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Kinetics and thermodynamics of heat inactivation of 'oha' (*Pterocarpus mildraedii*) polyphenol oxidase activity

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Abstract
Studies were carried out to determine the effect of heat treatment on the activity of 'oha' (Pterocarpus mildraedii) polyphenol oxidase over a range of temperatures, 50 to 80^oC. Heat denaturation of this enzyme, calculated as loss in activity, could be narrated as a simple first-order reaction where k-values ranged from 0.005 to 0.279min^{-1}. There was a decrease in D-values and a corresponding increase in k-values, as temperature increases, implying a rapid inactivation of polyphenol oxidase at elevated temperatures. Results indicate that polyphenol oxidase is a somewhat heat-stable enzyme with Z and Ea values of 16.2^oC and 135.97 kJ/mol, respectively. The range of Gibbs free energy ΔG values was from 91.43 to 87.55 kJ/mol at 323 – 353^oK. The thermodynamic evaluation result showed that the oxidation reactions were: not spontaneous ($\Delta G > 0$), slightly endothermic ($\Delta H > 0$) and not reversible ($\Delta S > 0$). The elevated figures observed for enthalpy variation imply that a large quantity of energy was needed to kick-start denaturation, presumably resulting from the molecular structure of polyphenol oxidase.

Key Words: *'Oha'(Pterocarpus mildbraedii), Polyphenol oxidase (PPO).*

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I. Introduction

Many researchers have stated that the availability of endogenous enzyme residues in raw as well as processed vegetable products may result in quality loss during storage[1,2].

'Oha' (*Pterocarpus mildbraedii*) tree, of Leguminosae family[3], grows predominantly in the eastern zone of Nigeria where the leaves are locally known as 'oha' leaves. The leaves are amongst the vegetables that are widely consumed especially by the Ibo tribe in Nigeria[4]. Customarily, the fresh and immature leaves of this plant are used as vegetables for soup making[5]. According to Uchegbu *et al.* [4], the leaves of 'oha' are rich in alkaloids, flavonoids and tannins. Hence, 'oha' may possess analgesic, antispasmodic, anti-oxidant, anti-inflammatory and diuretic properties [6]. In addition, the leaves of 'oha' tree possess large amounts of vitamins A, B^1, B^2, B^5, B^6 and C, and sufficient quantities of minerals [7]. Notwithstanding the nutritional, sensory, storage and market potentials of this vegetable, it is still limited by the occurrence of enzymatic browning caused by an enzyme, polyphenol oxidase (PPO)[8].

As noted by Diko *et al.* [9], horticultural products sustain both qualitative and quantitative losses brought about by phenolic compound oxidation or action of unwanted enzymes such as PPO which impair these products market value. Patterns of thermal inactivation of PPO enzymes presents a kinetics model of the first order, in which the time for inactivation vary with the product under study. Out of many works on PPO inactivation by heat, just a handful incorporated the Arrhenius calculation, the kinetic and thermodynamic properties of PPO inactivation by heat, and these works were mainly done on apple, apricot, mango and yam[10,11,12,13,14,15].

Little or no work has been done on PPO inactivation in 'oha' leaves. Therefore, this work covers more elaborate study of the kinetics and thermodynamic properties of 'oha' (*P. mildbraedii*) PPO enzyme inactivation by heat.

II. Materials and Methods

2.1 ***Laboratory resources***

'Oha' leaves that were used for this study were gotten from Owerri main market in fresh condition. PPOo substrate, catechol, was procured from Mikenis Reagent Company. Triton X-100 and polyvinylpyrrolidone were obtained from Sigma Chemical Company (Germany). All other reagents used were of analytical grade.

