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Research Article

Effects of post-harvest storage on cyanide content and rhodanese activities from bitter cassava (*Manihot utilissima*) tubers

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Abstract

This research investigated the effects of postharvest storage on cyanide content and rhodanese isolated from bitter cassava (*Manihot Utilissima*) tubers. Cyanide content of freshly harvested sample, samples stored for 4 days and samples stored for 8 days were estimated by silver nitrate titration method. Rhodanese was purified using 80% ammonium sulphate precipitate and ion-exchange chromatography on CM-Sephadex C-25. Cyanide content from freshly harvested sample was 345.6 mg HCN/kg while 237.6 mg HCN/kg and 108 mg HCN/kg were obtained for samples stored for 4 days and samples stored for 8 days respectively. Specific activity of rhodanese from freshly harvested sample was 3.411 RU/mg while 5.92 RU/mg and 5.35 RU/mg were obtained for samples stored for 4 days and samples stored for 8 days respectively. The Km values of rhodanese for KCN were 3.18 mM, 2.40 mM, 0.25 mM for freshly harvested sample, samples stored for 4 days and samples stored for 8 days respectively. The optimum temperature from freshly harvested sample and sample stored for 4 days was 70°C while samples stored for 8 days was 50°C. An optimum pH of 4.0 was obtained from the 3 samples. Rhodanese play plausible role in cyanide reduction during the postharvest storage.

Keywords: postharvest storage, cyanide, rhodanese, bitter cassava, ion-exchange chromatography, specific activity, ammonium sulphate precipitate

Abbreviations: $AgNO_3$ - silver nitrate; CM - carboxyl methyl; FHS - freshly harvested sample; HCl - hydrogen chloride; HCN - hydrogen cyanide; KCl - potassium chloride; Km - Michaelis constant; NaOH - sodium hydroxide; $Na_2S_2O_3$ - sodium thiosulphate; RU - rhodanese unit; SS4 - sample stored for four days; SS8 - sample stored for a period of eight days; Vmax - maximum velocity

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Introduction

Bitter cassava (*Manihot utilissima, Euphorbiaceae*) is a commonly grown variety of cassava in west – Africa. It is generally considered bitter cassava due to its undoubtedly high contents (≥ 100 mg HCN/kg) of cyanogenic glycosides (Mckey et al. 2010).

Despite the enormous role of bitter cassava in providing food security (by daunting foraging rodents and pests), consumption of its products with high cyanide levels may cause acute intoxications, aggravate goitre, induce paralytic diseases, loss of consciousness, lung injury and respiratory failure and sometimes death (Burns et al. 2012; Lambri et al. 2013). To avoid dietary cyanide exposure, cassava must undergo efficient processes. In recent years, different processing methods such as peeling, fermentation, boiling, drying, milling and sieving are employed to reduce residual cyanide (Njoku and Obi 2010; Oke et al. 2015).

Post-harvest handling of cassava begins with uprooting (pulling up from the soil) of the fresh tuber, transportation, immediate cooking/ consumption or subject to complex preparatory processing methods into quality products (Opara and Mditshwa 2013). Shortly after harvest, physiological conditions occurring in cassava may include increasing enzyme activities (linamarase, glucanase, proteinase and polyphenol oxidase), root respiration, water loss and attack by pest/microbes on cut surface(s) resulting postharvest deterioration (Iyer et al. 2010).

Deterioration that usually causes spoilage of the cassava tuber in postharvest can be categorized into three factors; mechanical damage, physiological (primary deterioration) and microbial deterioration (secondary deterioration). When tubers are mechanically damaged during or after harvest, they normally respond to healing processes for about four days under temperature between 30 - 40°C and 90 - 100% relative humidity. This curing process has been observed to reduce the onset of microorganism infestation, primary deterioration thereby extending the shelf life of the fresh root (Sánchez et al. 2010; Osunde and Fadeyibi 2011; Opara et al. 2015).

Rhodanese (thiosulphate:cyanidesulphurtransferase EC 2.8.1.1) is an enzyme that catalyses the bio formation of thiocyanate from free cyanide and a sulphur donor compound. It is a ubiquitous enzyme with significant activities in microorganisms, plants, and animals (Ezzi et al. 2003; Adetuyi et al. 2017). Rhodanese from plants has been proposed to play a significant role in cyanide detoxification beside other physiological functions such as providing sulphide for the formation of iron-sulphur centres, maintaining sulphane pool, thioredoxin oxidase activity (Aussignargues et al. 2012). To the best of our knowledge, there is paucity of information on the effects of post-harvest storage on the activity of cyanide degrading enzyme in bitter cassava. The aim of this study was to investigate the cyanide content and the activities of rhodanese in bitter cassava as affected by post-harvest storage at room temperature.

