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Original Research

Characterization of Thiosulphate: Cyanide sulphur transferase from the gut and body segments of Earthworm (*Hyperiodrilus africanus*)

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ABSTRACT: Cyanide compounds that are by products of industrial activities are known to pose serious environmental pollution. The use of these cyanide compounds by the mining industry, along with limitations in the analysis and monitoring of these compounds, raises serious concerns regarding environmental protection and public safety. *Hyperiodrilus africanus* (earthworm) is directly employed within bioremediation strategies to promote biodegradation of organic contaminants and thus could be employed to rejuvenate cyanide contaminated soils. Cyanides detoxification could also prevent the risk of cyanide poisoning in poultry animals by converting cyanides in forages to a less toxic compound. This work is designed to extract and characterize rhodanese (thiosulphate: cyanide sulphur transferase, (EC 2.8.1.1) from the gut and body segments of *H. africanus* collected from the swampy area along Uren bank river in Ikenne community of Ogun State, Nigeria. Our results show total rhodanese activities of 1434.50 RU and 2274.28 RU and specific activities of 108.01 RUmg^{-1} and 83.1901 RUmg^{-1} in the gut and body segments of *H. africanus* respectively. The optimum temperature of 25 °C and optimum pH of 10.5 were obtained for both the gut and body segments enzymes. The enzyme obeyed Michaelis-Menten kinetics and the kinetic constants, K_m and V_{max} in the gut segment were 33.33 mM and 62.50 RU/ml for KCN substrate and 22.22 mM and 41.67 RU/ml for $Na_2S_2O_3$ substrates. In the body segment, the K_m and V_{max} were 33.33 mM and 83.33 RU/ml; 15.38 mM and 4.00 RU/ml for the KCN and $Na_2S_2O_3$ substrates respectively. Hence, we conclude that the enzyme is more specific for $Na_2S_2O_3$ than KCN as substrates, though maximum activity was observed in the body segment for KCN substrate. Ca^{2+}, Mg^{2+}, Ba^{2+}, K^+, Na^+, Cu^{2+} and Zn^{2+} metal ion salts activated the body segment rhodanese at 1 mM and 5 mM concentrations while they have no effect on the gut segment rhodanese from earthworm. On the basis of these findings we conclude that earthworm could detoxify cyanide-containing wastes/forages and therefore promote biodegradation.

KEYWORDS: Rhodanese, earthworm*, environmental protection, cyanide detoxification, bioremediation.

INTRODUCTION

The principal pathway for cyanide detoxification is catalyzed by the enzyme rhodanese. Rhodanese (cyanide: thiosulphate sulphur transferase: EC.2.8.1.1) probably acts as a general sulphur transferase enzyme (Sorbo, 1953; Lee *et al.*, 1995), catalyzing the formation of less toxic thiocyanate from cyanide (Lang, 1933). It was hypothesized that rhodanese enzyme catalyzes the transfer of the outer sulphate of thiosulphate to cyanide forming the products thiocyanate and sulphide (Sorbo, 1975). The conversion of cyanide to thiocyanate was first demonstrated by Lang (1894). The enzyme originally found in the mitochondrion is widely distributed in both plants and animal species and has been

reported to be located in the cytosol and other organelles [6,7] and its presence in the liver tissue of different animals had been reported (Sorbo, 1953; Blumenthal & Heinrikson, 1971; Agboola et al., 2006).

Cyanide is a highly toxic compound that is readily absorbed and causes death by preventing the use of oxygen by tissues (Egezeke & Oehme, 1980). This toxicant is widespread in the environment. Cyanide compounds are widely used by the mining industry to assist in the extraction of metals from rock. The use of these cyanide compounds by the mining industry, along with limitations in the analysis and monitoring of these compounds, raises serious concerns regarding environmental protection and public safety at mine sites. These compounds find their way into streams and rivers through accidental spills, discharges, dam overflows, water run-off and also seeping into groundwater (Leduc, 1984) resulting in massive kill of fishes, amphibians, aquatic insects and aquatic vegetation (Leduc, 1984; Eisler, 1991; Dave, 2004) while also contaminating soils. Many naturally occurring substances as well as industrial products contain cyanide with more than 2,000 species of plants known to contain cyanogenic glycosides (Vennesland et al., 1982).