2.2 ***Extraction and partial purification of PPO***

The procedure outlined by Galeazzi *et al.* [16] was used, although with some alterations. Prior to extraction, the 'oha' leaves were kept at 4^oC for 24 hours to achieve temperature homogeneity in the entire leaves. After the pre-cooling, the precooled leaves were blended, at 4^oC, with solution, containing 100ml of 0.2M phosphate buffer (pH 6.8), 1.5% polyvinylpyrrolidone (PVP) and 0.5% Triton X-100, for 30 sec, in a blender. Centrifugation of the generated blend was done, in a refrigerator, at 4^oC for 15 min at 12000rpm (GenFuge 24D centrifuge). The supernatant contains the crude enzyme. In order to prevent enzyme denaturation by oxidation products from phenols, polyvinylpyrrolidone(PVP), which removes phenols, was included in the extraction solution during isolation stage [17]. Crude enzyme extract activity was evaluated using 1ml 0.05M catechol, 0.05ml of enzyme extract and 0.2M phosphate buffer (pH 6.8) in a final volume of 5ml. The assay was performed at a temperature of 30^oC. Under the environment of the determination, one unit (U) of PPO activity is that produced by a quantity of enzyme that increases absorbance by $0.001min^{-1}$[8].

The entire fractional purification process, comprising precipitation with acetone and dialysis, were done at 4^oC. Precipitation with acetone was accomplished by pouring in 2 volumes of cold acetone followed by recentrifugation at 12000rpm (25000g) for 15 min at 4^oC. After recentrifugation, one volume of 0.2M phosphate buffer (pH 6.8) was used to re-extract the precipitate. In the dialysis stage, the solution that was re-extracted, carrying the crude enzyme that was precipitated with acetone, was transferred into a dialysis sack (Sigma Dialysis 'Sacks' D6066-25EA) for dialysis. Dialysis was effected in 0.2M phosphate buffer (pH 6.8) at 4^oC for 12hrs with two replacements of dialysis broth. Evaluation of the enzymatic activity of the dialyzed extract was accomplished in a reaction mixture containing 1ml 0.05M catechol, 0.05ml of dialyzed extract and 0.2M phosphate buffer (pH 6.8) making up a final volume of 5ml. The assay was performed at a temperature of 30^oC. Under the environment of the determination, one unit (U) of PPO activity is that produced by a quantity of enzyme that increases absorbance by $0.001min^{-1}$[8].

2.3 ***Enzyme assay***

Activity of PPO was evaluated by observing the increase in absorbace at 420nm (Techmel&Techmel, UV-VIS spectrophotometer model 752 D). Dialyzed enzyme solution was diluted 1/5 (v/v) with phosphate buffer. Contained in the reaction mixture are1ml 0.05M catechol, varying quantities of enzyme extract and 0.2M phosphate buffer (pH 6.8) making up a final volume of 5ml. The evaluation was carried out at a temperature of 30^oC. The slope of the linear activity curve was used to calculate the reaction rate. Under the environment of the determination, one unit (U) of PPO activity is that produced by a quantity of enzyme that increases absorbance by $0.001min^{-1}$[8].

2.4 ***Thermal inactivation of enzyme extracts***

Thermal inactivation of PPO in the extracted enzyme was studied at the designated temperatures, 50, 60, 70 and 80^oC, for various times, 2, 4, 6, 8, 10 and 12 min, respectively, using screw-cap tubes following the method of Sophie *et al.* [19] but with slight alteration. To forestall temperature lag prior to addition of 0.5 ml aliquot of enzyme solution, preheating of the screw-cap tubes to the designated temperatures was carried out. After attaining designated temperatures, the tubes with the enzyme samples were pulled out of the water bath and, instantly, conveyed to an ice bath to stop the process of heat inactivation. Following the ice bath cooling of the sample, is the determination of the residual activity of the heat treated enzyme involving the mixing of 0.1 mL of heat treated enzyme with 0.9 mL of cathecol and measuring spectrophotometrically using a wavelength of 420 nm (Techmel & Techmel, UV-VIS spectrophotometer model 752 D). These determinations were replicated for each experiment. Under normal test atmosphere, the residual activity of enzyme at 30^oC, witnessed in both conditions, were reported as zero-time percentage activity control of unheated enzyme sample. An untreated enzyme sample served as blank (A_0). Sample's activity was compared with activity of unheated samples to determine their residual percentage activity. The gradient of the curve of natural logarithm (In) of A/A_0 vs. time graph was used to determine the first order inactivation constant (k_D).

