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Original Article

# Purification and partial characterization of glyceraldehyde-3-phosphate dehydrogenase from the ciliate *Tetrahymena thermophila*

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**In the present study, we purified the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is involved in cellular energy production and has important housekeeping functions, from the ciliate *Tetrahymena thermophila* using a three-step procedure. The enzyme was purified ∼68 folds by ammonium sulfate precipitation, followed by two steps of column chromatography (DEAE-cellulose and Mono-S). The purified enzyme is a homotetramer with a molecular weight of ∼120 kDa. Isoelectric focusing analysis showed the presence of only one basic GAPDH isoform with an isoelectric point of 8.8. Western blot analysis showed a single 32-kDa band corresponding to the enzyme subunit using a monospecific polyclonal antibody against the *T. thermophila* GAPDH. The maximum of enzyme activity occurred at pH 8.0 and at 30–35°C. The apparent $K_m$ values for both $NAD^+$ and D-glyceraldehyde-3-phosphate were 0.102 ± 0.012 and 0.360 ± 0.018 mM, respectively. The maximal velocity ($V_{max}$) was 39.40 ± 2.95 U/mg. The *T. thermophila* GAPDH is inhibited by oxidative and nitrosative stress reagents.**

*Keywords* *Tetrahymena thermophila*; glyceraldehyde-3-phosphate dehydrogenase; purification; enzyme characterization; stress reagents

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## Introduction

Glycolysis is a central metabolic pathway and is present, at least in part, in all organisms. Individual enzymes of the pathway are exceptionally well characterized, both in terms of enzymic properties and in terms of detailed structures. Glycolytic enzymes are among the most known highly conserved enzymes. In general, ∼5% of the residues of glycolytic enzyme changes every 100 million years. The most conservative enzyme is glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), with only 3% of its catalytic domain changing in the same period of time. GAPDH catalyzes the reversible oxidative phosphorylation of D-glyceraldehyde-3-phosphate (D-G3P) into 1,3-bisphosphoglycerate [1], and it is constituted of four identical subunits. Each subunit contains four cysteines; two of them (Cys-149 and Cys-153) are located in the catalytic site of each GAPDH subunit. The catalytically active Cys-149 interacts with a histidine to form a highly reactive thiolated group (Cys-S2) which is required for GAPDH activity. GAPDH is not only a simple classical metabolic protein involved in energy production, but also a multifunctional protein with defined functions in numerous subcellular processes, such as DNA replication and repair, regulation of histone gene translation, maintenance of telomere structure, modulation of the cytoskeleton, and nuclear membrane fusion [2]. It is also involved in various diseases, especially neurodegenerative disorders, e.g. β-amyloid precursor protein in Alzheimer's disease [3], prostate cancer [4], and aging [5].

Oxidative stress is a potent phenomenon able to affect a number of parameters in the cell. One of the possible targets of oxidative stress is glycolysis [6]. Indeed, several studies have shown that oxidative stress induced by apoptogenic dose of hydrogen peroxide ($H_2O_2$) follows a temporary block of glycolysis from ADP-ribosylation of GAPDH [6]. This inactivation of GAPDH is caused mainly by interferences of radicals with the cysteine residue present in the active site, which has been reported to be *S*-thiolated by $H_2O_2$ [7] and *S*-nitrosylated by nitric oxide [8]. Oxidative modification of proteins has been observed to increase in a large number of pathophysiological conditions, including neurodegenerative diseases [9] and aging [10]. Further understanding of GAPDH may aid novel therapeutic strategies for many disorders.

We report here, for the first time, the purification and some physical and kinetic properties of GAPDH from *Tetrahymena thermophila*. The effect of different stress reagents on purified *T. thermophila* GAPDH was also discussed. The ciliated protozoan *T. thermophila* has been

used as a microbial model due to its typical eukaryotic characterization. Its ultrastructure, cell physiology, development, biochemistry, genetics, and molecular biology have been extensively studied [11,12]. In addition, the *T. thermophila* genome is being sequenced [13].

## Materials and Methods

### Culture of *T. thermophila*

The *T. thermophila* strain SB1969, kindly supplied by Professor Juan Carlos Gutiérrez, Universidad Complutense de Madrid, was grown axenically at 32°C for 72 h without shaking to exponential phase in a broth medium containing 1.5% (w/v) proteose-peptone, 0.25% (w/v) yeast extract [14]. Cultures were inoculated with 1% (v/v) preculture in the same medium.

