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Thermodynamics and Kinetics of Thermal Inactivation of Polyphenol Oxidases and Peroxidase of Three Tissues (Cambium, Central Cylinder, Internal Bark) of Cassava Roots (*Manihot esculenta CRANTZ*) varietie Bonoua 2, Cultivated in Côte d'Ivoire

Yapi Jocelyn Constant[1]*, Djina Yves[2], Gnanwa Mankambou Jacques[1] and Kouame Lucien Patrice[2]

[1]Department of Biochemistry and Microbiology, Agroforestry Unit, University Lorougnon Guede, BP 150 Daloa, (Côte d'Ivoire)
[2]Department of Biochemistry and Food Technology, University Nangui Abrogoua-02 BP 801 Abidjan 02, (Côte d'Ivoire)

**Corresponding author*

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A B S T R A C T

The effect of heat treatment on polyphenoloxidases and peroxidase activities in three tissues (cambium, central cylinder and internal bark) of the varietie of cassava roots (*Manihot esculenta CRANTZ*) Bonoua 2 were studied over a range of 30 to 70 °C, using pyrogallol, 4-methylcatechol, dopamine and pyrocatechol as a substrates for polyphenol oxydase and guaiacol as a substrate for peroxidase. Optimal conditions for enzymatic studies were determined to between pH 8.6 and 9.6 for polyphenoloxydases and pH 6.6 and 7.6 for peroxidase and 25-45 °C. $T_{1/2}$-values of enzymatic activities are between 2.22 and 4.27 min at 70 °C, they decreased with increasing temperature, indicating a difference thermostability of each enzyme. D- and k-values decreased and increased, respectively, with increasing temperature, indicating faster of these enzymes inactivation at higher temperatures. Results suggested that polyphenoloxydases and peroxidase were relatively thermostable enzymes with a Z-value which vary from 21.49 at 37.76 °C and Ea of 75.84 at 139.17 $kJmol^{-1}$. Thermodynamic parameters were also calculated. The Gibbs free energy ΔG values range from 47.33 to 265.85 kJ/mol. These kinetic data can be used to predict prevention of browning in the cassava roots (*Manihot esculenta CRANTZ*) by thermal inactivation of enzymes.

Introduction

Cassava (*Manihot esculenta*), is a root crop originating from South America and is one of the major staple food crops in West Africa. It is one of the most important sources of dietary energy in tropical and sub-tropical regions of the world; an estimated 700 million people

obtain more than 500 cal day^{-1} from cassava (Maziya-Dixon *et al.*, 2009).

According to FAO (2013), approximately 215 million tons of cassava is cultivated in the world. And the contribution of Africa is estimated at 52.88% of the worldwide production. In Cote d'Ivoire, the annual production of cassava is estimated at 2.50 million tons (FAOSTAT, 2009).

The crop is grown almost exclusively by low-income smallholder farmers and consumed and processed at the household level (Eriksson, 2013). Cassava is drought resistant and can be grown all year round and as such determines the food supply for many smallholder farmers and low-income families (Nnedimma, 2015). In spite of its importance in the food (human and animal), the tuberose root of cassava is unfortunately hampered by a phenomenon of enzymatic browning during postharvest storage or processing (Mohapatra *et al.*, 2010). These browning reactions have been linked to mechanical damage during handling and processing, abrasions, washing, senescence, and bacterial infestations. Polyphenoloxidase (PPO) and peroxidase (POD) have been identified as responsible for browning reactions (Tomàs-Barberàn and Espin, 2001).

Cassava PPO (EC 1.14.18.1), which is a copper-containing enzyme, has been associated with the conversion of phenolic compounds to quinones and their products' polymerization. PPO enzymes lead to the formation of undesirable brown pigments and off-flavoured products (Yemenicioglu *et al.*, 1999). Two kinds of reactions generated by PPO are the hydroxylation of monophenols to O-diphenol and the oxidation of O-diphenol to O-quinone (Tomas-Barberan and Espin, 2001). POD (EC 1.11.1.7), mostly in isoenzyme forms and also one of the most thermostable enzymes, is responsible for performing single-electron oxidation on a wide variety of compounds, in the presence of hydrogen peroxide. An empirical interdependence between prevention of off-flavour development and inactivation of POD enzyme in frozen vegetables has been highlighted (Yemenicioglu *et al.*, 1999). Furthermore, the synergistic activity of PPO and POD is due to the generation of hydrogen peroxide during the oxidation of phenolic compounds in PPO-catalysed reactions (Khan and Robinson, 1994; Sugai and Tadini, 2006).

