**International Journal of Bioorganic Chemistry**
2021; 6(2): 21-25
http://www.sciencepublishinggroup.com/j/ijbc
doi: 10.11648/j.ijbc.20210602.12
ISSN: 2578-9384 (Print); ISSN: 2578-9392 (Online)

# Properties of Partially Purified Rhodanese from Leaves of Cassava in Owo Southwestern Nigeria

**Omowumi Funke Adedugbe, Oluwatosin Desola Owolala, Olutosin Samuel Ilesanmi***

Department of Chemical Sciences, Achievers University, Owo, Nigeria

## Email address:
olutosinilesanmi@yahoo.com (O. S. Ilesanmi)

*Corresponding author

## To cite this article:
Omowumi Funke Adedugbe, Oluwatosin Desola Owolala, Olutosin Samuel Ilesanmi. Properties of Partially Purified Rhodanese from Leaves of Cassava in Owo Southwestern Nigeria. *International Journal of Bioorganic Chemistry*. Vol. 6, No. 2, 2021, pp. 21-25.
doi: 10.11648/j.ijbc.20210602.12

**Received**: August 10, 2021; **Accepted**: August 25, 2021; **Published**: November 19, 2021

**Abstract:** Introduction**:** Rhodanese is a transferase enzyme that catalyses detoxification of cyanide. Cyanide is popular for its presence in a wide variety of food materials that are consumed by animals especially man. In this work, we investigated the partially purified rhodanese from cassava leaves in order to ascertain its suitability for cyanide detoxification in cassava tuber. Crude rhodanense was subjected to 80% $(NH_4)_2SO_4$ precipitation, the percentage yield and purification fold obtained was 68% and 4 respectively. The degree of substrate preference was $Na_2S_2O_3$>2-mercaptoethanol>ammonium persulphate>sodium metabisulfite>sodium sulfite. Apparent $K_m$ and $V_{max}$ were 18.2 mM and 13.9 RU/ml respectively. While that of $Na_2S_2O_3$ were 10.9 mM and 5.8 RU/ml respectively. The substrate specificity $V_{max}/K_m$ revealed that KCN was a better substrate. The optimum pH and temperature of rhodanese from cassava leaves were 6.0 and 40°C respectively. Result: The information provided by this study can be exploited for understanding cyanide distribution and detoxification of cassava.

**Keywords:** Rhodanese, Cyanogenic Glycosides, Detoxification, Cyanophoric Plant, *Manihot esculenta*

## 1. Introduction

Rhodanese (thiosulfate: cyanide sulfurtransferase; EC 2.8.1.1) catalyzes the transfer of the reduced sulfur atom in a sulfane-containing compound to a thiophilic acceptor. *In vivo,* the enzyme is multifunctional [1], clearly it act to detoxify cyanide [2]. This function is more prominent in mammals where highly cytotoxic cyanide is converted to a less toxic thiosulphate and excreted through the kidney. The principal detoxification pathway of cyanide is that catalysed by a liver mitochondrial rhodanese [3]. It is a ubiquitous enzyme that is active in all living organisms, from bacteria to humans [4]. Beside cyanide detoxification, many other physiological functions have been proposed for the enzyme. It supplies sulphide for the formation of iron–sulphur centres, thiamine biosynthesis, maintains the sulphane pool, participates in selenium metabolism, and functioning as a thioredoxin oxidase [5]. Several studies on its intracellular distribution have revealed that it is present in the cytosol, mitochondrion and nucleus [6]. A close relationship exists between rhodanese activity and cyanogenesis, this gives an indication that the enzymes provide a mechanism for cyanide safe biotransformation in plants [7].

Cyanide is a potent cytotoxic agent that act by inhibiting mitochondrial cytochrome oxidase [8]. It is popular for its presence in a wide variety of food materials that are consumed by animals especially man [9]. Many plants and plant products used for food in tropical countries contain cyanogenic glycosides. These plants include cassava [10]. Cyanide detoxification mechanism is activated when ingested, resulting into biotransformation of cyanide into less toxic thiocyanate [11].

