# Purification and characterization of a thermostable glutamate dehydrogenase from a thermophilic microorganism from Deception Island, Antarctica

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**Abstract**    Glutamate dehydrogenase (GDH) catalyzes the oxidative deamination of glutamate to $\alpha$-ketoglutarate and ammonium ions. Currently the determination of ammonium and glutamate is carried out using a bovine GDH enzyme, which lacks optimal thermostability for long term storage at room temperature.  From samples of Deception Island, Antarctica, we obtained the thermophilic bacteria PID 15 belonging to the *Bacillus* genera with high GDH specific activity. This new enzyme exhibited NAD$^+$ dependent activity and no activity was observed when NADP$^+$ was used. This enzyme shows a specific activity of 4.7 U·mg$^{-1}$ for the oxidative deamination reaction and 15.4 U·mg$^{-1}$ for the reduction of $\alpha$-ketoglutarate. This enzyme has an optimum temperature of 65°C and pH of 8.5 for the oxidative deamination. For the reduction of $\alpha$-ketoglutarate, the optimum temperature is 60°C, with a pH of 8.0. One of the most important characteristics of this enzyme is its ability to retain more than 60% of its activity when it is incubated for 8 h at 65°C. The enzyme is also able to retain full activity when it is incubated for 48 d at 4°C and over 80% of its activity when it is incubated at 25°C. Characterization of its kinetics suggests that it primarily catalyzes the formation of $\alpha$-ketoglutarate. This enzyme has an important biological role in the catabolism of glutamate and may have some interesting biotechnological applications based on its thermostable properties.

**Keywords**    Antarctica, glutamate dehydrogenase, thermophile

## 1  Introduction

Glutamate dehydrogenase (GDH) is an enzyme that plays an important role in the metabolism of carbon and nitrogen[1-4]. This enzyme belongs to the oxidoreductase family and is widely distributed among eukaryotes, bacteria and archaea. Its function is to reversibly catalyze the oxidative deamination of glutamate to $\alpha$-ketoglutarate and ammonia[5-7].

All GDHs reported so far are oligomeric and depending on the number of subunits, can be classified as homohexameric and homotetrameric proteins with an approximate subunit size of 50 kDa and 115 kDa, respectively[7]. GDHs are divided into three classes based on their coenzyme specificity, NAD$^+$ (NAD-specific) which function in glutamate catabolism, NADP$^+$ (NADP-specific) related to ammonia assimilation, and finally those with dual specificity for NAD$^+$ and NADP$^{+[8]}$.

Glutamate dehydrogenases are widely used as biosensors. In the medical industry, ammonia  is an important marker for liver diseases and many protocols for quantifying ammonia in biological fluids are based on the redox reaction of NAD(P)$^+$ mediated by GDH. In these tests, the amount of NAD(P)$^+$ is directly related to the amount of ammonium generated[9-10]. Therefore, the disappearance of NAD(P)H

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is determined by measuring the absorbance at 340 nm. In the food industry, the presence of ammonia is an important marker for bacterial degradation of proteins, peptides and amino acids; consequently, GDH has been used for many years to determine the degree of food decomposition[11].

However, it is well known that the use of mesophilic enzymes as biosensors is relatively problematic due to their instability under temperature extremes, organic solvents, and denaturing agents as well as their decreased stability during long term storage[12-14].

In particular, enzymes produced by thermophiles and hyperthermophiles (thermoenzymes) are of interest in both academic and industrial research, mainly because of their optimal activity at high temperatures, high thermal stability and resulting resistance to inactivation at high temperatures and[10,12,15-16]. These characteristics facilitate the application of thermoenzymes in industrial processes or products that require not only high temperatures, but also high stability and long term storage. Generally, these enzymes are adapted to function optimally at the growth conditions of the microorganism that produces them. Therefore, in many cases the conditions in which the microorganism was isolated define the operative boundaries of the enzyme of interest [5,17-18].

One of the most thermostable GDHs reported to date was purified from the microorganism *Pyrococcus furiosus*, a marine archaea that grows optimally at temperatures near 100°C. This enzyme has a half-life of 12 h at 100°C[19]. By comparison, a stable GDH isolated from *Geobacillus* GWE1, a thermophilic bacterium identified from a sterilization oven, maintains more than 70% of its relative activity when incubated for 5 h at 100ºC[20]. Nonetheless, despite the high thermal stability, none of these enzymes display sufficient specific activity at room temperature, to be useful as biosensors.

