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CHARACTERIZING PARTIALLY PURIFIED RHODANESE ENZYME FROM THE WEST AFRICAN MUD CREEPER

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Abstract: Indiscriminate release and deposition of hazardous substances continue to produce streams of pollutants into the environment. Research efforts geared towards the development of new low-cost and eco-friendly agents capable of reducing, detoxifying and eliminating pollutants are ongoing. Certain enzymes, extracted from natural sources, have shown great promise in restoring polluted environment due to their enormous ability to transform and detoxify wide range of pollutants. This study was conducted to characterize partially purified rhodanese, a known cyanide detoxifying enzyme, from the West African mud creeper (*Tympanaotonos fuscatus*) or periwinkle. The enzyme was partially purified using ammonium sulphate [$(NH_4)_2SO_4$] precipitation and gel filtration chromatography and some of its specific biochemical characteristics assessed using standard methods. Results showed a yield of 2.58 U/mg protein and 4.42 U/mg protein for the $(NH_4)_2SO_4$ precipitation and gel filtration chromatography respectively. The purification efficiency from gel filtration chromatography was observed to be approximately twice that obtained for the $(NH_4)_2SO_4$ precipitation. The kinetic parameters evaluated showed that the Km for KCN and $Na_2S_2O_3$ were 39.76mM and 18.37mM respectively while the Vmax were 1.67µM/min and 2.10µM/min respectively. Substrate specificity study showed that the enzyme exhibited high affinity for sodium thiosulphate (100%), sodium metabisulphite (76%) and 2-mercaptoethanol (47%). The optimum pH was in the range of 8-10 while the optimum temperature was 50°C. The enzyme was most stable at 30°C, its activity was inhibited at the different concentrations of metal ions and the estimated molecular weight was found to be 39,700 Dalton. This study demonstrated that the biochemical characteristics of rhodanese extracted from periwinkle is comparable to the activities of rhodanese from other organisms and tissues. The rhodanese enzyme from this organism may be exploited as eco-friendly agent for extreme cyanide detoxification and bioremediation.

Keywords: Rhodanese, cyanide detoxification, enzyme, *Tympanotonos fuscatus*, environment

Introduction

Indiscriminate release and deposition of hazardous and harmful substances continue to produce streams of pollutants which contaminate the environment (Rao and Gianfreda, 2010). Research efforts have been geared towards the development of new low-cost technology and ecofriendly agents capable of reducing, detoxifying and eliminating these pollutants using several biological agents (Whiteley and Lee, 2006). Among these biological agents are enzymes, which have been identified as very effective in restoring polluted environment due to their enormous ability to transform and detoxify wide range of pollutants (Rao and Gianfreda, 2010). Such enzymes which act intracellularly or extracellularly can be utilized as free enzymes in solution or when immobilized on different support media or solid matrices (Gianfreda and Rao,

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2004). They carry out extensive transformations of structural and toxicological properties of different environmental pollutants converting them to less toxic products.

Enzymes represent a good alternative for direct use of microorganisms because they are not halted by inhibitors of microbial metabolism. They can be used under extreme conditions that limit microbial activity and are effective in the presence of microbial predators or antagonists. They also exhibit increased mobility than microorganisms because of their unique characteristics (Gianfreda and Bollag, 2002).

Cyanide is a toxic substance, containing covalently bonded carbon and nitrogen, present in the environment (Rao and Gianfreda, 2010). The presence of this substance in biological systems causes death by sequestering cyanide into tissues instead of oxygen (Logue *et al*., 2010). Organisms detoxify cyanide by the use of Rhodanese, an enzyme involved in the conversion of cyanide to the less toxic thiocyanate (Saidu *et al.*, 2005; Ehigie *et al*., 2013). Therefore, production of rhodanese enzyme is a protective mechanism adopted by organisms against cyanide toxicity. The sequence of rhodanese mediated action takes place by a double displacement (ping-pong) mechanism resulting in the formation of a stable persulphide-containing enzyme complex (Ehigie *et al*., 2018)**.** The reaction which occur in two stages begin with the transfer of sulphane sulphur from the substrate to the active site cysteine to form a persulphide enzyme intermediate and the thiophilic acceptor then attacks the enzyme-bound persulphide intermediate forming product and regenerating the free form of the enzyme (Saidu *et al*., 2005; Ehigie *et al*., 2018). The presence of this enzyme has been detected in different tissues and organisms (Okonji *et al*., 2015; Oluwatosin *et al*., 2017).

The marine ecosystem contains an enormous biodiversity of organisms. The survival of these organisms depends on a wide variety of biologically active compounds (bioactive molecules) which can be exploited for the environmental bioremediation. This study aims at characterizing partially purified rhodanese enzyme from the West African Mud Creeper (*Tympanotonos fuscatus*), a brackish water snail commonly referred to as periwinkle

Materials and Methods

Chemicals

The ammonium sulphate, potassium cyanide, and sodium thiosulphate used were from Sigma Chemical Company, St. Louis, USA. Sephadex G-100 was from Pharmacia Fine Chemical, Sweden. All other reagents used were of analytical grade and obtained from Rivers State University and other reputable firms.

Collection and Identification of Samples

The *Tympanotonos fuscatus* (periwinkles) were obtained from local dealers in Kula Community in Akuku-toru Local Government Area, Rivers State, Nigeria and were identified at the Department of Animal and Environmental Biology, Rivers State University, Port Harcourt, Nigeria.

