PURIFICATION AND CHARACTERIZATION OF RHODANESE FROM THE HEPATOPANCREAS OF GARDEN SNAIL, *Limicolaria flammea.*

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ABSTRACT

Rhodanese from the cytosolic fraction of the hepatopancreas of garden snail, *Limicolaria flammea* was purified by 70% ammonium sulphate precipitation, Reactive Blue-2 Agarose affinity chromatography and CM-Sephadex C-25 ion-exchange chromatography. The pure enzyme had a specific activity of 4.57 μmol of thiocyanate formed per milligram of protein with a purification fold of 4.20 and a percentage yield of 19.73%. The native molecular weight was 33.1 kDa, while the subunit molecular weight of the enzyme was estimated to be 32.8 kDa. The K_m of rhodanese from the hepatopancreas of *Limicolaria flammea* for sodium thiolsulphate ($Na_2S_2O_3$) and potassium cyanide (KCN) were 12.3 mM and 9.1 mM respectively, while their V_{max} were 10.5 RU/ml/min and 7.4 RU/ml/min respectively. The substrate specificity study showed the percentage utilization of the various substrates to be: 2-mercaptoethanol (2-MCPE) (57.8%), ammonium persulphate ($(NH_4)_2S_2O_8$) (37.6%), ammonium sulphate ($(NH_4)_2SO_4$) (46.8%) and Sodium metabisulphite ($Na_2S_2O_5$) (48.1%). The K_m of rhodanese for the sulphur donor substrates were 13.3 mM, 15.4 mM, 15.9 mM and 13.5 mM respectively in the same order as above. The optimum temperature was 50°C at a pH of 8.0 and the activation energy values were 19.618 kcal/mol and 76.834 kcal/mol. The heat stability result showed the enzyme to be stable up to 50°C. The inhibition study on the enzyme by salts ($BaCl_2$, $CaCl_2$, $CoCl_2$, $HgCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$ and $ZnCl_2$) showed no significant effect. In summary, this study showed the presence of rhodanese activity in hepatopancreas of garden snail, *Limicolaria flammea*. The presence of rhodanese in the hepatopancreas of *Limicolaria flammea* suggests that the enzyme may possess functional cyanide detoxification mechanism necessary for the survival of the animal in the environment.

Keywords: Cyanide, Detoxification, Rhodanese, *Limicolaria flammea,* Hepatopancreas.

INTRODUCTION

Cyanides are produced naturally by numerous organisms, including bacteria, fungi, algae, insects and plants (Harborne, 1993; Cipollone *et al.*, 2008). Cyanides are found in certain seeds and fruits like apple, mango, peach, and bitter almonds (ATSDR, 2006). In plants, cyanides are usually bound to sugar molecules in the form of cyanogenic glycosides and help to defend the plant against herbivores (Vetter, 2009). Cyanide is a highly toxic compound that is readily absorbed by living cells and causes death by preventing the use of oxygen by tissues (Broderick *et al.*, 2006). It is a potent cytotoxic agent that kills the cell by inhibiting cytochrome oxidase of the mitochondrial electron transport chain. Cyanide poisoning occurs when a living organism is exposed to a compound that produces cyanide ions (CN^-) in aqueous solution (Peter *et al.*, 2013).

Rhodanese is a ubiquitous enzyme that is known to be responsible for the biotransformation of cyanide to thiocyanate using thiosulphate as the donor substrate (Sorbo, 1953a; Saidu, 2004; Cipollone *et al.,* 2008; Okonji *et al.*, 2010a).

$$\underset{\text{Thiosulphate ion}}{SSO_3^{2-}} + \underset{\text{Cyanide ion}}{CN^-} \longrightarrow \underset{\text{Sulphite ion}}{SO_3^{2-}} + \underset{\text{Thiocyanide ion}}{SCN^-}$$

Although the enzyme is localized in the mitochondria, it is also found in the cytosol in different tissues and the species and tissue distributions of rhodanese are highly variable (Aminlari and Gilanpour, 1991; Nasir *et al.*, 2003; Aminlari *et al.*, 2007; Okonji *et al.*, 2010a; Baghshani and Aminlari, 2012). Rhodanese uses a double displacement mechanism to catalyse the transfer of a sulphur atom from suitable donors to nucleophillic acceptors. During the catalytic process the enzyme cycles between a sulphur-free and a persulfide-containing form, via formation

of a persulfide linkage to a catalytic Cys residue (Domenico *et al*., 2000). Rhodanese has been crystallized (Sorbo, 1953a), sequenced and its three dimensional structure determined by X-ray crystallography (Bergsma *et al*., 1975; Ploegman *et al*., 1978). The active site of rhodanese contains a tryptophan residue in close proximity with essential sulphydryl groups of cysteine residues (Saidu, 2004). The sulphydryl group of cysteine-247 has been identified as the site of covalent attachment of sulphane sulphur in sulphur-rhodanese (Domenico *et al*., 2000). Several biological functions have been attributed to this ubiquitous enzyme which include cyanide detoxification such as construction of iron-sulphur centres in proteins (Ugulava *et al*., 2000); selenium metabolism (Ray *et al*., 2000); thioredoxin metabolism (Nandi *et al*., 2000) and also in the supply of sulphur for key enzymes in sulphur energy metabolism (Aussignargues *et al*., 2012).

Garden snails feed on some plants which may contain cyanogenic glycosides. There is paucity of information on the properties of rhodanese from garden snails. This study therefore intends to investigate the properties of rhodanese from the hepatopancreas of garden snail, *Limicolaria flammea*.

MATERIALS AND METHODS

Materials

Potassium cyanide, sodium thiosulphate, boric acid, sodium borate, formaldehyde, ferric nitrite, nitric acid, citric acid, sodium citrate and ε-amino-n-caproic acid were obtained from BDH Chemical Limited, Poole, England. Glycerol, sodium acetate, sodium dodecyl sulphate (SDS), low molecular weight calibration kit for electrophoresis, ethylenediamine tetraacetic acid (EDTA), Coomassie Brilliant-Blue, Blue Dextran, Reactive Blue-2 Agarose and Bovine Serum Albumin (BSA) were obtained from Sigma Chemical Company, St. Loius, Mo., USA. Biogel P-100 was purchased from Bio-Rad Laboratories Inc., Benicia Ca., USA. Other chemicals used were of analytical grade and were procured from reputed chemical firms.