### Crude extract preparation

Protozoan cells from 10 l of culture were harvested by centrifugation at 6000 *g* for 15 min at 4°C, washed three times with 20 mM Tris-HCl buffer (pH 7.5), and suspended in the extraction buffer: 50 mM Tris-HCl (pH 7.5) containing 1 mM ethylenediamine tetraacetic acid (EDTA), 10 mM 2 β-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride, and 1% (v/v) glycerol (buffer A) at a ratio of 3 ml/g (wet weight). Cells were then disrupted in the cold by sonication (90% output, 20 s, 12×) using a Bandelin Sonopuls Sonifier. Cellular debris and unbroken cells were removed by centrifugation at 15,000 *g* for 45 min at 4°C using a Sigma 2-16K refrigerated centrifuge. The supernatant obtained constitutes the crude extract (soluble protein fraction).

### Purification procedure

The enzyme was purified to electrophoretic homogeneity from crude extract by a three-step procedure carried out at 4°C.

#### *Ammonium sulfate precipitation*

Ammonium sulfate precipitates and protects proteins in solution from denaturation and bacterial growth. Crude extract was brought to 55% saturation with solid ammonium sulfate [$(NH_4)_2SO_4$] and stirred for 1 h. Precipitated proteins were removed by centrifugation at 15,000 *g* for 45 min at 4°C, and for the supernatant more ammonium sulfate was added to yield 88% saturation. The fraction of precipitated proteins between 55% and 88% saturation was recovered by centrifugation (15,000 *g* for 45 min at 4°C), resuspended gently in a minimal volume of buffer A, and dialyzed twice against 5 l of the same buffer over an 8-h period to remove residual ammonium sulfate.

#### *Anion-exchange chromatography*

The dialyzed fraction was applied to a DEAE-cellulose (Fluka, Buchs, Switzerland) column (1.5 × 12 cm) previously equilibrated with two bed volumes of buffer A. The enzyme was then eluted with equilibrating buffer at a flow rate of 12 ml/h. Fractions of 1 ml were collected and those that showed GAPDH enzyme activity were pooled.

#### *Cation-exchange chromatography*

The enzyme preparation from above was applied at a flow rate of 12 ml/h to a Mono-S column (HR 5/5) previously equilibrated with buffer A. The column was extensively washed at the same flow rate with equilibrating buffer to remove unbound proteins. Elution was performed with a linear gradient of potassium chloride (KCl) (0–300 mM; total volume of 130 ml) prepared in the same buffer. Fractions of 1 ml were collected and those which showed the highest GAPDH activity were pooled.

### Assay of GAPDH activity

The activity of GAPDH was determined spectrophotometrically at 30°C, using a Jenway 6405 UV/Visible spectrophotometer (Bibby Scientific Ltd, Stone, UK) by monitoring NADH generation at 340 nm [15]. In general, enzyme preparation was added to the mixture assay containing 50 mM Tricine buffer (pH 8.0), 10 mM sodium arsenate, 1 mM $NAD^+$, and 2 mM D-G3P. The total volume of the reaction mixture was 1 ml. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the reduction of 1 μmol $NAD^+$ per minute. All experiments and assays were carried out in triplicate.

### Protein concentration

Protein concentration was estimated according to the Bradford procedure [16] using bovine serum albumin (BSA) as a standard.

### Denaturing polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli [17] on one-dimensional 12% polyacrylamide slab gels containing 0.1% (w/v) SDS. Gels were run on a miniature vertical slab gel unit (Mini-Protean 3 cell; Bio-Rad, Hercules, USA). After electrophoresis, gels were stained with 0.2% (w/v) Coomassie brilliant blue R-250 in methanol/acetic acid/water (4 : 1 : 5, v/v/v) for 45 min at room temperature. Distaining was done in methanol/acetic acid/water (4 : 1 : 5, v/v/v). The apparent subunit molecular weight was determined by measuring relative mobilities and comparing with the pre-stained SDS–PAGE molecular weight standards (Precision Plus Protein Standards, Bio-Rad).

### Native molecular weight determination

It was performed with a Superdex HR 200 column attached to a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, Uppsala, Sweden). Separation was achieved with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and 5 mM $MgCl_2$, at a flow rate of 0.3 ml/min. Gel filtration markers used to calibrate the column and to set up a standard curve were ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa), and ovalbumin (43 kDa). Proteins were detected by ultraviolet (UV) absorbance at 280 nm.

### Isoelectric focusing

It was carried out with the same electrophoresis system in 5% polyacrylamide slab gels holding ampholite-generated pH gradients in the range 3.5–10 (Sigma-Aldrich, St Louis, USA) [18]. Cathode and anode solutions were 25 mM sodium hydroxide and 20 mM acetic acid, respectively. The isoelectric point (p*I*) protein markers kit used was the Bio-Rad IEF standards p*I* range 4.4–9.6 for isoelectric focusing.

