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Effect of heat treatment on edible yam (*Dioscorea cayenensis-rotundata* cv *Longbô*) polyphenol oxidase activity: kinetic and thermodynamic analysis

Sophie Nadège Gnangui, Edmond Ahipo Dué, Jean-Parfait Eugène N'guessan Kouadio, Lucien Patrice Kouamé*

Laboratoire de Biochimie et Technologie des Aliments de l'Université d'Abobo-Adjamé 02 BP 801 Abidjan 02, Côte d'Ivoire

**Corresponding author email:* kouame_patrice@yahoo.fr *; Tel: (225) 07412701 Fax (225) 20304256*

Key words
Yam, *Dioscorea cayenensis-rotundata*, polyphenol oxidase, kinetic and thermodynamic analysis

1 SUMMARY

The effect of heat treatment on edible yam (*Dioscorea cayenensis-rotundata* cv *Longbô*) polyphenol oxidase activity was studied over a range of 35 to 75°C. Denaturation of this enzyme, measured by loss in activity, could be described as a first-order reaction with k-values between 0.004 and 0.108 min^{-1}. D- and k-values decreased and increased, respectively, with increasing temperature, indicating faster polyphenol oxidase inactivation at higher temperatures. Results suggested that polyphenol oxidase is a relatively thermostable enzyme with a Z-value of 29.41°C and Ea of 67.67 kJ mol^{-1}. The Gibbs free energy ΔG values range from 86.524 to 88.938 kJ/mol at 308-348°K. The results of the thermodynamic investigations indicated that the oxidation reactions were: (1) not spontaneous ($\Delta G > 0$), (2) slightly endothermic ($\Delta H > 0$) and (3) reversible ($\Delta S < 0$). The high value obtained for the variation in enthalpy indicated that a high amount of energy was required to initiate denaturation, probably due to the molecular conformation of polyphenol oxidase.

2 INTRODUCTION

Enzymes become inactive at temperatures above a critical level due to unfolding of the molecules (LapanJe, 1978). This process is usually reversible for most enzymes but prolonged heating results in irreversible loss of catalytic activity involving destruction of various covalent and noncovalent interactions (Ghosh & Nanda, 1993). Comparative studies of thermophilic and mesophilic enzymes have demonstrated that weak interactions such as hydrogen bonds (Macedo-Ribeiro *et al.*, 1996), disulfide bonds (Hopfner *et al.*, 1999), ion pairs (Vetriani *et al.*, 1998), salt bridges (Criswell *et al.*, 2003), hydrophobic interactions (Elcock, 1998), and compactness (Russell *et al.*, 1997) are of importance for stability. However, no universal basis of stability has been recognized because the stability of different enzymes has different origins. For this reason, more extensive studies have been performed to elucidate the fundamentals of protein stability in terms of macromolecular interactions based on thermodynamics (Hansen *et al.*, 1999; Ramstein *et al.*, 2003).

In general, exposure of polyphenol oxidase (PPO) to temperatures of 60-90°C destroys their catalytic activity (Vamos-Vigyazo, 1981). Of the studies on heat inactivation of PPO, only a few have included the calculations of Arrhenius, the kinetic and thermodynamic

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parameters of heat inactivation of PPO from various foods. These include apple (Strubi *et al*., 1975), Sultana grapes (Aquilera *et al*., 1987), apricot (Heil *et al*., 1988), rice (Ansah, 1989) and mango (Askar *et al*., 1994).

Recently, Yapi (2008) purified to homogeneity a polyphenol oxidase (PPO_Y) from edible yam (*Dioscorea cayenensis-rotundata* cv *Longbô*) cultivated in Côte d'Ivoire. The enzyme was stable at 30°C and its pH stability was in the range of 6.0-7.0. Maximal PPO_Y activity occurred at 30°C and pH 6.6. The values V_{max}/K_M showed that the enzyme was a dopamine oxidase having diphenolase activities. There is no report on the inactivation of PPO_Y by heat treatment.

The objective of this study was to determine the effect of heat treatment over a range of temperatures from 35 to 75°C, on PPO_Y activity. This method permits accurate determination of heating times and consequently, accurate calculations of kinetic and thermodynamic parameters.

