PARAOXONASE ACTIVITY IN LIVER OF PACU, *PIARACTUS MESOPOTAMICUS* HOLMBERG (CHARACIDAE)

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ABSTRACT. Enzymatic production of p-nitrophenol in liver of *Piaractus mesopotamicus* Holmberg, 1887 was consistently assayed at pH 8.5 using 7.5 mM paraoxon as substrate. This pacu liver paraoxonase activity was activated by NaCl. Apparent values of K_M were 2.42 x 10^{-3} M in the presence of 0.5 M NaCl and 8.99 x 10^{-3} M without NaCl. Apparent maximum velocity values calculated in the absence and presence of 0.5 M NaCl were 1.09 x 10^{-3} µmoles/min/mg of proteins and 1.29 x 10^{-3} µmoles/min/mg of proteins, respectively. These V_{max} values are fifty-fold the value described for trout (*Salmo trutta* Linnaeus, 1758) liver paraoxonase. Paraoxonase activity of pacu liver homogenates was recovered as much in cytosolic as in particulate cellular subfractions, but the particulate subfractions showed higher specific activities. The data presented here indicate that hepatic hydrolysis of organophosphorous pesticides may not be an important detoxification process in pacu.
KEY WORDS. Detoxication, esterase, fish, organophosphate

As a result of crop defensive practices aquatic animals may absorb pesticides from contaminated water drained off into rivers from surrounding farmland. Absorbed pesticides may harm fish. On the other hand, organophosphorous insecticides have been used to protect fish against parasites in fish-farming tanks (GRAVE *et al.* 1991a,b). Therefore there is a need to learn steadily more about fish toxicant metabolism.

Organophosphorous insecticides are toxic because they inhibit nerve synaptic acetylcholinesterase. Many organophosphorous substances have a phosphorothionate structure (P=S group) that cannot efficiently bind to cholinesterase: they must be bioactivated to produce the corresponding phosphate (P=O group) that is able to inhibit acetylcholinesterase by binding to the esterasic site of the enzyme. Several authors have referred to metabolization of xenobiotics by fish liver (PEDERSEN *et al.* 1976; BURNS 1976; HINTON *et al.* 1978, 1985; NOWAK 1996). In fact, the

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liver is the major organ involved in the organophosphorous bioactivation process (SULTATOS *et al.* 1985). Measuring acetylcholinesterase inhibition *in vitro*, we showed that parathion (O,O-diethyl, O-p-nitrophenyl phosphorothionate) is activated to paraoxon (O,O-diethyl, O-p-nitrophenyl phosphate) through a monooxigenase system existing in liver of *Hypostomus punctatus* Valenciennes, 1840, a Brazilian suckermouth catfish (CUNHA BASTOS *et al.* 1992). Liver homogenates of pacu, *Piaractus mesopotamicus* Holmberg, 1887, also showed the ability to produce inhibition of acetylcholinesterase during incubation with parathion *in vitro* (not published data from our laboratory).

As described in rat, before reaching the brain, where it causes toxic acetylcholinesterase inhibition, paraoxon produced from parathion in the liver undergoes several detoxicating chemical interactions (NEAL & DUBOIS 1965; PLA & JOHNSON 1989; POND *et al.* 1995). Among them, paraoxonase activity renders paraoxon ineffective by splitting it into p-nitrophenol and diethyl-phosphate (GAINES *et al.* 1966; NEAL 1967). Thus, high levels of paraoxonase are considered protective against poisoning caused by active metabolites of organophosphorous pesticides (GELDMACHER-VON MALLINCKRODT & DIEBGEN 1988; LI *et al.* 1995). In spite of what has already been published on mammals paraoxonase activity, we still have scarce biochemical information about any fish liver detoxicating esterase activities. In view of these facts, we felt the need to determine the enzymatic capability that liver of *P. mesopotamicus* could have of hydrolyzing paraoxon. The extent of such a reaction would allow the assessment of the efficiency with which liver of pacu minimizes the effect of organophosphate intoxication on the nerve system.

This paper describes procedures to establish the main optimal assay parameters of paraoxonase activity we found in *P. mesopotamicus* liver. Biochemical kinetic constants were calculated as long as their values give a good approximation regarding the magnitude of paraoxon hydrolysis in pacu liver. To better characterize the paraoxon esterase activity in pacu liver we studied how this activity is affected by NaCl, $CaCl_2$ and EDTA. Distribution of paraoxonase activity among liver cellular subfractions obtained trough differential centrifugation was investigated as an attempt to verify whether this activity could be typical of some intracellular organelle.

