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PURIFICATION AND CHARACTERIZATION OF RHODANESE FROM THE LEAVE OF BITTER MELON (*MOMORDICA CHARANTIA*)

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ABSTRACT

The *Mormodica charantia* leaves used in this work was obtained from Ile-Ife, Osun State. *Mormodica charantia* rhodanese was purified using 80% ammonium sulphate precipitation and affinity chromatography technique. The enzyme had a specific activity of 5.4 Rhodanese Unit per milligram of protein (RU/mg) with a purification fold of 2.42 and a percentage yield of 8.78%. The K_m of rhodanese from *Mormodica charantia* for sodium thiosulphate ($Na_2S_2O_3$) and potassium cyanide (KCN) were 16.67mM and 20mM respectively, while their V_{max} were 0.2RU/ml/min and 0.24RU/ml/min respectively. The substrate specificity showed that the enzyme was not inhibited by 2-mercaptoethanol, ammonium persulphate and sodium metabis- sulphite. The optimum temperature was 60°C at a pH of 7.0. The enzyme was not inhibited by salts (KCl, NaCl, $NiCl_2$, $MnCl_2$, $ZnCl_2$ and BaCl).

KEYWORDS: Rhodanese, *Mormodica charantia*, Properties, Sulphur Compounds

INTRODUCTION

Cyanide is toxic to all living things including humans. Cyanide reduces ferric cytochrome oxidase (an iron-containing metalloprotein) to ferrous cytochrome oxidase which transfers electron to oxygen (Jeong *et al*., 2005). Many naturally occurring substances as well as industrial products contain cyanide (Egekeza and Oehme, 1980). More than 2,000 species of plants are 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). Many studies report the death of birds from cyanide poisoning through several routes, including exposure to cyanide salts or ingestion of cyanogenic plants (Wiemeyer *et al*., 1986).

Rhodanese, a thiosuphate: cyanide sulphurtransferase (EC 2.8.1.1), is an enzyme that catalyses the transfer of the sulphane sulphur from thiosulphate to cyanide, forming the less toxic thiocyanate and sulphite (Westley, 1981; Westley *et al*., 1983).

$$SSO_3^{2-} + CN^- \rightarrow SO_3^{2-} + SCN$$

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 and Nishino 1996; Agboola and Okonji, 2004). Various plant and microbial sources also demonstrate rhodanese activity (Smith and Urbanska, 1986). Rhodanese has also been investigated in tapioca leaf (Boey, *et al*.,1976).

Momordica charantia (Cucurbitaceae) is a creeping or climbing annual weak herb. It is extensively cultivated in India, East Africa, Central and South America, China and other parts of South East Asia. A multitude of plants have been used for the treatment of diabetes throughout the world. One such plant is *Momordica charantia*, whose fruit is known as Karela or bitter gourd (Celia *et al*., 2003). This plant has been reported to have anti-leukaemia and antiviral activities (Ng *et al*., 1994). *Mormodica charantia* is a plant with many medicina use (Sathish *et al*., 2010), and very rich in nutrients like thiamine, beta-carotene and minerals etc (Leatherdale *et al*., 1981; Ahmad *et al*., 1999).

This paper discusses the enzymatic properties of rhodanese enzyme isolated from leaves of *Momordica charantia*, bitter melon which will possibly explain its role in the treatment of ailments and diseases.

MATERIALS AND METHODS

Materials

Potassium cyanide, sodium thiosulphate, boric acid, sodium borate and mercaptoethanol were all purchased from Sigma Aldrich Chemicals, USA. All reagents used were of analytical grades.

Methods

Collection of Samples

The leaves of *Mormodica charantia* was collected in the in Ile-Ife town, Osun State, South-Western region of Nigeria. The leaves were freshly collected from their natural environment and stored in an ice container before transporting to the laboratory where they were stored at temperature in the refrigerator until ready for use. They were identified at the Department of Botany, ObafemiAwolowo University, Ile-Ife, Nigeria.

Preparation of Leaves

Prior to homogenization, the leaves were washed to remove debris. Leaf extracts were prepared by homogenizing 150 g (w/v) of the leaf in 3 volume of homogenization buffer (phosphate buffer, pH 7.2). The suspensions were centrifuged for 20 min at 4,000 rpm in a Microfield Centrifuge Model 800 D. The supernatant was used as the source of enzyme.

