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# Extraction, partial purification and characterization of *Xanthosoma caracu* polyphenol oxidase

Ebizimor Wodu *, Abraham Sisein Eboh, Ayibaene Frank-Oputu and Ufuoma Creda Okeze

*Department of Biochemistry, Faculty of Basic Medical Sciences, Niger Delta University Wilberforce, Island Bayelsa State. Nigeria.*

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## Abstract

Polyphenol oxidase, a group of copper-containing enzymes responsible for the oxidation of monophenols to diphenols and diphenols to quinones are very important as their action sometimes produces undesirable results. In this study, polyphenol oxidase was extracted from *Xanthosoma caracu* tuber and its characteristics elucidated. The activity of *X. caracu* polyphenol oxidase was assayed by monitoring the increase in absorbance at 420nm at 30sec interval for 5mins using a spectrophotometer. The partially purified preparation of polyphenol oxidase extracted from *X. caracu* was purified 2.094 fold, recovered 37.33% andhad a specific activity of 1.60u $min^{-1}mg^{-1}$. The km and Vmax values, were found to be 27.03mM±4.31 and 0.17$umin^{-1}$for catechol, 51.64mM±10.12 and 011$umin^{-1}$ for p-cresol, and 31.25mM±3.58 and 0.14$umin^{-1}$forpyrogallol.Maximum activity of *X. caracu* polyphenol oxidase was observed at temperature of 40°C and pH 7.0 with catechol and pyrogallol substrates, while with p-cresol substrate was 45°C and pH 6.5.The results on inhibition studies revealed mixed type inhibition by citric acid, while ascorbic acid was noncompetitive. Inhibitor constant with ascorbic and citric acids was 0.65mM and 1.7mM respectively. The presence of the browning enzyme, polyphenol oxidase was established in *X. caracu* with kinetic characteristics similar to those in other plant sources. Catechol was the best substrate, while ascorbic acid was the most potent inhibitor. At appropriate concentrations citric and ascorbic acids may be used to prevent enzymatic browning in *X. caracu* due to the action of polyphenol oxidases.

**Keywords:** *Xanthosoma caracu*; Polyphenol oxidases; Inhibitors; Kinetic constants

## 1. Introduction

*Xanthosoma caracu*, a tuber crop, is a type of cocoyam belonging to the family acaceae (figure 1). It is commonly called odu by the Ijaws in southern Nigeria. It grows between 1.5 to 1.8 metres. The plant is known to contain a toxin that is destroyed during cooking [1]

Phenolic compounds are phyto-metabolites found in foods and their presence confers biological activities on plant parts containing them. These phenolics are subject to attack by enzymes when these foods are cut open or bruised. The action of these enzymes reduces the food quality; hence the activity of these enzymes sometimes produces undesirable results. Polyphenols are acted upon by a group of copper-containing enzymes referred to as polyphenol oxidases. They catalyze the oxidation of hydroxyl groups in phenolics, resulting in the formation of reactive compound, quinones, which then polymerise to form complexes with proteins or amino acids leading to brown pigment [2,3,4].

Polyphenol oxidases, mostly found in the chloroplast of photosynthetic cells and leucoplast in storage cells [5] catalyze three types of reactions, 1) hydroxylation of monophnols to o-diphenols, 2) oxidation of o-diphenols to o-quinones

*Corresponding author: Ebizimor Wodu

[6,7,8] and 3) the oxidation of p-diphenols to p-quinones [8]. The oxidation of o-diphenols to o-quinones can also be catalysed by laccase [9].

Polyphenol oxidases are ubiquitous and are widely distributed in animals and plants. They have been studied in many plants and plant tissues. Polyphenol oxidases have been studied in many organisms including *Bacillus spp*[10], edible mushroom [11], bitter Leaf [12],*Colocasia esculenta* [13,14] and *Musa paradisiaca* [15]. They have been shown to have varying kinetic and other properties depending on the enzyme source. Despite the wealth of information available to the scientific community on the characteristics of polyphenol oxidases, the enzyme in *Xanthosoma caracu*is rarely explored. Thus, this investigation focuses on elucidating the characteristics of polypnenol oxidase extracted from *Xanthosoma caracu*.

