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Some Biochemical Properties of Liver Rhodanese (E. C. 2. 8. 1.1) Isolated from a Typical Marine Fish *(Lutjanus goreensis)*

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Abstract: ***Rhodanese is a multifunctional, mitochondrial enzyme which primary function is the protection of the electron transport system from the deleterious effect of cyanide. It carries out the said primary function, by catalyzing the conversion of the cyanide to thiocyanate, a less toxic compound. The enzyme is also implicated in preventing the formation of inorganic sulfide, a highly neurotoxic substance and in the synthesis of sulfur- containing macromolecules. The activity of the ubiquitous enzyme has been reported in soil, bacteria, plants and animal tissues. The aim of this research is to establish the presence of the cyanide detoxifying enzyme, Rhodanese, in marine aquatic life, especially in the liver of a typical marine fish, Lutjanus goreensis that subsists on other fishes and shrimps which in turn, feed on planktons and some lower organisms of the aquatic food chain. The fish liver Rhodanese, purified 11.4 fold with activity recovery of 9.03%, had a specific activity of 19.7µMmin^{-1}mg^{-1} enzyme protein. The invitro cyanide detoxifying enzyme, thiosulphate sulphur transferase (E.C. 2.8.1.1), followed a non-sequential ping-pong mechanism in the formation of thiocyanate from cyanide and thiosulphate. From the kinetic studies, the V_{max} of the enzyme catalysis was 5.0µM/minute while the Km values for thiosulfate and cyanide were 5.88mM and 11.11mM respectively. The optimum pH of the fish liver Rhodanese enzyme was 8.2, with the pH range of 8.0 to 8.4. The optimum temperature of the enzyme was 35^{0}C. The molecular weight of the fish liver Rhodanese was 37,153 daltons, using the gel filtration technique. From the foregoing, it is evident that the Rhodanese enzyme is present in the liver of the tropical marine fish with an appreciable activity level that is possibly attributable to the feeding habit of the fish.***

Keywords: Marine fish Liver, Rhodanese, cyanide and thiocyanate

1. Introduction

The metalloenzyme of Iron with the odd suffix for enzymes and named, Rhodanese (thiosulphate sulphur transferase, E. C. 2.8.1.1) was first discovered in bovine liver by Lang, (1933). From the bovine liver, the enzyme was crystalized and characterized by Sorbo, (1953). This initial isolation of the enzyme, opened the research avenue for several investigators to carry out numerous studies on the Rhodanese enzyme with the primary aim of elucidating the physiological importance of the mitochondrial enzyme. The current concept is that the iron-sulphur Centre enzyme protein is implicated in the synthesis of sulphur-containing macromolecules. The second understanding is that Rhodanese plays a vital role in the protection of cytochrome oxidase of the electron transport system from the attack of the cyanide molecule as per the accounts of the protective effect of Rhodanese on respiration by Augrica and Koj, (1975). Isom and Jaine, (1976) and Tabita *et al*., (1969) documented the presence of the Rhodanese enzyme in bovine liver and in bacteria, *Ferrobacillus ferroxidan,* respectively. Other investigators that reported on the activity of the enzyme in various other sources include; Anosike and Jack (1982a and b) in liver and kidney of guinea pig (*Lepus caniculus)* and albino rat (*Mus muscusculus*), (Westley, 1959) in beef liver and kidney Rhodanese enzyme activity comparative study. Recently, Lee *et al*. (1995) in mouse liver, Agboola and Okonji (2004) in fruit bat liver, Ezzi *et al ., (2003)* in *Trichoderma sp*, Aladesanmi *et al.,* (2009) in tortoise liver, Akinsiku *et al*.,(2010) in the liver of African catfish, Okonji *et al*.,(2011) and(2010) in mudskipper liver and in soldier termite, respectively.

