sciendo

Nova Biotechnol Chim (2019) 18(2): 102-114
DOI: 10.2478/nbec-2019-0013

Nova Biotechnologica *et* Chimica

Physicochemical properties, kinetics and thermodynamic studies of polyphenol oxidase from sorghum (*Sorghum bicolor* (L.) Moench) for potential use in industry

Toluwase Hezekiah Fatoki and David Morakinyo Sanni

Enzyme Biotechnology Unit, Department of Biochemistry, Federal University of Technology Akure, PMB 704 Akure, Ondo State, Nigeria

Article info

Article history:
Received: 27th July 2019
Accepted: 10th November 2019

Keywords:
Kinetics
Physicochemical properties
Polyphenol oxidase
Sorghum bicolor
Thermodynamics

Abstract

Advances Polyphenol Oxidase (PPO) from *Sorghum bicolor* (white and yellow varieties) grains were investigated for optimum processing condition. The partially purified enzyme was obtained from two varieties of *Sorghum bicolor* by step-wise separation through ion-exchange and size-exclusion chromatography. The final purification gave a yield of 7.33 % and 12.3 % for PPO from white and yellow sorghum respectively. The PPO has V_{max} and K_m of 2.66 U.mL^{-1} and 19.72 mM for white sorghum, 1.33 U.mL^{-1} and 12.92 mM for yellow sorghum. The optimal pH of PPO activity was found at pH 4 and pH 7 for white and pH 4 and pH 8 for yellow sorghum. The pK_a 7.4 and 8.7 were obtained for PPO from white sorghum, and pK_a 5.4, 7.4 and 8.5 for yellow sorghum. The PPO residual activity were above 70 % at 5 hours of incubation within the neutral pH ranges for white sorghum, while those of yellow sorghum were below 40 %. The optimum temperature of 40 ºC and 30 ºC for white and yellow sorghum PPO respectively. The average value of enthalpy (ΔH), entropy (ΔS) and Gibbs free energy (ΔG) obtained at 20 min of incubation and temperature 50 – 80 °C were respectively 49.03 kJ.mol^{-1}, - 129.52 J.mol^{-1}.K^{-1}, and 92.81 kJ.mol^{-1} for white sorghum PPO, and 90.1 kJ.mol^{-1}, - 9.29 J.mol^{-1}.K^{-1}, and 93.37 kJ.mol^{-1} for yellow sorghum PPO. Zn^{2+}, Fe^{2+} and ascorbic acid inhibited PPO while Cu^{2+}, Na^{+} and K^{+} activated the enzyme. The results suggest the processing parameters for controlling PPO in potential industrial application of white and yellow sorghum grains.

University of SS. Cyril and Methodius in Trnava

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is indigenous to Africa and is a member of the grass family *Poaceae* and has high morphological variations (Deu and Hamon 1994; Hariprasanna and Patil 2015). Sorghum is ranked as the fifth most important grain in terms of production, preceded by wheat, rice, maize and barley (Sanni and Fatoki 2017a). Nigeria is the third largest world producer after the United States and India (FAOSTAT 2011), accounting for about 71 % of the total sorghum production in West Africa which could be of different varieties, with two best known species which are *Sorghum vulgare* and *Sorghum bicolor* (L.) Moench (Ogbonna 2011). Sorghum is a dietary staple for about 500 million people in over 30 countries of the semi-arid tropics (Dahlberg *et al*. 2011). Sorghum find its application in human food, such as bread, malt drinks and beer; livestock feed; and renewable energy source such as bioethanol (Dahlberg *et al*.

Corresponding author: hezekiahfatoki@gmail.com, dmsanni@futa.edu.ng

2011). It is a source of nutrients and bioactive compounds, especially 3-deoxyanthocyanidins, tannins and polycosanols, which beneficially modulate, *in vitro* and *in vivo*, the metabolic markers for varieties of diseases. (Sanni and Fatoki 2017a).

Polyphenol oxidases (PPO; 1, 2-benzenediol: oxygen oxidoreductase; EC 1.10.3.1) are copper containing oxidoreductases that catalyze the hydroxylation and oxidation of phenolic compounds in the presence of molecular oxygen. They are widely found in plants, animals and microorganisms (Yoruk and Marshall 2003). The three types of polyphenol oxidases which were classified according to their ability to oxidizes different types of phenolic compounds are catecholase (1,2-benzenediol: oxygen oxidoreductase; E. 1.10.3.1), laccase (EC 1.10.3.2) and tyrosinase (monophenol monooxygenase; EC 1.14.18.1) (Sanchez-Ferrer *et al*. 1995; Erhan 2003; Mayer 2006). PPO cause oxidative browning reactions in many foods of plant origin, which result into deterioration in food quality by changing nutritional and organoleptic properties (Martinez and Whitaker 1995), but PPO has its advantage in the processing of tea and malt.

The PPO has been characterized either partially or fully, from different plant sources which include sweet potato (Manohan and Wai 2012) and yams (Sanni and Fatoki 2017b). Also, PPO of most grains of industrial application has been studied which include wheat (Interesse *et al*. 1980; Kihara *et al*. 2005; Naqvi *et al*. 2013), barley (Huynh and Jerumanis 1977). The potential industrial application of sorghum in Africa include large scale production of beverages (such as malt, pito/dolo, burukutu, kunu etc.), breads (such as kisra, injera, sourdough etc.) and porridges.

Although polyphenol oxidase content has been screened in grains of from fifty different sorghum varieties (Dicko *et al*. 2002) as well as the activity of PPO during malting of sorghum (Sanni and Fatoki 2017a). The understanding of thermodynamics, kinetics activities and physicochemical properties of purified PPO of sorghum will enhance the processing and production of sorghum-based products. Reduction in heating time and optimization of heating temperatures to minimize damage of nutrients and sensory components are part of the objectives of the current food processing technologies (Sant'Anna *et al*. 2012). The activity and thermostability of enzymes are important issues often considered when assessing the economic feasibility of enzyme-based industrial processes. High stability is generally considered an economic advantage for a desirable enzyme because of reduced enzyme consumption (Souza *et al*. 2015) whereas low thermostability favors the inhibition of undesirable enzyme in an industrial process. The thermodynamic and kinetic studies can provide valuable information about the thermostability of enzymes at the operating temperature. Hence, this present work focused on the investigation of substrate specificity, kinetics parameters, effect of pH, pH stability, effect of pH on kinetics parameter, optimum temperature, thermal stability, and effect of salts on the activities of polyphenol oxidase from two varieties of *Sorghum bicolor* grains (Fig. 1) toward advancement of industrial utilization.

Fig. 1. Photograph of studied sorghum samples (**A**) White Sorghum (**B**) Yellow Sorghum.

Experimental

Sample collection

Fresh sorghum (white and yellow) was purchased from Oba market in Akure, Nigeria and dried at room temperature. The sample was identified and authenticated at the Department of Crop, Soil and Pest management (CSP) of the Federal University of Technology, Akure, Nigeria.

Preparation enzyme crude extract

One hundred and fifty grams (150 g) of sorghum grains were weighed and homogenized with 450 mL of chilled phosphate buffer (25 mM, pH 6.8) containing 0.5 % Triton X-100 and 0.01 % ascorbic acid, for 5 min and filtered through cheese cloth. The filtrate was centrifuged at 6,000 rpm at 4 ºC for 30 min. The supernatant was used as crude extract for further analysis.