2.5 ***Computation of enzyme parameters***

For the studied enzyme, the reliance of temperature on the rate constant of the reaction was the basis for the formulation of the Arrhenius equation[20].

$\ln [A_t/A_0] = -kt$ (Eq. 1)

With A_t being the enzyme residual activity at time t, and

A_0 being the enzyme initial activity;

k is the rate constant of the reaction (min^{-1}) for a particular environment. The slope of regression line of $\ln [A_t/A_0]$ against time was presented as k.

The time needed to decrease the amount of compounds being evaluated by 90% of its original figure is described as D-value. Eqn. 2 was used to calculate the decimal reduction time (D) as stated by Stumbo[21].

$D = 2.303/k$ (Eq. 2)

The temperature rise needed to cause a 90% decrease in decimal reduction time (D) is denoted as Z (^oC) value, and traces the equation:

$\text{Log } [D_1/D_2] = [T_2-T_1]/Z_T$ (Eq. 3)

The lower and higher temperatures, in ^oC or ^oK, are denoted as T_1 and T_2, respectively.

The values of decimal reduction time (D) at the lower and upper temperature, in minutes, are presented as D_1 and D_2.

From the linear graph of log D and temperature (T), the Z-values were extrapolated. A relationship, consistent with Arrhenius equation[20] was established between the treatment temperature and denaturation function rate constant:

$k = Ae^{(-Ea/RT)}$ (Eq. 4)

Eq. 4 can be transposed to:

$\ln k = \ln A – Ea/R \times T$ (Eq. 5)

With

The value of rate constant of the reaction being denoted as k,

Arrhenius constant is denoted as A;

The activation energy (the energy needed to realize the inactivation) is denoted as Ea,

The gas constant (8.31 J mol^{-1} K^{-1}) is denoted as R,

The absolute temperature, in Kelvin, is denoted as T.

The gradient of the linear regression of 'ln k' vs. 'inverse of absolute temperature (1/T)'was used to obtain the activation energy along with the ordinate intercepts that is consistent with 'ln A' [22,23].

The various thermodynamic properties were calculated from the values of Arrhenius constant and activation energy[24], gotten. The thermodynamic properties calculated are changes in enthalpy (ΔH), entropy (ΔS) and Gibb's free energy (ΔG) as represented with the following statements [25]:

$\Delta H^{\#} = Ea – RT$ (Eq. 6)

$\Delta S^{\#} = R (\ln A – \ln K_B/ hp – \ln T)$ (Eq. 7)

$\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#}$ (Eq. 8)

With

Boltzmann constant (1.38×10^{-23} J/K) denoted as K_B,

Planck constant (6.626 × 10-34 J.s) denoted as Hp, and

Absolute temperature denoted as T.

III. Result and Discussion

It has been reported that enzyme activity typically rises with rise in temperature till a threshold is attained where enzyme inactivation is initiated, followed by a decline in activity [26,27]. Therefore, a maximum enzyme activity, commonly, denoted as optimum temperature that varies from one enzyme to another is witnessed[28]. The activity of polyphenol oxidase was maximal at a temperature of 30^oC [12].

Presented in Table 1 is the heat tolerance profile for this oxidase. At 50^oC, activation of enzyme was observed until after 25 min of heating, with the maximum enzyme activity, 109.3%. Denaturation of enzyme was observed after 20 min of heating at 60^oC (72.09%), after 10 min of heating at 70^oC (86.05%) and after 5 min of heating at 80^oC (67.4%). The unfolding of the tertiary arrangement of protein (enzyme) may be responsible for the fall in percentage residual activity witnessed at elevated temperatures. Notwithstanding that keeping the temperature at 70^oC for 25 min produced a fragmental (51.16%) denaturation, heating at 80^oC for equal time substantially (11.63%) inactivated the enzyme.

Table 1: Heat tolerance profile of polyphenol oxidase from 'oha' (*Pterocarpus mildbraedii* Harms).

