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Kinetic analysis of the thermostability of peroxidase from African oil bean (*Pentaclethra macrophylla Benth*) seeds

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Abstract

A major problem in the storage and marketing of processed African oil bean seeds is its high deterioration rates due to the undesirable activity of peroxidases. The effect of heat treatment on the activity of peroxidase (POD) from African oil bean (*Pentaclethra macrophylla*) seeds was studied over a range of 30°C to 80°C. A simple first-order reaction which assumes a biphasic thermoinactivation curves was used to study the denaturation of this enzyme. Results suggested that peroxidase is a stable enzyme with a Z-value as low as 0.0147. This indicates that it is more sensitive to increase in temperature than to the duration of the heat treatment making high temperature short time treatment a technique to be used for its inactivation.

The results of the thermodynamic investigations indicated that the oxidation reactions were: (a) not spontaneous ($\Delta G > 0$) for peroxidase at $323^{o}K$, and at all the temperatures (2) slightly endothermic ($\Delta H > 0$) at 323 K and (3) reversible ($\Delta S < 0$) at all the temperatures under study.

Keywords: *Pentaclethra macrophylla*, peroxidase, purification, kinetics, thermostability, thermo-inactivation.

Introduction

African oil bean (*Pentaclethra macrophylla* Benth) seeds are among the tropical underutilized seeds that have recently attracted worldwide attention. This is because of their high nutrient potentials, especially with regard to solving the prevalent protein energy malnutrition (PEM) in less developed parts of the world (Enujiugha and Akanbi, 2008).These seeds are eaten in the form of salad (Chilaka et al. 1993), across different African countries. Although the seeds retained their whitish colour after processing, they eventually undergo browning during soaking and fermentation (Njoku and Okemadu, 1989). Jood et al. (1987) observed that these browning reactions cause loss of nutritive value in food products.

Despite the importance of African oil bean seeds as an African delicacy, there is a dearth of information on the preservation technique for these seeds in order to control loss of quality arising from browning.

Ngoddy and Onuoha (1985) ruled out the possibility of non-enzymic browning due to reaction between amino acid and sugars before fermentation in African oil bean seeds. Browning of processed oil bean seeds could only be as a result of the reactivation of enzymes involved in browning reactions (Chilaka et al. 2005). Presence of residual endogenous enzymes in processed vegetables causes quality changes and nutritional losses during storage (Chilaka et al. 2002; Anthon and Barrett 2002). Polyphenol oxidase (o-diphenol; O_2 oxidoreductase EC 1.10.3.2) and peroxidase (donor; H_2O_2 oxidoreductase EC 1.11.1.7) are known to be involved in browning of freshly damaged and processed plant materials (Chilaka et al. 1993; Lopez-Serrano and Barcelo 1998).

Polyphenol oxidase has been ruled out in the browning process as it doesn't reactivate after inactivation (Eze and Chilaka 2002). Thus this qualifies peroxidase as an enzyme of study for the browning of processed African oil bean seeds given its reported ability to reactivate (Eze and Chilaka 2002) after inactivation.

Regeneration of activity after thermoinactivation is common among plant peroxidases and has been associated with deterioration of food quality during storage. Because of their physiologically significant roles in plants, peroxidase has become the subject of a broad range of biochemical and molecular studies.

Thermal processing has been applied in the preservation of the fermented African oil bean seeds slices with remarkable improvements in nutrient bio-availability, digestibility and functionality (Enujiugha and Akanbi 2005).

As investigation has shown that thermal processing is an achievable technique for the maintenance of the preferred form and quality of the processed oil bean seeds, and at the same time ensure the elongation of its shelf life (Enujiugha and Akanbi 2005). It has

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become necessary to analyze the thermoinactivation of peroxidase already implicated in the deterioration of these seeds during processing and storage. This will contribute knowledge on the use of thermal processing as a veritable tool in the preservation of this value added product.

Materials and Methods

Isolation of Peroxidase from African oil bean seeds

Fresh and uninjured African oil bean seeds were purchased from local dealers from Orba modern market in Udenu Local Government Area of Enugu state, Nigeria.

The method used here was adapted from Chilaka et al (2005) with slight modification. About 100g of the seeds were homogenized in 500ml of ice cold 0.025M Tris-Hcl buffer at pH 7.5 with a warring blender. The solution was filtered through 4 layers of cheese cloth and centrifuged at 10,000xg for 30min at 4°C. The supernatant was collected as the crude enzyme and stored at 4°C until further use.

