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Purification and Characterization of Glyceraldehyde-3-phosphate Dehydrogenase from European Pilchard *Sardina pilchardus*

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Abstract The NAD^+-dependent cytosolic glyceralehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) was purified from the skeletal muscle of European pilchard *Sardina pilchardus* and its physicochemical and kinetic properties were investigated. The purification method consisted of two steps, ammonium sulfate fractionation followed by Blue Sepharose CL-6B chromatography, resulting in an approximately 78-fold increase in specific activity and a final yield of approximately 25%. The Michaelis constants (K_m) for NAD^+ and *D*-glyceraldehyde-3-phosphate were 92.0 μM and 73.4 μM, respectively. The maximal velocity (V_{max}) of the purified enzyme was estimated to be 37.6 U/mg. Under the assay conditions, the optimum pH and temperature were 8.0 and 30 ºC. The molecular weight of the purified enzyme was 37 kDa determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Non-denaturing polyacrylamide gels yielding a molecular weight of 154 kDa suggested that the enzyme is a homotetramer. Polyclonal antibodies against the purified enzyme were used to recognize the enzyme in different sardine tissues by Western blot analysis. The isoelectric point, obtained by an isoelectric focusing system in polyacrylamide slab gels, revealed only one GAPDH isoform (pI 7.9).

Keywords glyceraldehyde-3-phosphate dehydrogenase; *Sardina pilchardus*; skeletal muscle; purification; kinetics

During the past 20 years, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) has been recognized as playing an integral role in glycolysis [1]. It is not only a simple classical metabolic protein involved in energy production, but also a multifunctional protein with defined functions in numerous subcellular processes [2].

A number of new additional functions for GAPDH have been described. These new roles include its requirement for transcriptional control of histone gene expression, its essential function in nuclear membrane fusion, its necessity for the recognition of fraudulently incorporated nucleotides in DNA, and its mandatory participation in the maintenance of telomere structure [2,3]. These new functions require GAPDH association into a series of multi-enzyme complexes. Although other proteins in those complexes are variable, GAPDH remains the single constant protein in each structure.

This enzyme is broadly distributed in nature in a variety of organisms ranging from bacterial cells to human tissues [4–7]. It is found for the most part in the cytosol, mitochondria, and chloroplasts in plants. Generally, these molecules are all tetramers composed of apparently identical subunits [6,8,9] and have molecular weights ranging from 120 to 200 kDa [6,10].

This glycolytic protein has been extensively studied and widely characterized because of its central role in the intermediary metabolism, its high concentration in muscle tissue [11,12], its ease of preparation, and its conservation during evolution. That is, the rate of evolution of the catalytic domain is only 3% per 100 million years. Thus, these domains in eukaryotic and prokaryotic enzymes are 60% identical [13].

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In the present study, we wondered if some of the distinguishing characteristics of other GAPDHs are in any way evident in the features of sardine GAPDH. The reasons for choosing the enzyme GAPDH are manifold. GAPDH has been well studied and a considerable amount of work has been done on this enzyme, providing a useful model to study the context of oogenesis and embryonic development from teleost [14]. It has also been studied as an allozymic polymorphic marker in the investigation of the stock structure of different marine fishes [15,16].

Here, we report the isolation and biochemical characterization of skeletal muscle GAPDH from *Sardina pilchardus* Walbaum on the basis of the apparent native and subunit molecular weights, isoelectric point (pI), Western blot analysis, and physicochemical and kinetic characterizations of the purified enzyme.

Materials and Methods

Animals

The European pilchard *S. pilchardus* (Walbaum 1792) is one of the most important species of small pelagic fishes of the north-west coast of Africa [17]. Its distribution extends from Cap Blanc (21º N) to Cap Spartel (35º45′ N), and this pelagic species are very active all day. The samples were original from the Moroccan coastline. Animals used in this study were purchased from fishermen operating small fishing vessels, and they were immediately frozen at –20 ºC until use.

Enzyme purification

The enzyme was purified from skeletal muscle using the previously described procedure [18,19], which was modified according to our conditions. All steps were carried out at 4 ºC.

Pieces of dorsal muscle tissue of sardine (approximately 30 g, fresh weight) were ground and homogenized using an Ultra-Turrax homogenizer T25 basic (Fisher Bioblock Scientific, Illkirch, France) in 25 mM Tris-HCl buffer (pH 7.5) containing 5 mM EDTA and 10 mM 2 β-mercaptoethanol, at a ratio of 3 ml/g fresh tissue. The supernatant (soluble protein fraction), obtained after centrifugation at 15,000 *g* for 45 min, was considered as the crude extract.