Temperature	Mean of relativeactivitity (%) at each process time (min)					
°C	5*	10	15	20	25	30
50	76.75	83.72	102.33	97.68	109.30	76.75
60	102.33	118.61	100.00	72.09	69.77	67.44
70	104.65	86.05	72.09	62.79	51.16	41.86
80	67.44	27.91	11.63	9.30	11.63	-2.33

*Process times in minutes

Table 2: Enzyme parameters for heat inactivation of 'oha'(*Pterocarpus mildbraedii* Harms) polyphenol oxidase at temperature range (50 – 80°C).

Temperature(°C)	Kinetic attributes				
	k (min^{-1})		D (min)	Z (°C)	Ea (kJ/mol)
	value	R^2			
50	0.005	1.000	460.60		
60	0.011	0.548	209.36	16.20	135.97
70	0.110	0.991	20.94		
80	0.279	0.997	8.25		

The linear logarithmic association between activity of PPOo and process time prevailing from 50 – 80°C traced first-order kinetics (Figure 1) which was coherent with patterns determined in previous works in fruits and vegetables [29,30,31]. Premised on this result (Table 2), it is evident that the enzyme was less heat tolerant at elevated temperatures (70 – 80°C) inasmuch as an elevated rate constant is indicative that the enzyme was less heat tolerant[32].

After "ln" conversion, the inactivation rate of PPOo decayed linearly with the reciprocal of temperature (Figure 2). This association was elucidated by the equation: ln k= -16362(1/T) + 45.13 (R^2=0.95), where T depicts absolute temperature (K). Within the range of 50 to 80°C, the value of activation energy (Ea) for heat inactivation of PPOo was computed to be 135.97 kJ/mol (Table 2). This Ea value was high compared to that observed for kiwifruit (33.67kJ/mol[33], rice (23.3 kJ/mol[34] and plantain (18 kJ mol^{-1})[35]; but, however, was low compared to apple (241 – 323 kJ/mol)[36] and whole banana (413 kJ/mol)[37]. Elevated activation energy connotes a higher tolerance of PPO to change in temperature [38,11]. Therefore, the PPO of 'oha' (*Pterocarpus mildbraedii*) has higher tolerance to heat than those of kiwifruit, rice and plantain but with lower tolerance to heat than those of apple and banana.

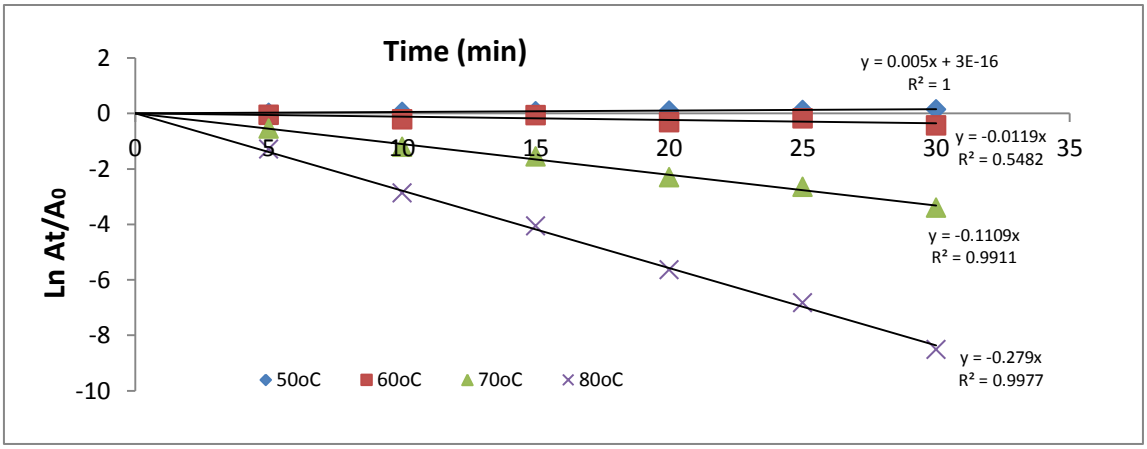

Figure 1: The curves for heat inactivation of polyphenol oxidase from 'oha' (*Pterocarpus mildbraedii*) in phosphate buffer (pH 6.8)in the temperature range 50 – 80°C. A_0 is the initial enzymatic activity and A_t the activity at each holding time.