Peroxidase assay and protein determination

Peroxidase activity was assayed using the modified McLellan and Robinson (1981) method. The change in absorbance at 460nm due to the oxidation of o-dianisidine in the presence of H_2O_2 and the enzyme at 30°C was monitored using a JENWAY 640 UV/VIS spectrophotometer (Beckman/Instruments, Inc., Huston Texas). The standard assay solution contains 0.1ml of 0.2% o-dianisidine, 0.1ml of 30% H_2O_2, and 2.7ml of 0.05M sodium acetate buffer, pH5.4 and 0.1ml of suitably diluted enzyme extract in a total of 3ml. One unit of enzyme activity was defined as the amount of enzyme that gives an absorbance change of 0.1AU/min at 30°C. The protein content was determined by the Lowry et al. (1951) method, unless where otherwise stated.

Estimation of kinetic parameters

The Michaelis-Menten constants (K_m) and maximum velocity (V_{max}) for the oxidation of o-dianisidine in the presence of hydrogen peroxide and the enzyme at 30°C were determined by measuring the activity rates (under the assay conditions) at substrate concentrations ranging from 0 - 2.67mM H_2O_2 with a fixed amount of the enzyme in 0.05M acetate buffer, pH 5.4. These Michaelis-Menten constants were derived from Lineweaver-Burk plot using equation 1 below:

$$\frac{1}{v} = \left(\frac{1}{Vmax}\right) + \left(\frac{Km}{Vmax}\right).\frac{1}{S} \quad (1)$$

The Michaelis-Menten equation shown in equation 2 bellow,

$$v = \frac{Vmax\,[S]}{Km + [S]} \quad (2)$$

Could be rewritten as

$$v = \frac{Kcat\,[Et][S]}{Km + [S]} \quad (3)$$

Where

$$Vmax = Kcat\,[Et] \quad (4)$$

thereby allowing the catalytic efficiency (i.e. the ratio V_{max}/Km) and other kinetic constants (K_{cat}, K_{cat}/Km) to be determined. K_{cat} was calculated from equation 2 below:

$$Kcat = \frac{Vmax}{[Et]} \quad (5)$$

Where $[E_t]$, = Total molar concentration of enzyme. K_{cat}/K_m represents the catalytic efficiency of the enzyme.

K_{cat}/K_m is a fundamental kinetic constant for catalysis and it is a traditional measure of enzyme performance (Eisenthal et al. 2007).

Thermal treatment

The kinetic and thermodynamic parameters for the thermal inactivation of peroxidase from African oil bean seeds were determined on the basis of isothermal inactivation experiments for varying periods of time in a temperature controlled water bath. The enzyme solution was placed in a pre-warmed tube at the specified temperature (30, 40, 50, 60, 70 and 80°C), and aliquots were withdrawn using a micropipette at 30min time intervals. Afterwards, the samples were immediately cooled in ice water 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. The incubation was carried out in screw capped tubes to prevent change of volume of the sample and hence, the enzyme concentration due to evaporation.

Calculations

Most of the calculations and analysis carried out here were adapted from Ajay and Farhath (2010). The data obtained from the thermal stability profile were used to analyze thermodynamic parameters of the enzyme. The experimental points were plotted based on a first-order kinetic model according to equation 6 (Eagerman and Rouse 1976) below:

$$\ln\frac{At}{Ao} = K.t \quad (6)$$

Where Ao is the initial activity, A_t is the residual activity after heat treatment for a given period of time, K is thermal inactivation rate constant (min^{-1}) and t is the exposure time (min). The inactivation rate constant can be estimated by linear regression analysis of the natural logarithm of activity versus treatment time. The half-life of the peroxidase ($t_{1/2}$, min^{-1}) was determined from the relationship

$$t\frac{1}{2} = \frac{\ln 2}{K} \quad (7)$$

Espachs-Barroso et al. (2006) observed that in food processing, it is common to characterize first-order reactions in terms of the thermal death concepts (D and Z-values). It is important to reduce the number of deleterious enzymes in foods/food products to ensure proper safety. This is most often done by thermal processing. Time-temperature measurements of residual enzyme activity reduction are determined by thermal process parameters such as D- and Z- values. The D-values (decimal reduction time or time required to reduce the activity of an enzyme at a given temperature by 90%) was calculated from the following relationship

$$D - Value = \frac{ln\,10}{K} \quad (8)$$

The Z-value which reflects the temperature dependence of D-value equals the temperature increase necessary to obtain a 10-fold decrease of the D-value (Espachs-Barroso et al, 2006). The Z-value