The crude extract was subjected to protein precipitation in the 66%–88% (*W*/*V*) saturation range of ammonium sulfate. The final pellet was dissolved in a minimal volume of 25 mM Tris-HCl buffer (pH 7.5), containing 5 mM EDTA and 10 mM 2 β-mercaptoethanol (buffer A). The protein solution was dialyzed twice against 2 l of the same buffer overnight.

The dialyzed enzyme preparation was applied to a Blue Sepharose CL-6B column (1 cm×10 cm) equilibrated with 100 ml buffer A (approximately 10 times bed volume). The column was washed with 10 bed volumes of buffer A and 5 bed volumes of the same buffer adjusted to pH 8.5 (buffer B). The enzyme was eluted with buffer B containing 10 mM NAD^+ at a flow rate of 10 ml/h. Active fractions were collected, pooled and preserved in 50 % glycerol (*V*/*V*) at –20 ºC.

GAPDH activity determination

GAPDH activity was determined spectrophotometrically at 25 ºC by monitoring NADH generation at 340 nm [20]. The reaction mixture (1 ml) contained 50 mM tricine-NaOH buffer (pH 8.5), 10 mM sodium arsenate, 1 mM NAD^+ and 2 mM *D*-G3P. During enzyme purification, a coupled assay in which aldolase (1 U/ml) produced the stoichiometric breakage of *D*-fructose 1-6 biphosphate (2 mM) to dihydroxyacetone-phosphate and *D*-G3P that was the actual substrate of the oxidative reaction [21], was usually used.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [26] on 12% polyacrylamide slab gels containing 0.1% SDS. Gels were run on a miniature vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, USA). After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 at 0.2% (*W*/*V*) in the mixture of methanol/acetic acid/water (4:1:5, *V*/*V*/*V*) for 30 min at room temperature.

The apparent subunit molecular weight was determined by measuring and comparing relative mobility of the following pre-stained SDS-PAGE molecular weight standards (Low Range MW; Bio-Rad): myosin, 212 kDa; β-galactosidase, 116 kDa; phosphorylase B, 97.4 kDa, BSA, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; trypsin inhibitor, 20 kDa; and lysozyme, 14.4 kDa.

Determination of native molecular weight was carried out by electrophoresis on non-denaturing polyacrylamide slab gels (Bio-Rad, Hercules, USA) using the following protein standards: amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; dimeric BSA, 132 kDa; monomeric BSA, 66 kDa; ovalbumin, 45 kDa; and lactalbumin, 14.2 kDa. As described by the method of

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Hedrick and Smith [24], a calibration curve can be calculated from the relative mobility of standard proteins on non-denaturing polyacrylamide gels with different acrylamide concentrations (6%, 8%, 10%, and 12%, *W*/*V*). By constructing the Ferguson plot [Log (R_f×100) versus the concentration of polyacrylamide gels (%)], the resulting slopes versus the known molecular weights of standard native proteins allowed the determination of the native molecular weight of purified GAPDH.

Isoelectric focusing [25] was carried out with the same electrophoresis system in 5% polyacrylamide slab gels holding ampholite-generated pH gradients in the range 3.5−10 (Pharmacia Biotech, Uppsala, Sweden), using 25 mM NaOH and 20 mM acetic acids as cathode and anode solutions, respectively. The standard protein marker (Bio-Rad) was applied to estimate the pI of the purified enzyme.

Preparation of polyclonal antiserum

Polyclonal antiserum was raised in New Zealand White rabbits to GAPDH that had been purified to electrophoretic homogeneity from *S. pilchardus* skeletal muscle. The purified protein (approximately 0.5 mg) was mixed with Freund's complete adjuvant and injected subcutaneously into rabbits in multiple places, as described by Vaitukaitis [27]. Rabbits were boosted three times at 3-week intervals and bleeding was done 10 days later.

