

# Purification and characterization of a thermostable glutamate dehydrogenase from a thermophilic bacterium isolated from a sterilization drying oven

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**Glutamate dehydrogenase from axenic bacterial cultures of a new microorganism, called GWE1, isolated from the interior of a sterilization drying oven, was purified by anion-exchange and molecular-exclusion liquid chromatography. The apparent molecular mass of the native enzyme was 250.5 kDa and was shown to be an hexamer with similar subunits of molecular mass 40.5 kDa. For glutamate oxidation, the enzyme showed an optimal pH and temperature of 8.0 and 70°C, respectively. In contrast to other glutamate dehydrogenases isolated from bacteria, the enzyme isolated in this study can use both NAD<sup>+</sup> and NADP<sup>+</sup> as electron acceptors, displaying more affinity for NADP<sup>+</sup> than for NAD<sup>+</sup>. No activity was detected with NADH or NADPH, 2-oxoglutarate and ammonia. The enzyme was exceptionally thermostable, maintaining more than 70% of activity after incubating at 100°C for more than five hours suggesting being one of the most thermoestable enzymes reported in the family of dehydrogenases. [BMB reports 2012; 45(2): 91-95]**

## INTRODUCTION

Glutamate dehydrogenase (GDH) is an enzyme that relates carbon and nitrogen metabolism, catalyzing the reversible oxidative deamination of L-glutamate to  $\alpha$ -ketoglutarate and ammonia using NAD<sup>+</sup> or NADP<sup>+</sup> as cofactors (1-3). They are involved in ammonia assimilation and catabolism of amino acid in a wide range of organisms. All GDHs reported to date are oligomeric enzymes and regarding the subunit composition, can be grouped as hexameric GDHs (with six identical subunits) or tetrameric GDHs (with four identical subunits) (4). Depending on the coenzyme involved in the reaction, GDHs are classified into three

different types: the NADP-specific enzymes (EC 1.4.1.4) which are used for anabolic functions, such as ammonia assimilation, the NAD-specific enzymes (EC 1.4.1.2) which takes part in glutamate catabolism and the NAD(P)-dependent enzymes (EC 1.4.1.3) which can use both coenzymes (5). In general, GDH enzymes from vertebrates are able to use both coenzymes, whereas the enzymes from bacteria are specific for either NAD<sup>+</sup> or NADP<sup>+</sup> (6).

To date, several thermostable GDHs have been isolated from different hyperthermophilic microorganisms (2, 7-9); being the most thermostable GDH reported to date the one purified from *Pyrococcus furiosus* (10, 11). So far, microbial GDHs have been purified from many microorganisms, but all of them from natural origin environment. The isolation of any enzyme from a microorganism obtained from an anthropogenic environment such as a sterilization oven has never been reported.

We examined the activity of glutamate dehydrogenase in the cell homogenate of GWE1, a Gram-positive microaerophilic microorganism, belonging to the genera *Geobacillus*, which had an optimum temperature of 70°C. This extreme thermophile was isolated from a sterilization drying oven which is characterized for having different extreme conditions like high temperatures as well as dehydration and rehydration cycles. Therefore, microorganisms found under these conditions can be considered to be true extremophiles. The importance of this work lies on the novel properties that the enzymes of this kind of microorganisms could have, such as a high thermostability (12, 13).

Here we describe the purification and biochemical characterization of the NAD(P)-dependent glutamate dehydrogenase from GWE1.