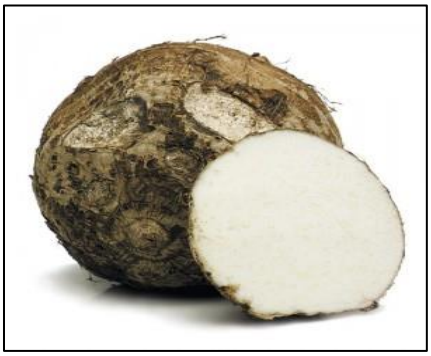

**Figure 1** *Xanthosoma caracu [16]*

## 2. Materials and Methods

### 2.1. Reagents

All reagents used were of analytical grade.

#### *2.1.1. Enzyme Extraction*

Freshly harvested tuber of *X.caracu* was washed clean with distilled water, peeled and cut quickly into tiny sizes. Exactly 200g was soaked in 300ml of cold acetone and kept in a refrigerator for 12hrs and then homogenized. The homogenate was filtered through double layer of cheese cloth. The residue was dried at room temperature to obtain the acetone powder which was stored in the refrigerator prior to aqueous extraction. Exactly 20g of acetone was dissolved in cold 0.2M potassium phosphate buffer (pH 6.8) and was centrifuged using a universal centrifuge (320R Hectti) at 4000rpm for 25mins at 4°C to obtain the supernatant as aqueous crude extract.

#### *2.1.2. Acetone Precipitation*

To precipitate the enzyme, ice cold acetone was added gradually to the supernatant in the ratio 1.5:1, with gentle stirring for 60minutes in ice bath. The precipitate was collected by centrifuging the mixture at 4000rpm for 25mins at 4°C. Exactly 10ml of extraction buffer was used to dissolve the precipitate, which was then dialyzed overnight at 4°C with same buffer. The buffer was changed three times during dialysis.

#### *2.1.3. Ammonium Sulphate Precipitation*

The solution from acetone precipitation was brought to 70% ammonium sulphate saturation by adding ammonium sulphate salt with continuous stirring to dissolve all the salt. Enzyme precipitate was collected by centrifuging at 4000rpm for 25mins at 4°C. The precipitate obtained was re-dissolved in 5ml of extraction buffer and was dialyzed as above.

#### *2.1.4. Protein Estimation and Polyphenol Oxidase Assay*

Protein was estimated using the method by Bradford [17] with bovine serum albumin as standard.

The activity of *X.caracu*polyphenol oxidase was assayed in a 3ml reaction mixture that contained 2.8ml of substrate (prepared in 0.1M phosphate buffer, pH 6.8) and 0.2 ml of enzyme solution. The increase in absorbance was monitored at 30sec interval for 5mins using a Spectrum lab spectrophotometer (755a). Substrate solution was used as blank.

Initial velocity was obtained from the slope of the absorbance versus time graph. One unit of polyphenol oxidase activity was defined as the amount of enzyme that caused a 0.001 increase of absorbance per minute.

*2.1.5. Kinetic constants Estimation*

The maximum rate and Michaelis-Menten's constant of *X. caracu*polyphenol oxidase were estimated by measuring the enzyme activity at varying substrate concentrations using catechol, p-cresol and pyrogallol. Vmax and km were obtained through the double reciprocal plot [18].

*2.1.6. Temperature and pH effects*

Temperature effect on *X. caracu*polyphenol oxidase was done by measuring the enzyme activity at a temperature range of $20^0$C-$70^0$C. The standard assay procedure was used at different temperature in the range studied.

Effect of pH on *X. caracu*polyphenol oxidase was investigated by measuring the enzyme activity through pH of 4.0 - 9.0. For pH 4.0 - 5.5 0.2M acetate buffer was used, while for pH 6.0 - 9.0 0.2M phosphate buffer was used. The *X. caracu* polyhenol oxidase activity was measured with the standard reaction procedure and changing the buffer.