Materials and Methods

Materials. Sodium thiosulphate, potassium cyanide, boric acid, sodium borate, formaldehyde, ferric nitrite, nitric acid were obtained from BDH Chemical Limited, Poole, England. Sodium acetate, blue dextran and bovine serum albumin were purchased from Bio Rad Laboratories Inc., Benicia Ca., USA. CM-Sephadex C-25 was obtained from Pharmacia Fine Chemical, Uppsala, Sweden. Disodium hydrogen phosphate and monosodium dihydrogen phosphate were products of Kermel Reagent Company Limited, Tianjin China.

Sample collection. Freshly harvested bitter cassava tubers of about fifteen month old were collected from a local farm along Irele road, Okitipupa, Ondo State, Nigeria. The bitter cassava was firstly identified by a local farmer who tasted the tuber and clearly scrutinizes the stem and leaves. It was further authenticated at the Biological Science Department, Ondo State University of Science and Technology (OSUSTECH). The bitter cassava samples were collected in a polythene bag and transported to the Laboratory for analysis.

Research grouping. Selected samples used for the research were without blemish. They were thoroughly cleaned and rinsed in a running tap water. The bitter cassava samples were grouped into

three categories (Samples). Stored samples were kept at ambient temperature (30 ± 2°C)

a = represent the freshly harvested samples (FHS)

b = sample stored for four days (SS4).

c = sample stored for a period of eight days (SS8).

Determination of cyanide content. Cyanide content was determined using the method of Yeoh and Oh (1979). Ten g of the cassava sample was homogenized in 200 ml of distilled water using an electric blender, the homogenate was transferred into a clean round bottle flask and left to soak for 4 h. The sample was filtered using a double cheese cloth to collect the filtrate and then steam distilled to obtain 150 ml of distillate in an Erlenmeyer flask containing 50 ml of 1 % alcoholic NaOH. About 2 drops of an indicator (1% dietherzone) was added to the distillate yielding an orange color solution. The mixture obtained was titrated against silver nitrate (0.02 M $AgNO_3$) until pinkish – purple end point was obtained.

Crude enzyme extraction. One hundred g of bitter cassava sample was sliced into bits, homogenized in 300 ml of 0.1 M phosphate buffer (pH = 7.2) using a mechanical blender and filtered using a double cheese cloth. The filtrate was centrifuged at 4000 x g for 30 min to obtain a supernatant which was used as crude enzyme.

Protein concentration determination. Protein concentration determination followed the method of Bradford (1976) using bovine serum albumin as standard protein. The reaction medium includes 10 µl of the enzyme solution and 1000 µl of Bradford reagent. The absorbance was taken at 595 nm and protein concentration was deduced from the standard protein graph.

Rhodanese assay. Rhodanese activity was measured using the method of Lee (1995) as modified by Agboola and Okonji (2004). The reaction medium 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 of volume of 1.0 ml. The mixture was incubated for 60 s at 37°C and the reaction was terminated by adding 0.5 ml of 15% formaldehyde, followed by the addition of 1.5 ml of Sorbo reagent. The absorbance was read at 460 nm. One Rhodanese unit (RU) is expressed as the amount of rhodanese that will convert one micromole (1 µmol) of cyanide ion to thiocyanate in one minute.

Enzyme purification

Ammonium sulphate precipitation and dialysis. The crude enzyme was brought to 80% ammonium sulphate saturation by slow addition of solid ammonium sulphate for a period of one hour with occasional stirring until all added salt had completely dissolved. This was allowed to stand for a period of 12 h at 4°C, it was then centrifuged at 4,000 x g for 30 min. The supernatant was discarded and the pellet (precipitate) was re-suspended in 50 mL of 0.1 M phosphate buffer (pH 7.2) containing 2.5 mM sodium thiosulphate. This was then dialyzed against several changes of 0.1 M solution of phosphate buffer (pH 7.2) containing 2.5 mM sodium thiosulphate. The dialysate was centrifuged for 30 min at 4,000 rpm to remove contaminant and the supernatant collected and assayed for rhodanese activity and protein concentration.

