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Physicochemical Properties of 3-Mercaptopyruvate Sulfurtransferase: A Cyanide Detoxifying Enzyme from the Hemolymph of *Limicolaria flammea* (Garden Snail)

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Abstract

Background: 3-Mercaptopyruvate sulfurtransferase (3-MST) is a multifunctional, mitochondrial and cytoplasmic sulphurtransferase that catalyses the detoxification of cyanide to a less toxic thiocyanate. *Limicolaria flammea* feeds majorly on green leaves, plants and other cyanide containing foods.

Methods: 3-MST from the hemolymph of *Limicolaria flammea* was purified by 70 % ammonium sulphate precipitation and ion exchange chromatography. The purified enzyme was characterized at different levels such as optimal activity, inhibitors, substrate preference, thermal stability and analysis of kinetic parameters.

Results: 3-MST from the hemolymph of *Limicolaria flammea* had a yield of 0.75 % with specific activity of 0.42 μ /mg/ml. The K_m values for the substrates; KCN and 2-Mercaptoethanol were 1.09 and 2.83 mM, while the V_{max} values were 3.08 μ mol/mol/min and 6.17 μ mol/mol/min respectively. The optimum pH and temperature of the enzyme were 5.0 and 60° C respectively. The metals (Al^{3+} , Ca^{2+} , and K^+) demonstrated inhibitory activity in a concentration dependent manner. The substrate specificity study showed that sodium sulphite, ammonium per sulphate and ammonium sulphite showed enzymatic interference.

Conclusion: This study affirmed the presence of 3-MST activity in the hemolymph of *Limicolaria flammea*, an indication that the enzyme possesses functional cyanide detoxification mechanism necessary for the survival of the animal in the environment.

Keywords: 3-mercaptopyruvate sulfurtransferase, cyanide, detoxification, *Limicolaria flammea*

1.0 INTRODUCTION

The enzyme 3-mercaptopyruvate sulfurtransferase (MST, EC 2.8.1.2) catalyzes the chemical reactions of 3-mercaptopyruvate. It belongs to the family of transferases, specifically the sulfurtransferases [1]. 3-MST has been known to catalyze endogenous cyanide (CN⁻) detoxification due to ability to transfer sulfur from the sulfur donor, 3-mercaptopyruvate (3-MP), to cyanide (CN⁻), to generate thiocyanate (SCN) which is less toxic [2]. 3-MST is widely distributed in nature amongst prokaryotes and eukaryotes [3]. Interestingly, 3-MST is localized in the cytoplasm and mitochondria, however, it is not present in all cell types [4]. 3-MST is active in the detoxification of cyanide, a potent toxin of the mitochondrial respiratory chain [5].

Although 3-mercaptopyruvate sulfurtransferase (3-MST) is a protein closely-related to rhodanese with striking similarity in active site amino acid sequence there exists some biochemical and molecular variations between them [6, 7]. The two different sulfurtransferase (Str) enzymes have been identified and localised in different species. The two sulfurtransferases in mammals have been demonstrated to have 3-mercaptopyruvate and thiosulfate-specific activities and are characterized by different Km values for both substrates [8]. The 3-mercaptopyruvate sulphurtransferase enzyme was localized to the cytoplasm and the mitochondria by immunogold-labeling and immunoblotting, whereas thiosulfate-specific sulphurtransferase was detected exclusively in mitochondria and particularly in liver cells. 3-mercaptopyruvate Str might catalyses cyanide detoxification cyanide in the cytosol while both Str enzymes would function to protect cytochrome c oxidase effectively in the mitochondria [6]. To unveil the relationship between the two enzymes, some workers in Japan examined both Thiosulphate sulphurtransferase (TST) activity and mRNA expression in MST knockout mice [9]. It was revealed that there was overexpression of rhodanese in the animals lacking MST. This gives an insight to complementary roles played by sulfurtransferases in handling cyanide detoxification. These enzymes seem to function principally in metabolism of sulfur compounds [10]. Various accounts of 3-MST physiological functions including cyanide detoxification [11], trans-sulfuration, H₂S and polysulfides production, anti-oxidative stress and redox sensing [12], possibly SO₂ production, and anti-anxiety behaviour have been reported by scientists [13].