Assay for polyphenol oxidase activity

PPO enzyme activity was determined with a spectrophotometer by measuring an increase in absorbance at 420 nm at room temperature by using catechol as substrate (Eq. 1). The reaction mixture consisted 0.1 mL freshly prepared enzyme extract and 2.9 mL of 20 mM catechol in 20 mM phosphate buffer pH 6.8 while the blank contained the buffer and the substrate. One unit (U) of PPO activity was defined as the amount of the enzyme that increased the absorbance by 0.001 per minute under the conditions of the assay (Sanni 2016) (Eq. 1).

$$Activity = \frac{Absorbance}{Incremental \times Time \times Volume} \quad (1)$$

Activity (U.mL^{-1}) of polyphenol oxidase, incremental is 0.001, time (min) of assay is 10 and the volume (mL) of assay is 3 (Eq. 2).

$$Specific\ activity = \frac{Acivity}{Protein\ concentration} \quad (2)$$

Specific activity (U.mg^{-1}) of polyphenol oxidase, activity (U.mL^{-1}) and protein concentration (mg.mL^{-1}).

Determination of protein concentration

Protein concentration was measured according to the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Purification of Enzyme

Ammonium sulphate precipitation

The crude extract obtained from each species was brought to 30 – 80 % ammonium sulphate $(NH4)_2SO_4$ saturation by slowly dissolving the precise amount of the solid salt evaluated by Encorbio ammonium sulphate calculator using magnetic stirrer. After the mixture stood for 2 h, the precipitate was separated by centrifugation at 6,000 rpm for 30 min at 4 ºC. The precipitate was dissolved in 15 mL of 20 mM phosphate buffer (pH 6.8) and dialyzed against the same buffer at 4 ºC for 72 h, with three changes of buffer. The dialyzed samples (partial extract) were further centrifuge at 14,000 rpm, and the resulting supernatant were used as the enzymes source in further experiments.

Purification on DEAE-Sephacel chromatography

The certain amount of pre-swollen DEAE-Sephacel stored in ethanol was poured into a clean 500 mL beaker and stirred with addition of distilled water to form a slurry. This was carefully loaded into column (2.5 × 20 cm) and washed with 500 mL of distilled water and later equilibrated with about 1 L phosphate buffer (20 mM, pH 6.8). Fifteen milliliters (15 mL) of dialyzed sample was loaded onto the column and washed with the same buffer at the flow rate of 18 mL.h^{-1} until the absorbance of the fractions at 280 nm was nearly zero. The bound protein was eluted with linear gradient of 0.0 – 0.5 M NaCl in 20 mM phosphate buffer pH 6.8 and collected in fractions of 5 mL. The absorbance of the fractions was measured at 280 nm and assayed for PPO activity. The fractions with PPO activity were pooled and concentrated by dialyzing against 4 M sucrose.

Purification on Sephadex G-100

Fifteen grams of Sephadex G-100 was soaked and allowed to swell in 20 mM phosphate buffer pH 6.8 for 72 h at room temperature. This was poured carefully into the column. (2.5 × 70 cm) and allowed to packed very well without space. It was later equilibrated with 1 L of 20 mM phosphate buffer pH 6.8. 15 mL of the concentrated pooled fraction from the DEAE-Sephacel step was carefully applied to the column. The proteins were eluted using the same buffer and 5 mL fractions were collected at a flow rate of 12.0 $mL.h^{-1}$. The absorbance of the fractions collected was measured at 280 nm and were assayed for PPO activity while those that contained PPO were pooled and used for further analysis.

Physicochemical properties, kinetics and thermodynamic of PPO

Substrate specificity of PPO

Substrate specificity was determined using 2 different substrates; L-DOPA and Catechol. The assay for PPO was carried out using 20 mM of L-DOPA at 475 nm, as well as 20 mM of catechol at 420 nm, as earlier described.

Determination of kinetics parameter

The catechol concentrations (30, 25, 20, 15, 10 and 5 mM) were prepared in 20 mM phosphate buffer (pH 6.8). PPO activity was assayed as according to standard assay procedure earlier described. Kinetics parameter; K_m and V_{max} were evaluated using Lineweaver-Burk plot.

Effect of pH on PPO activity

Effect of pH on polyphenol oxidase activity was determined by incubating 0.1 mL of purified PPO with 0.9 mL of 20 mM catechol prepared in each buffer pH range 2.0 – 9.0 using 20 mM Glycine/HCl (pH 2.0 – 3.0), 20 mM sodium acetate buffer (pH 4.0 – 5.0), 20 mM phosphate buffer (pH 6.0 – 7.0) and 20 mM Tris/HCl buffer (pH 8.0 – 9.0) at room temperature. The enzymes activity was determined at 420 nm.

Effect of pH on PPO stability

The effect of pH on stability was determined by incubating 0.2 mL enzyme solution in 1.8 mL of the buffer solution between pH range 4.0 – 9.0, using 20 mM Na-Acetate buffer (pH 4.0 – 5.0), 20 mM phosphate buffer (pH 6.0 – 7.0) and 20 mM Tris/HCl buffer (pH 8.0 – 9.0) at room temperature. The residual polyphenol oxidase activity was determined according to the standard assay procedure.

Effect of pH on kinetics parameter

Effect of pH on kinetics parameters was determined according to the method of Kolawole *et al.* (2005). Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) were determined using catechol as substrates. Substrate concentrations (30, 25, 20, 15, 10 and 5 mM) was prepared in different buffer pH between 4 – 9, using 20 mM Na-acetate buffer (pH 4.0 – 5.0), 20 mM phosphate buffer (pH 6.0 – 7.0) and 20 mM Tris-HCl buffer (pH 8.0 – 9.0); Polyphenol oxidase activity was assayed according to standard assay procedure earlier described. Data were plotted according to the method of Lineweaver-Burk. The V_{max}/K_m value was evaluated and plotted against the pH. The apparent pK_a of ionized amino acids was obtained from the peak of the plot of V_{max}/K_m against pH.

Effect of temperature on PPO activity

Effect of temperature on PPO activity was carried out between 30 °C to 90 °C at 10 °C interval. The assay mixtures consisting of 0.2 mL of the enzyme and 1.8 mL of 20 mM catechol were incubated at each temperature for 10 min, and the absorbance was read at 420 nm according to the standard assay procedure earlier described.

Effect of temperature on PPO stability

Thermal stability of the PPO was determined by incubating purified enzyme at different temperatures range of 30 – 90 ºC for 60 min. At 10 min interval the residual activity was determined according standard assay procedure.

Thermal inactivation was evaluated in the temperature range of 50 – 80 °C after 20 min of enzyme incubation (Manohan and Wai 2012), and the residual PPO activity (*A*) was determined according to the standard procedure described earlier. The data obtained were used to evaluated activation energy (E_a), enthalpy (ΔH), entropy (ΔS) and Gibbs free energy (ΔG) using Equation 1 to 11.