was calculated from the slope of graph between log D versus T (°C). The activation energy (Ea) for peroxidase denaturation was determined by an Arrhenius plot of log denaturation rate constants (lnk) versus reciprocal of the absolute temperature (K) The change in enthalpy (ΔH, $KJmol^{-1}$), free energy (ΔG, $KJmol^{-1}$), and entropy (ΔS, $Jmol^{-1}\ K^{-1}$) for thermal denaturation of peroxidase was determined using equation 9, 10, and 11 below:

$$\Delta H = Ea - RT \quad (9)$$

$$\Delta G = -RT\ ln\ \frac{Kh}{Kb}T \quad (10)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (11)$$

Where Ea is the activation energy for denaturation, T is the corresponding absolute temperature (K), R is the gas constant ($8.314Jmol^{-1}K^{-1}$), Kh is the plancks constant (11.04×10^{-36} $Jmin^{-1}$), Kb is the Boltzmann constant ($1.38x10^{-23}JK^{-1}$) and K is the deactivation rate constant (min^{-1}).

Results and Discussion

Kinetic parameters

The Michaelis-Menten constants K_m and V_{max} for the oxidation of o-dianisidine in the presence of hydrogen peroxide at 30°C were 1.937 mM ml^{-1} and 2.370 mM $ml^{-1}min^{-1}$respectively (Fig 1). The K_m value was 5 times lower than 9.731mM obtained by Thongsook and Barrett (2005), for basic peroxidase from Broccoli (*Brassica oleracea* Var. Italica), but about 5 times higher than 0.33mM obtained by Vernwal et al. (2006), for peroxidase from *Solanum melongena* fruit juice indicating that POD from Oil bean seeds has higher affinity for hydrogen peroxide than POD from Broccoli. The turnover number (K_{cat}), was $0.90min^{-1}$, which is more than that of 0.148 sec^{-1} obtained by Guida et al. (2011), for peroxidase from the leaves of *Phytolocca dioica* L. The apparent second-order rate constant (k_{cat}/K_m) is $0.47min^{-1}mM^{-1}$ as shown in Table 1. K_{cat}/K_m allows direct comparison of the effectiveness of an enzyme towards different substrates. The (V_{max}/K_{cat}) value of POD from Oil bean seeds is $2.63\mu molmin^{-1}$ substrate. This notation is used to describe catalytic efficiency of enzymes and is useful for measuring the efficiency of the enzyme in commercial applications. The V_{max}/K_m of 1.22 obtained in this work (Table 1), compares with the values of 1.12±0.19 (o-dianisidine) and 0.93±0.33 (hydrogen peroxide), obtained by Mbassi et al, (2011) for peroxidases from *Vigna species* (V).

Thermal stability

The rate of heat inactivation was investigated at different temperatures 30-80°C. A biphasic first-order inactivation curves were constructed (Fig 3), with different thermal stabilities. The pattern suggests the existence of isoenzymes (Ghaemmaghami et al. 2010). In this study, the reduction of peroxidase activity was more evident for higher temperature and within the first 5min. This is as a result of the presence of the heat labile fraction, which was inactivated faster at higher temperatures then followed by a much slower inactivation phase. A pseudo-first-order plot was applied to determine the kinetics of the thermal inactivation (Figure 4). The plot between (lnA/Ao and time), was observed to follow the first order kinetic equation.

Results of the thermoinactivation of peroxidase were presented in Table 2. It was observed that the rate of enzyme inactivation (k) increases with increase in temperature. Higher rate constant means the enzyme is less thermostable at higher temperature (Pal and Khanum 2010). The peroxidase was found to be stable at 40, 50, and 60°C with calculated half-life ($t_{1/2}$) values of 4.33, 3.85 and 3.01min respectively (Its stability dropped drastically at 70 °C and 80°C with half-life values of 1.65min and 0.81min respectively. This suggests that the use of high temperature –short time treatment will inactivate the enzyme within the conditions of the study though not completely. Vieille and Zeikus (2001) observed that an enzyme's kinetic stability is often expressed as its half-life ($t_{1/2}$) at defined temperatures. The 6 fold decrease in half-life with increase in temperature (Table 2), indicates that the enzyme is unstable at high temperatures. The result obtained for peroxidase from *Penthaclethra macrophylla* could be compared to that earlier obtained in our