### Preparation of polyclonal antibodies

A New Zealand white rabbit, weighing ~1.5 kg, was injected intracutaneously in multiple places with 500 μg of the purified *T. thermophila* GAPDH in incomplete Freund's adjuvant (1 : 1, v/v) after collecting 10 ml of rabbit blood that was used for preparing a negative control serum (preimmune serum). After 3 weeks, the rabbit was given a second booster injection and a week later injected with the final booster and bled 1 week thereafter. Antiserum was separated by letting collected blood coagulate 1 h at 30°C and then overnight at 4°C. After centrifugation, the obtained antiserum containing monospecific anti-GAPDH polyclonal antibodies was supplied with 0.02% (w/v) of sodium azide and stored at −20°C until use.

### Western blot analysis

Immunoblot assays of protein samples were carried out after SDS–PAGE on 12% polyacrylamide slab gels. Proteins were electroblotted onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany), the non-specific sites were blocked by soaking the membrane in 5% (w/v) BSA prepared in Tris-buffered saline (TBS) overnight, and then incubated with 1 : 500-fold diluted antiserum in TBS. The membrane was washed three times (15 min each) with TBS and then once with TBS containing 0.05% (v/v) Tween 20 (TBST) (15 min) and incubated for 45 min with a goat anti-rabbit immunoglobulin G antibody-peroxidase conjugate (1 : 1000) (Promega, Madison, USA). After three 15-min rounds of washing with TBST and once 15 min with TBS, the nitrocellulose membrane was developed under a mixture of TBS, 2 mM $H_2O_2$, and 10 mM 4-chloro-3-naphtol in methanol.

### Determination of optimal pH and temperature of purified GAPDH

The influence of pH on the GAPDH activity was studied over a wide range of pH (from 4.0 to 9.5) using a mixture of different buffers that have different p$K_a$ (Tris, 4-morpholineethanesulfonic acid, Hepes, sodium phosphate, and sodium acetate) adjusted to the same ionic strength as the standard reaction mixture.

Thermal activation experiments were carried out by measuring the activity in 50 mM Tricine buffer at a temperature range from 5 to 65°C. Thermal denaturation experiments were carried out by enzyme incubation over a temperature range from 5 to 65°C. After 10 min of incubation, the enzyme was added to the reaction mixture to initiate the enzymatic reaction.

### Kinetic studies of purified GAPDH

Initial velocities of the enzymatic reaction were performed by varying the concentration of one substrate, $NAD^+$ (from 0.02 to 0.32 mM) or D-G3P (from 0.02 to 0.64 mM), while the concentration of the other substrate was kept constant (D-G3P or $NAD^+$). Experiments were carried out in the same conditions as described above using 50 mM Tricine buffer (pH 8.0) at 30°C. Values of the Michaelis constants ($K_m$) and maximal velocity ($V_{max}$) were determined graphically from Lineweaver–Burk double reciprocal plots [19].

### Effect of oxidative and nitrosative stress on GAPDH activity

To study the effect of two types of stress on *T. thermophila* GAPDH, the purified enzyme was incubated with various concentrations of $H_2O_2$ (from 0 to 25 mM) and sodium nitroprusside (SNP) (from 0 to 75 mM). An increasing set of concentrations of each stressor ($H_2O_2$ and SNP), prepared in 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, was added to the purified GAPDH. After 30 min of incubation at 30 ± 1°C, the GAPDH activity was measured spectrophotometrically at 340 nm using 50 mM Tricine buffer (pH 8.0).

## Results

### Purification of *T. thermophila* GAPDH

The purification of the GAPDH from *T. thermophila* was performed by ammonium sulfate precipitation, followed by two steps of column chromatography (DEAE-cellulose and Mono-S). **Table 1** summarizes a representative purification protocol. A total amount of ~986 mg of protein, corresponding to ~642 units of GAPDH, was obtained from

