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Physicochemical Properties of Mudskipper (*Periophthalmus Barbarus* Pallas) Liver Rhodanese.

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Abstract: Cyanide is highly toxic and its ineffective detoxification may result in inhibition of respiration (through iron complexation in cytochrome oxidase), ATP production and other processes dependent on ATP, subcutaneous hemorrhaging, liver necrosis, and hepatic damage in aquatic organisms. Rhodanese a cyanide detoxifying enzyme, was isolated and partially purified from the liver of mudskipper using ammonium sulphate fractionation and Reactive Blue-2 agarose affinity chromatography. The enzyme had a specific activity of 8.4 RU per milligram of protein. The K_m values of the substrates (KCN and $Na_2S_2O_3$) were 33.3 mM and 14.29 mM respectively. The results of substrate inhibition showed that the enzyme was inhibited considerably by sulphide (S^{2-}), sulphate (SO_4^{2-}) and sulphite (SO_3^{2-}) while showing specificity for thiosulphate ($S_2O_3^{2-}$). The reaction of the enzyme with heavy metals did not show any inhibition. The optimum pH and temperature of the enzyme activity were 8.0 and 50 ^oC respectively.

Key words: Kinetics, inhibition, rhodanese, detoxification, *Periphthalmus barbarous*, aquatic environment.

INTRODUCTION

Numerous accidental spills of sodium cyanide or potassium cyanide into rivers and streams have resulted in massive kills of fishes, amphibians, aquatic insects, and aquatic vegetation (Leduc, 1984; Eisler, 1991). Cyanides are ubiquitous in industrial effluents, and their increasing generation from power plants and from the combustion of solid wastes results in elevated cyanide levels in air and water (Leduc, 1984). Hydrogen cyanide (HCN) is a common industrial pollutant and frequently occurs in water at concentrations between 0.1 and several milligrams per liter of free HCN (Leduc, 1978; Leduc *et al*., 1982; Eisler, 1991). In water, cyanides occur as free hydrocyanic acid, simple cyanides, easily degradable complex cyanides such as $Zn(CN)_2$, and sparingly decomposable complex cyanides of iron and cobalt; complex nickel and copper cyanides are intermediate between the easily decomposable and sparingly degradable compounds (Towill *et al*., 1978; Eisler, 1991). Free cyanide is the primary toxic agent in the aquatic environment. Free cyanide refers to the sum of molecular HCN and the cyanide anion (CN^-), regardless of origin (Eisler, 1991).

Mudskippers (*Periophthalmus barbarus*) are found in tropical, subtropical, and temperate regions including the atlantic coast of West Africa (Murdy, 1989). They are complete amphibious fish (having the ability to use their pectoral fins to walk on land) (Swanson and Gibbs, 2004). They are uniquely adapted to intertidal habitats and thermoregulates (Tyller and Vaughan, 1983). Reports have shown that mudskippers can tolerate high levels of toxic substances such as cyanide (Tyller and Vaughan, 1983). Intertidal zone organisms are able to adapt to environment of harsh extremes with varying temperature from very hot with full sun to near freezing colder climate. In this study, the use of affinity chromatography in the partial purification of rhodanese from the liver of mudskipper is reported, and also to demonstrate the mechanism of cyanide detoxification in this organism.

Rhodanese (thiosulphate: cyanide sulphurtransferase EC. 2.8. I.I) is present in all living organisms, from bacteria to humans and it's thought to play a central role in cyanide detoxification (Westley, 1973; Westley, 1981; Nagahara *et al*., 1999; Aminlari *et al*., 2002). Rhodanese activity has been demonstrated in most mammalian tissues, with the greatest activity present in liver and kidney (Horowitz and DeToma, 1970). Rhodanese catalyses the transfer of the outer sulphate of thiosulphate to cyanide forming the products thiocyanate and sulphide (Lee *et al*., 1995). The enzyme originally found in the mitochondrion has now been reported to also be located in the cytosol and other organelles (Nagahara *et al*., 1999; Agboola and Okonji, 2004). Its presence in the liver tissues of different animals have been demonstrated (Sorbo, 1953a; Jarabak and

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Westley, 1974; Blumenthal and Heinrikson, 1971; Lee *et al*., 1995; Nagahara *et al*., 1996; Agboola and Okonji, 2004; Akinsiku *et al*., 2009). The aim of the study therefore, is to determine the physicochemical properties of rhodanese in mudskipper (*Periophthalmus barbarus* Pallas) which will possibly explain its role in the survival of the mudskipper (*Periophthalmus barbarus* Pallas) considering its environment.

