academicJournals

Vol. 9(2), pp. 9-15, February, 2017
DOI 10.5897/JABSD2016.0274
Article Number: 1CF52DA63509
ISSN 2141-2340

http://www.academicjournals.org/JABSD

Journal of Agricultural Biotechnology and Sustainable Development

Full length Research Paper

Comparative studies on the partial purification and characterization of rhodanese from seed and mesocarp of snake tomatoes (*Trichosanthes cucumerina* Linn.)

R. E. Okonji[1], B. S. Fagbohunka[2], L. O. Ehigie[3] and Z. A. Ayinla[1*]

[1]Department of Biochemistry and Molecular Biology, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Nigeria.
[2]Department of Biochemistry, Faculty of Basic Medical Science, Olabisi Onabanjo University, Ago-Iwoye, Nigeria.
[3]Department of Biochemistry, Faculty of Basic Medical Science, Ladoke Akintola University, Ogbomoso, Nigeria.

Received 16 October, 2016; Accepted 1 February, 2017

Rhodanese enzyme was partially purified and characterized from seed and mesocarp of snake tomato (*Trichosanthes cucumerina*) using 80% ammonium sulphate precipitation. The enzyme isolated from *T. cucumerina* mesocarp had a specific activity of 8.8 RU/mg with a percentage yield of 87.8%, while that from *T. cucumerina* seed had a specific activity of 19.7 RU/mg with a percentage yield of 14.6%. The K_m of rhodanese from *T. cucumerina* mesocarp and seed for sodium thiosulphate ($Na_2S_2O_3$) and potassium cyanide (KCN) were 12.5 and 10 mM, respectively. The enzyme from both mesocarp and seed was not inhibited by ammonium persulphate and sodium metabisulphite. The optimum temperature for both the seed and the mesocarp rhodanese was 50°C, while the optimum pH was 8.0 and 7.0, respectively. Rhodanese from *T. cucumerina* mesocarp was not inhibited by the metal ions at concentrations of 0.01 and 0.001 M, while rhodanese from the seed was inhibited by the metal ions at the two concentrations of each of the salts (KCl, $MnCl_2$, NaCl, $NiCl_2$, $ZnCl_2$ and $BaCl_2$).

Key words: Rhodanese, *Trichosanthes cucumerina*, seed, mesocarp.

INTRODUCTION

A recently discovered crop, *Trichosanthes cucumerina*, is of high importance in some parts of Africa, majorly Nigeria and Ghana. This is because of the red fruit pulp which can be substituted for the commonly used tomato sauce. This part of the crop protects against free radicals which are harmful and has been linked with lowered risk of Alzheimer's disease, cancer, cardiovascular diseases, cataracts and diabetes (Cohen et al., 2000; Sahlin et al., 2004). The plant has been reported to contain high percentage of anti-oxidant compounds such as β-carotene, carotenoids, flavonoids and phenolics (Zhang and Hamauzu, 2004). The medicinal uses of the plant have also been reported (Nadkani, 2002; Madhava et al., 2008).

*Corresponding author. E-mail: mailnike2001@yahoo.co.uk or zsalimanu@oauife.edu.ng.

Cyanide is cytotoxic and kills the cell by inhibiting the mitochondrial electron transport chain enzyme, cytochrome oxidase; it is, thus, an inhibitor of cellular respiration (Ohlen et al., 2016). Through cytochrome oxidase inhibition, cyanide adversely affects higher plants. Certain host plants also produce cyanogenic compounds in the form of cyanogenic glycosides as chemical defenses, hence exposing herbivorous insects that feed on such plant to cyanide (Gleadow and Møller, 2014). Cyanide is released to the environment through death and decomposition of plants, but the rate of such production is unknown. With cyanogenic plants, various animals and wildlife are at risk, especially species that feed on such plants and an efficient means of detoxifying cyanide is important to the survival of such animals (Wybouw et al., 2014). Different species of bacteria, fungi, algae and higher plants may serve as natural sources of cyanide if they produce and excrete cyanide (Way, 1984). Food plants such as *Manihot esculenta* (cassava), *Panicum miliaceum* (millet), *Bambusa* species (bamboo shoots), *Ipomoea batatas* (sweet potatoes), *Zea mays* (corn), *Phaseolus lunatus* (lima beans) and *Linum* species (linseed) have been reported to contain cyanide (Way, 1984).

