

Isolation, Partial Purification and Characterization of Polyphenol Oxidase from Two Species of African Mango Seeds (*Irvingia gabonensis* and *Irvingia wombolu*)

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Abstract: Polyphenol oxidases are oxido-reductases that catalyses oxidation of phenolic compounds in the presence of oxygen to form corresponding quinone intermediates which spontaneously polymerize to form undesirable pigments. Polyphenol oxidase was isolated and characterized in *Irvingia species (gabonensis and wombolu)* kernel, a tropical African fruit. Extracts were partially purified with ammonium sulphate. The effects of optimum pH and temperature were investigated while the pH and thermal stability were carried out. The K_m , V_{max} and the activity on both monophenol and diphenol substrates were determined. The optimum pH and optimum temperature of activity were found out to be 7.0 and 60°C in both species. The enzyme was stable at pH 7.0 and pH 8.0, and also at temperatures of 25, 30, 40, 50 and 60°C, it however loses stability beyond 60°C. K_m and V_{max} for *Irvingia gabonensis* were 2.51mM and 0.0411 Unit/min while K_m and V_{max} for *Irvingia wombolu* were 2.55mM and 0.0415 Unit/min respectively. Higher activity was observed with diphenol substrate L-DOPA than with monophenol - tyrosine. It can therefore be deduced from this study that the storage of the seeds of African mango at a room temperature above 60°C will prolong its shelf-life.

Keywords: Enzyme Activity, African Mango, Polyphenol Oxidase

1. Introduction

Polyphenol oxidase (PPO; EC 1.10. 3.1) is widely found in nature [37]. It is typically present in majority of plant tissues [40, 31]. Fruits and vegetables frequently undergo browning as a consequence of mechanical injury suffered during harvest, transport, storage and processing. Browning is attributed to the oxidation of phenolic substances by the enzyme polyphenol oxidase to quinones and their eventual polymerization to melanin pigments [29, 37]. Due to its involvement in adverse browning of plant products, polyphenol oxidase has received much attention from researchers in the fields of plant physiology and food science.

The oxidative browning reactions in many foods of plant origin generally cause deterioration in food quality by changing nutritional and organoleptic properties [21]. These reactions significantly diminish consumer's acceptance, storage life and value of the plant product. Thus highly

prized and economically valuable products are extremely vulnerable to deteriorative enzymatic browning also referred to as melanosis. Owing to its tremendous economic impact to the food industry, inhibition of polyphenol oxidase in food product has been widely studied [15].

It was suggested that polyphenol oxidase might be involved in the development of necrosis around damaged leaf surface and in defence mechanism against insects and plant pathogen attack [3] while melanin biosynthesized in animals mainly by polyphenol oxidase acts as a skin and hair pigment-protecting agent against harmful solar radiation [12].

Irvingia gabonensis and *Irvingia wombolu* are highly valuable and extensively utilised tropical African trees. An important food product in Africa is the *Irvingia gabonensis*, commonly known as "African mango", "Dika nut" or "bush mango". The juicy fruit pulp of *Irvingia gabonensis* is rich in vitamin C and it is widely reported to be consumed as a dessert fruit or snack throughout Western and Central Africa [9, 33].

The kernels of *Irvingia gabonensis* is classed as an oilseed. They are grounded with pestle and mortar or on a stone into a paste or cake called 'dika bread', which is used as soup, stew or sauce additive, for flavouring and thickening [1, 17]. The kernels are highly valued for the slimy consistency they produced. Okafor [24] notes that whilst kernels from *Irvingia* spp. are used in soup making, Dika bread may be sun-dried so that it can be stored [34]. *Irvingia* kernels form an important part of the West and Central African diet, providing carbohydrate and protein [25]. Agbor [1] reports that the kernels may be roasted to enhance their flavouring effect, and that crushed pieces of the roasted kernels may be used in frying vegetables. Fat extracted from the kernels could be used for food applications, such as in margarine or cooking oil, and it is also suitable for soap, cosmetics and pharmaceuticals [8]. Flour produced by milling the seed testa has been used in feeds formulation for livestock [9]. Proper understanding of the polyphenol oxidase from African mango would go a long way in the development of enhanced storage techniques and in the utilization of commercial food processor.