In the thermodynamic study, the percentage residual PPO activity is calculated by comparison to the initial enzyme activity (A_0). The rate constants *k* for first-order inactivation was determined from the slopes of the inactivation time courses according to the following equation (Eq. 3).

$$\log\left(\frac{A}{A_0}\right) = -\left(\frac{k}{2.303}\right)t \qquad (3)$$

Where A_0 is the initial enzyme activity (before heating where time *t* is zero) and *A* is the activity after heating for time *t*. A useful indication of the rate of a first-order chemical reaction is the half-life $t_{1/2}$, of a substance, the time it takes for its concentration to fall to half the initial value. The time for [*A*] to decrease from [A_0] to ½[A_0] in a first-order reaction. The half-life of the enzyme ($t_{1/2}$) calculated according to the following equation (Eq. 4).

$$t_{1/2} = \frac{(\ln 2)}{k} \qquad (4)$$

The main point to observe about this result is that, for a first-order reaction, the half-life of a reactant is independent on its initial concentration. In addition, decimal reduction time (*D*-value) is defined as the time required to pre-incubate the enzyme at a given temperature to maintain 10 % residual activity or reduce the initial activity by 90 % (Sant'Anna *et al.* 2012; Manohan and Wai 2012; Wong and Lee 2014), and was estimated from the relationship between *k* and *D* according to the Eq. 5.

$$D = \frac{\ln 10}{k} \qquad (5)$$

The *Z*-value is the temperature increase required for one-log_{10} reduction (90 % decreases) in *D*-value or the temperature needed to vary *D*-value one log unit. It was determined from a plot of log *D* versus temperature (ºC). The slope of the graph is equal to -1/*Z* (Sant'Anna *et al.* 2012; Wong and Lee 2014).

The temperature of treatment and the rate constant (*k*) in a denaturation process was related according to the Arrhenius equation (Eq. 6).

$$k = Ae^{-\frac{Ea}{RT}} \qquad (6)$$

Equation 2.4 can be transformed as in Eq. 7

$$\ln k = \ln A - \frac{Ea}{T} \qquad (7)$$

$$-R\ln k = -R\ln A + \frac{Ea}{T} \qquad (8)$$

where *R* is the universal gas constant (8.314 J.mol^{-1}.K^{-1}), *k* is the reaction rate constant value, *A* is the Arrhenius constant, E_a is the activation energy (energy required for the inactivation to occur), and *T* is the absolute temperature in Kelvin. Slopes were calculated by linear equation ($y = mx + c$). The energy of activation of denaturation (E_a) was calculated from the slopes of these Arrhenius plots, ($-R \ln k$) values versus reciprocal of absolute temperatures ($1/T$) according to Eq. 8, and the ordinate intercept corresponds to $-R \ln A$.

The values of the activation energy (E_a) and Arrhenius constant (*A*) allowed the determination of different thermodynamic parameters such as variations in enthalpy (ΔH; Eq. 9), entropy (ΔS; Eq. 10) and Gibbs free energy (ΔG; Eq. 11), respectively (Sant'Anna *et al.* 2012), according to the following expressions.

$$\Delta H^* = Ea - RT \qquad (9)$$

$$\Delta S^* = R\left(\ln A - \ln\left(\frac{K_B}{h_p}\right) - \ln T\right) \qquad (10)$$

$$\Delta G^* = \Delta H^* - T\Delta S^* \qquad (11)$$

Where K_B is the Boltzmann constant (1.38×10^{-23}J.K^{-1}), h_P is the Planck constant (6.626×10^{-34} J.s), and *T* is the absolute temperature (K).

Effect of salts and ascorbic acid

The effect of salts such as copper sulphate, zinc sulphate, iron sulphate, sodium chloride, potassium chloride and ascorbic acid, at three different concentrations (10, 30 and 50 mM) on enzyme activity were examined. The salts and ascorbic acid were each prepared in 20 mM catechol. The assay

Table 1. Purification of PPO from white and Yellow Sorghum.

Samples	Purification Steps	Volume [mL]	Protein [mg.mL^{-1}]	Total protein [mg]	Activity [U.mL^{-1}]	Total activity [U]	Specific activity [U.mg^{-1}]	Yield [%]	Fold
White	Crude	345.00	4.47	1,540.25	2.24	773.84	0.50	100.00	1.00
	Ammonium sulphate precipitation	33.00	66.07	2,180.34	5.24	172.92	0.08	22.35	0.16
	Ion-exchange chromatography on DEAE-Sephacel	80.00	6.70	535.68	2.60	164.40	0.31	21.24	0.61
	Gel filtration on Sephadex G-100	45.00	1.60	47.70	1.26	56.70	1.19	7.33	2.37
Yellow	Crude	325.00	8.93	2,901.93	2.56	832.00	0.29	100.00	1.00
	Ammonium sulphate precipitation	35.00	59.22	2,072.81	5.30	185.50	0.09	22.30	0.31
	Ion-exchange chromatography on DEAE-Sephacel	100.00	3.75	339.40	1.40	140.00	0.41	16.83	1.44
	Gel filtration on Sephadex G-100	55.00	0.06	2.97	1.86	102.30	34.44	12.30	120.00

was done at room temperature as earlier described above. The percentage effect (inhibition or activation) was calculated (Eq. 12).

$$Effect\ [\%] = \left[\frac{A_t - A_0}{A_0}\right] \times 100 \qquad (12)$$

Where A_0 is initial PPO activity (without salt/additive); A_t is PPO activity with salt/additive, where (-) is inhibition, (+) is activation at a baseline of zero (0).

Data analysis

All experiments were performed in triplicates and average of the data sets were processed using Microsoft excel 2010.

Results and Discussion

The results of PPO activity obtained from two varieties of *Sorghum bicolor* in this project, confirmed the occurrence and distribution of PPO in sorghum. Reports by other authors have shown the presence of PPO in both the leaves and the grain of sorghum (Luthra *et al.* 1988; Gowda *et al.* 1989; Dicko *et al.* 2006). The crude activity of PPO was found to be high in malted and non-malted yellow sorghum grains than in white counterpart (Sanni and Fatoki 2017a).

Purification Parameter of the PPO

The polyphenol oxidase activity of white sorghum was found to be 2.234 U.mL^{-1} and 2.055 U.mL^{-1} for crude and ammonium sulphate precipitation samples respectively while that of yellow sorghum was 2.560 U.mL^{-1} and 5.300 U.mL^{-1} for crude and ammonium sulphate precipitate samples respectively (Table 1). The elution profile of both varieties showed the same pattern. Two activity peaks were obtained from DEAE-Sephacel chromatography, one in flow through fractions and the other in the gradient elution fractions. Overall, the polyphenol oxidase (PPO) activities of yellow sorghum were found to be slightly higher than that PPO of the white sorghum.

The purification of PPO from many plants to homogeneity has remained difficult possibly due to the high phenolic content and irreversible binding of phenolics to PPO during purification steps (Mishra and Gautam 2016). In this study, there was an obvious difference in the purification results obtained from two varieties of *Sorghum bicolor*. These variations in fold purification and yield could be due to differences in the concentration and types of phenolics affecting their overall binding to the PPO protein (Yoruk and Marshall 2003).