3 MATERIALS AND METHODS

3.1 Materials: Mature tubers of *Dioscorea cayenensis-rotundata* **(cv** *Longbô*) were harvested from the Biological Garden University of Abobo-Adjamé (Abidjan, Côte d'Ivoire) and stored at -20°C until used. PPO_Y substrate dopamine was procured from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All other chemicals and reagents were of analytical grade.

3.2 Methods

3.2.1 Preparation of polyphenol oxidase: Freshly peeled tubers (150 g) were homogenized for 10 min in 300 ml of cold NaCl 0.9% (w/v). The resulting homogenate was centrifuged at 20000g for 10 min at 4°C. The supernatant represented the crude extract.

This enzymatic solution (20 ml) was loaded onto a DEAE-Sepharose CL-6B gel (2.4 cm x 6.5 cm) that had been equilibrated previously with 100 mM phosphate buffer pH 6.6. The unbound proteins were removed from the column by washing with two column volumes of the same buffer pH 6.6. Proteins were eluted using a stepwise gradient with 0.3, 0.5 and 1 M NaCl in 100 mM phosphate buffer pH 6.6. Fractions (3 ml each) were collected at a flow rate of 180 ml/h and assayed for enzyme activity. The active fractions were pooled and saturated overnight by 80 % ammonium sulphate in a cold room. The precipitated pellet was then separated by centrifugation at 20000 g for 30 min and dissolved in 1 ml of 100 mM phosphate buffer pH 6.6. The enzyme solution was loaded directly into a Sephacryl S-100 HR (1.6 cm x 64 cm), which was pre-equilibrated with the same buffer pH 6.6. Proteins were eluted at a flow rate of 20 ml/h using 100 mM phosphate buffer pH 6.6. Fractions of 1 ml were collected and active fractions were pooled together. The pooled fraction from the previous step was saturated to a final concentration of 1.7 M ammonium sulphate and applied on a Phenyl-Sepharose CL-6B column (1.4 cm x 7.5 cm) previously equilibrated with 100 mM phosphate buffer pH 6.6 containing 1.7 M ammonium sulphate. The column was washed with equilibration buffer and the proteins retained were then eluted using a stepwise gradient with 0.9, 0.7, 0.5, 0.3, 0.1 and 0 M ammonium sulphate in 100 mM phosphate buffer pH 6.6. Fractions of 1 ml were collected at a flow rate of 15 ml/h and active fractions were pooled together. The pooled fraction was dialysed against 100 mM phosphate buffer pH 6.6 overnight in a cold room.

3.2.2 Enzyme assay and protein determination: Under the standard test conditions, the activity of PPO_Y was determined with dopamine as substrate using a modification of the method of Wong *et al*. (1971). A mixture (2 ml) consisting of a 100 mM phosphate buffer pH 6.6, 5 mM dopamine and enzyme solution was incubated at 30°C for 10 min. 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 (U) of enzymatic activity. One unit of enzymatic activity was defined as an increase in absorbance of 0.001 per min (Bartolo & Birk, 1998).

Protein was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

3.2.3 Thermal inactivation: The thermal inactivation of the enzyme was determined at constant temperature between 35 and 75°C after exposure to each temperature for a period of 5 to 30 min. The enzyme (15 U) was incubated in 100 mM phosphate buffer (pH 6.6). Aliquots were

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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.

3.2.4 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):

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

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 to 90% 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:

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

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 estimated from the linear regression of logD 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:

$$\ln k = \ln A - Ea/R \times T . \quad \text{(Eq. 5)}$$

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 & Owusu, 1997):

$$\Delta H^{\#} = Ea - RT \quad \text{(Eq. 8)}$$

$$\Delta S^{\#} = R\,(\ln A - \ln K_B/h_P - \ln T) \quad \text{(Eq.9)}$$

$$\Delta G^{\#} = \Delta H^{\#} - T\,\Delta S^{\#} \quad \text{(Eq. 10)}$$

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.