Protein and Enzyme Assays

Bradford method (1976) was used to measure the protein concentration of the enzyme using bovine serum albumin (BSA) as standard. Rhodanese was assayed by the method of Agboola and Okonji (2004). The reaction mixture contained 50 mM sodium thiosulphate, 50 mM potassium cyanide, 0.25 mM borate buffer, pH 9.4 and 10 µl of enzyme solution in a final volume of 1.0 ml. The reaction was carried out for 1 min at 37°C and stopped by adding 0.5ml 15% formaldehyde and 1.5 ml of Sorbo reagent (which is made up of ferric nitrate solution containing 0.025 g $Fe(NO_3)_3.9H_2O$ in 0.74 ml water and 0.26 ml concentrated nitric acid). Absorbance was measured at 460 nm. The unit of enzyme activity was defined as micromoles thiocyanate formed per minute at 37°C and pH 9.2.

Enzyme Purification

The supernatants were then pooled together and brought to 80% ammonium sulphate concentration and left for 12 hr. The resulting precipitate obtained after centrifugation at 4,000 rpm for 30 min. was dialyzed against several changes of 0.1 M solution of phosphate buffer (pH 7.2) containing sodium thiosulphate at 4°C for 18 h. The dialysate was centrifuged at 12,000 rpm at 10°C for 30 min to remove insoluble materials and the supernatant was assayed for rhodanese activity and protein. This was used for the affinity chromatography step. The Reactive Blue-2 agarose was equilibrated with 50 mM citrate buffer pH 5.0. The gel was packed into a 1.5 × 10 cm column. The enzyme fraction from the previous step was layered on it. Fractions of 1.0 ml were collected from the column. The protein was monitored using Bradford method. The fractions were also assayed for rhodanese activity. The fractions with high enzyme activities were pooled and preserved in 50 % glycerol-citrate buffer solution.

Determination of Kinetic Parameters

The kinetic parameters (K_m and V_{max}) of the enzyme were determined by varying concentrations of 250 mM KCN between 0.05 M and 0.005 M at fixed concentration of 0.1 M $Na_2S_2O_3$. Also, the concentration of 250 mM $Na_2S_2O_3$, ammonium persulphate, sodium metabissulphite, 3-mercatoethanol was varied between 0.05 M and 0.005 M at fixed concentration of 0.1 M KCN. Plots of the reciprocal of initial reaction velocity (1/V) versus reciprocal of the varied substrates 1/[S] at each fixed concentrations of the other substrate were made according to Lineweaver and Burk (1934).

Substrate Specificity and Kinetics

The substrate specificity of the enzyme was determined by using different sulphur compounds such as sodium thiosulphate, 2-mercaptoethanol, ammonium persulphate, and sodium metabisulphite in a typical rhodanese assay mixture. The activity was expressed as a percentage activity of the enzyme using sodium thiosulphate which was the control.

Effect of Metals on the Enzyme Activity

The method of Lee *et al.* (1995) was used to study the effect of various metal ions on the activity of *Momordica charantia* leave rhodanese. The salts of the cations include $MgCl_2$, $CaCl_2$, $NiCl_2$, $MnCl_2$, $SnCl_2$, $BaCl_2$ and KCl and at concentrations of 1.0 mM and 10 mM. A typical enzyme assay with 1 ml of reaction mixture contained 0.5 ml 50 mM borate buffer pH 9.4, 0.2 ml of 250 mM KCN, 0.2 ml of 250 mM $Na_2S_20_3$, 0.05 ml of the respective salt solution and 0.05 ml enzyme solution.

Effect of pH on the Enzyme Activity

The effect of pH on the enzyme activity was performed according to the methods of Agboola and Okonji (2004). The enzyme was assayed using different buffers and pH: 50 mM of citrate (pH 3-5); phosphate (6-8) and borate (pH 9-11). The rhodanese activity was assayed as described in the assay section.

Effect of Temperature on the Enzyme Activity

The enzyme was assayed at temperatures between 30ºC and 70ºC to investigate the effect of temperature on the activity of the enzyme and to determine the optimum temperature of the enzyme. The assay mixture was first incubated at the indicated temperature for 10 min before initiating reaction by the addition of an aliquot of the enzyme which had been equilibrated at the same temperature. The rhodanese activity was assayed routinely as previously described.

RESULTS

Purification of Rhodanese

The results of the purification of rhodanese from *Mormodica charantia* are summarized in Table 1. The elution profiles after reactive blue affinity chromatography is shown in Figure 1.