Cassava (*Manihot esculenta* Crantz) plant is very important to the economy of Nigeria as well as Africa, being the most common staple food [12]. Furthermore, Nigeria is one of the highest producer of the food crop accounting for 73% of the world production [13]. It has been a raw material for global industrial production of textiles, papers, adhesives, pharmaceuticals and various food products (sugar, fructose syrups, bread, wine, brandy and others) because it is rich in carbohydrate with high energy density and has generated great impact in world economics [14]. However, cyanogenesis in cassava tubers has been a limiting factor in

its acceptance, exploitation and utilization which can be complemented by cassava leaves [15]. Cassava leaves are rich in protein and beta-carotenoids, they are also consumed as a vegetable and forage (fresh or dehydrated meal) in various parts of the world [16]. Cassava, a cyanophoric plant contains cyanogenic glycosides in its edible roots and leaves. The regular exposure of body system to cassava cyanogens may leads to the development of protein deficiency diseases. Rhodanese activity has been established in the leaves and in the flesh of tuberous part of *Manihot esculenta.*

Although, some workers have reported the presence rhodanese in cassava [17, 18]. Different species are cultivated across the world and humans are exposed to different levels of cyanide concentration because of diversity in the species of cassava. Whether the characteristics of rhodanese in all these species of cassava are the same or not, it is not clear. We have therefore decided to establish the properties of rhodanese from cassava commonly grown in Owo, Southwestern, Nigeria. The information provided may be exploited for the understanding of diversity of cyanide levels and rhodanese concentrations in cassava.

# 2. Materials and Methods

### *2.1. Materials*

Potassium cyanide, ammonium sulphate, sodium thiosulphate, formaldehyde, sorbo reagent, nitric acid, ferric nitrate, boric acid and ethanol were obtained. All other reagents were of analytical grade.

### *2.2. Methods*

#### *2.2.1. Collection of Samples*

Fresh Cassava leaves and tubers were obtained from a local farm around Achievers University, Owo, Southwestern, Nigeria and was authenticated in the Department of Plant Science and Biotechnology, Achievers University, Owo.

#### *2.2.2. Enzyme Extraction and Homogenization*

Thirty grams (30 g) of fresh Cassava leaves were weighed and homogenized with 0.1M phosphate buffer pH 6.5 at 4°C to obtain 30% homogenate. The homogenate was squeezed using cheese cloth and centrifuged at 3000 rpm for 30 mins at 4°C. The supernatant was then taken to be the crude rhodanese and it was stored at -20°C.

#### *2.2.3. Rhodanese Activity Assay*

Rhodanese activity was measured according to the method of [19], as modified by [20]. Briefly, 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 20 µl of the enzyme solution in a total mixture of 1.0 ml. The mixture was incubated for 1 minute at room 37°C and the reaction was stopped by adding 0.5 ml of 15% formaldehyde, followed by the addition of 1.5 ml of Sorbo reagent [21]. The absorbance was taken at 460 nm. One Rhodanese Unit (RU) is defined as the amount of the enzyme that will convert one micro-mole (1 µmol) of cyanide to thiocyanate in one minute at 37°C.

Ɛthiocyanate @ 460nm = 1.08M-1cm-1 1.08 = Extinction Coefficient at 460nm.

#### *2.2.4. Protein Determination*

Protein concentration was determined by the method of Bradford [22] using Bovine Serum Albumin (BSA) as the standard.

#### *2.2.5. Enzyme Purification*

#### *(i). Ammonium Sulphate Precipitation*

The supernatant obtained after centrifugation of the crude homogenate was subjected to 70% ammonium sulphate saturation by the slow addition and stirring of solid ammonium sulphate. This was done for one hour with occasional stirring until all the salt had dissolved completely in the supernatant. The mixture was left overnight at 4°C, followed by centrifugation at 1,600 g for 30 min at 10°C. The supernatant was discarded and precipitate was collected and resuspended in a small amount of 0.1 M Phosphate buffer (pH 7.2).

#### *(ii). Desalting by Dialysis*

The ammonium sulphate precipitate was dialysed against 0.1 M Phosphate Buffer (pH 7.2) for about 24 hr. The dialysate was centrifuged at about 1,600 g for 30 min to remove insoluble materials.

#### *2.2.6. Determination of Kinetic Parameters*

The kinetic parameters ($K_m$ and $V_{max}$) of the enzyme were determined by varying concentrations of 250 mM KCN between 0.0125 M and 0.05 M at fixed concentration of 0.2 M $Na_2S_2O_3$. Also, the concentration of 250 mM $Na_2S_2O_3$ was varied between 0.0125 M and 0.05 M at fixed concentration of 0.2 M KCN.

