose	16.8 ± 0.3	209.5 ± 5.0	0.1

3.6 Effect of pH on enzyme activity

For TmGNDH, activity was determined with D-gluconate at pH 4.0–10.0 (see the Materials and Methods section for detail description of the buffers used). There was almost no detected activity at pH 4.0. The optimal pH of TmGNDH was approximately 9.0 (Figure 3a), which was different from GNDH from *G. liquefaciens*, *G. oxydans*, and *G. suboxydans* (optimal pH, 10.0).

3.7 Effect of temperature on enzyme activity

For TmGNDH, activity was determined with D-gluconate at temperatures of 30 ℃–90℃. The maximum activity of TmGNDH was observed at 60℃ (Figure 3b), which was higher than the optimal temperature of GNDH from *G. suboxydans* and *G. liquefaciens* (50℃). The enzyme activity decreased sharply when the temperature exceeded 80℃. Significant precipitation was observed when TmGNDH was incubated at 90℃ for 20 min. The enzyme showed thermostability at 60℃ within 12 h (Figure 3c).

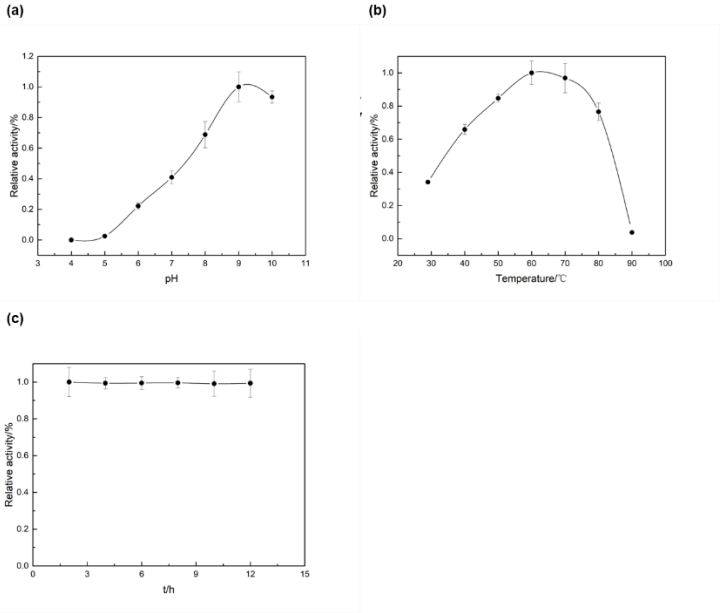


Figure 3: (a) Effect of pH on the activity of TmGNDH. (b) Effect of temperature on the activity of TmGNDH. (c) Thermostability of TmGNDH.

4. Conclusion

We described the biochemical characterization of a thermostable GNDH from *T. maritima* MSB8. The enzyme is a member of the short-chain dehydrogenase/reductase family. Compared with reported GNDH, TmGNDH has some differences in cofactor specificity, kinetic property with D-gluconate as substrate, substrate specificity, optimum pH, optimum temperature, and stability. The reported GNDH showed activity only for NADP as the cofactor, while TmGNDH showed activity for both NADP and NAD. And the enzyme preferred NADP to NAD as its cofactor. D-glucose and D-sorbitol were not oxidized by GNDH from *G. suboxydans* and *G. oxydans*, while TmGDNH showed efficiencies for the two substrates. Curiously, GNDH from *G. liquefaciens* IFO 1238 and *G. suboxydans* lost all of activity within a few days, while TmGNDH remains all of activity for at least one month. The thermostability of TmGNDH suggests that it can be used as an industrially effective tool for synthesizing 5-KGA.

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