Enzyme Extraction and Partial Purification

Samples of *Tympanotonos fuscatus* (periwinkle) were gently washed to remove surface dirt, and their shells were carefully opened. 100g of the tissues of the periwinkles were crushed into small pieces and homogenized in three volumes of 50mM phosphate buffer of pH 7.2 containing ε-amino-n-caproic acid with warring blender. The homogenate was filtered using a double layered cheese cloth and then centrifuged at 12,000 rpm for 30 minutes. The supernatant was brought to 80% ammonium sulphate saturation by the addition of 51.1 g of solid ammonium sulphate with continuous stirring over a period of 1 hour and then left overnight. The resulting precipitate was collected by centrifugation at 12,000 rpm for 30 minutes and re-suspended in a small amount of the buffer solution. The ammonium sulphate precipitate was dialyzed against several changes of phosphate buffer at 4^0C for 18 hours and centrifuged at 12,000 rpm for 30 minutes. The supernatant collected was used as enzyme source.

Determination of Protein Concentration

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Protein concentration was determined by the method of Bradford (1976) using Bovine Serum Albumin (BSA) as the standard. The absorbance of the protein was extrapolated from the standard protein calibration curve. The reaction mixture consists of 10 µl of the enzyme solution and 2 ml of Bradford reagent. The absorbance was read at 595 nm (Agboola and Okonji, 2004).

Determination of Rhodanese Activity

Rhodanese activity in the sample was measured according to the method described by Okonji *et al*., (2015). The reaction mixture had 0.5 ml of 0.05M borate buffer (pH 9.4), 0.2 ml of 0.25 M KCN, 0.2 ml of 0.25 M $Na_2S_2O_3$ and 0.5 ml of the enzyme solution. The mixture was incubated at 37°C for 1 min. The reaction was stopped by adding 0.5 ml of 15% formaldehyde, followed by the addition of 1.5 ml of Sorbo reagent (ferric nitrate solution containing 10 g $Fe(NO_3)_3$. $9H_2O$ in 20 ml concentrated nitric acid and 80 ml of distilled water). The absorbance was recorded at 460 nm. Reaction velocity was computed from linear slopes of absorbance-time curve. One Rhodanese unit (RU) was taken as the number of micromoles of product (thiosulphate) formed in one minute under the specified experimental conditions (Sorbo, 1951).

Native molecular weight Determination

The molecular weight of native rhodanese in the sample was determined on Sephadex G-100 (2.5 x 90 cm) that was calibrated with the buffer for 72 hours. The Sephadex-packed column was calibrated with standard marker proteins of known molecular weights; Cytochrome C (13,000 Daltons; 5mg/ml), α-Chemotrpsin (25,000 Daltons; 5mg/ml), Oval Albumin (44,000 Daltons; 5mg/ml) and Bovine Serum Albumin (65,000 Daltons; 5mg/ml). The elution volume of Blue Dextran (5 mg/ml) was used to estimate the void volume (Vo) of the column. The elution volume of rhodanese was estimated by layering 10 ml of the enzyme solution on the same column. The elution was with the same buffer. Fractions of 5 ml were collected from the column at a flow rate of 20 ml/h and monitored for protein and rhodanese activity.

Determination of Kinetic Parameters

The enzyme kinetic parameters (K_m and V_{max}) were determined using KCN and $Na_2S_2O_3$ as substrates. K_m and V_{max} values were determined using Lineweaver and Burk plots obtained by varying the concentration of KCN in the reaction medium between 20 mM and 100 mM at a fixed concentration of $Na_2S_2O_3$. Concentration of $Na_2S_2O_3$ was also varied between 50 mM and 250 mM at a fixed concentration of KCN. (Lineweaver and Burk, 1934).

Determination of Substrate specificity

Varying Sulphur containing compounds (sodium thiosulphate, sodium metabisulphite, ammonium persulphate and 2-mercaptoethanol) were used as substrates for the typical rhodanese activity measured as earlier described. The relative activities were compared with sodium thiosulphate as control. The activity was expressed as a percentage activity of the enzyme (Ejeme *et al*., 2015).

Effect of pH on the enzyme Activity

The effect of pH on the enzyme activity was performed using varying buffers solutions and pH. 50 mM citrate buffer (pH 3-5); 50 mM phosphate buffer (pH 6 - 8) and 50 mM borate buffer (pH 9-11) were used. The rhodanese activity was assayed as described earlier.

Effect of Temperature on the Enzyme Activity

The enzyme was assayed at temperatures between 30°C and 70°C to assess the effect of temperature on the activity of the enzyme and determine the optimum temperature of the enzyme. The assay mixture was first incubated at the indicated temperature for 10 minutes before starting the reaction by adding an aliquot of the enzyme equilibrated at the same temperature. The rhodanese activity was subsequently assayed as described earlier.

Effect of Metals on the Enzyme Activity

The effect of metals on the activity of the rhodanese was performed using different metal chlorides (NaCl, $CaCl_2$, $BaCl_2$,$ZnCl_2$, $MnCl_2$ and $NiCl_2$) at 0.5mM and 0.1mM from stock solutions of 0.5mM in a typical rhodanese assay mixture. The metallic chlorides were dissolved in distilled

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water. The reaction mixture without the salts was taken as control with 100% activity All assays were done in triplicates and the mean values were reported (Ejeme *et al*., 2015).

Thermal stability of rhodanese

Thermal stability of the crude rhodanese was investigated by incubating an aliquot volume of enzyme at different temperatures (30, 40, 50, 60 and 70 ^{o}C) for up to 70 minutes in a temperature-controlled water bath and measuring the activity at room temperature after cooling. The incubation was carried out in sealed vials to overcome change of volume and enzyme concentration by evaporation.