**Table 1 Purification of GAPDH from *T. thermophila***

| Fraction | Total protein (mg) | Specific activity (U/mg of protein) | Total activity (U) | Purification factor (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 986.06 | 0.651 | 642.02 | 1.00 | 100.00 |
| Ammonium sulfate (55%–88%) | 76.23 | 4.992 | 380.61 | 7.66 | 59.28 |
| DEAE-cellulose | 12.15 | 9.197 | 111.75 | 14.12 | 17.40 |
| Mono-S | 1.20 | 44.500 | 53.40 | 68.34 | 8.31 |

crude extract of disrupted cells of *T. thermophila* (26 g wet weight). After ammonium sulfate fractionation (55%–88%), which eliminated 92% of contaminating proteins, the dialyzed fraction was applied to DEAE-cellulose and the enzyme was eluted at a flow rate of 12 ml/h, this step permit to obtain 380.61 units of GAPDH in 76.23 mg of protein. The elution volume of the fraction with maximal activity was ~15 ml. The recuperated enzyme solution was loaded onto Mono-S column, which was extensively washed to remove contaminating proteins, and then the GAPDH was eluted with a linear gradient of KCl (0–300 mM) at a flow rate of 12 ml/h. A value of ~44.5 U/mg of protein was obtained for the purified enzyme with a yield of ~8% and a purification factor of ~68 folds (**Table 1**).

The SDS–PAGE analysis of the different fractions obtained during the purification procedure showed a progressive enrichment in 32-kDa protein [**Fig. 1(A)** ]. Only this protein band, which corresponds to the putative GAPDH subunit, was seen in the electrophoretically homogenous final enzyme preparation [**Fig. 1(A),** lane 4]. This protein band corresponds to the *T. thermophila* GAPDH subunit whose molecular weight could be estimated at 32,000 ± 1000 Da.

### Western blot analysis

Rabbit polyclonal antibodies have been produced against purified *T. thermophila* GAPDH. These antibodies selectively reacted by the immunoblotting procedure with a single immunoreactive band in both crude extract and purified preparations. **Figure 1(B)** shows the reaction GAPDH-antibodies which clearly recognize a single band of 32 kDa, corresponding to the GAPDH subunit. No protein bands were detected using the preimmune serum as the primary antibody.

### Molecular weight determination

To determine the molecular weight of the native enzyme, FPLC gel filtration was performed and yielded a value of ~120 kDa corresponding to the molecular weight of the native *T. thermophila* GAPDH (**Fig. 2**). This result compared with that obtained from SDS–PAGE, which shows a single band corresponding to the 32-kDa protein, suggesting that GAPDH purified from *T. thermophila* should have a homotetrameric structure.

### p*I* determination

Isoelectric focusing of the protein separation according to p*I* values showed a single protein band at 8.8 (the estimated p*I* for the enzyme) (**Fig. 3**). This result indicates that only one basic isoform of the enzyme occurs, and strongly suggests that a single copy of *gapC* gene is expressed.

### Effect of pH and temperature on the purified GAPDH activity

The pH activity profile of purified GAPDH was determined in a pH range from 4.0 to 9.5 using a mixture of different buffers. The enzyme has a typical bell-shaped profile covering a broad pH range [**Fig. 4(A)**]. The maximum enzymatic activity occurred at ~pH 8.0.

The influence of temperature on enzymatic activities was determined between 5 and 65°C. Studies on the effect of temperature on enzyme activity (thermal activation) revealed that an increase in temperature increases enzyme activity until an optimal value of 30–35°C [**Fig. 4(B)**]. Preincubation of *T. thermophila* GAPDH for 10 min at temperature range varying from 5 to 40°C (thermal denaturation) did not irreversibly affect the enzyme activity. Thermal inactivation did, however, occur above 40°C. The increase in temperature after 40°C does not increase the kinetic energy of the enzyme but instead disrupts the forces maintaining the shape of the molecule; the enzyme is gradually denatured causing changes in the shape of the active site. Temperatures >60°C completely denature the enzyme.

### Kinetic studies of purified GAPDH

Initial velocities of the enzymatic reaction were determined by varying the concentration of substrates $NAD^+$ (from 0.02 to 0.32 mM) or D-G3P (from 0.02 to 0.64 mM), since GAPDH catalyzes a two-substrate reaction. Apparent $K_m$ values for $NAD^+$ and D-G3P were estimated to be 102 ± 12 and 360 ± 18 μM, respectively. The $V_{max}$ of the purified protein was estimated to be 39.40 ± 2.95 U/mg (**Table 2**). The $K_m$ $NAD^+$ of *T. thermophila* GAPDH is