We examined the GDH activity in the cell homogenate of PID 15, a bacterium belonging to the *Bacillus* genera with an optimal growth temperature of 50°C. This extremophile was isolated from a terrestrial geothermal site located in Deception Island, Antarctica. Samples were collected during Antarctic Chilean Expedition 46 (ECA 46). The full sequence of the 16s rDNA from PID 15 (accession number JQ965669) shares 98% identity with *Bacillus gelatini* LMG 21880[T] (accession number AJ551329).

In this work, we report the purification and characterization of a novel GDH enzyme from PID15. This enzyme showed promising thermostability and activity at a wide range of temperatures, characteristics that should make it applicable for use in biotechnology processes requiring different temperatures and long lasting stability.

# 2 Materials and methods

## 2.1 Bacterial growth and preparation of the crude extract

Axenic cultures of PID15 microorganism were microaerobically grown in liquid media Luria Bertani medium which contained (g·L^{-1}) 10 g tryptone, 5 g of yeast extract and 10 g NaCl at pH 8.0. Cultures were incubated for 22 h at 50°C and 120 rpm in a Lab Companion (SI-300) culture oven. Cells were collected by centrifugation (9000 × g for 20 min) and stored at −20°C for further downstream processing.

The biomass of PID15 was resuspended in 2 volumes of 50 mM Tris HCl pH 8.5, containing 2 mM DTT, 0.5 mM PMSF and 10% glycerol (Buffer A). This cell suspension was incubated for 1 h at 37°C with 1 mg·mL^{-1} of lysozyme and subsequently disrupted using a sonication (10–20 cycles of 1 min; Brandson 1510R-MT). The cell lysis was monitored by optical microscopy. Finally, the supernatant was centrifuged for 20 min at 8300 × g and then clarified by ultracentrifugation (Hitachi, HIMAC CP80WX) at 25000× g for 30 min. Crude cell extracts were used for subsequent purification steps.

## 2.2 Enzyme purification and electrophoresis

A chromatographic separation protocol was designed and a FPLC (Pharmacia LKB) equipped with an UV detector (280 nm) to control each purification step (flow rate was 1 mL·min^{-1} at 23ºC). Specific activity was measured in all collected fractions in the direction of glutamate deamination.

Anion exchange chromatography was performed first using Q-Sepharose (Pharmacia, XK 16/20). Fractions with GDH activity were collected and loaded onto an anion exchange DEAE-Sepharose column (Pharmacia HiPrep 16/10). Q-Sepharose and DEAE-Sepharose columns were equilibrated with Buffer A and eluted with a 0–1 M NaCl gradient over 150 min. This was followed by affinity chromatography using Blue-Sepharose (Pharmacia, XK 16/20), eluted with 10 mM Tris HCl pH 7.5 and 1 M NaCl over 45 min. Fractions with GDH activity were concentrated by ultrafiltration using a membrane filter of 30000 Da (Millipore, Amicon). The concentrated fraction was loaded on a gel filtration Superdex-200 column (GE Healthcare Tricorn 10/600) and eluted with 50 mM Tris HCl pH 8.5 containing 2 mM DTT, 0.5 mM PMSF, 10% glycerol and 0.2 M NaCl.

SDS-PAGE was routinely performed accordingly to Laemmli[21] using 10 μg of protein from the active purified fractions, loaded onto 12% SDS-PAGE and stained with Coomassie blue. The molecular weight marker Benchmark[TM] 26–180 kDa (Invitrogen) was used as a reference.

## 2.3 Molecular mass determination

The apparent molecular mass of PID15 GDH was estimated by gel filtration chromatography on a column (GE Healthcare, Tricorn 10/600) of Superdex-200 (Pharmacia Biotech) equilibrated with buffer A and calibrated using lysozyme (14.3 kDa), egg ovalbumin (45.0 kDa), bovine serum albumin (66.0 kDa), bovine glutamate dehydrogenase (300.0 kDa) and urease (545.0 kDa) as the standard proteins.

## 2.4 Protein identification

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

## 2.5 Enzyme assay

GDH activity was determined by measuring the increase in absorbance at 340 nm using a spectrophotometer (Shimadzu UV-1700) coupled to a thermo-controlled water bath that allows activity measurements from 10 to 95°C.