Plants are exposed to cyanide from soil contaminated with various industrial wastes (Henny et al., 1994). Also, when 1-amino-cyclopropane-1-carboxylic acid is converted to ethylene, cyanide is produced in equimolar quantities as ethylene, thus serving as an endogenous source of cyanide in plants and such cyanide level increases drastically during ripening of fruit and senescence (Yip and Yang, 1988).

Overtime, in plants and animals, there has been the evolution of a number of mechanisms to overcome the adverse effects of toxicity caused by compounds such as cyanide and various heavy metals (Most and Papenbrock, 2016). Rhodaneses (Cyanide: Thiosulphate Sulphur Transferase; E.C.2.8.1.1) are sulphur transferases which are comprised of a group of enzymes found widely in plant and animal species which catalyze the transfer of a sulfur atom to nucleophilic sulfur acceptors from suitable sulfur donors (Papenbrock et al., 2010). This ubiquitous enzyme, using thiosulphate as the donor substrate, biotransforms cyanide to thiocyanate (Westley, 1981). The physiological role played by rhodanese in animal tissues and plants is controversial, particularly its function in the detoxification of acute cyanide exposure (Sylvester and Sander, 1990). Rhodanese, and other sulphur transferases, play a vital role in L-cysteine metabolism (Most and Papenbrock, 2016). When heavy/toxic metals actively bind with the sulfhydryl groups of cysteine residues, the catalytic activity of these enzymes are decreased (Kaczor-Kamińska and Sura, 2013).

Many organisms, ranging from bacteria to animals have been reported to produce rhodanese (Lee et al., 1995; Agboola and Okonji, 2004; Akinsiku et al., 2009). It has also been reported that there is an established relationship between cyanogenesis and rhodanese activity in plants. This is an indication that the enzyme present in cyanogenic plants provides a mechanism of detoxification of cyanide (Smith and Urbanska, 1986). Overtime, it has been reported that exposure to cyanide ions increases the overall mortality of organisms involved (Eisler, 1991), as many industrial products and naturally occurring substances contain cyanide (Egekeza and Oehme, 1980). Social insects, such as the soldier termites, feed off grasses, decaying woods and plant debris which contain cyanogenic glycosides, leading to the release of cyanide upon ingestion (Gerozisis and Hadlington, 2001; Conn, 2008). The death of grazing animals can result from the ingestion of such cyanogenic glycosides naturally present in forage crops (Keeler et al., 1978). Similarly, the death of birds can also result from cyanide poisoning which may be through cyanide salts exposure or cyanogenic plants ingestion (Wiemeyer et al., 1986). Aquatic lives are also not left out, as exposure to cyanide ions can cause stress and significantly increase the metabolic load on the aquatic organisms (Eisler, 1991).

This research aims to compare the properties of rhodanese enzyme isolated from the seed and mesocarp of snake tomato (*T. cucumerina*), and to understand better the mechanism of cyanide detoxification in the plant.

MATERIALS AND METHODS

Boric acid, sodium chloride, phosphoric acid, manganese chloride tetrahydrate, zinc chloride, magnesium chloride, nickel chloride, potassium and ammonium sulphate were purchased from BDH Chemical Limited, Poole England. Potassium chloride, nitric acid, ferric nitrate, potassium cyanide and sodium thiosulphate were purchased from Sigma Chemical Company, St. Louis, USA. All other reagents were of analytical grade. Snake tomato (*T. cucumerina*) was collected from South-Western part of Nigeria.