Due to the importance of colour preservation of the cassava roots before any processing, the inactivation of naturally-occurring enzymes including PPO and POD is key. The aim of the present study was to inactivate the PPO and POD of cassava roots using the effect of heat treatment over a range of temperatures from 30 to 70°C on these enzymes in order to predict their behavior.

Materials and Methods

Enzyme sources

Mature roots (six months) of Manihot esculenta CRANTZ (varietie Bonoua 2) were harvested from the Biological Garden of Soumalekro (Bonoua 5°16'20'' North ; 3°36'3'' West, Côte d'Ivoire) and stored until used. The PPO substrates pyrogallol, dopamine and pyrocatechol and POD substrate gaïacol of were obtained from Sigma Chemical Compagny (Saint-Quentin Fallavier France). All other chemicals and reagents were of analytical grade.

Measurement of the enzymatic activities

Extraction of enzymes

In order to obtain the enzymatic extract, 150 g of each tissue (cambium, central cylinder and internal bark) of cassava roots were ground

using a blender in 300 mL NaCl solution 0.9% (w/v).

The mixture was centrifuged (TGL-16M, USA) at 8000 x g for 20 min at 4°C and the pellet discarded. The supernatant obtained contained the enzymes. polyphenoloxidases and peroxidase activity.

Polyphenoloxidases activity assay

Under the standard test conditions, the activities of PPO were determined with pyrogallol, dopamine and pyrocatechol as a substrate using a modification of the method of Wong *et al.,* (1971). An assay mixture (2 mL) consisting of a 100 mM phosphate or (glycine or tris HCl) buffer to between pH 8.0 and 9.6, 10 mM dopamine or (pyrocatechol and 5 mM pyrogallol) and enzymes solutions were incubated at 28°C for 10 min. After incubation, the activity was determined by measuring the absorbance of the reaction mixture at 480 and 410 nm. Experiments were performed in triplicate, and the results expressed as units of enzymatic activity per mg of protein. One unit of enzymatic activity was defined as an increase in absorbance of 0.001 per min (Cong *et al.,* 2005).

Peroxidase (POD) activity assay

Under the standard test conditions, the activity of POD was determined with gaïacol as a substrate. A 0.1 ml aliquot of enzyme extract was added to 1.9 ml of 0.1 M phosphate buffer (glycine and tris HCl) (pH 6.6 at 8) which contained 0.2 ml of 0.1 M guaiacol and 0.1 ml of 0.05 M hydrogen peroxide. The assay mixture was incubated at 30 °C for 20 s. After incubation, the activity was determined by measuring the absorbance of the reaction mixture at 480 nm. Experiments were performed in triplicate, and the results expressed as units of enzymatic activity per mg of protein. One unit of enzymatic activity was defined as an increase in absorbance of 0.001 per min.

Determination of proteins

The proteins concentration of the different enzymatic samples was measured using the Lowry method (Lowry *et al.,* 1951) with serum albumin bovine (SAB) as protein standard.

Characterization of PPO and POD

Optimum pH

The PPO and POD activities were determined in a pH range of 2.6 – 10.6 in 0.1 M phosphate, citrate-phosphate, tris HCl, and glycine buffers using pyrogallol, dopamine and pyrocatechol as a substrates for polyphenol oxydase and guaiacol as a substrate for peroxidase. PPO and POD activities were assayed by using the standard reaction mixture and changing the buffers. Calculated PPO and POD activities were expressed in the form of relative activity (%) at the optimum pH. The optimums pH obtained for each enzyme was used in all other experiments.

Optimum temperature

The optimum temperatures were determined by measuring PPO and POD activities at different temperatures ranging from 10 °C to 80 °C. Enzymes were added after equilibration of the standard reaction mixture at the selected temperatures and PPO and POD activities were measured. Calculated PPO and POD activities were expressed in the form of relative activity (%) at the optimum temperature.