Ion-exchange chromatography on CM-Sephadex C25. CM-Sephadex C25 cation exchanger (resin) was pre-treated firstly by soaking 5 g of the resin in 500 mL of distilled water to swell for 48 h (Agboola and Okonji 2004). The resin was washed severally with 1 M KCl in a series of stirring and decantation processes. It was later washed with equal volumes (1 L) of 0.1 M KOH and 0.1 M HCl, rinsed intermittently with distilled water between each washing for total clearance of traces of KOH and HCl. The resin was gently packed into a 2.5 x 40 cm chromatography column, equilibrated with 0.1 M Phosphate buffer (pH = 7.6). Dialysate (enzyme solution) from the preceding step was layered on the column and washed with 0.1M Phosphate buffer (pH 7.6) to remove unbound protein, followed by elution with 1.0 M NaCl in the same buffer solution. Fractions of 2 ml were collected from the column at a rate of 36 ml per hour. The active fractions eluted from the column were pooled and assayed for rhodanese activity and protein concentration.

Kinetic studies. The kinetic parameters (Km and Vmax) of partially purified rhodanese from the samples were determined by varying concentrations

of KCN between 25 mM and 250 mM at a fixed concentration of 0.25 M $Na_2S_2O_3$, while the concentration of $Na_2S_2O_3$ was varied between 25 mM and 250 mM at a fixed concentration of 0.25 M KCN. The linear equation from a double reciprocal (1/V0 and 1/[S]) plot of Lineweaver-Burk was used to calculate the apparent Km and the Vmax of the enzyme (Lineweaver and Burk 1934; Lee et al., 1995).

Effect of temperature on enzyme activity. Assay to determine the effects of temperature and evaluate the optimum temperature of rhodanese from bitter cassava samples were carried out at different temperatures (30 - 100ºC). The assay mixture was first incubated at an indicated temperature for 10 min before initiating reaction by the addition of 200 µl of enzyme sample which had been equilibrated at the same temperature.

Effect of pH on rhodanese activity. Assay to determine the effects of pH on rhodanese from bitter cassava were carried out using three buffer solutions with different pH values: 50 mM of citrate buffer (pH 3.0 to 5.0), 50 mM phosphate buffer (pH 6.0 to 8.0) and 50 mM borate buffer (pH 9.0 to 10.0). Activity of rhodanese was assayed as described in the enzyme assay section.

Results and Discussion

Results

Table 1. The cyanide content of cassava from tested samples

Cassava samples	Cyanide concentration, mg/kg
Freshly harvested (Sample a)	345.6
After 4 days of storage (Sample b)	236.7
After 8 days of storage (Sample c)	108

Average of 3 titre values for each samples were used to calculate their respective total concentration of cyanide.

Cyanide contents obtained from freshly harvested sample (FHS), sample stored for 4 days (SS4) and sample stored for 8 days (SS8) are presented in Table 1. Cyanide content obtained from FHS was found to be 345.6 mg HCN/kg while 237.6 mg HCN/kg and 108 mg HCN/kg were obtained from SS4 and SS8 respectively.

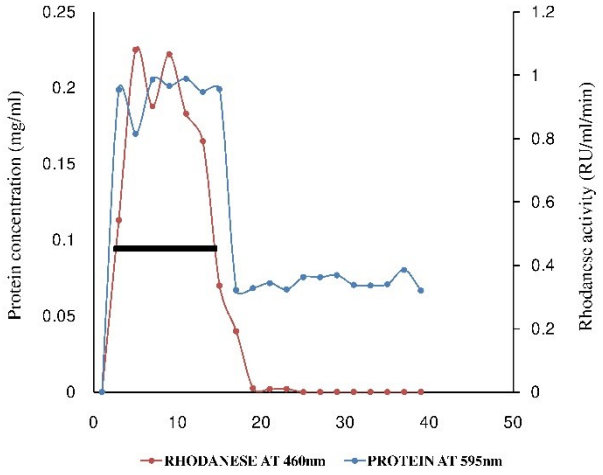


Figure 1a. Elution profile of rhodanese from freshly harvested sample.
▬ - indicate pooled fractions