MATERIALS AND METHODS

Materials:

Sucrose, sodium chloride, phosphoric acid, manganese chloride tetrahydrate, zinc chloride, magnesium chloride, tin chloride and ammonium sulphate, were purchased from BDH Chemical Limited, Poole England. Potassium chloride and Sephadex G series are from Pharmacia Fine Chemicals, Uppsala, Sweden. Nitric acid, ferric nitrate, potassium cyanide and sodium thiosulphate were purchased from Sigma Chemical Company, St. Louis, USA. All other reagents were of analytical grade.

Mudskippers were collected from University of Lagos Lagoon Front (a swampy margin on the Lagos Lagoon), Akoka, Lagos, Nigeria.

Enzyme Extraction and Purification:

Thirty-two mudskippers were collected from the University of Lagos Lagoon Front and transported in a cooling container to the Laboratory. The mudskippers were cut opened to remove their livers. These were kept frozen till required. The liver tissues weighing about 20 g was homogenised in a Warring Blender in nine volumes of the homogenisation buffer containing 0.25 M sucrose in 0.1 M acetate glycine buffer containing 10 mM sodium thiosulphate. The homogenate obtained was subjected to centrifugation at 6,000 rpm using Beckman Optima LE-80K Ultracentrifuge for 30 min. The supernatant was filtered using a double-layered cheese cloth and the precipitate was rehomogenised in 2 volumes of homogenisation buffer and centrifuged under the same condition. The supernatants were then pooled together and brought to 70% ammonium sulphate concentration, and left for 12 hr. The resulting precipitate obtained after centrifugation at 6,000 rpm for 30 min. was dialyzed against several changes of 50 mM citrate buffer, pH 5.0 containing 10 mM $Na_2S_2O_3$. The dialysate was centrifuged at 6,000 rpm for 30 min to remove insoluble material .This was used for the affinity chromatography step.

The Reactive Blue-2 agarose was equilibrated with 50 mM citrate buffer pH 5.0 according to the manufacturer's manual. The gel was packed into a 2.5 × 20 cm glass column. The enzyme fraction from the previous step was layered on it. Fractions of 5 ml were collected from the column at the rate of 36 ml per hour. The protein was monitored at 280 nm. The fractions were also assayed for rhodanese activity. The fractions with high enzyme activities were pooled and preserved in 50 % glycerol-citrate buffer solution (Okonji *et al*., 2010).

Enzyme Assay and Protein Concentration Determination:

Principle of the rhodanese assay was based on the colorimetric method for the determination of thiocyanate formation. Rhodanese activity was measured using sodium thiosulphate ($Na_2S_2O_3$) as substrate, as previously described by Agboola and Okonji (2004).

Bradford method (1976) was used routinely to measure the protein concentration of the enzyme using bovine serum albumin (BSA) as a standard.

Kinetic Studies:

The K_m and $V_{max,}$ were determined by varying the concentration of KCN (between 50 mM and 250 mM) at fixed concentrations of $Na_2S_2O_3$ (50 mM) and also varying the concentration of $Na_2S_2O_3$ (between 50 mM and 600 mM) at fixed concentrations of KCN (50 mM). The kinetic parameters were then estimated from the double reciprocal plots according to the plot of Lineweaver and Burk (1934).

Inhibition Studies:

Different sulphur compounds (sodium thiosulphate, sodium sulphate, sodium sulphite and sodium sulphide) were used as substrates at 0.1 M and 1.0 M final concentration in a typical rhodanese assay mixture.

Effect of Heavy Metals on Rhodanese Activity:

The effect of the following chloride salts: manganese chloride, mercuric chloride, nickel chloride, cobalt chloride, sodium chloride, tin chloride, magnesium chloride and zinc chloride on the activity of rhodanese was

investigated by assaying the enzyme at 0.1 mM 0.2 mM and 0.5 mM final salt concentrations in the reaction mixture.