Results

Partial Purification of rhodanese from *Tympanotonos fuscatus*

The result for the partial purification of rhodanese from *Tympanotonos fuscatus* is presented in Table 1. The enzyme was partially purified using ammonium sulphate precipitation and gel filtration chromatography. Specific activity gave 2.58 U/mg protein and 4.42 U/mg protein respectively. The purification fold from gel filtration chromatography was observed to be approximately twice that obtained for the ammonium sulphate precipitation fraction.

Table 1: Summary of Purification Profile of Rhodanese from TF

Fractions	Total Protein (mg)	Total Activity (U/ml)	Specific Activity (U/mg protein)	Recovery (%)	Purification fold
Crude enzyme source	74.54	107.83	1.45	100	1
Ammonium Sulphate (80%)	26.28	67.88	2.58	63	1.78
SephadexG-100 Chromatography	4.67	20.63	4.42	19.13	3.05

Kinetic Studies

The kinetic parameters evaluated for the rhodanese from *Tympanotonos fuscatus* as obtained from Lineweaver-Burk plots is shown in Figure 2. The Km obtained for KCN and $Na_2S_2O_3$ were 39.76mM and 18.37mM respectively while the Vmax were 1.67µM/min and 2.10µM/min respectively.

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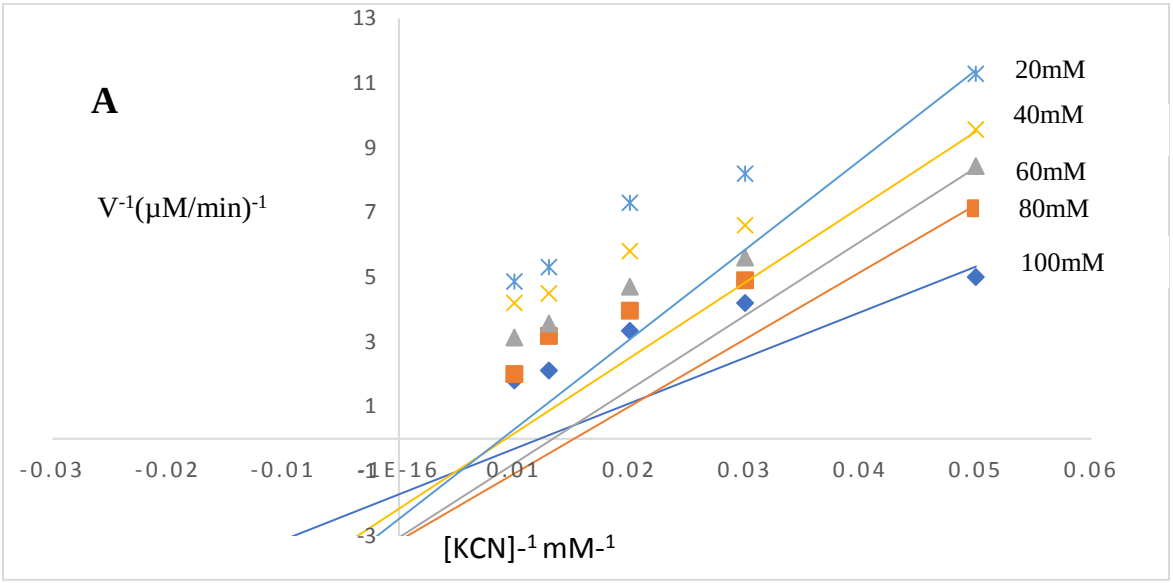

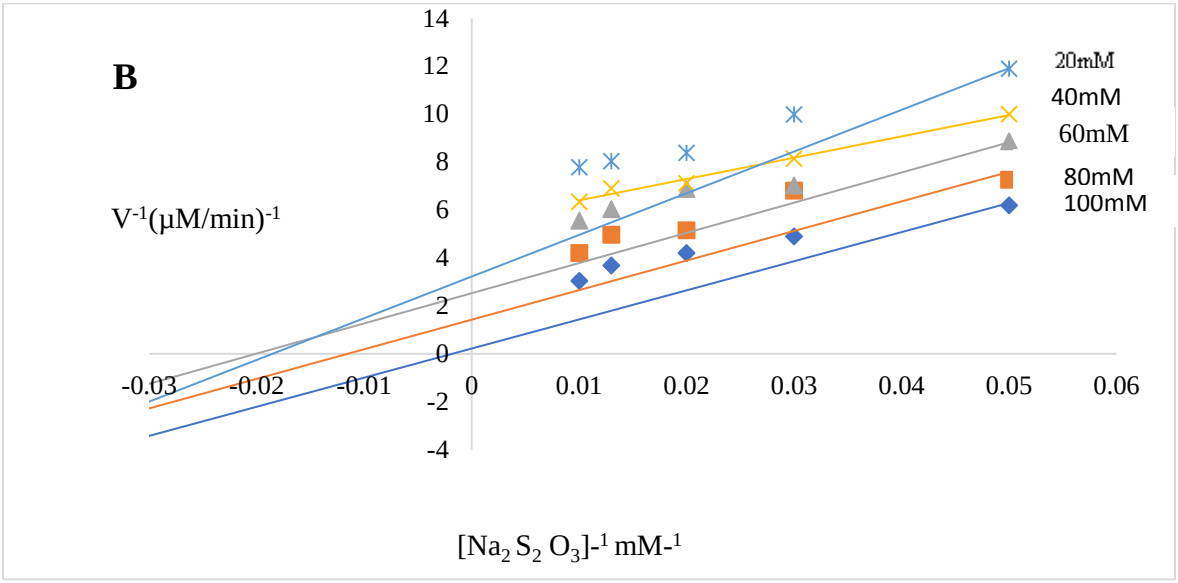

Figure 2: Lineweaver-Burk Plots for Rhodanese enzyme from*Typanotonos fuscatus*.
A: At Varying Concentrations of KCN and fixed Concentration of $Na_2S_2O_3$.
B: At Varying Concentrations of $Na_2S_2O_3$ and Fixed Concentration of KCN**.**