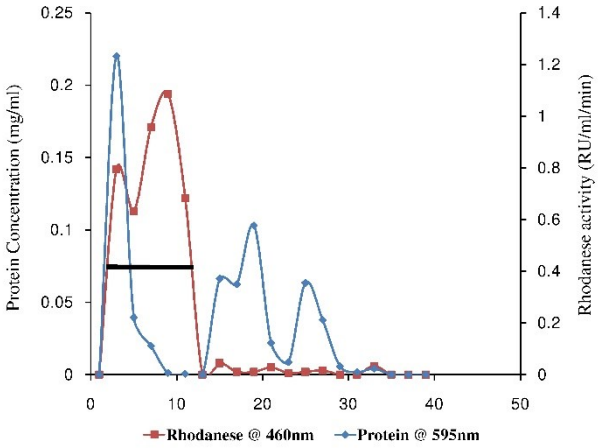


Figure 1b. Elution profile of rhodanese from sample stored for 4 days (SS4)

The results of the purification steps of rhodanese from FHS, SS4 and SS8 are summarized in Table 2. Partially purified rhodanese from FHS, SS4 and SS8 using ammonium sulphate precipitation and ion exchange chromatography had specific activities of 3.41 RU/mg, 5.92 RU/mg and 5.35 RU/mg. The purification folds after purification were 2.37, 5.28 and 5.10 with the recovery yield of 39.11%, 42.55% and 51.00% for FHS, SS4 and SS8 respectively.

Table 2. The purification profile of rhodanese from bitter cassava samples

Purification steps	Samples	Total protein, mg	Total activity, RU	Specific activity, RU/mg	Yield, %	Purification fold
Crude Extract	FHS	1115.37	1610.29	1.44	100.00	1.00
	SS4	914.20	1102.61	1.21	100.00	1.00
	SS8	882.04	927.54	1.05	100.00	1.00
80% Ammonium sulphate precipitation	FHS	525.10	1271.01	2.42	78.93	1.68
	SS4	288.55	938.42	3.25	85.10	2.69
	SS8	263.51	791.32	3.00	85.31	2.86
CM Sephadex C-25 Ion exchange chromatography.	FHS	184.2	629.77	3.41	39.11	2.37
	SS4	79.29	469.19	5.92	42.55	5. 28
	SS8	63.22	338.51	5.35	51.00	5.10

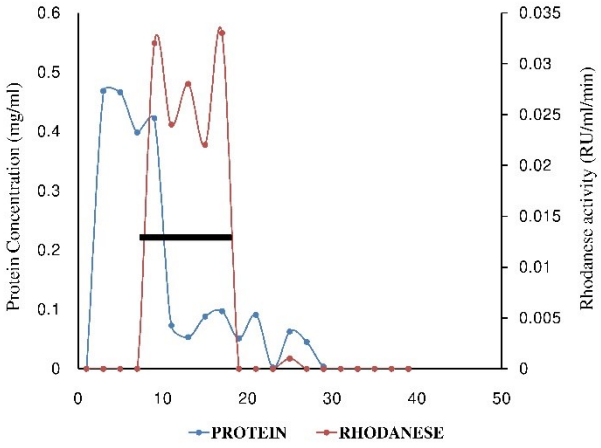


Figure 1c. Elution profile of rhodanese from sample stored for 8 days (SS8)

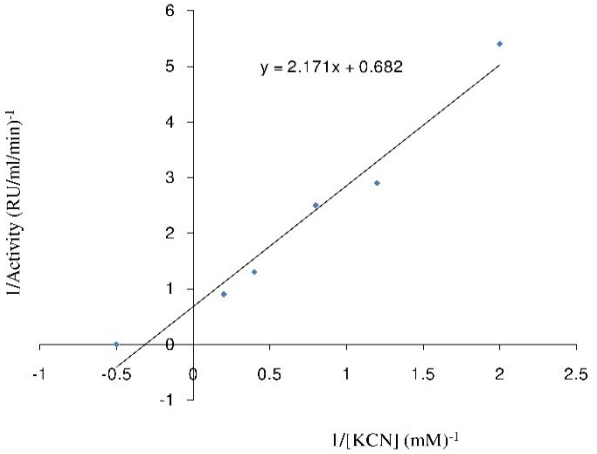


Figure 2a. Double reciprocal plot (Lineweaver-Burk) of rhodanese on varying concentration of KCN at constant $Na_2S_2O_3$ for freshly harvested sample (FHS).