2. Materials and Methods

2.1. Sample Collection

African mango (*Irvingia gabonensis* and *Irvingia wombulu*) seed both commonly known as "Dika nut" were bought from *Oja Oba* market in Akure, Ondo State of Nigeria.

2.2. Preparation of Crude Extract

African mango seeds, 45g of each species 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 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 6,000rpm for 30 min at 4°C. The supernatant obtained was stored in a refrigerator and used as crude extracts for further studies.

2.3. Partial Purification of Polyphenol Oxidase from *Irvingia* Species

The supernatant obtained from each species was brought to 80% Ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$ saturation with solid Ammonium sulphate. The precipitated enzyme (polyphenol oxidase) was separated by centrifugation at 6,000rpm for 30 minutes. The precipitate was dissolved in small amount of 0.1M phosphate buffer (pH 6.8) and dialyzed at 4°C overnight with three changes of buffer.

2.4. 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 [5]. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 per minute. The sample cuvette

contained 0.7 ml of 10mM 3,4-dihydrophenylalanine solution in 0.1 M phosphate buffer (pH 6.8) and 0.3 ml of the enzyme solution while the blank contained only 0.7 ml of 10mM 3,4-dihydrophenylalanine and 0.3ml of 0.1M buffer solution.

2.5. Effect of pH on Polyphenol Oxidase Activity

The effect of pH on polyphenol oxidase was investigated using pH range of pH 4.0–9.0, 0.1 M acetate buffer (pH 4.0–5.0), 0.1 M phosphate buffer (pH 6.0–7.0) and 0.1M Tris/HCl buffer (pH 8.0–9.0). Each of the buffer solution was used to prepare the substrate used in determining the activity of the enzyme at a particular pH. Enzymatic activity was measured according to the standard assay procedures.

2.6. Effect of Temperature on Polyphenol Oxidase Activity

The assay mixture was incubated at different temperatures varying from 30 to 80°C for 10mins in a regulated water bath. The activity assay was determined at 10°C temperature interval while the enzymatic activity under each temperature condition was expressed in relative form as the percentage of the highest activity reached.

2.7. Effect of pH Stability on Polyphenol Oxidase

The effect of pH on the stability of the enzyme was carried out by incubating the enzyme in a buffer solution at different pH in room temperature. Four fold dilution of the enzyme was prepared using the buffer solution. Enzymatic activity was determined at 0mins and at 1hour interval for six hours period using the standard assay procedure.

2.8. Thermal Stability

Thermostability of polyphenol oxidase was investigated using the enzyme solution incubated for 1 hour in regulated Gallenkamp water bath at a particular temperature within the temperature range of 30 to 80°C at 10°C interval. Aliquot was withdrawn and cooled for the determination of enzyme activity at 10mins interval.

2.9. Kinetic Parameters

Polyphenol oxidase activities were determined using varying concentration of dihydrophenylalanine under standard conditions. Michaelis constant (K_m) and maximum velocity (V_{max}) was measured using the double reciprocal plot.

2.10. Protein Determination

Protein concentration was measured in all the stages of purification according to the method of Lowry *et al.*, [19] using bovine serum albumin (BSA) as a standard.

3. Results

3.1. Activity of Enzyme

The activities of the crude enzyme of the two species were 86.4 and 100 units/ml while the activities of the partially

purified were 34 and 40 units/ml for *gabonensis* and *wombolu* respectively. The protein contents of the partially purified *Irvingia gabonensis* and *Irvingia wombolu* were 1.6mg/ml and 1.0mg/ml while the specific activities were 4.1 and 2.13 units/ml/mg respectively.

