



## **Partially Purified 3-Mercaptopyruvate Sulphurtransferase Obtained from the Intestine of Cane Rat (*Thryonomys swinderianus*) as a Detoxifier of Cyanide**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. Author AAS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors OMI and GBO managed the analyses of the study. Author BAO managed the literature searches. All authors read and approved the final manuscript.*

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### **ABSTRACT**

Cane rat (*Thryonomys swinderianus*) is an herbivorous animal which feeds on plant materials, including cassavas that are known to contain cyanogenic glycosides. Cyanide or cyanogenic glycosides are known to be toxic for animal consumption. Therefore, Cane rat must have an inherent mechanism for detoxifying cyanide to be able to survive on its food. Previous works on 3MST have been done on several tissues of cane rats other than the intestine. In this work, we characterized and explored a partially purified 3MST (cyanide detoxifying enzyme) from the intestine of a cane rat for a possible therapeutic source against cyanide poisoning in other mammals that are susceptible to the toxin. 3MST from the intestine of *T. swinderianus* had a yield of 10.3% with specific activity of 0.21Umg<sup>-1</sup>. The Km and Vmax values of the 3-MST were determined to be 40 mM and 0.20µmol/ml/min respectively for KCN (Potassium Cyanide); also 33.3 mM and 0.15 µmol/ml/min for mercaptoethanol. 3-MST presents in the intestine of *T. swinderianus* plays a significant role in detoxification of cyanogenic compounds, which makes it an effective target for cyanide poisoning therapy.

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## 1. INTRODUCTION

Cyanide is a compound molecule which is poisonous to living organisms when consumed in food materials, it exist naturally in bacteria, fungi and plants as cyanide glycosides, also found in the environment from human activities such as industrial waste seepage into agricultural farms whereby it contaminates food produce and becomes deleterious to mammals and its surroundings [1,2]. Many plants acts as a precursor for cyanogenic glycosides, notably cassava [3] on which cane rats extensively feed. Natural source of cyanide complex – cyanide glycosides (derivative from ethylene synthesis) is from bacteria, fungi, plants and it has been reported that the range of 2000 - 2500 diversity of plants species contain twenty-five cyanogenic glycosides [1], (Cipollone et al., 2008).Cane rat consumes cassava, grasses and plant debris containing cyanide glycosides [4]. Disruption in cellular activity caused by cyanide is detoxified in-vitro in the mitochondria of the cell and detoxification is done in-vitro by group of enzymes termed Sulphutransferases (EC. 2.8.1). These enzymes catalyse substitution reaction by transfer of a sulphane atom, from a donor molecule to a thiophilic acceptor substrate in vitro. Such enzymes are found generally in animal, bacteria and plants. Rhodanese (RHD) (EC.2.8.1.1), 3-mecaptopyruvate-cyanide sulphurtransferase (MST) (EC.2.8.1.2) and thiosulphate-thiol transferase (EC.2.8.1.3) are subclasses, member groups of sulphutransferases [1]; Cipollone et al., 2008). 3-mecaptopyruvate-cyanide sulphurtransferase (3-MST) (EC.2.8.1.2) detoxifies cyanide by a transfer of sulphur ion using 3-mercaptopyruvate as to cyanide or to other thiol compounds. 3-MST necessitates thiosulphate biosynthesis (Cipollone et al., 2008).

Several studies claimed in-vivo utilization of sulphurtransferase for detoxifying cyanide is not known and other physiological functions of sulphurtransferase are claimed. However, since plants and animals are the man's source of food, humans are exposed to cyanide poisoning more often through oral route from contaminated plant crops and livestock hunt [5,6]. Cyanide salts ingested from food expose mammals to sodium cyanide (NaCN) and potassium cyanide (KCN) which has potential to disrupt cellular activity [7,8]. Cane rat scientifically known as (*Thryonomys swinderianu*), is well known for its

high quality protein diet consumption in West African nations (Akinola et al.,2014). It possesses a short nose, rounded ears, blunt bristle hair, and a big rump back with hind feet bigger than forefeet. Cane rat inhabit densely populated herbaceous vegetation in swamps and along bank of rivers within shallow burrow Aendem et al. (2012) reported that Cane rat easily acclimatize to different habitats and forest which depends on climate conditions in Nigeria.