In plants, the activity of the Rhodanese enzyme was reported in tapioca leaves by Chew and Boey, (1972) and by Anosike and Ugochukwu, (1981) in cassava leaf, peels and flesh where the enzyme distribution correlated with the concentration of the cyanogenic glucoside, estimated on the premise of hydrogen cyanide (HCN) released. Most recently, the activity of Rhodanese has also been reported in the leaves of bitter melon (*Momordica Charantia*) by Ehigie *et al*. (2015)

However, not much investigation has been reported in literature, concerning the presence of the cyanide detoxifying enzyme in various tropical marine aquatic lives. This study was, therefore, undertaken to demonstrate the presence of the Rhodanese enzyme in the liver of a typical marine fish, (*Lutjanius goreensis*) and to reveal some of the inherent biochemical properties associated with the enzyme for the advancement of knowledge on the ubiquitous Rhodanese enzyme which diverse physiological functions are increasingly being appreciated, such as preventing the formation of inorganic sulphide (a highly neurotoxic substance), the synthesis of sulfur- containing macromolecules and most importantly, the protection of the electron transport chain from the deleterious effect of cyanide.

2. Materials and Methods

The marine fish which liver was implicated in this study was purchased from the Creek Road waterside local market in Port-Harcourt, Nigeria. All other reagents, chemicals and their sources are as listed below;
Folin-Ciocalteu reagent, Bovine Serum Albumin (BSA) and Disodium hydrogen phosphate were products of E. Merch (Darstadt). Hydrochloric acid, Sodium hydroxide, potassium thiocyanate were products of East Anglia Chemicals. Sodium hydrogen phosphate, Formaldehyde, Tris (hydroxyethyl) amino methane and potassium cyanide were products of BDH.

2.1 Protein Estimation

Protein was estimated, using the method of Lowry *et al*.,(1951) with bovine serum albumin as the standard.

2.2 Enzymes assay

For routine assay of the enzyme activity, the method of Tomati *et al*., (1974) was used. In which the intensity of the reddish-brown color formed in the course of enzyme catalysis, was proportional to the concentration of thiocyanate produced and was estimated spectrophotometrically at 460nm, using Bausch and Lomb spectronic 20 Spectrophotometer. The enzyme activity was expressed as µmole KCSN formed per min per mg enzyme protein.

2.3 Effect of pH on fish liver Rhodanese enzyme

The optimum pH of the marine fish liver Rhodanese was determined, using 0.01M Tris-HCl (pH 5.0-7.0) and 0.01M Sodium phosphate (pH 7.5-9.5) as buffer systems. A suitable aliquot (1.5mls) of pH buffer at a particular pH was used for assay of the enzyme activity in the manner of using 0.01M Sodium Phosphate buffer (pH 8.2) for routine enzyme assay (Tomati *et al.,* 1974). The enzyme assay was carried out with 8.33mM of $Na_2S_2O_3$, 8.33mM of KCN and 2.5mM of each type of buffer.

2.4 Effect of temperature on fish liver Rhodanese

The effect of temperature on the marine fish liver Rhodanese catalyzed reaction was tested over the temperature range of 20-60^oC with 5^oC interval. For temperature 25^oC, the reaction tubes were incubated in temperature regulated water bath by the addition of ice chips. At each temperature of the test, the tube was duplicated; the reagent blank tube with which the spectrophotometer was zeroed and the other in which the actual effect of temperature on the enzyme was measured. This nullified the formation of thiocynate by temperature rather than by enzyme catalysis.

2.5 Molecular weight of fish liver Rhodanese determination

The enzyme extract was separately applied to a column of 1cm x 34cm, packed with Sephadex G150-120 that was equilibrated for 72 hours in 10.0mM Tris-HCl buffer (pH7.6). The elution was with the same buffer and 4.0mls (42drops) was collected with the aid of a fraction collector that was the product of Rikakika company limited. Tokyo, Japan

The sephadex-packed column was calibrated with standard marker proteins of known molecular weights; Cytochrome C (13,000 Daltons), Pepsin (34,000 Daltons), Egg Albumin (44,000 Daltons) and Bovine Serum Albumin (65,000 Daltons).

3. Results

The results of kinetic study of the fish liver Rhodanese are as shown in table 1 of the double reciprocal plot. Fig 3 shows the pH profile of the fish liver Rhodanese in relation to percentage Vmax, while fig 4 shows the effect of temperature on the enzyme in relation to percentage Vmax. Fig 5 shows the plot of Log mol.wt versus elution volume with which the Mol. wt of the fish liver Rhodanese enzyme was determined by extrapolation.

