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Inhibitory Studies of Peroxidase from Infected African Eggplant (*Solanum aethiopicum*) Fruit

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ABSTRACT: The effect of two parameters: inhibitors (potassium cyanide, salicylic acid and urea) and heat on peroxidase from the infected *Solanum aethiopicum* grown within the Nsukka Area of Enugu State, Nigeria was studied. The inhibitory and heat studies were carried out using standard procedures. The thermal stability of the enzyme was monitored using thermodynamic parameters after heating the enzyme over a temperature range of 30-70°C for 90 min. Potassium cyanide and salicylic acid and urea inhibited the enzyme in a concentration dependent manner. The inhibition of the enzyme by salicylic acid was an indication that the enzyme is a heme-protein. A high half-life of 64.78 mins was observed when the enzyme was heated at 50 °C for 90 mins. The free energy change (ΔG) values of 55.142, 58.731, 60.472, 60.227 and 64.296 KJ/mol and entropy (ΔS) values of -196.45, -179.07, -178.49, -195.66 and -195.43 were obtained. Similarly, low Z-value was obtained. The thermal stability results implied that high amount of energy was required to initiate the enzyme denaturation at the temperatures studied.

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Solanum aethiopicum, (garden egg) is known as igba in Yoruba, gauta in Hausa and añara in Igbo and belongs to the family of *Solanaceae* (Agoreyo *et al*., 2012). It is a good source of minerals and vitamins (Soydam-Aydin *et al*., 2015). Because of its importance, there is increase in its cultivation in Nigeria. *Solanum spp* is one of the major agricultural crops cultivated in Nsukka which hosts University of Nigeria. It is better consumed when fresh and green. It is susceptible to spoilage due to pest infestations thereby leading to loss and generation of agro-waste. Plants tend to produce more antioxidant enzymes while trying to overcome or tolerate the harsh conditions resulting from pest infestation (Baby and Jini 2010). The utilization of garden egg is hampered by pest infestation which induces cellular damage and oxidative effects on it (Aydin *et al*., 2013; Munns, 2011. Clarke *et al*. (2013) opined that antioxidant scavenging system (enzymatic) plays important role in preventing or alleviating the stress and subsequent damage. Plants secrete some intrinsic compounds which exert some physiological functions to ameliorate unfavorable conditions. Salicylic acid occurs naturally in plants and had been implicated in plant defense actions against infection by various pathogens (Hayat and Ahmad, 2007). Salicyclic acid could contribute to maintaining cellular redox homeostasis through the regulation of antioxidant enzymes activity (Slaymaker *et al*., 2002), such as peroxidase. It is generated internally by the metabolism of cyanogenic glycoside and bye-product of ethylene biosynthesis as reported by Siegien and Bogatek, (2006) and functions as signaling molecule and inhibitor of various intracellular enzymes involved in many important metabolic pathways (Siegien and Bogatek, 2006).Peroxidases (EC 1. 11. 1. 7) are house-keeping enzymes responsible for the detoxification of hydrogen peroxidase in a living cell that catalyze the oxidation of phenolic compounds. They are the most studied protein in plant, since they have been found in every major plant division (Tayefi-Nasrabadi and Asadpour, 2008).

Peroxidase has found applications in diverse bio-transformational purposes (Husain and Husain, 2008; Chaurasia *et al*., 2013; Osuji *et al*., 2014) and has been extracted from various higher plants (Osuji *et al*., 2014; Zia *et al*., 2011; Mikami*et al*., 2013; Bania and Mahata, 2012, Bari *et al*., 2013) and *Solanum aethiopicum* fruit (Omeje and Eze, 2018). There is a dearth of information in literature on the inhibitory patterns of these inhibitors and chaotropic agent involved in plant regulatory responses and defense. Hence, the objectives of this research was to evaluate the inhibitory patterns of these inhibitors and

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thermodynamics of peroxidase involved in plant defense.

MATERIALS AND METHODS

Solanum aethiopicum was harvested from a farm in Edem-ani, Nsukka L. G. A. of Enugu state, Nigeria in May, 2018. o-dianisidine was product of Sigma (England). All other chemicals and reagents were of analytical grade. Salicylic acid and urea were products of BDH chemicals (England) and potassium cyanide was a product of Analyticals (Carlo erba, Milano).

Extraction of peroxidase: Peroxidase was extracted from *Solanum aethiopicum* according to the method of Eze *et al.* (2010). Protein content was estimated according to the method described by Lowry *et al*. (1951), Bovine serum albumin was used as standard protein.