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 40 mM and 16.6 mM respectively while the Vmax values for the two substrates (KCN and $Na_2S_2O_3$) were 0.3 and 0.4 RU/ml/min respectively. The result of the kinetic studies for sodium metabissulphite, ammonium persulphate and 3-mercaptoethanol is shown in Figures 4, 5 and 6. Values for the K_m and V_{max} of each sulphur compund is shown in Table 2

Substrate Specificity

Sulphur compounds such as sodium thiosulphate, sodium metabissulphite, ammonium persulphate and 2-Mercaptoethanol were used in the determination of substrate specificity. The result showed that all the sulphur compounds showed rhodanese activity. The result is shown in Table 3.

Effect of Salts on Rhodanese from leave of *Mormodica charantia*

The results of the effect of salts show that the activity of the enzyme was not inhibited by the salts (Table 4).

Effect of pH and Temperature on Rhodanese from leave of *Mormodica charantia*

The maximum activity of rhodanese from leave of *Mormodica charantia* was found to be at pH 6.0 (Figure 7). While the highest activity was obtained at 60ºC (Figure 8)

Table 1: Summary of Purification of Rhodanese from the Leaf of *Mormodica charantia*

Fraction	Total Activity(RU)	Total Protein (mg)	Specific Activity	Yield %	Purification Fold
Crude	334.7	150	2.23	100	1
Ammonium sulphate	180.3	18	5.008	53.87	1.783
Affinity Chromatography	29.7	5.4	5.4	8.78	2.42

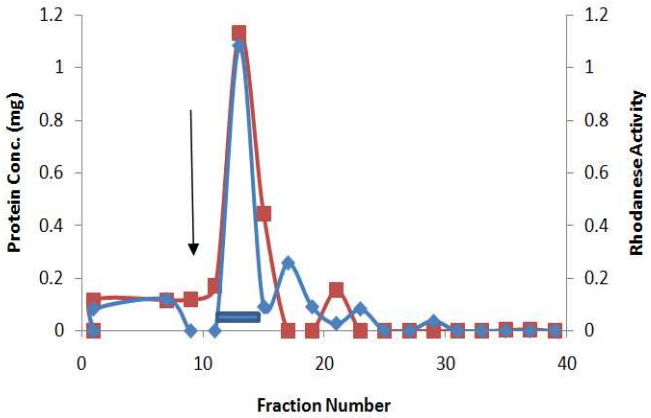

Figure 1: Purification of the *Mormodica charantia* Rhodanese Using Reactive Blue-2 Agarose Affinity Chromatography