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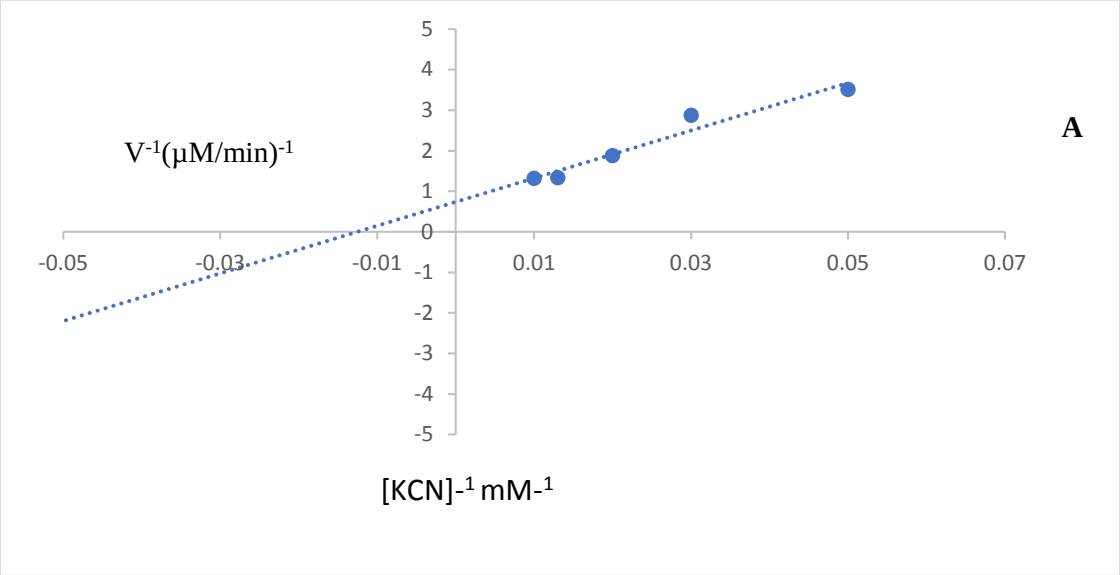

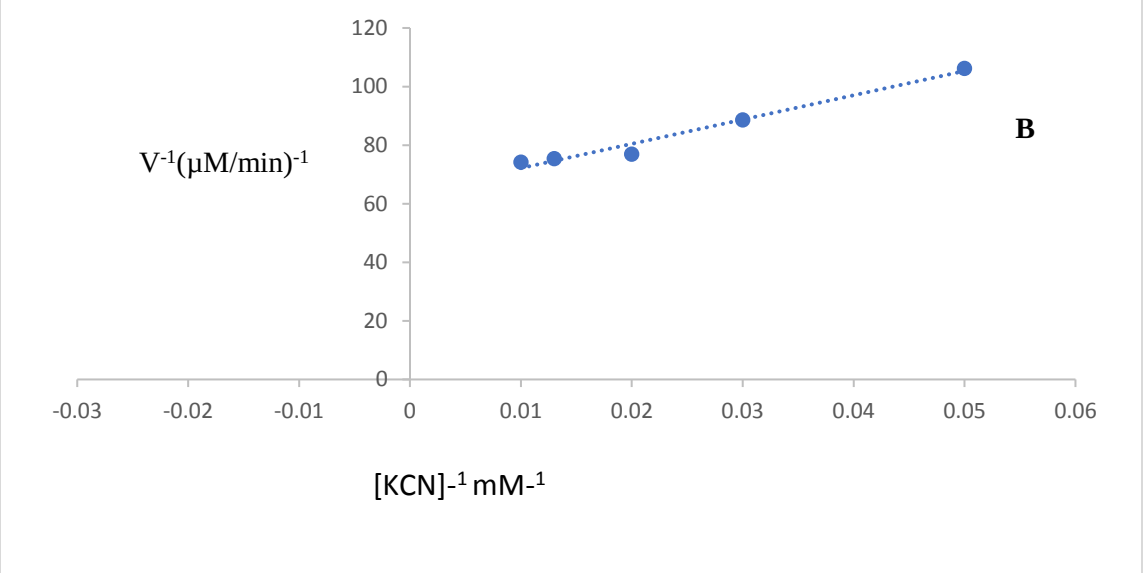

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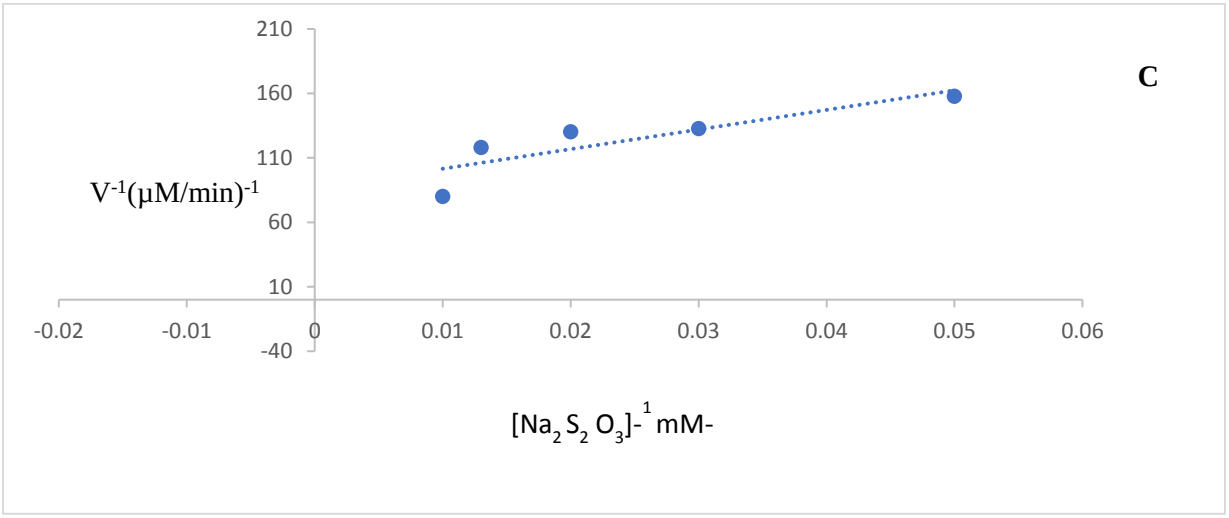