*Thryonomys swinderianus* is an herbivore with phytophage feeding habit. [9] reported that persistent increase in death of livestock results from cyanogenic glycoside present in feed materials. Cyanide glycoside in plants is catalyzed by plant enzymes and stress such as drought. Tendency to toxicity of Cane rat by cyanide glycoside contained feed materials is significant on consumption through hydrolyzing enzymes [10]. This toxicity in *Thryonomys swinderianu*, is initiated from ingestion by chewing and in the large intestinal organ (ceacum) where negatively charged group  $CN^-$  of cyanide bounds with hydrogen ion  $H^+$  thus, produce acidic poisonous gas, hydrogen cyanide (HCN) also known as hydrocyanic acid. Beta-glycoside and Hydroxynitrile lyase are enzymes catalyzing hydrolysis in the intestinal tract of cane rat [1,11]. This interrupts metabolic enzymes to dysfunction, a cause for inhibition of cellular growth forming stable complexes. Three inhibitory mechanisms involved in cellular growth from cyanide reactivity are; chelation of divalent and trivalent metals in metalloenzymes, stable nitrile derivatives from Schiff-base intermediates reaction and cyanohydrin derivatives from keto-compound reactions [11]. Previous works have been done on 3MST and rhodanese from different tissues and organisms; however, this study attempts to partially purify and characterize 3MST from the intestine of cane rat and determine its significance on detoxification of cyanide toxins in cane rat.

## 2. MATERIALS AND METHODS

The reagents used for this study are Phosphate buffer (pH 7.2), Tris buffer (pH 8.0), 0.5M Potassium cyanide, 0.3M Mercaptoethanol, 38% Formaldehyde, Sorbo reagent (ferric nitrite and nitric acid), Bradford reagent. All Chemicals used were of analytical grade and purchased from reputed chemical firms.

## 2.1 Extraction of Intracellular 3MST

*Thryonomys swinderianus* was obtained by hunters from Abagboro village located at Ile-ife, Osun state, Nigeria. The village inhabitants are basically farmers and hunters, they basically cultivate cassava and other cyanide rich food crops, therefore the cane rats in this location are effective for this experiment as they will have the 3MST enzyme in abundance in their intestine and other tissues. The cane rat was transported to the laboratory in less than three hours after it was slaughtered, and then dissected. The intestines were removed and rinsed with distilled water, 30g was weighed and the crude extract was prepared by homogenization using laboratory sized pestle and mortar in 3 volumes of 0.1M Phosphate buffer (pH 7.2), according to the method of [12]. The crude extract was filtered using muslin cloth and both the residue and filtrates were collected, and the residue was further homogenized to get more crude extract. The suspensions were centrifuged for 30min at 4,000rpm in a Micro field Centrifuge Model 800D and the supernatants were collected and precipitated with 80% ammonium sulphate for protein extraction. Subsequently, the precipitated protein was then centrifuged again and the pellet was collected and desalted by dialysis. After dialysis (desalting), the dialysate was layered on the pre-treated CM Sephadex c-50 cation exchanger and the column was washed with 0.1M Tris buffer at pH 7.2 to remove unbound protein, followed by a step-wise elution with 1.0M NaCl in the buffer. Fractions were collected at a rate of 5 ml per 12 mins from the column. The active fractions from the column were pooled and used as enzyme source with the 3-MST activity and protein concentration assayed for, from the pooled fractions.

## 2.2 3-MST Assay

With the modified method of [13], 3-MST activity was determined through the measurement of thiocyanate produced from the catalytic reaction of potassium cyanide and mercaptoethanol. The reaction mixture contained 250  $\mu$ l Tris HCl buffer, 100  $\mu$ l mercaptoethanol, 100  $\mu$ l potassium cyanide and 50  $\mu$ l of enzyme solution. The reaction was incubated for 15 minutes at 37°C. The reaction was terminated by the addition of 750 $\mu$ l 38% formaldehyde and 750  $\mu$ l of Sorbo reagent and the optical density was read at 460 nm. The unit of enzyme activity was defined as micromoles thiocyanate formed per minute at 37°C.

## 2.3 Protein Assay

Bradford method (1976) was used to measure the protein concentration of the enzyme using bovine serum albumin (BSA) as standard. 1 ml of the Bradford Reagent was pipetted and added to 0.05 ml of the enzyme, with the blank containing 1 ml of Bradford Reagent and 0.05ml of distilled water without the enzyme sample. The absorbance was read at 595nm immediately.