Live garden snails (*Limicolaria flammea*) were collected from the Botanical Garden of the Obafemi Awolowo University, Ile-Ife, Nigeria. The garden snails were identified and authenticated at the Department of Zoology, Obafemi Awolowo University, Ile-Ife.

Enzyme Extraction

Live snails gently washed to remove surface dirt, and their carapaces were carefully split. The hepatopancreas was excised and weighed. One hundred grams (100 g) of the hepatopancreas was homogenized in three volumes of 100 mM phosphate buffer, pH 7.2 containing 10 mM sodium thiosulphate and 10 mM ε-amino-n-caproic acid with a Warring Blender. The homogenate was filtered through a double layer of cheese cloth and then centrifuged at 12, 000 rpm at 10°C for 30 min using Centurion cold centrifuge (R-1880). The pellets were discarded and an aliquot of the supernatant was then assayed for rhodanese activity and protein concentration.

Enzyme Assay

Rhodanese activity was measured according to the method of Lee *et al*. (1995) as described by Agboola and Okonji (2004). The reaction mixture consists of 0.5 ml of 50 mM borate buffer (pH 9.4), 0.2 ml of 250 mM KCN, 0.2 ml of 250 mM $Na_2S_2O_3$ and 0.1 ml of the enzyme solution in a total volume of 1.0 ml. The mixture was incubated at 37°C for 1 min and the reaction was stopped by adding 0.5 ml of 15% formaldehyde, followed by the addition of 1.5 ml of Sorbo reagent (made up of ferric nitrate solution containing 10.1 g $Fe(NO_3)_3.9H_2O$ in 20 ml concentrated nitric acid and made up to 100 ml with distilled water). The absorbance was taken at 460 nm. One rhodanese unit (RU) was taken as the amount of enzyme, which under the given conditions produced an optical density reading of 1.08 at 460 nm per min (Sorbo, 1951).

Determination of Protein

Protein concentration was determined by the method of Bradford (1976) using Bovine Serum Albumin (BSA) as the standard, where the protein absorbance was interpolated from a standard protein curve. The reaction mixture consists of 10 µl of the enzyme solution and 1.0 ml of

Bradford reagent. The absorbance was read at 595 nm.

Ammonium Sulphate Precipitation

The supernatant of the crude enzyme was brought to 70% ammonium sulphate saturation by slow addition and stirring of solid ammonium sulphate. This was done for 1 h with occasional stirring until all the salt had dissolved completely in the supernatant. The mixture was left for 12 h at 4°C, followed by centrifugation at about 12,000 rpm at 10°C for 30 min. The supernatant was discarded and the precipitate collected and re-suspended in a small amount of 0.1 M phosphate buffer (pH 7.2). The ammonium sulphate precipitate was dialysed against several changes of 0.1 M solution of phosphate buffer (pH 7.2) 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.

Affinity Chromatography on Reactive Blue-2 Agarose

Fifteen millilitres (15 ml) of Reactive Blue-2 Agarose resin was packed into a 1.5 x 10 cm glass column and equilibrated with 0.05 M citrate buffer (pH 5.0). Three millilitres (3 ml) of enzyme solution from the preceding step was then layered on the column. The column was then washed with the buffer to remove unbound protein, followed by a step-wise elution with 1.0 M NaCl solution in the same buffer. Fractions of 2 ml were collected from the column at a rate of 12 ml per h. The active fractions from the column were pooled and dialyzed against 100 ml 50% glycerol in 0.1 M phosphate buffer, pH 7.5. The dialyzed fraction was assayed for rhodanese activity and protein.

Ion-Exchange Chromatography on CM-Sephadex C-25

CM-Sephadex C-25 cation exchanger was pretreated by boiling five grams (5 g) of the resin in distilled water for 1 h. This was followed by the addition of 100 ml 0.1 M HCl for 30 min, after which the acid was decanted and the resin was washed with distilled water several times to ensure the total removal of the acid. Thereafter, 100 ml of 0.1 M NaOH was added to the resin, which was decanted after 30 min, followed by the thorough rinsing of the resin with distilled water to remove all traces of the base. The resin was then equilibrated with 0.1 M phosphate buffer (pH 7.6) before it was packed into a 2.5 x 40 cm column. Six millilitres (6 ml) of the enzyme solution from the preceding step was then applied on the column. The column was washed with 0.1 M phosphate buffer (pH 7.6) to remove unbound protein, followed by a step-wise elution with 1.0 M NaCl in the same buffer. Fractions of 3 ml were collected from the column at a rate of 36 ml per h. The active fractions from the column were pooled and dialyzed against 50% glycerol in 0.1 M phosphate buffer, pH 7.5. The dialyzed fraction was assayed for rhodanese activity and protein.

Determination of Native Molecular Weight

The native molecular weight was determined on a Biogel P-100 column (2.5 x 90 cm). The standard proteins were bovine serum albumin (M_r 66000; 5 mg/ml), ovalbumin (M_r 45000; 5 mg/ml) and chymotrypsinogen-a (M_r 25,000; 5mg/ml). Total sample volume of each of the protein markers applied to the column was 5 ml. The proteins were eluted with phosphate buffered saline pH 7.2. Fractions of 5 ml were collected and monitored by measuring absorbance at 280 nm for the protein. The void volume (V_o) of the column was determined by the elution volume (V_e) of Blue dextran (2 mg/ml). A 5 ml aliquot of the enzyme solution was then applied to the same column and the elution volume of the rhodanese was determined.

Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Weber and Osborn (1975) to determine the subunit molecular weight of the enzyme on a 10% slab gel apparatus with a notched glass plate. Gels of 1.5 mm thickness were prepared by using perplex spacers of same size.