*2.1.7. Inhibitor effects*

Inhibitor effect was done using citric and ascorbic acids. Three different inhibitor concentrations were used at four different substrate concentrations. The type of inhibition was deduced using primary plots of $1/V_0$ versus 1/[S], while the ki was estimated using secondary plots of $1/V_{max}$ versus [I].

## 3. Results and Discussion

Polyphenol oxidases no doubt have been characterised in many plant species, information on *Xanthosoma caracu* polyphenol oxidase however, is rarely available. The presence and characteristics of *X. caracu* polyphenol oxidase was the focus of this study. The partially purified preparation of polyphenol oxidase extracted from *X. caracu* was purified 2.094 fold, recovered 37.33% with a specific activity of 1.60u $min^{-1}mg^{-1}$.

### 3.1. Kinetic constants and substrate specificity

The kinetic constants with which *X. caracu* polyphenol oxidase oxidizes polyphenols to o-quinones were studied with catechol, cresol and pyrogallol substrates. The km values for catechol, p-cresol and pyrogallol were found to be 27.03mM±4.31, 51.64mM±10.12, and 31.25mM±3.58 respectively. Km of polyphenol oxidases from plant sources differ widely ranging from 0.12mM for honeydew peach polyphenol oxidase [19] and 0.56mM for gooseberry fruit polyphenol oxidase [20] to 3113mM for tea leaves polyphenol oxidase [21] However, the km values recorded for *X. caracu* polyphenol oxidase compares well with 25mM reported by [22] for blackberry PPO and 52.63, 25.0, and 40.0 reported by Frank-Oputu and Wodu, [23] for polyphenol oxidases from Orange flesh, purple peel, and brown peel sweet potatoes respectively.

**Table 1** Kinetic properties of polyphenol oxidase extracted from *Xanthosoma caracu*

| Substrate | Km (mM) | Vmax ($u\ min^{-1}\ mg^{-1}$) | Vmax/Km ($u\ min^{-1}\ mg^{-1}\ mM^{-1}$) | Temp. Optimum ($^0$C) | pH Optimum |
|---|---|---|---|---|---|
| Catechol | $27.03\pm4.31^a$ | $0.17\pm0.01^a$ | $0.006\pm0.0^a$ | $40\pm0.20^a$ | $7.0\pm0.0^a$ |
| Pyrogallol | $31.25\pm3.58^a$ | $0.14\pm0.01^b$ | $0.003\pm0.0^b$ | $40\pm4.6^a$ | $7.0\pm0.46^a$ |
| p-cresol | $51.64\pm10.12^c$ | $0.11\pm0.01^b$ | $0.002\pm0.0^b$ | $45\pm2.31^a$ | $6.5\pm0.72^a$ |

Values are represented as mean±sd of triplicate determinations. The means with different superscripts letters in column are significantly different ($P \leq 0.05$).

The maximum rate (Vmax) for polyphenol oxidases also vary considerably. The Vmax value of $0.17umin^{-1}$ for catechol was significantly higher than $0.14umin^{-1}$, and $011umin^{-1}$ estimated for pyrogallol and p-cresol respectively. The Vmax

values estimated in the present study were in line with the values reported for polyphenol oxidases from other sources. Eljack*et al*., [24] reported 0.125au $ml^{-1}min^{-1}$ for tamarind polyphenol oxidase, while 0.55unit $min^{-1}$ was reported by Adeseko and Fatoki [25] for melon seed polyphenol oxidase.