Plots of the reciprocal of initial reaction velocity (1/V) versus reciprocal of the varied substrates 1/[S] at each fixed concentrations of the other substrate were made according to Lineweaver and Burk [23].

#### *2.2.7. 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 [20]. The enzyme was assayed using different buffers and pH: 50 mM of citrate (pH 3-6); phosphate (7-9) and carbonate (pH 10 and 11). The rhodanese activity was assayed as earlier described.

#### *2.2.8. Effect of Temperature on the Enzyme Activity*

The enzyme was assayed at temperatures between 40°C and 100°C. 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 residual enzyme was then assayed routinely.

#### *2.2.9. Substrate Specificity and Kinetics*

Sulphur compounds such as sodium sulfite, sodium meta-bisulphate, ammonium persulphate and 2-mercaptoethanol were used as substrates at 0.25M final concentration in a typical rhodanese assay mixture. The kinetic parameters of the individual substrates were also estimated and compared, relative with constant KCN concentration, using the Line weaver and Burk plot.

## 3. Results and Discussion

Rhodanese activity assay was carried out on both leaves and tuber homogenate of cassava. The result of the activity assay indicated cassava tubers lacked rhodanese enzyme. The crude rhodanese activity for cassava leaves was calculated to be 12.75 RU/ml/min. Anosike and Ugochukwu [17] in their work discovered that rhodanese play a role in detoxification of cyanide in cassava though inhibited by cyanide in the absence of cysteine or thiosulphate. Zidenga and his co-workers [24] did not detect rhodanese activity in cassava roots whereas sufficient activity of the enzyme was detected in its leaves which is in agreement with this current study. Therefore, concentration level of rhodanese cannot be directly related but can be inversely correlated to cyanogenic glycoside levels in these tissues. That explains complete lack activity of rhodanese in the flesh of cassava and its sufficient levels observed in this work. The inverse relationship between rhodanese activity and concentration of cyanogenic glucosides suggest that the enzyme is involved in the cellular control of cyanide concentration. The level of cyanogenic glycosides contained in these tissue as it correlates with differences in overall activity levels of the enzyme rhodanese, obviously show that rhodanese play a role in cyanide detoxification.

*Cassava leaves*

***Table 1.*** *Purification Summary of Crude Rhodanese obtained from Manihot esculenta leaves.*

| Fraction | Volume (ml) | Activity (RU/ml/min) | Total Activity (RU) | Protein (mg/ml) | Total Protein (mg) | Specific Activity (RU/mg) | Yield (%) | Purification Fold |
|---|---|---|---|---|---|---|---|---|
| Crude | 225 | 12.75 | 2868.75 | 25.94 | 58275.5 | 0.492 | 100 | 1 |
| Ammonium Precipitate (80%) | 49 | 39.91 | 145.5 | 196.9 | 965.3 | 2.03 | 68.2 | 4.1 |

### *3.1. Extraction and Partial Purification of Rhodanese*

Cassava leaves homogenate with rhodanese activity was subjected to partial purification by precipitation with 80 % ammonium sulphate. The enzyme preparation used for this study is therefore partially purified rhodanese. Table 1 shows the summary of purification. The yield and purification fold obtained were 68.2 % and 4.1. Some of the unwanted proteins was soluble in 80% ammonium sulphate, hence leaving rhodanese proteins as precipitate at this salt concentration. The purification fold revealed that some of the unwanted proteins were separated from the rhodanese to other soluble phase. Rhodanese is an industrial enzyme required for several processes, as such it does not need to be extremely pure.

Table 2 summarizes the result of residual activity of different sulphur containing substrates in percentage. This substrate specificity study revealed that rhodanese obtained from cassava leaves preferred sodium thiosulpate for its maximal catalytic activity. This result is similar to the result obtained from the work done by Itakorede et al. [25], wherein sodium thiosulphate has 100% specificity, however, 65%, 90% were obtained for ammonium persulphate, and sodium sulfite substrate respectively. Howeverer, the rhodanese was inhibited by 2-mercaptoethanol and sodium metabisulfite in the same study. Cassava leaves rhodanese was not inactivated by any of the substrates owing to 60% activity maintained all the sulphur containing compounds studied. This is an indication that cyanide metabolism and concentration levels is a not a factor in its detoxification but possibly absence of cysteine or thiosulphate, as all the sulphur containing compound that were explored gave an appreciable percent activity (not less than 60%). The substrate specificity study involving the use of other sulphur substrates showed that the rhodanese from the cassava leaves had preference for thiosulphate while it can still use other sources of sulphur for its catalytic activity, which is in line with the studies reported by other researchers [18]. This is a useful property in industrial apllications.