Computed Z-values for PPOo from 'oha' leaves was 16.2°C within 50 – 80°C (Table 2) which is huge compared to results obtained for fruits that was within a range of 8.5 to 10.1°C [13,39]. Typically, high Z-values are believed to imply elevated tolerance to duration of heat process, and low Z-values imply enhance tolerance to temperature increase[41]. Compositional differences may be accountable for the differences in heat activation kinetics of PPO from different products, which is a reflection of the variety, climatic and agronomic condition in which they were cultivated[11].

D-value is an index that demonstrates the relationship between treatment time and enzyme activity [42]. Kinetic attributes for PPOo inactivation work determined, as contributed by pre-treatment temperature, are

presented in Table 2. As the pre-heating temperature was raised from 50 – 80^{o}C for PPOo (Figure 3), the D-values gotten from Eqn. (2) reduced linearly (R^2= 1) from 460.6 – 8.25 min. D-values from 'oha' were comparatively high (Table 2) relative to the D-value of POD, which is believed to be the most heat tolerant enzyme in vegetables [36]. Broadly, as the structure of an enzyme is becoming big and complicated, the more prone it is to yield to high temperatures[40].

Presented in Table 3 are the thermodynamic attributes for heat inactivation of PPOo from 'oha'. Within a temperature range of 50 – 80^{o}C, the mean values of ΔH, ΔS and ΔG were 133.16 (kJ/mol), 129.2 (J mol^{-1}K^{-1}) and 89.49 (kJ/mol), respectively. Positive values of ΔH show that the oxidation reaction is endothermic. The high value of change gotten for the various treatment temperatures reveals that enzyme experiences a significant structural modification during denaturation. The ΔH value gotten for PPOo was higher than that of potato (98.02 kJ mol^{-1})[43], *Lepista nuda* (13 ± 1 kJ mol^{-1}) and *Hypholoma fasciculare* (36 ± 2 kJ mol^{-1}) [44]. This result implies that PPOo could withstand more heat than *Lepsita nuda* and *Hypholoma fasciculare*, due to the elevated ΔH value for inactivation.

The ΔS value obtained for PPOo was lower than that of potato (145 J mol^{-1} K^{-1}) [43]; therefore, following the transition state development, the aggregate change in disorder of enzyme was higher, leading to a reduction in ΔG value.

The variation in entropy, witnessed, had positive values, implying that substantial levels of aggregation occurred[45]. We also noticed that as temperature increased, the free energy of PPOo decreased. The free energy of inactivation was positive at all temperatures, showing that the oxidation reaction was not spontaneous.

From our findings, it is established that first-order kinetic model may define heat inactivation profile of polyphenol oxidase. The values of D-, Z-, k and the high values gotten for activation energy and enthalpy change implies that initiating denaturation of PPOo will require a great amount of energy, which most possibly, is as a result of its stable molecular structure. This high heat stability should be considered where heat processes are applied in producing products gotten from 'oha' leaves (*Pterocarpus mildbraedii*).