One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzes the formation of one µmol of NADH per minute. The reaction was carried out in a final volume of 1 mL containing the reagents for the oxidative deamination reaction: 10 mM glutamate, 0.4 mM NAD$^+$ and 100 mM EPPS pH 8.0 and for reduction of α-ketoglutarate: 10 mM α-ketoglutarate, 100 mM EPPS pH 8.0, 50 mM NH$_4$Cl and 0.2 mM NADH.

The protein concentration was estimated by the Bradford method[22] using the commercial Bio-Rad protein assay and BSA (Sigma) as the standard.

## 2.6 Effect of ionic strength

The ionic strength in the GDH enzymatic reaction mixture was varied by the addition of NaCl in the reaction buffer at final concentrations of 0.2, 0.3, 0.4 and 0.5 M. These assays were performed only in the oxidative deamination direction under the same conditions detailed above at temperatures of 37 and 65°C.

## 2.7 Temperature and pH optimum

The best fraction with PID15 GDH activity obtained during the purification process was used for the determination of the effects of temperature and pH.

The buffers used for the optimum pH determination were 100 mM MES pH 6.0; 100 mM HEPES pH 7.0; 100 mM EPPS pH 8.0; 100 mM EPPS pH 8.5; 100 mM Tris-HCl pH 9.0; 100 mM Tris-HCl pH 9.5; 100 mM CAPS pH 10; 100 mM CAPS pH 11. The specific activity for both oxidative deamination of glutamate and reduction of α-ketoglutarate were determined at 65°C and 60°C respectively.

For the optimal temperature activity of the enzyme was examined over the range of 25 to 80°C. The enzyme was placed in small tubes with O-ring-sealed caps and incubated for 5 min at the selected temperature in a dry bath (Major Science, MD-02N-220). The activity was measured at the same incubation temperature. All determinations were performed for both the oxidative deamination of glutamate and the reduction of α-ketoglutarate

## 2.8 Thermostability

To test its thermostability, the protein was placed in small tubes with O-ring-sealed caps and incubated at 65°C in a dry bath (Major Science, MD-220-02N). The activity was measured as described above at different incubation intervals over 8 h.

To investigate long term stability, the purified PID15 GDH was incubated at temperatures of 4 and 25°C for a period of 48 d. The thermostability of the purified enzyme was compared with the thermal stability of a commercial GDH (GDH bovine G7882, lot 061M7001V, Sigma) incubated under the same conditions. For both enzymes, the activity was measured in the direction of oxidative deamination as described above at 65ºC for PID15 GDH and 37ºC for bovine GDH.

## 2.9 Kinetics parameters

The Michaelis-Menten constant ($K_m$) and maximum velocity ($V_{max}$) for the oxidative deamination of glutamate and the reduction of α-ketoglutarate were determined. GDH activity was measured as described above. The kinetic constants for PID15 GDH were obtained using different concentrations of substrates. The apparent $K_m$ and $V_{max}$ were obtained from the double reciprocal plot (Lineweaver-Burk plot).

# 3 Results

## 3.1 Purification and molecular mass determination of GDH from the PID15 bacterium

The purification process was initiated with 22 g (w/w) of PID15 biomass. During the first stage of purification, the crude extract was loaded onto a Q-Sepharose column. It is important to mention that the total activity increased significantly after Q-Sepharose purification compared with

**Table 1** Purification profile of a novel GDH from PID 15. The specific activity was determined in the direction of oxidative deamination of glutamate

| Purification step | Volume/mL | Total protein /mg | Total activity /U | Specific activity /( U·mg$^{-1}$) | Yield/% | Purification (Fold) |
|---|---|---|---|---|---|---|
| Cell-free Extract | 43 | 714.8 | 95.6 | 0.13 | 100 | 1 |
| Q-Sepharose | 18 | 154.5 | 198.8 | 1.29 | 207.9 | 9.9 |
| DEAE-Sepharose | 10 | 44.7 | 70.6 | 1.58 | 73.8 | 12.2 |
| Blue-Sepharose | 4 | 6.8 | 21.8 | 3.19 | 22.8 | 24.5 |
| Superdex-200 | 2 | 1.6 | 7.7 | 4.76 | 8.1 | 36.6 |

that of the crude extract (Table 1), possibly as a result of the removal of inhibitors that were decreasing enzyme activity. The purification step using the Blue Sepharose column allowed to achieve the highest purification fold. This result was expected because the affinity resin was chosen to be compatible with enzymes that require adenine cofactors such as PID15 GDH.

