

## Full Length Research Paper

# Catalase activity of cassava (*Manihot esculenta*) plant under African cassava mosaic virus infection in Cape coast, Ghana

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African cassava mosaic virus has caused an immersed low yield of the cassava crop. The virus impacts stress on the cellular metabolism of the plant producing a lot of reactive oxygen species and increases the expression of the antioxidant enzymes. The activity of catalase as a response to oxidative stress was investigated in this research. Cassava leaf extract infected with the African cassava mosaic virus and uninfected were prepared and used as crude catalase extract. The total protein was determined by Biuret method and activity of the crude catalase was compared using hydrogen peroxide as substrate. Infected leaf extract had  $141.02 \pm 3.536$  mg/mL protein, apparent Michealis constant ( $K_{mapp}$ ) of  $26.7 \mu\text{M}$  and maximum rate of reaction ( $V_{max}$ ) of  $54.50 \mu\text{M}/\text{min}$  compared to the uninfected leaf extract with  $75.04 \pm 0.560$  mg/mL protein,  $K_{mapp}$  of  $39.61 \mu\text{M}$  and  $V_{max}$  of  $143.06 \mu\text{M}/\text{min}$ . The activation energy of the infected extract was  $0.1578$  J/mol compared to  $0.2181$  J/mol obtained for the uninfected extract. Activity of the crude catalase in the viral infected leaf extract from the study was higher than that in the uninfected one and confirms the response to the stressful condition imposed by the viral infection.

**Key words:** Cassava leaf extract, African cassava mosaic virus (ACMV), reactive oxygen species (ROS), catalase, hydrogen peroxide,  $K_{mapp}$ ,  $V_{max}$ .

## INTRODUCTION

Cassava (*Manihot esculenta*) is a woody shrub of the Euphorbiaceae that can grow for more than two years. Although, cassava has some features that allow it to cope with stress better than other crops, such as high stomatal

sensitivity to environmental humidity, deep rooting capacities and quick recovery after stress under these conditions, productivity is sub-optimal and unstable (El-Sharkawy, 2012). Cassava has therefore become African

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**Abbreviations:** CBSD, Cassava brown streak disease; ACMV, African cassava mosaic virus; ROS, reactive oxygen species;  $\text{H}_2\text{O}_2$ , hydrogen peroxide; BSA, bovine serum albumin.

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continent's most important food security crop and a major source of carbohydrates. In Ghana, cultivation of cassava is a reserve crop against lean periods, because it can survive all weather conditions and it is the last resort of food, when there is severe shortage of other food crops. Several items of foods are processed from cassava, examples are: fufu, gari, cassava dough, starch, bread, biscuits and cassava flour (konkonte).

Cassava productivity is threatened by abiotic factors such as temperature, soil conditions, pH and rainfall and also biotic factors such as bacterial and viral diseases, as well as arthropod pests. The most important diseases include cassava bacterial blight, *Xanthomonas campestris manihoti*, African cassava mosaic disease and cassava brown streak disease (CBSD), the two viral diseases (Parkes et al., 2013) both of which their infection are thought to have risen from viruses already present in the crop through propagation (Njock, 2014). In Africa, this is extremely detrimental to the production of subsistence farming. Cassava plants infected with the African cassava mosaic virus (ACMV) express a range of symptoms which depend on the virus strain, environmental conditions, and the sensitivity of the cassava host. African cassava mosaic virus (ACMV) is a single strand DNA pathogenic virus transmitted by the whitefly *Bemisia tabaci*. ACMV causes either a mosaic appearance to plant leaves or chlorosis and a loss of chlorophyll. In *M. esculenta* (cassava), a highly valuable African food crop, the virus is the only known plant virus that causes cassava mosaic disease (CMD). When plant is under a stress condition, like a viral infection, there is increased rate of photosynthesis and respiration. These results in the release of reactive oxygen species (ROS) mainly hydrogen peroxide ( $H_2O_2$ ) which causes membrane damage that eventually leads to cell death which is fatal to plant life. When there is an imbalance (either by abiotic or biotic factors) in the cellular compartment between ROS production and antioxidant defense, there is dramatic physiological challenges which results in oxidative stress. The generation of  $H_2O_2$  seems to be mediated by a membrane-bound NADPH oxidase complex in plants.  $H_2O_2$  may further activate defense genes such as proteinase inhibitors as it diffuses to adjacent cells. Environmental stresses that cause oxidative stress include drought, salt stress, extreme temperatures, air pollution, oxidant-forming herbicides, heavy metals, wounding, UV light, and high intensity light conditions that stimulate photo-inhibition of photosynthesis.