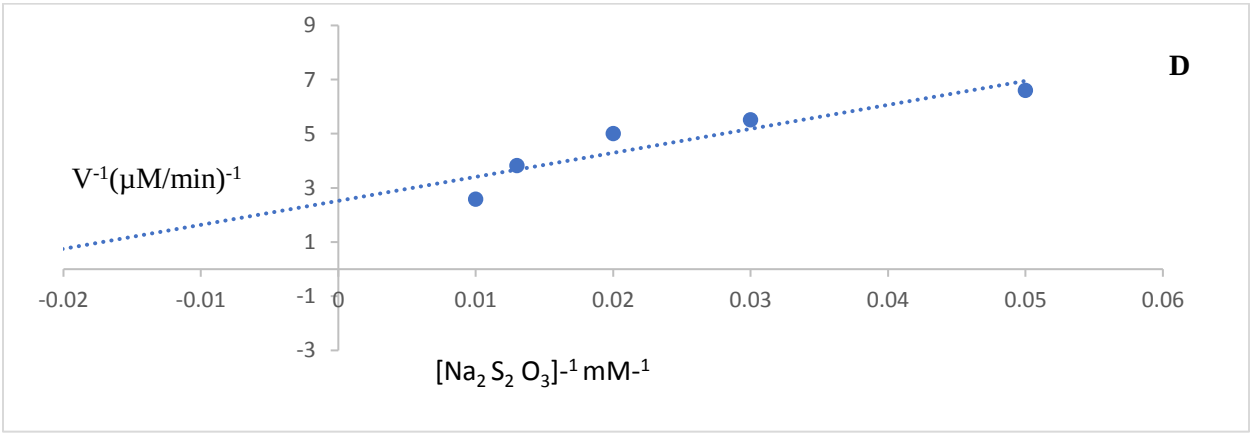

Figure 3: Reciprocal Plots.
A: Intercept plot at varying concentrations of KCN and fixed concentration of $Na_2S_2O_3$.
B: Slope plot at varying concentrations of KCN and fixed concentration of $Na_2S_2O_3$.
C: Slope plot at varying concentrations of $Na_2S_2O_3$ and fixed concentration of KCN
D: Intercept plot at varying concentrations of $Na_2S_2O_3$ and fixed concentration of KCN.

Molecular Weight

The calibration curve for the determination of native molecular weight of rhodanese from *Tympanotonos fuscatus* is presented in Figure 3. The molecular weight estimated was found to be 39,700 Dalton.

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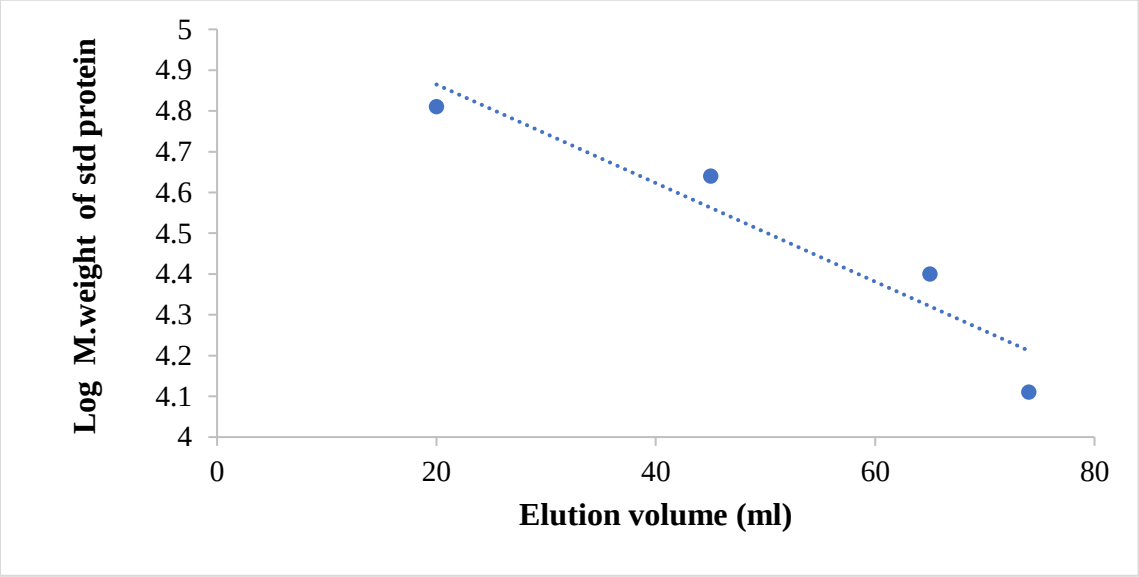

Figure 3: Graph of Log Molecular weight of Standard Protein Versus Elution Volume

Effect of Substrate Specificity

The result for the effect of substrate specificity for the enzyme is given in Figure 4 and expressed as % activity using $Na_2S_2O_3$ as control. The result showed that the enzyme exhibited high affinity for sodium thiosulphate (100%) followed by sodium metabisulphite (76%). However, 2-mercaptoethanol showed lowest activity (47%).