A mixture of the enzyme sample and sample buffer containing SDS (10%), bromophenol blue, β-mecaptoethanol and glycerol was prepared in a concentration of 1:1, and was then heated in boiling water bath for 3 minutes and then allowed to cool. Fifteen microlitre (15 µl) of the mixture along with the same volume of protein standards

were applied to different wells on the slab and electrophoresed at a constant voltage of 70 volts to allow stacking of the proteins. The standard proteins that were used for the calibration of the gel were bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), trypsin inhibitor (20,100 Da) and lysozyme (14,300 Da), which had also been denatured by mixing with SDS and heating for 3 minutes. After stacking was achieved, the voltage was increased to 100 volts to allow separation of the proteins in the resolving gel.

After electrophoresis, the gels were stained in 0.04% Coomasie brilliant blue R-250 in 3.5% perchloric acid solution for 1 h, followed by destaining in a solution containing 7.5 % acetic acid to increase the sensitivity of detection. After destaining, the lengths of the gels as well as the distance of migration by the different protein bands were measured.

Kinetic Studies

The kinetic parameters (K_m and V_{max}) of the enzyme were determined by varying concentrations of KCN between 12.5 mM and 50 mM at a fixed concentration of 25 mM $Na_2S_2O_3$. Also, the concentration of $Na_2S_2O_3$ was varied between 12.5 mM and 50 mM at a fixed concentration of 25 mM 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

The substrate specificity of the enzyme was determined by using different sulphur compounds such as 2-mercaptoethanol, ammonium persulphate, ammonium sulphate 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. The kinetic parameters of the different compounds were also estimated using the Lineweaver-Burk plot, by varying the concentrations of the individual substrates between 12.5 mM and 50 mM at a constant KCN concentration of 25 mM.

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 (what year?). The enzyme was assayed using different buffers and pH: 50 mM citrate buffer (pH 3-5); 50 mM phosphate buffer (pH 6-8) and 50 mM borate buffer (pH 9-10). The rhodanese activity was assayed as described in the assay section with the assay buffer being replaced by these buffers.

Effect of Temperature on the Enzyme Activity

The enzyme was assayed at temperatures between 30°C and 70°C 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.

Determination of Heat Stability

The heat stability of the enzyme was determined by incubating 1 ml of the enzyme for 1 h at 30°C, 40°C, 50°C, 60°C and 70°C respectively. From the incubated solution, 0.1 ml was withdrawn at 10 min intervals and assayed for residual activity. The activity at 40°C, 50°C, 60°C and 70° C was expressed as a percentage of the activity of the enzyme incubated at 30°C which was the control.

Effect of Salts on the Enzyme Activity

The method of Lee *et al.* (1995) was used to study the effect of various salts on the activity of the rhodanese from the hepatopancreas of *Limicolaria flammea*. The salts tested were $BaCl_2$, $CaCl_2$, $CoCl_2$, $HgCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$ and $ZnCl_2$ at 0.05 mM, 0.1 mM and 0.2 mM from stock solutions of 0.5 mM in a typical rhodanese assay mixture. The metallic chlorides were dissolved in distilled water. The reaction mixture without the salts was taken as control with 100% activity.

RESULTS

Purification of Rhodanese

The results for the purification of rhodanese from the hepatopancreas of *Limicolaria flammea* are summarized in Table 1. The elution profiles after reactive blue-2 affinity chromatography and

CM Sephadex C-50 ion-exchange chromatography are shown in Figures 1 and 2 respectively. The pure enzyme had a specific activity of 4.57 RU/mg and a yield of 19.73%. The enzyme was purified using ammonium sulphate precipitation, affinity chromatography and ion-exchange chromatography.

Table 1: Summary of Purification for Rhodanese from the Hepatopancreas of *Limicolaria flammea*

Fractions	Total Protein (mg)	Total Activity (RU)	Specific Activity (RU/mg)	Yield (%)	Purification Fold
Crude	336.60	367.44	1.09	100	1
70% Ammonium Sulphate ppt	168.44	368.33	1.98	55.39	1.82
Affinity Chromatography	97.02	336.13	3.47	28.82	3.18
Ion Exchange Chromatography	66.42	303.42	4.56	19.73	4.20

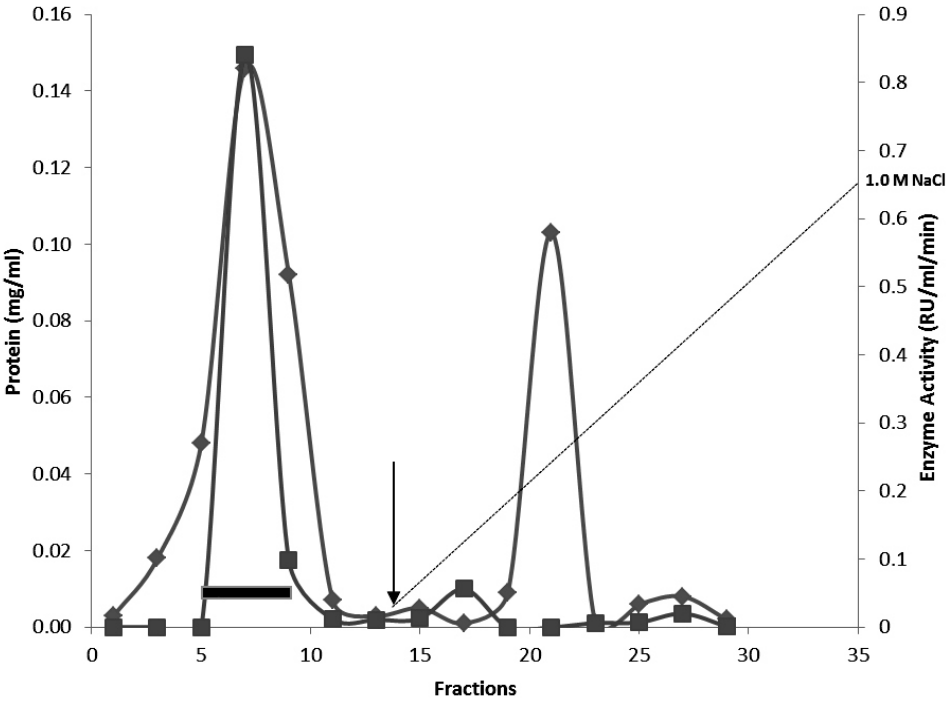

Fig. 1. Reactive Blue-2 Agarose Affinity Chromatography of Rhodanese from the Hepatopancreas of *Limicolaria flammea*

The dialysed sample was applied to the column and the column was washed with 0.05 M citrate buffer (pH 5.0) and later eluted with 1.0 M NaCl in 0.05 M citrate buffer (pH 5.0). Fractions of 2 ml were collected at a flow rate of 12 ml/h from the column.