Table 1: The Double reciprocal plot (Lineweaver-Burk plot).

(KCN)-^{1}mM -^{1}	0.12	0.15	0.2	0.3	0.6
$(Na_2S_2O_3)$-^{1}mM-^{1} 0.12	V^{-1}= 1.6	1.9	2.4	3.20	5.9
0.15	V^{-1}= 1.76	2.07	2.76	3.87	7.25
0.20	V^{-1}= 2.13	2.52	3.2	4.5	8.5
0.30	V^{-1}= 2.5	3.0	3.87	5.2	9.7

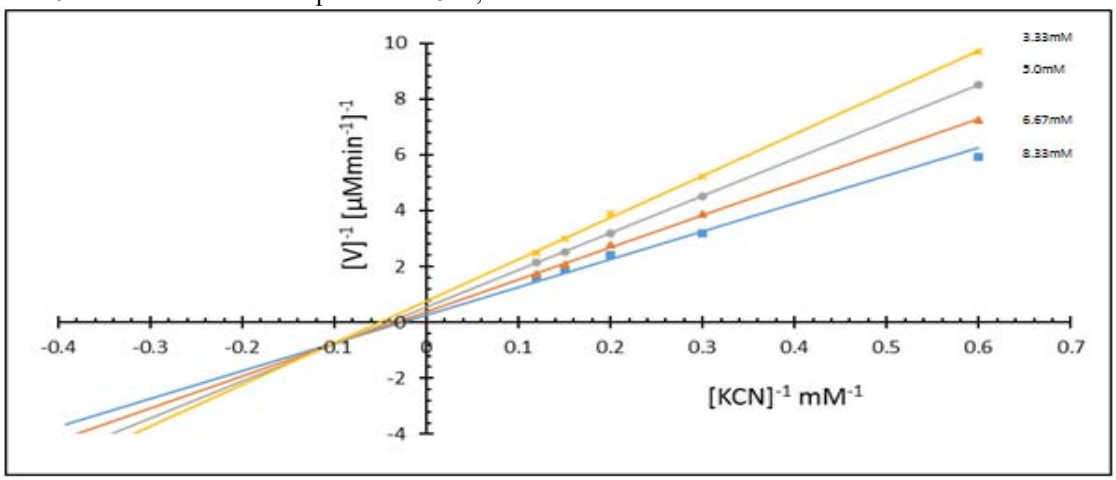


Figure 1a: A plot of reciprocal initial rate versus varying concentration of $[KCN]^{-1}mM^{-1}$

A plot of reciprocal initial rate $(V)^{-1}(\mu M.min^{-1})^{-1}$ of enzyme catalyzed reaction Vs reciprocal of potassium cyanide concentration $[KCN]^{-1}$ mM^{-1}. At various fixed concentration of sodium thiosulphate $[Na_2s_20_3]$ 8.33mM; 6.67mM 5.0mM; 3.33mM.

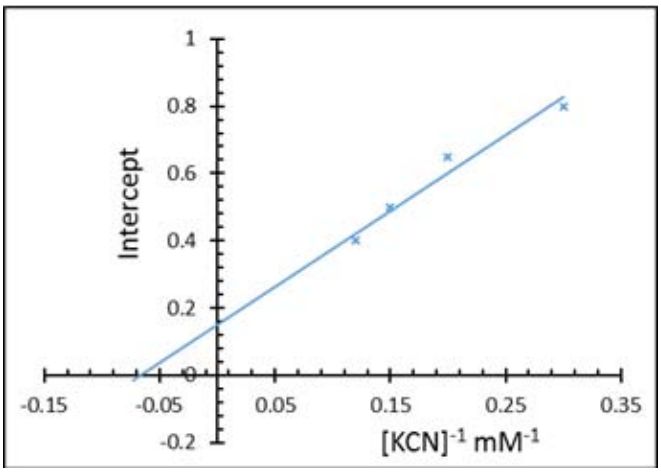


Figure 1b: A plot of intercept versus varying Concentrations of $[KCN]^{-1}mM^{-1}$