Thermal inactivation

The thermal inactivation of each enzyme

(polyphenoloxidases and peroxidase) was investigated on the standard condition at various constant temperatures from 30 to 70°C after exposure to each temperature for a period of 5 to 180 min. Each enzyme was incubated in 100 mM (phosphate, glycine, tris HCl and gaïacol) buffer (pH 6.6 at 10). Aliquots were drawn at intervals and immediately cooled in ice-cold water. Experiments were performed in triplicate. The residual enzymatic activity, determined in both cases at 30 °C under the standard test conditions, was expressed as percentage activity of zero-time control of the untreated enzyme.

Kinetic data analysis

The temperature dependence of the reaction rate constant for the studied enzyme served as the basis for fitting to the Arrhenius equation (Arrhenius, 1889):

$$\text{Ln } [A_t/A_0] = -kt \quad \text{(Eq.1)}$$

Where;

A_t is the residual enzyme activity at time t,

A_0 is the initial enzyme activity;

k is the reaction rate constant (min^{-1}) at a given condition.

k values were obtained from the regression line of ln $[A_t/A_0]$ versus time as -slope.

The D-value represents the time required to reduce the concentration of the component under examination to90% of its initial value.

The decimal reduction time (D) was calculated according to Stumbo (1973) as:

$$D = 2.303/ k \quad \text{(Eq. 2)}$$

Z (°C) is temperature increase needed for a 90% reduction in D-value (temperature sensitivity parameter), and follows the equation:

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

Where;

T_1 and T_2 represent the lower and higher temperatures, °C or °K;

D_1 and D_2 are D-values at the lower and higher temperatures in minutes.

The Z-values were determined from the linear regression of log (D) and temperature (T).

The temperature of treatment and the rate constant in a denaturation process was related according to the Arrhenius equation (Arrhenius, 1889)

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

Eq. 4 can be transformed to: $\ln k = \ln A - Ea/R \times T$. (Eq. 5)

Where;

k is the reaction rate constant value,

A is the Arrhenius constant,

Ea is the activation energy (energy required for the inactivation to occur),

R is the gas constant (8.31 $Jmol^{-1}K^{-1}$),

T is the absolute temperature in Kelvin.

When the "ln" of "k" is plotted against the reciprocal of the absolute temperature, a linear relationship should be observed in the temperature range studied. The slope of the line obtained permitted to calculate the activation energy and the ordinate intercept corresponds to ln A (Dogan *et al.*, 2000;2002).

The values of the activation energy (Ea) and Arrhenius constant (A) allowed the determination of different thermodynamic parameters (Marin *et al.,* 2003) such as variations in enthalpy, entropy and Gibbs free energy, ΔH, ΔS and ΔG, respectively, according to the following expressions (Galani and Owusu, 1997):

$\Delta H^{\#} = Ea - RT$ (Eq. 6)

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

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

Where;

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

h_P is the Planck constant (6.626×10^{-34} J.s),

T is the absolute temperature.

Results and Discussion

Characterization of PPO

Optimum pH

The effect of pH on the activities polyphenoloxidases and peroxidase of three tissues (cambium, central cylinder and internal bark) of the cassava roots varietie bonoua 2were investigated in the range of 2.6-10.6.

As seen in table 1, the optimum pH of each enzyme was determined to between pH 8.6 and 9.6 for polyphenoloxydases and pH 6.6 and 7.6 for peroxidase. The enzymes activities decreased rapidly at pHs below orabove the optimum. PPO and POD optimums pH varies from pH 4.0-8.5 as reported in the literature, depending on the origin of the material, the purity of enzyme, extraction method, the type of buffer used and substrate (Dincer *et al.,* 2002). In general, fruits, vegetables and roots show maximum activity at or near neutral pH values.

Optimum temperature

The relatives activities of the PPO and peroxidase of the crude extracts of the three cassava tissues in a range of temperature was shown in Table 2. It was found that crude extracts of the three cassava tissues PPO and peroxidase had an optimum temperature between 25 and 45°C. It has been reported that the optimum temperatures for PPO of tuber yam *Dioscorea cayenensis rotundata* cv Kponan is 25°C (Yapi *et al.,* 2014). At 60°C, approximately 80 % of PPO and peroxidase activities were lost but these enzymes were not completely inactivated. It appears that the crude enzymes are sensitive to the increase in assay temperature.