Enzyme assay Peroxidase activity was assayed using the method of Eze (2012) by measuring the change in absorbance as it oxidizes the o-dianisidine on addition of hydrogen peroxide. The assay mixture (3 ml) contained 2ml of 100 mM phosphate buffer (pH 7.0), 0.3 ml of substrate (o-dianisidine), and 0.4ml of crude enzyme. The reaction was initiated by adding 0.3 ml of hydrogen peroxide. The peroxidase activity was monitored by change in absorbance (470 nm) due to the oxidation of o-dianisidine using Jenway 6305 UV/VIS spectrophotometer.

Inhibitory Studies: Various concentrations (1, 5 and 10 mM) of the inhibitors were prepared in 100 mM phosphate buffer pH 7.0. Each reaction mixture (3.0 ml) contained 1.50 ml buffer, 0.4 ml of the enzyme solution, 0.3 ml of guaiacol and 0.5 ml of the different inhibitor solutions and 0. 3 ml of H_2O_2. The mixtures were incubated for 30 min at pH 6.5 and 40 °C while the control was assayed without including any inhibitor solutions. The residual activity was determined as described previously.

*Thermal treatment :*Kinetic parameters were estimated as described by Eze *et al*. (2010). The kinetics of thermal inactivation of peroxidase from the fruit of *Solanum aethiopicum* were determined based on inactivation experiments after enzyme incubation for a period of 90 min in a temperature controlled water bath (Gallenkamp, England). The enzyme solution was placed in a pre-heated tube at temperatures of 30, 40, 50, 60 and 70 °C, and aliquots were withdrawn using a micropipette at 10, 20, 30, 60 and 90 mins respectively. Afterwards, the samples were immediately cooled on ice to stop the thermal inactivation process. The residual enzyme activity was then measured as described in the peroxidase assay section. The stability of the enzyme was expressed as percentage residual enzyme activity.

Calculations: Calculations were carried out as outlined by Marangoni (2003), using data derived from some parameters of the enzyme**;**

$$t\frac{1}{2} = ln\ 2/kd \qquad (1)$$

The decimal reduction time *(D-value*), which is the time required for 90 % reduction in the concentration or activity of the reacting species (Maragoni, 2003), was calculated using the first order rate constant (*kd*).

$$\text{D-value} = 2.303/Kd \qquad (2)$$

Similarly, Z-value, the temperature increase required to cause 90 % reduction in the *D*-value was calculated from a plot of log $_{10}$ D versus temperature. The change in enthalpy, entropy and free energy of denaturation was calculated directly from the following equations

$$\Delta H = Ea - RT \qquad (3)$$

$$\Delta G = -RT\ln K_h/ K_b \qquad (4)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \qquad (5)$$

Ea is the activation energy of the reaction; T is temperature (K); R is the gas constant (8.314 $Jmol^{-1}K^{-1}$), k_h is the Planck's constant (11.04×10^{-36} $Jmin^{-1}$), k_b = Boltzmann constant ($1.38\times10^{-23}JK^{-1}$).

RESULTS AND DISCUSSION

The percentage residual activity and ln percentage activity of peroxidase from the infected fruit of *Solanum aethiopicum* is shown in Figures 1 and 2. It was observed that more than 60 % peroxidase activity was retained after incubating the enzyme for 30 mins. Further heating beyond 30 mins showed a decrease in the residual enzyme activity as shown in Fig. 1. Thermodynamic stability is a central requirement for protein function and one goal of protein engineering is improvement of stability for biotechnological applications (Zieske *et al*., 2016). When proteins are placed in an environment with temperature higher than their optimum condition, they lose their biological activity (Petsko and Ringe, 2004).The results (Table 1) indicated that INGe peroxidase retained more than 50% of its activity after its incubation at 70 °C for 90 mins. This could be attributed to the adjustment that is been carried out in the protein modification as the enzyme tries to control and get the cells rid of hydrogen peroxide. The $t\frac{1}{2}$ data obtained from INGe peroxidase was similar to that obtained for sorghum

peroxidase (Eze and Chilaka, 2007). It could be observed from the results that the half-life of the purified peroxidase reduced significantly at 343 K. The data obtained were higher than the t ½ values obtained by Eze, (2012) for African oil bean seed peroxidase. The results indicated an increase in enzyme inactivation with temperature (Eze and Chilaka, 2007).The INGeperoxidase showed *ΔG* values of 55.142, 58.731, 60.472, 60.227 and 64.296 KJ/mol at 303, 313, 323, 333 and 343 K (Table 1). Change in entropy of inactivation (*ΔS)* values of -196.45, -179.07, -178.49, -195.66 and -195.43 were obtained for partially purified INGeperoxidase at 303, 313, 323, 333 and 343 K as shown in Table 1. The obtained D-values showed that successive increase as the temperature was increasing in all the enzymes assayed. Eze*et al*. (2010) reported that corresponding decrease with increasing temperature is indicative of fast enzyme inactivation at higher temperature. The *D*-values were increasing as the temperature (K) was increasing this implies faster enzyme inactivation at higher temperature. Also, the Z-value was extrapolated from the plot of Log. D vs temperature (Fig. 3).