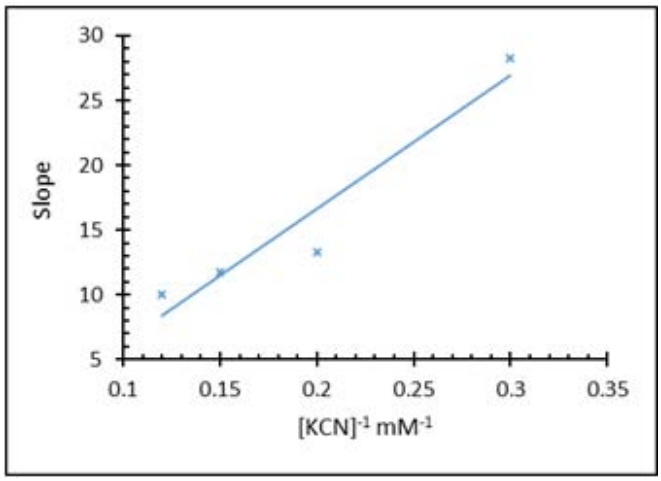


Figure 1c: A plot of slope versus varying concentrations of $[KCN]^{-1}mM^{-1}$

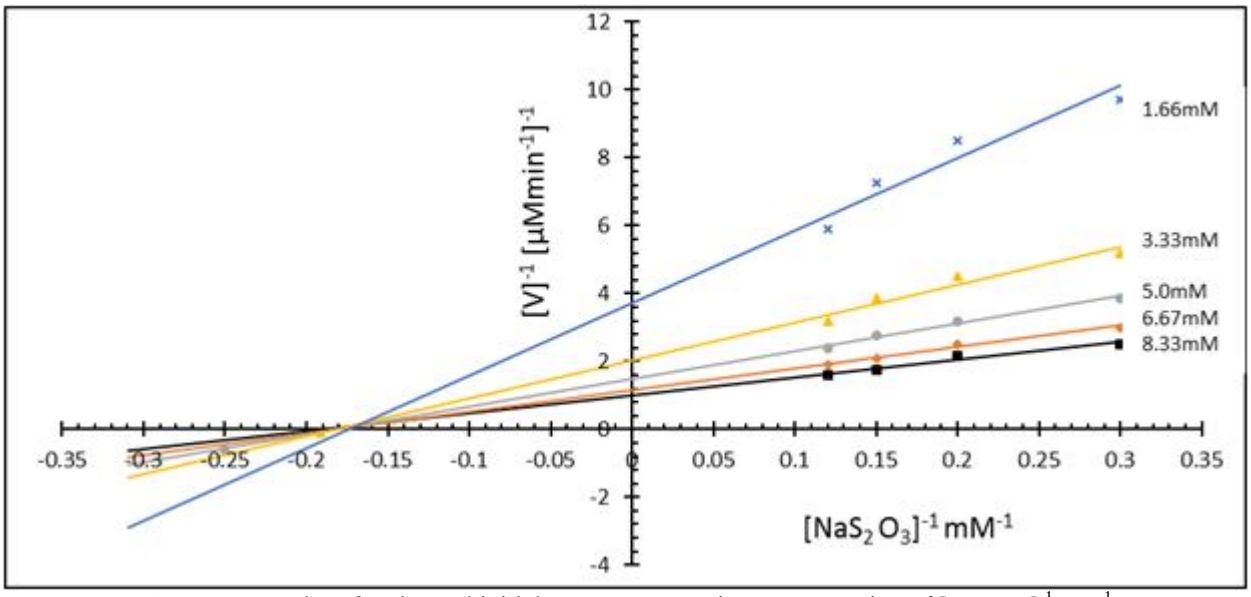


Figure 2a: A plot of reciprocal initial rate versus varying concentration of $[NaS_2O_3]^{-1}mM^{-1}$

A plot of reciprocal initial rate $(V\ \mu M.\ min^{-1})^{-1}$ of enzyme catalyzed reaction versus reciprocal of sodium Thiosulphate concentration $[Na_2s_20_3]^{-1}$ mM^{-1}. At various fixed concentration of Potassium Cyanide [KCN] 8.33mM, 6.67mM, 5.0mM, 3.33mM, 1.66mM