Kinetic Analysis of Thermal Denaturation

The increase in temperature from 30 to 70°C leads to a decrease in the polyphenoloxidase and peroxidase activities of the crude extracts of the three root tissues of cassava (internal bark, Cambium, central cylinder). Therefore, the half-life of enzymatic activities decreases with increasing temperature thus was showing the difference in thermostability of each enzymatic activity. The half-life ($t_{1/2}$) values of the polyphenoloxidases and peroxidase activities of crude cassava root extracts decreased to values between 2.22 and 4.21 minutes at 70°C (data not shown). Gouzi *et al.,* (2012) found half-life values of 1.45 minutes for the polyphenoloxidase of the fungus *Agaricus bisporus*. The low half-life times obtained show that the enzymes responsible for the polyphenoloxidic and peroxidase activities studied are sensitive to the temperature of 70°C. The decimal reduction times (D) of the polyphenoloxidase and peroxidase activities of the three cassava

root tissues *Manihot esculenta* CRANTZ determined between 30 and 70°C decrease as the temperature increases (data not shown). These results confirm the instability of these biocatalysts responsible for polyphenoloxidase and peroxidase activities at the studied cassava roots. Z values were calculated, ranging from 21.49 to 37.76°C for all enzymatic activities studied (Table 3).

This reveals that the enzymes responsible for these polyphenoloxidases and peroxidase activities in the studied cassava roots are sensitive to the heat of the medium. The high values of the different Z obtained show that these enzymes are heat stable at temperatures between 30 and 70°C (Barrett *et al.,* 1999).

The activation energy values of the polyphenoloxidases and peroxidase activities are positive. They oscillate between 75.84 and 139.17 kJ mol^{-1}(Table 4). These high positive values indicate a sensitivity of these enzymes responsible for these activities to the increase in temperature (Chutintrasri and Noomhorm, 2006). This suggests that the denaturation process requires a high energy input to the enzyme substrate complex to initiate denaturation probably due to a possible compact structure of the enzymes and the strength of the thiol groups (SH) or disulfide bond at the active site (Björck, 1992). These activation energies are higher than those of the potato peroxidase (27.11 kJ mol^{-1}) reported by Yu *et al.,* (2011).

Thermodynamic Analysis of Thermal Denaturation

The calculation of the thermodynamic parameters of inactivation provides information on the enzyme thermal stability for each step of the heat-induced denaturation process. This could help in detecting any secondary stabilization or destabilization effects that would go unnoticed if only the half-life times were considered (Longo and Combes, 1999).These parameters include $\Delta G^{\#}$, the Gibbs free energy change considered as the energy barrier for enzyme inactivation, the enthalpy ($\Delta H^{\#}$) change measuring of the number of bonds broken during inactivation, and the entropy ($\Delta S^{\#}$) change that indicates the net enzyme and solvent disorder. They were calculated in the temperature range 30 to 70 °C from experimental data using eqs 6-8 (data not shown).

The values of enthalpy ($\Delta H^{\#}$) and free energy ($\Delta G^{\#}$) are positive, while those of entropy ($\Delta S^{\#}$) are negative for the polyphenoloxidases and peroxidase activities(data not shown).

The high values of enthalpy obtained during thermal inactivation of these enzymes from the three tissues (cambium, central cylinder and internal bark) of cassava roots varietiebonoua 2 indicate that these enzymes undergo substantial conformational changes to be under their activated forms during the heat treatment (Gnangui *et al.,* 2009). Moreover, positive values of the enthalpies suggest that the endothermic nature of the thermal inactivation reactions (Ozdes *et al.,* 2009).

However, the negative values observed for the variation in entropy indicate that there are no significant processes of aggregation, since, if this would happen, the values of entropy would be positive (Anema and McKenna, 1996). They also indicate that these reactions are reversible (Gnangui *et al.,* 2009).

Negative values of entropy ($\Delta S^{\#}<0$) obtained during thermal inactivation, naturally leads to higher values of free energy ($\Delta G^{\#}$).

