Comparative physicochemical properties of polyphenoloxidase from white and red kolanuts (Cola nitida subspecies alba and rubra)

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ABSTRACT: Polyphenol oxidase is a generic name for a group of enzymes capable of catalyzing reactions for several phenols to produce o-quinones and it is responsible for the darkening of cut surfaces of fruits, vegetables and plants. Polyphenoloxidase was extracted and partially purified from two sub-species of Cola nitida, alba (white cola) and rubra (red cola) using ammonium sulphate precipitation. The effect of pH and temperature on the activity of enzyme was investigated while the thermal stability, pH stability and the kinetic parameter were determined. The optimal pH and temperature activity of polyphenoloxidase from both samples were found to be pH9.0 and 50°C. The enzyme was very stable at pH 4-6 for 2hours in the two cola while also maintaining 100% stability at 30 – 40°C for 1 hour. About 60% and 45%, 55% and 30%, 40% and 25% residual activity were observed for white (alba) and red (rubra) respectively at 50°C, 60°C and 70°C after 10minutes inubation time. The Michealis-Menten constant, Km for Cola nitida subsp. alba and rubra were 2.33mM and 5.63mM respectively while Vmax were 77unit/min/ml and 70unit/min/ml respectively. The enzyme showed activity toward both monophenol and diphenol substrate but with a higher activity for monophenol.

Keywords: Activity, Extract, Kolanut, Polyphenoloxidase.

INTRODUCTION

Polyphenoloxidase is a bifunctional, copper containing oxidase having catecholase and cresolase activity (Whitaker, 1996). It is responsible for the development of the characteristic golden colour in dried fruits such as raisins prunes, dates (Ensiminger and Vamos-Vigyazo, 1995). Polyphenoloxidase catalyses the initial step in the polymerization of phenolics to produce quinones, which undergoes further polymerization to yield black insoluble polymers referred to as melanin. Due to its involvement in adverse browning of plant products, Polyphenoloxidase has received much attention from the researchers in the field of plant physiology and food science. The catalytic action of polyphenol oxidase has an enormous impact on the quality of several fruit and vegetable crops and result in alteration of colour, flavor, texture, and nutritional value (Zawistowski et al., 1991). In addition to general occurrence in plants, Polyphenol oxidase is also found in seafood (crustacean) products, such as shrimp (Chen et al., 1997) and lobster (Ali et al., 1994). These highly prized and economically valuable products are extremely vulnerable to deteriorative enzymatic browning, also referred to as melanosis (Kim et al., 2000).

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Kolanut are consumed by breaking them open and into pieces, then chewing the kola nut pieces as one chews gum. It forms an integral part of the social life of the people and has been accorded many attributes, this include its use as stimulant, astringent and diuretic. It increases energy and strength due to its stimulating property, it also dispels drowsiness, allays thirst, aids digestion and starves off hunger, and it also has healing and aphrodisiac qualities (Trindall, 1997). Kola nuts contain large amounts of caffeine and theobromine and are therefore used as a stimulant (Jayeola, 2001) to keep awake and withstand fatigue (Purgesiove, 1977). It was considered a substitute for alcohol in people dependent on drink, and for nicotine in smokers. It is also used as antidepressant because of its stimulating effect on the central nervous system, the caffeine content may be useful in relieving migraine headaches; the phenolic and anthocyanin component can induce antioxidant activity (Goodman et al., 1990). Browning reaction occurs when Kolanut is cut into pieces before chewing, hence the need to investigate and determine various physicochemical properties of polyphenoloxidase from the most commonly consumed Kolanuts owing to the tremendous economic importance of Kolanut as food and resources in other allied industry.

MATERIALS AND METHODS

Collection of Samples
The kolanuts (C. nitida subsp. alba and rubra) were purchased at Isinkan market, Akure, Ondo state, Nigeria.