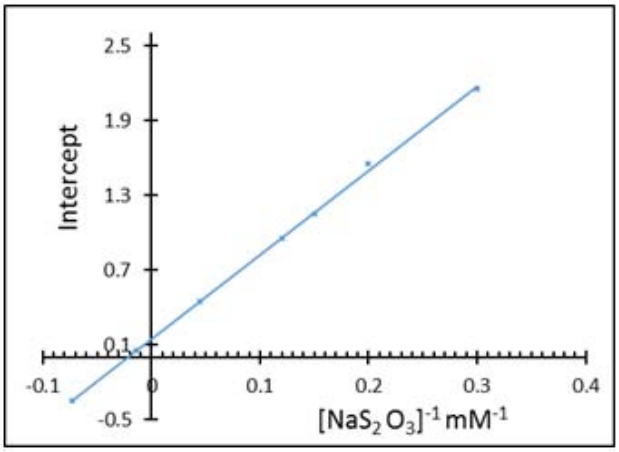


Figure 2b: A plot of intercept versus varying Concentration of $[Na_2S_2O_3]^{-1}mM^{-1}$

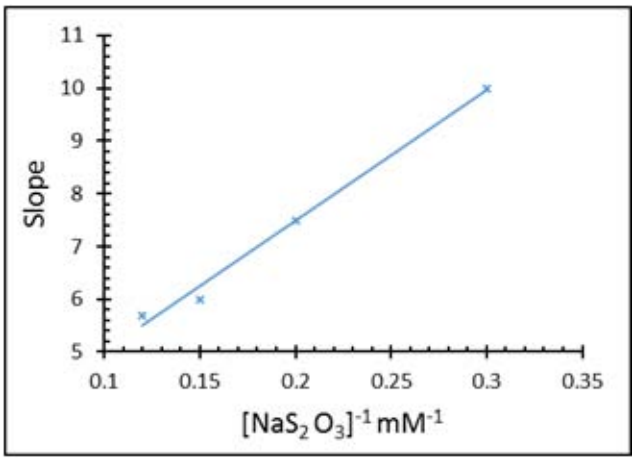


Figure 2c: A plot of slope versus varying concentration of $[Na_2S_2O_3]^{-1}mM^{-1}$

3.1 Effect of pH on fish liver Rhodanese enzyme

From the results obtained and with the plot of percentage Vmax versus pH, as shown in fig 3 , it was deduced that the optimum pH for the marine fish liver Rhodanese was 8.2, with optimum pH narrow range of 8.0-8.4.

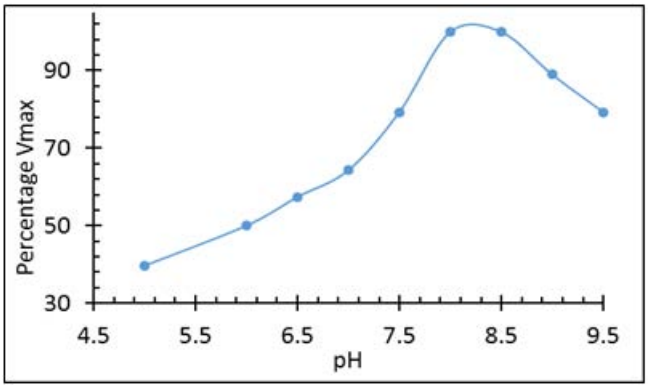


Figure 3: A plot of percentage Vmax versus pH

3.2 Effect of temperature on fish liver Rhodanese enzyme

From the results of the study, upon which the percentage Vmax versus temperature was plotted, as shown in fig 4, it was observed that the optimum temperature, at which point the marine fish liver Rhodanese exhibited maximum enzyme activity, was 35°C.