Table.1 Optimum pH of polyphenoloxidase and peroxidase activities of different tissues of cassava roots (*Manihot esculenta* CRANTZ) varietie Bonoua 2

pH					
Tissues	**Pyrogallol**	**Dopamine**	**Pyrocatechol**	**4-methylcatechol**	**Guaiacol**
Internal b	Glycine pH 8.6	Tris pH 9	Tris pH 8.6	Tris pH 8.6	Citrate pH 6.6
Camb	Tris pH 9	Glycine pH 9.6	Glycine pH 9.6	Glycine pH 8.6	Tris pH 7.6
C cylinder	Tris pH 8.6	Glycine pH 9	Glycine pH 9.6	Tris pH 8.6	Phosphate pH 7.6

Internal bark (Internal b); Cambium (Camb); Central Cylinder (C Cylinder)

Table.2 Optimum oxidation temperatures of polyphenoloxidase and peroxidase activities in three cassava root tissues (*Manihot esculenta* CRANTZ) varietie Bonoua 2

Optimum temperatures (°C)					
Tissues	**Pyrogallol**	**Dopamine**	**Pyrocatechol**	**4-methylcatechol**	**Guaiacol**
Internal b	35	35	40	40	30
Camb	45	45	40	30	25
C cylinder	45	45	40	30	30

Internal bark (Internal b); Cambium (Camb); Central Cylinder (C Cylinder)

Table.3 Thermal resistant constant of polyphenoloxidase and peroxidase activities in three tissues of cassava roots (*Manihot esculenta* CRANTZ) varietie Bonoua 2

Values of Z (°C)					
Tissues	**Pyrogallol**	**Dopamine**	**Pyrocatechol**	**4-methylcatechol**	**Guaiacol**
Internal b	34.22 ±0.1[a]	29.52±0.16	34.77±0.38	35.21±0.42	30.87±0.42
Camb	27.33 ±0.5	24.66±0.12	28.68±0.4	37.28±0.25	28.11±0.12
C cylinder	23.56±0.2	21.49±0.17	25.93±0.1	37.76±0.25	27.81±0.18

Internal bark (Internal b); Cambium (Camb); Central Cylinder (C Cylinder), ND: not determined. [a]Mean (±SD) for triplicate experiments

Table.4 Activation energy of polyphenoloxidic and peroxidase activities of three tissues of cassava roots (*Manihot esculenta* CRANTZ) varietie Bonoua 2

Activation energy (kj/mol)					
Tissues	**Pyrogallol**	**Dopamine**	**Pyrocatechol**	**4-methylcatechol**	**Guaiacol**
Internal b	123.35±2.1[a]	139.17±0.89	87.51±0.5	119.67±2.1	79.15±0.8
Camb	109.17±1.6	101.22±1.4	96.55±0.45	87.38±0.6	75.84±0.5
C cylinder	96.58±0.8	86.15±0.76	79.74±0.77	107.43±1.0	88.55±0.7

Internal bark (Internal b); Cambium (Camb); Central Cylinder (C Cylinder), ND: not determined. [a]Mean (±SD) for triplicate experiments

These values are positive, indicating that thermal inactivation of these reactions do not occur spontaneously. These reactions must be initiated by adding energy in the form of heat, hence the high activation energy in these reactions. For cons, the values of entropy (ΔS#) are positive for perosidase in central cylinder. Positive values of entropy (ΔS#) indicate that this enzyme is found in a chaotic state at the end of the reaction. This shows that he suffered a distortion pronounced between 30 and 7à°C (Anema and McKenna, 1996).

Catalytic activities of all enzymes decrease in thermal inactivation between 30 and 70 °C.

These temperatures destroy about half of the enzyme activity during the time of treatment. These results meet the target that was partially destroying the enzyme activity to slow the browning process and not completely stop this process. Therefore, treatment of enzyme crude extract at 49°C for 90 min could meet the target. This result is confirmed by the work of Djioua (2010).

The thermal inactivation of the enzymes of the three tissues from four varieties of cassava roots (*Manihot esculenta CRANTZ*) could be described by a firs-order kinetic model. D-, Z-, k values and the high values obtained for activation energy and change in enthalpy indicated that a high amount of energy was needed to initiate denaturation of the polyphenoloxidases and peroxidase activities in the three tissues (cambium, central cylinder and internal bark) of cassava roots varietie bonoua 2, most likely due to its stable molecular conformation.

This high thermostability may be taken into account when thermal treatments are used to obtain processed products derived the enzymes from the three tissues of the varietie of cassava roots (*Manihot esculenta CRANTZ*) Bonoua 2 and preserve tuber quality.

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