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Cytosolic glutathione peroxidase from liver of pacu (*Piaractus mesopotamicus*), a hypoxia-tolerant fish of the Pantanal

V.L.F. Cunha Bastos [a], J.B. Salles [b], R.H. Valente [c], I.R. León [c], J. Perales [c], R.F. Dantas [a], R.M. Albano [a], F.F. Bastos [a], J. Cunha Bastos [a,*]

[a] *Department of Biochemistry, UERJ, Av. Professor Manuel de Abreu, 444, Rio de Janeiro, RJ 20550-170, Brazil*
[b] *Laboratory of Chemistry and Function of Proteins and Peptides, North Fluminense State University, Rio de Janeiro, Brazil*
[c] *Department of Physiology and Pharmacodynamics, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil*

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Abstract

Pacu (*Piaractus mesopotamicus* Holmberg, 1887, Characiformes) dwells in waters of Pantanal, in which it has adapted for alternate concentrations of dissolved oxygen. Intracellular antioxidant protection should be vital for such an adaptation. Accordingly, we found that cytosol from liver of pacu has the highest antioxidant glutathione peroxidase activity so far reported for fish and murine species. To clarify whether this activity was due to a selenium independent glutathione *S*-transferase or to a glutathione peroxidase, we purified it and studied its kinetics. The substrates cumene hydroperoxide and hydrogen peroxide were promptly reduced by the enzyme, but peroxidized phosphatidylcholine had to undergo previous fatty acid removal with phospholipase A_2. Augmenting concentrations (from 2 to 6 mM) of reduced glutathione activated the pure enzyme. Curves of velocity versus different micromolar concentrations of hydrogen peroxide in the presence of 2, 4 or 8 mM reduced glutathione indicated that at least 2.5 mM reduced glutathione should be available *in vivo* for an efficient continuous destruction of micromolar concentrations of hydrogen peroxide by this peroxidase. Molecular exclusion HPLC and SDS–polyacrylamide gel electrophoresis indicated that the purified peroxidase is a homotetramer. Data from internal sequences showed selenocysteine in its primary structure and that the enzyme was a homologue of the type-1 glutathione peroxidase found in rat, bull, trout, flounder and zebra fish. Altogether, our data establish that in liver cells of pacu, a hypoxia-tolerant fish from South America, there are high levels of a cytosolic GPX-1 capable of quenching hydrogen peroxide and fatty acid peroxides, providing an effective antioxidant action.

Keywords: Glutathione peroxidase; GPX; Hypoxia; Pacu; Pantanal

1. Introduction

Piaractus mesopotamicus is a fish species (locally called ''pacu'') of the South American Pantanal which evolved in waters with high and low oxygen concentrations that recur throughout the inundation pulse, the annual flood that acts as the driving force behind this ecosystem of 140,000 square kilometres. In water of several lagoons, which remain in lowland areas of Pantanal until the next flood, the amount of dissolved oxygen can vary from normoxia to hypoxia or fully anoxia. Pacu migrates to smaller streams, channels and the floodplain during the flood season. When the flood recedes, pacu moves to the riverbeds where it stays in deep pools until the subsequent inundation period. Many hundreds of them, though, fall behind in lakes and ''bacias'', which are miles-long shallow lakes, where they survive under oxygen concentrations that can reach less than 1 mg L^{-1} [1,2].

Oxygen is highly important to many physiological processes in fish [3], but seasonal and daily changes in dissolved oxygen expose fish to potentially harmful oxidative cycles. Following anoxia, sudden exposure of fish to well-oxygenated water may submit their cells to an abrupt oxidative stress.

* Corresponding author. Tel.: +55 21 2587 6429; fax: +55 21 2587 6136.
E-mail address: jbastos@uerj.br (J.C. Bastos).

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Interestingly, it has been reported that a sudden increase in the intracellular oxidative potential is tolerated by carp [4], goldfish [5] and turtles [6].

Oxidative stress occurs when reactive oxygen species, such as superoxide ion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen (O_1) react with lipids, proteins, carbohydrates or nucleic acids producing malfunctioning derivatives responsible for several biochemical injuries [7,8]. In order to avoid an increase in the concentrations of reactive oxygen species beyond a tolerable threshold, organisms rely on non-enzymatic and enzymatic antioxidants [9,10]. The selenium-dependent reductive split of H_2O_2 into two molecules of H_2O, carried out by glutathione peroxidase (GPX; EC 1.11.1.9) isoenzymes, is considered an important enzymatic activity for the antioxidant quenching of H_2O_2 in many aerobic organisms including aquatic animals [5,11]. Noteworthily, the levels of GPX activity in kidney, gill and liver from specimens of the catfish *Wallago attu* kept in water with 2.0 mg L^{-1} dissolved oxygen did not parallel those kept in water with 7.7 mg L^{-1} dissolved oxygen; in the oxygen-depleted water *W. attu* showed an increased GPX activity in kidney and gill, but diminished in liver [12]. Peroxidase activity of some alpha glutathione *S*-transferase isoenzymes (alpha-GST) is also considered an enzymatic defence against oxidative injury [13,14]. With regard to this, an alpha-GST from plaice has been shown to decompose organic peroxides [15] and an alpha-GST from largemouth bass, capable of conjugating the unsaturated cytotoxic 4-hydroxynonenal produced during lipid peroxidation [16], has already been cloned [17].