Extraction of enzyme and partial purification

The seed and mesocarp were gotten from the snake tomato. Seed and mesocarp were prepared by homogenizing each, separately, in 3 volumes of homogenization buffer (phosphate buffer, pH 7.2). These suspensions were centrifuged for 20 min at 4,000 rpm. The supernatants served as the enzyme source. The supernatants were brought to 80% ammonium sulphate saturation, and left for 24 h. The resulting precipitate obtained after centrifugation at 4,000 rpm for 20 min was dialyzed against several changes of phosphate buffer, pH 7.2 containing 10 mM $Na_2S_2O_3$. The dialysate was centrifuged at 4,000 rpm for 20 min. The supernatant was used as source of the enzyme.

Determination of protein concentration and enzyme assay

Protein concentration was measured using the Bradford method (1976) with bovine serum albumin (BSA) as standard. The method of Agboola and Okonji (2004) was used to assay for rhodanese.

Table 1. Summary of the partial purification process of rhodanese obtained from snake tomato (*Trichosanthes cucumerina*) seed and mesocarp.

Fractions		Total activity (RU)	Total protein (mg)	Specific activity (RU/mg)	Yield (%)	Purification fold
Crude	SD	283.8	35.4	8.0	100	1
	MC	93.1	37.1	2.5	100	1
80% Ammonium sulphate precipitation	SD	41.3	2.1	19.7	14.6	2.46
	MC	81.7	9.3	8.8	87.8	3.52

SD: Seed; MC: Mesocarp.

The reaction mixture contained 0.25 M sodium thiosulphate, 0.25 M potassium cyanide, 50 mM borate buffer, pH 9.4 and 100 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 0.5 ml 15% formaldehyde and 1.5 ml of Sorbo reagent (which was 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. One unit of activity is represented as the RU and 1RU is the amount of enzyme that gave an optical density of 1.08 (Sorbo, 1953).

Kinetic parameters determination

The kinetic parameters (K_m and V_{max}) of the enzyme were determined by varying concentrations of KCN between 5 and 50 mM at fixed concentration of 25 mM $Na_2S_2O_3$. Also, the concentration of $Na_2S_2O_3$ was varied between 5 and 50 mM at fixed concentration of 25 mM KCN. Plots of the reciprocal of initial reaction velocity (1/V) versus reciprocal of the varied substrate, 1/[S], at each fixed concentrations of the other substrate were made according to Lineweaver and Burk (1934).

Effect of salts 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 snake tomato (*T. cucumerina*) rhodanese. The salts of the cations include $NiCl_2$, $MnCl_2$, $ZnCl_2$, NaCl, $BaCl_2$ and KCl at concentrations of 1.0 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.

Substrate specificity

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

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 buffers of different pH, thus, 50 mM of citrate (pH 3-5), phosphate (pH 6 to 8) and borate (pH 9 to 11). The rhodanese activity was assayed as described previously.

Effect of temperature on the enzyme activity

The enzyme was assayed at temperatures between 30 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.

RESULTS AND DISCUSSION

Rhodanese from seed and mesocarp of snake tomato (*T. cucumerina*) was partially purified using 80% ammonium sulphate precipitation. The enzyme isolated from mesocarp had a specific activity of 8.8 RU/mg with a purification fold of 3.52 and a percentage yield of 87.8%. The enzyme isolated from the seed had a specific activity of 19.7 RU/mg with a purification fold of 2.46 and a percentage yield of 14.6% (Table 1). Akinsiku et al. (2009) obtained a specific activity value of 73 and 72 RU/mg for catfish rhodanese I (cRHDI) and catfish rhodanese II (cRHDII), respectively. Agboola and Okonji (2004) reported a value of 136.6 RU/mg for fruit bat liver rhodanese. Sorbo (1953) who independently worked on bovine liver rhodanese, reported a value of 256 RU/mg.