### 2.3.1 Determination of kinetic parameters

The kinetic parameters ( $K_m$  and  $V_{max}$ ) of the enzyme were determined for 3-MST. The  $K_m$  of cyanide ion was determined by varying the concentration of KCN between 50 mm and 200 mm at 100 mm Mercaptoethanol. The kinetic parameters were then estimated from the double reciprocal plots according to the plot of [14].

### 2.3.2 Determination of pH and temperature effects on 3 mst activity

The effect of pH on 3MST was determined during 3MST assay with the use of buffers of varying pH at constant concentration: citrate buffer (50 mm, pH 3-5), phosphate buffer (50 mm, pH 6-7), 50 mm Tris-HCl buffer (pH 8) and borate buffer (50 mm, pH 9-10). The temperature effect was determined through 3MST assay at varying temperatures ranging from 30 - 100°C.

### 2.3.3 Determination of Substrate Specificity

The substrate specificity of 3MST was carried out by substituting mercaptoethanol for different sulphur containing compounds while the KCN was constantly added throughout the assay. The compounds used are mercaptoethanol, sodium meta-bisulphate, sodium thiol-sulphate, ammonium sulphate and copper sulphate. The activity of 3MST with these substituted compounds were compared to that of mercaptoethanol. The comparison was analysed in form of percentage by checking 3MST activity when mercaptoethanol is used as control.

## 3. RESULTS

### 3.1 Purification and Elution Profile of 3 MST

The purification profile of 3MST from the intestines of a cane rat resulted in overall yield of 10.3% and a purification fold of 3.0. The specific activity obtained after the purification process was 0.21U/mg. The total protein, enzyme activity, total enzyme activity, specific activity, yield and

purification fold of 3MST obtained at the extraction of the crude enzyme are 348.3 mg/ml, 0.63  $\mu\text{mol/ml/min}$ , 27.09  $\mu\text{mol/min}$ , 0.07  $\mu\text{mol/min/mg}$ , 100% and 1 respectively. Also, the total protein, enzyme activity, total enzyme activity, specific activity, yield and purification fold of 3MST obtained from dialysis after 80% ammonium sulphate precipitation are 79.5 mg/ml, 0.29  $\mu\text{mol/ml/min}$ , 7.25  $\mu\text{mol/min}$ , 0.091  $\mu\text{mol/min/mg}$ , 26.7% and 1.3 respectively. During ion exchange chromatography, the total protein, enzyme activity, total enzyme activity, specific activity, yield and purification fold of 3MST are 12.8 mg/ml, 0.20  $\mu\text{mol/ml/min}$ , 2.8  $\mu\text{mol/min}$ , 0.21  $\mu\text{mol/min/mg}$ , 10.3% and 3.0 respectively (Table 1). The elution profile of 3MST after CM Sephadex c-50 ion exchange chromatography shows the enzyme is present in fraction numbers between 15 and 25 (Fig. 1).

### 3.2 Kinetic Parameters of 3MST from the Intestine of *T. swinderianus*

The double reciprocal plots show the effect of varying concentrations of mercaptoethanol at fixed concentration of KCN and vice versa (Figs. 2. and 3). The  $K_m$  of the 3MST from the intestine of a cane rat for mercaptoethanol and KCN were estimated to be 33.3 mm and 40 mm respectively; while the  $V_{max}$  were estimated to be 0.15 MU and 0.20 MU respectively (Table 2).

### 3.3 Effect of pH and Temperature and Substrate Specificity of 3MST

The optimum pH of 3MST from the intestine of *T. swinderianus* was determined to be 6.0 (Fig. 4.) and the optimum temperature was determined to be 40°C (Fig. 5.). The activity and percentage of 3-MST specificity of sulfur containing substrates are given as: Mercaptoethanol = 0.032MU and 100% respectively; Sodium meta-bisulphate = 0.009MU and 28% respectively; Sodium thiol-sulphate = 0.003MU and 9% respectively; Ammonium Sulphate = 0.002MU and 25% respectively; Copper sulphate = 0.001MU and 10% respectively (Table 3).