***Table 2.*** *Substrate specificity.*

| Sulphur Compounds | ENZYME ACTIVITY (%) |
|---|---|
| Sodium thiosulphate | 100 |
| 2-Mercaptoethanol | 80.96 |
| Ammonium persulphate | 78.21 |
| Sodium metabisulfite | 70.18 |
| Sodium sulfite | 59.87 |

### *3.2. Kinetic Parameters*

Table 3 shows the summary of estimated kinetic parameters for partially purified rhodanese from cassava leaves. The $k_m$ and $V_{max}$ for KCN as substrate were 18.2 ± 1.0 µM and 13.9 ± 2.1 RU/ min respectively while that obtained for $Na_2S_2O_3$ as substrate were 10.9 ± 0.5 µM and 5.8 ± 1.1 RU/ min respectively. Earlier reports on the $K_m$ value for potassium cyanide were higher than the value obtained in this work. This is an indication that the cassava leaves rhodanese has a higher affinity for thiosulphate as compared with rhodanese from other sources therefore, it requires less substrate to get to $V_{max}$.

***Table 3.*** *Summary of Kinetic Parameters of Partially Purified Rhodanese from Cassava leaves.*

| Substrate | $K_m$ (mM) | $V_{max}$ (RU/ml) | $V_{max}/K_m$ |
|---|---|---|---|
| KCN | 18.2 ± 2.5 | 13.9 ±1.0 | 0.7637 ± 0.2 |
| $Na_2S_2O_3$ | 10.9 ± 0.5 | 5.8 ± 0.3 | 0.5321 ± 0.1 |

The effect substrate concentration was carried out where

the concentration of $Na_2S_2O_3$ was varied and the other substrate KCN was kept constant. Lineweaver-Burk plot of the two experiment for the estimation of kinetic parameters are as shown in Figures 1 and 2.

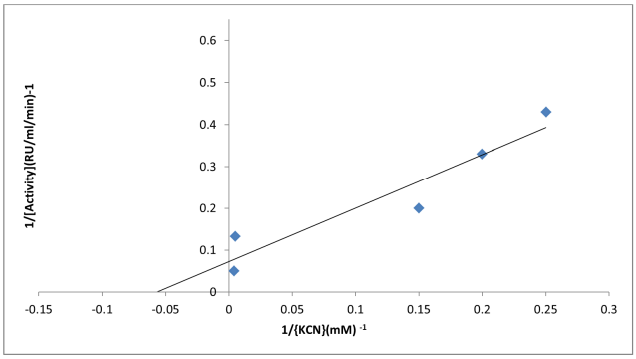


***Figure 1.*** *Lineweaver-Burk plot for varying concentration of potassium cyanide and $Na_2S_2O_3$ kept constant.*

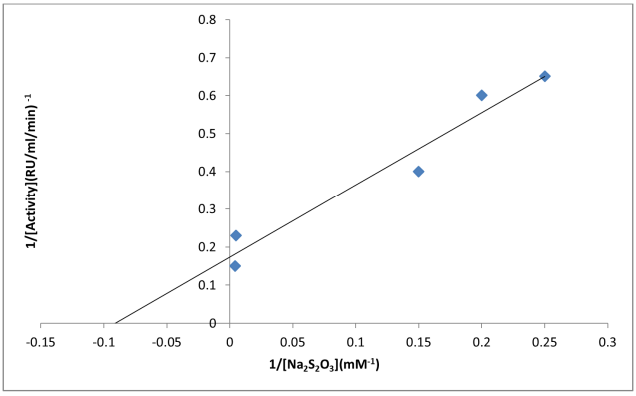


***Figure 2.*** *Line weaver-Burk plot for varying concentration of sodium thiosulphate.*

Figures 1 and 2 shows that rhodanese activity follows the normal Michaelis-Menten pattern. The double reciprocal plots were linear for all values of KCN and thiosulphate.