Tables 3 depicts a summary of the kinetic parameter values (K_m and V_{max}) obtained from the Lineweaver-Burk plots as shown in Figures 1a, 1b, 2a, 2b, 3a, 3b, 4a and 4b. The enzyme from *T. cucumerina* seed and mesocarp had a similar K_m value of 10 mM for KCN and also a similar K_m value of 12.5 mM for $Na_2S_2O_3$ in both seed and mesocarp. According to Okonji et al. (2008), K_m values of 10.0 and 2.56 were reported for KCN and $Na_2S_2O_3$, respectively from hepatopancreas of *Macrobrachium rosenbergii* (freshwater prawn). Also, Akinsiku et al. (2009) reported values of 25.4 and 18.6 mM for KCN and $Na_2S_2O_3$, respectively for catfish liver. On the other hand, K_m values of 33.3 and 14.29 mM for KCN and $Na_2S_2O_3$ were obtained for mudskipper liver rhodanese by Okonji

Table 2. Effect of different substrates on rhodanese from seed and mesocarp of snake tomato (*Trichosanthes cucumerina*).

Substrates	Activity (%)	
	Seed	Mesocarp
Sodium thiosulphate	100.00	100.00
Sodium metabisulphite	80.00	68.51
Ammonium persulphate	66.60	54.71
2-Mercaptoethanol	37.53	35.78

Table 3. Kinetics parameters for rhodanese from seed and mesocarp of snake tomato (*Trichosanthes cucumerina*).

Substrates	Seed		Mesocarp	
	K_m (mM)	V_{max} (RU/ml/min)	K_m (mM)	V_{max} (RU/ml/min)
KCN	10.00	0.10	10.00	0.15
$Na_2S_2O_3$	12.50	0.08	12.50	0.12
$Na_2S_2O_5$	50.00	0.40	16.67	0.18
$(NH_4)_2S_2O_8$	13.33	0.26	13.33	0.26

et al. (2011). The lower the K_m value, the higher the affinity of the enzyme for the substrate and the more efficient the catalysis of the detoxification reaction. Considering the environment, the plant will be exposed to different cyanide-containing compounds and sulphur donors, hence, the study of the kinetic parameters using different sulphur donors. Sodium thiosulphate ($Na_2S_2O_3$) has been reported to be highly specific for rhodanese (Sorbo, 1953; Westley, 1980) and the result obtained in this study is in agreement with this finding. Of all three sulphur donor substrates studied ($Na_2S_2O_3$, $Na_2S_20_5$ and $(NH_4)_2S_2O_8$), $Na_2S_2O_3$ had the lowest K_m value of 12.50 mM in both seed and mesocarp rhodanese, while the others showed higher K_m values (Table 3). This further confirms that the rhodanese has higher affinity for $Na_2S_2O_3$ than the other sulphur donors.

Rhodanese from the seed and mesocarp had the same optimum temperature of 50°C (Figure 6a and b), while the pH values were different. The mesocarp had an optimum pH of 7.0 (Figure 5b) while the seed had an optimum pH of 8.0 (Figure 5a). This could be as a result of varying level of exposure of the different components of the plant fruit to various environmental conditions. These results are in agreement with results reported for rhodanese from different sources. Sorbo (1953) reported an optimum temperature of 50°C for bovine liver rhodanese. A wide temperature optimum of 35 to 55°C for rhodanese from different strains of *Trichoderma* was reported by Ezzi et al. (2003), while Okonji et al. (2011) obtained an optimum temperature of 50°C for mudskipper liver rhodanese. The optimum pH ranges of 8.0 to 11.0 have been reported for different organisms (Lee et al., 1995; Agboola and Okonji, 2004; Saidu, 2004; Okonji et al., 2008). In recent works by Okonji et al. (2008) and Akinsiku et al. (2009), an optimum pH as low as 6.0 and 6.5 for giant fresh water prawn (*M. rosenbergii*) hepatopancreas and catfish liver, respectively, was reported, with both organisms being of aquatic origin.