It has been reported that ingestion of cyanogenic glycosides in forage crops can result in the death of grazing animals (Keeler et al., 1978). It is also present in a wide variety of food materials that are consumed by mammals (Montgomery, 1965).

Hyperiodrilus africanus (earthworm) is employed directly within bioremediation strategies to promote biodegradation of organic contaminants (Hickman & Reed, 2008). Thus, a cyanide-contaminated soil/stream can be rejuvenated or rid-off respectively of this contaminant by employing this class of annelids. Moreover, the potential value of earthworms as a protein source had been established by various researchers [(Stafford & Tacon, 1988; Edwards & Niederer, 1988; Orozco et al., 1988; Ortega et al., 1996; Mattson et al., 2002).

It has been suggested that earthworm could provide substantial nutrients to the animals consuming them. Furthermore, some studies (Albarran, 1996; Dynes, 2003; Vielma-Rondon et al., 2003) have shown that not only could earthworm serve as a rich protein source but also as a source of essential amino acids, especially lysine which is limiting in many basic foodstuffs and that the amino acid

Table 1: Purification Table for the Earthworm Gut Segment Rhodanese

Purification Steps	Total Volume (ml)	Total Protein (mg)	Total Activity (Ru)	Specific Activity (Ru/Mg)	Yield (%)	Purification Fold
Crude Homogenate	260.00	478.40	22687.60	47.42	100.00	1.00
Ammonium Sulphate Fractionation	90.00	252.00	12972.70	51.48	57.18	1.09
Ion-Exchange on CM-Sephadex	136.80	131.33	10260.00	78.13	45.22	1.65

Table 2: Purification Table for Earthworm Body Segment Rhodanese

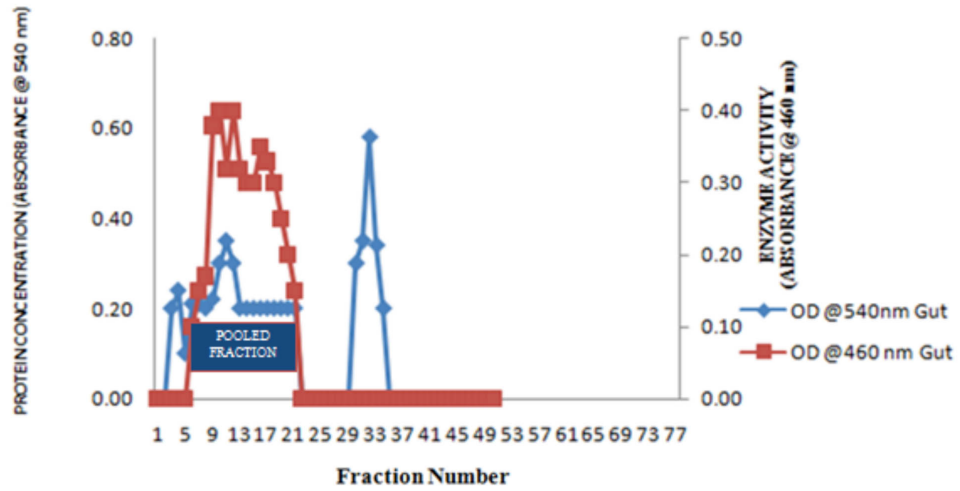| Purification Steps	Total Volume (ml)	Total Protein (mg)	Total Activity (Ru)	Specific Activity (Ru/Mg)	Yield (%)	Purification Fold
Crude Homogenate	1135.00	1816.00	100629.10	55.41	100.00	1.00
Ammonium Sulphate Fractionation	165.00	693.47	62336.01	97.48	61.95	1.76
Ion-Exchange on CM-Sephadex	298.00	326.31	46915.69	143.78	46.62	2.59