During oxidative stress, the plant protects itself against reactive oxygen species by antioxidant enzymes as well as a wide array of non-enzymatic antioxidants (Das and Roychoudhury, 2014). Superoxide dismutase is considered to be the first line of defense against ROS and is the major  $O_2^-$  scavenger. Its enzymatic action results in  $H_2O_2$  and  $O_2$  formation. The  $H_2O_2$  produced is then scavenged by catalase and several classes of peroxidases. Peroxidases decompose  $H_2O_2$  by the oxidation of phenolic compounds. The catalase is a tetrameric heme protein,

found in peroxisomes, cytosol and mitochondria (Krych et al., 2014). This enzyme has hyperoxidase activity which catalyzes the dismutation of hydrogen peroxide into water and oxygen. Catalase is nearly ubiquitous among organisms that can grow in the presence of oxygen. The major function of catalase within cells is to prevent the accumulation of toxic levels of hydrogen peroxide formed as a by-product of metabolic processes - primarily that of the electron transport pathway.

The objectives of the study was to compare protein levels in virus infected and uninfected leaf extracts of cassava plants and also to determine the kinetic parameters:  $K_{mapp}$  and  $V_{max}$  and the activation energy of crude catalase extract from the different portions of the cassava leaf under study.

## MATERIALS AND METHODS

### Collection and handling of cassava leaves

Leaves of the infected cassava ("Obotan": local name for the type of cassava used) showing the symptoms of mosaic virus and the non-mosaic patterns were collected from the University of Cape Coast, Department of Agriculture School Farm. The plants were specific for CMD from the Agricultural Department and free of co-infection. The two leaf samples were collected from different positions of the branches on the same plants with the disease. They were transported to the laboratory in labeled rubber bags where they were washed with water and stored in the refrigerator prior to use.

### Homogenization of cassava leaves

The vein of the leaf was cut out and 3.0 g of each leaf sample weighed on a balance. With a chilled mortar and pestle, the weighed leaves were macerated in 20 mL each of 0.1 M universal buffer containing 0.1 M citric acid- $H_2O$ , 0.1 M potassium phosphate monobasic, 0.1 M sodium tetraborate- $10H_2O$ , 0.1 M Tris and 0.1 M potassium chloride adjusted to pH 7. The homogenate was then centrifuged at 12000 rpm for 30 min at 0°C. The supernatant was decanted and aliquoted into Eppendoff tubes. The aliquots were stored frozen prior to use as the crude enzyme extract.

### Determination of protein in the crude enzyme extract of the leaf

The concentration of protein in the crude extract was determined from an absorbance-concentration calibration graph using standard bovine serum albumin (BSA) solutions prepared from a stock solution of 15 mg/mL.

### The kinetics of crude catalase activity

Preliminary investigations were made to determine the appropriate reaction times as well as the amount of the crude enzyme extract to be used for the kinetic studies. From the investigations, a reaction time of 5 min and crude enzyme extract of 0.3 mL were used for the subsequent investigations. A set of eight clean test tubes were obtained and into each tube, 1.7 mL of the universal buffer at pH of 7 and 0.3 mL of the crude enzyme extract was added. A water-bath was set and tubes were incubated at 90°C for 5 min to denature the enzyme present. The test tubes were then removed after the 5 min

**Table 1.** Crude protein determination in leaf extracts.

Leaf extract	Protein concentration (mgmL <sup>-1</sup> )
Obotan with virus	141.02 ± 3.536
Obotan without virus	75.04 ± 0.560