It was observed that the effect of heavy metals ($BaCl_2$, $NiCl_2$, KCl, NaCl, $ZnCl_2$ and $MnCl_2$) at concentrations of 1 and 10 mM in the reaction mixture on the rhodanese from seed and mesocarp was different. The rhodanese from seed was inhibited by metals at both concentrations (result not shown), while rhodanese from the mesocarp was not appreciably inhibited by any of the metals at both concentrations (Table 4). The inhibition of rhodanese from seed by these metals may be as a result of the metal ions interacting with the sulfhydryl groups present at the enzyme catalytic site or as a result of changes being induced in the secondary and tertiary structure of the enzyme. Structural changes such as these have been reported after incubating proteins with metal ions (Tayefi-Nasrabadi et al., 2006).

The effect of different substrates (sodium thiosulphate, sodium metabisulphite and ammonium persulphate) showed that rhodanese from both mesocarp and seed was more specific for sodium thiosulphate (Table 2). The result showed that only sodium thiosulphate gave 100% activity, while the other sulphur compounds, to some extent, inhibited rhodanese activity. This might be due to the specificity of the enzyme for sodium thiosulphate.

Conclusion

This study showed the presence of rhodanese in the

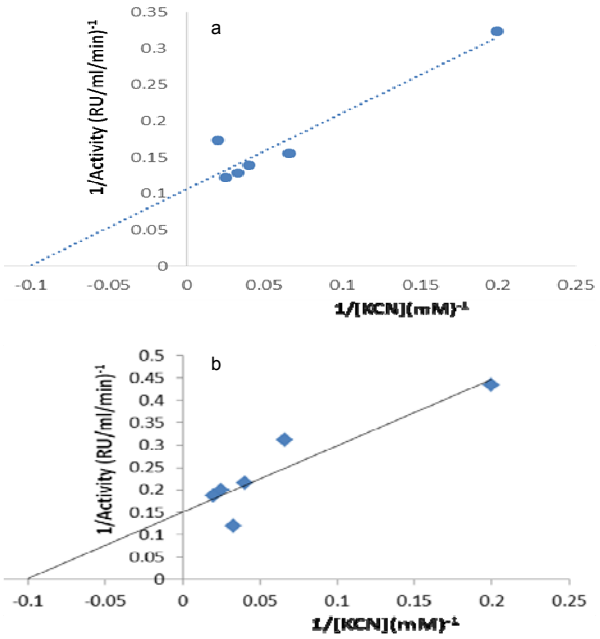

Figure 1. Lineweaver-Burk plot for varying concentrations of potassium cyanide in (a) seed and (b) mesocarp of snake tomato (*Trichosanthes cucumerina*). Plot of 1/V against 1/[S] at varying concentrations of KCN between 5 and 50 mM and a constant concentration of $Na_2S_2O_3$ at 25 mM.

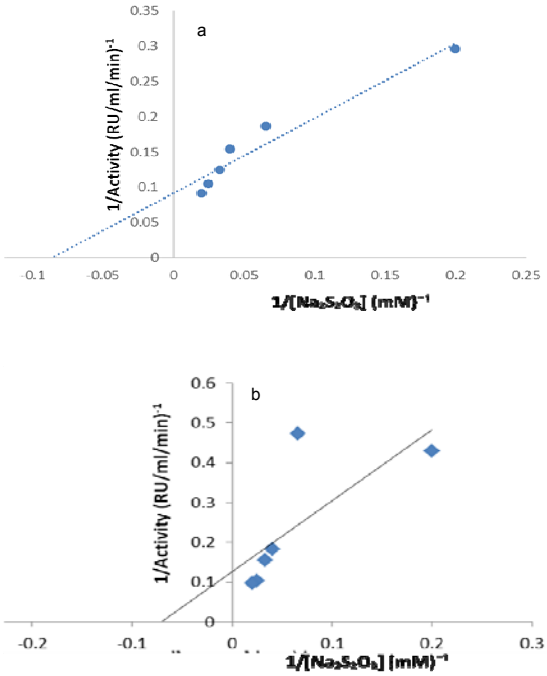

Figure 2. Lineweaver-Burk Plot for varying concentration of sodium thiosulphate in (a) seed and (b) mesocarp of snake tomato (*Trichosanthes cucumerina*). Plot of 1/V against 1/[S] at varying concentrations of $Na_2S_2O_3$ between 5 and 50 mM and a constant concentration of KCN at 25 mM.