The catalytic efficiency (Vmax/Km) of polyphenol oxidase extracted from *X. caracu* was used to determine the best substrate for the enzyme. Catechol had a significantly lower km value indicating that the *X. caracu* enzyme had higher affinity for it than for pyrogallol and p-cresol. The Vmax/km ratio of 0.006 for catechol, was significantly higher than those of 0.003 for pyrogallol and 0.002 for p-cresol (table 1). From the results, catechol was a better substrate as the enzyme was more specific to it. Polyphenol oxidases from plant sources differ in their activities depending on the phenolic substrate. The number and position of OH groups on the substrate have a remarkable effect on the polyphenol oxidase activity [12]. High specificity for catechol has being reported by other researchers. Azzouzi *et al*., [22] reported higher specificity of blackberry polyphenol oxidase for pyrocatechol than pyrogallol, thus the enzyme had higher catalytic efficiency for pyrocatechol compare to pyrogallol. Siddiq and Dolan, [26], Eljack*et al*., [24] and Wodu and Frank-Oputu, [15] also reported higher specificity for catechol than pyrogallol for polyphenol oxidases extracted from blueberry, Tamarindus indica and Musa paradisiaca respectively. Polyphenol oxidases from the above sources and that being studied in this article have higher specificity for o-diphenols than o-triphenols. This may be due to the size of their active sites [3]. In other studies however, polyphenol oxidase had higher affinity and specificity for other phenolic compounds other than catechol. Polyphenol oxidase from two species of bush mango [27] and melon seed [25] were most specific to L-DOPA. Also, sunflower seed polyphenol oxidase was more specificity to pyrogallol and gallic acid [28] than catechol.

### 3.2. Temperature and pH Optimum

Figure 2 revealed that *X. caracu* polyphenol oxidase was active optimally at $40^0$C when catechol and pyrogallol substrates were used and $45^0$C with p-cresol. Also as shown in figure 3, maximum activity of the enzyme was observed at pH 7.0 with catechol and pyrogallol substrates, while with p-cresol substrate was at 6.5. Polyphenol oxidases from other plant sources have exhibited the same optimum temperatures with the *X. caracu* enzyme using the same and other substrates. Benaceur *et al*., [29] reported $40^0$C for pyrogallol and 45°C for catechol for truffles (*Terfezia arearia*) polyphenol oxidase. Su *et al*., [30] also reported 45°C for apricot (*Primus ArmeniaL*) with catechol as substrate. Temperature Optimum of 50°C have been reported by Ilesanmi *et al*., [31] for bitter leaf; by Lee *et al*., [32] for tomato polyphenol oxidase and Adeseko *et al*., [33] for African bush mango (*Irvingia gabonesis*) using catechol as substrate. Very low optimum temperature values of $20^0$C was reported by Guo *et al*., [34] for polyphenol oxidase extracted from areca nut kernal and 15°C was reported by Wang *et al*., [35] for lili cotton polyphenol oxidase.

Optimum pH values for polyphenol oxidase studied for plant species mostly fall within the range 5.5-7.5. The pH of 7.0 reported in the present study was same with those reported for polyphenol oxidases studied in chestnut [36], borage [37], lotus seed [38], areca nut kernel [34] and tamarind pulp [24] when catechol was used as substrate. Also, pH optimum of 6.5 with p-cresol as substrate in the present study was reported for polyphenol oxidases studied in soursop with catechol substrate [39], walnut leaves with L-tyrosine substrate [40], truffles with pyrogallol substrate [29] and melon seed [25].

