



Partial Purification and Characterisation of Polyphenol oxidase from Two Species of Bitter Yam (*Discorea dumentorum*)

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To cite this article:

David Morakinyo Sanni. Partial Purification and Characterisation of Polyphenol oxidase from Two Species of Bitter Yam (*Discorea dumentorum*). *Biochemistry and Molecular Biology*. Vol. 1, No. 2, 2016, pp. 11-16. doi: 10.11648/j.bmb.20160102.11

Received: June 1, 2016; **Accepted:** June 12, 2016; **Published:** July 6, 2016

Abstract: Polyphenol oxidases (PPO) are group of copper proteins, widely distributed phylogenetically from bacteria to mammals that catalyze the oxidation of phenolics to quinones which produces brown pigments in wounded tissues. PPO was isolated and partially characterized in white and yellow bitter yam (*Discorea dumentorum*). Extracts were partially purified using ammonium sulphate. The effects of optimum pH and temperature were investigated, while thermal and pH stability was also determined. The substrate specificity, kinetic and inhibition studies were also carried out. The optimal pH in the absence and presence of SDS were found to be 5.0 and 6.0, and 6.0 and 8.0 for white and yellow bitter yam respectively, while the optimum temperature for both white and yellow bitter yam was at 30°C. The enzyme was stable with over 90% activity after 3 hours incubation time at pH of 4.0 and 5.0, and 4.0 for yellow and white bitter yam respectively. The enzyme revealed over 70% remaining activities at all temperature investigated for yellow and at 30, 40 and 50°C for white bitter yam after 1 hour incubation. The K_m values of 6.8×10^{-2} mM and 8.0×10^{-2} mM, and 3.3×10^{-1} mM and 4.0×10^{-1} mM with L-DOPA and tyrosine as substrates for PPO from white and yellow bitter yam respectively. Higher activity was observed with L-DOPA than with tyrosine.

Keywords: Activity, Bitter Yam, Polyphenol oxidase, Partial Purification

1. Introduction

Bitter yam (*Discorea dumentorum*) is of major importance as food in Nigeria and many other tropical countries. They are perennial through root system but are grown as annual crops [2]. Bitter yam is more popular as vegetables in parts of West Africa than any other yam species in the World. It is a native to West Africa, today the *dumentorum* remains one of the principal yam cultivated in west Africa because its requires less labour than other yams. It is the third most important tropical root and tuber crop after cassava and sweet potato [9]. The crop is of major importance in the diet and economic life of people in West Africa, the Caribbean islands, parts of Asia, and Oceania [11, 23]. Besides fulfilling some physiological needs, it is capable of delivering health benefit due to the presence of antioxidant properties [1]. The yam tuber, which is the most important part of the plant, can be stored longer than other root and tuber crops, ensuring food security even at times of general scarcity. However,

damage incur by yam tubers in the cause of harvesting, storage and processing leads to adverse oxidative browning, cause by polyphenol oxidases as being an economic problem for both the producers and consumers.

Polyphenol oxidases (PPOs) are a group of copper-proteins, widely distributed phylogenetically from bacteria to mammals, that catalyze the oxidation of *ortho*-phenolics compounds such as caffeic acid and catechol to their respective quinones which produce brown pigments in wounded tissues [16]. These highly reactive PPO-generated quinones polymerize with other quinones, amino acids and proteins, leading to the production of black, brown and red pigments usually observed in damaged plant tissues and plant extracts. The obvious pigments are generally undesirable and responsible for unpleasant sensory qualities as well as losses in nutrient quality in food products. This explains why phenolic content and PPO activity have been considered major determinants in the quality of fruits and vegetables [14]. In intact tissues, PPO and its polyphenolic substrates

are found in separate compartments, plastids and vacuoles, respectively [16], thus no reaction occurs. The disruption of the cell integrity that occurs naturally during senescence and as a result of physical damage plant tissues bring PPO into contact with its substrates producing the so-called enzymatic browning observed in overripe fruits, bruised tissues, freshly cut fruits and vegetables and also in diseased and physiopathy tissues [27].