4 RESULTS AND DISCUSSION

The rate of enzyme catalysis generally increases with increase in temperature until a critical point is reached when denaturation of the enzyme is initiated and the reaction rate begins to decrease (Skujins, 1967; Tabatabai, 1982). Thus, a peak in enzyme activity is observed, which is usually referred to as the optimum temperature and which varies for different enzymes (Trasar-Cepedaa *et al*., 2007). The temperature at which the activity of polyphenol oxidase from edible yam (*Dioscorea cayenensis-rotundata* cv *Longbô*) was maximal was 30°C (Yapi, 2008). The thermal stability profile for this oxidase, presented in the form of the residual percentage activity, is shown in Table 1. At temperatures between 35-75°C, heat-denaturation of the enzyme occurred after 5 min of incubation (97.81 to 53.00 %). The drop in percentage residual activity at high temperatures was most likely due to the unfolding of the tertiary structure of the enzyme to form the secondary structure. Although heating at 60°C for 15 min resulted in partial (49.73%) inactivation, heating at 75°C for the same period strongly inactivated the enzyme (16.39%).

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Table 1: Effect of treatment temperature and time on the inactivation of polyphenol oxidase from edible yam **(***Dioscorea cayenensis-rotundata* cv *Longbô*).

Temperature (°C)	Relative activity (%) at each treatment time (min)					
	5*	**10**	**15**	**20**	**25**	**30**
35	97.81	95.08	92.90	91.26	89.07	86.34
40	94.54	90.71	86.89	84.15	81.42	78.69
45	93.98	86.89	80.87	76.50	73.77	69.40
50	83.61	74.86	65.03	60.66	52.46	47.54
55	81.42	72.13	63.39	56.83	48.63	43.72
60	73.22	60.11	49.73	37.70	33.33	24.59
65	65.03	54.10	40.98	30.05	25.68	18.03
70	62.30	42.62	29.51	20.22	15.85	10.38
75	53.00	30.05	16.39	9.83	6.56	4.92

***Treatment time in minutes.

Table 2: k, D-, Z- and Ea-values for thermal inactivation of edible yam (*Dioscorea cayenensis-rotundata* cv *Longbô*) polyphenol oxidase at temperature range (35–75°C).

Temperature (°C)	Kinetics parameters				
	k (min^{-1})		D (min)	Z (°C)	Ea (kJ/mol)
	value	R^2			
35	0.004	0.9892	575.75		
40	0.008	0.9667	287.88		
45	0.012	0.9539	191.92		
50	0.025	0.9720	92.12		
55	0.028	0.9742	82.25	29.41	67.67
60	0.046	0.9824	50.07		
65	0.057	0.9726	40.40		
70	0.077	0.9857	29.91		
75	0.108	0.967	21.32		

The logarithmic linear relationship between PPO_Y activity and treatment time for the temperature range of 35-75°C followed first-order kinetics (Figure 1) and was consistent with the relationships found in earlier studies on fruits and vegetables (Dogan *et al.*, 2005; Ditchfield *et al.*, 2006; Rapeanu *et al.*, 2006). Based on results (table 2) it is clear that the enzyme was less thermostable at higher temperatures (70-75°C) since a higher rate constant means that the enzyme was less thermostable (Marangoni, 2002).

The rate of PPO_Y inactivation, after ln transformation, decreased linearly with the inverse of temperature (Figure 2). This relationship was described by the equation: $\ln k = -8144\ (1/T) + 21.26$ ($R^2 = 0.98$), where T represents absolute temperature (K). From 35 to 75°C, the activation energy (Ea) value for thermal inactivation of the PPO_Y was calculated to be 67.67 kJ/mol (Table 2), which was much higher than that of catalysis (32.30 kJ/mol) (Yapi, 2008). Therefore, the effect of temperature on the denaturation rate was much greater than the effect on catalysis. This activation energy was much higher than that reported for rice **(**23.3 kJ/mol (Aquilera *et al.*, 1987), kiwifruit **(**33.67 kJ/mol) (Park & Luh, 1985) and plantain **(**18 kJ mol^{-1} (Ngalani *et al.*, 1993), but lower than that for whole banana **(**413 kJ/mol (Dimick *et al.*, 1951), cranberry **(**116 kJ/mol (Lee *et al.*, 1983), apple **(**241–323 kJ/mol, (Yemenicioglu *et al.*, 1997) and taro **(**84 kJ/mol (Yemenicioglu *et al.*, 1999). High activation energy reflects a greater sensitivity of PPO to temperature change (Weemaes *et al.*, 1998; Chutintrasri & Noomhorm, 2006). Thus, the PPO of edible yam (*Dioscorea cayenensis-rotundata* cv *Longbô*) is more sensitive to heat than wild rice, plantain and

kiwifruit but less than the other fruits and vegetables examined to date.