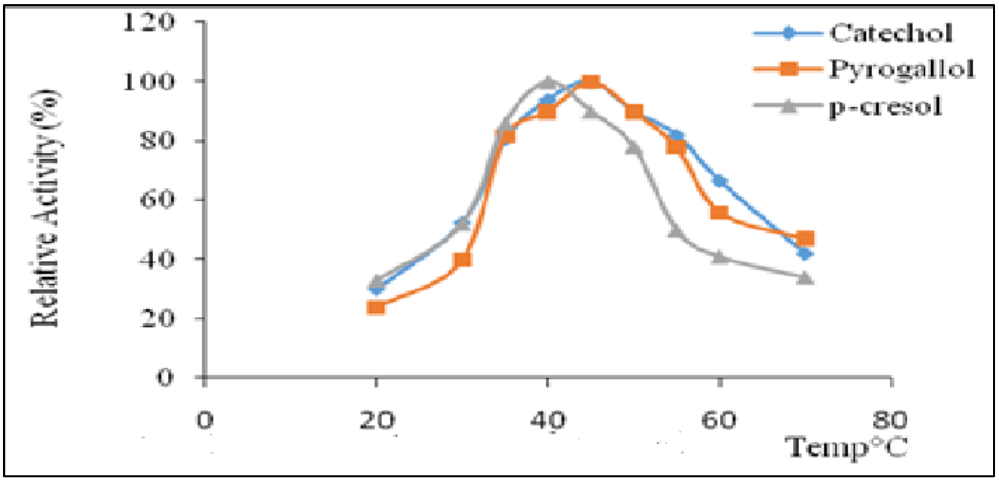


**Figure 2** Effect of temperature on polyphenol oxidase activity

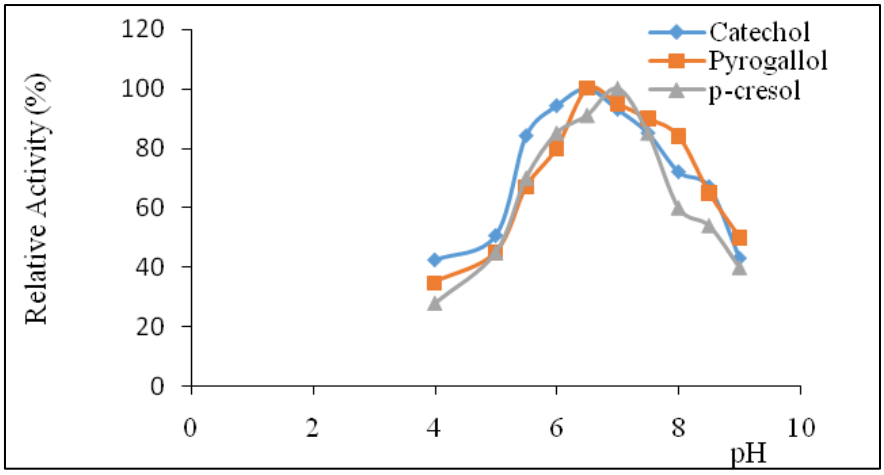


**Figure 3** Effect of pH on polyphenol oxidase activity

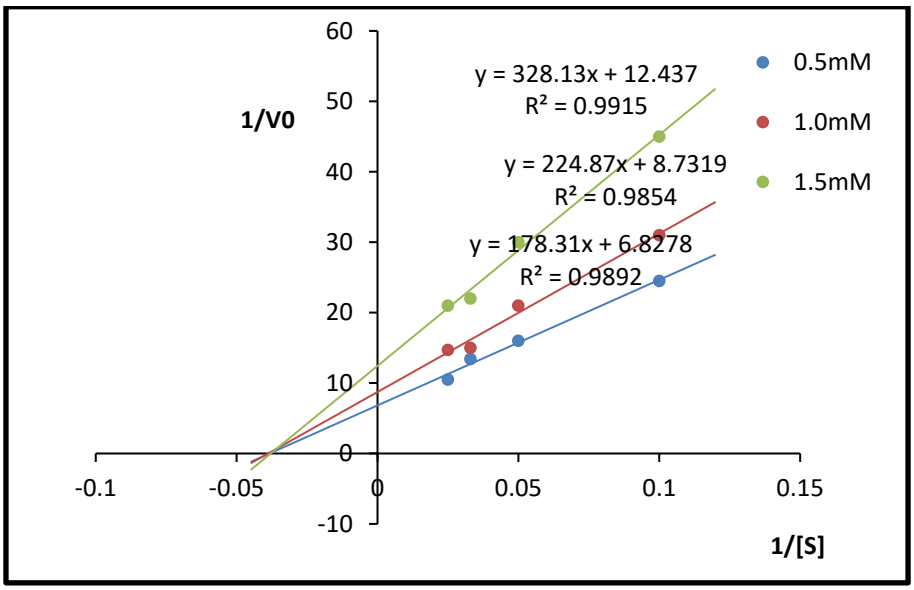


**Figure 4** Lineweaver-Burk plot showing the effect of ascorbic acid on *X. Caracu* polyphenol oxidase with catechol as substrate