## RESULTS

### Purification and molecular mass determination of GDH

Table 1 shows the results of the purification method developed for GWE1 GDH. The enzyme was purified 22-fold compared to the crude extract with a 4% recovery after subjecting it to three successive steps. During the purification, the use of a gel filtration column eliminated non-specific bands, resulting, however, in a pronounced loss of activity. On the basis of SDS-PAGE, the purified enzyme was found to be homogeneous. The apparent molecular

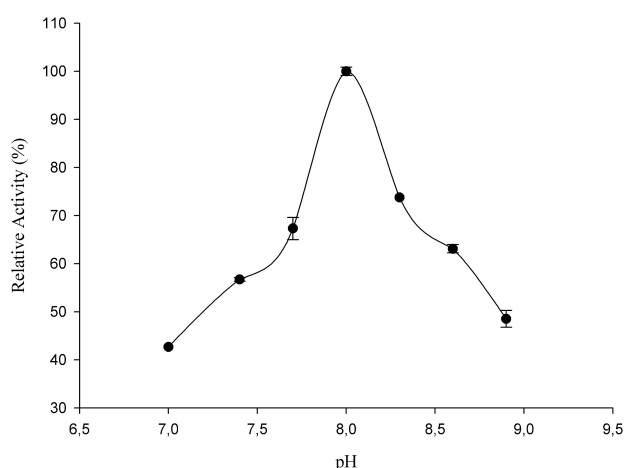
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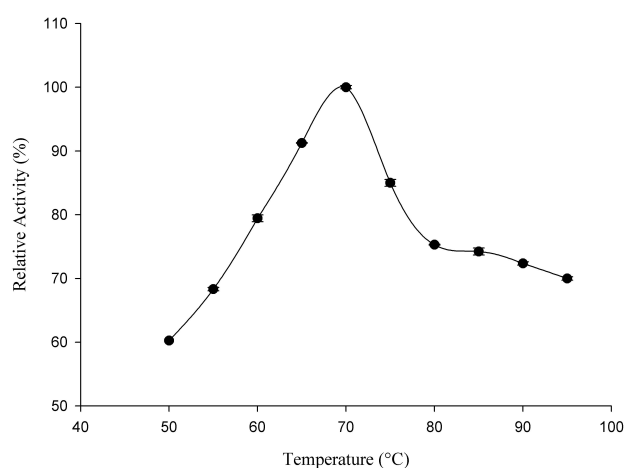
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**Table 1.** Purification of GDH from a drying oven's microorganism

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	2,960.0	10,360.0	3.5	100	1.0
Q-sepharose FF	382.7	9,612.8	25.1	92.8	7.2
DEAE-sepharose FF	110.4	4,137.8	37.5	39.9	10.7
Superdex-200	5.2	412.8	79.4	4	22.7



**Fig. 1.** pH dependence of GWE1 GDH. GDH activity was measured by monitoring glutamate oxidation at 70°C. The buffer used was 100 mM EPPS (pH 7.0 to 8.9).



**Fig. 2.** Temperature dependence of GWE1 GDH. GDH activity was measured by monitoring glutamate oxidation at 50, 55, 60, 65, 70, 75, 80, 85, 90 and 95°C. The buffer used was 100 mM EPPS (pH 8.0).

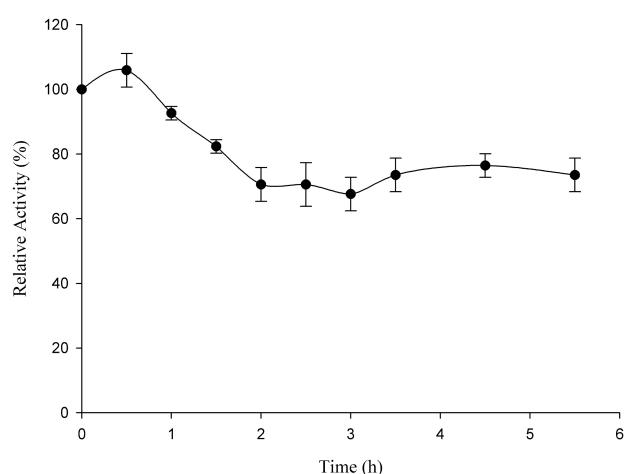
mass of the native enzyme was 250.5 kDa as estimated by size exclusion chromatography using a Superdex 200 column. From SDS-PAGE the subunit molecular mass was estimated as 40.5 kDa. These results suggest that the native enzyme has a hexameric structure composed of six identical or similar subunits. On MALDI TOF/TOF analysis, the experimentally obtained masses were compared with the theoretical peptide masses of proteins stored in the NCBI nr data base using the mass search program Mascot. The result of the peptide mass fingerprinting showed that the enzyme matched with the information reported for the NAD-specific GDH from *Geobacillus kaustophilus* HTA426.