Two peaks were obtained from ion exchange profile of PPO activity in both sorghum varieties,

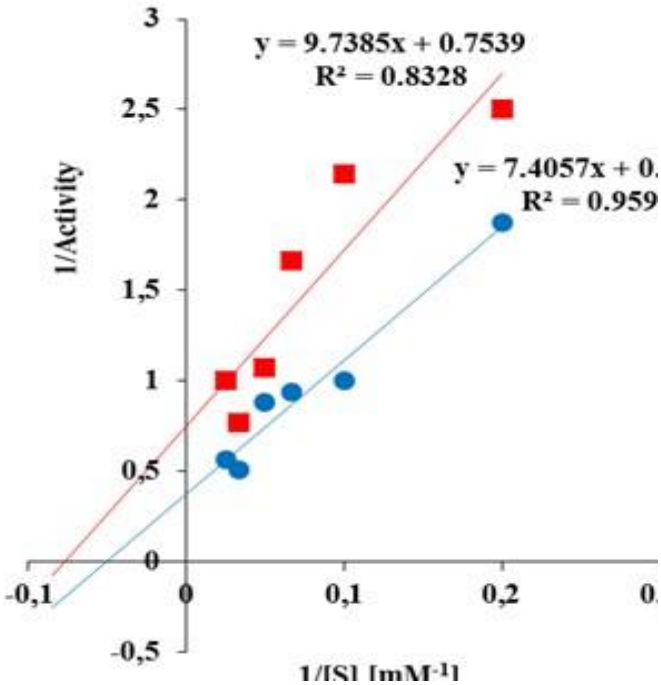

Fig. 2. Lineweaver-Burk Plot for PPO from White (round) and Yellow (square).

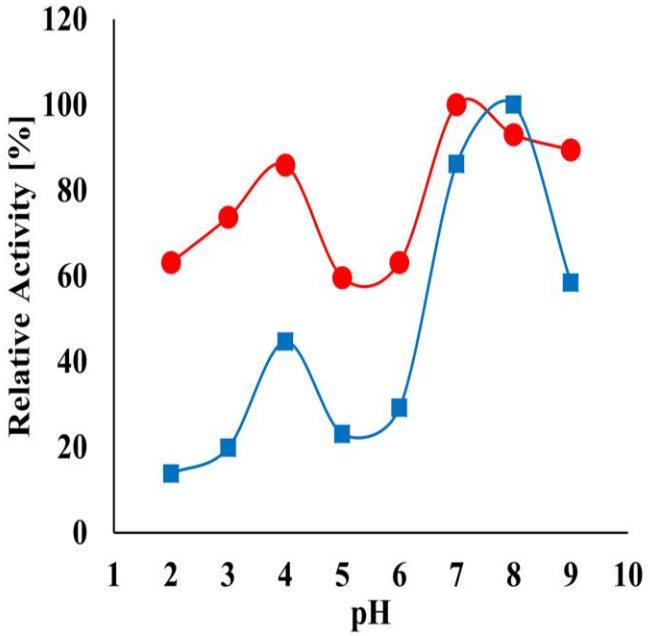

Fig. 3. Effect of pH on PPO from White (round) and Yellow (square). The result was computed relative to the alkaline optimum pH expressed as 100 % for each PPO respectively

and it may possibly be an indication that the PPO consist of two isoforms. Polyphenol oxidases isolated from barley, steeped barley, and green malt were resolved into two active forms on Sephadex G-200 column (Huynh and Jerumanis 1977). The two isoform of PPO obtained for each varieties of sorghum grains in this study, align with the evidence of three isoforms of crude PPO found in the sorghum leaves (Dicko *et al.* 2006).

Substrate specificity of PPO

PPO has higher affinity using catechol than L-DOPA in both samples while yellow sorghum showed more activity than white sorghum in both substrates. The PPO from both white and yellow sorghum in this study showed greater specificity for catechol than L-DOPA and this indicates di-phenolase activity. Mishra and Gautam (2016) have reported the substrate specificity of PPO to be in the order of 4-methyl catechol > tert-butylcatechol > dihydrocaffeic acid > pyrocatechol > L-DOPA, D-DOPA > caffeic acid > chlorogenic acid > pyrogallol. Moreover, Dicko *et al*. (2006) observed difference in mono-phenolase and *o*-diphenolase activities of sorghum varieties and found that sorghum PPOs are more active with *o*-diphenols than with monophenols, and corroborated other findings from most plant PPOs (Martinez and Whitaker 1995). Previous study has shown that germination decreases the *o*-diphenolase activity and slightly increases the monophenolase activity of PPO, while the zymography revealed that germination does not induce new PPO isoenzymes in sorghum grain (Dicko *et al.* 2006). Since the activities of PPO can be kinetically controlled, during possessing the modulation of these activities will have interesting applications in the food and chemical industries (Dubey *et al.* 1998; Espin *et al.* 2001).

Determination of kinetics parameters

The kinetics parameters obtained using Lineweaver-Burk plot were V_{max} and K_m are 2.66 U.mL^{-1} and 19.72 mM for white sorghum PPO and 1.33 U.mL^{-1} and 12.92 mM for yellow sorghum PPO respectively using catechol substrate as shown in Fig. 2. The V_{max} and K_m showed that PPO from white sorghum have much better capacity and more affinity for the substrate used than that of yellow sorghum. K_m is a measure of the amount of enzyme that is bound in any form whatsoever to the substrate. The results showed that the rate of polyphenol oxidase activity

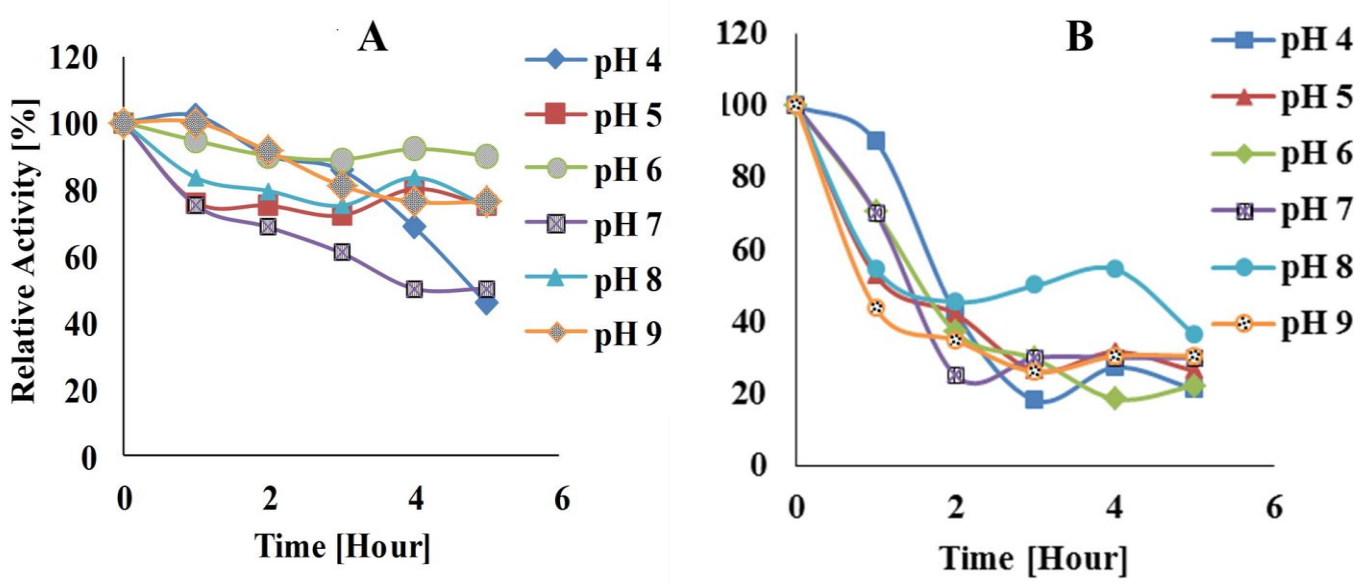

Fig. 4. Effect of pH on stability of PPO from (**A**) White Sorghum and (**B**) Yellow Sorghum. The result was expressed as percent relative activity to the activity at 0 hour (100 %).

in white sorghum was better than that of yellow sorghum.