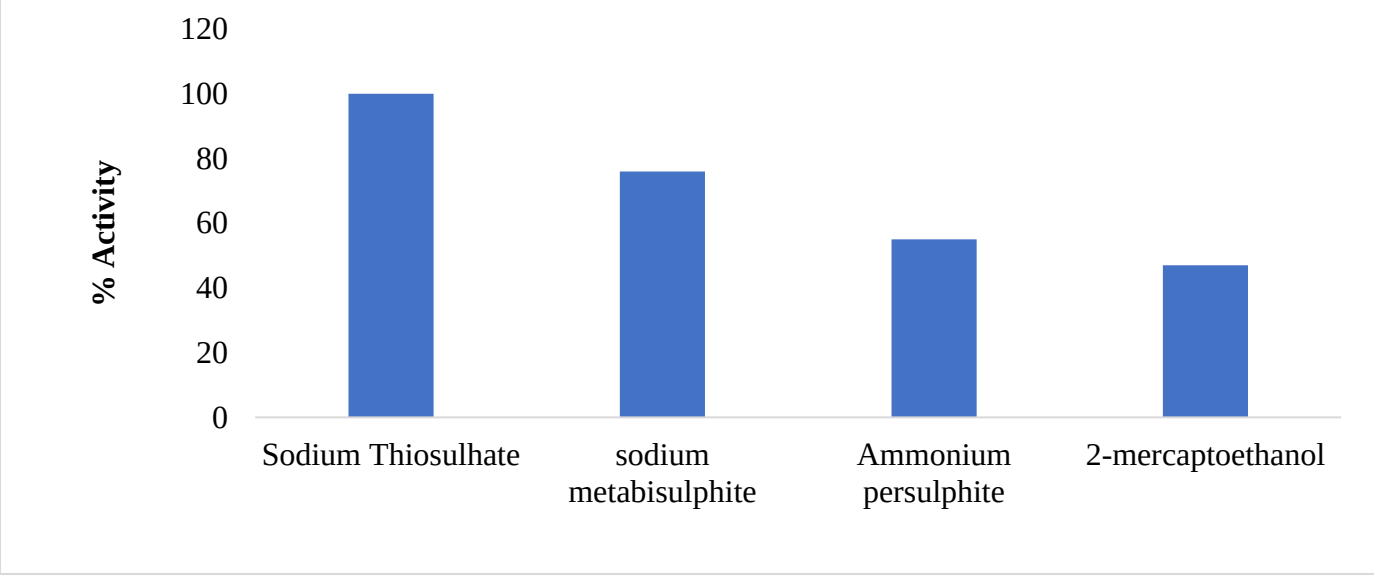

Figure 4. Percentage substrate activity of various sulphur compound

Effect of pH and Temperature on rhodanese enzyme from *Tympanotonos fuscatus*.

The result obtained for the effect of pH and Temperature is presented in Figure 5. Effect pH was assayed using buffers ranging from 3-11. While the effect of temperature was assayed ranging from 30-70^{o}C. The optimum pH was in the range of 8-10 while the optimum temperature was 50^{o}C.

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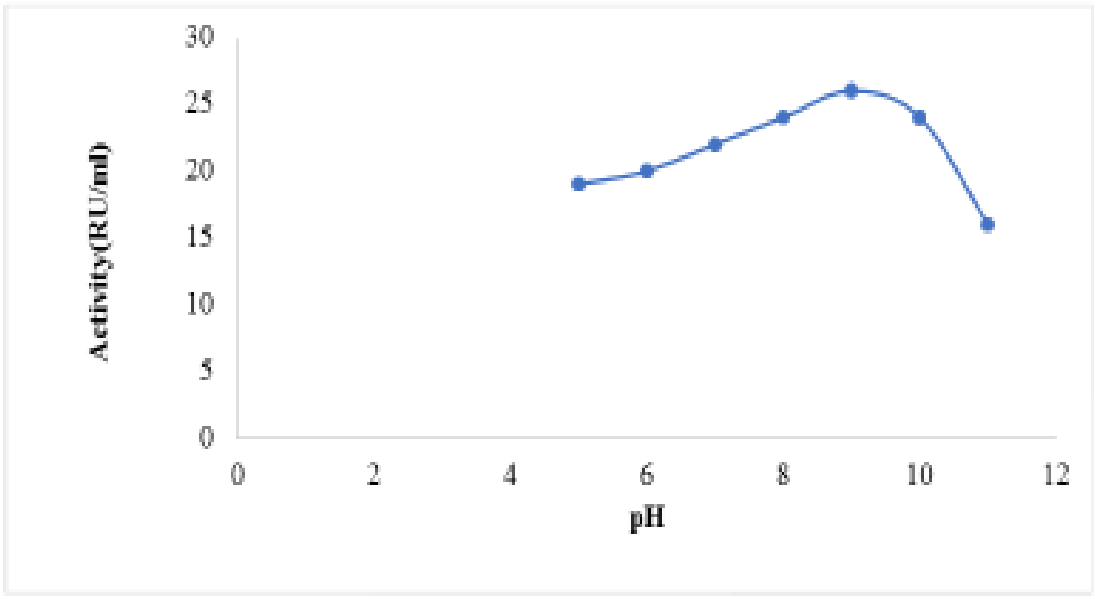