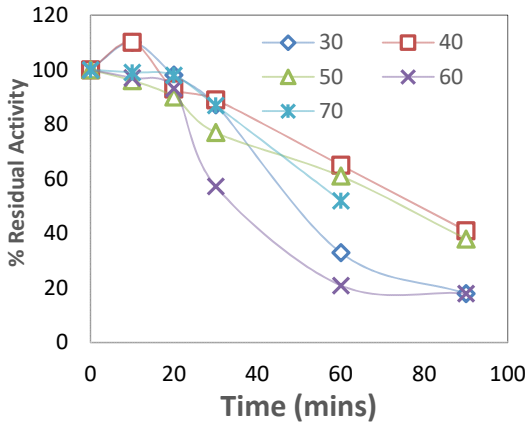

Fig. 1: % Residual activity of INGE peroxidase

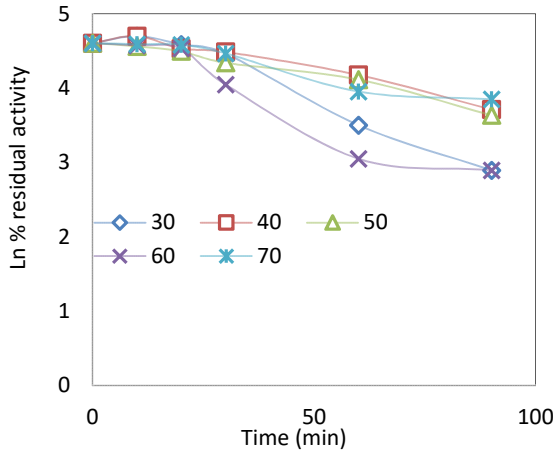

Fig. 2: Ln of percentage residual activity of INGE peroxidase

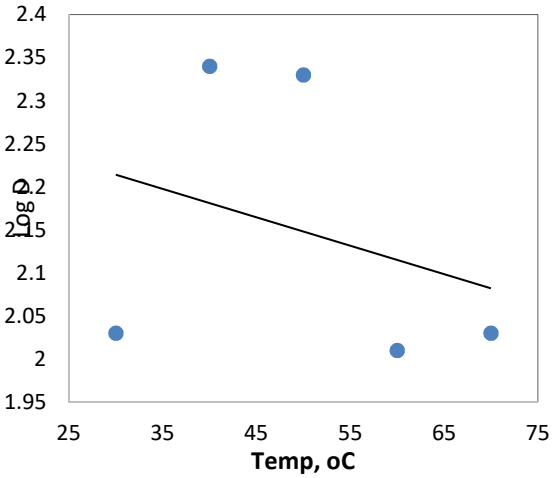

Fig. 3: Z-value of purified peroxidase from Infected NsukkaGarden Egg

Free energy (*ΔG*) is defined as the maximum amount of energy available to a thermodynamic process that can be converted into useful work (Hames and Hooper, 2011). The change in free energy (*ΔG*) can be used to predict the direction of a reaction at constant temperature and pressure (Harvey and Ferrier, 2011). If *ΔG* has a negative value, there is a net loss of energy, and the reaction occurs spontaneously, if *ΔG* has a positive value, there is a net gain of energy, and the reaction does not go spontaneously, if *ΔG* value is equal to zero, the reactants are in equilibrium (Hames and Hooper, 2011). Enzyme molecule with high or positive *ΔG* is considered to be stable (Eze, 2012). This indicates that values obtained for INGe peroxidase were stable at 313 and 323 K, which suggested that the peroxidase would not disintegrate non-spontaneously, hence, the enzyme could be said to be stable. Previous study on *Solanum aethiopicum* peroxidase showed that it is thermally stable at temperature range of 50 – 60 °C (Omeje *et al*., 2017). Eze *et al*. (2010) described *Ea* as the parameter that evaluates the rate of enzyme inactivation at any temperature. Also, activation energy of 67.67 Kjmol^{-1}(16.17 Kcal) was obtained by Nadege *et al*. (2009). Similarly, activation energy of -2.983 Kcal/mol was obtained for sorghum peroxidase (Eze and Chilaka, 2007). Enthalpy change of inactivation for sorghum peroxidase was -2937.83kcal/mol^{-1} (Eze and Chilaka, 2007). The activation energy (*Ea*) for peach fruit peroxidase was reported as 7.97 Kcal/mol, *Ea* values of peach fruit peroxidase increased with increasing concentration of sucrose (Neves an d Lourenco, 1998). Activation energy of potato peroxidase was obtained as 27.114 KJ/mol(Yu *et al*., 2010). A large value of *Ea* is indicative that more energy is required to inactivate the enzyme (Eze*et al*., 2010). They further stated that high *Ea* is indication that the process is strongly temperature dependent, at lower temperature; this rate