The summary of the kinetic studies of rhodanese from FHS, SS4 and SS8 are shown in Table 3. While Fig. 2 - 4 shows Lineweaver-Burk plots for FHS, SS4 and SS8. From the linear equation of the Lineweaver-Burk plot, the Km values of rhodanese for potassium cyanide (KCN) were 3.18 mM, 2.40 mM, 0.25 mM while Km for sodium thiolsulphate ($Na_2S_2O_3$) were 0.09 mM, 3.48mM, 0.72mM for FHS, SS4 and SS8 respectively. The Vmax of KCN and Na2S2O3 were 1.46 RU/ml/min, 0.68 RU/ml/min, 1.96 RU/ml/min and 1.59 RU/ml/min, 1.02 RU/ml/min, 1.24 RU/ml/min for FHS, SS4 and SS8 respectively.

Table 3. The kinetic parameters (Km and Vmax) of rhodanese for KCN and $Na_2S_2O_3$

Substrate	Samples	K_m, mM	V_{max}, RU/ml/min
KCN	FHS	3.18	1.46
	SS4	2.40	0.68
	SS8	0.25	1.96
$Na_2S_2O_3$	FHS	0.09	1.59
	SS4	3.48	1.02
	SS8	0.72	1.24

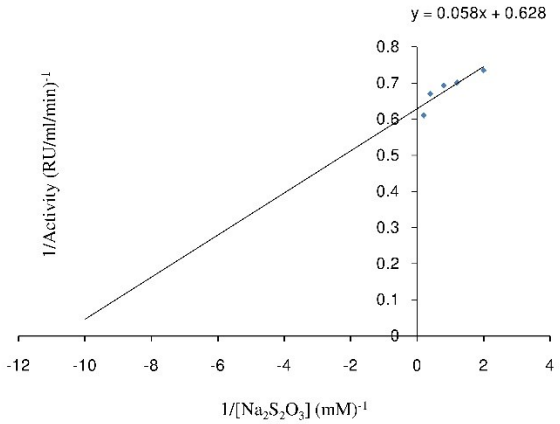


Figure 2b. Double reciprocal plot of rhodanese on varying concentration of $Na_2S_2O_3$ at constant concentration of KCN for freshly harvested sample (FHS)

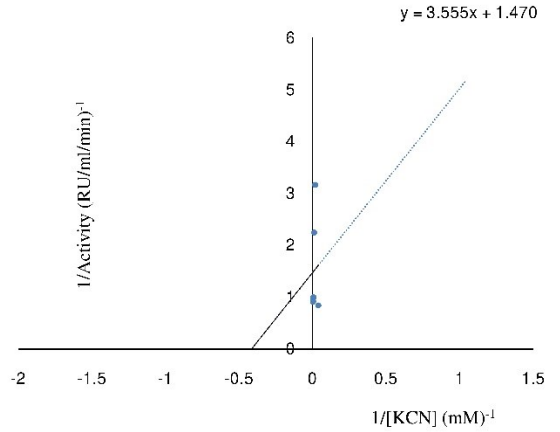


Figure 3a. Lineweaver-Burk plot of rhodanese on varying concentration of KCN at constant $Na_2S_2O_3$ for SS4

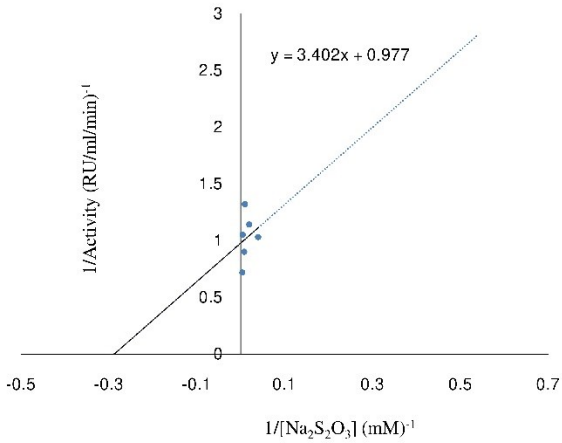


Figure 3b. Lineweaver-Burk plot of rhodanese on varying concentration of $Na_2S_2O_3$ at constant KCN for SS4