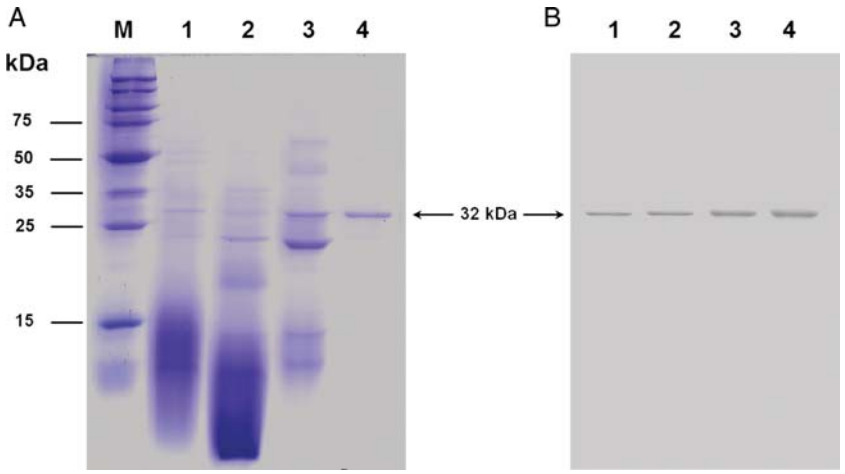


**Figure 1 Purification of GAPDH from *T. thermophila*** (A) Coomassie-stained SDS–PAGE showing the protein patterns of different fractions obtained during GAPDH purification from *T. thermophila*. M, standard proteins; 1, crude extract (soluble protein fraction); 2, 55%–88% ammonium sulfate protein fraction; 3, DEAE-cellulose fraction pool; 4, Mono-S eluate activity pool. A similar amount of protein (~30 μg) was applied to each lane. (B) Western blot analysis using polyclonal antibodies specific to *T. thermophila* GAPDH. 1, crude extract (soluble protein fraction); 2, 55%–88% ammonium sulfate protein fraction; 3, DEAE-cellulose fraction pool; 4, Mono-S eluate activity pool. The arrow points to the 32-kDa GAPDH subunit band.

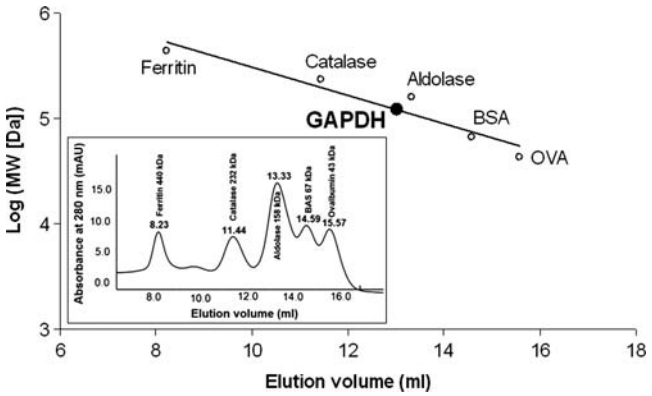


**Figure 2 Determination of native *T. thermophila* GAPDH molecular weight by Superdex 200 HR column** Proteins were applied to a Superdex column. Gel filtration markers used to calibrate the column and to set up a standard curve were ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa), and ovalbumin (43 kDa). Proteins were detected by UV absorbance at 280 nm.

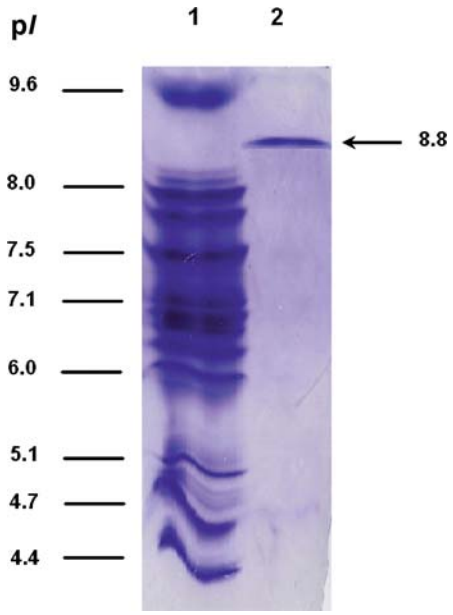


**Figure 3 Isoelectric focusing of *T. thermophila* GAPDH** Isoelectric focusing is carried out in polyacrylamide slab gel (5% w/v acrylamide) holding an ampholite-generated pH gradient (pH range, 3.5–10). 1, standard protein; 2, purified *T. thermophila* GAPDH (30 μg). The arrow points to the GAPDH isoelectric point (p*I*) of 8.8.

clearly lower, therefore suggesting a higher affinity for the nucleotide co-enzyme.