#### Effects of pH and temperature on the enzyme activity

The effects of pH and temperature on the rate of glutamate oxidation are shown in Fig. 1 and 2 respectively. Optimal activity occurred at pH 8.0 (at 70°C) (Fig. 1) and at temperatures around 70°C (at pH 8.0) (Fig. 2). The activity of the enzyme increased as the temperature changed from 50 to 70°C, diminishing at higher temperatures. Activities could not be determined at temperatures above 95°C because of the instability of the coenzyme.

#### Coenzyme specificity and kinetics constants

GWE1 glutamate dehydrogenase can use both NAD<sup>+</sup> and NADP<sup>+</sup> as electron acceptors, presenting catalytic efficiency values of



**Fig. 3.** Thermostability of GWE1 GDH at 100°C. Reaction rate was monitored at 70°C as described in Materials and Methods.

$1.41 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  and  $5.26 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  respectively. The apparent kinetic constants of GWE1 glutamate dehydrogenase, with respect to glutamate, NAD<sup>+</sup> and NADP<sup>+</sup> (forward reaction) were 0.3, 0.4 and 0.1 for *K<sub>m</sub>* (mM) and 10.8, 13.9 and 12.6 for *v<sub>max</sub>*

( $\mu\text{M min}^{-1} \text{mg}^{-1}$ ) respectively. From  $K_m$  values, estimated from Lineweaver-Burk plots (14), it was determined that GDH has a higher kinetic affinity for  $\text{NADP}^+$  than for  $\text{NAD}^+$ . No activity was detected with NADH or NADPH, 2-oxoglutarate and ammonia.

### Thermostability

The thermostability of the GWE1 GDH was examined. Fig. 3 shows the remarkable resistance of this GDH to thermal inactivation. The enzyme maintained its full activity after a 30 min incubation at  $100^\circ\text{C}$  and retained more than 70% of its activity even after 5 h incubation at  $100^\circ\text{C}$  at an enzyme concentration of 4.2 mg/ml.

### DISCUSSION

In this study GDH enzyme from the sterilization oven microorganism GWE1 has been purified and characterized. To date, few enzymes of the dehydrogenase family from thermophiles and hyperthermophiles have been studied. This is the first report on the purification and characterization of a GDH from a thermophilic bacterium isolated from an anthropogenic environment.

Like GDH from *P. furiosus* (10, 15, 11), *Pyrococcus woesei* (2) and *Sulfolobus solfataricus* (1), of which all are hyperthermophilic archaeas, GDH from the GWE1 bacterium presented dual specificity for the use of coenzyme, being able to use both  $\text{NAD}^+$  and  $\text{NADP}^+$ , in contrast to other GDH purified from bacteria which are specific for either  $\text{NAD}^+$  or  $\text{NADP}^+$  (6). GWE1 GDH shows therefore a coenzyme specificity more similar to archaeal and eukarial, than to bacterial GDHs; an important difference to already reported GDHs. The kinetic properties of GWE1 GDH suggest that it functions *in vivo* catabolizing glutamate, since no activity was detected in the reductive amination direction. This correlates well with the growth requirements of GWE1 which grows in the presence of proteins or peptides.

From molecular mass results, it was determined that GWE1 GDH is an oligomeric enzyme of hexameric structure, composed of six identical subunits. As other GDHs reported to date (4), this enzyme belongs to the Hexameric GDHs group.

The apparent molecular mass of the native enzyme and its subunits is only slightly smaller than that found in *P. furiosus* (10, 15, 11), *P. woesei* (2), *T. litoralis* (7) and *T. profundus* (8), suggesting that the enzyme is highly conserved in terms of molecular structure among thermophiles and hyperthermophiles.