MATERIAL AND METHODS

Reagents

Trichloroacetic acid (TCA), Trizma® (2-amino-2-[hydroxymethyl-1,3-propanediol]), hydrochloric acid (HCl), paraoxon (O,O–diethyl O–p–nitrophenyl phosphate), EDTA (ethylenediamine tetracetic acid disodium salt) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. Hexane specific for pesticide analysis was purchased from Grupo Química Industrial Ltda., Rio de Janeiro, Brazil. All other chemicals were of analytical grade.

Fish

Specimens of *Piaractus mesopotamicus* (pacu) were obtained from the National Centre for Research on Tropical Fish (CEPTA) of the Brazilian Environment and Renewable Natural Resources Institute (IBAMA). Sexually mature

pacus measured around 23 cm from snout to tail and weighed approximately 150 g. The animals were kept in 500 L aerated tanks at room temperature. The tanks contained dechlorinated water and were equipped with biologic filter. All fish were acclimated for at least 10 days before being used. Fish were fed on the appropriate pellets produced in CEPTA and were exposed to normal light-dark day cycles.

Preparation of cellular subfractions

Fish were sacrificed by decapitation and their livers were removed, weighed, minced and homogenized in four volumes of cold 0.1 M potassium phosphate buffer, pH 7.0, containing 0.25 M sucrose. The homogenate was fractionated by centrifugation under refrigeration at 15,000 x g for 30 min in order to separate intact cells and nuclei. The nuclear pellet (P1) was set apart and the supernatant (S1) was centrifuged at 105,000 x g for 90 min. The second supernatant (S2) was decanted and set apart. The deposited microsomal fraction (P2) was washed once and suspended in the potassium phosphate buffer. Each subfraction was taken for protein and enzymatic activity determinations. Protein was determined by the modified (FARIA & BRASIL 1959) Greenberg method (GREENBERG 1929).
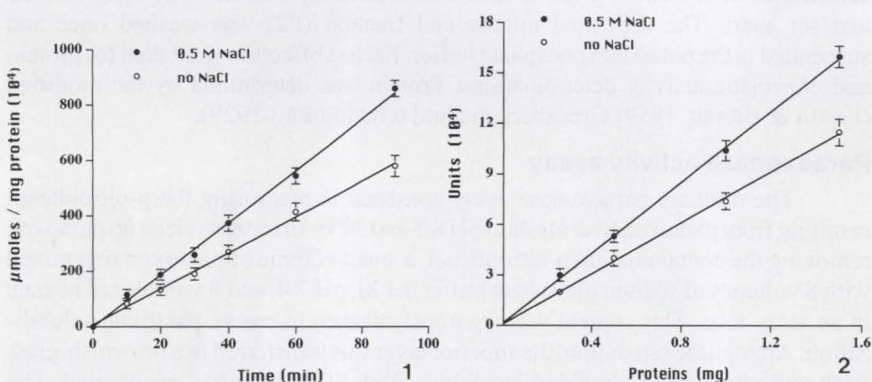
Paraoxonase activity assay

The ordinary paraoxonase assay consisted in measuring the p-nitrophenol resulting from paraoxon hydrolysis at pH 8.5 and 30°C. In order to clean up paraoxon removing the contaminating p-nitrophenol, a small volume of paraoxon was mixed with 8 volumes of sodium phosphate buffer 0.1 M, pH 7.4, and 8 volumes of hexane in an assay tube. This content was vigorously shaken to ensure paraoxon solubilization. After phase separation, the superior layer was transferred to a brownish-glass flask and nitrogen was bubbled inside this flask till hexane had evaporated. The residue was used as paraoxonase substrate. This simple procedure proved to be extremely useful to assure low blank absorbance values. Substrate was prepared as a 45 mM paraoxon solution made by mixing 20 mg of the repurified paraoxon with 726 µL of DMSO and 888 µL of 0.1 M Tris-HCl buffer, pH 8.5 (substrate solution). The assay contained a volume of sample corresponding to the required protein amount and 0.1 M Tris-HCl buffer solution, pH 8.5, to reach a final incubation volume of 300 µL. A 50 µL volume of the substrate solution were pipetted to start the enzymatic reaction. When salts were needed in the assays this substrate Tris-HCl buffered paraoxon solution also contained 3 M NaCl and 12 mM $CaCl_2$. Enzyme activity at different pH values and the velocity versus substrate concentration curves were performed as already described (CUNHA BASTOS *et al.* 1998). Different paraoxon concentrations were used in the range of 0.015 to 7.5 mM to perform v x [S] curves, in a constant concentration of DMSO (7.5%). To stop the reaction 0.9 mL of a 3% TCA (in water) were pipetted into the tubes at the end of each incubation time. In the blanks, a volume of 50 µL of the substrate solution was pipetted into the tubes (using solution which had been left along the entire reaction time under the incubation temperature) just after TCA addition. Then, the tubes were centrifuged at 4,000 rpm for 15 min and 0.9 mL aliquots of each supernatant were mixed with 0.2 mL of a 0.3 M Tris-HCl buffer solution, pH 8.0. Absorbance measurement in each tube was registered at 400 nm using a Shimadzu UV-160A spectrophoto-