### *3.3. Effect of pH*

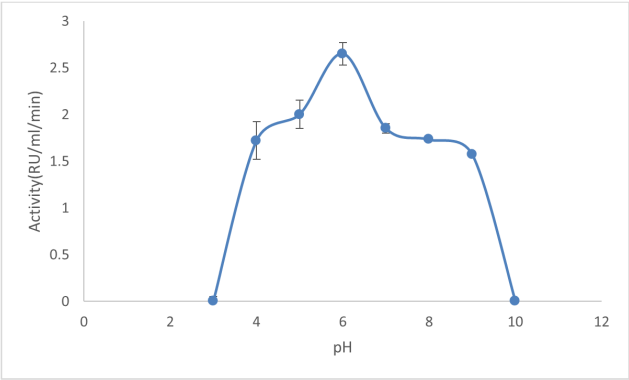


***Figure 3.*** *Effect of pH on Rhodanese obtained from Manihot esculenta leaves.*

Figure 3 revealed an optimum pH of 6.0 where there was highest rhodanese activity. There was progressive decrease in the activity at pH below or higher. The observation was in good agreement with the result obtained for pH in rhodanese activity by other workers. It was also observed that earlier researchers reported alkaline optimum pH for rhodanese from different sources. It is significant, however, that the many earlier workers used only crude preparations. The enzyme obtained in this work tend to be more active in acidic pH, which is an advantage in acidic industrial applications.

### *3.4. Effect of Temperature*

The optimum temperature of the enzyme studied were at 40°C. Earlier reported rhodanese from different sources usually have pH optimum ranging between 50 - 60°C. The rhodanese obtained in this work is active in a wider range of temperatures making it a suitable industrial enzyme suitable for many biotechnological applications.

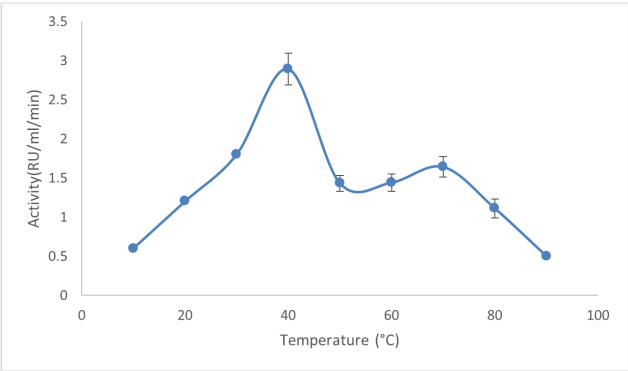


***Figure 4.*** *Effect of Temperature on Enzyme Production.*

## 4. Conclusions and Recommendations

Rhodanese has been shown to be present in the chloroplasts of several plants and its activity has been found to correlate with the labile sulphide concentration in these plants [17]. Rhodanese has also been purified from other plants [26]. This study made an attempt to characterize rhodanese from cassava leaves; the enzyme was partially purified by ammonium sulphate precipitation. The presence of rhodanese was first reported in higher plant tissues other than the leaves by Anosike and Ugochukwu [17] and other workers [18]. Rhodanese activity has been established in the leaves, peels and in the flesh of *Manihot esculenta* [27].

In conclusion, the presence of the enzyme, rhodanese in cassava leaves may due to the exposure of these leaves through ages to high level of cyanogenic substances in the environment. It is apparent that the enzyme might as well be employed for detoxification of the tubers to be free of cyanide. This study is able to affirm various researches that was conducted on the leaves suggesting that it contains rhodanese enzyme which suggests its safety for consumption than the tubers. Also, the information provided by this study can be exploited for understanding cyanide distribution in the tissues of *Manihot esculenta*. The properties exhibited by cassava leaves rhodanese obtained in this work is an indication that the partially purified enzyme will be an

excellent tool in industrial and biotechnological applications.

It is recommended therefore, that more studies should be conducted in the area of detoxification of cassava tuber with cassava leaves rhodanese, since it is the most widely consumed part of cassava plant. The results obtained in this study is indicative that cassava tubers can be fortified with the leaves to obtain a more industrially acceptable cassava tubers suitable for various biotechnological applications.

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