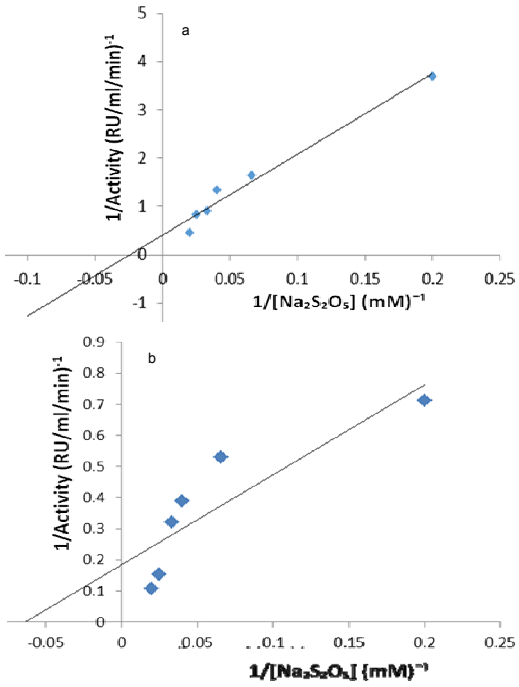

Figure 3. Lineweaver-Burk Plot for varying concentration of sodium metabisulphite in (a) seed and (b) mesocarp of snake tomato (*Trichosanthes cucumerina*). Plot of 1/V against 1/[S] at varying concentrations of $Na_2S_2O_5$ between 5 and 50 mM and a constant concentration of KCN at 25 mM.

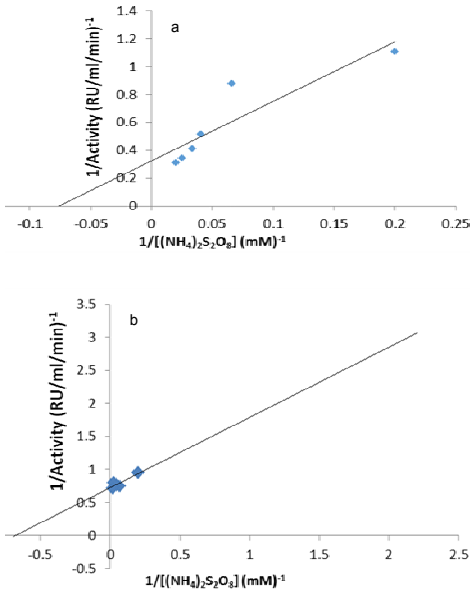

Figure 4. Lineweaver-Burk Plot for varying concentration of ammonium persulphate in (a) seed and (b) mesocarp of snake tomato (*Trichosanthes cucumerina*). Plot of 1/V against 1/[S] at varying concentrations of $(NH_4)_2S_2O_8$ between 5 and 50 mM and a constant concentration of KCN at 25 mM.