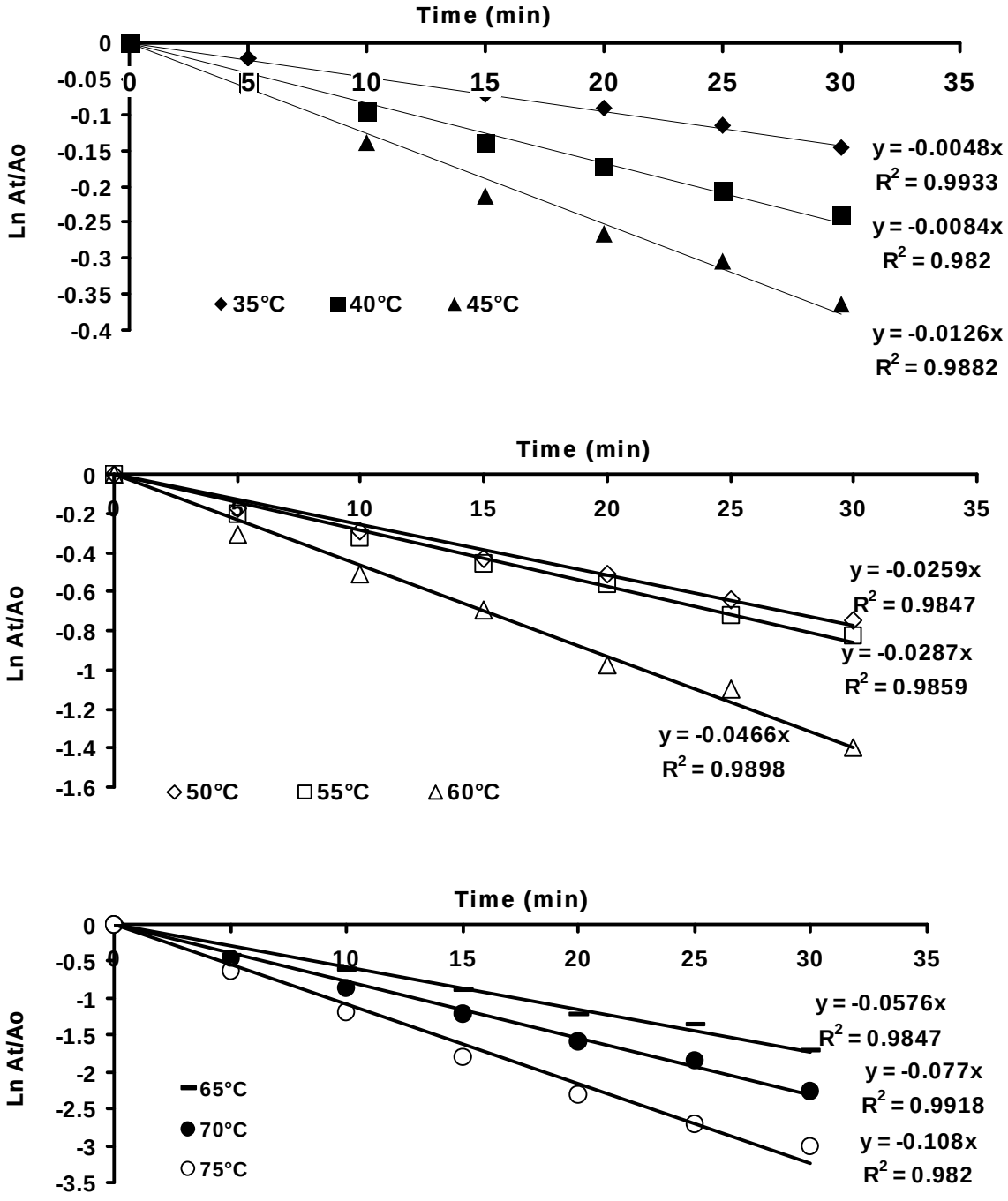

Figure 1: Thermal inactivation curves of polyphenol oxidase from edible yam (*Dioscorea cayenensis-rotundata* cv *Longbô*) in sodium phosphate buffer (pH 6.6) in the temperature range 35-75°C. A_0 is the initial enzymatic activity and A_t the activity at each holding time.