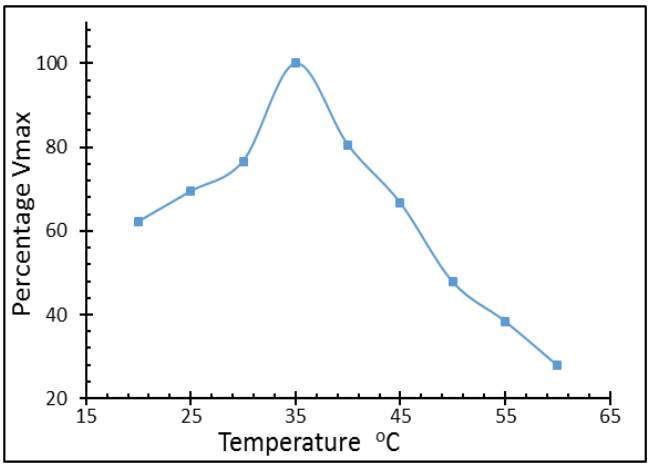


Figure 4: A plot of percentage V_{max} versus temperature

3.3 Molecular weight of fish liver Rhodanese

From the plot of log molecular weight against elution volume, as shown in fig 5, with the results from the study, it was observed that the molecular weight of the fish liver Rhodanese was extrapolated to be 37,153 Dalltons from the elusion volume of 60 mls for the enzyme.

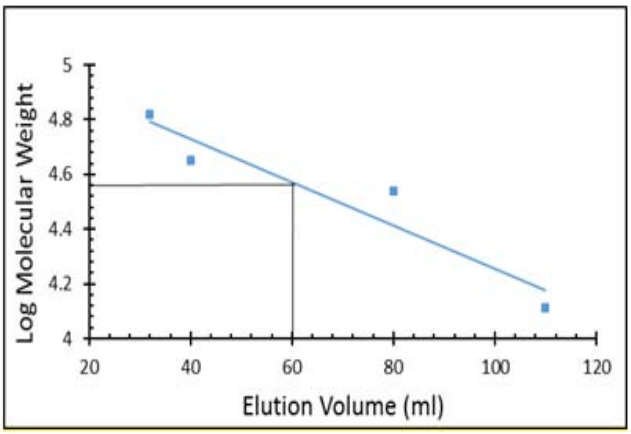
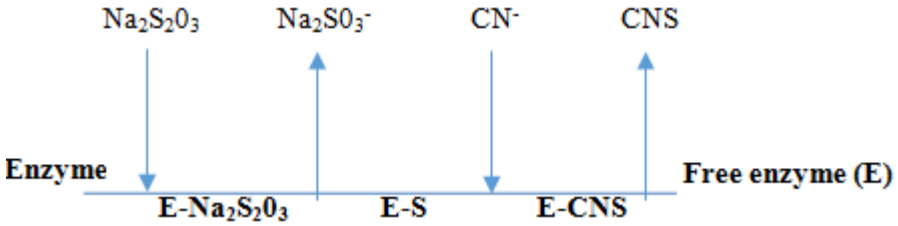


Figure 5: A plot of log. Molecular weight versus Elution Volume

4. Discussion

From the Kinetic study and the Lineweaver-Burk plots, as shown in figs 1a and 2a, the Vmax of the marine fish liver Rhodanese was observed to be 5.0µM/minute. This is a unique attribute of the marine fish liver Rhodanese under the condition of the study. Several investigators have reported different values of Vmax for the same enzyme from different sources. The Km values of the fish liver Rhodanese enzyme catalyzed reaction, indicated higher affinity for thiosulphate than for cyanide. This observation is consistent with the findings of Ehigie *et al.*,(2015) for the Rhodanese enzyme from the leaves of bitter melon, Tayefi-Nasrabadi and Rahmani, (2011) for the Rhodanese from the liver of Rainbow Trout, Gafar *et al*., (2014) for the Rhodanese from the liver of Tilapia, Tabita *et al*., (1969) for the Rhodanese from *Ferrobacillus ferroxidan*, Chew *et al.,* (1979) for the Rhodanese from tapioca leaf, Akinsiku *et al*.,(2010) for the Rhodanese from the liver of the African catfish, Okonji *et ai* ., (2010) and (2011) for the Rhodanese enzymes from the soldier termites and from the mudskipper liver, respectively. However, the km values of this study, are not consistent with the reported findings of Anosike & Jack, (1982a and b) for Rhodanese enzymes from rat and guinea pig kidneys and that of Agboola and Okonji, (2004) for the Rhodanese in the cytosolic fraction of the fruit bat liver where the enzymes were found to have higher affinity for cyanide than for thiosulphate. In all cases of catalysis, the Rhodanese enzyme followed a non-sequential ping-pong mechanism as was initially reported by Sorbo, (1953) for bovine liver Rhodanese and by Saidu (2004) reviewed account on Rhodanese. The mechanism entails the binding of thiosulphate ($S_20_3^{=}$) to the enzyme molecule that causes an intermolecular protein conformational change which appears to break the S-S bond with the extrusion of the sulfite ($S0_3^{=}$) radical. The suphonite- enzyme complex (E-S), then binds with the available cyanide (CN^-). During this process, the