——→ Salt concentration gradient
-■-■-■- Enzyme Activity at 460nm
-♦-♦-♦- Protein
—— Pooled Fractions

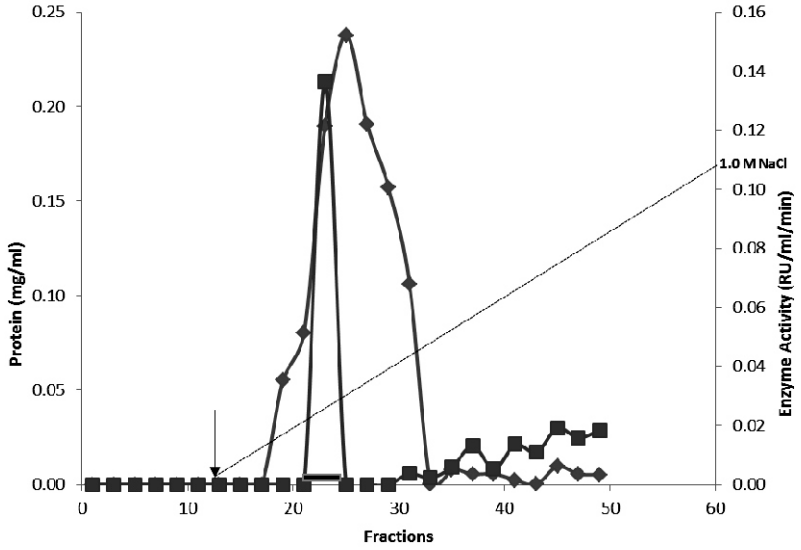

Fig. 2. Ion-Exchange Chromatography on CM-Sephadex C-25 of Rhodanese from the Hepatopancreas of *Limicolaria flammea*

The dialysed sample from the preceding step was applied to the column and washed with 0.1 M phosphate buffer (pH 7.6) and later eluted with 1.0 M NaCl in the same buffer at a flow rate of 36 ml/h. Each fraction of 3 ml was collected from the column

——→ Salt concentration gradient
-■-■-■- Enzyme Activity at 460nm
-♦-♦-♦- Protein
—— Pooled Fractions

Molecular Weights

Gel filtration on Biogel P-100 resulted in an estimated molecular weight of approximately 33,100 Dalton. The calibration curve on Biogel P-100 for the determination of the native molecular weight is shown in Figure 3. Figures 4 and 5 show the electrophoretogram and calibration curve respectively, obtained for the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight estimated from the SDS-PAGE was 32, 800 Dalton.

Kinetic Parameters

The Lineweaver-Burk plot for the determination of the kinetic parameters (K_m and V_{max}) of rhodanese from the hepatopancreas of *Limicolaria flammea* are presented in Figures 6 and 7 respectively. Summary of the kinetic data is also shown in Table 2.

Substrate Specificity

The percentage relative activity and kinetic parameters of the different substrates is shown in Tables 3 and 4.

Effect of pH on Rhodanese from the Hepatopancreas of *L. flammea*

The activity of rhodanese from the hepatopancreas of *Limicolaria flammea* was found to be optimum at pH 8.0. The result is shown in Figure 8.

Effect of Temperature on Rhodanese from the Hepatopancreas of *L. flammea*

The assay done to investigate the effect of temperature on activity of rhodanese from the hepatopancreas of *L. flammea* showed the enzyme to have its optimum temperature at 50°C, as shown in Figure 9. The activation energy values as determined using the Arrhenius plot were 19.618 kcal/mol and 76.834 kcal/mol.

Heat Stability Study on Rhodanese from the Hepatopancreas of *L. flammea*

The enzyme was found to be stable to 50°C. Figure 10 showed the plot of percentage residual activity of the enzyme against time.

Effect of Salts on Rhodanese from the Hepatopancreas of *L. flammea*

The results of the effect of salts show that the activity of the enzyme was not inhibited by the investigated salts (Table 5).

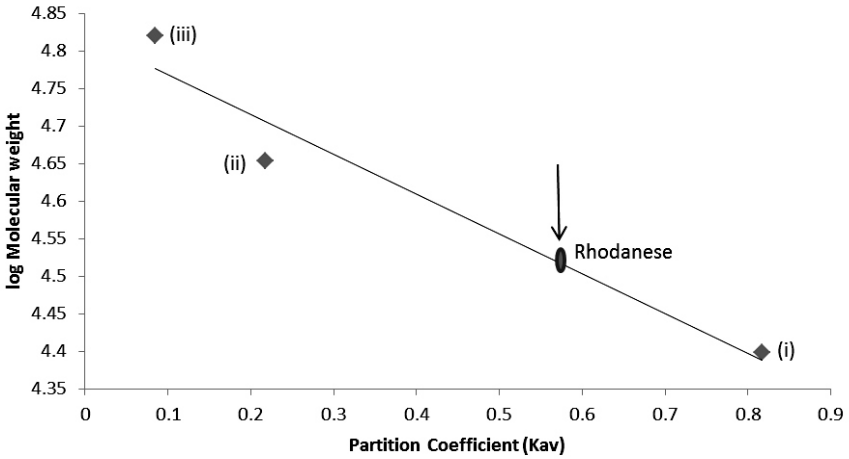

Fig. 3. Calibration Curve for the Determination of Native Molecular Weight of Rhodanese from the Hepatopancreas of *Limicolaria flammea*

The molecular weight of rhodanese from the hepatopancreas of *Limicolaria flammea* was determined on Biogel P-100 column (2.5 × 90 cm). The standard proteins were:

i = Chymotrypsinogen-a (25 kDa), ii = Ovalbumin (45 kDa) and iii = BSA (66 kDa).