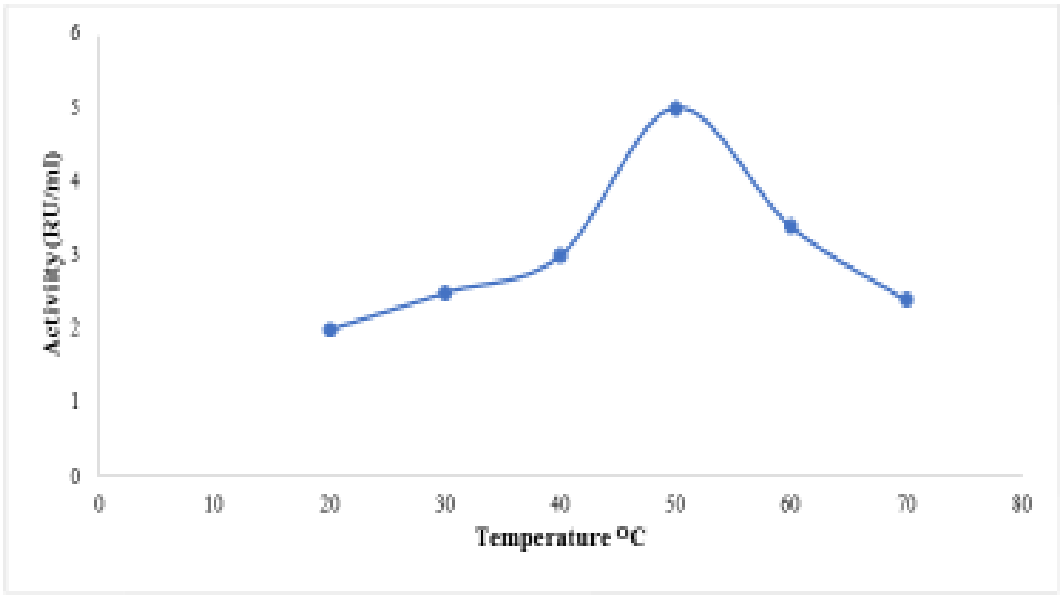

Figure 5. Effect of pH and Temperature on *Tympanotonos fuscatus* rhodanese activity

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Effect of Heat on *Tympanotonos fuscatus* rhodanese enzyme activity

The result obtained for the effect of heat on the *Tympanotonos fuscatus* rhodanese enzyme activity is presented in Figure 6. It was found that the enzyme was stable at 30 ºC for about 1 hour and unstable at temperatures above 30ºC losing about 20, 50, 55 and 70 % of activity after 20 minutes of incubation at 40, 50, 60 and 70 ºC respectively. However, relative stability of its activity was established (57, 45 and 39 % respectively) at 40, 50 and 60 ºC when incubated for 70 minutes.

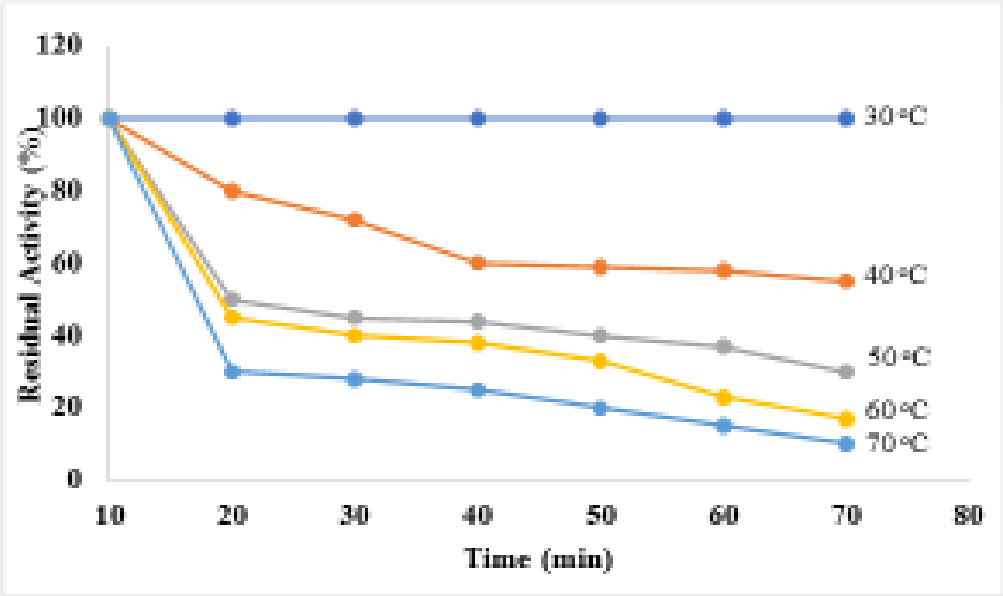

Figure 6: Effect of heat treatment on *Tympanotonos fuscatus* rhodanese activity as a function of treatment time at different temperatures. Residual activity was expressed as a percentage of activity of the enzyme incubated at 25°C

Effect of Metal ions on rhodanese from TF

The result obtained for different concentrations of 0.5mM and 0.1mM of the metals using their chlorides is presented in Table 2. The result showed that the activity of the enzyme was inhibited at the different concentrations of metal ions with $BaCl_2$ showing highest inhibition (50 %) in 0.1mM, while NaCl showed lowest inhibition (98%) in 0.5mM concentration of the metal ion.