Preparation of Crude Enzyme
About 120g of each kolanut were thoroughly washed and homogenized in 360ml of 25mM ice- cold phosphate buffer (pH6.8) containing 10mM ascorbic acid by using a blender for 3min each followed by a 60secs resting period to avoid local elevation in temperature. The mixture was filtered using layers of cheesecloth and glass wool. The filtrate obtained was centrifuged in a refrigerated centrifuge at 6,000rpm for 30 min at 4°C. The supernatant obtained was stored in refrigerator and used as crude extracts for other studies.

Partial Purification of kolanut Polyphenoloxidase
200ml of the crude extract of each kolanuts was measured into a beaker and brought to 80% (NH4)2SO4 saturation with solid (NH4)2SO4. The precipitated polyphenoloxidase was separated by centrifugation at 6,000rpm for 30 min. The precipitate was dissolved in 0.1M phosphate buffer (pH 6.8) and dialyzed in the same buffer for 72 hours at 4°C with three changes of the buffer during dialysis. The dialyzed sample was used as the polyphenoloxidase enzyme source for investigation.

Activity assay
Enzyme activity was determined by measuring the initial rate of quinone formation, as indicated by an increase in absorbance at 475 nm (Cosenteng and Lee, 1978). The assay mixture contained 0.7 ml of 10mM of 3, 4-dihydrophenylalanine solution in 0.1 M phosphate buffer (pH 6.8) and 0.3 ml of the enzyme solution. This was incubated at room temperature for 10min. The reference mixture contained 0.7 ml of 10mM substrate and 0.3ml of 0.1M phosphate buffer solution. The absorbance was taken at 475nm. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 per min.

Effect of pH on the Activity of Polyphenol oxidase
0.1M buffers of different range, pH4.0-9.0 was prepared using different buffer systems. Acetate (pH 4.0–5.0), phosphate (pH 6.0–7.0) and Tris/HCl (pH 8.0-9.0) buffers. Each buffer solution was used in
preparation of the substrate used for the assay mixture. Enzymatic activity was measured and determined according to the standard assay procedure (Coseteng and Lee, 1978).

**Effect of pH on the stability of Polyphenoloxidase**  
The effect of pH on the stability of the enzyme was carried out according to the method of Janovitz-klapp et al., (1989). Three fold dilution of the enzyme solution was prepared using the different buffer solution and the solution was incubated at room temperature for three hours. At one hour interval, the mixture was assayed for residual activity under standard assay conditions.

**Effect of temperature on the Activity of Polyphenoloxidase**  
A mixture of 0.7ml of 3, 4 dihydrophenylalanine and 0.3ml of the enzyme solution was incubated at different temperature of 30°C - 90°C for 10 minutes in a temperature regulated Gallenkamp water bath. The mixture was assayed for residual activity under standard assay condition.

**Effect of temperature on the stability of the Polyphenoloxidase**  
The thermal stability of the enzyme was determined by incubating enzyme fraction at various temperatures between 30°C-70°C without the substrate for 60 minutes. The activity of the enzyme was carried out at 10minutes interval.

**Kinetic parameters and substrate specificity**  
Polyphenoloxidase activities were determined using varying concentration value of dihydrophenylalanine under standard conditions. Km and Vmax was measured using the double reciprocal plot. Substrate specificity was determined by using two substrates of L-DOPA and tyrosine. The assay mixture was incubated at room temperature for 10 minutes and the absorbance was taken at 475nm (3, 4-dihydroxyphenylalanine) and 300nm (tyrosine). Protein concentration was measured according to the method of Bradford (1976) using bovine serum albumin as the standard.

**RESULT**

**Enzyme Activity**  
The partially purified enzyme of *Cola nitida* subspecies showed an activity of 19.4 unit/ml and 30.3unit/ml for White (*alba*) and Red (*rubra*) respectively indicating 64% percentage relative activity for *alba* compared to *rubra* as shown in figure 1.

**Effect of pH and temperarure on the activity of Polyphenoloxidase**  
The effect of pH and temperature on the activity of polyphenoloxidase from *alba* and *rubra* is as illustrated in Figure 2 and 4. The optima pH and temperature activity for the two *cola nitida* was found to be at pH 9 and 50°C. A steady increase in the activity of the enzyme toward the alkaline pH was observed with over 90% relative activity at pH10 and pH11.