There is no information on the biochemistry of the antioxidant enzymes that might occur in pacu cells to facilitate its adaptation to waters with different oxygen concentrations. In our hands, activity on reducing cumene hydroperoxide (CHP) was 10 to 15 times higher in cytosol of liver than in cytosol of gut, kidney, heart and brain of pacu. Since CHP is a substrate for assaying both GPX and alpha-GST, we thought it was important to investigate this enzymatic CHP-reducing activity in order to know more about the enzymatic antioxidant defences that pacu could carry out in response to hypoxia-normoxia cycles. Accordingly, this paper describes the purification and the biochemical characterization of a cytosolic glutathione peroxidase from liver of pacu, and presents its main kinetic characteristics.

2. Material and methods

2.1. Reagents and apparatus

All chemicals were purchased from Sigma Chemical Co. with the exception of the following. Phospholipase A_2 purified from snake venom was kindly donated by Dr Geórgia Atella from the Institute of Medical Biochemistry of the Rio de Janeiro Federal University. TEMED (*N,N,N′,N′*-tetramethylethylenediamine), 2-mercaptoethanol, ampholytes (Bio-Lyte®), the Rotofor® Cell and relative molecular mass (M_r) standards (M_r from 25,000 to 670,000) were obtained from Bio-Rad Laboratórios, Rio de Janeiro, RJ, Brazil. Polybuffer™ and Mono P HR 5/20 mm column for chromatofocusing were from GE Healthcare, São Paulo, SP, Brazil. A 7.9 mm × 25 cm Shim-pack Diol-150-Shimadzu column was purchased from Shimadzu do Brasil Ltd., São Paulo, SP, Brazil. Sep-Pak C18 cartridges were purchased from Waters Associates, Milford, MA, USA. Water for chromatography coupled to mass spectrometry was HPLC grade from Mallinckrodt Baker, Phillipsburg, NJ, USA. Trypsin was purchased from Promega, Madison, WI, USA, and alpha-cyano-4-hydroxycinnamic acid was from Applied Biosystems, São Paulo, SP, Brazil. All other chemicals were of analytical grade.

2.2. Fishes

Pacu specimens (*Piaractus mesopotamicus* Holmberg, 1887; Characiformes, Characidae) and tambaqui specimens (*Colossoma macropomum* Cuvier, 1816; Characiformes, Characidae) weighing 400–500 g (around 30 cm total length) were kindly donated by Morro Grande farm located in the municipality of Cachoeiras de Macacu, Rio de Janeiro, Brazil. Tilapia specimens (*Oreochromis niloticus* [Linnaeus, 1758]; Perciformes, Cichlidae) weighing 200–350 g (around 20 cm total length) were kindly donated by Sendas farm located in the municipality of Magé, Rio de Janeiro, Brazil. After arriving, these specimens were maintained in 1000 L (4.5 mg O_2 L^{-1}) tanks for 10 days with no food. The seawater tainha and parati specimens (*Mugil* sp.) weighing 280–310 g (around 26 cm total length) were caught from the shore waters of the south coast near Rio de Janeiro city and immediately euthanized.

2.3. Cytosolic fractions preparation

After anesthetizing the fish, their vertebral columns were severed. Their livers were excised through an abdominal incision, rapidly bathed in a cold 0.9% (w/v) NaCl solution and immediately frozen in liquid nitrogen until use. Immediately after being thawed, livers were mopped up quickly with filter paper, weighed and homogenized (1 g per 4 ml) in cold 0.1 M potassium phosphate buffer, pH 7.0, containing 0.25 M sucrose, using a Potter–Elvehjem tissue homogenizer [18]. Homogenates were centrifuged at 12,000 × *g* for 30 min. The supernatant was centrifuged at 105,000 × *g* for 90 min. Centrifugation steps were carried out at 5 °C. The 105,000 × *g* supernatant, named the cytosolic fraction, was kept frozen in liquid nitrogen until enzyme assays were carried out.

2.4. Determination of glutathione in pacu liver homogenates

Reduced glutathione (GSH) and oxidized glutathione (GSSG) amounts in liver of pacu were determined using the DTNB-GSSG reductase recycling assay [19]. Briefly, 0.18 g of liver was homogenized in 0.89 ml of 5% (w/v) sulphosalicylic acid, centrifuged at 12,000 × *g* for 5 min and the supernatant used as sample. Glutathione reductase reduced all GSSG to GSH, which in turn reacted with DTNB to produce thionitrobenzoic acid. The appearance of thionitrobenzoic