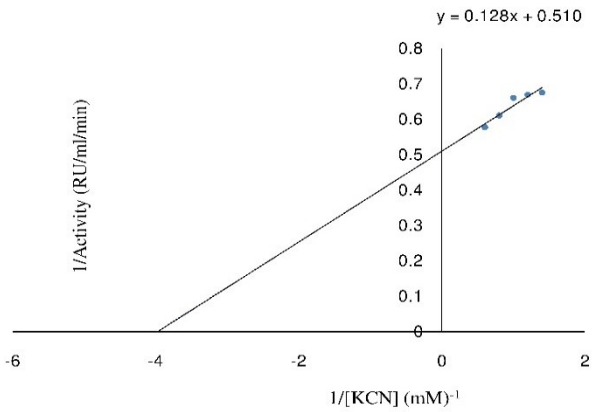


Figure 4a. Lineweaver-Burk plot for varying concentrations of potassium cyanide (KCN) at constant concentration of $Na_2S_2O_3$ for rhodanese from SS8

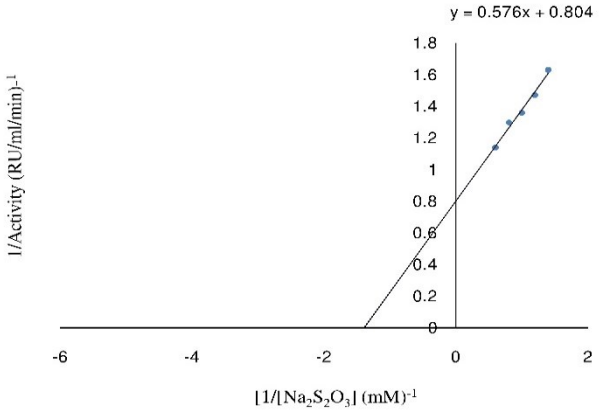


Figure 4b. Lineweaver-Burk plot for varying concentrations of $Na_2S_2O_3$ at constant concentration of KCN for rhodanese from SS8

Rhodanese from FHS, SS4 had an optimum temperature of 70°C while rhodanese from SS8 was 50°C (Fig. 5). The optimum pH of rhodanese from FHS, SS4 and SS8 was 4.0 (Fig. 6).

Discussion

The reduction in cyanide contents with days is presumably due to activity of endogenous enzyme that is responsible for cyanogenic glycosides metabolisms. The two proposed routes of cyanide degradation in plants are β cyanoalanine – synthase and rhodanese pathway that convert HCN into thiocyanate (Zagrobelny et al. 2004; Papenbrock et al. 2011).

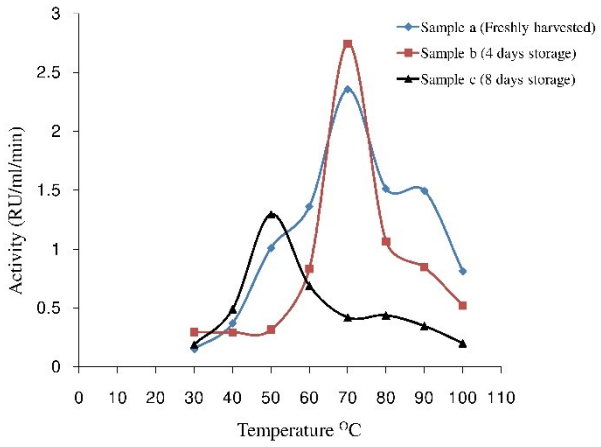


Figure 5. Effects of Temperature on rhodanese isolated from bitter cassava samples

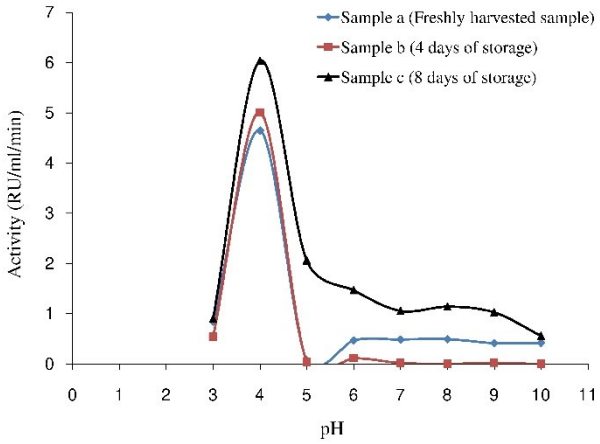


Figure 6. Effects of pH on rhodanese isolated from Bitter cassava (*Manihot utilissima*) samples.