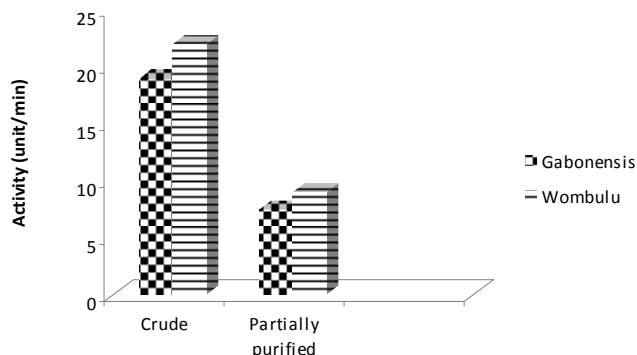


Figure 1. Activity of Crude and partially purified Polyphenol oxidase from *Irvingia* species.

3.2. Effect of Temperature on the Activity of Polyphenol Oxidase

Optimal temperature of 60°C was observed for polyphenol oxidase activity of both *Irvingia gabonensis* and *Irvingia wombolu*. The same pattern of a steady increase in activity of polyphenol oxidase was observed in both *Irvingia gabonensis* and *Irvingia wombolu* as the assay temperature was increased, until an optimal temperature was reached followed by a gradual decrease in the enzyme activity after 60°C as shown in figure 2. for *gabonensis* and *wombolu*.

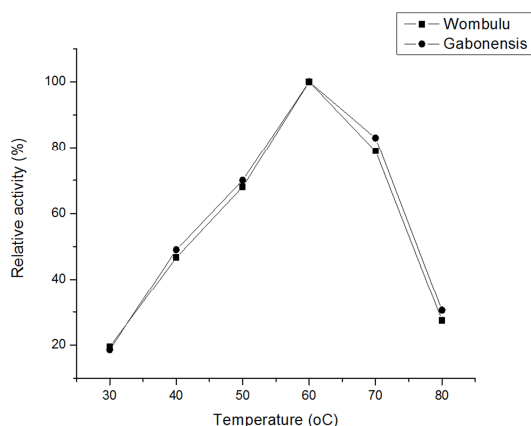


Figure 2. Effect of Temperature on the activity of *Irvingia gabonensis* and *Irvingia wombolu* polyphenol oxidase.

3.3. Effect of pH on the Activity of Polyphenol Oxidase

The effect of pH on the activity of partially purified polyphenol oxidase from *Irvingia gabonensis* and *wombolu* are illustrated in figure 3. The enzyme showed activity in all the pH investigated, (pH 4.0-9.0). The enzyme from the two species gave an optimal pH at pH 7.0. The figure showed a gradual increase in the activity of the enzyme but showed a decline after attaining the optimal pH activity at pH 7.0.

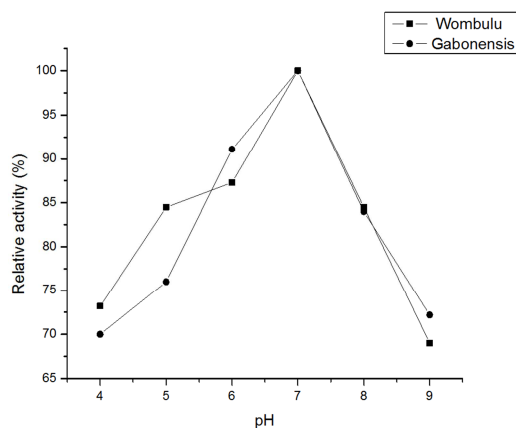


Figure 3. Effect of pH on the activity of Polyphenol oxidase from *Irvingia gabonensis* and *Irvingia wombolu*

3.4. Thermal Stability

The effect of temperature on the stability of polyphenol oxidase from *Irvingia gabonensis* is illustrated in figure 4a, while that of *Irvingia wombolu* is illustrated in figure 4b. The enzyme in both figures showed a 100% Relative activity at 25, 30 and 60°C. A very high relative activity of above 90% was observed both species at 40°C and 50°C. The enzyme was not stable at 70°C and 80°C showing a relative activity of 35 and 12%, and 35% and 15% for *gabonensis* and *wombolu* respectively after 1 hour incubation period.