Effect of pH:

The enzyme preparation was assayed at pH between 6 and 11 using 50 mM citrate buffer (pH 5.0-6.5), 10 mM phosphate buffer (pH 7.0-8.0) and 10 mM borate buffer (pH 8.5-11.0). The assay mixture of 1.0 ml contained 0.1 M of the required buffer, 0.05 M KCN, 0.05 M $Na_2S_2O_3$ and 20 µl of the enzyme solution.

Effect of Temperature:

The enzyme was assayed at temperatures between 30 °C and 60 °C to investigate the effect of temperature on the activity of the enzyme. The assay mixture was first incubated at the indicated temperature for 10 min before the addition of a 20 µl aliquot of the enzyme.

Results:

Purification of Rhodanese:

The results of the purification of rhodanese from mudskipper are summarized in Table 1. The elution profiles after reactive blue affinity chromatography is shown in Figure 1. The enzyme was partially purified using ammonium sulphate precipitation and Affigel Blue 2- agarose chromatography.

Table 1: The Purification Profile of Soldier Termite Rhodanese.

	Total Protein (mg)	Total Activity (RU)	Specific Activity (RU/mg)	Purification Fold	Yield (%)
Crude Extract	14161.58	21242.4	1.5	1.0	100
70% Ammonium Sulphate	7364	20284.9	4.4	2.9	58.3
Reactive Blue-Affinity Chromatography	1557.7	13084.7	8.4	5.6	11

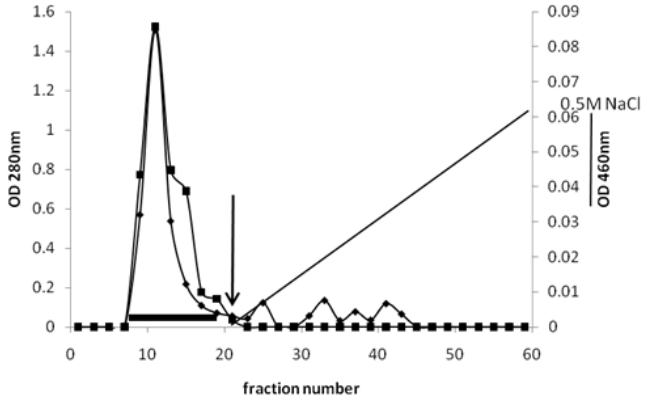

Fig. 1: Purification of the mudskipper liver rhodanese using Reactive Blue-2 agarose affinity chromatography. See text for the conditions. Fractions pooled (▬▬) and used for the other steps. Absorbance at 280 nm (■-■-■) and 460 nm (▸-▸-▸)

Kinetic Parameters and Inhibition Studies:

Figures 2 and 3 shows the Lineweaver-Burk plots for fixed concentration of thiosulphate and KCN respectively. The K_m values for KCN and $Na_2S_2O_3$ were 33.3 mM and 14.29 mM respectively while the V_{max} values for the two substrates (KCN and $Na_2S_2O_3$) were 0.77 and 1.0 RU respectively. The result of the inhibition studies with sulphur compound is presented in figure 4. The enzyme was found to be specific for thiosulphate as substrate.

Effects of Heavy Metals:

The effect of heavy metals on the activity of mudskipper liver rhodanese was presented as means ± Standard Deviation (SD). Data were analyzed by one-way ANOVA by using SAS/PC soft ware to examine whether there was any statistical difference among groups. A *P* value less than 0.05 was considered statistically significant. The effects of different concentrations of the various heavy metal ions on mudskipper liver rhodanese activity were found not to be significant (0.1 mM: p ? 0.0826; 0.2 mM: p ? 0.0812 and 0.5 mM: p ? 0.116) (Figure 5).

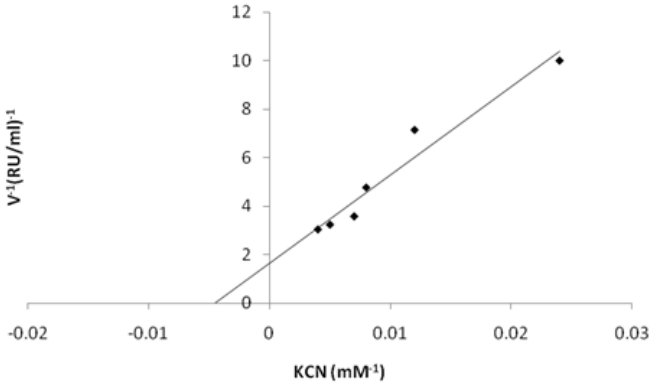

Fig. 2: Lineweaver-Bulk plots of liver rhodanese of mudskipper showing the effect of varying concentration of potassium cyanide (25 mM-200 mM) at fixed concentration of sodium thiosulphate ($Na_2S_2O_3$). The pH of the assay was 9.2.