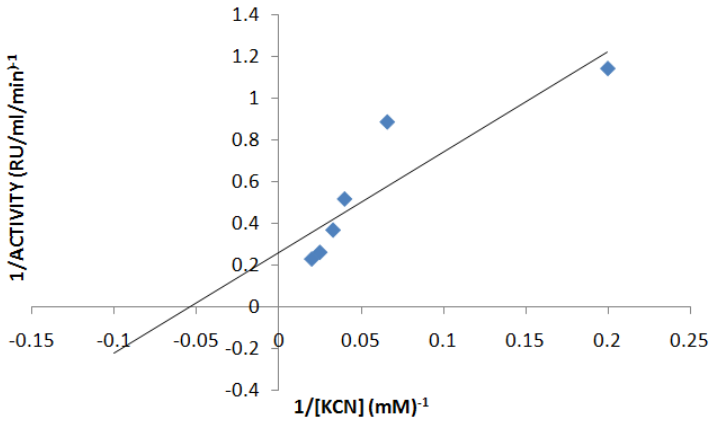

Figure 2: Lineweaver-Burk Plot for Varying Concentration of Potassium Cyanide

Lineweaver-Burk plot of 1/V against 1/[S] at varying concentrations of KCN

Between 5 mM and 50 mM and a constant concentration of $Na_2S_2O_3$ at 25 mM

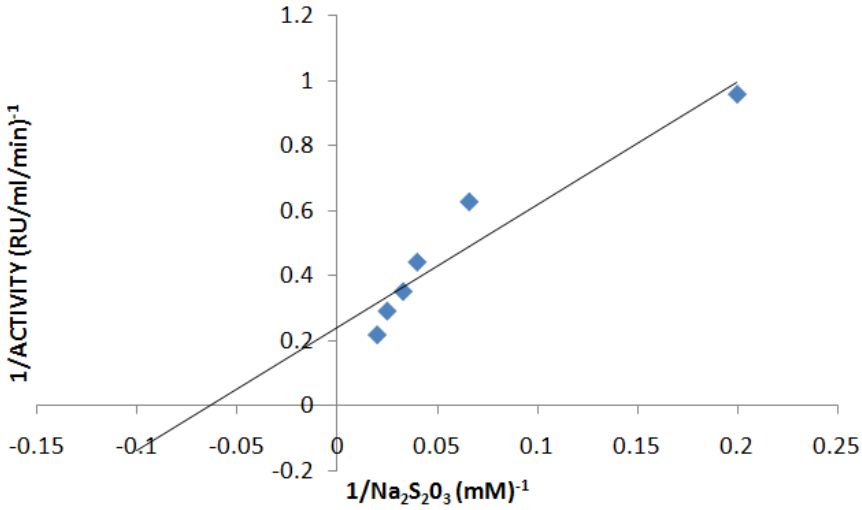

Figure 3: Lineweaver-Burk Plot for Varying Concentration of Sodium Thiosulphate

Lineweaver-Burk plot of 1/V against 1/[S] at varying concentrations of $Na_2S_2O_3$

Between 5 mM and 50 mM and a constant concentration of KCN at 25 mM

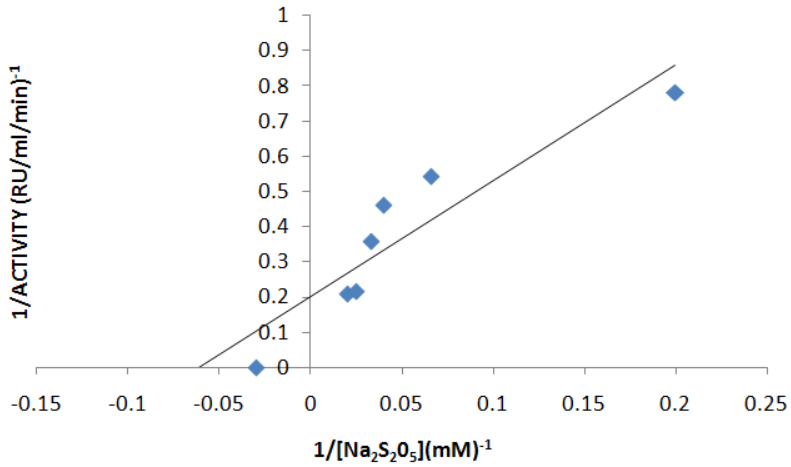

Figure 4: Lineweaver-Burk Plot for Varying Concentration of Sodium Metabissulphite

Lineweaver-Burk plot of 1/V against 1/[S] at varying concentrations of $Na_2S_2O_5$

Between 5 mM and 50 mM and a constant concentration of KCN at 25 mM

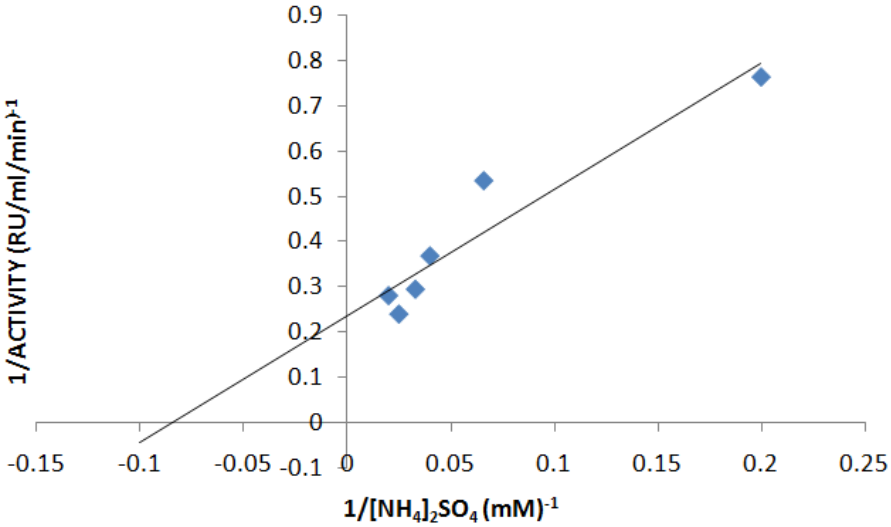

Figure 5: Lineweaver-Burk Plot for Varying Concentration of Ammonium persulphate

Lineweaver-Burk plot of 1/V against 1/[S] at varying concentrations of $[NH_4]_2SO_4$