Effect of pH on PPO activity

The two varieties of *Sorghum bicolor* PPO exhibited two optimal pH of activity each. Optimal pH of 4 and 7 were obtained for white sorghum PPO while that of yellow sorghum were found to be pH 4 and pH 8 as shown in Fig. 3. PPO from white sorghum showed high enzymatic activity of 93 % and 89 % at alkaline pH of 8.0 and 9.0, while that of yellow revealed a sharp reduction in activity after the optimal at pH 9.0. However, white sorghum PPO revealed a high percent relative activity of 85.95 % at pH 4.0 while yellow sorghum PPO revealed a lower percent relative activity of 44.62 % at pH 4.0.

These results indicate the possible occurrence of two PPO isoforms in both varieties of *Sorghum bicolor*, having different pH for optimal activity. However, the optimum pH of both varieties of sorghum was similar to the pH optima of partially purified PPO from two species of eggplants that have optimum activity at pH 4.0 – 4.5 and 7 (*Solanum depressum*), as well as pH 4 and 8 (*Solanum gilo*) for the oxidation of catechol (Sanni 2016). The optimum pH depends on the amino acid components of active site, while the isoelectric point depends of the full enzyme primary and tertiary structure (Fatoki 2016).

Effect of pH on PPO stability

The PPO residual activity were found to be relatively stable above 50 % at 2 h of incubation in all the pH ranges for white sorghum while those of yellow sorghum were below 50 % as shown in Fig. 4. White PPO stability were found in both acidic region (pH 5 and 6) with percent relative activity of 80 % and 90 % as well as in alkaline region (pH 8 and 9) with relative activity of 75 % and 76 % respectively after 5 h of incubation. The white sorghum PPO was found to be relatively stable at 5 hours of incubation retaining over 70 % of its initial activity within the neutral pH while that of yellow sorghum were below 40 %. This showed that PPO from white sorghum is highly stable within the neutral pH than that yellow sorghum. This may be an indication of possible difference in the amino acid residues composition at the active site of the PPO from these two varieties of sorghum.

Effect of pH on kinetics parameters

The dependencies of pH on kinetics parameters showed that activity of PPO from white sorghum was optimal at pH 8 independent of the substrate concentrations as shown in Fig. 5 while PPO from yellow sorghum was optimal at pH 6 – 7 independent of the substrate concentration. The slight difference observed in the PPO activity

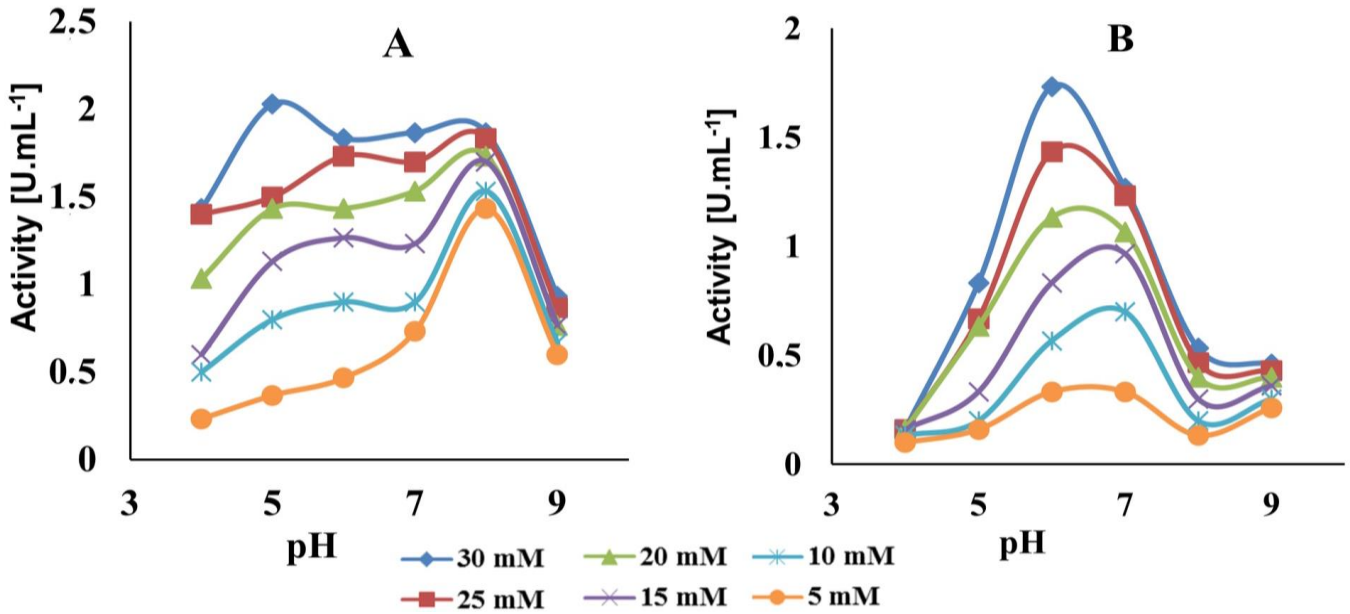

Fig. 5. Effect of pH and Substrate Concentration on Activity of PPO from (**A**) White Sorghum and (**B**) Yellow Sorghum.

may be due to difference in variety of sorghum. At concentration 25 mM and 30 mM, the optimum activity was in close range of pH 7 – 8. Lineweaver-Burk plot was used to evaluate the kinetics parameter at various pH for both sorghum. varieties (data not shown) from which V_{max} and K_m were obtained. The plot of V_{max}/K_m against pH gave *pKa* 7.4 and 8.7 for PPO from white sorghum and *pKa* 5.4, 7.4 and 8.5 for PPO from yellow sorghum (data not shown). The effect of pH on the kinetic parameter showed that pH dependencies of PPO activity at independence of the substrate concentrations, better provide direct useful insight for the evaluation of the *pKa* of an enzyme. Though a *pKa* value does not represent the microscopic ionization of a particular group but is a combination of this value and various equilibrium constants between different conformational states of the molecule. These results suggest the occurrence of cysteine (thiol group) and histidine (imidazole group) at active site of sorghum (*Sorghum bicolor*) polyphenol oxidase. Kolawole *et al.* (2005) earlier predicted the occurrence of cysteine and histidine at the active site of Fonio millet seed (*Digitaria exilis*) beta-amylase.