Western blot analysis

Proteins were separated by SDS-PAGE as described previously. Separated protein bands were electrophoretically transferred from the slab gel to a nitrocellulose membrane (Schleicher and Schuell, Keene, USA) using a Bio-Rad Trans-Blot system. Transferred proteins were then visualized by pre-staining in 0.2% (*W*/*V*) Ponceau red in trichloroacetic acid. The nitrocellulose membrane was then incubated for 1 h in blocking solution containing 5% (*W*/*V*) non-fat dry milk, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.01% (*W*/*V*) NaN_3, and 0.05% (*V*/*V*) Tween-20, followed by incubation with the anti-GAPDH antiserum (1:1000 dilution) as a primary antibody. Western blots were visualized by coupled immunoreaction with peroxidase-conjugated goat anti-rabbit immunoglobulin G (1: 1000; Boehringer Mannheim, Hamburg, Germany) as a secondary antibody using 4-chloro-1-naphthol as a chromogenic substrate.

Tissue distribution

Proteins were prepared from skeletal muscle, liver, brain, and ovary as described previously and subject to Western blot analysis.

Kinetic studies

For kinetic studies, initial velocities of the enzymatic reaction were carried out by varying the concentration of the substrates, *D*-G3P (from 0.04 to 10 mM) or NAD^+ (from 0.02 to 2 mM). Values of the Michaelis constants (K_m), dissociation constants (K_D), and maximal velocity (V_{max}) were obtained by mathematical calculation according to the method of Cleland [22]. One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of 1 μmol NADH/min under the conditions used. Protein concentrations were estimated by the method of Bradford [23] using bovine serum albumin (BSA) as a standard. Activity levels in cell-free extracts were expressed as specific activity (U/mg protein).

Influence of pH and temperature on purified GAPDH activity

To determine optimal pH, enzymatic activity was measured over a wide range of pH values (4–11) using a mixture of different buffers with different pKa (Tris, MES, HEPES, potassium phosphate at 50 mM, and sodium acetate at 180 mM) adjusted to the same ionic strength than the standard reaction mixture.

Temperature effects were characterized by activation and denaturation processes. For activation, the tricine-NaOH buffer (50 mM; pH 8.5) was incubated for 10 min at temperatures from 15 ºC to 85 ºC using a thermostated cuvette holder connected with a refrigerated bath circulator. Then 2 mM NAD^+, 200 mM sodium arsenate, and 10 μg purified GAPDH were added to the mixture. The reaction was started immediately by addition of 10 mM *D*-G3P. For denaturation, 10 μg purified GAPDH was incubated at temperatures from 15 ºC to 85 ºC for 10 min in the 50 mM tricine-NaOH buffer. Then 2 mM NAD^+ and 200 mM sodium arsenate were added. The enzymatic activities were measured after 2 min incubation at 25 ºC immediately started by addition of 10 mM *D*-G3P.

Results

Enzyme purification and characterization

GAPDH was purified from a soluble protein fraction of the European pilchard *S. pilchardus* skeletal muscle to electrophoretic homogeneity using a simple procedure involving only one chromatography step, namely dye-affinity chromatography on Blue Sepharose. **Table 1**

Table 1 Purification of GAPDH from skeletal muscle of *Sardina pilchardus* (Walbaum 1792)

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude extract	4700	2176.1	0.46	1.00	100
Ammonium sulfate (66%–88%)	840	1300.0	1.54	3.34	60
Blue Sepharose CL-6B	15	536.3	35.75	77.71	25

summarizes a representative purification protocol. Approximately 36 U/mg protein was obtained for the specific activity of the purified enzyme with a yield of 25% and an approximately 78-fold increase of purification.

SDS-PAGE analysis of the different fractions obtained during the purification procedure showed a progressive enrichment of a 37 kDa protein [**Fig. 1(A)**]. Only this protein band, the same size as the GAPDH subunit monomer, was seen in the electrophoretically homogeneous final enzyme preparation [**Fig. 1(A)**, lane 4].

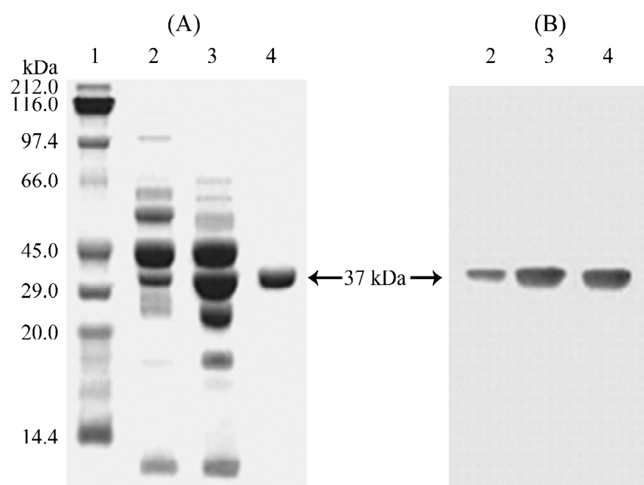

Fig. 1 Purification of GAPDH from *Sardina pilchardus* skeletal muscle detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (A) and Western blot analysis (B)