**Table 2.** Change in absorbance of H<sub>2</sub>O<sub>2</sub> for extracts.

H <sub>2</sub> O <sub>2</sub> (mg/mL)	Absorbance	
	ΔAb H <sub>2</sub> O <sub>2</sub> Used for reaction (uninfected virus extract)	Δ Ab H <sub>2</sub> O <sub>2</sub> Used for reaction (virus infected extract)
0.04	0.0679	0.0714
0.08	0.1298	0.1324
0.12	0.1681	0.1707
0.16	0.2785	0.2789
0.20	0.3061	0.3053
0.24	0.3446	0.3428
0.28	0.4126	0.4107
0.32	0.4853	0.4832

and allowed to cool to room temperature. Serial dilutions of the 0.3% H<sub>2</sub>O<sub>2</sub> were done. 2 mL of each dilution was measured into labelled test tubes and the absorbance read at a wavelength of 240 nm. Into another set of eight test tubes were added, 2 mL of the H<sub>2</sub>O<sub>2</sub> serial dilutions and 1.7 mL of the buffer. 0.3 mL of the thawed crude extract (frozen activated extract) was added into each tube at time interval of 5 min at room temperature. At the 5<sup>th</sup> minute, the absorbance of each reaction was recorded at 240 nm. The absorbance of the second set of test tubes (activated; enzymes present) was subtracted from the first set of test tubes (inactivated; enzymes denatured) to remove the effects of the color of the extract due to H<sub>2</sub>O<sub>2</sub>. The final absorbance obtained was then subtracted from that of the initial H<sub>2</sub>O<sub>2</sub> concentration to obtain the change in absorbance.

#### ***K<sub>m</sub>* and *V<sub>max</sub>* of crude catalase activity of leaf extracts**

The change in absorbance obtained was converted to concentration (μM) using Beer-Lambert's law,  $A = \epsilon Cl$ . The concentration obtained was divided by the reaction time (5 min) to obtain the rate of the reaction. Specific activity was also determined by dividing the rate by the total protein concentration of each crude enzyme extract. The reciprocal of both the substrate concentration and the rate of activity were determined and Lineweaver-Burk plot was plotted from which the *K<sub>m</sub>* and *V<sub>max</sub>* were determined.

#### ***Activation energy of the crude catalase activity in the leaf extracts***

The effect of temperature on the activity of the crude catalase extracts were investigated within a temperature range of 30 to 60°C. A serial dilution (0.2 mg/mL) of the stock solution of 0.3% H<sub>2</sub>O<sub>2</sub> was prepared and 2 mL was added to 1.7 mL of the universal buffer at pH of 7 and placed in a set, water bath at 30°C. When the mixture has attained the temperature of the water bath (30°C), 0.3 mL of the crude extract was added and allowed to react for 5 min after which the absorbance was read at 240 nm. The procedure above was repeated for a denatured enzyme extract (heated at

90°C for 5 min). The absorbance of the first test tube (activated) was subtracted from the second test tube (inactivated) to remove the effect of the color of the extract on the reaction. The procedure as described above was also carried at temperatures 40, 50 and 60°C, respectively.

The rate and specific activity of the enzymatic activity was determined by converting the absorbance to molar concentration using Beer-Lambert's law and the log of the rate found. The Arrhenius relation was plotted from which the activation energy of the crude catalase extract was extrapolated.

## **RESULTS**

### **Protein determination in crude leaf extracts**

To determine the protein content in the Obotan leaf extracts, the absorbance readings were plotted and the concentration of the leaf extracts were extrapolated from the standard BSA absorbance plot. Values of concentration with their means were determined (±the standard deviation) as shown in Table 1.

### **Crude catalase activity in leaf extracts**

The absorbance of H<sub>2</sub>O<sub>2</sub> left after reaction was subtracted from initial absorbance to attain the change in absorbance of H<sub>2</sub>O<sub>2</sub> used for the reaction and the result shown below for the crude extracts (Table 2).