Overall, PID15 GDH enzyme was purified 37 fold with a final specific activity of 4.8 U·mg$^{-1}$ in the oxidative deamination direction and 15.7 U·mg$^{-1}$ for the reduction of $\alpha$-ketoglutarate. The amount of protein recovered was 1.6 mg and was used for the subsequent enzyme characterization. The purified enzyme showed no activity when NADP(H) was used as the cofactor, either in the oxidized or reduced form, confirming that this enzyme is a NAD(H) dependent GDH.

The apparent molecular mass of the native enzyme was 295 kDa as estimated by size exclusion chromatography using a Superdex 200 column. From SDS-PAGE, the estimated subunit molecular mass was 49 kDa (Figure 1). By TOF/TOF mass spectrometry analysis, the experimentally obtained masses were compared with the theoretical peptide masses of proteins stored in the NCBInr database using the mass search program Mascot. The result confirmed that this enzyme is a homohexamer.
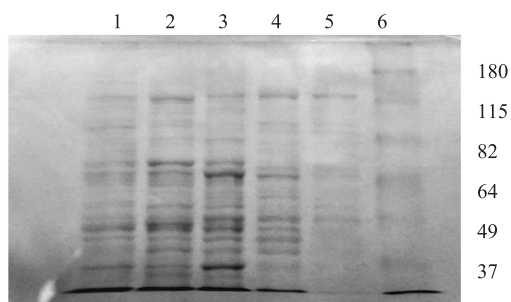


**Figure 1** Purification profile of PID15 GDH. Denaturing SDS polyacrylamide gel was stained using colorimetric Coomassie Blue method. Lane 1: Crude extract; Lane 2: Q-Sepharose fraction 5; Lane 3: DEAE-Sepharose fraction 5; Lane 4: Blue Sepharose fraction 2; Lane 5: Superdex 200 fraction 3; Lane 6: Molecular weight marker BenchmarkTM 26–180 kDa.

### 3.2 Biochemical characterization

For the oxidative deamination reaction, the optimum pH and temperature were 8.5 and 65°C respectively. For the reduction of $\alpha$-ketoglutarate, the optimum temperature and pH were 60°C and pH 8.0 (Figures 2 and 3). It was observed that this enzyme possesses similar optimal temperature and pH values regardless of the catalytic direction. In the direction of $\alpha$-ketoglutarate, activity was conserved over a wide range of temperatures, retaining over 40% of its specific activity when it was incubated at 25°C.

The thermostability of the enzyme was measured at the optimum temperature for the oxidative deamination direction. As shown in Figure 4, the enzyme retained over
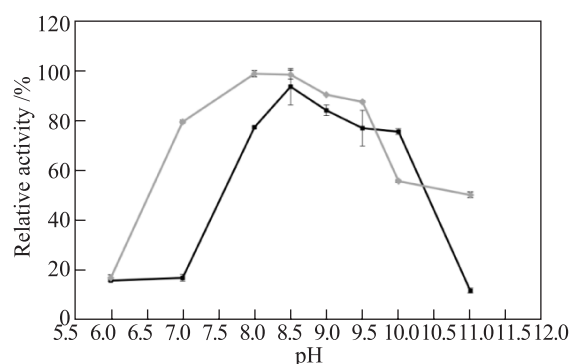


**Figure 2** Optimal pH of PID15 GDH. The specific activities for both the oxidative deamination of glutamate (black) and reduction of $\alpha$-ketoglutarate (grey) were determined at 65°C and 60°C, respectively. The relative activity at 100% corresponds to the highest specific activity (U·mg$^{-1}$) obtained. Error bars represent the standard error determined from three technical replicates.
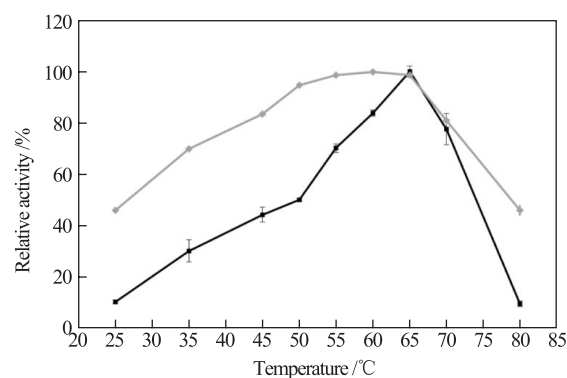