Figure 1: Elution profile of rhodanese from earthworm gut on CM-Sephadex Ion-exchange Chromatography.
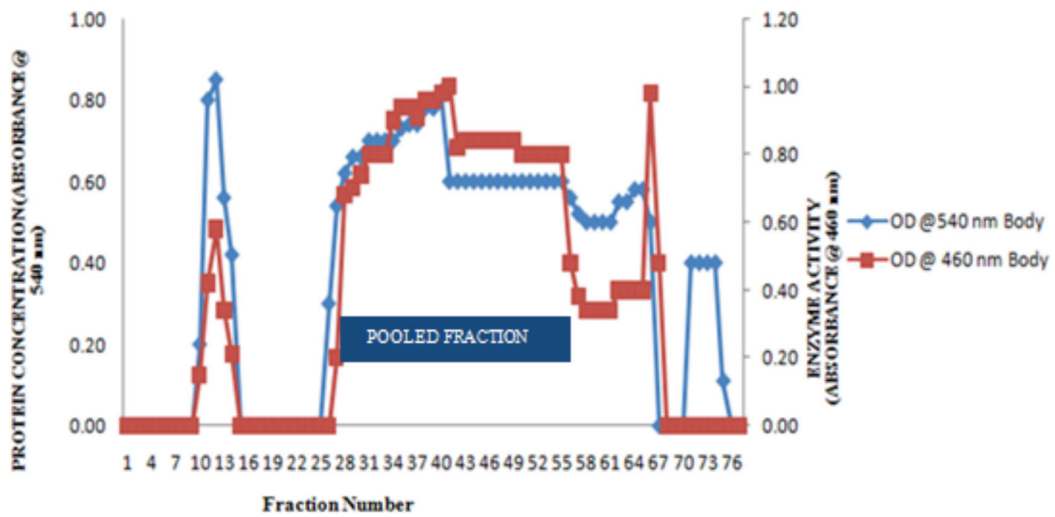

Figure 2: Elution profile of rhodanese from earthworm body on CM-Sephadex Ion-exchange Chromatography.

composition of earthworm meal is very similar to that of fishmeal and potentially superior to that of meat meal. Thus, it could serve as a cheaper source of protein to livestock while also preventing the risk of cyanide poisoning in these animals by converting cyanides in forages to a less toxic compound.

Although, the enzyme rhodanese has not been isolated and characterized from earthworms, it is plausible to assume that the earthworm possesses an efficient mechanism for cyanide detoxification since they feed on soil. An investigation of the distribution of the enzyme activities in the gut and body segments of *Hyperiodrilus africanus* could provide useful information on the metabolism of these toxicants by the organism.

MATERIALS AND METHODS

Mature earthworms were collected from the swampy sites along the Uren bank River, Ikenne, Ogun State, Nigeria between March 2010 and September 2010. The earthworms were brought to the laboratory with some of the earth materials and carefully placed in a clean jar and thereafter identified by the zoologist. All chemicals and reagents used were of analytical grade.

Enzyme Extraction

The earthworms were copiously washed in ordinary water to remove dirt and sand. They were placed in ice-bath for 30 minutes and then divided into three (3) regions, the head (segments 1-8), gut (segments 9-17) and body (the posterior end) segments. Each was washed in cold normal saline and homogenized in 0.10 M acetate-glycine buffer, pH 7.8 to give a 10% homogenate. The resulting homogenate was centrifuged at 3,000 rpm for 5 minutes. The supernatant (crude enzyme) was collected and the pellet was discarded. The volume of supernatants from each region was recorded.

Enzyme Assay

Rhodanese activity was measured routinely in the three divisions of *H. africanus* according to the method of Lee *et al.* (1995) to detect the concentration of thiocyanate (SCN^-) formed by the reaction of cyanide and sodium thiosulphate. The reaction mixture consisted of 0.60 ml of 50 mM borate buffer pH 9.4, 0.20 ml of 0.25 M KCN, 0.20 ml of 0.25 M of $Na_2S_2O_3$ and 20 µL of appropriately diluted enzyme extract. The mixture was incubated for 1 minute at room temperature and reaction stopped by the addition of 0.5 ml of 15% formaldehyde and 1.50 ml of 10% ferric nitrate solution (Sorbo reagent). The absorbance was read at 460 nm. The activity was expressed in rhodanese unit (RU). One unit of rhodanese enzyme is defined as the amount of enzyme which produces an optical density reading of 1.08 at 460 nm per minute (Sorbo, 1951).

Protein Concentration Determination

The protein concentrations of crude enzyme and at various levels of purification were determined by Biuret method (Gornall *et al.*, 1949) using bovine serum albumin as standard.