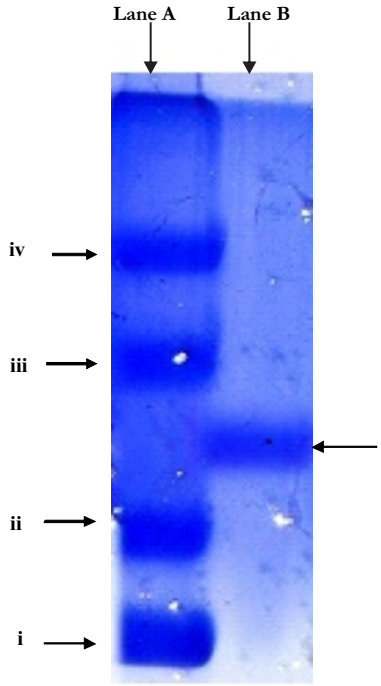

Fig. 4 SDS-PAGE for the Determination of Subunit Molecular Weight of *Limicolaria flammea* Hepatopancreas Rhodanese

SDS-PAGE of purified rhodanese from *Limicolaria flammea* hepatopancreas.
Lane A =Standard Proteins; The standard molecular weight markers include: i = Lysozyme(14.3 kDa), ii = Trypsin inhibitor (20.1 kDa), iii = Ovalbumin (45.0 kDa), and iv = BSA (66.0 kDa)
Lane B = Purified Rhodanese from the Hepatopancreas of *Limicolaria flammea*

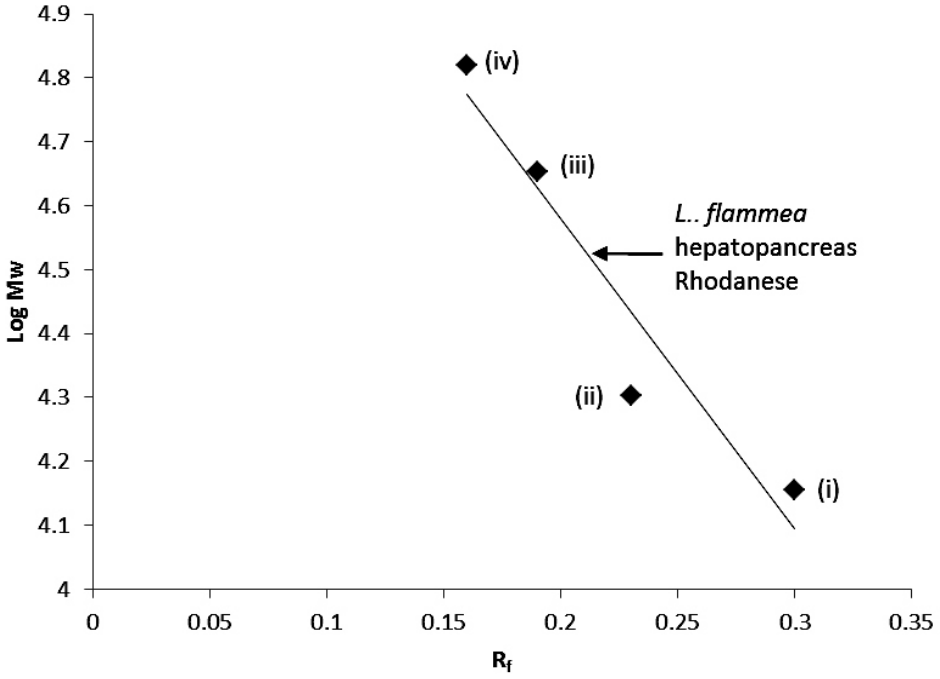

Fig. 5. Calibration Curve for the Determination of Subunit Molecular Weight of Rhodanese from the Hepatopancreas of *Limicolaria flammea*

The standard molecular weight markers include:
i = Lysozyme (14.3 kDa), ii = Trypsin inhibitor (20.1 kDa), iii = Ovalbumin (45.0 kDa) and iv = BSA (66.0 kDa).

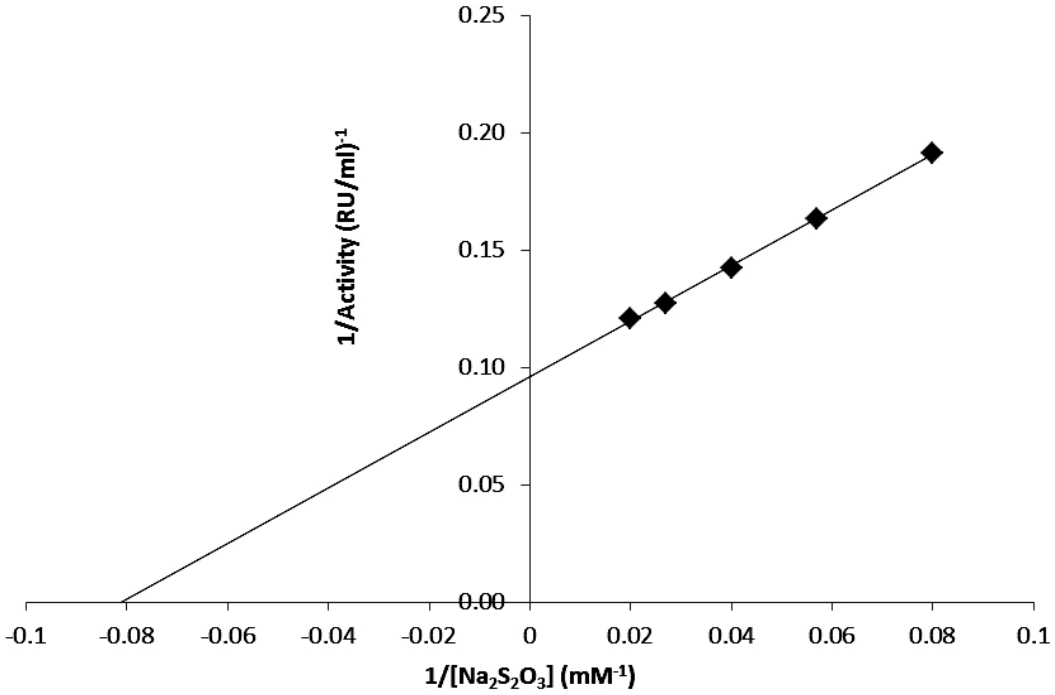

Fig. 6. Lineweaver-Burk Plot for Varying Concentration of Sodium Thiosulphate

Lineweaver-Burk plot of 1/V against 1/[S] at varying concentrations of $Na_2S_2O_3$ between 12.5 mM and 50 mM and a constant concentration of KCN at 25 mM.