Purification was carried out from dorsal skeletal muscle. Proteins at each purification step were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue (A) or subjected to Western blot analysis (B) using polyclonal antibodies specific to sardine GAPDH. Lane 2, crude extract (20 μg protein); lane 3, protein preparation from the ammonium sulfate fractionation (20 μg); lane 4, Blue Sepharose fractions pool (20 μg protein). The positions and apparent molecular weights of pre-stained standard proteins loaded in lane 1 [**Fig. 1(A)**] have been indicated. Bound antibody was located by coupled immunoreactions with peroxidase-conjugated goat anti-rabbit immunoglobulin G. The arrow indicates the band corresponding to the 37 kDa GAPDH.

Non-denaturing PAGE showed that the native molecular weight of obtained protein was approximately 154 kDa (**Fig. 2**). The value of pI was found with the isoelectric focusing technique that showed a single protein band with a pH value of 7.9 (the estimated pI for the enzyme) (**Fig. 3**).

Western blot analysis

We have produced rabbit polyclonal antibodies using purified sardine muscle GAPDH as immunogen. These

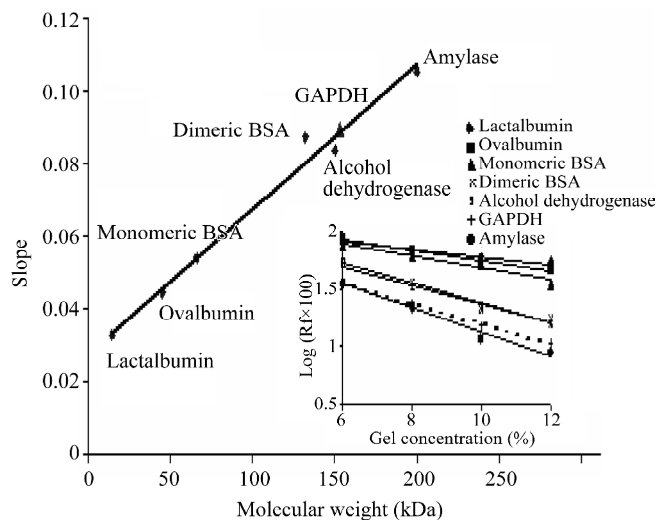

Fig. 2 Determination of GAPDH from the molecular weight native *Sardina pilchardus* by non-denaturing polyacrylamide gel electrophoresis

Proteins were electrophoresed on various acrylamide concentration gels (6%, 8%, 10%, and 12%) under non-denaturing conditions. Molecular weight marker proteins were amylase (200 kDa), alcohol dehydrogenase (140 kDa), dimeric bovine serum albumin (BSA; 132 kDa), monomeric BSA (66 kDa), and lactalbumin (14.2 kDa). Relative mobility of proteins plotted as Log R_f versus acrylamide concentration is indicated on the inset. A plot of the obtained slopes versus molecular weight was linear and used to determine native sardine GAPDH molecular weight.

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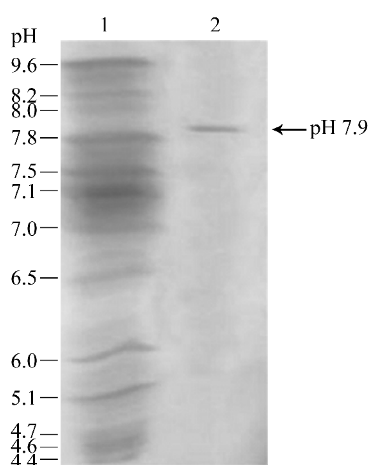

Fig. 3 Isoelectric focusing of GAPDH from *Sardina*
Isoelectric focusing was carried out in 5% polyacrylamide slab gels holding an ampholyte-generated pH gradient (pH range, 3.5–10.0). 1, standard protein; 2, purified GAPDH (approximately 25 μg).

antibodies selectively reacted by the immunoblotting procedure with a single immunoreactive band in both crude extract and purified preparations. **Fig. 1(B)** shows that the relative molecular weight of the detected protein was the same as the expected one for the GAPDH monomer (37 kDa).