Limicolaria flammea commonly called Garden snail is a small land animal found predominantly in Sub-saharan

Africa. It is a species of tropical air-breathing land snails, a terrestrial pulmonate gastropod mollusk in the family Achatinidae [14]. This species is indigenous to West Africa, Nigeria. It consumes a wide range of green plants, which are known to contain cyanogenic glycosides. It is therefore reasonable for us to assume that the animal possesses a good and efficient cyanide detoxifying mechanism. Cyanide is known to be a potent inhibitor of cytochrome oxidase of the mitochondrial electron transport chain [15]. When consumed or inhaled, cyanide activates the innate detoxifying system, resulting in the conversion of cyanide into a less harmful compound known as thiocyanate. 3-mercaptopyruvate sulfurtransferase is one of the major enzymes of cyanide detoxification [16]. This study investigates the properties of 3-mercaptopyruvate sulfurtransferase from the hemolymph of *L. flammea*.

2.0 METHODOLOGY

2.1 Material

The reagents below were used during the course of the research work; 0.1 M Phosphate buffer (pH 7.2), 0.3 M Tris buffer (pH 7.8), 0.5 M Potassium cyanide, 0.3 M Mercaptoethanol, Sodiumthiosulphate, 15% Formaldehyde, Sorbo reagent (ferric nitrite and nitric acid), Ammonium sulfate, Bradford reagent. Other chemicals used were of analytical grade and were obtained from reputable chemical firms. Garden snails (*Limicolaria flammea*) were collected and authenticated from the school farm, Faculty of Agriculture, Obafemi Awolowo University Ile-Ife, Osun state, Nigeria.

2.2 Enzyme Extraction and Ammonium Sulfate Precipitation

The samples were safely taken to the laboratory and carefully washed to remove sand and dirt. The shell of the garden snail was carefully broken at the bottom and the hemolymph was collected into a clean beaker. The hemolymph was filtered over a double layer cheese cloth to remove broken shell sediments and kept in a freezer for further analysis.

The hemolymph (47 ml) was brought to 70% ammonium sulphate saturation as described by Fagbohunka *et al.*, [17] by slow addition and stirring of 42.05 g solid ammonium sulphate. This was done for 60 minutes with intermittent stirring until all the salt had solubilised completely in the supernatant. The mixture was left for 12 hours at 4°C. The solution from the ammonium sulfate precipitation was centrifuged at about 4,000 rpm for 30 min. The pellet was washed with a small amount of 0.1 M

phosphate buffer (pH 7.2) and was dialyzed against several changes of 0.1 M solution of phosphate buffer (pH 7.2) at 4°C for 8 hours using a dialysis bag. The dialysate was subjected to centrifugation at 14,000 rpm for 15 min and the supernatant was assayed for enzyme activity and protein content.

2.3 The 3-MST Assay

The 3-MST activity was measured according to the method described by Taniguichi and Kimura [18] and Okonji and Popoola [19]. The reaction mixture contained 0.25 ml 0.38 M Tris-HCl buffer, pH 7.8, 0.1 ml of 0.5 M KCN, 0.1 ml of 0.3 M mercaptoethanol and 0.1 ml of the enzyme solution in a final volume of 0.55 ml. The mixture was incubated at 37°C for 1 min and the reaction was stopped by adding 0.25 ml of 15 % formaldehyde, followed by the addition of 0.75 ml of Sorbo reagent. The absorbance was taken at 460nm. One unit of enzyme activity (U) was taken as the amount of enzyme [20]. Protein content was determined by the method of Bradford [21] and standard curve was generated using Bovine Serum Albumin (BSA). The reaction mix consists of 10µl of the enzyme solution and 1.0 ml of Bradford reagent. The absorbance was read at 595nm

2.4 Ion-Exchange Chromatography on CM-sephadex C-25

CM-sephadex cation exchanger was used in purification of the enzyme solution obtained from preceding step according to the method of Fagbohunka *et al.*, [17]. CM-Sephadex C-25 was prepared by the following steps: 10 g of the CM-Sephadex C-25 was dissolved in 100 ml of the buffer or distilled water. It was allowed to swell for 48 hours (sephadex rises 4.5 fold). 100 ml 0.1 M HCl acid was added for 30 minutes after which the acid was decanted and the resin was washed with distilled water several times to ensure total removal of the acid. 100 ml 0.1 M NaOH was added to the resin which was decanted after 30 minutes followed by thorough rinsing 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 the column. Two millilitres (2 ml) of sample was then layered on the column and the fractions were collected using 30 elution tubes, 2 ml per tube. Immediately the column was layered with sample, 0.1 M phosphate buffer was continuously added to the column to remove unbound protein. Stepwise elution was then done by changing the elution buffer from 0.1 M phosphate buffer to phosphate buffer containing 0.1 M NaCl salt. The elutes were then assayed for protein concentration and enzyme activity. The active elutes were pooled.