**Effect of pH on the stability of Polyphenoloxidase**  
The influence of pH on the stability as revealed in Figure 3 showed that the percentage residual activity of 70% to 82% for red (*rubra*) is higher than 45% to 60% observed in white (*alba*) in all the pH investigated. However, the highest residual activity was measured at pH 7.0 and pH 8.0 for polyphenol oxidase from white and red respectively while the lowest residual activity of about 45% was measured at pH 4 and 5 for polyphenoloxidase from the two Kolanut samples.
DOPA was used as substrate with the partially purified enzyme. Assay was carried out according to the standard procedure.
The enzyme activity was measured at various pH. The assay mixture contained 0.3ml of enzyme solution and 0.7ml of 10mM 3,4-dihydroxyphenylalanine solution prepared by dissolving the substrate in the buffer with the pH of interest. The enzyme activity was determined according to the standard assay procedure. The activities were expressed relative to the maximum activity obtained at pH 9 which was taken as 100%.

![Graph: Effect of pH on the stability of polyphenol oxidase from white (alba) and red (rubra) Kolanut nitida.](image)

**Figure 3: Effect of pH on the stability of polyphenol oxidase from white (alba) and red (rubra) Kolanut nitida.**

Three fold dilution of the enzyme were prepared using different pH solution (pH4 – pH9). This was incubated at room temperature and the residual activity was determined after 3 hours.

The enzyme activity was measured at various temperatures. The assay mixture which consisted of 0.7ml of 10mM DOPA and 0.3ml of the enzyme solution was incubated at different temperature for 10 minutes. Assay was carried out according to the standard assay procedure. The relative activity at each temperature was expressed as a percentage of the activity at 50°C.

**Thermal stability of polyphenoloxidase**

The effect of temperature on the stability of polyphenoloxidase is presented in Figure 5a and 5b. The residual activity of the polyphenoloxidase after 10minutes incubation time was observed to be 60% and 45%, 55% and 30%, 40% and 25% for white (alba) and red (rubra) respectively at 50°C, 60°C and 70°C. However, 50% and 30% activity was retained after 1hour incubation time at 50°C for white and red samples while a minimal polyphenoloxidase activity was observed after 1hour at 70°C for both samples.

**Kinetic parameters and substrate specificity**

The Micheal-Menten constant, Km was estimated from the (Lineweaver- Burlk) double reciprocal plot from *Cola nitida* subspecies *alba* and *rubra*. The Km values were 2.33mM and 5.63mM while the Vmax values gave 78unit/min and 70unit/min for white and red Kolanut respectively. The activities of the polyphenoloxidase from red and white Kolanuts were 65unit/ml and 45.7unit/ml, 50unit./ml and 35.2 unit/ml using Tyrosine and DOPA as substrate respectively. The double reciprocal plot and effect of substrate on the enzyme activity is presented in Figure 6 and 7.
Figure 4: Effect of temperature on the activity of polyphenol oxidase from white (*alba*) and red (*rubra*) Kolanut.

Figure 5a: Effect of temperature on the stability of polyphenol oxidase from white (*alba*) Kolanut.
About 4ml each of the enzyme solution were incubated at temperature of (◊) 50°C, □ 60°C, (Δ) 70°C for 60 minutes 0.3ml was withdrawn at an interval of 10 minutes after which it was allowed to cool at room temperature and assay was carried according to standard assay procedure.

![Graph: Effect of temperature on the stability of polyphenol oxidase from Red (rubra).]

Figure 5b: Effect of temperature on the stability of polyphenol oxidase from Red (rubra).

About 4ml each of the enzyme solution were incubated at temperature of (◊) 50°C, □ 60°C, (Δ) 70°C for 60 minutes 0.3ml was withdrawn at an interval of 10 minutes after which it was allowed to cool at room temperature and assay was carried according to standard assay procedure.