Between 5 mM and 50 mM and a constant concentration of KCN at 25 mM

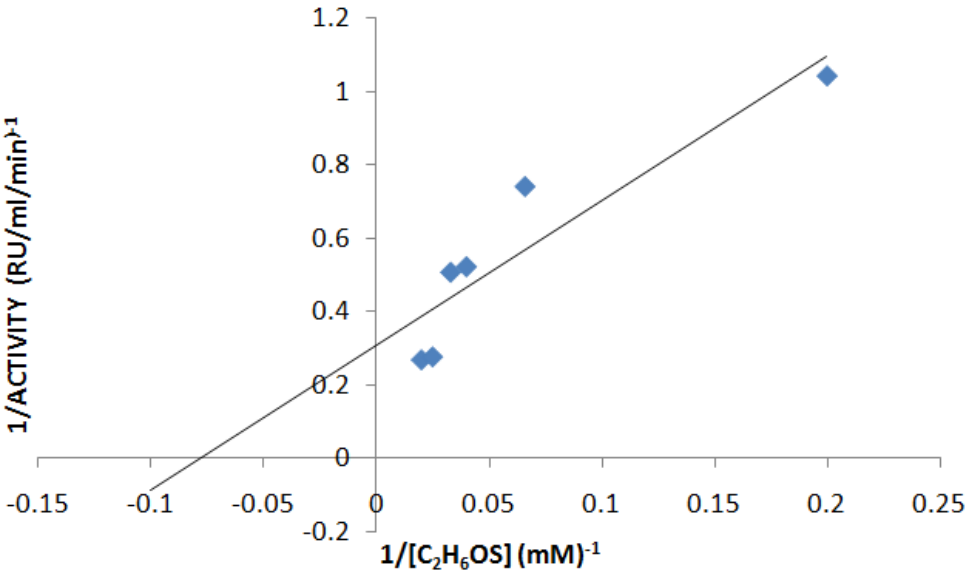

Figure 6: Lineweaver-Burk Plot for Varying Concentration of 3-Mercatoethanol

Lineweaver-Burk plot of 1/V against 1/[S] at varying concentrations of $C_2H_6O_5$

Between 5 mM and 50 mM and a constant concentration of KCN at 25 mM

Table 2: Showing the Kinetic Values of the KCN and Some Sulphur Compounds

Sulphur Compounds	K_m	V_{max}
KCN	20	0.24
Sodium thiosulphate	16.67	0.2
Sodium metabissulphite	33.33	0.2
Ammonium persulphate	12.5	0.24
3-Mercaptoethanol	12.5	0.3

Table 3: Showing the Percentage Substrate Specificity of each Sulphur Compound

Substrate	% Activity
Sodium thiosulphate	100
Ammonium persulphate	25.39
2-Mercaptoethanol	100
Sodium metabissulphite	100

Table 4: Effect of Metals on Rhodanese from *Mormodicacharantia*

Metals	% Enzyme Activity	
	0.01M	0.001M
$BaCl_2$	89.37 ± 0.625	86.3 ± 13.7
NaCl	96.5 ± 2.7	93.15 ± 6.85
$NiCl_2$	93.8 ± 3.3	84.85 ± 15.15
$MnCl_2$	85.9 ± 14.1	71.4 ± 10.00
KCl	86.3 ± 0.8	100
$ZnCl_2$	91.3 ± 2.9	100