2.5 Kinetic parameters and Substrate Specificity

Kinetic parameters (K_m and V_{max}) of the enzyme were determined by varying concentration of Mercaptoethanol between 30 mM and 300 mM at a fixed concentration of 0.5 M KCN and also the concentration of KCN between 50 mM and 500 mM at a fixed concentration of 0.3 M Mercaptoethanol. Graphical 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-Burk model [22]

The substrate specificity of 3-mercaptopyruvate sulfurtransferase was investigated by testing its activity towards structurally related compounds. The most commonly used substrate for 3-MST is 2-mercaptoethanol. The substrate specificity of the enzyme was determined by replacing 2-mercaptoethanol with different sulphur-containing compounds such as ammonium sulphate, ammonium per sulphate, sodium thiosulphate, in a typical 3- MST assay mixture. The calculated activity was expressed as a percentage (%) activity of the enzyme using mercaptoethanol which was the control substrate.

2.6 Effect of pH, Temperature and Metallic Salts on the Enzyme Activity

The Purified enzyme was assayed using different buffers of varying pH which includes: 50 mM citrate buffer (pH 4 -5); 50 mM phosphate buffer (pH 6-7); 50 mM tris buffer (pH 8) and 50mM borate buffer (pH 9-10). The 3-MST activity was assayed as described earlier with the assay buffer being replaced by these buffers.

The enzyme was assayed at temperatures between 40° C to 90° C to investigate the effect of change in 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 15 min before initiating reaction by the addition of an aliquot of the enzyme which had been equilibrated at the same temperature.

The inhibition effect of various metal ions on the activity of 3-MST was also studied. The salts used were: Aluminium chloride ($AlCl_3$), Calcium chloride ($CaCl_2$), and Potassium chloride (KCl) at concentrations of 1 mM, 5 mM and 8 mM each.

2.7 Inhibitory Study and Heat Stability

The inhibitory effect of Urea and EDTA was determined at concentrations of 1.0 mM, 3.0 mM, 5.0 mM, and 10 mM each.

Table 1. Quantification of the Purification Protocol for 3-Mercaptopyruvate Sulfurtransferase from Hemolymph of *L. flammar*

Fractions	Total protein (mg) ^a	Total Activity (U) ^b	Specific Activity (U/mg/ml)	% yield	Purification fold
Crude	164	584.5	3.56	100	1
70% ammonium sulphate precipitation	53.32	68.25	1.28	11.68	0.36
CM Sephadex C-25 ion exchange (pooled peak)	10.55	4.38	0.42	0.75	0.12

^a Protein concentration determined by Bradford assay using BSA as a standard protein^b Enzyme activity measured as described in methods section

Thermal stability of the enzyme was determined by incubating 2 ml of the enzyme at 40°C, 50°C, 60°C, and 70°C for 10, 20, 30, 40, 50, and 60 minutes of each temperature respectively. From the incubated solution, 0.1 ml was withdrawn at 10 min intervals and assayed for residual activity.

3.0 RESULTS

3.1 Purification profile of 3-MST from Hemolymph of *L. flammar*

The purification profile for 3-mercaptopyruvate sulfurtransferase from the hemolymph of *L. flammar* produced a yield of 0.75 % with a specific activity of 0.42 μ /mg/ml. The purification protocol is summarized in Table 1. Figure 1 shows elution profile of Ion exchange chromatography on CM-Sephadex C-25

Figure 1. Elution Profile on CM-Sephadex-C-25 Ion exchange Chromatography for 3-MST from Hemolymph of *Li-micolaria flammar*

3.2 Kinetic parameter of 3-MST from Hemolymph of *L. flammar*

The kinetic parameter for hemolymph of garden snail (K_m and V_{max}) were determined using the double reciprocal (Lineweaver-Burk) plot. This was achieved by varying the concentration of mercaptoethanol and potassium cyanide and then measuring the activity. Figures 2 and 3, and Table 2 shows the kinetic parameters