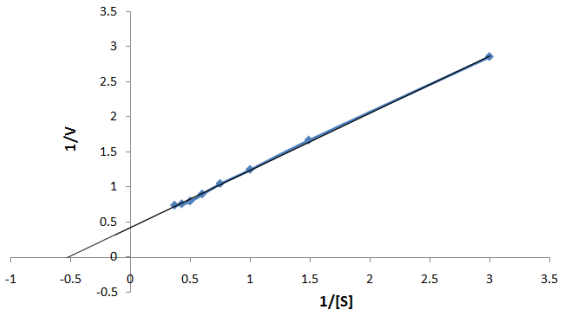

Figure 1: Lineweaver-Burk plot of o-dianisidine oxidation with hydrogen peroxide (H_2O_2) catalyzed by peroxidase from African oil bean seeds (*Pentaclethra macrophylla* Benth). The Km and Vmax values as determined here are 1.931mMml^{-1} and 2.370mM ml^{-1} min^{-1} respectively.

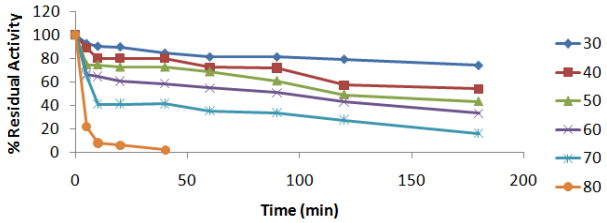

Figure 2: Biphasic inactivation curves for the thermal inactivation of African oil bean seeds (*Pentaclethra macrophylla* Benth) peroxidase at different temperatures.

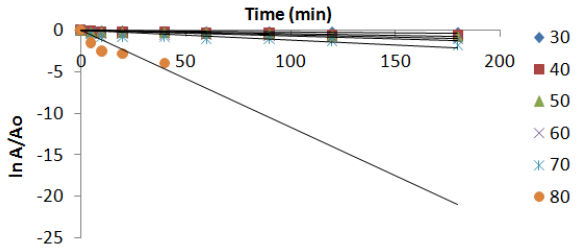

Figure 3: Psudo-first-order plot for thermal inactivation of African oil bean seeds (*Pentaclethra macrophylla* Benth) peroxidase

laboratory for peroxidase from yam (Eze et al. 2010). This is an indication that yam peroxidase may be more stable than peroxidase from *Penthaclethra macrophylla.* The time needed to reduce the enzyme activity by 90%, ie D-value, which is comparatively high, shows the thermostability of peroxidase. The results confirm the thermostable property of the peroxidases extracted from African oil bean seeds.

The Z-value, calculated from the slope from Figure 4 was 0.0147. Tayefi-Nasrabadi and Asadpour (2008), observed that the high magnitude of Z-values mean more sensitivity to the duration of heat treatment and lower Z-values mean more sensitivity to increase in

temperature, therefore the low Z-value of 0.0147 obtained for this peroxidase from African oil bean seeds indicates that the peroxidase

Table 1: Kinetic constants for peroxidase from *Pentaclethra macrophylla*

Kinetic parameters	Values
K_m [a]	1.937mM/ml
V_{max}	2.370 mM $ml^{-1}min^{-1}$
K_{cat} [b]	0.90min^{-1}
K_{cat}/K_m [c]	0.47min^{-1} mM^{-1}
V_{max}/K_m	1.22min^{-1}
V_{max}/K_{cat} [d]	2.63mmolmin^{-1}

[a] Michaelis constant for substrate affinity; [b] Turnover number; [c] Second-order rate constant; [d] Catalytic efficiency;

is more sensitive to increase in temperature. This suggests that the activity of peroxidase which causes browning in oil bean seeds might be as a result of its regeneration of activity.

Table 2: Thermoinactivation parameters for POD from oil bean seeds

T	K	T1/2	D	ΔH^*	ΔG^*	ΔS^*
303	0.15	4.62	15.35	-6719.14	-79027.67	-238.64
313	0.16	4.33	14.39	-6802.28	-81552.39	-238.81
323	0.18	3.85	12.79	-6885.56	-83926.05	-238.51
333	0.23	3.01	10.01	-6951.56	-85930.16	-237.17
343	0.42	1.65	5.48	-7051.35	-86877.80	-232.73
353	0.86	0.81	2.68	-9644.48	-87391.68	-220.24