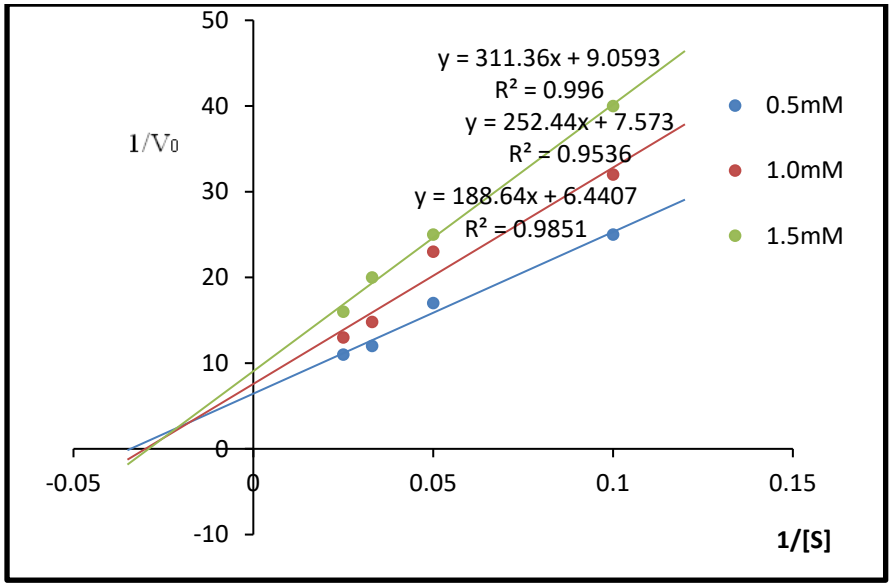


**Figure 5** Lineweaver-Burk plot showing the effect of citric acid on *X. Caracu* polyphenol oxidase with catechol as substrate

### 3.3. Inhibitor Effect

In the investigation of the nature of inhibition exhibited by citric and ascorbic acids on *X. caracu* polyphenol oxidase, catechol was used as substrate. The results revealed that the type of inhibition exhibited by citric acid was mixed inhibition, while ascorbic acid was noncompetitive inhibition. Inhibitor constant (table 2) as determined from a secondary plot of 1/Vmax' versus [I] revealed that ascorbic acid with ki of 0.65mM was a stronger inhibitor of *X. caracu* polyphenol oxidase compared to citric acid with ki of 1.7mM. In a similar study, the type of inhibition exhibited by ascorbic acid on purslane polyphenol oxidase was non-competitive, while that by citric acid was uncompetitive. Similar to the result of the present study, citric acid was found to exhibit mixed type of inhibition on eggplant polyphenol oxidase, but ascorbic acids had been shown to exhibit competitive inhibition [41]. Citric acid also was shown to exhibit a competitive inhibition on Chinese parsley polyphenol oxidase [42]. The type of inhibition is dependent on structural and functional properties of polyphenol oxidases from different species [43], hence the difference in the type of inhibition exhibited by polyphenol oxidase from different plant source. This observation was also noted by Guven *et al*., [44] for purslane polyphenol oxidase.

**Table 2** Inhibition properties of *Xanthosoma caracu* polyphenol oxidase

| Inhibiton | Ki (mM) | Type of Inhibition |
|---|---|---|
| Citric acid | 1.70 | Mixed |
| Ascorbic acid | 0.65 | Non-competitive |

## 4. Conclusion

In this investigation, polyphenol oxidase was extracted from *X. caracu* and some characteristic elucidated. The presence of the browning enzyme polyphenol oxidase was established in *X. caracu* and the properties are similar to polyphenol oxidases extracted from other plant sources. The enzyme is most efficient and specific to catechol. The optimum pH range was 6.5-7.0 and optimum temperature range was 40°C-45°C. Both ascorbic and citric acids inhibited *X. caracu* polyphenol oxidase, with ascorbic acid being more potent as it has a lower ki value of 0.65mM. The type of inhibition for ascorbic acid was non-competitive, but citric acid exhibited mixed type inhibition. At appropriate concentrations citric and ascorbic acids may be used to prevent enzymatic browning in *X. caracu* due to the action of polyphenol oxidases.

## Compliance with ethical standards

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*Disclosure of conflict of interest*

Authors declare no conflict of interest.

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