Effect of temperature on PPO activity

The optimum temperature of PPO from both white and yellow sorghum were found at 40 ºC and 30 ºC respectively. The PPO from both *Sorghum bicolor* varieties exhibited a similar pattern of gradual reduction in enzymatic activity after the optimal temperature were obtained. However, PPO from white sorghum revealed a higher percentage activity of 90 and 100 % compare to 100 and 94 % obtained for yellow sorghum PPO at 30 and 40 °C respectively as shown in Fig. 6.

The optimum temperature of 40 ºC and 30 ºC obtained for PPO activity in white and yellow sorghum was followed by a decrease in relative enzyme activity as the temperature increase above the optimum value. This result probably favored increase activity of PPO during germination process in beverage production. Sanni and Fatoki (2017a) reported increase in PPO activity in germinating sorghum in the malting floor during the early stage of brewing process while kilning and mashing at temperature of about 120 ºC will ensured total loss of PPO activity and favors the late stage of brewing process. The decrease in activity at high temperature was possibly owing to the thermal denaturation of PPO.

Effect of temperature on PPO stability and dynamics

The result showed that PPO from both white and yellow sorghum varieties were most stable at 30 – 40 ºC as the PPO showed over 60 % residual

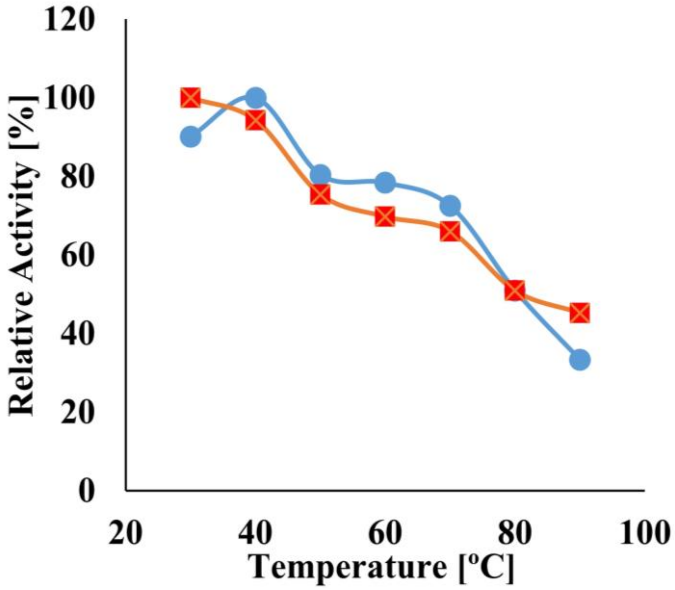

Fig. 6. Effect of temperature on PPO from White (round) and Yellow (square). The result was computed relative to the optimum temperature expressed as 100 % for each PPO respectively

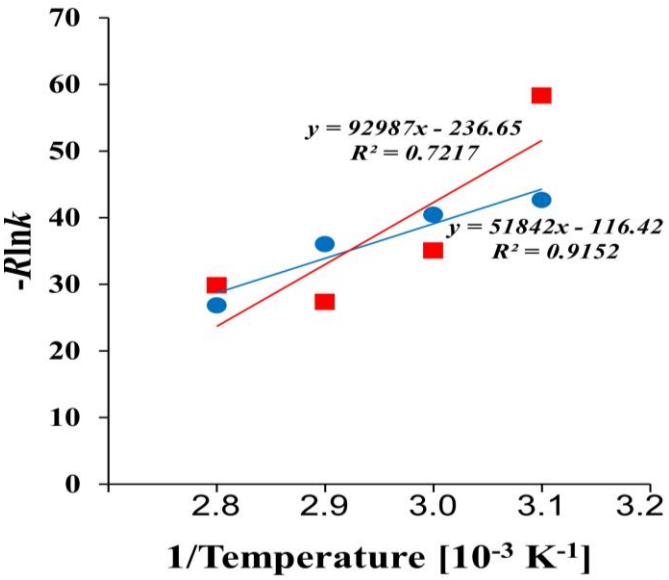

Fig. 7. Arrhenius Plot for PPO from White (round) and Yellow (square) Sorghum between 50 – 80 ºC at 20 min of incubation. The slope equals activation energy (Ea).

activity were observed between 30 – 50 ºC after 60 min of incubation in white and yellow sorghum. The PPO from white sorghum showed 37 % and 0 % relative activity after 60 min of incubation at 60 ºC and 70 – 90 ºC respectively, while over 20 % relative activity was observed after 60 min of incubation at 60 – 80 ºC for yellow sorghum. The result showed that PPO from yellow sorghum is thermally stable than PPO of white sorghum. The moderate thermal stability of yellow sorghum PPO will favor the development of flavors and color during the curing in malt production but will be a detrimental in confectionary processes if not adequately control at onset with another parameter such as additives.

The inactivation constant (K_{inact}) increased from 5.8×10^{-3} to 39.5×10^{-3} min^{-1} for white sorghum PPO, and 0.9×10^{3} to 27.5×10^{3} min^{-1} for yellow sorghum PPO, as the temperature increases from 50 – 80 ºC respectively. It was observed that half-life ($t_{1/2}$) of the PPO from both sorghum varieties, decreases as temperature increases. *D*-value is the temperature sensitivity parameter and *Z*-value is the temperature increase needed for a 90 % reduction in *D*-value. These results of kinetic study showed that the thermal inactivation of sorghum's PPO using catechol as substrate followed first-order kinetics However, the inactivation constant (k_{inact} or *k*-value) of PPO from white sorghum was linear while that of yellow sorghum was hyperbolic or non-linear. *D*-value and k-values decreased and increased, respectively with increasing temperature in both varieties, indicating faster polyphenol oxidase inactivation at higher temperatures. Manohan and Wai (2012) described denaturation of sweet potato PPO as a first-order reaction with *k* values between 0.0075 and 0.0657 min^{-1}. The half-life ($t_{1/2}$) value of sorghum PPOs in this study was between 117.46 to 17.54 min for white sorghum PPO and 770.0 to 25.20 min for yellow sorghum PPO.

Z-value is the temperature increase needed to vary *D*-value one log unit. The *Z*-value of 36.9 °C and 20.58 °C, obtained for white and yellow sorghum PPO respectively. In general, high *Z*-values mean more sensitivity to the duration of heat treatment and lower *Z*-values mean more sensitivity to increase in temperature (Barrett *et al*., 1999). Differences in the kinetics of heat activation of the two varieties of sorghum PPOs 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 and Noomhorm 2006). The Arrhenius plot for the two varieties of sorghum PPO were shown in Fig. 7. The results thus suggest that white and yellow PPO are relatively thermo-

Table 2. Thermodynamics parameter of PPO from white and yellow sorghum after 20 minutes of incubation.