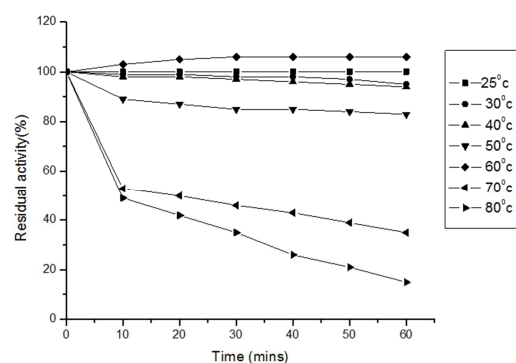


Figure 4a. Thermal stability of polyphenol oxidase from *Irvingia gabonensis*. The activity of the enzyme was expressed relative to the activity of the enzyme at 0 minutes taken at 100%.

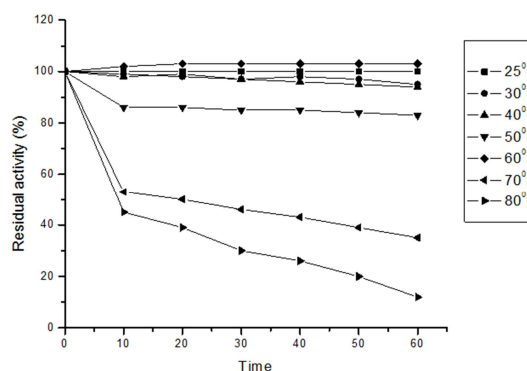


Figure 4b. Thermal stability of polyphenoloxidase from *Irvingia wombolu*. The activity of the enzyme was expressed relative to the activity of the enzyme at 0 minutes taken at 100%.

3.5. Effect of pH on the Stability of Polyphenol Oxidase

The influence of pH on the stability of polyphenol oxidase from *Irvingia gabonensis* and *Irvingia wombolu* are shown in figures 5a and 5b. The enzyme from both *Irvingia* spp was very stable at pH 7.0 with 100% relative activity after 6 hours of incubation period. The two enzymes were also fairly stable at pH 8, 6 and 5 showing a relative activity of 70 and 68%, 52 and 83%, and 70 and 50% for *Irvingia gabonensis* and *wombolu* respectively. However, there was a drastic decline in activity of the enzymes at pH 4 and 9 showing a relative activity of 10 and 7%, and 4 and 7% respectively for *Irvingia gabonensis* and *wombolu*.

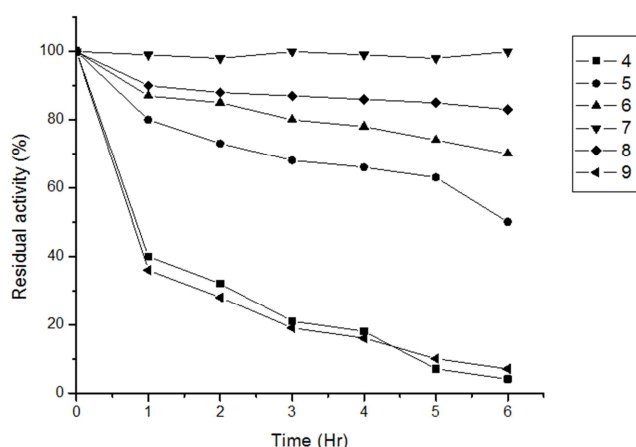


Figure 5a. pH stability of *Irvingia gabonensis* polyphenoloxidase. The activity of the enzyme was expressed relative to the activity of the enzyme at 0 minutes taken at 100%.

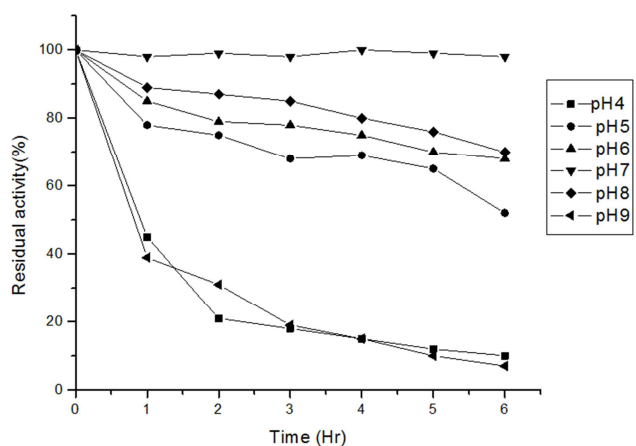


Figure 5b. pH stability of *Irvingia wombolu* polyphenoloxidase. The activity of the enzyme was expressed relative to the activity of the enzyme at 0 minutes taken at 100%.