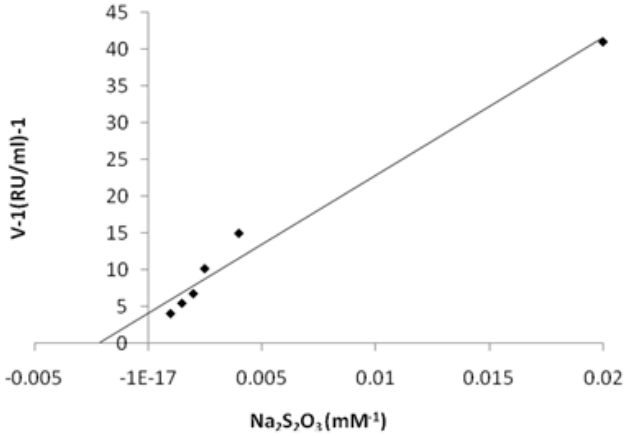

Fig. 3: Lineweaver-Bulk plots of liver rhodanese of mudskipper showing the effect of varying concentration of sodium thiosulphate (100 mM-500 mM) at fixed concentration of potassium cyanide (KCN). The assay pH was 9.2.

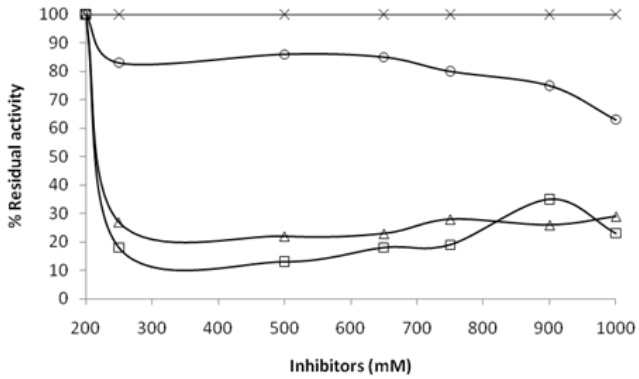

Fig. 4: The enzyme assay was carried out using standard assay mixture containing each salt at final concentrations between 0.1 M and 1.0 M. Thiosulphate (×-×-×), sulphate (**o-o-o**), sulphite (▲-▲-▲) and sulphide (□-□-□).

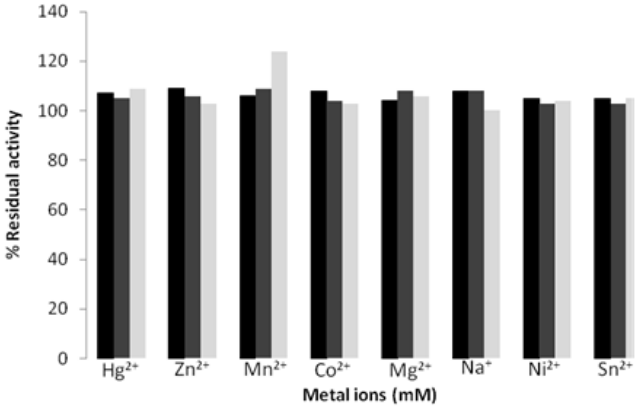

Fig. 5: The effect of heavy metals on the liver rhodanese of mudskipper. See text for assay conditions. The final concentrations of each metal in the assay mixtures were 0.1 mM ( ), 0.2 mM ( ) and 0.5 mM ( ). 0.1 mM: p ? 0.0826; 0.2 mM: p ? 0.0812 and 0.5 mM: p ? 0.116.

Effects of pH and Temperature on Enzyme Activity:

There was an increase in the enzyme activity between pH 7.0 and 8.5. An optimum pH of 8.0 was observed (Figure 6). The maximum activity of the enzyme was obtained at a temperature of 50 ^{0}C (Figure 7).