Enzyme Purification

Ammonium Sulphate Fractionation

The crude enzyme extracts were precipitated overnight at 70% ammonium sulphate at 4 ^{o}C. The precipitated protein was recovered by centrifugation at 3000 rpm for 5 minutes at room temperature and was reconstituted with 50 mM citrate buffer (pH 5.0) containing 10% ammonium sulphate and 1% PMSF. The samples were saved at 4 ˚C for further analysis.

Dialysis

The precipitated samples were desalted using pretreated dialysis tubing immersed in 50 mM citrate buffer at pH 5.0.

Ion-Exchange Chromatography

Each dialysate was loaded on pre-treated CM-Sephadex C-50 column (2.5 x 40 cm) which had been pre-equilibrated with 50 mM citrate buffer, pH 5.0 containing 10 mM sodium thiosulphate ($Na_2S_2O_3$). Fractions (5 ml) were collected at a flow rate of 30 ml/hr. The bound proteins were eluted with 0.50 M KCl in the same buffer. Enzyme assay and protein concentration were routinely determined in each of the fractions collected.

Determination of kinetic parameters

The kinetic parameters (K_m and V_{max}) were determined by varying the concentrations of KCN and $Na_2S_2O_3$ (between 10 mM and 100 mM at fixed concentrations of 50 mM for each of the substrates) respectively. The kinetic parameters were then estimated from the double-reciprocal plot of Lineweaver and Burk (1934).

Effect of pH

The optimal pH values were determined by assaying for the activity of the enzyme in the gut and body segments of *H. africanus* using three buffer systems which included 0.2 M citrate buffer (pH 5.0-6.5), 0.2 M phosphate buffer (pH 7.0-8.5), and 0.2 M borate buffer (pH 9.0-11.0).

Effect of Temperature

Optimal temperatures of the enzyme were determined by assaying for the activity of the enzyme from the gut and body segments of *H. africanus* at temperatures between 20 ˚C and 75 ˚C. The reaction mixture was first incubated at the selected temperature for 10 minutes before the addition of the aliquot of enzyme that had been equilibrated at the same temperature to the reaction mixture.

Effects of Cations

The effect of metal ions salts on the activity of the gut and body segments rhodanese were investigated at 1 mM and 5 mM concentrations. The following salts were used in assaying for the effect of the cations: Calcium chloride ($CaCl_2$), Magnesium chloride ($MgCl_2$), Barium chloride ($BaCl_2$), Potassium chloride (KCl), Sodium chloride (NaCl), Copper sulphate ($CuSO_4$), and Zinc sulphate ($ZnSO_4$).

RESULTS

Partial purification of Enzyme

Activities were detected only in the gut and body segments while the head segment had no activity and was discarded. The results of the purification procedure are summarized in Tables 1 and 2. The total activities were calculated and the total protein concentrations were interpolated from the protein standard calibration curve (not shown) in the two segments. The procedure yielded rhodanese with specific activity of 78.13 RU/mg and 143.78 RU/mg for the gut and body segments respectively.

Ion-Exchange Chromatography

The enzyme activity and protein concentration of the eluents collected were determined and the results are shown in Figures 1 and 2.

Kinetic Parameters

The plots of activity of the enzyme (V) versus substrate concentration [S] (KCN and $Na_2S_2O_3$) were hyperbolic (Figures not shown). The kinetic parameters (K_m and V_{max}) were estimated from Lineweaver-Burk's plots for the gut (Figures 3 and 4) and body (Figures 5 and 6) segments respectively. In the earthworm's gut segment, the K_m values of 33.33 and 22.22 mM were obtained while the V_{max} values determined were 62.50 and 41.67 RU/ml for the KCN and $Na_2S_2O_3$ substrates respectively.. In the body segment, the same value of 33.33 mM was obtained for the K_m(KCN) while 15.38 mM was obtained for the K_m ($Na_2S_2O_3$); the V_{max} of 83.33 and 4.00 RU/ml were observed for KCN and $Na_2S_2O_3$ substrates respectively (Table 3). The overall result showed that the enzyme was more specific for the $Na_2S_2O_3$ substrate than the KCN although highest value of V_{max} was obtained in the body segment of the organism for KCN substrate.