sulphur group, combines with the electrophilic nitrogen centre of the cyanide (CN^-), forming a thiocyanate-Enzyme Complex (E-CNS) that rapidly breaks down to thiocyanate (CNS) and free Rhodanese enzyme molecule (E).



From the plot of the percentage maximum activity (Vmax) of the fish liver Rhodanese versus pH, as shown in fig3, it is deduced that the marine fish liver rhodanese had an optimum pH of 8.2, with the optimum pH narrow range of 8.0-8.4. This report is in agreement with the documented finding of Okonji *et al*., (2010) for the soldier termite Rhodanese of optimum pH of 8.0, with a wide optimum pH range of 8-11. It is also consistent with the reports of Okonji *et al*.,(2011) and Yanhua *et al*., (2012). Both of whom reported an optimum pH value of 8.0 for Rhodanese enzyme from mudskipper liver and for the cyanide degrading enzyme from *Alcaligene* sp.DN25 with a narrow optimum pH range of 7.0-8.0, respectively. The optimum pH of 8.0 for the marine fish liver Rhodanese of this study, is not in agreement with the optimum pH value of 6.0, reported for giant freshwater prawn Rhodanese by Okonji *et al*.,(2008) and optimum pH of 6.5 reported by Lee *et al.,* (1995) for mouse liver Rhodanese, Akinsiku *et al*.,(2011) for African catfish liver Rhodanese and Gafar *et al*., (2014) for tilapia liver Rhodanese. It is also not consistent with the reported optimum pH as high as 10.5 with the optimum pH wide range of 5-12 for the rainbow trout liver Rhodanese by Tayefi-Nasrabidi and Rahmani, (2011). The optimum pH range of 8.0-8.4 for marine fish liver enzyme of this study, compares well with optimum pH range of 8.0-9.0, 7.5-9.0 and 7.0-8.0 reported for bovine liver, *F. ferroxidan* and guinea pig and rat kidney enzymes by Anosike & Jack,(1982a and b); Sorbo, 1953; Tabita *et al*.,(1969) respectively. However, it is in contrast with the optimum pH range of 10.2 -11.0 and10.2-10.4, reported for tapioca leaf Rhodanese enzyme by Chew and Boey, (1972) and for the *thiobacilus* A_2 Rhodanese enzyme by Silver and Kelly (1976) respectively. The intertidal marine habitat has a pH range of 6.0-9.0, with an average of approximately 8.0 (Gordon *et al*., 1978). This is said to be physiologically significant for the survival of the mudskipper in the environment (Okonji *et al*.,2011) as maximum bicarbonate (HCO_3^-) level for the buffering of the physiological system of most marine teleost fishes occur within alkaline pH range of 8.4-9.0 (Wilson *et al.,* 2002). So, the optimum pH of 8.2 possibly enables *Lutjanus goreensis, the* snapper, to thrive as it displays estuarine dependence and habitat flexibility (kafayat *et al.,* 2015) even where the oxygen concentration could be low (hypercapnia environment).