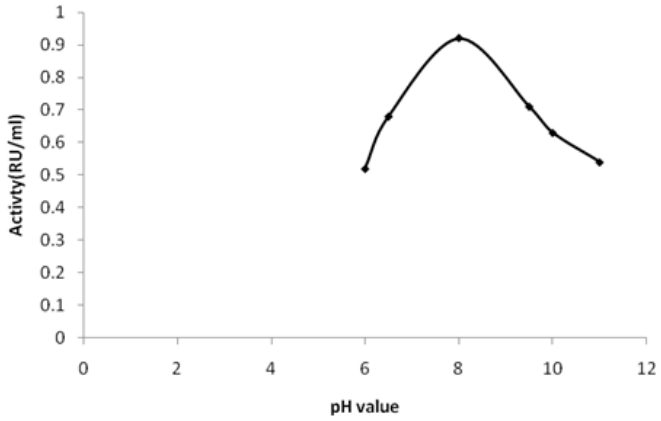

Fig. 6: The determination of pH optimum of mudskipper liver rhodanese. This was carried out as described in the text. The pH was varied between 6 and 11.

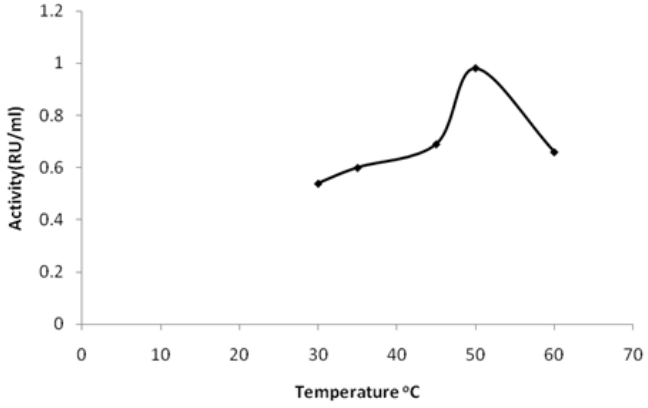

Fig. 7: The activity-temperature profile indicating the optimum temperature. Assays were as described in the text.

Protein values were determined by the method of Bradford (1976). One Rhodanese unit is taken as the amount of enzyme which under the given conditions will produce an optical density reading of 1.08 at 460 nm. The specific activity is expressed as µmoles of thiocyanate formed per min per mg of protein at 37^{o}C.

Discussion:

Anthropogenic sources of cyanide in the environment include industrial processes, laboratories, fumigation operations, cyanogenic drugs, fires, cigarette smoking, and chemical warfare operations (Marrs and Ballantyne, 1987). Cyanides are present in many industrial wastewaters, especially those of electroplaters; manufacturers of paint, aluminium, and plastics; metal finishers; metallurgists; coal gasification processes; certain mine operations; and petroleum refiners (Towill *et al*., 1978; Egekeze and Oehme, 1980). Cyanides are present in effluents from iron and steel processing plants, petroleum refineries, and metal-plating plants, and constitute a hazard to aquatic ecosystems in certain waste-receiving waters (Smith *et al.,* 1979; Eisler, 1991). Rhodanese has been identified as the main enzyme system responsible for the transulphuration process of cyanide (Westley, 1980). Rhodanese is widely distributed in the body, but activity levels in mammals are highest in the mitochondrial fraction of liver (Jarabak and Westley, 1974; Lee *et al*., 1995; Agboola and Okonji, 2004). Indeed, recent studies have clearly shown that rhodanese is involved in energy metabolism through its participation in regulation of mitochondrial electron transport and in the biosynthesis of iron-sulphur centres (Toohey, 1989; Aminlari *et al*., 1994).

In this work, rhodanese was partially purified by ammonium sulphate precipitation and Reactive Blue-2 agarose affinity chromatography from the liver of mudskipper (*Periophthalmus barbarus*). The enzyme had a specific activity of 8.4 RU per mg of protein and a 11.0 % recovery. Agboola and Okonji (2004) obtained 136.6 RU/mg for fruit bat liver rhodanese, Sorbo (1953a) and Himwich and Saunders (1948) obtained 256 RU/mg and 131 RU/mg respectively for bovine liver rhodanese. Akinsiku *et al.* (2009) obtained a value of 73 and 72 RU/mg for catfish rhodanese I (cRHDI) and catfish rhodanese II (cRHDII) respectively.