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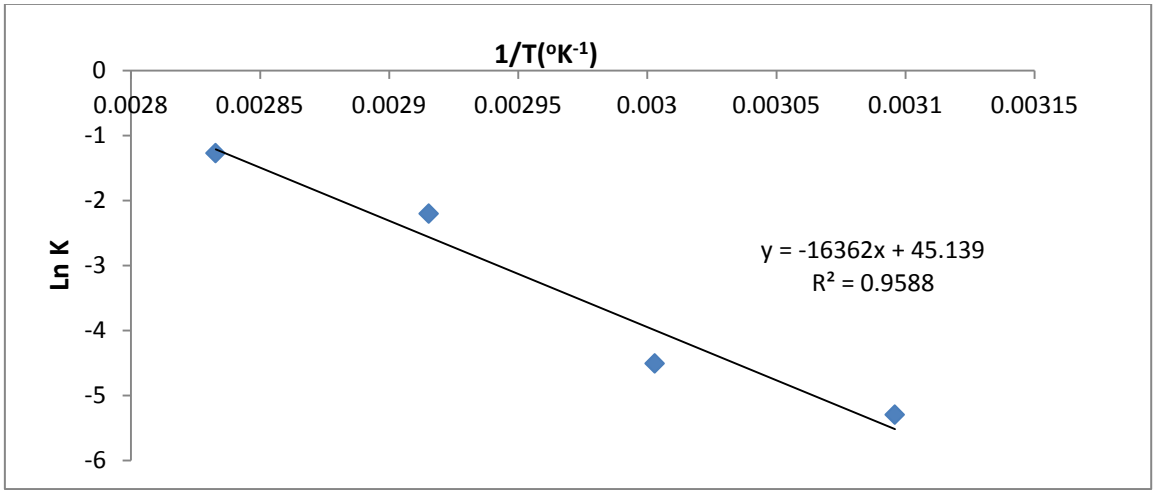

Figure 2: Effect of temperature variation on D-values for inactivation of 'oha' (*Pterocarpus mildbraedii*) polyphenol oxidase activity.

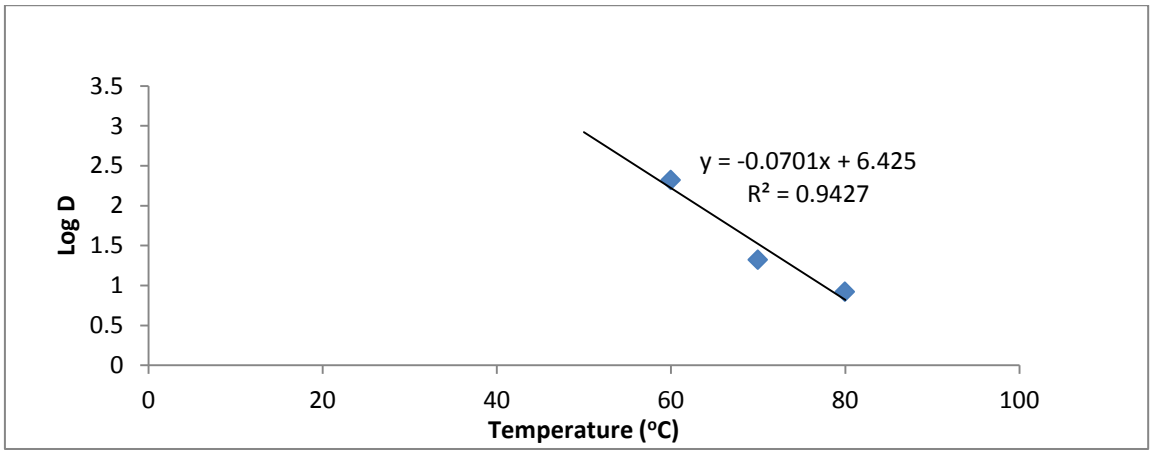

Figure 3: Temperature reliance of rate constant of inactivation for heat inactivation of 'oha'(*Pterocarpus mildbraedii*) polyphenol oxidase. 1/T represents the inverse of absolute temperature.

Table 3: Thermodynamic attributes for 'oha' (*Pterocarpus mildbraedii*) polyphenol oxidase under heat treatment ranging from 50 – 80°C (presuming a first order model)

Temperature (°C)	Thermodynamic attributes		
	$\Delta H^{\#}$ (KJ/mol)	$\Delta S^{\#}$ ($Jmol^{-1}K^{-1}$)	$\Delta G^{\#}$ (kJ/mol)
50	133.286	129.58	91.433
60	133.203	129.32	90.138
70	133.120	129.08	88.846
80	133.037	128.84	87.557
Mean	133.161	129.20	89.493

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