T=Temperature (K); K (First order rate constant of denaturation) (min^{-1}); $t_{1/2}$ (half-life) = 0.693/k (min^{-1}); $\Delta H - E_a$ (10.31 $KJmol^{-1}$) – RT; E_a (Activation energy of denaturation) is calculated from Figure 6.; ΔG = -RT. ln (k.h /k_bT); $\Delta S = (\Delta H - \Delta G) / T$;E_a= -4200; D-Value = Decimal reduction time = ln 10/k; Z-value = 0.0147

The thermodynamic parameters like thermal stability, the energy of activation (Ea), and the thermal denaturation rate were found by plotting the Arrhenius equation (Fig 5). The Ea for peroxidase from oil bean seeds was found to be -4200kcal mol^{-1}. The values of enthalpy of denaturation (ΔH^*) and free energy of denaturation (ΔG^*) were -6802.282$kJmol^{-1}$ and -81552.394$kJmol^{-1}$ at 40°C respectively (Table 2).

The dependence of the inactivation rate constants on temperature fitted adequately to the Arrhenius equation (Figure 5). The thermal resistance of plant peroxidases largely depends on its origin. At 80°C, the value of ΔG^* was -87391.6875$Jmol^{-1}$ indicating that oil bean POD showed high resistance to thermal unfolding. Thermodynamically, the enzyme molecule with high ΔG^* is considered to be stable. Moreover, the value of entropy of inactivation (ΔS^*) at 80°C was slightly increased (-220.247$Jmol^{-1}K^{-1}$), over the value of -238.642$Jmol^{-1}K^{-1}$ at a lower temperature of 30°C suggesting that there was negligible disorder in the configuration of oil bean POD at higher temperature (Maisuria et al. 2010), though, negative ΔS^* suggests that there is an aggregation process in which a few inter- and/intramolecular bonds are formed. When, enzyme proceeds to the aggregated complex, the state of order of the system increases (Arema and Mckenn 1996). These results are in accord with those of Polygalacturonidase from *Erwinia carotovora* subsp. *Carotovora* BR1 which was found to be more ordered at higher temperature as revealed by their negative ΔS^*. Aledo and Jimenez-Riveres (2010), observed that only positive values for activation energies can be expected for enzymes working under saturation conditions but not for those working at sub saturating concentrations of their substrates thereby substantiating the negative values of activation energy obtained in this report. The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with a concomitant increase in the enthalpy of inactivation (Strivastava et al 2005).

Conclusion

Experimental results indicate that the biphasic first-order model provides an adequate description for the non-linear thermal inactivation curves of POD from African oil bean. POD from African oil bean (*Pentaclethra macrophylla* Benth), seeds is catalytically efficient for the oxidation of o-dianisidine/H_2O_2 substrate pattern. The kinetic and thermodynamic parameters and

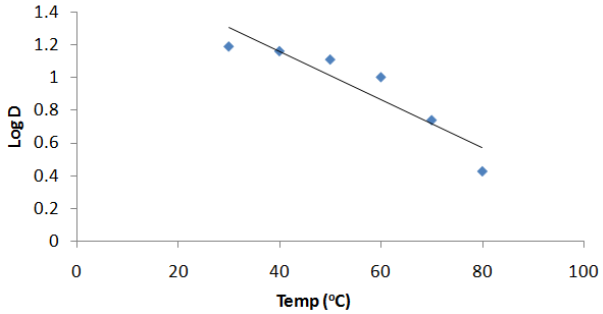

Figure 4: Temperature dependence of the decimal reduction of peroxidase to calculate Z-value

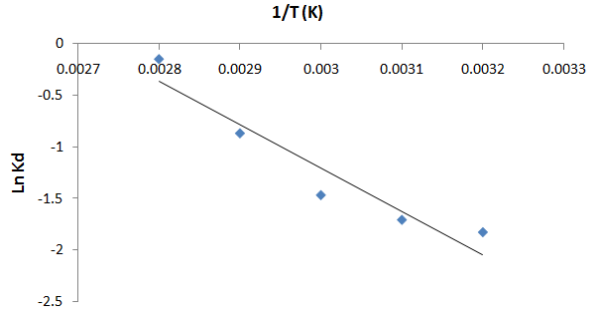

Figure 5: Arrhenius plot for the thermal inactivation of African oil bean seeds (*Pentaclethra macrophylla* Benth) peroxidase. Ea values were calculated from this figure.

higher activation energies suggest that this enzyme might be more suitable for industrial applications. A low Z-value of 0.0147 obtained for this enzyme suggests that it is more sensitive to increase in temperature than to the duration of the heat treatment making high temperature short time treatment a panacea to its observed effect.

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