The K_m values of 33.3 mM and 14.29 mM were obtained for KCN and $Na_2S_2O_3$ respectively. These values compares very well to previous work reported on the enzyme. In our recent work, we obtained a much lower values of 10.0 and 2.56 for KCN and $Na_2S_2O_3$ respectively from hepatopancreas of freshwater prawn (*Macrobrachium rosenbergii*) (Okonji *et al*., 2008). Also, Akinsiku *et al*. (2009) obtained 25.4 mM and 18.6 mM for KCN and $Na_2S_2O_3$ respectively for catfish liver. The values reported for mudskipper is an indication that the enzyme has high affinity for the substrate and it would catalyse the detoxification reaction more efficiently considering the level of exposure of the organism to cyanide in its aquatic environment.

The study of the ability of different thiosulphates, (e.g benzene-thiosulphonate, ethane thiosulphonates, and n-butane-thiosulphonates) to replace thiosulphate in rhodanese reaction was first reported by Sorbo (1953b). The inhibitory studies of mudskipper liver rhodanese showed that mudskipper rhodanese was considerably inhibited by persulphide, sulphide and sulphite. The enzyme was observed to show specificity towards thiosulphate as substrate. Cyanide and thiosulphate were reported to be acceptor substrates (Sorbo, 1953b; Westley, 1980), while sulphite, persulphide and sulphinates were reported to serve as donor substrates (Sorbo, 1957; Villarejo and Westley, 1963; Nagahara *et al*., 1999). The inhibitory effects of sodium sulphide, dithiobiuret and cystein have been reported to be due to the blocking of the enzyme active site (Himwich and Saunders, 1948).

Our findings on the effect of some heavy metals on the activity of mudskipper liver rhodanese showed that the three different concentrations of metals used did not affect the activity of the enzyme (Figure 5). This is could be explained by the presence of high amount of these heavy metals both as free metal ions and metallocyanide complex in the aquatic habitat (Eisler, 1991). For example, formation of the nickelocyanide complex markedly reduces the toxicity of both cyanide and nickel at high concentrations in alkaline pH (Towill *et al*., 1978; Eisler, 1991). Trace metals such as Copper (Cu), Zinc (Zn), Cadmium (Cd) and Iron (Fe) were found to bioaccumulate in liver followed by gills and muscles in fish (Bu-Olayan and Thomas, 2008). Also the findings of Eisler (1991), show that cyanides occur as free hydrocyanic acid, simple cyanides, easily degradable complex cyanides such as $Zn(CN)_2$, and sparingly decomposable complex cyanides of iron and cobalt.

An optimum temperature of 50 ^{o}C was obtained for mudskipper liver rhodanese. This result is in good agreement with the results reported for rhodaneses from different sources. Sorbo (1953a) reported an optimum temperature of 50 ^{o}C for bovine liver rhodanese. Ezzi *et al.* (2003) obtained a wide temperature optimum of 35–55 °C for rhodanese enzyme in all different *Trichoderma* strains. Organisms in the intertidal zone are

adapted to an environment of harsh extremes with varying temperature (Tyller and Vaughan, 1983). The high optimum temperature of 50 ^{O}C obtained for mudskipper liver rhodanese could be an adaptive feature by the organism to survive the harsh environment. Murdy (1989) had shown that mudskipper were uniquely adapted to intertidal habitat and went further to report there thermoregulatory ability.

The intertidal marine habitat has been reported to have a pH range of 6-9 with an average of approximately 8.0 (Gordon *et al*., 1978). Some aquatic plants such as mangrove were reported to grow in seawater and tolerate salinity. The Mudskipper liver rhodanese showed maximum activity at pH 8.0. Different optimum pH values in the range of 8.0-11.0 have been reported for different organisms (Jarabak and Westley, 1974; Lee *et al.*, 1995; Agboola and Okonji, 2004; Saidu, 2004). There is a differential salinity in the lagoon due to the effect of the Atlantic Ocean and it fluctuates both seasonally and semi-diurnal. This and the pollution by urban and industrial waste/discharge contribute to the alkalinity of the Lagoon, which could explain the alkaline pH of mudskipper liver rhodanese.

The present data show that the liver rhodanese partially isolated from the mudskipper possesses an efficient enzyme mechanism for the detoxification of cyanide (which has been reported to be more toxic to fish) and has adapted features for its survival in the harsh environment.

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