pH and Temperature Profile

The optimum pH of 10.5 was obtained for the gut and body segments rhodanese (Figures 8 and 9) while the optimum temperature of 25 °C was obtained in the two segments (Figures 10 and 11).
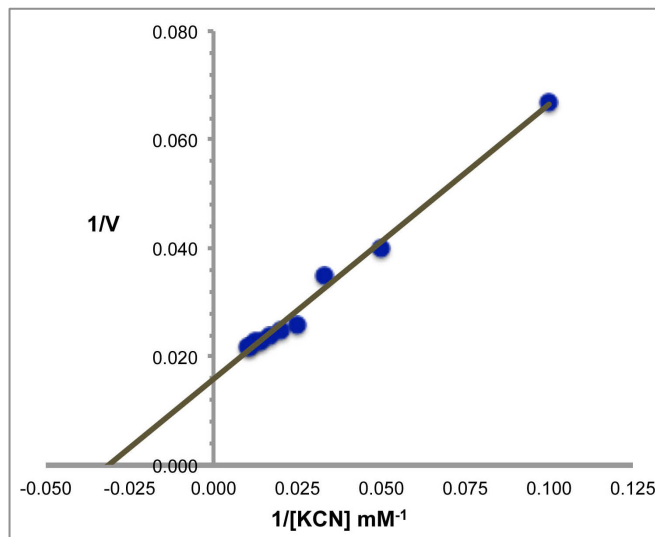

Figure 3: Lineweaver–Burke's plot using KCN as substrate of partially purified rhodanese from earthworm gut.
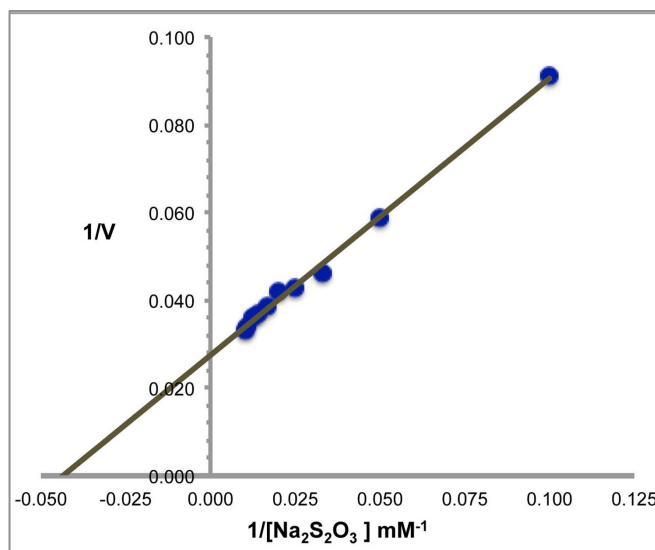

Figure 3: Lineweaver–Burke's plot using $Na_2S_2O_3$ as substrate of partially purified rhodanese from earthworm gut.