The same protein band was recognized by the anti-GAPDH antiserum in the different tested tissues [**Fig. 4 (A)**] including skeletal muscle, liver, brain, and ovary. No protein bands were detected by non-immune rabbit serum, confirming specificity. The data show that the skeletal muscle, compared with any other tested organs, presents the highest expression level and relative activity of GAPDH [**Fig. 4(B)**].

Kinetic properties and influence of pH and temperature on purified GAPDH activity

Because GAPDH catalyzes a two-substrate reaction, the K_m values for NAD^+ and *D*-G3P were determined by varying the concentration of one of the substrates (*D*-G3P or NAD^+) and keeping a constant concentration of the other. And the K_m values for NAD^+ and *D*-G3P were determined to be 92.0 and 73.4 μM, respectively (**Table 2**). The value for V_{max} was 37.6 U/mg.

Table 2 Kinetic constants (K_m and V_{max}) for oxidation reaction of GAPDH from *Sardina pilchardus*

	K_m G3P (μM)	K_m NAD^+ (μM)	K_D NAD^+ (μM)	V_{max} (U/mg)
GAPDH	73.4±8.1	92.0±7.4	173.1±28.2	37.6±2.9

Pre-incubation of sardine GAPDH for 10 min at temperatures varying between 15 ºC and 30 ºC did not irreversibly affect the enzyme activity. Thermal inactivation, however, occurred above 35 ºC and resulted in total activity loss at 60 ºC [**Fig. 5(A)**]. Studies on the effect of temperature on enzyme activity revealed an optimal value in the 28–32 ºC interval. The optimal value of pH for the oxidative reaction was approximately 8 [**Fig. 5(B)**] with considerable GAPDH activity in the range 7.5–8.5.

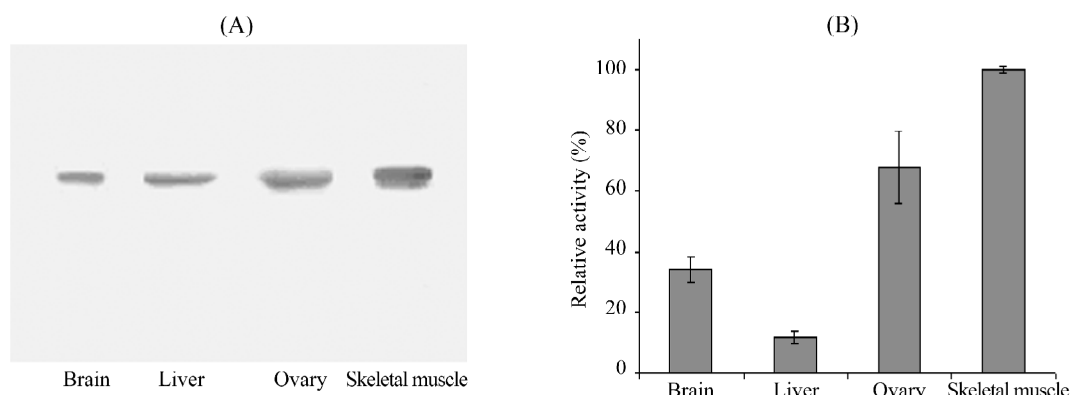

Fig. 4 Tissue distribution of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in *Sardina pilchardus*
Tissue distribution of GAPDH was detected by Western blot analysis (A) and also shown as relative activity (%) (B).

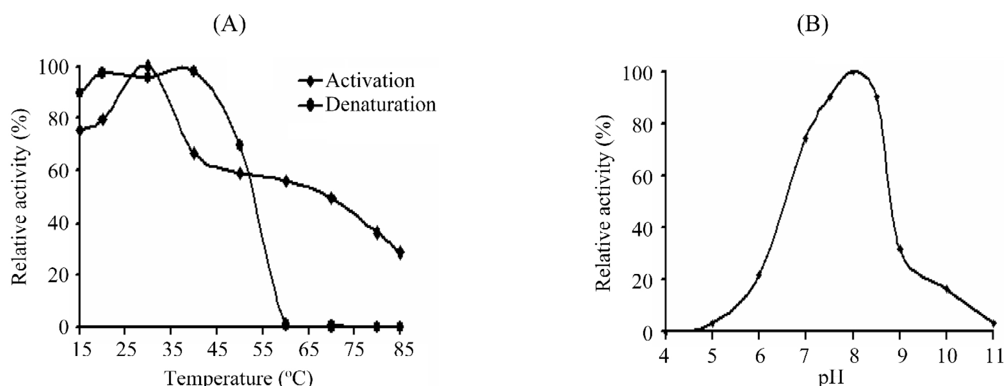

Fig. 5 Effects of temperature and pH on relative activity of GAPDH from *Sardina pilchardus*
(A) Relative activity of purified GAPDH from skeletal muscle of *Sardina pilchardus* after activation or denaturation at different temperatures (from 15 °C to 85 °C). (B) Relative activity of the purified GAPDH in the pH range 4–11 using a mixture of different buffers. Values are given as means of three separate experiments.