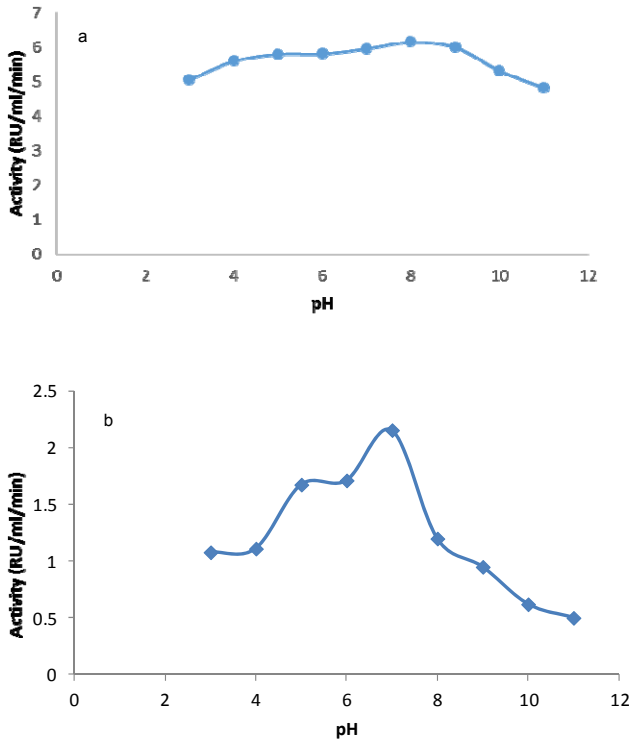

Figure 5. Effect of pH on rhodanese from (a) seed and (b) mesocarp in snake tomato (*Trichosanthes cucumerina*).

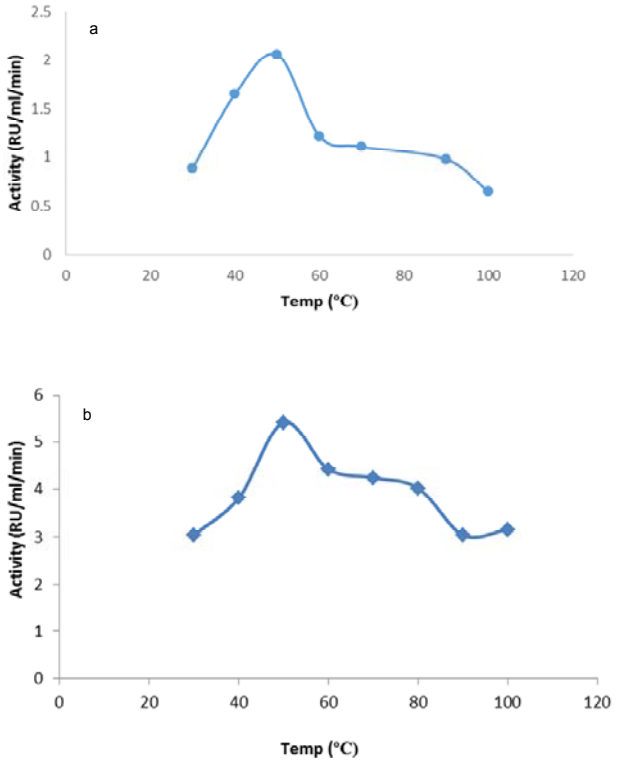

Figure 6. Effect of temperature on rhodanese from (a) seed and (b) mesocarp in snake tomato (*Trichosanthes cucumerina*).

Table 4. Effect of salts on rhodanese from mesocarp of snake tomato (*Trichosanthes cucumerina*).

Salt	Enzyme activity (%)	
	0.01 M	0.001 M
$BaCl_2$	81.98 ± 11.46	74.16 ± 1.64
$NiCl_2$	100 ± 1.23	100 ± 0.12
KCl	79.72 ± 3.27	74.5 ± 3.27
NaCl	83.31 ± 2.94	76.42 ± 5.91
$ZnCl_2$	77.42 ± 2.29	70.58 ± 4.58
$MnCl_2$	73.21 ± 2.09	77.76 ± 7.84

seed and mesocarp of *T. cucumerina* and elucidated the physicochemical properties of the enzyme. The enzyme in both *T. cucumerina* mesocarp and seed showed specificity towards $Na_2S_2O_3$ as sulphur donor. The presence of rhodanese in the plant is a suggestion that the enzyme may be functional in many physiological activities, one of them being the detoxification of cyanide.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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