## 4. DISCUSSION

The partially purified 3MST from the intestine of a cane rat (*T. swinderianus*) using 70% ammonium sulphate precipitation and ion exchange chromatography on CM-Sephadex c-50 has a specific activity of 0.21U/mg and a yield of 10.3%. Different specific activity values were reported for 3-MST from different sources. [15]

reported 0.0028U/mg as the specific activity of 3MST from ginger, a report also revealed the specific activity of 3MST in tilapia mariae fish to be 0.19 U/mg [16]; these values are lower than the value obtained from cane rat in this study, this might be as a result of cane rat consumption of cassava, grasses and plant debris containing cyanide glycosides and therefore developed more of inherent cyanide-detoxification mechanism than those present in ginger and tilapia mariae fish [4]. [1] reported the specific activity of 3MST from different tissues (liver, kidney and heart) of local chicken with the range of 0.01 (Heart) - 0.21 (Liver); pigeon with the range of 0.04 (Heart) - 0.34 (Kidney). [16] reported that in poultry chicken, duck and fruit bat, 3MST activity was found to be highest in kidney, followed by the lungs in poultry chicken and liver in the duck. The Liver and kidney is usually the richest source of 3MST in animals [17], and this may be due to the fact that liver and kidney play pivotal role in detoxification processes. Interestingly, 3MST in the intestine of cane rat reported from this study is found to have higher specific activity than 3MST from liver and kidney of poultry chicken, local chicken, and duck reported by Agboola et al. [1]. This may be due to the fact that cane rat feeds extensively on plants rich in cyanide or cyanogenic glycosides (Uyoh et al., 2007), which makes the enzyme to be highly modified to specifically detoxify the glycosides for the survival of the herbivore.

The kinetic parameters show the enzymatic ability for cyanide detoxification in organisms. In this study, the  $K_m$  and  $V_{max}$  values of the 3-MST were determined to be 40 mm and 0.20  $\mu\text{mol/ml/min}$  respectively for KCN; and 33.3 mm and 0.15  $\mu\text{mol/ml/min}$  for mercaptoethanol. Ehigie et al. (2020) reported that the  $K_m$  and  $V_{max}$  values for Mearpctoethanol were 16.67 mm and 0.2  $\mu\text{mol/ml/min}$ ; and KCN are 20 mm and 0.24  $\mu\text{mol/ml/min}$  respectively in different plant tubers. In addition, [15] that the  $K_m$  value of 2.02 mm and  $V_{max}$  of 0.143  $\mu\text{mol/ml/min}$  were obtained for mercaptoethanol and 4.56 mm and 0.026  $\mu\text{mol/ml/min}$  respectively from Ginger (*Zingiber officinale*). As reported by Agboola and Okonji, (2004) this less effective enzymatic system may indicate lower exposure to cyanide. In contrast to mammalian animals (especially cane rat used in this study), which are continually exposed through their diet, plants are less vulnerable to cyanogenic toxins. Similar research on the garden snail shows that the  $K_m$  and  $V_{max}$  values for Mearpctoethanol is 2.83 mm and 6.17MU; and KCN is 1.09 mm and 3.08MU

respectively [18], this is lower ( $K_m$ ) than the values gotten from this study because garden snail feeds more extensively on substances from the soil which contain high level of cyanogenic glycosides and therefore require higher affinity for the cyanogenic substrate to help the animal survive on its food [19].

**Table 1. Purification Profile of 3-MST from *T. swinderianus***

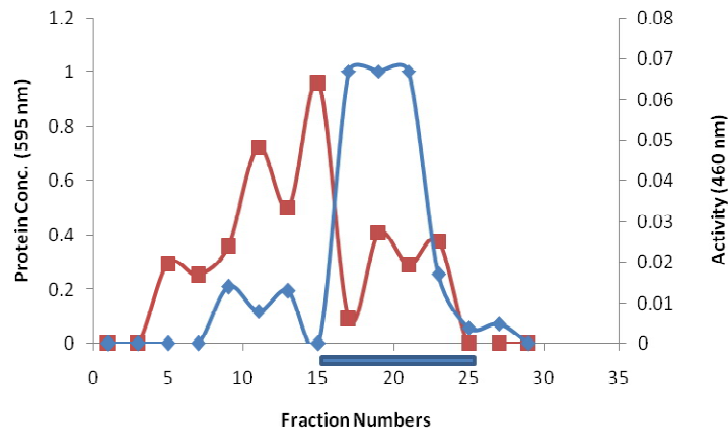
Purification Steps	Total protein (mg/ml)	Enzyme activity ( $\mu\text{mol/ml/min}$ )	Total Enzyme activity (MU)	Specific activity (Unit/mg)	Yield (%)	Purification Fold
Crude	348.3	0.63	27.09	0.07	100	1
80% Ammonium Sulphate	79.5	0.29	7.25	0.091	26.7	1.3
Ion exchange Chromatography	12.8	0.20	2.8	0.21	10.3	3.0