becomes insignificant (Eze*et al*., 2010). Entropy (S) is a measure of degree of randomness or disorder of a system; it increases (ΔS is positive) when the system becomes more disordered (Hames and Hooper, 2011). The rate of any chemical reaction is a function of the temperature and energy difference between the reactants and the activation energy Ea (Arcus *et al*., 2016).

Eze (2012) stated that a negative ΔS show there is an aggregation process in which a few inter or intra molecular bonds are formed. The results of this study are higher than those obtained for oil bean seed peroxidase (Eze, 2012). This implies a decrease rate of inactivation at higher temperature.High Z-value indicates that high amount of energy was required to initiate denaturation and vice versa (Eze *et al*., 2010), low Z-values was obtained for the enzyme. High Z-values indicates more sensitivity to the duration of heat treatment, while low Z-value implies more sensitivity to increase in temperature (Tayefi-Nasrabadi and Asadpour, 2008). Hence, low Z-value of the peroxidase implied that the peroxidase from infected fruit of Nsukka garden egg was more sensitive to temperature increase.

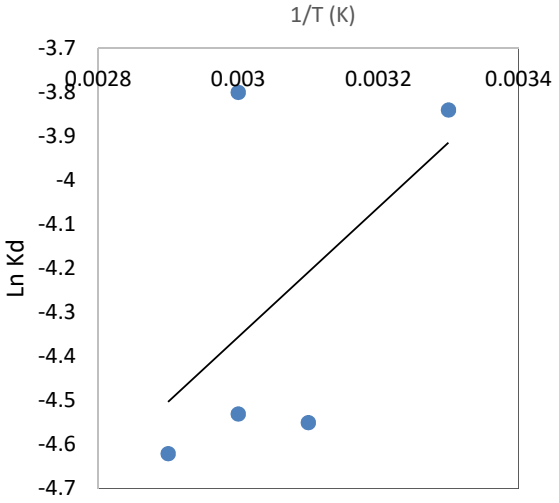

Fig. 4: Arrhenius plot for the determination of thermal inactivation of INGeperoxidase

Table 1: Half-life and Bioenergetic Parameters for Purified INGe peroxidase

T (ºK)	*Kd*	$t_{1/2}$(min)	D-value	ΔH(KJ/mol)	Ea (KJ/mol)	ΔG(KJ/mol)	ΔS(KJ/mol)
30 (303)	0.0214	32.39	107.59	-4383.30	-1864.16	55.142	-196.45
40 (313)	0.0105	66.01	219.29	2680.34	-78.06	58.731	-179.07
50 (323)	0.0107	64.78	215.19	2819.69	-134.27	60.472	-178.49
60 (333)	0.0223	31.08	103.25	-4928.03	-2159.47	60.227	-195.66
70 (343)	0.0098	70.72	234.95	-2737.64	114.06	64.296	-195.43