**DISCUSSION**

The result obtained showed the presence of polyphenoloxidase in *cola nitida* subspecies *alba* and *rubra*. Chikezie (2006) reported a polyphenoloxidase activity in crude extract of *cola nitida*, *cola acuminata* and *theobroma cacao* using catechol as substrate. However, a lower percentage residual activity of 64% was observed in polyphenoloxidase from subspecies *alba* (white kolanut) compared to rubra (red kolanut). This is an indication of a degree of interspecies variability in Kolanuts. Different authors had reported the occurrence of polyphenoloxidase in plants; apple (Barrett *et al.*, 1991), banana (Gooding *et al.*, 2001), cucumber (Miller *et al.*, 1990), grape (Cash *et al.*, 1976), Pineapple, (Das *et al.*, 1997), mango (Robinson *et al.*, 1993), Potato (Cho and Ahn 1999). The occurrence and wide distribution of enzyme substrate, the polyphenolic and monophenolic compounds and their derivatives in almost all plant tissues is probably the underlying reason for the presence of this enzyme in plant (Mayer and Harel, 1979; Vamos-Vigyazo, 1981). The level of polyphenol oxidase activity of a particular plant species is inextricably connected to physiological needs of the plant. Connie and Gina (1996) reported that plants which possess relatively high levels of polyphenoloxidase 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.

The polyphenol oxidase from the two cola is more active at the alkaline pH. The observed pH optimal of the enzyme is the same in both Kolanuts. This is higher than reported range of pH 4.0 – 8.0 for variety of plant sources (Yoruk and Marshall, 2003). Several fruit including almond, apricot, peach and plum...
generally have maximal polyphenoloxidase activities around pH 5 (Fraignier et al., 1995). Other plants showed pH optimal activity at alkaline region, Kiwifruit and spinach, pH 8.0 (Park and Luh, 1985; Sheptovitsky and Brudvig, 1996). The changes in ionization of prototropic groups on the active site of an enzyme at lower acid and higher alkali pH value may prevent conformation of the active site, binding of substrates, and/or catalytic of the reaction (Segel, 1976; Whitaker, 1994). 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. Red (rubra) polyphenoloxidase showed a higher pH stability compared to white (alba) but toward the alkaline pH.

The enzyme did not showed interspecies variation in the pH and temperature optimal activity as the temperature activity for cola nitida subspecies alba and rubra was found to be 50°C. This result is comparable to polyphenoloxidase from Strawberry (Serradell et al., 2000) and cucumber (Miller et al., 1990) that was reported to show a relatively high optimum temperature of 50°C using catechol and pyrocatechol as a substrate respectively. Optimal temperature of the activity is also affected by the substrate used in the assay (Yoruk and Marshall, 2003). However the optimum temperature of activity in this study is higher than that for apple (Zhou et al., 1993), banana (Yang et al., 2000) and mango.

The polyphenoloxidase was stable at 30°C – 40°C in the two kolanut for 1hour. Enzyme was unstable as from 50°C as there was a substantially inactivation after 10 minutes incubation time between 50°C - 70°C. White (alba) polyphenoloxidase is more resistant to thermal denaturation when compared with a more active enzyme from red (rubra). The variability in the Km and Vmax values of the polyphenoloxidase between the two subspecies of Cola nitida may relate to the absolute concentration of polyphenoloxidase present in the respective plant specimen, since absolute concentration of enzyme [E] shows direct proportionality with maximum enzyme activity (Vmax): [E] Vmax.

CONCLUSION

The same pH and temperature optimal activity of pH 9 and 50°C was observed for polyphenoloxidase from both samples. Red (rubra) Kolanut showed a higher polyphenoloxidase activity while white (alba) Kolanut a better thermal stability compared to red Kolanuts.

![Figure 6: Effect of different substrate on enzyme activity](image)
Tyrosine and DOPA was used as monophenol and diphenol respectively. Enzyme activity was carried out according to standard procedure.

![Double reciprocal plot](image)

**Figure 7: Double reciprocal plot**

**REFERENCES**


Physicochemical properties of polyphenoloxidase of white and red Kolanuts