Sorghum sample PPO	Temp. [°C]	K_{inact} [10^{-3} min^{-1}]	$t_{1/2}$ [min]	*D* [min]	*Z* [°C]	Temp. [K]	*Ea* [kJ.mol^{-1}]	ln*A*	$\Delta H^{\#}$ [kJ.mol^{-1}]	$\Delta S^{\#}$ [J.mol^{-1}.K^{-1}]	$\Delta G^{\#}$ [kJ.mol^{-1}]
White	50	5.90	117.46	390.27		323			49.16	-129.15	90.87
	60	7.70	90.00	299.04		333			49.07	-129.40	92.16
					36.90		51.84	14.00			
	70	13.10	52.90	175.77		343			48.99	-129.65	93.46
	80	39.50	17.54	58.29		353			48.91	-129.89	94.76
Yellow	50	0.90	770.00	2,558.43		323			90.30	-8.92	93.18
	60	14.70	47.14	156.64		333			90.22	-9.17	93.45
					20.58		92.99	28.46			
	70	37.10	18.68	62.06		343			90.14	-9.42	93.37
	80	27.50	25.20	83.73		353			90.05	-9.66	93.46

stable enzymes with the *Ea* of 51.84 kJ.mol^{-1} and 92.99 kJ.mol^{-1}, respectively, at 20 min of incubation. High activation energy reflects a greater sensitivity of the sorghum PPOs to temperature change as indicated for PPO from other sources (Weemaes *et al.* 1998; Chutintrasri and Noomhorm 2006). This suggests that the denaturation process requires a high energy input for the enzyme-substrate complex to initiate denaturation probably due to a possible compact structure of enzymes and the strength of the thiol groups (–SH) or disulfide bond (–S–S–) at the active site (Björck 1992).

Thermal inactivation of PPO from sorghum varieties in this study gave an average value of ΔH, ΔS and ΔG of 49.03 kJ.mol^{-1}, - 129.52 J.mol^{-1}.K^{-1}, and 92.81 kJ.mol^{-1} for white sorghum PPO, and 90.1 kJ.mol^{-1}, - 9.29 J.mol^{-1}.K^{-1}, and 93.37 kJmol^{-1} for yellow sorghum PPO respectively at 20 min of incubation and temperature of 50 – 80 °C as shown in the Table 2. The high values of change in enthalpy obtained for the different treatment temperatures revealed that the enzyme undergoes a considerable change in conformation during denaturation. Positive values of ΔH of the PPO indicate the endothermic nature of the oxidation reactions. The negative values of entropy indicate that there are no significant processes of aggregation respectively, otherwise the values of entropy would be negative (Anema and McKenna 1996). The Gibbs free energy (ΔG) is a measure of the spontaneity of the inactivation processes. The free energy of the PPO of both varieties of sorghum increases slightly with increasing temperature, and the result of this study showed Gibbs free energy obtained for PPO from both sorghum varieties were the same and positive, which showed that the reactions or inactivation processes of PPO were not spontaneous which could be due to presence of disulphide and imidazole linkage at the active sites (Dietler and Lerch 1982).

Effect of salts and ascorbic acid on PPO activity

The effect of salts and additives on the PPO activity at three different concentrations from the two sorghum varieties is given in Table 3. Ascorbic acid, Zn^{2+} and Fe^{2+} have increased inhibitory effect on PPO activity from both white and yellow sorghum varieties in concentration dependent manner (from 10 to 50 mM). Similar activation effect was observed for Cu^{2+}. Highest activation effect was obtained at 30 mM for Na^{+} and 50 mM for K^{+} in both varieties. This result corroborates the evidence of full activity of yam polyphenol oxidases (PPO1 and PPO2) in the presence of Na^{+} and Cu^{2+} (Yapi *et al.* 2014). The involvement of copper as a prosthetic group in PPO is essential for enzyme activity since the active site of the enzyme consists of two copper atoms each in the ligand field with three conserved histidine residues. Copper-chelating reagents inactivate enzyme, while activity can be restored by the addition of excess copper (Yoruk and Marshall 2003). Previously, strong inhibitory effect of ascorbic acid has been reported for PPO from sweet potato (Manohan and Wai 2012), cassava (Wong and Lee 2014), eggplant (Sanni 2016), and *Dioscorea* (Sanni and Fatoki 2017b).

Table 3. Effects of salts and ascorbic acid on activity of PPO from white and yellow sorghum.

Salt/Additive	Effect on PPO activity [%]					
	White sorghum			Yellow sorghum		
	10 mM	30 mM	50 mM	10 mM	30 mM	50 Mm
$ZnSO_4$	-39.65	-47.72	-61.35	9.90	14.99	-14.99
$CuSO_4$	34.08	38.58	54.53	29.99	34.93	44.98
$FeSO_4$	-81.63	-83.65	-95.90	-41.75	-58.25	-83.25
NaCl	0.00	69.44	20.43	100.00	250.00	41.75
KCl	59.22	65.34	70.50	25.00	41.75	125.00
Ascorbic Acid	-51.01	-42.87	-46.91	-58.25	-25.00	-33.25

(-) inhibition, (+) activation. Zero was taken as the baseline value for the control (PPO activity in the absence of salt or ascorbic acid).

Conclusions

Polyphenol oxidase (PPO) was successfully extracted from the grains of white and yellow varieties of sorghum (*Sorghum bicolor* (L) Moench). The results on the enzyme properties obtained in this study provide a good starting point for processing and potential use of sorghum grains for large scale production of edible foods, such as beverages (such as malt, pito/dolo, burukutu, kunu etc.), bread (such as kisra, injera, sourdough etc.), pharmaceutical excipient, and others. Operating at the optimal parameters can inhibit browning activity of PPO and enables maintaining sensory and nutritional properties of sorghum during industrial processing.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

Anema SG, McKenna AB (1996) Reaction kinetics of thermal denaturation of whey proteins in heated reconstituted whole milk. J. Agric. Food Chem. 44: 422-428.

Arogba SS, Ajiboye OL, Ugboko LA, Essienette SY, Afikabi PO (1998) Properties of polyphenol oxidase in mango (*Mangifera indica*) kernel. J. Agric. Food Chem. 77: 459-462.

Arslan O, Temur A, Tozlu I (1997) Polyphenol oxidase from *Allium sp*. J. Agric. Food Chem. 45: 2861-2863.

Barrett NE, Gryison AS, Lewis MJ (1999) Contribution of the lactoperoxidase system to the keeping quality of pasteurized milk. J. Dairy Res. 66: 73-80.

Björck L (1992) Indigenous enzymes in milk Lactoperoxidase. Vol. 1. *In* Advanced Diary Chemistry, 1st Ed., Fox, P.F. (Ed.), Springer, London, p. 323-338.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analyt. Biochem. 72: 248-254.

Chutintrasri B, Noomhorm A (2006) Thermal inactivation of polyphenoloxidase in pineapple puree. Lebensmittel-Wissenschaft. 39: 492-495.

Dahlberg J, Berenji J, Sikora V, Latković D (2011) Assessing sorghum [*Sorghum bicolor* (L) Moench] germplasm for new traits: food, fuels and unique uses. Adv. Access Public. Maydica. 56: 1750.

Deu M, Hamon P (1994) The genetic organization of sorghum. Agric. Développ. 3: 25-30.

Dicko MH, Gruppen H, Traoré AS, Voragen AGJ, van Berkel WJH (2006) Phenolic compounds and related enzymes as determinants of sorghum for food use. Biotech. Mol. Biol. Rev. 1: 21-38.