Figure 2. Lineweaver-Burk plot for 3-MST from Hemolymph of *L. flammar* at varying Concentration of Mercaptoethanol **Figure 3.** Lineweaver-Burk Plot for 3-MST from Hemolymph of *L. flammar* at Varying Concentration of Potassium Cyanide (KCN)

Table 2. Kinetic Parameters of 3-MST from Hemolymph of *L. flammae*

Substrates	V _{max} (μmol/ml/min)	K _m (mM)
KCN	3.08	1.09
MERCAP-TOETHANOL	6.17	2.83

3.3 Effect of Temperature, pH and Metals on Activity of 3-MST from Hemolymph of *L. flammae*

The activity of 3-mercaptopyruvate sulfurtransferase in the hemolymph of garden snail was assayed at a temperature between 40°C to 90°C. The effect of temperature on the enzyme activity shows that the enzyme has maximum activity at 60°C for 50 mins (Figures 4 and 5)

The activity of 3-mercaptopyruvate sulfurtransferase was determined in the assayed buffer pH range from 4 to 10 at 37°C. The optimum pH of 3-MST was at pH 5.0. This is depicted in Figure 6. The assay was done to determine the effect of metals at different concentrations of 1, 5 and 8mM using Al³⁺, Ca²⁺ and K⁺. The metals demonstrated a slight inhibition in a concentration dependent manner (Figure 7).

Figure 4. Temperature-activity Profile of 3-MST from Hemolymph of *L. flammae*.

Figure 5. Heat Stability Profile of 3-MST from Hemolymph of *L. flammae*

Figure 6. pH-activity profile of 3-MST from the Hemolymph of *L. flammae*.

3.4 Substrate Specificity of 3-MST from Hemolymph of *L. flammae*

Substrate specificity of 3-MST was investigated by testing its activity towards structurally related sulfur compounds in a typical 3-MST assay mixture. The sulfur compounds tested drastically decreased the enzyme activity relative to the control which is mercaptoethanol (Table 3). Figure 8 shows the degree of the inhibitory effect on the enzyme activity in the order; ammonium sulfite>sodium sulfite>ammonium persulphate

Table 3. Effect of Sulfur Compounds

Substrate	% Residual activity
Control	100
Ammonium Sulphite	26.93
Sodium Sulphite	28.87
Ammonium per sulphate	29.94

Figure 7. Effect of Metals on the Activity of 3-MST from Hemolymph of *L. flammae*.

Figure 8. Effect of Sulfur Compounds on the Activity of 3-MST from Hemolymph of *L. flammea*

Figure 9. Effect of Urea and EDTA on the activity of 3-MST from the Hemolymph of *L. flammea*

3.5 Inhibitory Effects of Urea and EDTA on Activity of 3-MST from Hemolymph of *L. flammea*

The assay was done to determine the effect of Urea and EDTA at different concentrations of 1, 3, 5 and 8 mM (Figure 9)

4.0 DISCUSSION

3-mercaptopyruvate sulfurtransferase was isolated and partially purified from the hemolymph of *L. flammea* using ammonium sulphate precipitation and CM-Sephadex C-25 ion exchange chromatography. The specific activity of the partially purified 3-MST from the hemolymph of *L. flammea* was approximately 0.42 μ/mg/ml with a yield of 0.75 %. Different specific activity values were reported for 3-MST from different sources. The specific activity of 3-MST from onion bulb was 540 μ/mg/ml [23]. Activity of 3-MST from liver of chicken, duck, and pigeon are 0.21 μ/mg/ml, 0.09 μ/mg/ml and 0.19 μ/mg/ml respectively [24]. Fagbohunka and Agboola [17] in 2006 obtained a

specific activity of 0.19 μ/mg/ml from the gut of *Tilapia mariae* fish and 0.29 μ/mg/ml from the gut of *Tilapia zilli* fish. They also reported that the 3-MST from gills of *Tilapia mariae* and *Tilapia zilli* have a specific activity of 0.035 μ/mg/ml and 0.12 μ/mg/ml respectively.