**Table 2. Summary of kinetic parameters deduced from the plots (Figs. 2. and 3.)**

PARAMETERS	KCN	MERCAPTOETHANOL
$K_m$ (mm)	40	33.3
$V_{max}$ (MU)	0.20	0.15

**Table 3. Substrate Specificity of 3MST from *T. swinderianus* intestine**

Sulfur-containing Substrates	Activity (MU)	Percentage of 3-MST Specificity (%)
Mercaptoethanol	0.032	100
Sodium meta-bisulphate	0.009	28
Sodium thiol-sulphate	0.003	9
Ammonium Sulphate	0.002	25
Copper sulphate	0.001	10



OD460-----Protein assay  
absorbance @ 595nm

OD595-----Enzyme assay  
absorbance @ 460nm

**Fig. 1. Elution profile of 3 MST from the intestines of *T. swinderianus* on a CM selphadex C-50 ion exchange chromatography**

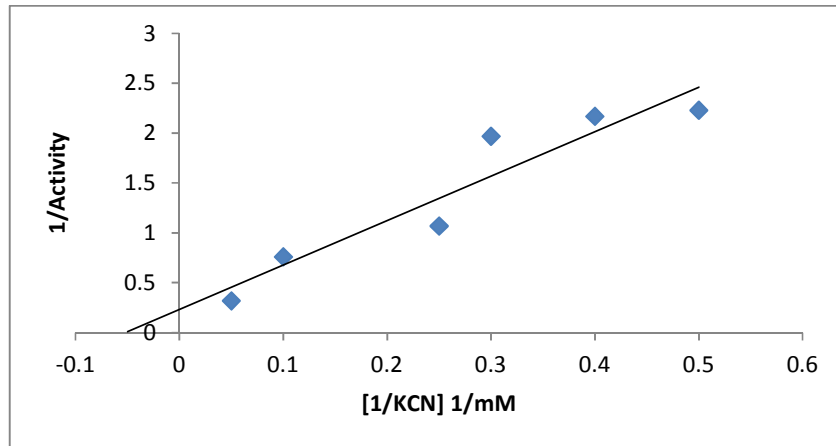


Fig. 2. Double reciprocal plot for varying concentration of KCN between 10 mM and 60 mM and a fixed concentration of mercaptoethanol

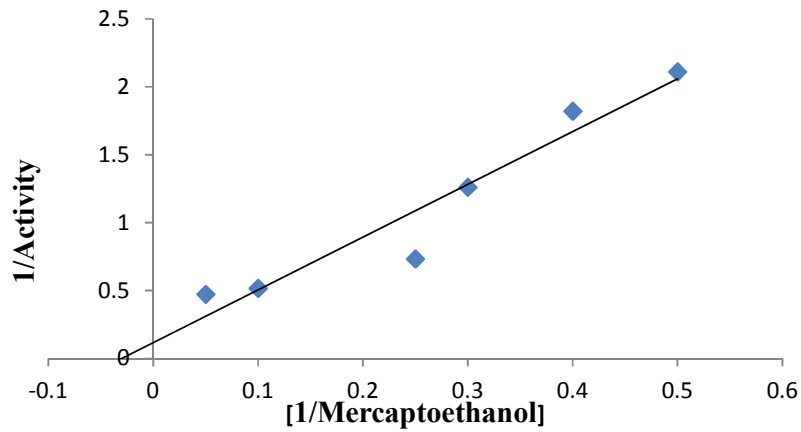


Fig. 3. Double reciprocal plot for varying concentration of mercaptoethanol between 10 mM and 60 mM and a fixed concentration of KCN