Discussion

GAPDH is the most highly conserved protein of all glycolytic enzymes. It plays a key role in central carbon metabolism and shows both genetic and post-translational regulations [2]. This enzyme is responsible for the oxidative phosphorylation of G3P in the presence of NAD^+ and inorganic phosphate. In this work, GAPDH from *S. pilchardus* skeletal muscle was purified to electrophoretic homogeneity from a soluble protein fraction of approximately 4700 mg corresponding to approximately 2176.1 U GAPDH, and purification fold of 78.

The purification of the enzyme was carried out by a simple procedure involving only one chromatography step. As for other NAD^+ dependent GAPDHs [18,28,29], dye-affinity chromatography on Blue Sepharose was very effective for this purification and no additional steps were required to obtain homogeneous GAPDH samples. As stated above, SDS-PAGE of the purified enzyme showed a single band corresponding to a 37 kDa protein [**Fig. 1(A)**, lane 4]. This result, compared with the native molecular weight (154 kDa), suggests that the enzyme has a homotetrameric structure like other GAPDHs [10,12,18]. However, the pilchard enzyme subunit had an estimated molecular weight of approximately 37 kDa, somewhat higher than the one reported for the GAPDH subunit of different organisms like bacterial specie *Bacillus cereus* (35 kDa) or amphibian *Xenopus* sp. (36 kDa) [30,31]. The production of the polyclonal antibodies, from the purified GAPDH sardine as immunogen, has confirmed that the relative molecular weight of the detected protein is the expected one for the GAPDH monomer (37 kDa) in the different tested tissue, skeletal muscle, liver, brain, and ovary, with a significantly high expression level and relative activity in muscle compared with any other organs, as reported for several species in other works [4,12]. The pI value was estimated by the isoelectric focusing technique. A single protein band was observed at pH 7.9, the estimated pI for the enzyme (**Fig. 3**). It strongly suggests that a single *gapC* gene is expressed in this tissue. Therefore, this result indicates that only one slightly basic isoform of the enzyme occurs in skeletal muscle of the teleost sardine, as in other aquatic species such as *Electrophorus electricus* [32]. A single GAPDH isoform has been also found in some other animal tissues and microorganisms, both prokaryotes and eukaryotes [4,12,31]. However, it does not seem to be a general rule, as the presence of several GAPDH isoforms has been reported in organisms phylogenetically very different [3,4,19,20,33].

The obtained value for V_{max} was 37.6 U/mg. The K_m of *D*-G3P was approximately similar to those found for cytosolic GAPDHs purified from other unicellular organisms such as *Tetrahymena pyriformis* [29] or mammalians like *Jaculus orientalis* and human tissues [20, 34]. Whereas the K_m of NAD^+ of sardine GAPDH is clearly higher, suggesting therefore a lower affinity for the nucleotide co-enzyme, as it was observed for pleurodeles GAPDH [12]. This indicates a possible difference in the mechanism of the catalytic reaction of sardine GAPDH with a relatively higher V_{max} value compared to those of *Tetrahymena*, jerboa liver, and human tissues. The kinetic parameter values obtained for the purified GAPDH differ in a number of instances from those described previously [18,29,33,34], reflecting protein differences between species.

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In order to confirm these differences, we are planning to sequence the protein corresponding gene and establish phylogenetic relationships for the sardine enzyme, the first GAPDH from a teleost fish studied so far, with other GAPDHs of eukaryotic and prokaryotic organisms.

These results are the first to be reported on characterization of GAPDH purified to homogeneity from *S. pilchardus*. The procedure used for this purification was rapid and straightforward involving one chromatographic step, namely dye-affinity on Blue Sepharose. The kinetic parameter values differed in a number of instances from those described previously from other sources, reflecting protein differences between species [12,18,32–34]. The physicochemical properties of this GAPDH, being characterized, could be used in further studies for the differentiation and discrimination of natural populations of *S. pilchardus*.

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