## Effect of $H_2O_2$ and SNP on the purified *T. thermophila* GAPDH

In order to investigate the physiological effect of oxidative and nitrosative stress on *T. thermophila*, we selected GAPDH as $H_2O_2$ and NO-sensitive enzyme, based on previous studies in animal cells, *Tetrahymena pyriformis* [20] and *Saccharomyces cerevisiae* [21]. The purified *T. thermophila* GAPDH was incubated at various concentrations of two agents of stress; $H_2O_2$ and SNP a NO-donating compound. **Figure 5** shows the dose-effect curve on GAPDH activity. Both oxidative and nitrosative stress decreased the GAPDH activity until complete inhibition at 25 and 75 mM, respectively. Thus, the obtained results indicate that 1 mM $H_2O_2$ could inhibit the GAPDH activity up to 50% at 30°C within 30 min of incubation [**Fig. 5(A)**], while SNP decreased GAPDH activity by 50% within 5 mM at the same time period [**Fig. 5(B)**]. The $H_2O_2$ effect on GAPDH activity exhibited a standard monotonic sigmoid dose–response curve [**Fig. 5(A)**],

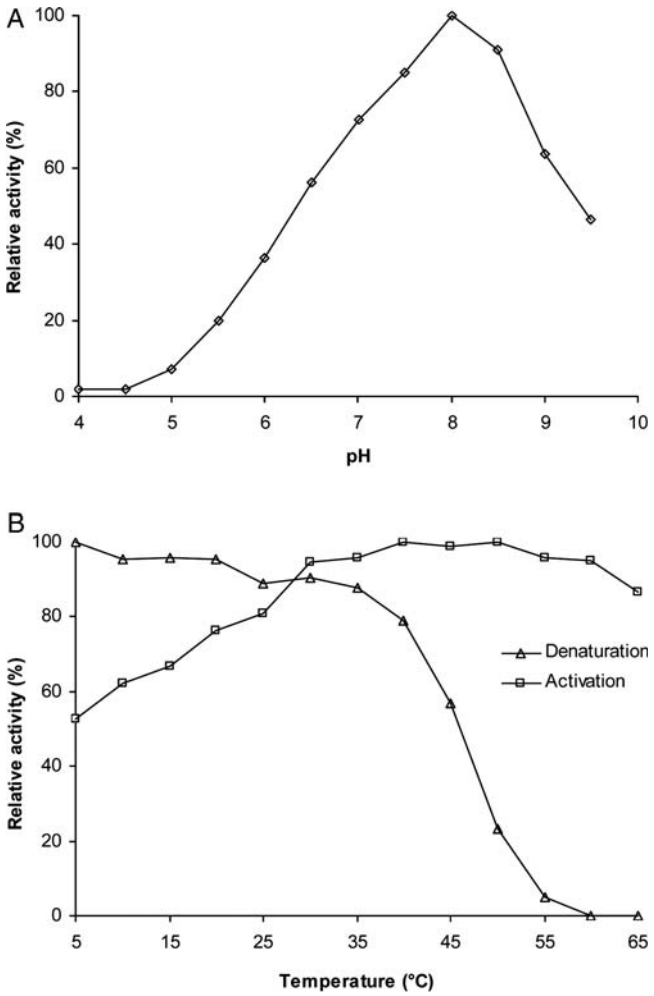


**Figure 4 Effect of pH and temperature on enzymatic activity of purified GAPDH from *T. thermophila*** (A) Enzymatic activity of purified GAPDH from *T. thermophila* in the pH range 4.0–9.5 using a mixture of different buffers. (B) Enzymatic activity of purified GAPDH at temperature range 5–65°C; effects of the essay temperature (activation) and the enzyme pre-incubating temperature (denaturation). Values are given as means of three separate experiments.