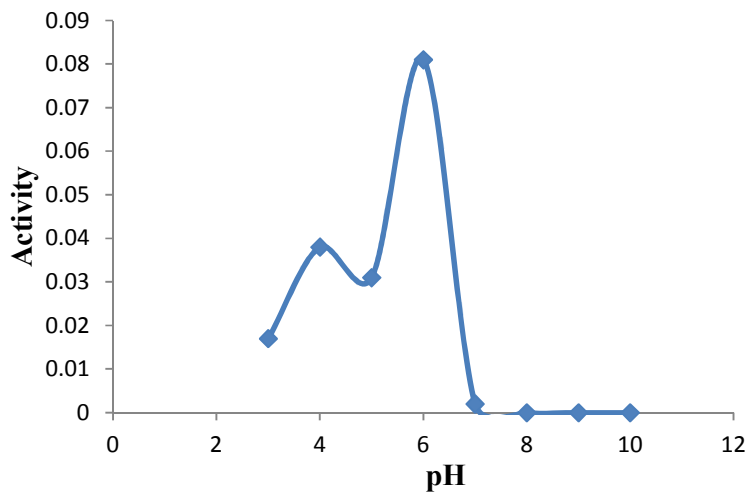
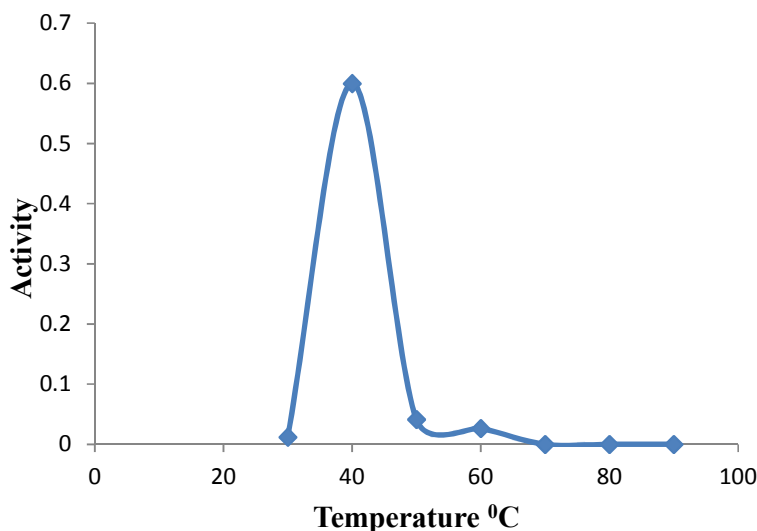


Fig. 4. Effect of pH on 3MST from *T. swinderianus* intestine



**Fig. 5. Effect of temperature on 3MST from *T. swinderianus* intestine**

From this study, 3 mercaptopyruvate sulfurtransferase from the intestine of cane rat (*T. swinderianus*) has an optimum pH and temperature of 6.0 and 40°C respectively. As pH increased from 6.0 to 7.0 and the temperature from 40°C upward, a sharp decline was observed in the enzyme activity suggesting its sensitivity to pH and temperature change. [18] reported the sensitivity to pH and temperature increase for hemolymph's 3- MST of *Limicolaria flammae*, the study showed that MST from *Limicolaria flammae*'s (hemolymph) has optimum pH of 5.0 and temperature of 60 °C. Other studies also reported different pH and temperature values for the enzyme, gotten from different organisms. [20] reported that human MST has optimum pH of 8.2 while *Escherichia coli* have optimum pH and temperature of 9.3-9.6 and 45°C-50°C respectively [21]. Beyond the optimum temperature, the enzyme loses its activity (thus becomes inactive) and is ultimately denatured. The loss of activity comes first before denaturation because the active site of an enzyme tends to more flexible than other parts of the enzyme [22]. The ability of pH to affect ionic and hydrogen bonds necessary for enzyme shape and activity might be responsible for the loss of activity of the enzyme at pH other than the optimum [23].

Using a range of sulphur compounds, the substrate specificity study of 3-MST of *T. swinderianus* revealed that the enzyme has little affinity for other sulphur compounds compared to mercaptoethanol, that is 3MST cannot use any of

the sulphur containing compounds as a better substitute for mercaptoethanol as the activity of the enzyme was drastically low when other substrates were used. [24] in a bid to understand what chemical properties of 3-MP makes it the only known sulfur-donor substrate for MST, studied two mercaptic acids (2-mercaptopropionic and 3-mercaptopropionic acids) which are structurally related to mercaptopyruvate. The result showed that none of these two compounds could serve as sulfur-donor substrate for MST. It was inferred from the study that the  $\alpha$ -keto group of 3-MP was important for its use as a substrate and it was also suggested that the position of sulphur on these substrates may influence their binding to the enzyme. The absence of the hydroxyl group (-OH) in these sulphur compounds which makes them non-polar compared to mercaptoethanol might be responsible for their inhibition of this enzyme (Ehigie et al., 2020).

## 5. CONCLUSION

The study established the presence of 3-MST activity in the intestine of cane rat, also that 3-MST plays a significant role in cyanide-detoxification mechanism of Cane rat thereby helping the animal survive on its food.

## DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely



no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

### ETHICAL APPROVAL

Animal ethic committee approval has been collected and preserved by the author.

### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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