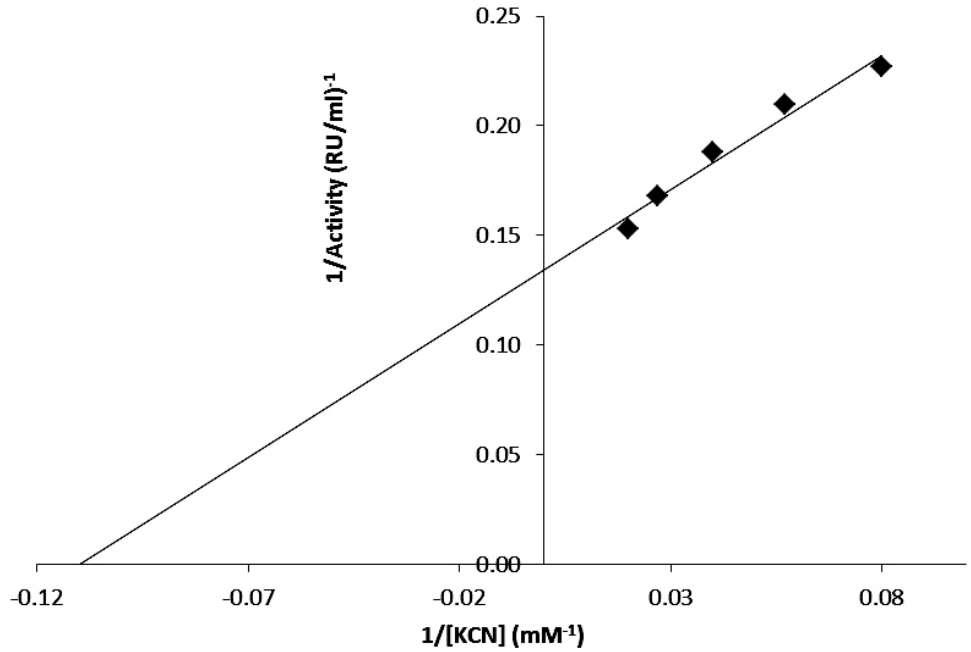

Fig. 7. Lineweaver-Burk Plot for Varying Concentration of Potassium Cyanide

Lineweaver-Burk plot of 1/V against 1/[S] at varying concentrations of KCN between 12.5 mM and 50 mM and a constant concentration of $Na_2S_2O_3$ at 25 mM.

Table 2: Summary of Kinetic Parameters of Rhodanese from the Hepatopancreas of ***L. flammea*** **with KCN and $Na_2S_2O_3$ Substrates**

Substrate	K_m (mM)	V_{max} (RU/ml/min)
KCN	9.1 ± 0.6	7.4 ± 0.7
$Na_2S_2O_3$	12.3 ± 0.2	10.5 ± 0.4

Table 3: Percentage Relative Activity for Different Sulphur Donour Substrates

SUBSTRATE	% RELATIVE ACTIVITY
Sodium thiosulphate ($Na_2S_2O_3$)	100.0
2-mercaptoethanol ($CH_2(SH)CH_2(OH)$)	57.8
Ammonium persulphate ($(NH_4)_2S_2O_8$)	37.6
Ammonium sulphate ($(NH_4)_2SO_4$)	46.8
Sodium metabisulphite ($Na_2S_2O_5$)	48.1

Table 4: Summary of Kinetic Parameters of Rhodanese from the Hepatopancreas of *L. flammea* with Different Sulphur Donour Substrates

SUBSTRATE	K_m (mM)	V_{max} (RU/ml/min)
2-mercaptoethanol ($CH_2(SH)CH_2(OH)$)	13.3	8.7
Ammonium persulphate ($(NH_4)_2S_2O_8$)	15.4	9.1
Ammonium sulphate ($(NH_4)_2SO_4$)	15.9	8.3
Sodium metabisulphite ($Na_2S_2O_5$)	13.5	8.2

Rhodanese activity was assayed as described in the method replacing the sodium thiosulphate with the respective sulphur donour substrates.

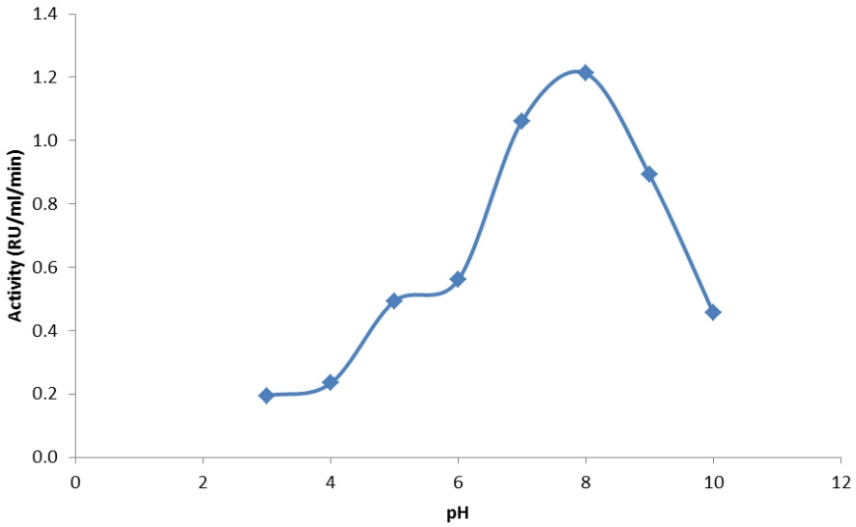

Fig. 8. Effect of pH on Rhodanese Activity from the Hepatopancreas of *L. flammea*.