meter in 1 cm optical path cuvettes. A p-nitrophenol standard curve (1.5 to 75 nmoles concentration range) was used for calculation of the amount of p-nitrophenol. Statistical calculations were performed using Student's unpaired t test. One unit of enzyme activity corresponded to 1 μmol of p-nitrophenol produced within one minute of reaction.

RESULTS

As it can be seen in figures 1 and 2 the velocity of paraoxonase reaction in pacu liver homogenates shows a linear progress within the time intervals and protein concentrations chosen. Less than 7% of the 7.5 mM added paraoxon was utilized over the 20 min assay time by 1 mg of liver homogenate proteins.


Figs 1-2. Paraoxonase liver activity. (1) As a function of incubation time and (2) as a function of protein (enzyme) amounts. Assays were performed at 30°C using 0.1 M Tris-HCl buffer solution, pH 8.5, 0.5 M NaCl, 2 mM CaCl₂, 1 mg of protein in each tube (only figure 1) and 7.5 mM paraoxon. Each point is the mean of five assays ± S.E.M.

The velocity curve versus pH of the incubation medium shows that the best pH to assay pacu liver paraoxonase activity was 8.5 (Fig. 3). Activities stimulated by 0.5 M NaCl were higher from pH 7.0 to 8.0 when compared to activities assayed without NaCl.

In figures 4 and 5 it is seen that both, NaCl and CaCl₂, activate liver paraoxonase activity. Adding sodium to the incubation medium increases paraoxonase activity around 25% to 43%. Concentrations between 0.5 M and 2.0 M NaCl produce a significant ($p < 0.05$) activation (Fig. 4). Calcium activates paraoxonase liver activity nearly two-fold. Significantly similar levels of activation are observed between 1 mM and 10 mM CaCl₂ (Fig. 5). EDTA is effective in inhibiting the paraoxonase activity of liver of pacu. It can be seen in figure 6 that activity is reduced to 25% in the presence of 0.25 mM EDTA.

Figures 7A and B clearly show that 0.5 M NaCl provokes a decrease in the affinity of liver paraoxonase for paraoxon. The apparent K_M value without NaCl in the assays (8.99 mM) is at least three-fold the apparent K_M calculated in the presence