3.6. Substrate Specificity

The oxidizing ability of the partially purified polyphenol oxidase from the two *Irvingia* spp as shown in figure 6 was determined using tyrosine as monophenol and L-DOPA as the diphenol. Polyphenol oxidase from *Irvingia wombolu* and *gabonensis* showed a higher relative activity of 100% and 83% toward L-DOPA compared to 10 and 13% relative

activity toward tyrosine.

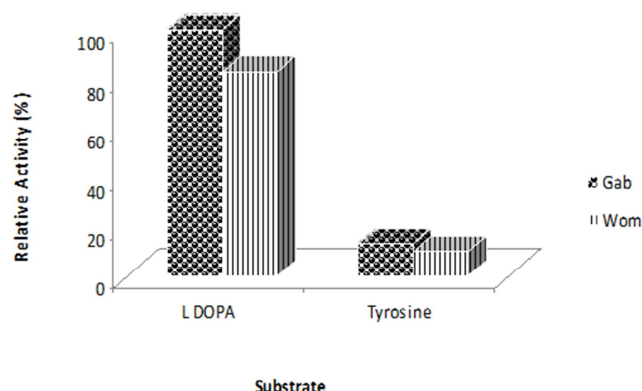


Figure 6. Activity of the enzyme using a model monophenol and diphenol. The figure shows the activity of polyphenol oxidase of *Irvingia* species using tyrosine and L-DOPA as substrates.

3.7. Kinetic Study

The lineweaver- burk plots analysis of this enzyme showed Michaelis constant (K_m) values of 2.51 mM and 2.55 mM, while maximum rate (V_{max}) values of 0.0411 Unit/min and 0.0415 Unit/min for *Irvingia gabonensis* and *Irvingia wombolu* polyphenol oxidase respectively.

4. Discussion

The result obtained indicated the presence of polyphenol oxidase in *Irvingia gabonensis* and *Irvingia wombolu*, with *Irvingia wombolu* specie showing more polyphenol oxidase activity than *Irvingia gabonensis* species. Several authors had reported the occurrence of polyphenol oxidase in plants; cucumber [23], grape [4], pineapple [6], mango [28]. Polyphenol oxidase is said to be involved in advance browning of plant products which generally causes deterioration in food quality by affecting its colour, flavour, texture and nutritional value [21]. It is a limiting factor in the processing of crops as peeled, sliced or injured crops rapidly undergo browning. The occurrence and wide distribution of polyphenolic and monophenolic compounds and their derivatives in almost all plant tissues is probably the underlying reason for the presence of this enzyme in plants [22]. The level of polyphenol oxidase activity of a particular plant species is inextricably connected to physiological needs of the plant. It has been reported that plants which possess relatively high levels of polyphenol oxidase activity are less susceptible to fungi and bacteria infections. This is obviously connected to the bacteriostatic properties of the brown products or pigments (melanin) of the enzyme action [21].

A wide range of pH optima for the activity of polyphenol oxidase ranging from pH 4.0 [26] in field bean seed to pH 8.5 [30] in *Ginkgo biloba* leaves had been reported in literature. The enzyme from the two species had optima activity at pH 7.0. This reveals that it is more active in a neutral pH. Some plants unlike *Irvingia* species show optimum pH in the acidic region; pH 4.5 for cherry and strawberry with methylcatechol