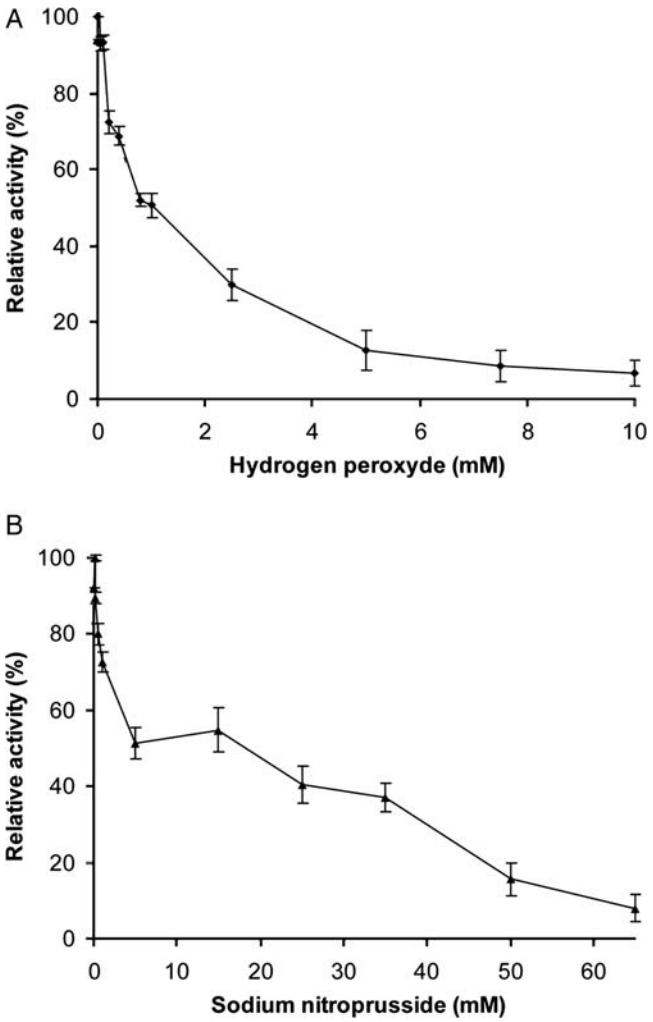


**Figure 5 Effect of oxidative and nitrosative stress on *T. thermophila* GAPDH activity** (A) Purified GAPDH inhibition by $H_2O_2$. GAPDH activity was measured following 30-min incubation with different concentrations of $H_2O_2$. (B) Purified GAPDH inhibition by SNP. GAPDH activity was measured following 30-min incubation with different concentrations of SNP. Values are given as means of three separate experiments.

**Table 2 Comparison of some kinetic and physicochemical parameters of GAPDH purified from *T. thermophila* and other organisms**

| Origin of GAPDH | $K_m$ (D-G3P, μM) | $K_m$ ($NAD^+$, μM) | $V_{max}$ (U/mg of protein) | Optimal pH | Optimal temperature (°C) |
|---|---|---|---|---|---|
| *T. thermophila* | 360 ± 18 | 102 ± 12 | 39.4 ± 2.9 | 8.0 | 30–35 |
| *T. pyriformis* [25] | 150 ± 5 | 5 ± 1 | 5.6 ± 0.8 | 8.5 | 35 |
| *S. pilchardus* [28] | 73.4 ± 8.1 | 92 ± 7.4 | 37.6 ± 2.9 | 8.0 | 28–32 |
| *P. waltl* [26] | 27 ± 11 | 60 ± 10 | 33.2 ± 5.7 | 8.5 | 40 |
| *C. dromedarius* [30] | 210 ± 80 | 25 ± 10 | 52.7 ± 5.9 | 7.8 | 28–32 |
| Human erythrocytes [29] | 20.7 | 17.8 | 4.3 | 8.5 | 40–45 |

while SNP exerted a biphasic dose-dependent inhibitory effect on the GAPDH activity as indicated by two different slopes in the curve (0–5 and 5–75 mM) [**Fig. 5(B)**]. Investigations are in progress to clarify these results.

## Discussion

GAPDH was purified to electrophoretic homogeneity, from crude cell extract of the ciliated protozoan *T. thermophila*,

using a procedure involving sulfate ammonium precipitation, followed by two chromatographic steps (anion exchanger and strong cation exchanger) which were carried out at 4°C to minimize protein degradation. As indicated above, SDS–PAGE and western blot analysis of the purified enzyme showed a single band corresponding to a 32-kDa protein (**Fig. 1**). This result, compared with the native molecular weight determined by FPLC gel filtration (120 kDa) (**Fig. 2**), suggests that the purified enzyme has a homotetrameric structure like other GAPDHs [22]. However, the molecular weight of the purified GAPDH subunit (32,000 Da) is somewhat lower than that estimated from the predicted protein (36,800 Da) from the same microorganism (*T. thermophila*) [23]. The nucleotide sequence of *T. thermophila* GAPDH gene (*gapC*) appear in GenBank with the following accession number AF319450.1. The difference observed between estimated and theoretical molecular weight can be the result of multiple types of post-translational events, such as alternative splicing, endoproteolytic processing, and post-translational modifications [24].

Analysis by isoelectric focusing technique of the purified GAPDH revealed only one isoform at 8.8 (the estimated p*I* for the enzyme) (**Fig. 3**). This result strongly suggests that a single copy of *gapC* gene is expressed. These data are in agreement with those reported by Zhao *et al*. [23]. A single *gapC* isoform has been found also in *T. pyriformis* [25] and other eukaryote and prokaryote organisms [26], but it seems not to be a general rule since the presence of several GAPDH isoforms has been reported in other organisms [27].