2. Methodology

2.1. Sample Collection

The white *Discorea dumetorum* and the yellow *Discorea dumetorum* both commonly known as “bitter yam (esuru)” were bought from farmers at Ilara town near Akure, Ondo state of Nigeria.

2.2. Preparation of Crude Extract

About 150g of each yam species (peeled) was thoroughly homogenized in 450ml of ice cold 25mM phosphate buffer (pH 6.8) containing 10mM ascorbic acid using a warring blender for 3min with a 60 seconds resting period to avoid local elevation in temperature. The mixture was filtered using four layers of cheese cloth. The filtrate obtained was centrifuged in a refrigerating centrifuge at 6,000 rpm (revolutions per minute) for 30 min at 4°C. The supernatant obtained was stored in a freezer and used as crude extracts for further studies.

2.3. Partial Purification of White and Yellow Bitter Yam

2.3.1. Ammonium Sulphate Precipitation

Supernatant obtained (175ml) from each species was brought to 80% ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$) saturation by slowly dissolving solid ammonium sulphate using magnetic stirrer. The precipitate was separated by centrifugation at 6,000 rpm for 30 minutes. The precipitate was dissolved in 0.1M phosphate buffer (pH 6.8) and dialyzed at 4°C overnight with three changes of buffer. The dialyzed samples were used as the polyphenol enzyme source in further experiments.

2.3.2. Determination of Polyphenol oxidase Activity

Polyphenol oxidase activity was determined by measuring the initial rate of quinone formation, as indicated by an increase in absorbance at 475 nm, using visible spectrophotometer. Change in absorbance was recorded after incubation at room temperature for 10mins [4]. The sample cuvette contained 0.7 ml of 10mM 3, 4-dihydrophenylalanine (DOPA) solution in 0.1 M phosphate buffer (pH 6.8) and 0.3 ml of the enzyme solution. The blank sample contained only 0.7 ml of 10mM 3, 4-dihydrophenylalanine and 0.3ml of 0.1M buffer solution. One unit of polyphenol oxidase activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 per minute.

2.3.3. Effect of PH on Polyphenol oxidase Activity in Presence and Absence of SDS

Effect of pH on polyphenol oxidase activity in presence

and absence of SDS was carried out according to the method of Escribano [6], in buffer pH range 4.0-9.0, using 0.1 M acetate (pH 4.0–5.0), 0.1 M phosphate (pH 6.0–7.0) and 0.1M Tris/HCl (pH 8.0-9.0). The reaction mixture in presence of SDS contains 0.69mM of the detergent while polyphenol oxidase activity was assayed according to standard assay procedure earlier described.

2.3.4. Effect of Temperature on Polyphenol oxidase Activity

The assay mixture containing the polyphenol oxidase and L-DOPA was incubated for 10mins at different temperature of 30 to 80°C at 10°C temperature interval. The absorbance reading was taken at 475nm according to standard assay procedure.

2.3.5. Thermal Stability

Thermal stability of the enzyme was determined by incubating the enzymes solution in 0.1 M phosphate buffer at pH 6.8 for 1 hour at different temperatures ranging from 30 to 80°C at 10°C temperature interval. The residual polyphenol oxidase activity was determined at 19mins time interval for 1 hour incubation period.

2.3.6. Kinetic Parameters

Michaelis Menten constant (K_m) and maximum reaction velocity (V_{max}) were determined using two different substrates (Tyrosine and L-DOPA). Substrate solutions were prepared in 0.1 M phosphate buffer (pH 6.8) at different initial concentrations (25, 20, 15, 10, 5 and 1 mM). Polyphenol oxidase activity was assayed according to standard assay procedure earlier described. Data were plotted according to the method of Lineweaver-Burk.