as substrate [10], pH 3.5-4.5 for apple and grape [20]. Several fruits including almond, apricot, peach and plum have been reported to show a general polyphenol oxidase optima pH around pH 5.0 [10]. However, polyphenol oxidase from subtropical fruits such as longan and pineapple is most active near neutral pH [6, 14]. The pH optima from the two species of *Irvingia* is comparable to pH 7.5 reported by Lima *et al.*, [18] for immature, unripe fruit of soursop and pH 7.0 for fully ripe fruits. Dogan *et al.*, [7] and Gomes *et al.*, [11] had also reported pH optima of 7.5 for aubergine, and 7.2 for beans polyphenol oxidase respectively. The changes in ionization of prototropic groups in the active site of an enzyme at lower acid and higher alkali pH values may prevent proper conformation of the active site, binding of substrates, and or catalysis of the reaction [37]. In addition, irreversible denaturation of the protein and or reduction in stability of the substrate as a function of pH could also influence the catalytic activity of enzymes [39].

Temperature is a very important factor that significantly influence the catalytic activity of polyphenol oxidase, as a decrease in temperature decreases kinetic energy and hence a low reaction rate [16]. Temperature optima as low as 20°C for bartlet pear [32] and as high as 60°C for *Thermomyces lanuginosus* has been observed [2]. An optima activity of 60°C observed in both *Irvingia* spp is high compared to the temperature optima of between 20°C-60°C generally observed for various plants using different substrate by Yoruk and Marshall [39] in his review of plant polyphenol oxidase. Yoruk and Marshall [39] however noted that optimum temperature of polyphenol oxidase varies in different plant sources and that nature of the substrate influences the optimum temperature. *Irvingia* spp polyphenol oxidase optimum temperature is higher than the 50°C observed in strawberry using pyrocatechol, cucumber using catechol [23] and 55°C observed in a thermophilic fungus *Chaetomium thermophile* [13].

The enzyme from the two species of *Irvingia* was thermally stable at 25°C, 30°C and 40°C while over 80% residual activity was observed at 50°C and less than 15% residual activity at 80°C. It demonstrated a higher thermal resistant when compared with Artichoke heads [3]. Polyphenol oxidases from different sources exhibit different heat resistance. Welsche-Ebeling and Montgomery [36] reported that to inhibit 99% strawberry polyphenol oxidase activity 4, 5 and 6 minutes heating was necessary at 95, 90 and 80°C respectively while at 70°C, 7 minutes heating was required to inhibit about 80% activity. Wakayama [35] also observed about 1.4 minutes to 2.4 minutes were required for 90% inhibition of the polyphenol oxidase from the core of six Japanese apple varieties.

The enzyme showed a higher catecholase activity and a very low cresolase activity. Polyphenol oxidase generally showed differences in activity for diphenol and monophenol substrate. Polyphenol oxidase from some seeds such as field bean seed [26] and sunflower seed [27] showed activity toward only o-diphenol while activity of polyphenol oxidase from apple on monophenol (tyrosine) is much lower than on o-diphenols. The

Michealis-menten constant (K_m) and maximum velocity (V_{max}) showed a low degree of interspecies variability.

5. Conclusion

The results from the present study demonstrated that the two species of African mango seeds contained significant polyphenol oxidase activity. The optima pH and temperature for polyphenol oxidase activity were found to be 7.0 and 60°C from both *Irvingia* spp. The thermal stability studies show that the enzyme from both *Irvingia gabonensis* and *Irvingia wombolu* were 100% relatively stable at 25, 30 and 60°C after 1.0 hour incubation period, while the pH stability studies of the enzyme from both *Irvingia* spp was 100% relatively stable at pH 7.0 after 6.0 hours incubation period. The enzyme show higher relative activities of 100% and 83% toward L-DOPA and lower relative activities of 10% and 13% toward tyrosine for *Irvingia wombolu* and *gabonensis* respectively. The kinetic studies of the enzyme show K_m and V_{max} values to be 2.51 mM and 0.0411 Unit/min, and 2.55 mM and 0.0415 Unit/min for *Irvingia gabonensis* and *Irvingia wombolu* respectively. Therefore, to extend the shelf-life of African mango seeds the enzyme (polyphenol oxidase) can be inactivated by brief heating to attain a temperature of 70°C above or pH adjustment.

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