Z-Value = 0.0033

The effects of salicylic acid and potassium cyanide on the activity of INGe peroxidase were assayed at different concentrations of 1, 5 and 10 mM. The primary plots obtained showed concentration dependent effect of the inhibitors. Potassium cyanide revealed to have inhibited peroxidase from INGe uncompetitive (Table 2 and 3). Similarly, when pyrogallol and o-dianisidine were used as substrates, the inhibition pattern showed by peroxidase was mixed inhibition, while INGe peroxidase showed an uncompetitive inhibitory pattern when ascorbate served as the substrate as shown in Table 2.The competitive inhibition indicated that the inhibitor could have similar structure that enable it mimic the actual substrate and compete with it at the active site. The uncompetitive inhibitors could have bound to the enzyme-substrate complexes, thereby forming a bulky complex that may not have the right shape, structure and orientation to get fix to the enzyme active site which reduces the rate of product formation and regeneration of the free enzyme (Copeland, 2000).Bari *et al*. (2013) observed that potassium cyanide strongly inhibited peroxidase in their study. Similar study by Neves and Lourenco (1998) on purified peach fruit peroxidase revealed a strong potassium cyanide inhibition of the enzyme activity. Nadler *et al.* (1986) identified potassium cyanide as a hemoprotein inhibitor, which indicates that the peroxidase purified in this study contains hemoprotein. On the other hand, *S. cyaneus* peroxidase, another hemoprotein peroxidase was inhibited by potassium cyanide (Pandey and Dwivedi, 2011). Also, Pandey and Dwivedi (2011) reported the activation of purified peroxidase from *L. leucocephala* by salicylic acid that inhibited the enzyme at high concentrations.In contrast, salicylic acid exhibited an inhibitory effect on all the peroxidase studied. Similarly, Srivastava and Dwivedi, (2000) reported a complete inhibition of guaiacol peroxidase. The sensitivity of peroxidase purified in this study to salicylic acid implicates its role in the plant defense system (Pandey and Dwivedi, 2011). Peroxidase has been reported to be inhibited by salicylic acid (Durner and Klessig, 1995). *In vitro* inhibitory study showed that salicylic acid inhibited peroxidase from the infected fruit of *Solanumspp*, this could suggest that there is reduction in the biosynthesis of salicylic acid *in vivo*.Also, the inhibitory pattern of salicylic acid, a specific inhibitor for peroxidase was studied. INGe peroxidase showed noncompetitive inhibitory patterns when guaiacol was used as the substrate (Table 3). INGe showed mixed patterns when pyrogallol was used as substrate. When

ascorbate and o-dianisidine were used as substrate to determine the inhibitory pattern in the peroxidase, the results revealed mixed and competitive inhibition for INGe respectively. Furthermore, the inhibitory strength of potassium cyanide was assayed on INGe peroxidase. The *Ki* value of -10.82 was obtained for INGe peroxidase when o-dianisidine was used as substrate. The inhibitor showed strongest affinity on INGe peroxidase (-10.82) as indicated in Table 4.

Table 2: Inhibition Patterns of Potassium Cyanide (KCN) on INGe Peroxidase

Enzyme	Inhibition type/ Pattern	Substrate
INGe	Uncompetitive	Guaiacol
INGe	mixed	Pyrogallol
INGe	Uncompetitive	Ascorbate
INGe	mixed	o-dianisidine

Table 3: Inhibition Patterns of Salicylic acid on INGe Peroxidase

Enzyme	Inhibition type/ Pattern	Substrate
INGe	Uncompetitive	Guaiacol
INGe	mixed	Pyrogallol
INGe	Mixed	Ascorbate
INGe	Uncompetitive	o-dianisidine

Table 4: Inhibitory Kinetic Parameters (*Ki, Vmax*) of Potassium Cyanide on Peroxidase Activity

Enzyme	*Ki (Inhibitory Constant)*	*Vmax*
INGe	-10.82	0.90 (o-dianisidine)
INGe	-10.00	0.60 (Guaiacol)
INGe	18.10	0.68 (Ascorbate)
INGe	-3.10	0.73 (pyrogallol)

Table 5: Inhibitory Kinetic Parameters (*Ki,Vmax*) of Salicylic acid on Peroxidase Activity

Enzyme	*Ki (Inhibitory Constant)*	*Vmax*
INGe	-14.80	0.10 (o-dianisidine
INGe	-2.00	0.31 (pyrogallol)
INGe	-0.13	0.10 (Ascorbate)
INGe	-7.10	0.36 (Guaiacol

Subsequently, guaiacol was used as substrate to assay for the inhibitory strength of potassium cyanide on the peroxidase. INGe peroxidase yielded *Ki* values of -10.00. Also, INGe peroxidase was incubated with potassium cyanide using ascorbate as the substrate. The *Ki* value of 18.10 was obtained. The INGe peroxidase was inhibited by salicylic acid yielding the *Ki* value -14.80 (Table 5). It was observed that salicylic acid inhibited INGe peroxidase more (*Ki* = -14.80) when o-dianisidine served as the substrate. The inhibitory constant -2.00 was obtained for INGe peroxidase when pyrogallol was used as substrate. Also, INGe peroxidase showed inhibitory constant of -0.13, when ascorbate was used as substrate. The INGe peroxidase showed *Ki* value of -7.10 when guaiacol was used as the substrate. The result indicated that salicylic acid inhibited INGe peroxidase strongly.

Conclusion: In vitro inhibitory study showed that salicylic acid inhibited peroxidase from the infected fruit of *Solanumaethiopicum*, this could suggest that there is reduction in the biosynthesis of salicylic acid *in vivo* during pathogenic attacks or infestation. The thermal stability results implied that high amount of energy was required to initiate the enzyme denaturation at the temperature ranges studied.

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