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Calculated Z-values for PPO_Y from edible yam was 29.41°C at 35–75°C (Table 2) which is high relative to values reported for fruits which range from 8.5 to 10.1°C (Strubi *et al*., 1975; Vamos-Vigyazo, 1981). The Z-value of 29.41°C for PPO_Y inactivation (Table 2) was in agreement with the Z-value of PPO from taro of 25.5°C, (Yemenicioglu *et al*., 1999). In general, low Z-values are thought to indicate greater sensitivity to heat (Barrett *et al*., 1999). Differences in the kinetics of heat activation of PPO for different products may result from differences in their composition, which is reflective of their variety or the agronomic and climatic conditions under which they were grown (Chutintrasri & Noomhorm, 2006).

In order to establish the link between treatment time and enzyme activity, the D-values were calculated (Riener *et al*., 2008). Kinetic data from the PPO_Y inactivation studies measured as a function of preheating temperature are summarised in Table 2. At a pre-treatment temperature of 35°C, the D-values obtained using the rate constant data (k) and Eq. (2) decreased in a linear manner (R^2 = 0.98) from 575.75 to 21.32 min, when the pre-treatment temperature was increased from 35 to 75°C for PPO_Y (Figure 3). D-values of PPO_Y from yam were relatively high (Table 2) compared to the D-values of POD, which is considered to be the most heat stable enzyme from vegetables (Yemenicioglu *et al*., 1999). In general, the larger an enzyme and the more complex its structure, the more susceptible it is to high temperature (Yang *et al*., 2004).

The thermodynamic parameters for thermal inactivation of PPO_Y from edible yam are shown in Table 3. At temperatures of 35–75°C, the average values of ΔH, ΔS and ΔG were respectively 64.950 (kJ/mol), -68.91 (J mol^{-1} K^{-1}) and 87.557 (kJ/mol). The ΔH value of PPOy was similar to that of *Armillaria mellea* **(**64 ± 2 kJ mol^{-1} (Yang & Wang, 2008), implying that the number of noncovalent bonds broken in forming a transition state for enzyme inactivation are similar. The high values of change in enthalpy obtained for the different treatment temperatures indicate that enzyme undergoes a considerable change in conformation during denaturation. Positive values of ΔH indicate the endothermic nature of the oxidation reaction. This energy was smaller than that of potato PPO **(**98.02 kJ mol^{-1} (Duangmal & Owusu, 1999) and was much higher than that of *Lepista nuda* **(**13±1 kJ mol^{-1} and *Hypholoma fasciculare* **(**36±kJ mol^{-1} (Yang & Wang, 2008).

These results suggest that PPO_Y was more heat-resistant than that *Lepista nuda* and *Hypholoma fasciculare*, apparently as a result of the larger ΔH value for inactivation. The ΔS value of PPO_Y was smaller than that of potato PPO (145 J mol^{-1} K^{-1} (Duangmal & Owusu, 1999), hence the net change in disorder of enzyme and solvent accompanying the transition state formation was larger and induced reduction of ΔG value.

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 & McKenna, 1996). We observed that the free energy of PPO_Y increased slightly with increasing temperature. At all temperatures, it was positive and revealed the fact that oxidation reaction was not spontaneous.

Based on our results, it is concluded that thermal inactivation of polyphenol oxidase 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 PPO_Y, 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 from edible yam (*Dioscorea cayenensis-rotundata* cv *Longbô*).