From the thermal stability experiment and the plot of percentage Vmax verse temperature, as shown in fig 4, it was observed that the optimum temperature for the fish liver Rhodanese, where it had the maximum enzyme activity, was 35^0C. This finding, is in good agreement with results for the Rhodanese enzymes from different sources; especially the report of Agboola and Okonji, (2004) for the Rhodanese in the cytosolic fraction of the fruit bat liver that was observed to have similar optimum temperature of 35°C. Wang *et al*., (2012) and Tayefi-Nasrabadi and Rahmani, (2011) reported optimum temperature of 30°C for the cyanide-degrading enzyme from *Alcoligene* sp DN25 and for the Raindow Trout liver Rhodanese enzymes ,respectively, which accounts are slightly lower in value than the finding of this study. A lower optimum temperature of 25°C was reported for *F. ferrioxidan* and guinea pig and rat kidney Rhodanese enzymes by Tabita *et al*., (1969) and Anosike and Jack, (1982a and b), respectively. The optimum temperature of 40°C has also been reported for the mouse and tilapia liver Rhodanese enzymes by Lee *et al*., (1995) and Gafar et al. (2014) respectively. While Okonji *et al*.,(2011) and (2010) reported 50°C and 55°C as optimum temperature for the activity of the mudskipper liver and the soldier termite Rhodanese enzymes, respectively, Chew and Boey, (1972) and Ehigie *et al*., (2015) documented an optimum temperature as high as 59°C and 60°C for the tapioca leaves and the bitter melon leaves Rhodanese enzymes, respectively. The observed optimum temperature of 35°C for the marine fish liver Rhodanese by this study, is within the lower optimum temperature range of 35-55°C obtained for Rhodanese enzymes in all *Trichoderma* strains by Ezzi *et al*., (2003). It is also within the upper optimum temperature range of 25-35°C reported for Bovine liver Rhodanese enzyme by Sorbo (1953). However, the value is completely not in agreement with the optimum temperature range of 50-55°C documented for cabbage Rhodanese enzyme by Tomati *et al*., (1974). The remarkable difference in optimum temperature of the enzymes from other sources, especially from plants, may well be as a result of the temperature difference of the environment to which the organism or the plant is adapted. This confers an appreciable higher thermal stability on the enzymes from these sources.

The molecular weight of the marine fish liver Rhodanese was found to be 37,153daltons, using the gel filtration technique. This realization is very similar to the molecular weight of 37,154 daltons for the native soldier termite Rhodanese by Okonji *et al*., (2010). It is also consistent with the value of 37,000 daltons that was reported for the bovine liver Rhodanese enzyme by Sorbo, (1953), the human liver Rhodanese by Jarabak and Westley,(1974) and the guinea pig kidney Rhodanese enzyme by Anosike and Jack (1982b). It compares well with the 36000 daltons for the fruit bat liver Rhodanese reported by Agboola and Okonji,(2004) However, it is in contrast with the value of 32000 daltons for rat kidney Rhodanese by Anosike and Jack (1982a), mouse liver Rhodanese by Lee *et al*., (1995), cabbage leaf Rhodanese by Tomati *et al*., (1974); the value of 33000 daltons for tapioca and cassava leaves Rhodanese

enzymes by Chew and Boey, (1972) and Anosike and Ugochukwu, (1981), respectively, as well as with the value of 35460 daltons for the tilapia liver Rhodanese by Gafar *et al*., (2014). It would be inferred, therefore, that the molecular weight of the Rhodanese enzyme protein molecule, from most sources, is between 32,000 to 37,000 daltons.

5. Conclusion

This research has shown cyanide detoxifying enzyme, Rhodanese, to be present in the liver of the typical marine fish, *Lutjanus goreensis* (snapper) that mainly feeds on other fishes and shrimps, within the rocky and sandy estuary, which in turn subsist on proteinic remains of other aquatic lives, planktons and some lower aquatic organisms such as diatoms. So, the feeding habit of the tropical marine fish may have contributed to having a high activity of the Rhodanese enzyme protein, especially in the liver of the fish which is the detoxification organ, with the characteristic of having a higher affinity for the thiosulfate than for cyanide, the toxicant. The more hypolimion- dwelling marine fish is adapted to the prevailing warm temperature of the sandy sea bed (hypolimion) which confers a high thermal stability on the inherent Rhodanese enzyme. The alkaline optimum pH of the marine fish liver Rhodanese is an indication of the physiological optimum pH that enables the fish to thrive even at low oxygen tension where the pH could be low (hypercapnia environment).

It is not surprising, therefore, that the molecular weight of the tropical marine fish liver Rhodanese is similar to that of the human liver, soldier termites, fruit bat liver, bovine liver and guinea pig kidney Rhodanese enzymes. The two latter animals have a common feeding habit of subsisting on cyanogenic glycosides-containing elephant grass *(Andropogon spp)* while the termites feed on decaying wood.

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