2.3.7. Substrate Specificity

Substrate specificity was determined by using 2 different substrates (Tyrosine and L- DOPA). 10mM of each substrate was prepared in 0.1 M phosphate buffer (pH 6.8). Enzyme activity was assayed as according to standard assay procedure earlier described.

2.3.8. Protein Determination

Protein concentration was measured according to the Bradford method [3] using bovine serum albumin (BSA) as a standard.

2.3.9. Effect of Inhibitors and Activator on the Enzyme Activity

Polyphenol oxidase activity was measured in the presence of reagents (ascorbic acid, EDTA disodium salt, benzoic acid and sodium dodecyl sulfate) at three concentrations (10, 5 and 1 mM) prepared in 10mM DOPA (pH 6.8). Polyphenol oxidase activity was assayed according to standard assay procedure as earlier described.

3. Results and Discussion

3.1. Activity of Enzyme

The activity of the crude enzyme (Polyphenol oxidase) from white *Discorea dumetorum* and yellow *Discorea*

dumentorum were 29 and 45.1 units/ml while the activity of the partially purified were 43 and 117.5 units/ml respectively. The protein contents of partially purified enzyme from white and yellow *Discorea spp.* were 0.34mg and 0.37mg while its specific activities and percentage yield were found to be 126.5 and 39.3%, and 317.6 and 67% unit/mg respectively. This is in agreement with the report of Muzac-Tucker [21] who stated reports from several authors explain the variances of PPO activity within the same species.

3.2. Effect of pH in the Presence and Absence of SDS on the Activity of Polyphenol oxidase

The effect of pH in the absence and presence of SDS on the activity of the Polyphenol oxidase of white and yellow *Discorea dumentorum* were shown in figure 1a, 1b, 2a and 2b. Optimum pH of activity for polyphenol oxidase from white bitter yam was observed at pH 5.0 while two optima pH of 6.0 and 8.0 was observed for polyphenol oxidase from yellow bitter yam. Yoruk and Marshall [37] reported that pH

optimum of polyphenol oxidase varies widely with plant source but is generally in the range of 4.0 - 8.0. Some plants show optimum pH in the acidic region for example, pH 4.5 for cherry and strawberry with 4-methyl catechol as substrate [8], pH 3.5-4.5 for apple and grape [17], pH optimal reported by Fraignier [8] for fruits including almond, apricot, peach and plum is similar to that observed for Polyphenol oxidase from white bitter yam in this study (pH 5.0). Harel [12] and Wong *et al.*, [33] stated that the two pH optima in yellow bitter yam maybe due to PPO isoforms. The optimum pH for maximum PPO activity was also subject to changes when assayed in the presence of the modulator, SDS. With few exceptions, most enzymes are inactivated by SDS. Some of these exceptions include enzymes which are activated by SDS, such as pancreatic lipase [5], pyruvate oxidase [27], tyrosinase [35], and polyphenol oxidase [31, 14, 7]. Broad bean polyphenol oxidase, exists in a latent state and can be activated several fold by SDS [7].

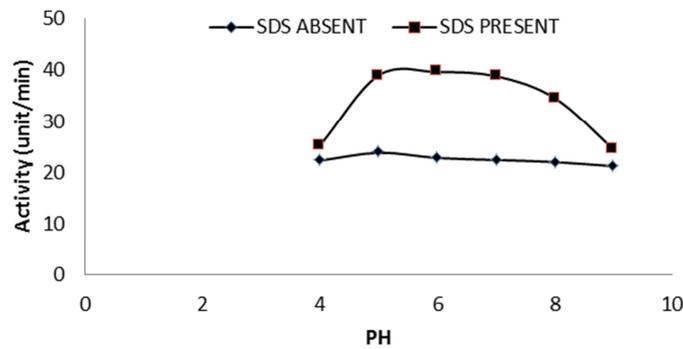


Figure 1a. Show the effect of pH on the activity of the enzyme in presence and absence of SDS.