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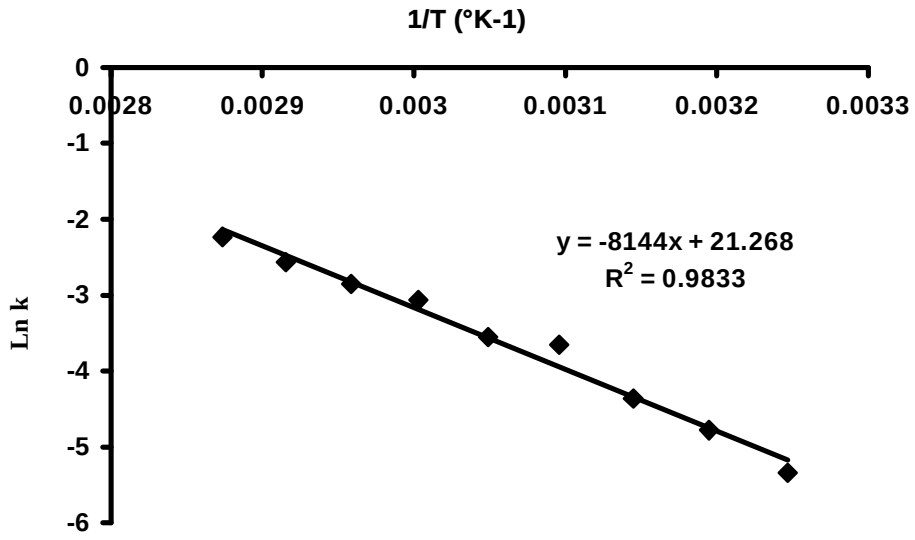

Figure 2: Effect of temperature on D-values for inactivation of edible yam (*Dioscorea cayenensis-rotundata* cv *Longbô*) polyphenol oxidase activity.

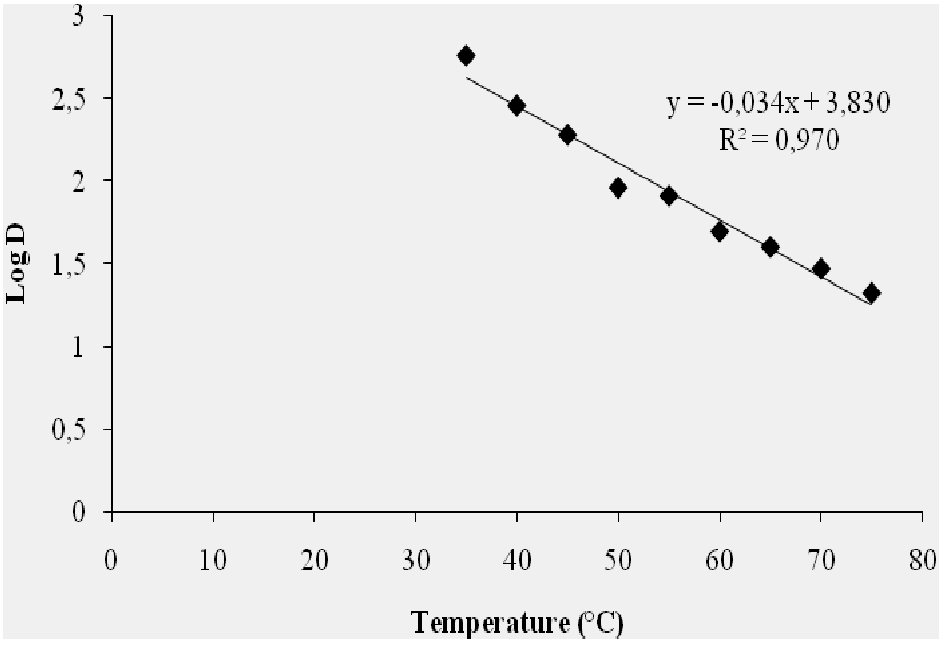

Figure 3: Temperature dependence of inactivation rate constant for thermal inactivation of edible yam (*Dioscorea cayenensis-rotundata* cv *Longbô*) polyphenol oxidase. 1/T represents the reciprocal of the absolute temperature .

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Table 3: Thermodynamic parameters for edible yam (*Dioscorea cayenensis-rotundata* cv *Longbô*) polyphenol oxidase under heat treatment between 35 to 75°C (assuming a 1st-order kinetic model).

Temperature (°C)	Thermodynamic parameters		
	$\Delta H^{\#}$ (kJ/mol)	$\Delta S^{\#}$ (J mol^{-1} K^{-1})	$\Delta G^{\#}$ (kJ/mol)
35	65.117	-68.39	86.181
40	65.075	-68.52	86.524
45	65.034	-68.66	86.867
50	64.992	-68.79	87.210
55	64.950	-68.91	87.555
60	64.909	--69.04	87.899
65	64.867	-69.16	88.245
70	64.826	-69.29	88.591
75	64.784	-69.41	88.938
Mean	64.950	-68.91	87.557

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