DISCUSSION

In this study, we characterized the rhodanese enzyme in the gut and body segments of the earthworm. Several soil organisms are able to thrive on cyanogenic plants and soil, due primarily to inherent cyanide detoxifying mechanisms of the organism. Rhodanese and 3-mercaptopyruvate sulphurtransferase (3-MST) represent the chief enzymes of cyanide detoxification (Westley, 1980; Nagahara & Nishino, 1996). Rhodanese has been reported in many organisms
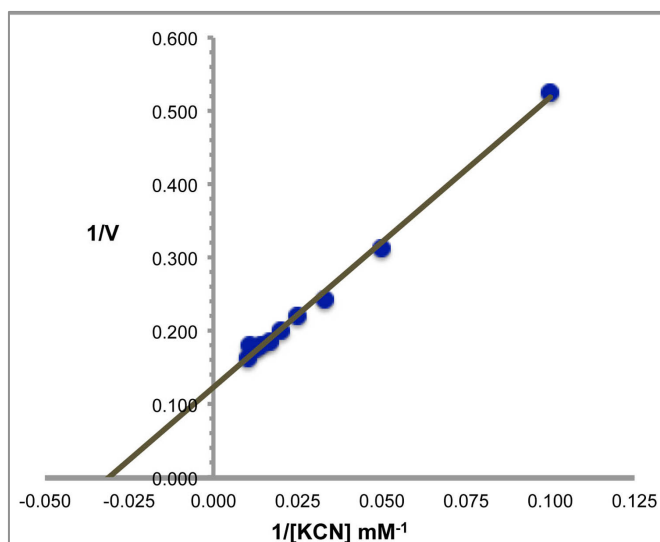
Figure 5: Lineweaver–Burk's plot using KCN as substrate of partially purified rhodanese from earthworm body segment.
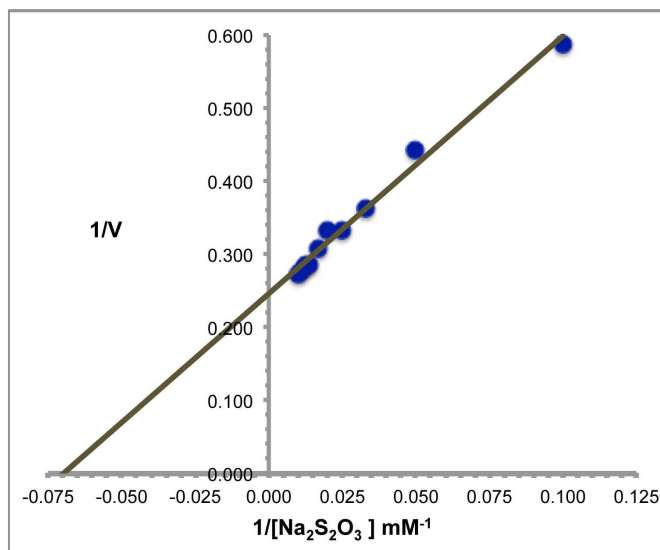

Figure 6: Lineweaver –Burk's plot using Na₂S₂O₃ as substrate of partially purified rhodanese from earthworm body.

Table 3: Kinetic Parameters of gut and body segments of the Earthworm

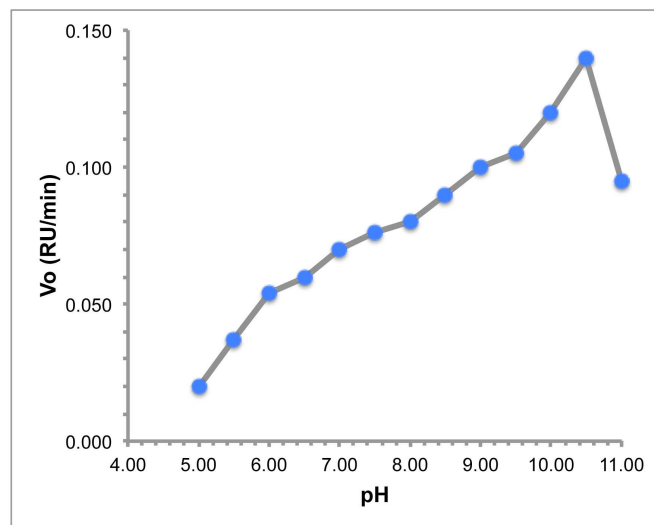| Kinetic Constants	KCN		Na₂S₂O₃	
	Gut	Body	Gut	Body
K_m (mM)	33.33	33.33	22.22	15.38
V_{max} (RU/ml)	62.50	83.33	41.67	4.00

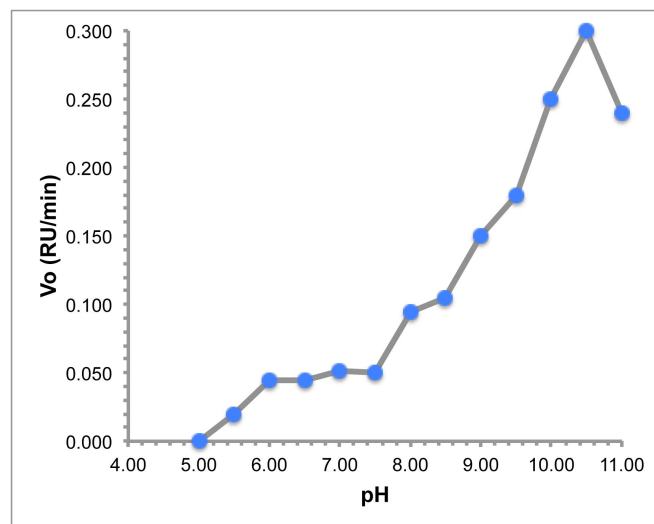**Figure 7: Effect of pH on the activity of Rhodanese Activity (RU) in the earthworm gut segment**