Dicko MH, Hilhorst R, Gruppen H, Traoré AS, Laane C, van Berkel WJH, Voragen AGJ (2002) Comparison of content in phenolic compounds, polyphenol oxidases and peroxidases in grains of fifty sorghum varieties from Burkina Faso. J. Agric. Food Chem. 50: 3780-3788.

Dietler C, Lerch K (1982) Reaction inactivation of tyrosinase. *In* Oxidases and Related Redox Systems, King, TE Mason, HS, Morrison, M (Eds.), Pergamon Press, New York. p. 305-317.

Doğan S, Ayyildiz Y, Doğan M, Alan Ü, Diken ME (2013) Characterisation of polyphenol oxidase from *Melissa officinalis* L. subsp. *officinalis* (lemon balm). Czech J. Food Sci. 31: 156-165.

Duangmal K, Owusu ARK (1999) A comparative study of polyphenol oxidases from taro (*Olocasia esculenta*) and potato (*Solanum tuberosum* var. Romano). Food Chem. 64: 351-359.

Dubey S, Singh D, Misra RA (1998) Enzymatic synthesis and various properties of poly (catechol)-bilirubin oxidase. Sorghum malts for the production of a lager. Enzym. Microb. Tech. 23: 432-437.

Erhan A (2003) Production and biochemical characterization of polyphenol oxidase from *Thermomyces lanuginosus*. M.Sc Thesis, Department of Biotechnology. The Graduate School of Natural and Applied Sciences of the Middle East Technical University, Ankara, Turkey.

Espin JC, Soler-Rivas C, Cantos E, Tomas-Barberan FA, Wichers HJ (2001) Synthesis of the antioxidant hydroxytyrosol using tyrosinase as biocatalyst. J. Agric. Food Chem. 149: 1187-1193.

FAOSTAT (2011) Food and Agricultural Organization of the United Nations, ProdSTAT, Production Crops. HYPERLINK \l "ancor" \o "Current Document" Accessed: September 10, 2017.

Fatoki TH (2016) Comparative evaluation of computational and experimental analysis of polyphenol oxidase from sweet potato (*Ipomoea Batatas*). J. Microb. Biotech. Res. 6: 39-46.

Gowda P, Bhavanishankara S, Bhat SG, Bhat SS (1989) Peroxidase and polyphenol oxidase activities in sorghum and *Peronosclerospora sorghi* interaction. Current Sci. 58: 1037-1039.

Hariprasanna K, Patil JV (2015) Sorghum: Origin, Classification, Biology and Improvement. *In* Sorghum Molecular Breeding. Madhusudhana, R *et al.* (Eds.), Springer India, p. 15-20.

Huynh NV, Jerumanis J (1977) Study on barley and malt polyphenol oxidase I. Chromatography of barley, steeped barley and malt polyphenol oxidase. ASBC Journal. 35: 153-159.

Interesse FS, Ruggiero P, D'Avella G, Lamparelli F (1980) Partial purification and some properties of wheat (*Triticum aestivum*) *o*-diphenolase. J. Sci. Food Agric. 31: 459-466.

Kihara T, Murata M, Homma S, Kaneko S, Komae K (2005) Purification and characterization of wheat (*Triticum aestivum*) polyphenol oxidase. Food Sci. Tech. Res. 11: 87-94.

Kolawole AO, Sanni DM, Ajele JO (2005) Fonio millet seed (*Digitaria exilis*) *β*-amylase: possible involvement of histidine and cysteine in its catalytic mechanism. Biosci. Res. Comm. 16: 61-65.

Luthra YP, Ghandi SK, Joshu UN, Arora SK (1988) Total phenols and their oxidative enzymes in sorghum leaves resistant and susceptible to *Ramulispora sorghicola* Harris. Acta Phytopath. Entomol. 23: 393-400.

Manohan D, Wai WC (2012) Characterization of polyphenol oxidase in sweet potato (*Ipomoea batatas* (L.)). J. Adv. Sci. Art. 3: 14-30.

Martinez MV, Whitaker JR (1995) The biochemistry and control of enzymatic browning. Trends Food Sci. Tech. 6: 195-200.

Mayer AM (2006) Polyphenol oxidases in plants and fungi: going places? A review. Phytochemistry 67: 23188-2331.

Mishra BB, Gautam S (2016) Polyphenol oxidases. Biochemical and molecular characterization, distribution, role and its control. Enzyme Eng. 5:141-149.

Naqvi SMH, Batool I, Farooq FU, Deeba F, Hyder MZ, Mahmood T (2013) Polyphenol oxidase activities in wheat (*Triticum aestivum* L.) grain. Pak. J. Bot. 45: 407-410.

Ogbonna AC (2011) Current developments in malting and brewing trials with sorghum in Nigeria: A Review. J. Inst. Brew. 117: 394-400.

Oktay M, Kocaçaliskan KI, Sakiroglu H (1992) Polyphenol oxiades from Amasaya apple. J. Food Sci. 60: 494-496.

Sanchez-Ferrer A, Rodriguez JN, Garcia-Canovas F, Garcia-Carmona F (1995) Tyrosinase: a comprehensive review of its mechanism. Biochem. Biophy. Acta 1247: 1-11.

Sanni, DM (2016) Partial purification and characterisation of polyphenol oxidase from two commonly consumed eggplants (*Solanum mellongena depressum* and *Solanum gilo*) in Nigeria. Biochem. Mol. Biol. 1: 1-10.

Sanni DM, Fatoki TH (2017a) Evaluation of malting properties and activities of three enzymes from sorghum (*Sorghum bicolor*) during malting. Afr. J. Food Sci. Tech. 8: 090-098.

Sanni DM, Fatoki TH (2017b) Biochemical evaluation of polyphenol oxidase activities in yellow yam (*Dioscorea cayenensis*) and water yam (*Dioscorea alata*). Amer.-Euras. J. Agric. Environ. Sci. 17: 401-409.

Sant'Anna V, Cladera-Olivera F, Brandelli A (2012) Kinetic and thermodynamic study of thermal inactivation of the antimicrobial peptide P34 in milk. Food Chem. 130: 84-89.

Souza PM, Aliakbarian B, Filho EXF, Magalhães PO, Junior AP, Converti A, Perego P (2015) Kinetic and thermodynamic studies of a novel acid protease from *Aspergillus foetidus.* Int. J. Biol. Macromol. 81: 17-21.

Weemaes CA, Ludikhuyze LR, Broeck IV, Hendrickx ME (1998) Effect of pH on pressure and thermal inactivation of avocado polyphenol oxidase: a kinetic study. J. Agric. Food Chem. 46: 2785-2792.

Wong CW, Lee PLA (2014) Characterization of polyphenol oxidase from cassava leaf (*Manihot esculenta* crantz). Int. J. Plant Animal Environ. Sci. 4: 546-553.

Yapi JC, Gnangui SN, Ahi AP, Kouassi-Koffi JD, Kouamé LP (2014) Biochemical characterization of two polyphenol oxidase purified from edible yam (*Dioscorea cayenensis-rotundata* cv. Kponan) Cultivated in Côte d'Ivoire. Annual Res. Rev. Biol. 4: 3532-3548.

Yoruk R, Marshall MR (2003) A survey on the potential mode of inhibition for oxalic acid on polyphenol oxidase. J. Food Sci. 68: 2479-2485.