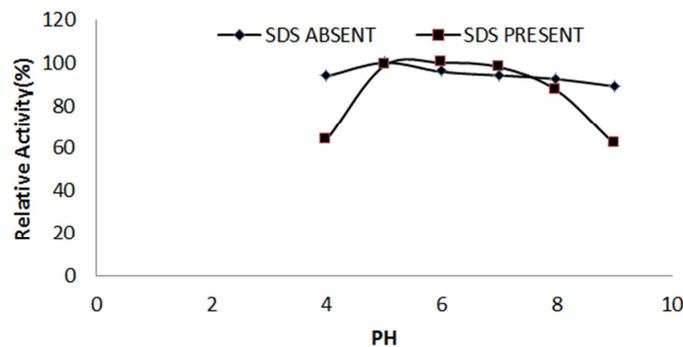


Figure 1b. Show the effect of pH on the enzyme relative activity in the presence and absence of SDS.

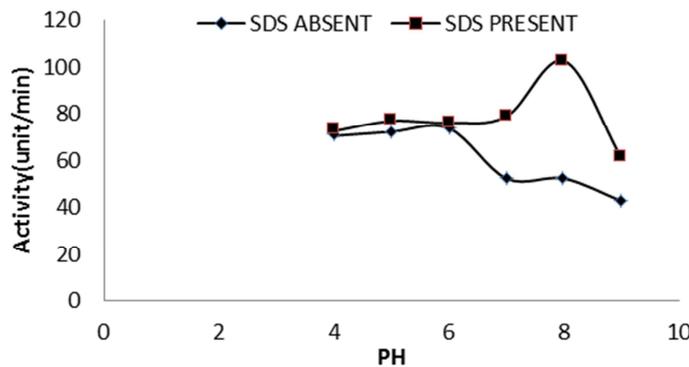


Figure 2a. Show the effect of pH on the activity of the enzyme in presence and absence of SDS.

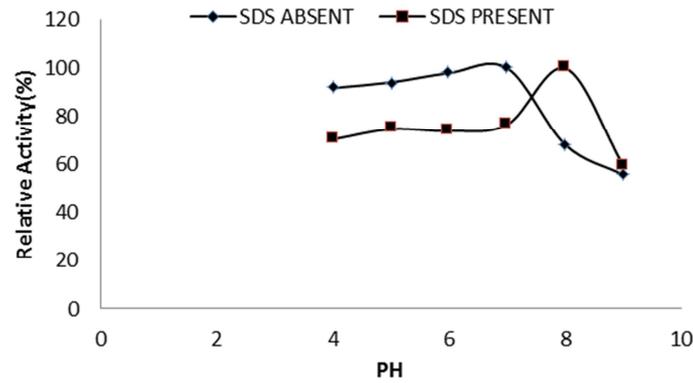


Figure 2b. Show the effect of pH on the enzyme relative activity in presence and absence of SDS.

3.3. Effect of Temperature on the Activity of Polyphenol oxidase

The influences of temperature on the enzyme activity of PPO from white and yellow *Discorea dumetorum* was shown in figure 3. Optimal temperature of polyphenol oxidase from both white and yellow *Discorea dumetorum* was found to be at 30°C. The majority of enzymes exhibit optimum temperature in the range of 30 to 40°C. Heating at 80°C inactivates some enzyme as absorbance sharply

decreases. The optima temperature for white and yellow bitter yam was similar to the optimum temperature of the polyphenol oxidase of another yam tuber species [10, 13]. Some plants have also been reported to show unusually high optimum temperature for example straw berry with an optimum temperature of 50°C using pyrocatechol as substrate [28] and cucumber with an optimum temperature of 50°C using catechol as substrate [19].