Figure 8: Effect of pH on the activity of Rhodanese Activity (RU) in the earthworm gut segment

from bacteria to animals (Sorbo, 1951; Jarabak & Westley, 1974; Anosike & Ugochukwu, 1981; Lee *et al.*, 1995; Agboola & Okonji, 2004; Saidu, 2004; Akinsiku *et al.*, 2009; Aladesanmi *et al.*, 2009).

The specific activities of this enzyme from gut and body segments were 78.13 RU/mg with a 45.22% yield and 143.78 RU/mg with 46.62% yield respectively. The higher specific activity of the enzyme in the body segment could be attributed to the concentration of intestinal (digestive)

enzymes in this region. Specific activity values of 73 and 72 RU/mg were obtained for catfish rhodanese I (cRHDI) and catfish rhodanese II (cRHDII) (Akinsiku *et al.*, 2009), 136.6 RU/mg was obtained for fruit bat liver rhodanese (Agboola & Okonji, 2004).
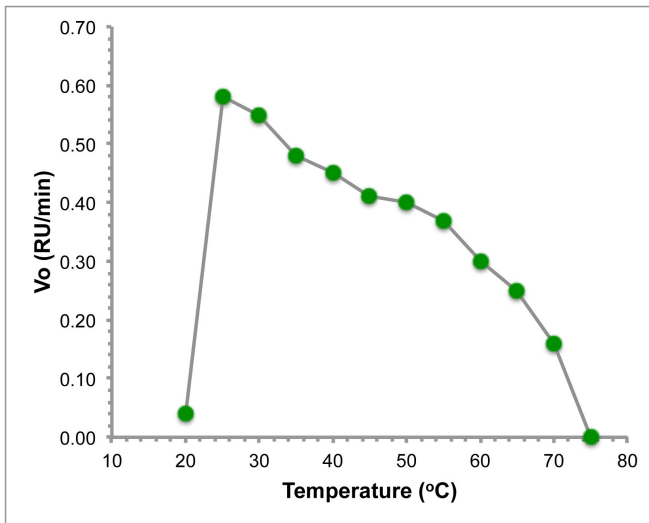
Figure 9: Temperature dependence of the activity of rhodanese from earthworm gut segment
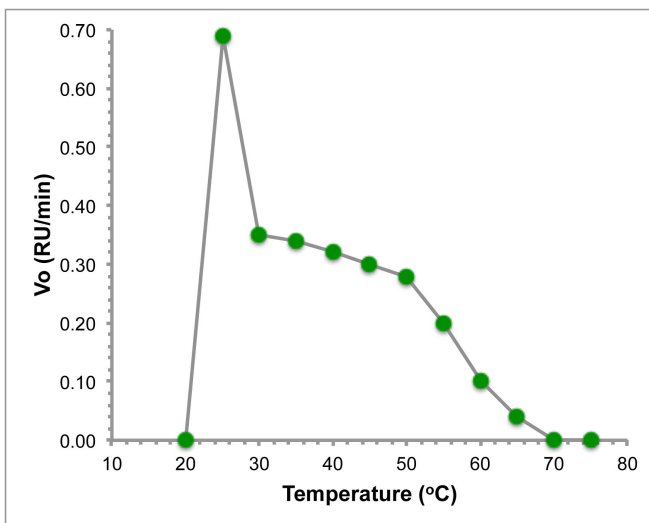
Table 4: Effect of metal ion salts on rhodanese from the gut segment of *Hyperiodrilus africanus* (earthworm)

Salts	Enzyme activity (%)	
	1mM	5mM
None	100 ± 0.00	72.89 ± 0.93
CaCl$_2$	71.50 ± 41.28	73.51 ± 0.21
MgCl$_2$	75.00 ± 0.46	66.20 ± 1.69
BaCl$_2$	71.89 ± 0.41	73.53 ± 1.09
KCl	74.68 ± 0.84	78.26 ± 0.76
NaCl	75.86 ± 0.35	82.33 ± 1.13
CuSO$_4$	81.91 ± 0.71	70.55 ± 0.55
ZnSO$_4$	73.26 ± 1.37	72.89 ± 0.93