GWE1 GDH is thermoactive, a property shared with enzymes from hyperthermophiles (16), showing an optimal activity at  $70^\circ\text{C}$  which correspond to the temperature of growth of the microorganism. Almost all enzymes show optimal temperature at or slightly above the growth temperature of the organism (16).

The purified GDH enzyme from GWE1 microorganism showed to be highly thermostable. To date, GDH from *P. furiosus* is the most thermostable enzyme reported, having a half-life of 10 h at  $100^\circ\text{C}$  (11). However, in contrast to *P. furiosus* which optimally grows at temperatures near to  $100^\circ\text{C}$  (17, 18) and has an optimal temperature for enzyme activity of  $95^\circ\text{C}$ , GWE1 microorganism

showed an optimal growth and enzyme activity at  $70^\circ\text{C}$ . In comparison to *P. furiosus*, GDH enzyme from GWE1 maintains more than 70% of its relative activity when incubated for 5 h at  $100^\circ\text{C}$ , this means  $30^\circ\text{C}$  above the optimal growth temperature of the microorganism. This characteristic might indicate a reversible change in its active protein conformation with temperature, which could relate the lower enzyme activity at temperatures above  $70^\circ\text{C}$  with the capacity to maintain its activity after the incubation at  $100^\circ\text{C}$ . This result suggests that GWE1 GDH is one of the most thermostable dehydrogenases described to date, with regard to retention of enzyme activity, as shown in Fig. 3. This property could make this GDH as a useful enzyme in industrial applications where high temperatures do not allow the use of mesophilic GDHs (12, 19).

This high thermostability could be associated to different structural determinants that affect thermal stability of proteins at high temperatures, such as a relatively small solvent-exposed surface area (20), an increased packing density that reduces cavities in the hydrophobic core (21-23), an increase in core hydrophobicity (24) and an increased number of ionic interactions (25), among others. However, enough experimental evidence has been accumulated on hyperthermophilic proteins to conclude that no single mechanism is responsible for the remarkable stability of thermophilic and hyperthermophilic proteins (26, 27). Further studies on this enzyme could reveal new insights into the structural basis for thermal adaptations of proteins at high temperatures and may provide a plausible explanation on how microorganisms can deal with extreme environments.

### MATERIALS AND METHODS

#### Microorganism and growth conditions

Axenic cultures of GWE1 microorganism were microaerobically grown in liquid media composed of modified marine media containing (g/l) 5.0 yeast extract, 3.0 maltose, 5.0 peptone, 1.2  $\text{NH}_4\text{Cl}$ , 35  $\text{NaCl}$ , 3.5  $\text{MgSO}_4$ , 2.75  $\text{MgCl}_2$ , 3.25  $\text{KCl}$ , 0.75  $\text{CaCl}_2$ , 1.5  $\text{H}_3\text{BO}_3$ , 0.0075  $\text{SrCl}_2$ , 0.005 Citrate, 0.005  $\text{KI}$ , 0.0165  $\text{FeCl}_3$ , 0.0075  $\text{MnSO}_4$ , 0.0045  $\text{Na}_2\text{WO}_4 \times 2 \text{H}_2\text{O}$ , 0.003  $\text{NiCl}_2$ , 0.0015  $\text{ZnSO}_4$ , 0.0015  $\text{Na}_2\text{MoO}_4$ , pH was adjusted to 5.8. Samples were grown in sealed bottles at  $70^\circ\text{C}$  for 24 hours. The cells were collected by centrifugation ( $9,000 \times g$  for 60 min) and stored at  $-20^\circ\text{C}$ .