The specific activities of rhodanese isolated from stored samples (SS4 and SS8) are higher than specific activity obtained from freshly harvested sample (Table 2). Under physiological conditions, tubers continue to respire after harvest resulting in diverse metabolic processes. The increasing activity may be due to activities of other enzyme(s) that requires rhodanese for their metabolic reactions. Besides its primary role in cyanide detoxification, secondary roles of rhodanese include biosynthesis of thiamine, selenium metabolisms, maintenance of sulphane pool and supplying sulphide to containing iron-sulphur centers (Papenbrock et al. 2011).

The specific activities obtained in this research are similar to specific activities of rhodanese isolated from other plant sources. Adetuyi et al. (2017), obtained specific activity of 5.07 RU/mg rhodanese from malted maize. Specific activity of 5.4 RU/mg was reported for rhodanese from *Momordica charantia* (Ehigie et al. 2015). Rhodanese isolated from *Citrus paradise*, *Carica papaya* and Citrus *sinensis* had specific activities of 4.09 RU/mg, 6.24 RU/mg and 4.21 RU/mg respectively (Okonji and Agboola 2014).

The Michaelis constant (Km) is the measures of affinity of an enzyme for its substrate. Enzyme activity with small Km values exhibit maximal catalytic efficiency (Okonji et al. 2017).

The lower Km values of rhodanese for KCN from SS4 and SS8 compared to Km value from FHS (Table 3) possibly indicate high affinity of rhodanese for cyanide hence the observed reduction of cyanide content during postharvest storage. The lower Km value of rhodanese for $Na_2S_2O_3$ from freshly harvested sample compared to stored samples (SS4 and SS8), KCN from FHS showed high affinity of rhodanese for $Na_2S_2O_3$ in a freshly harvested sample. This corroborates with findings that rhodanese plays secondary role in maintaining sulphur pool for sulphur energy metabolism. Cipollone et al. (2007) reported the contribution of rhodanese in the recovery of the native architecture of reconstituted iron-sulphur protein(s) by mobilizing sulphur for the formation of iron-sulphur clusters.

Aussignargues et al. (2012) also reported the activity of rhodanese in supplying sulphur to key enzymes involved in sulphur energy metabolism. Other reported roles of rhodanese in sulphur metabolism include its role in L-cysteine metabolism (Most and Papenbrock 2015); construction of iron – sulphur centres in proteins (Ugulava et al. 2000) and thioredoxin metabolism (Nandi et al. 2000).

The high optimum temperature (Fig. 5) from this research could presumably be due to adaptive attributes of cassava to ambient temperature. The enzyme might have been conditions to maintained its 3D structure and function at the temperature. The optimum temperature of purified rhodanese from the leaves of *Mormodica charantia* was found to be

60°C (Ehigie et al. 2015). Optimum temperature for both the seed and the mesocarp rhodanese from snake tomato was 50°C (Okonji et al. 2017). Rhodanese from the *hepatopanceas* of *Limicolaria flammea* had an optimum temperature of 50°C (Okonji et al. 2015).

The low optimum pH of 4.0 obtained in this study may be due to factors such as toleration of bitter cassava (*Manihot Utilissima*) to soil with low pH, presence of high concentration of cyanogenic glycosides and production of organic acids by lactic acid bacteria which constitute the dominant microflora in cassava after harvest (Islam et al. 1980; Kobawila et al. 2005; Oghenejoboh 2012). Several optimum pH values between 6.0 and 11.0 for rhodanese from different sources have been reported. Okonji et al. (2008) obtained an optimum pH as low as 6.0 for giant fresh water prawn. Optimum pH of 8.0 and 7.0 were obtained for rhodanese from seed and mesocarp of snake tomatoes respectively (Okonji et al. 2017).

Conclusions

From this research, postharvest contribute significantly to the reduction of cyanide content in bitter cultivar of cassava. One possible mechanism of reduction could presumably be due to the increase activity of rhodanese as postharvest progressed. Beside cyanide degradation, rhodanese from this study preferentially mobilize sulphane-sulphur.

With the explicit role postharvest storage in cyanide reduction of bitter cassava tuber, further study should be carried out to ascertain the nutritional quality and anti-nutrient status of bitter cassava during postharvest storage.

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