**Figure 3** Optimal temperature of PID15 GDH. The specific activities for the reaction of oxidative deamination of glutamate (black) and reduction of $\alpha$-ketoglutarate (grey) were determined in 100 mM EPPS pH 8.0. The relative activity at 100% corresponds to the highest specific activity (U·mg$^{-1}$) obtained. Error bars represent the standard error determined from three technical replicates.
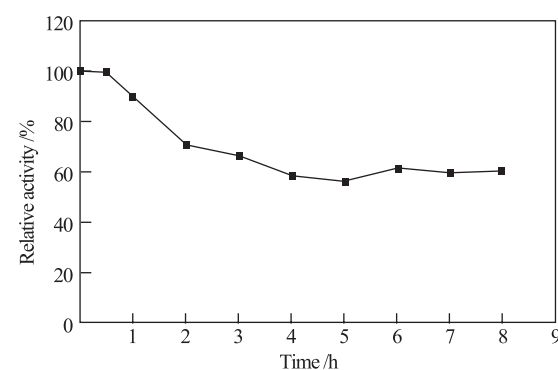


**Figure 4** Thermostability of PID15 GDH at 65°C. The specific activity was determined in the direction of oxidative deamination of glutamate at 65°C and 100 mM EPPS pH 8.5. The relative activity at 100% corresponds to the highest specific activity (U·mg$^{-1}$) obtained.

60% of its activity when incubated at 65°C for 8 h. We compared the thermostability of purified PID15 GDH with commercial bovine GDH G7882 at 4 and 25°C. Figure 5 shows that PDI15 GDH is considerably more thermostable than the commercial enzyme. When these two enzymes were maintained at 4°C for 48 d, PID15 enzyme retained its full activity. However, the bovine enzyme loses approximately half of its activity. A more dramatic difference was observed when these enzymes were incubated at room temperature (25°C) over the same period. The PID15 GDH retained more than 80% of its activity while the commercial bovine enzyme lost 50% of its activity after 15 d of incubation and 100% of its activity after 48 d of incubation.
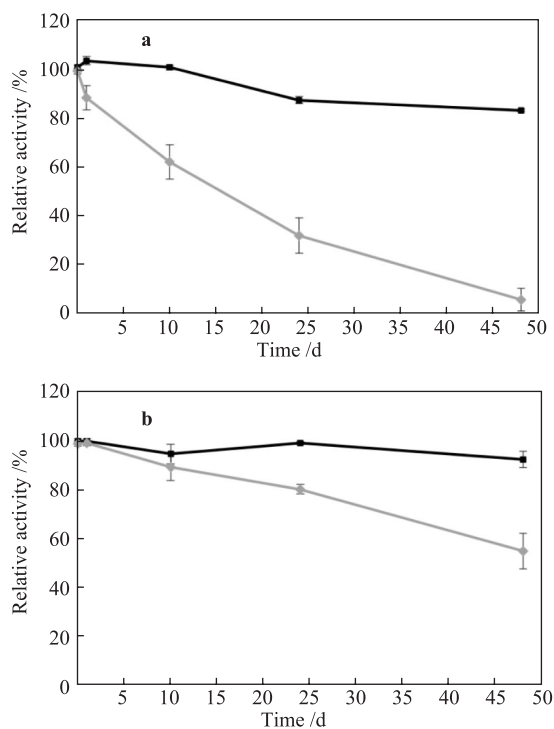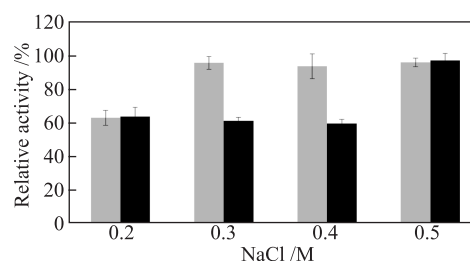


**Figure 5**　Thermostability of PID15 GDH at 25°C (a) and 4°C (b). The specific activity was determined in the direction of oxidative deamination of glutamate at 65°C (PID15 in black) and 37°C (bovine GDH in grey) in 100 mM EPPS pH 8.5. The relative activity at 100% corresponds to the highest specific activity (U·mg$^{-1}$) obtained.