Table 2. Effect of Metal ions on rhodanese from *Tympanotonos fuscatus*

Metal ions	% Residual Activity **(0.5mM)**	**(0.1mM)**
NaCl	98	88
$CaCl_2$	95	74
$BaCl_2$	70	50
$ZnCl_2$	65	60
$MnCl_2$	76	65

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Discussion

In this study, ammonium sulfate salt was used for the partial purification of rhodanese. The specific activity obtained (2.58 U/mg protein) was found to be higher than the crude extract (1.45 U/mg protein) but lower than the fraction obtained from Sephadex G-100 chromatography (4.42 U/mg protein) as shown in Table1. A specific activity of 8.4 U/mg protein was obtained for rhodanese from liver of mudskipper (*Periophthalmus barbarus*) (Okonji*et al.*, 2011), while a value of 0.206 U/mg protein was reported as the specific activity of rhodanese from rainbow trout liver (Nasrabadi and Rahmani, 2012). The increase in specific activity is indicative of an increase in purity of the enzyme as it increases during purification process (Marinou *et al.*, 2018).

Michaelis constants (Km) and maximum reaction velocities (Vmax) of rhodanese enzyme from *Tympanotonos fuscatus* were determined using Lineweaver-Burk plot as shown in Figures 2 and 3. The Km obtained for KCN and $Na_2S_2O_3$ were 39.76mM and 18.37mM respectively. A value of 36.81mM and 19.84mM for KCN and $Na_2S_2O_3$ respectively were reported for rhodanese enzyme from rainbow trout liver (Hossein and Reza, 2011). While 0.316mM and 0.408mM for KCN and $Na_2S_2O_3$ respectively were reported for rhodanese enzyme in *Thryonomyss winderianus* (Ehigie *et al*., 2019). Km is significance because it shows the affinity of the substrate for the enzyme and lower values indicate high affinity (Button, 2018). The values obtained from this study shows that the enzyme has high affinity for thiosulphate which will subsequently facilitate cyanide biotransformation into less toxic thiocyanate in the organism (Ehigie *et al.*, 2019).

A molecular weight of 35,700 Dalton was obtained for the rhodanese enzyme from *Tympanotonos fuscatus*. This value is within the molecular weight range of this enzyme (31,000 and 37,000 Dalton) (Okonji *et al*., 2015). Different molecular weights have been obtained for this rhodanese from different tissues and organisms. 36,000 Dalton was reported as the molecular weight of rhodanese from fruit bat (Fagbohunka *et al*., 2004) 36,800 Dalton was reported for catfish (*Clarias gariepinus*) rhodanese (Akinsikun *et al*., 2004) and 37,153 Dalton was reported in typical marine fish (*Lutjanus goreensis*) (Jack *et al*., 2015).

The substrate specificity of rhodanese from *Tympanotonos fuscatus* showed affinity for all substrates used (sodium thiosulphate, sodium metabisulphite, ammonium persulphite and 2-mercaptoethanol). Sodium thiosulphate had the highest affinity while 2-mercaptoethanol had the lowest affinity as shown in Figure 4. This is in agreement with previous studies that reported the utilization of sulphur from varying sulphur compounds as substrates for the rhodanese enzyme (Okonji*et al*., 2016; Ehigie*et al*., 2018).

The Effect of pH was assayed using buffers ranging from 3-11. While the effect of temperature was assayed ranging from 30-70^{o}C as shown in Figure 5. The optimum pH was found to be 9.0 while the optimum temperature was found to be 50^{o}C. Different optimum pH and temperature values in the range of 7.0-11.0 and 33-55 ^{o}C have been reported for rhodanese enzyme (Aboola and Okonji, 2004; Jack *et al*., 2015; Ehigie*et al*., 2019). The optimum temperature of rhodanese from *Typanatonos fuscatus* was found to be similar to those reported by Okonji *et al*. (2011) and Okonji *et al*., (2015). An optimum temperature of 30°C was reported for rhodanese enzyme from liver of rainbow trout (Hossein and Reza, 2011). The optimum pH and temperature give the highest activity of the enzyme (Sun *et al*., 2018).

The result obtained for the effect of heat on the *Tympanotonos fuscatus* rhodanese activity as presented in Figure 6 indicates that the enzyme was found to be unstable at temperatures above 30^{o}C losing about 20, 50, 55 and 70 % of activity after 20 minutes of incubation at 40, 50, 60 and 70^{o}C respectively. However, relative stability of its activity was established at 40, 50 and 60 ^{o}C (57, 45and 39

% respectively) when incubated for 70 minutes. This implies that the enzyme showed considerable heat sensitivity. Exposure of catalytic sites to temperatures above 30 ^{o}C may lead to inactivation of the enzyme (Hossein and Reza, 2011)**.**

The effects of metals ions on the enzyme as presented in Table 2 showed that the activity of the enzyme was inhibited at the different concentrations of metal ions with $BaCl_2$ showing highest inhibition (50%) in 0.1mM, while NaCl showed lowest inhibition (98%) in 0.5mM. The low inhibitory actions may be due to the natural habitat of the organism (brackish water) which contain NaCl. The absence of inhibitory effect of metal ions such as Na was reported in studies conducted on the physicochemical properties of rhodanese from the kidney of cane rat (*Thryonomyss winderianus*) (Ehigie *et al*., 2019). Effect of the metal ions on some functional groups present at the active site may have contributed to inhibitory activities of the enzyme (Hossein and Reza, 2011; Obasi *et al*., 2017).

Conclusion

The indiscriminate release and deposition of hazardous and harmful substances in the environment has continued to be a source of different pollutants. Among several biological agents utilized as eco-friendly bioremediation agent, enzymes have been discovered as most efficient tool in restoring polluted environment due to their enormous abilities in the transformation and detoxification of pollutants. This study demonstrated that the biochemical properties of rhodanese from *Tympanotonos fuscatus* is comparable to the activities of rhodanese from other organisms and tissues. The rhodanese enzyme from this organism may be exploited as eco-friendly agent for extreme cyanide detoxification and bioremediation.

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