#### Enzyme purification

Glutamate dehydrogenase was routinely purified from GWE1 cells at  $23^\circ\text{C}$ . A cellular disruption method was specially designed for thermophilic microorganisms. Frozen cells were thawed in 13 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 15 mM EDTA (pH 8.0), lysozyme (1 mg/ml) and DNase I (10  $\mu\text{g/ml}$ ) and were incubated at  $37^\circ\text{C}$  for 4 h. Then, the sample was disrupted using a sonicator (Branson sonifier 450) and a bead beater (Biospec Products, 1107900) alternatively. Cell debris was removed by centrifugation ( $9,000 \times g$  for 20 min) and the supernatant solution was used as the crude extract for the purification. The crude extract (10 ml) was loaded onto a column (Pharmacia, C 16/20) of Q-Sepharose Fast Flow (Pharmacia

Biotech) equilibrated with buffer A (50 mM Tris-HCl [pH 8.0] containing 10% [vol/vol] glycerol, 2 mM dithiothreitol, and 2 mM sodium dithionite). After the column was washed with the same buffer, the enzyme was eluted with a linear gradient (200 ml) of 0 to 1 M NaCl in buffer A. GDH activity started to elute as 0.3 M NaCl was applied to the column. The fractions containing GDH activity were combined and applied to a column (Pharmacia, C 10/10) of DEAE-Sepharose Fast Flow (Pharmacia Biotech) equilibrated with buffer A. The enzyme was eluted with a linear gradient (90 ml) of NaCl 0 to 1 M in buffer A. GDH activity started to elute as 0.22 M NaCl was applied.

The fractions from the DEAE-Sepharose Fast Flow column containing GDH activity were combined, concentrated to a volume of 0.5 ml by ultrafiltration (PM-30 membrane filter; Amicon), and applied to a column (GE Healthcare, Tricorn 10/600) of Superdex-200 (Pharmacia Biotech), equilibrated with buffer A containing 0.2 M NaCl. The fractions with GDH activity was concentrated by ultrafiltration (PM-30 membrane filter; Amicon), and were store at  $-20^{\circ}\text{C}$ . All columns were controlled by a Pharmacia FPLC system.

#### Enzyme assay

Glutamate dehydrogenase activity was measured spectrophotometrically at  $70^{\circ}\text{C}$  by measuring glutamate-dependent reduction of  $\text{NAD(P)}^{+}$  at 340 nm. Each reaction mixture (2 ml) contained 100 mM EPPS [N-(2-hydroxyethyl)piperazine-N'-(3-propanesulfonic acid)] (pH 8.0), 2 mM glutamate, and 0.4 mM  $\text{NAD(P)}^{+}$ . The reaction was started by the addition of the coenzyme. One unit (U) of GDH activity was defined as the formation of 1  $\mu\text{mol}$  of  $\text{NAD(P)H}$  per min. Protein concentration was measured by the method of Bradford (28) with a commercial assay kit (Bio-Rad) using BSA as the standard.

#### Molecular mass determination

The apparent molecular mass of the native GDH was estimated by gel filtration chromatography on a column (GE Healthcare, Tricorn 10/600) of Superdex-200 (Pharmacia Biotech) equilibrated with buffer A containing 0.2 M NaCl and calibrated using hemocyanin subunit  $\beta$  (350.0 kDa),  $\beta$ -amylase (200.0 kDa), alcohol dehydrogenase (150.0 kDa) and ovalbumin (45.0 kDa) as the standard proteins. The subunit molecular mass was determined by SDS-PAGE (12%) according to the method of Laemmli (29) using a wide range marker (Winkler, BM-1110) with the following proteins:  $\beta$ -galactosidase (166.0 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), restriction endonuclease Bsp 981 (25 kDa),  $\beta$ -lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa). Gel was stained for proteins with a silver stain protocol based on the method of Sammons *et al.* (30).

#### Protein identification

Glutamate dehydrogenase was identified by MALDI TOF/TOF in the National Center for Biotechnology, Spain.

#### Thermostability

For determination of GDH thermostability, the enzyme was placed in small tubes with O-ring-sealed caps and incubated for 0-5:30 hours in a dry bath (Major Science, MD-02N-220) at  $100^{\circ}\text{C}$ . Samples were taken at different times and assayed for enzyme activity. Residual activity was determined at  $70^{\circ}\text{C}$  under the conditions described in section Enzyme Assay.

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