### **Kinetics of crude catalase activity**

The absorbance was converted to molar concentration using the Beer Lambert's Law ( $A = \epsilon Cl$ ). The rate, specific

**Table 3.** Kmapp and Vmax for crude catalase activity.

Leaf extract	Kmapp ( $\mu\text{M}$ )	Vmax ( $\mu\text{M}/\text{min}$ )
Obotan with ACMV infection	26.70	54.50
Obotan without viral infection	39.61	143.06

**Table 4.** Arrhenius data of crude catalase extracts.

Temperature $1/T \times 10^{-3} \text{ K}^{-1}$	Leaves extract with virus infection Log R	Leaves extract without infection Log R
3.299	0.771	1.217
3.193	0.719	1.221
3.095	0.742	1.262
3.002	0.749	1.302

**Table 5.** Activation energy of crude catalase activity in leaf extracts.

Leaf extract	Activation energy (J/mol)
Obotan with ACMV infection	0.1578
Obotan without viral infection	0.2181

activity and the inverse of the rate were determined for each leaf extract. A double reciprocal (Lineweaver-Burk's) was plotted and the kinetic parameters determined from the plot. The line of equation for Obotan with virus and without virus were  $y = 4.899x + 0.1835$  and  $y = 2.7689x + 0.0699$ , respectively. These equations were compared with the equation of line for Lineweaver-Burk plot. The Kmapp and Vmax for both extracts were then determined as shown in Table 3. The change in absorbance used for the reaction was converted to molar concentration using the Beer Lambert's Law ( $A = \epsilon CI$ ). The rate, specific activity and the logarithm of the rate were determined for each leaf extract. The temperature in degree Celsius was also converted to degree Kelvin and the inverse determined. Arrhenius plot was done and the activation energy determined from the slope by comparing the line of equation with log (Tables 4 and 5).

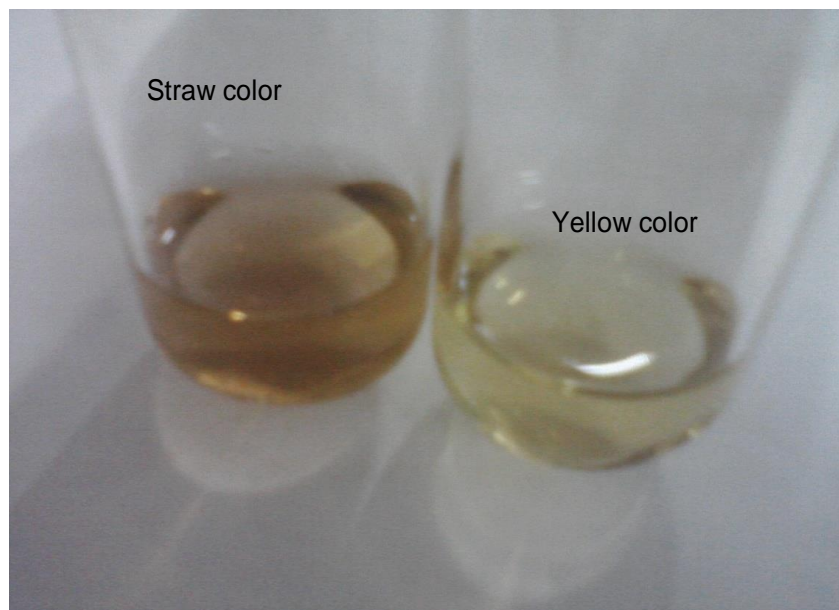
$$R = -E_a(1/T) + Y_o.$$

## DISCUSSION

Plants under stressful conditions tend to respond in order to survive these conditions homeostatically. Hydrogen peroxide is a primary metabolites produced excessively in green plants when under stress. At lower concentrations, they tend to protect the plant from pest. Therefore, to investigate the activity of catalase, the protein in the two