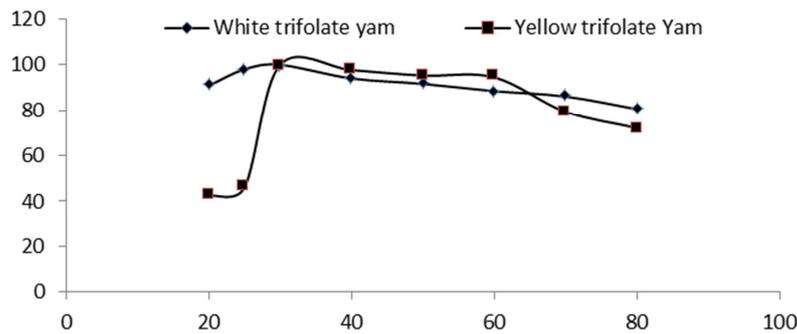


Figure 3. Effect of Temperature on the activity of polyphenol oxidase from white and yellow bitter yam.

3.4. Thermal Stability

The effects of temperature on the stability of polyphenol oxidase from white and yellow *Discorea dumetorum* were shown in figure 4a and 4b respectively. The partially purified enzyme from white bitter yam showed stability with over 70% residual activity at all the temperature investigated while stability at 70% residual activity was observed at 30°C, 40°C and 50°C and less than 40% residual activity at 60 and 70°C was observed for enzyme from yellow bitter yam. Therefore polyphenol oxidase from white bitter yam is more thermally stable than the one from yellow bitter yam. The result from white bitter yam is in line with the results of Ni Eidhin [23] who reported that PPO from apple show stability between 30 and 75°C, while above this temperature it becomes rapidly inactivated. The same behavior was observed for PPO from Chinese cabbage; it was thermally stable between 50 and 70°C and inactivated above this temperature [22]. In contrast, the result from yellow bitter yam agrees with Yang [36] results; PPO from banana was thermally stable at 30°C and even at temperatures greater than 60°C; the PPO displayed a partial residual activity.

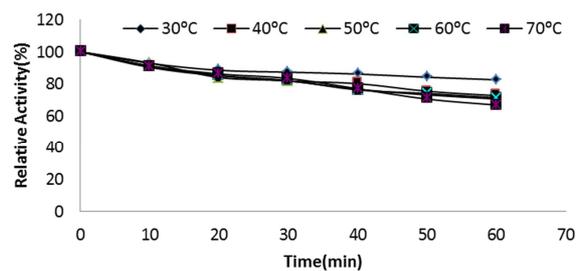


Figure 4a. Show the thermal stability of white bitter yam.

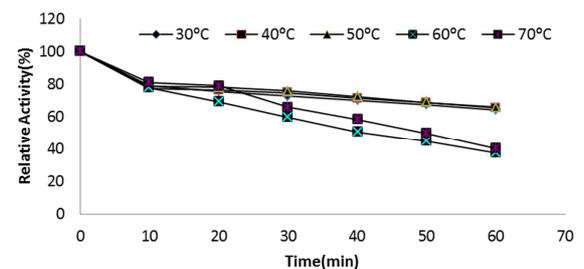


Figure 4b. Show the thermal stability of yellow bitter yam.

3.5. Substrate Specificity

The result of diphenol and monophenol on the polyphenol oxidase activity of white and yellow *Discorea dumetorum* was presented in figure 5. Polyphenol oxidase from white bitter yam was 36.6% and 17.7% compared to 100% and 36.9% for polyphenol oxidase from yellow bitter yam with L-DOPA and Tyrosine as substrates respectively. Sherman [30] reported that the level of polyphenol oxidase activity toward L-DOPA as a substrate varies widely in the plant kingdom. However, these differences could be due to the differing substrate specificity of polyphenol oxidase from different plants. This indicated that both white and yellow bitter yam polyphenol oxidase showed activity toward ortho-diphenols but low activity toward the monophenolic compound. The result from this research was similar to that of the work on polyphenol oxidase from different sources such as grape [16], field bean seed [24], strawberry [34], sunflower seed [26] and apple [38].