The kinetic parameters, K_m and V_{max} describes the affinity and the turnover number of the substrates. The K_m and V_{max} values as determined by the Lineweaver-burk plot for KCN and mercaptoethanol were 1.09 mM, 3.08 μmol/ml/min and 2.83 mM, 6.17 μmol/ml/min respectively. The relatively high kinetic data obtained for mercaptoethanol may reveal low physiological concentration of cyanide. Moreover, this may suggest that the enzyme has other roles to play aside cyanide detoxification. Report has shown various kinetic parameters for these substrates in various organisms: bovine liver, 19.0 and 6.5 [25] bat liver, 13.36 and 19.15 [17] and cat liver, 25.4 mM and 18.6 μmol/ml/min [26] for KCN and mercaptoethanol substrates respectively. The K_m and V_{max} of 3-MST in some plant tubers for sodium thiosulphate and potassium cyanide were 16.67 mM and 20 mM and 0.2 μmol/ml/min and 0.24 μmol/ml/min respectively [27]. The low values of the kinetic data as compared to the report of other workers may suggest that the organism has little exposure to cyanide compounds. It can be said that the kinetic parameters vary with the enzyme source and nature of the substrates used for the assay.

The enzymatic activity of *L. flammea*'s (hemolymph) 3-MST was optimum at pH 5.0. There was a rapid decline in enzyme function with increasing alkalinity. A unit increase of pH from 5 to 6 showed a sharp drop in the activity indicating that the enzyme was relatively sensitive to pH increases. Different pH values have been reported for the enzyme from different species of organism. Optimum pH for *L. major* and *L. Mexicana* was 6.9-7.6 [28]. Human 3-MST has optimum pH of 8.2 [1] while 9.3-9.6 was reported for *E. coli*'s 3-MST [23]. For suitable biotechnological application, an acidic condition must be considered. The product formation at different incubation temperature conditions showed an optimal activity at 60°C for *L. flammea*'s (hemolymph) 3-MST.

Bos. taurus' and *R. novergicus*' 3-MST have optimum temperature of 30°C [29] while 45°C-50°C was reported for the enzyme in *E. coli* with loss of activity at 60°C [23]. The thermal stability of the enzyme indicated that the enzyme was stable between 40°C and 60°C. The enzyme showed increase in activity for 50 minutes at 60°C. These results indicate that for application of the 3-MST from *L. flammea*'s (hemolymph), the suitable temperature range could be 40–60°C.

The substrate specificity study showed that the enzyme has little affinity for the tested sulphur compounds (sodium sulphite, ammonium per sulphate, and ammonium sulphite). Several factors come into play in the development and utilization of a compound as a sulfur donor substrate for 3-MST. According to Porter and Baskin [30] in comparing two mercaptic acids (3- mercaptopropionic acid and 2-mercaptopyruvic acid) that are structurally related to 3-Mercaptopyruvate, it was opined that the α -keto group of 3-Mercaptopyruvate was necessary for its utilization as a substrate, and that the position of the sulfur may also affect the binding of the tested Mercaptic acids these compounds to the enzyme. Moreover, the enzyme interference of the tested Sulphur compounds may be due to lack of hydroxyl group (-OH) which would make them relatively non-polar compared with mercaptoethanol. This property could be largely responsible for significant inhibition. However, further *in vitro* enzyme kinetic studies may be needed to increase the understanding of how difference in chemical properties of the substrates affects their utilization.

Specific binding sites for some metal ions have been crystallographically documented for sulphur transferases [19]. In some of these cases, the binding of the cation has been found to promote functional effects [31]. The inhibitory effects of the metal ions tested (Al^{3+} , Ca^{2+} and K^{+}) could be due to the interaction of these metal ions with sulphanyl groups at the active site or decrease in the availability of the substrate to the enzyme or the binding of the cations to an amino acid side chain involved in the binding or catalysis of the substrate. EDTA and Urea showed inhibitory properties against the enzyme which may be blocking the active site. The isolation, partial purification and characterization of 3-MST was carried out in order to understand the properties and activity of the enzyme. The biochemical parameters obtained confirmed that the enzyme 3-MST is present in the hemolymph of Garden snail (*L. flammea*) and indicates the presence of a powerful detoxification mechanism of cyanide as it functions in the conversion of cyanide to thiocyanate, thereby improving the survival of the animal feeding on plants containing cyanide.

Conflict of Interest

The authors declare that there is no conflict of interest.

Authors Contribution

AFE conceived and designed the study, performed data analysis and manuscript writing; **REO** performed data collection; **FDO** contributed to data collec-

tion and data analysis tools; **MOJ** contributed to data analysis; **LOE** contributed to study design, data analysis and writing of manuscript.

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