Figure 9: Temperature dependence of the activity of rhodanese from earthworm body segment

Table 4: Effect of metal ion salts on rhodanese from the gut segment of *Hyperiodrilus africanus* (earthworm)

Salts	Enzyme activity (%)	
	1mM	5mM
None	135.52 ± 1.18	110.05 ± 0.38
CaCl$_2$	101.22 ± 0.82	113.16 ± 1.09
MgCl$_2$	100.02 ± 0.02	105.60 ± 0.60
BaCl$_2$	106.81 ± 0.31	115.06 ± 0.26
KCl	184.28 ± 0.75	102.27 ± 1.77
NaCl	106.02 ± 0.43	102.27 ± 1.77
CuSO$_4$	91.53 ± 0.73	112.36 ± 0.91
ZnSO$_4$	135.52 ± 1.18	110.05 ± 0.38

Activity values of 256 RU/mg and 131 RU/mg were reported for bovine liver rhodaneses by Sorbo (1953a) and Himwich & Saundrs (1948) respectively. The K$_m$ values of 22.22 and 15.38 mM were obtained for the Na$_2$S$_2$O$_3$ in the gut and body segment of the earthworm respectively while a higher value of 33.33 mM was obtained for the KCN substrate in both segments of the organism. These values corroborated the specificity of the enzyme for these substrates as reported by other researchers: bovine liver, 19.0 and 6.5 (Sorbo, 1953a) bat liver, 13.36 and 19.15 (Agboola & Okonji, 2004) and cat liver, 25.4 mM and 18.6 mM (Akinsiku *et al.*, 2009) for KCN and Na$_2$S$_2$O$_3$ substrates respectively.

Sodium thiosulphate had been reported to be one of the antidotes employed in treating cyanide toxicity and being a sulfur donor for sodium thiosulphate:cyanide sulphur transferase (rhodanese) (Leung *et al.* 1986), it was not surprising that the enzyme is more specific for this substrate as observed in this work. It facilitates catalytic metabolism of cyanide to less toxic thiocyanate (Nagahara & Sawada, 2003). Cyanide and thiosulphate were reported to be acceptor substrates (Sorbo, 1953b; Westley, 1980).

Earthworms feed on a wide variety of plants and soil (Mutere, 1965; Halstead & Segun, 1975; Okon *et al.*, 1976). Many plants and soils contain prussic acid (hydrogen cyanide)

(Montgomery, 1965). In fact, the use of hydrogen cyanide sprays by farmers and yet the ability of earthworms to improve soil aeration thus provides evidence for the presence of a powerful mechanism for the detoxification of cyanide in this animal (Constantine, 1970). Therefore, the incorporation of earthworm into livestock feeds could make available more plants as foods for these animals while also serving as a cheaper source of protein to these animals. The metal ion salts reported in this work activated the enzyme in the body segment while the gut segment enzyme was unaffected by these ions. This activation could possibly be as a result of the diffusion of solutions in and out of the body of the earthworm because of the regular exposure of the organism to varying environmental factors. The optimum temperature of 25 ^{o}C obtained in the gut and body segments of the earthworm is lower than the values reported for the enzyme from different sources which had higher optimum temperature of 38 ^{o}C and 40 ^{o}C for bovine liver rhodanese (Himwich & Saunders, 1948), 50 ^{o}C for bovine liver (Sorbo, 1953a), and 55 ^{o}C for the soldier termites (Okonji et al., 2010). This large difference could be attributed to the natural ecology (damp environment) of the organism. The optimum pH obtained in this study was 10.5 and this is in good agreement with the optimum pH value of 8.0-11.0, which was reported for different organisms (Sorbo, 1951; Jarabak & Westley, 1974; Anosike & Ugochukwu, 1981, Lee et al., 1995; Agboola & Okonji, 2004; Saidu, 2004; Okonji et al., 2008).

Conclusion

In conclusion, this research confirmed the presence of rhodanese in the gut and body segments of the earthworm homogenates and therefore the rhodanese from could be employed where the conditions of temperature and pH specified are required in cyanide detoxification.

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