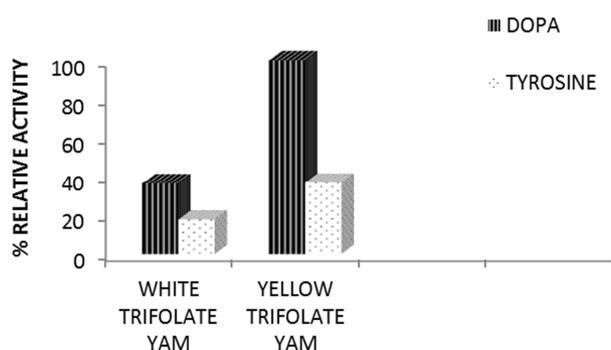


Figure 5. Effect of Diphenol and monophenol on the activity of polyphenol oxidase from white and yellow bitter (Trifoliolate) yam.

3.6. Kinetic Parameter

The K_m value of the white trifoliolate yam polyphenol oxidase using L- DOPA and Tyrosine was found to be $6.83 \times 10^{-5} M$ and $3.3 \times 10^{-4} M$ while that of yellow trifoliolate yam was $7.92 \times 10^{-5} M$ and $3.96 \times 10^{-4} M$ respectively. V_{max} for white trifoliolate yam was 2.3unit/ml and 2.2unit/ml, and yellow trifoliolate yam was 7.9unit/ml and 5.6unit/ml for L-DOPA and Tyrosine. The variability of V_{max} values amongst the species was related to the absolute concentration of polyphenol oxidase in their respective extracts, since absolute concentration of enzyme (E) shows direct proportionality with maximum enzyme activity (V_{max}). This result conforms to previous reports which noted significant differences and marked variations in polyphenol oxidase kinetic parameters between enzyme extract of different species of plants.

3.7. Effect of Inhibitor

Effect of different reagent on the polyphenol oxidase from yellow *Discorea dumetorum* and white *Discorea dumetorum* showed that of the inhibitors used, ascorbic acid was found to be the most effective, followed by benzoic acid, EDTA and SDS appears to activate the enzyme. The result of

this finding is in agreement with Umit Unal [32] results in which tea PPO was inhibited by ascorbic acid. However, SDS was found to activate PPO from both species of bitter yam in this research which is in agreement with finding of Moore and Flurkey [20]: that little activation occurred until 0.44mM SDS.

4. Conclusion

The specific activity of the partially purified polyphenol oxidase from white and yellow bitter yam (*Discorea dumetorum*) using ammonium sulphate was found to be 126.5 and 317.6unit/mg respectively. The optimal pH of polyphenol oxidase from white and yellow bitter yam (*D. dumetorum*) in the absent and presence of SDS were found to be at the pH 5.0 and 6.0, and pH 6.0 and 8.0 respectively, while the optimum temperature of the enzyme from both white and yellow bitter yam was at 30°C. The enzyme maintained stability of over 70% relative activities at all temperature investigated and at pH 4.0 and 5.0 for white bitter yam, but at 70% relative activity at 30°C, 40°C and 50°C and pH 5.0, while it retained about 40% relative activity at 60 and 70°C for yellow bitter yam. The affinity of enzyme from bitter yam was higher toward the substrate L-DOPA compare to tyrosine. The K_m and V_{max} values of PPO from white and yellow bitter yam using L-DOPA as substrate were 0.068mM and 0.080mM, and 6.7 and 21.35 unit/ml respectively, while K_m and V_{max} values of PPO from white and yellow bitter yam using tyrosine as substrate were 0.33mM and 0.40mM, and 6.47 and 15.1 unit/ml respectively. The activity of the partially purified PPO was strongly inactivated by ascorbic acid and stimulated in the presence of SDS even at a lower concentration. Therefore, the result obtained from this research regarding the purification, optimum pH and temperature, substrate specificity, inhibitors may give an insight to understanding the behaviour of polyphenol oxidases and in order to control this enzyme from white and yellow bitter yam during processing and storage.

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