　　　The activity of the enzyme was tested in the presence of four different concentrations of NaCl. Figure 6 shows that at 37°C, the highest activity occurred at the highest NaCl concentration tested. There was not a significant difference at lower concentrations. However, at the optimum temperature of the enzyme (65°C), the protein had similar activities at concentrations above 0.2 M NaCl (Figure 6).

### 3.3  Kinetic parameters

The catalytic constants $K_m$ and $V_{max}$ were calculated using



**Figure 6**　Effect of ionic strength on GDH activity. The specific activity was determined in the direction of oxidative deamination of glutamate at 65°C (grey) and 37°C (black) in 50 mM EPPS buffer pH 8.5. The relative activity at 100% corresponds to the highest specific activity (U·mg$^{-1}$) obtained. Error bars represent the standard error determined from three technical replicates.

both Michaelis-Menten and Lineweaver-Burk graphs (not shown). The smaller $K_m$ values obtained for the reaction using glutamate and NAD$^+$ (Table 2), suggest that the reaction is shifted toward the formation of α-ketoglutarate and ammonia. For the substrates tested, the enzyme showed a michaelian behavior. However, a sigmoidal curve was observed in the Michaelis-Menten graph when the dependence of enzymatic activity toward the NADH cofactor was examined. To calculate a more specific value of the catalytic constants $K_m$ and $V_{max}$ for NADH, it was necessary to graph the reciprocal of the velocity versus the reciprocal of the squared concentration of NADH. Then Eadie-Scatchard plot was used for the study of cooperative behavior.

**Table 2**　Summary of catalytic constants for PID 15 GDH

| Substrate | Concentrations range/mM | $K_m$ /mM | $V_{max}$/ (mM·min$^{-1}$) |
|---|---|---|---|
| Glutamate | 0.15−10 | 1.5 | 5.07 |
| NAD$^+$ | 0.06−4 | 0.4 | 6.07 |
| NH$_4$Cl | 0.78−50 | 10.7 | 20.96 |
| α-Ketoglutarate | 0.15−20 | 4.3 | 20.96 |
| NADH | 0.0031–0.4 | 0.2 | 24.44 |

## 4  Discussion

Relatively high levels of GDH were naturally produced by the Antarctic thermophile PID15 grown on rich LB medium. PID15 GDH displays coenzyme specificity for NAD(H) in concordance with bacterial GDHs, which are either specific for NAD(H) or NADP(H)[23]. The kinetic properties of PID15 GDH indicate a double catalytic function since high levels of activity were detected in the direction of oxidative deamination as well as for the reduction of α-ketoglutarate.

　　　Molecular mass results indicate that PID15 GDH has a homohexameric structure with a subunit mass of 49 kDa, similar to those GDHs commonly found in other bacteria [20]. Purified PID15 GDH is thermoactive with an optimal activity at 65°C for the oxidative deamination and 60°C for the

reduction of α-ketoglutarate, which is similar to the growth temperature of PID15. Almost all enzymes show optimal temperature at or slightly above the growth temperature of the organism they originated from[24-25]. Two of the most remarkable characteristics of PID15 GDH are that it is active over a broad range of pH and temperature range. This is very important for biotechnology applications because it allows the use of the enzyme in processes which require different operational temperatures without significant loss of activity. Purified PID15 GDH was more thermostable than commercial bovine GDH G7882 when incubated at 4 and 25°C for a period of 48 d.

High ionic strength enhances PID15 GDH catalysis at 37°C, but this enhancement was not observed at 65°C. This differential behavior may be a result of better folding of PID15 GDH at 65°C, also known as the "maturing" factor of thermophilic proteins[25]. This lack of maturity at 37°C may have been supplied by the highest concentration of salt in the buffer, indicating a higher ionic strength could help to improve the folding of the enzyme and enhance activity.

The kinetic parameters indicate that the reaction is shifted towards the formation of α-ketoglutarate and ammonia, suggesting that the enzyme has a more important role in the catabolism of glutamate than the assimilation of ammonium[7,13]. Furthermore, according to the Eadie-Scatchard plot, PID15 GDH shows an uncommon cooperative behavior that has been also reported for other enzymes, including Sulfolobus solfataricus GDH[1], and could be related to its regulation.

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