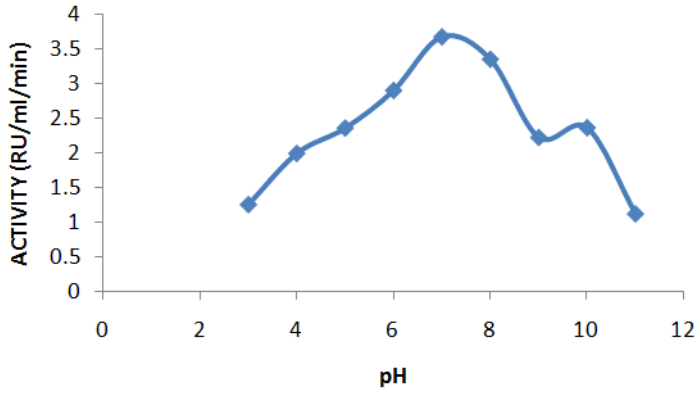

Figure 7: Effect of pH on Rhodanese Activity from *Mormodicacharantia*

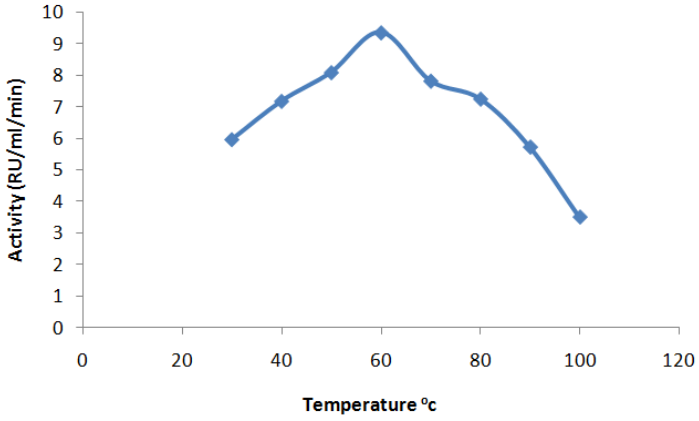

Figure 8: Effect of Temperature (°C) on Rhodanese Activity from *Mormodicacharantia*

DISCUSSIONS

Rhodanese produced from the leaves of the plant *Mormodica charantia* was purified and characterized by ammonium sulphate precipitation and Reactive Blue-2agarose affinity chromatography. The enzyme had a specific activity of 5.4 RU per mg of protein and 8.78% recovery (Table 1). Agboola and Okonji (2004) obtained136.6 RU/mg for fruit bat liver rhodanese, a yield of 7.8 was obtained in tapioca leave (Chew *et al*., 1972).

The apparent K_m values, as determined by Lineweaver-Burk plots for KCN and $Na_2S_2O_3$ were 20 and 16.67mM, respectively (Figure 2 and 3). 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). A rhodanese purified from tapioca leaves showed properties similar to that of bovine rhodanese (Boey *et al*., 1976; Hatzfeld and Saito, 2000)

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 substrate specificity study of rhodanese isolated from the leave of *Mormodica charantia*h as preference for sodium thiosulphate but can also use other sulphur compounds such as ammonium persulphate, sodium metabissulphite, and 3-mercaptoethanol for its catalytic activities (Table 3), which is in line with the studies reported by other researchers. Ehigie *et al*. (2013) and

Okonji *et al*. (2011) reported the use of different sulphur compounds from a sulphane pool by rhodanese. Other researchers have also shown the specificity of rhodanese for sulphur from thiosulphate source (Lee *et al*., 1995). Westley (1981) also reported the importance of sulphanesulphur pool in rhodanese cyanide detoxification mechanism.

The effect of heavy metals on rhodanese from *Mormodica charantia* showed that two different concentrations of metals used did not affect the activity of the enzyme metals (Table 4). This could be as a result of high content of heavy metals in the plant and or its environment.. Similar results have also been reported by Fagbohunka *et al.* (2004) and Okonji *et al.* (2011) on the rhodanese from the hepatopancreas of giant African snail and liver of mudskipper respectively. Agboola and Okonji (2004) and Hossein and Reza (2012) also reported the effect of metals on the fruit bat liver and rainbow trout rhodanese respectively, stating that the inhibition of fruit bat liver and rainbow trout liver rhodanese by Hg^{2+} and Ba^{2+} was probably due to the interaction of these metal ions with sulphydryl groups at the enzyme catalytic site or induction changes in the comformation of the enzyme (Ulmer and Vallee, 1972; Lee *et al*., 1995; Nagahara and Nishino, 1996).

The rhodanese from the leave of *Mormodica charantia* showed maximum activity at pH 7.0 (Figure 7). 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). Akinsiku *et al.* (2011) working on the African catfish liver obtained a pH value of 6.5.

An optimum temperature of 60°C was obtained for rhodanese isolated from the leaves of *Mormodica charantia* (Figure 8). Sorbo (1953a) reported an optimumtemperature of 50°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. The high temperature might be as a result of adaptation to harsh environmental condition. The rhodanese in the cytosolic fraction of fruit Bat liver was reported by Agboola and Okonji (2004) to have an optimal temperature of 35°C. Akinsiku *et al*. (2010) reported an optimum temperature of 40°C for rhodanese from the liver of the catfish in Asejire Lake. Okonji *et al*. (2011) also reported an optimum temperature of 50°C from mudskipper liver

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