The optimal pH value for the enzymatic reaction of the purified GAPDH was 8.0 [**Fig. 4(A)**]. An identical value was obtained for the GAPDH of *Sardina pilchardus* [28]. However, for *T. pyriformis* GAPDH the optimal pH was 8.5 [25] (**Table 2**). On the other hand, studies on the effect of temperature on *T. thermophila* GAPDH activity revealed an optimal value of 30–35°C [**Fig. 4(B)**]. Similar value was obtained for *T. pyriformis* GAPDH [25]. However, GAPDH from human erythrocyte [29] and *Pleurodeles waltl* [26] exhibit different values of optimal temperature (40–45°C and 40°C, respectively) (**Table 2**).

Since GAPDH catalyzes a two-substrate reaction, values of the Michaelis constants ($K_m$) and the maximum velocities ($V_{max}$) for the reduction of $NAD^+$ and the oxidation of D-G3P by the purified GAPDH were determined graphically from Lineweaver–Burk double reciprocal plots [19]. The $K_m$ values for $NAD^+$ and D-G3P were estimated to be $102 \pm 12$ and $360 \pm 18$ μM, respectively. The $V_{max}$ of the purified enzyme was $39.40 \pm 2.95$ U/mg (**Table 2**). Therefore, the $K_m$ $NAD^+$ of *T. thermophila* GAPDH is clearly lower, suggesting a higher affinity for the nucleotide co-enzyme, as it was observed for *T. pyriformis* [25] and *Camelus dromedarius* [30]. For purified GAPDHs from *S. pilchardus* and *P. waltl*, the $V_{max}$ (37.6 and 33.24 U/mg, respectively) was similar to that obtained for *T. thermophila* GAPDH, and the $K_m$ $NAD^+$ of *S. pilchardus* GAPDH [28] was also similar to that estimated for *T. thermophila* GAPDH, but the $K_m$ $NAD^+$ of GAPDH from *P. waltl* [26] was different. Indeed, the $K_m$ D-G3P was ∼similar to those found for *T. pyriformis* [25] and *C. dromedarius* GAPDHs [30], but was different from those reported for *S. pilchardius* [28] and *P. waltl* GAPDHs [26] (**Table 2**).

Investigations on the effect of oxidative and nitrosative stress on *T. thermophila* GAPDH showed that both $H_2O_2$ and SNP decreased the GAPDH activity until complete inhibition (**Fig. 5**). It has been reported that the inactivation of GAPDH is caused mainly by interferences of radicals with the cysteine residue present in the active site which has been described to be *S*-thiolated by $H_2O_2$ [7] and *S*-nitrosylated by nitric oxide [8]. Also, it has been established that $H_2O_2$ modifies cysteine residues at the catalytic site of GAPDH resulting in both inactivation of GAPDH enzyme activity and structural changes in GAPDH [31]. Nitric oxide inhibits GAPDH activity by modifications of the thiols, which are essential for this activity, and that the modification includes formations of sulfenic acid [32]. Nitrosylation of Cys-149 in the active site of GAPDH attenuates enzymatic activity [33]. Here, we showed that 1 mM $H_2O_2$ could inhibit the GAPDH activity up to 50% at 30°C within 30 min of incubation [**Fig. 5(A)**], while SNP decreased GAPDH activity by 50% within 5 mM at the same time period [**Fig. 5(B)**]. These observations are in accordance with those reported by Fourrat e*t al*. [20] for the same enzyme from *T. pyriformis*, and suggest that GAPDH could be used to characterize the physiological effect of oxidative and nitrosative stress. Study in U937 cells (human promonocytes derived from a histiocytic lymphoma) has found that GAPDH is inactivated by the hydrogen peroxide, as a part of a cell defense pathway against oxidative stress induced apoptosis [6]. The degree of inhibition depends on the nature of stress, thus $H_2O_2$ should be most toxic than SNP, since $H_2O_2$ acts at lower concentrations.

In conclusion, this work is the first, at our knowledge, to be reported on purification and characterization of GAPDH from *T. thermophila* strain. The procedure used for this purification was rapid and straightforward involving two chromatographic steps, namely DEAE-cellulose and Mono-S. The purified *T. thermophila* GAPDH has a homotetrameric structure. This GAPDH differ in a number of instances from those described previously from other sources [25,26,28–30]. Indeed, the effect of two types of stress (oxidative and nitrosative) on this enzyme was studied, showing that oxidative stress is most toxic than

nitrosative stress. The physicochemical properties of this GAPDH, being characterized, could be used to evaluate the effect of both agents of stress ($H_2O_2$ and SNP) on the protozoan *T. thermophila*, especially the events occurring during the stress-treatment at the physiological and growth level.

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