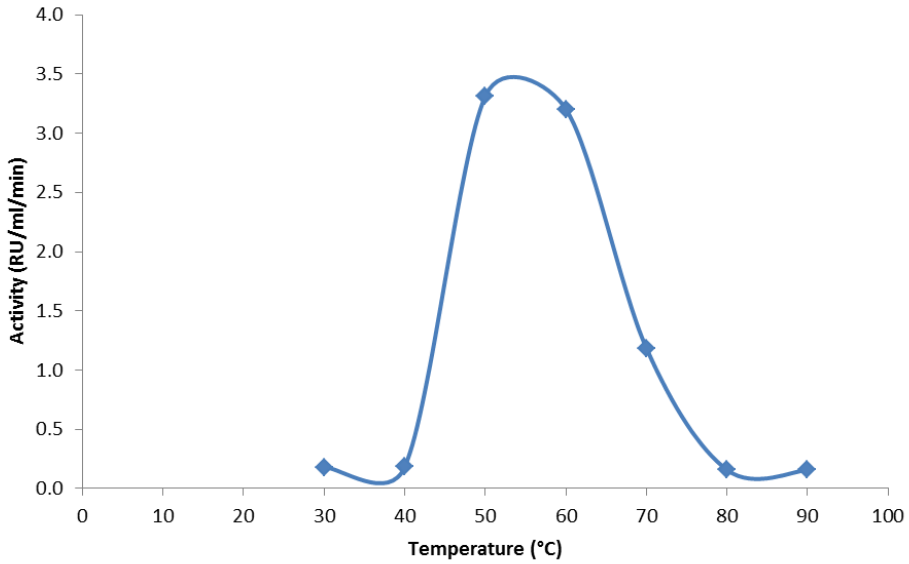

Fig. 9. Effect of Temperature on Rhodanese Activity from the Hepatopancreas of *L. flammea*.

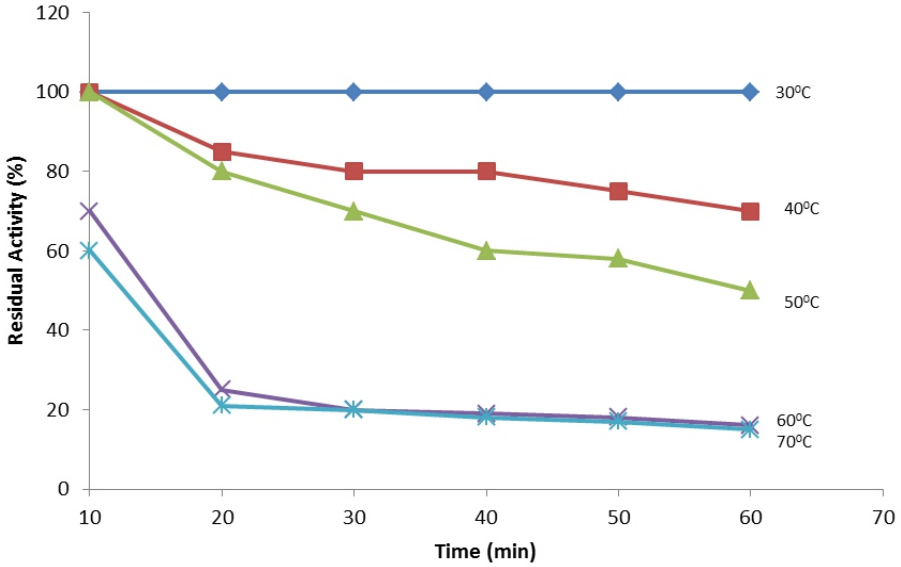

Fig. 10. Thermal Stability of Rhodanese from the Hepatopancreas of *L. flammea*.

Table 5: Effect of Salts on Rhodanese from the Hepatopancreas of *L. flammea*

SALTS	ENZYME ACTIVITY (%)		
	0.05 mM	**0.1 mM**	**0.2 mM**
$BaCl_2$	97.0 ± 0.654	96.8 ± 2.532	99.1 ± 0.707
$CaCl_2$	90.0 ± 0.272	88.8 ± 2.125	89.3 ± 0.191
$CoCl_2$	97.4 ± 0.106	92.0 ± 0.840	96.2 ± 0.113
$HgCl_2$	91.6 ± 0.492	91.0 ± 1.146	92.7 ± 1.004
$MgCl_2$	88.7 ± 0.842	90.2 ± 2.992	93.3 ± 0.980
$MnCl_2$	95.7 ± 0.764	95.1 ± 2.040	96.7 ± 1.280
$NiCl_2$	97.8 ± 1.114	95.5 ± 0.626	96.7 ± 0.332
$ZnCl_2$	91.9 ± 1.443	93.6 ± 0.516	92.9 ± 0.792

The various salts at the different specified concentrations were used in the reaction mixture. Rhodanese assay was routinely carried out but in the presence of the salts.