leaf extract was determined by the biuret method and the results obtained were compared. The protein concentration determined were  $141.02 \pm 3.536 \text{ mg/mL}$  in the extract of the viral infected leaves and  $75.04 \pm 0.560 \text{ mg/mL}$  in the healthy leaves. Studies by Coldebella et al. (2013) showed that cassava healthy leaves are rich in protein but difference in the protein concentration as observed in this study was because of increased viral protein on the infected leaves as compared to the uninfected one. Again the plant under study was infected by the virus hence lowering its protein content in leaves. The kinetic parameters of the crude enzyme extract from the viral infected and the healthy leaves were also studied. The solutions of the hydrogen peroxide reacted with the extract from the viral infected leaves changed from yellow to straw-like color after the reaction time (Figure 1) whilst that with the extract from the healthy leaves remained yellow. The change in color as observed was as a result of activity of the reactive oxygen species (ROS-  $\text{H}_2\text{O}_2$  and other free radicals) on the plant's pigment which could be investigated further. This might be due to a unique reaction which was not present in the non-infected extract since heat treatment was able to stabilize the color change. Hence, another preparation of the hydrogen peroxide and the crude extract from the viral infected leaves were made where the enzyme extract was heated at  $90^\circ\text{C}$  to denature the enzyme before adding to the hydrogen peroxide solution. The color change was still observed showing it was not as a result of enzymatic activity. The absorbance of the activity of the crude extract was then deducted from the denatured extract activity in order to obtain the activity on the hydrogen peroxide although it was not that efficient in completely removing the color effect.

From the results obtained, the Line-Weaver Burk plot that was made for each crude enzyme extract had a positive slope from which the Kmapp and the Vmax were determined. Also, the Kmapp for the viral infected leaf extract and the healthy leaf extracts were 26.7 and 39.61  $\mu\text{M}$ ,



**Figure 1.** Effect of hydrogen peroxide on the cassava leaf pigment.

respectively. The relative difference in activity measured showed a higher affinity for the hydrogen peroxide in the viral infected leaf compared to the healthy leaf extracts. This observation explains why there is increased expression of catalase in the stressed plant and this is very necessary to rapidly hydrolyze the elevated levels of the hydrogen peroxide so as to prevent any harmful effect and also to promote food security of cassava among the people of Cape Coast, Ghana since it is their most staple food. On the other hand, the  $V_{max}$  of 143.06  $\mu\text{M}/\text{min}$  as against 54.50  $\mu\text{M}/\text{min}$  was observed without viral leaf extract and viral leaf extract, respectively. A higher  $V_{max}$  shows a faster activity and the one with the lower  $K_{mapp}$  was expected to have a higher  $V_{max}$  as a result of the higher affinity. The deviation could have resulted due to the superimposing color of the reaction mixture.

The activation energy of 0.1578 J/mol was determined for the extract with the viral infection as compared to 0.2181 J/mol for the healthy leaf extracts. The result shows a higher activity at higher temperatures for the viral infected extract. The viral infected extract will be less sensitive to temperature than the viral uninfected extract. Since, the Arrhenius plot was a plot of log of rate against  $1/T$  ( $\text{K}^{-1}$ ), the peroxide concentration did not have a direct influence on the activation energy as it did on the  $V_{max}$  in the Lineweaver-Burk plot.

## Conclusion

From the results and the observations made from the study, it has been shown that there is a significant increase

in the protein concentration in the ACMV infected cassava leaves as compared to that in the uninfected one. It was also observed that a lot of hydrogen peroxide were produced in the stressed leaf and hence the increased expression of the catalase activity. This was evident by the lower  $K_{mapp}$  and the lower activation energy which were measured for the ACMV infected cassava leaf extract. It can therefore be concluded that there is an increased level of catalase expression in the cassava leaf infected with the Africa cassava mosaic virus (ACMV) relative to the uninfected one. The study investigators recommend that the plant DNA should be extracted, purified and quantified to ascertain the inducement of the catalase activity. Also, further other antioxidant enzymes such as superoxide dismutase and peroxidase should also be determined to investigate the infected plants. Lastly, the effect of the hydrogen peroxide on the plant pigment after extraction in the viral infected plants should be investigated.

## Conflict of interests

The authors did not declare any conflict of interest.

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