of 0.5 M NaCl (2.42 mM). Accordingly, the V_{max}/K_M ratio values in the presence and absence of 0.5 M NaCl are 4.9×10^{-4} and 1.2×10^{-4} $min^{-1} \times g^{-1} \times L$, respectively.
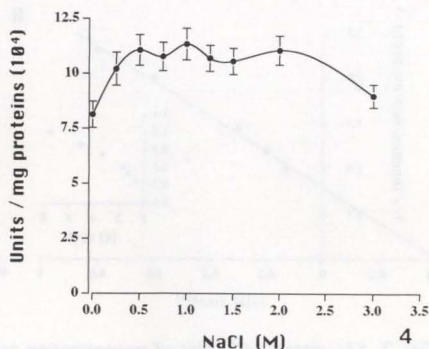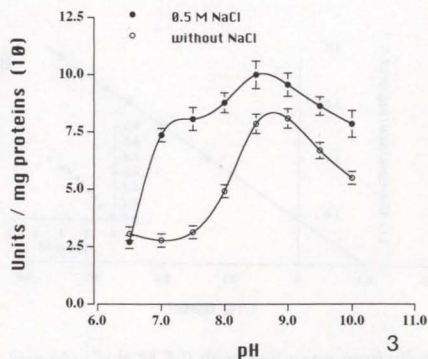
Figs 3-4. (3) Influence of pH on paraoxonase liver activity. Assays were performed at 30°C for 20 min using 1 mg of protein in each tube, 0.5 M NaCl, 2 mM $CaCl_2$ and 7.5 mM paraoxon. The buffers used were 0.1 M Sodium Phosphate for pH 6.5, 0.1 M Tris-HCl for pH values from 7.0 to 9.0 and 0.1 M glycine-NaOH for pH 9.5 and pH 10.0. Each point is the mean of five assays ± S.E.M.. (4) effect of NaCl concentration on paraoxonase activity of pacu liver. Assays were performed at 30°C for 20 min, using 0.1 M Tris-HCl buffer solution, pH 8.5, 1 mg of protein, 2 mM $CaCl_2$ and 7.5 mM paraoxon. Different volumes of a 3 M NaCl solution were used to obtain the plotted NaCl concentrations. Each point is the mean of five assays ± S.E.M.
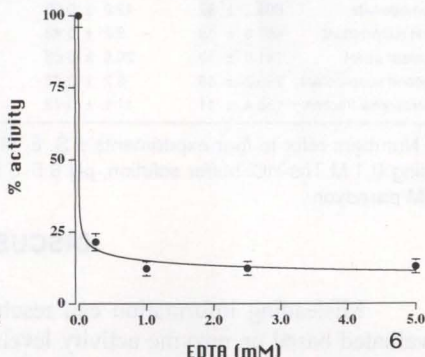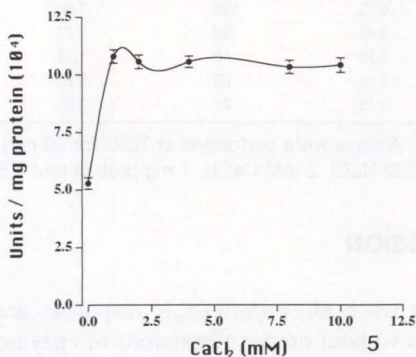
Figs 5-6. Effect of $CaCl_2$ (5) and EDTA (6) concentration on paraoxonase activity of pacu liver. (5) Assays were performed at 30°C for 20 min, using 0.1 M Tris-HCl buffer solution, pH 8.5, 1 mg of protein, 0.5 M NaCl and 7.5 mM paraoxon. Different volumes of a 12 mM $CaCl_2$ solution were used to obtain the plotted NaCl concentrations. Each point is the mean of 5 assays ± S.E.M.. (6) Assays were performed at 30°C with or without 0.5 M NaCl and 2 mM $CaCl_2$, using 1 mg of protein, 0.1 M Tris-HCl buffer solution, pH 8.5, and 7.5 mM paraoxon.

Table I shows that nearly 60% of the pacu liver paraoxonase activity occurs in the citoplasmatic cellular subfraction S1. The microsomal pellet contained about half of the activity recovered after ultracentrifuging S1 at 105,000 x g. Considering

the low amount of protein in pellets P1 and P2 when compared to the protein amount present in S1 and S2 it can be observed that the pellets fractions are the richest in paraoxonase activity.
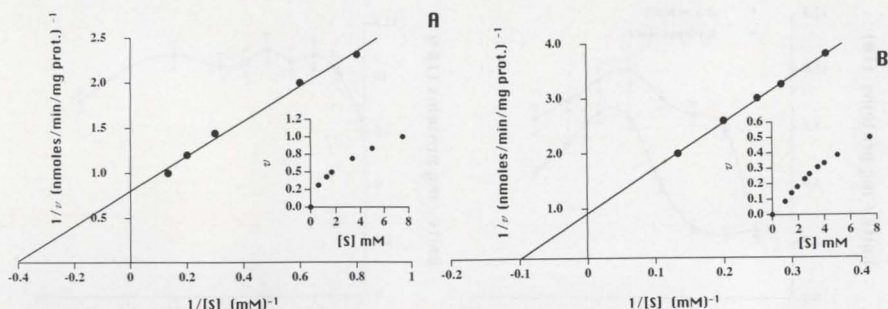

Fig. 7. 1/v versus 1/[S] plot of paraoxonase activity from pacu liver with 0.5 M NaCl (A) and without NaCl (B). The inserted graphics show the corresponding v versus [S] curves. Assays were performed at 30°C for 20 min, using 0.1 M Tris-HCl buffer solution, pH 8.5, 1 mg of protein, 0.5 M NaCl and 2 mM CaCl₂. Each point is the mean of six assays.

Table I. Paraoxonase activity in cellular subfractions of pacu liver.