DISCUSSION AND CONCLUSION

In this research work, rhodanese was isolated and purified from the hepatopancreas of garden snail (*Limicolaria flammea*) using 70% ammonium sulphate precipitation, affinity chromatography on Reactive Blue-2 Agarose column and ion-exchange chromatography on CM-Sephadex C-25. The results showed a specific activity of 4.57 RU/mg with a percentage recovery of 19.73. Specific activity of different values has been reported for rhodanese from different sources. Agboola and Okonji (2004) reported a value of 131 RU/ mg for rhodanese from the fruit bat liver, while Fagbohunka *et al.* (2004) obtained a value of 20.1 RU/mg for the Giant African snail. Akinsiku *et al.* (2010) obtained a value of 73 and 72 RU/mg for catfish rhodanese I (cRHDI) and catfish rhodanese II (cRHDII) respectively. Okonji *et al.* (2011) obtained a value of 8.4 RU/mg for mudskipper liver rhodanese. The molecular weight values reported for most rhodanese falls between 31, 000 to 37, 000 Dalton (Lee *et al.*, 1995; Agboola and Okonji, 2004; Akinsiku *et al.*, 2010). Sorbo (1953b) reported a molecular weight of 37,000 Dalton for bovine liver rhodanese. Lee *et al.* (1995) reported a molecular weight of 34,800 Dalton for the mouse liver rhodanese and Nagahara and Nishino (1996) obtained a molecular weight of 34,000 Dalton for rat liver rhodanese, while Agboola and Okonji (2004) reported a molecular weight of 35,700 Dalton for rhodanese from the fruit bat liver (*Eidolon helvum, Kerr*) and Fagbohunka *et al.* (2004) also reported rhodanese from the hepatopancreas of *Archachatina marginata* to be 35,481 Dalton. An apparent molecular weight of 34, 500 Dalton and 36, 800 Dalton were reported for rhodanese I (cRHD I) and rhodanese II (cRHD II) respectively from the liver of African catfish (*Clarias gariepinus*) (Akinsikun *et al.*, 2004). Okonji *et al.* (2010b) reported a molecular weight of 32, 210 Dalton for soldier termite (*Amitermes silvestrianus*) rhodanese. The apparent molecular weight of rhodanese from variegated grasshopper (*Zonocerus variegated*) as estimated by Igue and Agboola (2013) was 35, 400 Dalton.

The K_m values for both substrates (potassium cyanide and sodium thiosulphate) are similar to those obtained from some other animal sources: Watanabe *et al.* (1985) reported an apparent K_m of 35 mM and 13.5 mM for potassium cyanide and sodium thiosulphate respectively for rhodanese in *Euglena gracilis*. Keith *et al.* (1987) reported apparent K_m values of 78 mM and 17 mM for potassium cyanide and sodium thiosulphate respectively for rhodanese from *Escherichia coli*. Agboola and Okonji (2004) also reported K_m values of 13.5 mM and 19.15 mM for potassium cyanide and sodium thiosulphate respectively for rhodanese from fruit bat liver, while Hossein and Reza (2011) reported apparent K_m values of 36.81 mM and 19.84 mM for potassium cyanide and sodium thiosulphate respectively for rhodanese from rainbow trout liver.

The result from the K_m values shows that rhodanese from the hepatopancreas of *Limicolaria flammea* has a higher affinity for thiosulphate and a possible efficient cyanide catalytic mechanism.

The substrate specificity study involving the use of other sulphur substrates (Tables 3 and 4) showed that the rhodanese from the hepatopancreas of *Limicolaria flammea* had preference for thiosulphate while it can still use other sources of sulphur for its catalytic activity, which is in line with the stuidies reported by other researchers. 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. Westley (1981) also reported the importance of sulphane sulphur pool in rhodanese cyanide detoxification mechanism.

The affinity of rhodanese from the hepatopancreas of *Limicolaria flammea* for cyanide and sodium thiosulphate could explain how the organism is able to survive feeding on cyanogenic plants. The rhodanese from the hepatopancreas of *Limicolaria flammea* showed maximum activity at pH 8.0, a value which corresponds to the range of optimum pH values reported for the enzyme in other sources. Watanabe *et al.* (1985) and Hossein and Reza (2011) reported an optimum pH of 10.5 for rhodanese from *Euglena gracilis* and rainbow trout (*Oncorhynchus mykiss*) liver respectively. Most reports have also shown

optimum pH range of 6.0-11.0; Boey *et al.* (1976) from tapioca leaves, Lee *et al.* (1995) from bovine liver, Agboola and Okonji (2004) from fruit bat liver, Akinsiku *et al.* (2010) from African catfish and Okonji *et al.* (2011) from mudskipper liver.

Various optimum temperatures have been reported for the enzymes from different sources. The activity of rhodanese from the hepatopancreas of *Limicolaria flammea* was assayed at temperatures between 30°C and 70°C. The optimum temperature of the enzyme was found to be 50°C at pH 8.0. Similar results have been reported for rhodanese from other sources. Sorbo (1953a and b) reported an optimum temperature of 50°C from bovine liver. Ezzi *et al.* (2003) obtained a wide temperature optimum of 35–55°C for the rhodanese enzyme in different *Trichoderma* strains while 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 collected from Asejire Lake Okonji *et al.* (2011) also reported an optimum temperature of 50°C from mudskipper liver while an optimum temperature of 30°C was obtained for rhodanese enzyme from liver of rainbow trout (Hossein and Reza, 2011).

The high optimum temperature of 50°C obtained for rhodanese from the hepatopanceas of *Limicolaria flammea* could be an adaptive feature by the organism to survive harsh environment. The stability study showed the enzyme to be stable up to 50°C which conformed to the point of enzyme inactivation on the Arrhenius plot.

The results of the inhibition study on the rhodanese from the hepatopancreas of *Limicolaria flammea* showed that the salts used did not have any significant effect on the activity of the enzyme. This could be as a result of consistent exposure to these metals due to their presence in the 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. Other results showed little variations. Akinsiku *et al.* (2010) reported that land tortoise liver rhodanese was not affected by Mn^{2+}, Co^{2+}, Sn^{2+}, $Ni^{2+}$and NH_4^+, while Ba^{2+} and Zn^{2+} inhibited the enzyme. Agboola and Okonji (2004) and Hossein and Reza (2011) also reported the effect of metals on the fruit bat liver and rainbow trout rhodanese respectively. It could be stated 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 (Lee *et al*., 1995; Nagahara and Nishino, 1996; Ulmer and Vallee, 1972).

CONCLUSION

In summary, this study showed the presence of rhodanese activity in hepatopancreas of garden snail, *Limicolaria flammea* with similar characteristics to rhodaneses obtained from other sources. The presence of rhodanese in the hepatopancreas of *Limicolaria flammea* suggests that the enzyme may possess functional cyanide detoxification mechanism necessary for the survival of the animal in the environment.

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