Fraction	Protein (mg*)	Paraoxonase activity			
		Units/mg protein $(10^4)^*$	Total amount (mmoles/min)	Yield (%)	Purification factor (fold)
Homogenate	608.7 ± 32	12.0 ± 0.90	0.73	100	1.00
First supernatant	467.9 ± 19	9.2 ± 0.43	0.43	59	0.77
Nuclear pellet	141.0 ± 13	20.6 ± 0.58	0.29	40	1.72
Second supernatant	295.2 ± 16	6.2 ± 0.43	0.18	25	0.52
Microsomal fraction	136.4 ± 11	11.6 ± 0.72	0.16	21	0.97

*) Numbers refer to four experiments ± S. E. M. Assays were performed at 30°C for 20 min, using 0.1 M Tris-HCl buffer solution, pH 8.5, 0.5 M NaCl, 2 mM CaCl₂, 1 mg protein and 7.5 mM paraoxon.

DISCUSSION

Misleading information can result when physiopathologic responses are evaluated based on enzyme activity levels without proper adaptations of enzyme determinations. Enzymes may be characterized for their time course of reaction, proportionality of activity to enzyme concentration, effects of pH and ionic strength, substrate optimum concentration and other reaction parameters. Regrettably, too often it is seen that many laboratories simply reproduce procedures established elsewhere, for diverse organisms.

The results shown in figures 1 to 5 indicate that pacu liver paraoxonase activity was kinetically assayed using 1 mg of liver homogenate proteins in a Tris-HCl buffer, pH 8.5, containing 0.5 M NaCl and 2 mM CaCl₂ for 20, 40 or even 60 min of incubation. No significant deviation of the paraoxon hydrolysis from its initial velocities was verified under such conditions.

It has been established that two allozymic forms of esterases account for the paraoxonase activity present in human serum (PLAYFER *et al.* 1976; SMOLEN *et al.* 1991) and that the simplest way to reach qualitative distinction between them is to assay human serum in the presence and absence of 1 M NaCl (ECKERSON *et al.* 1983). As it happens with others serum enzymatic proteins, fish serum paraoxonase might also be produced in liver cells and secreted into serum. Thus, it is possible to hypothesize that the NaCl activation of the liver activity, clearly demonstrated by the ratio between apparent V_{max} and K_M, could be an indication of the presence of the two paraoxonase phenotypes in this fish species.

Earlier results concerning human serum arylesterase inhibition by EDTA (ERDÖS *et al.* 1959) showed that high levels of inhibition are observed at EDTA concentrations higher than 0.01 mM as a result of calcium chelation. Once paraoxonase has been classified as an arylesterase, the inhibition of the pacu liver paraoxonase activity caused by EDTA found here suggests that the enzyme molecule responsible for this activity in pacu liver could be, in fact, a paraoxonase protein.

Paraoxonase activity from trout (*Salmo trutta*) liver using less than 0.1 M NaCl in the assays has been reported as being of 2.0 nmol/g tissue/min (CHEMNITIUS *et al.* 1983). Calculating from our data, obtained in $1/v$ x $1/[S]$ plots, pacu liver showed activity levels higher than 100 nmoles/g tissue/min, even in assays without NaCl. Based on this, it is reasonable to conjecture that each fish species has different liver abilities to hydrolyze organophosphate pesticides.

Paraoxonase activity appeared more related to particulate cellular subfractions in pacu liver than to soluble ones (Tab. I). This agrees with previous findings in rat liver (POND *et al.* 1995) and seems to support the assumption that serum paraoxonase should be originated in liver cells and secreted via cellular mechanisms involving membrane organelles. However, if one is interested in a high purification yield, our findings indicate that a preliminary step to make paraoxonase more soluble from liver homogenate should be carried out in order not to loose a significant amount of the enzyme in the pellets.

Until now, there has been no information on K_M values of liver paraoxonase in any fish species. Determining K_M for paraoxonase activities in fish tissues may be of importance to assess what would be the range of paraoxon concentration that could be most effectively inactivated by hydrolysis. It seems reasonable to consider that *in vivo* concentrations of paraoxon in fish exposed to lethal and sublethal doses of parathion is likely below the K_M we observed for pacu liver paraoxonase activity. Thus, liver paraoxonase might not represent a true detoxicating step concerning organophosphate intoxication of pacu, for the enzyme would split paraoxon with half of its maximum velocity only at paraoxon concentrations higher than those already capable of affecting the cholinergic nerves of pacu.

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