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Extraction and Activity of Polyphenol Oxidase from Kolanuts (*Cola nitida* and C*ola acuminata*) and Cocoa *(Theobroma cacao)*

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ABSTRACT

The activity of polyphenol oxidase was investigated in kolanuts (Cola nitida and Cola acuminata) as well as cocoa (Theobroma cacao). Spectrophotometric method was used to assay the enzyme activity and the kinetic constants - maximum enzyme velocity (Vmax) and Michealis - Menten constant (Km). The maximum enzyme activity (Vmax) for the three plant species were 1.1 x10^{-3} O.D/Sec, 1.5 x 10^{-4} O.D/Sec and 2.5 x 10^{-3} O.D/Sec for C. nitida, C. acuminata and T. Cacao respectively. The Michealis – Menten's constant (Km) were 9.1x10^{-4}M, 9.1x10^{-4}M and 5.0x10^{-3}M for C.nitida, C.acuminata and T.cacao respectively. The variation in maximum enzyme activity (Vmax) reflects interspecies differences in the absolute quantity of enzyme present in the three plant tissues. However, the km values were the same for the two species of kolanuts but varied when compared with the km value of enzyme extract of T. Cacao. The differences in km and Vmax values showed that there are variations in the physicochemical characteristics and absolute quantity of polyphenol oxidase present in the three plant species.

Key words: *Polyphenol oxidase, Cola nitida, Cola acuminata, Theobroma cacao, Maximum enzyme activity, Michealis-Menten's constant.*

INTRODUCTION

In Nigeria, over forty cola species exist, out of which *Cola nitida* and *Cola acuminata* are of major economic and social importance (Egbe and Oladokun, 1987). *C.nitida* is by far the most important of these species and commonly referred to as 'gbarya" or "gworo" by natives of Nigeria. *C. acuminata* is bitterer and less disease – prone species, commonly referred to as 'abata" or "Oji Igbo". Proximate analysis has been carried out by AOAC, (1990) on these two species of kolanuts based on their mineral content and certain food functional properties. The presence of caffeine in kolanuts was demonstrated by Attfield, (1865), who reported the range 1 – 2% per gram of fresh nuts. The nutritional value of protein present was compromised by the large quantity of polyphenol oxidase content. Polyphenols form a caffeine – glucose – tannin complex, which breaks down upon drying under the influence of enzymes to form red substance known as kola red (tannin) and free caffeine (Connie and Gina, 1996). *T. cacao* is of economic importance among the several species of the genus (Uguru, 1981) and theobromine is the chief alkaloid principle of cacao (Ensiminger and Vamos – Vig azo, 1995).

Polyphenol oxidase (PPO) was first discovered in mushrooms and is widely distributed in nature (Vaughn and Duke, 1984). They appear to reside in the plastids and chloroplast of plants although freely existing in the cytoplasm of senescing or ripening plants (Mayer and

Harel, 1979). Polyphenol oxidase is a bifunctional, copper containing oxidase having both catecholase and cresolase activity (Ryden and Malmstron, 1968). It is responsible for the development of the characteristic golden colour in dried fruits such as raisins prunes, dates etc (Ensiminger and Vamos-Vigyazo, 1995). PPO 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. A variety of phenolic compounds are oxidized by PPO, the most important substrate are catechins, cinnamic acid esters, 3.4 hydroxyphenylalanine (DOPA) and tyrosine (Whitaker, 1972). The optimum pH for PPO activity is 5 – 7. The enzyme is relatively heat labile and can be inhibited by sulphites, halides ascorbic acid, aromatic acids (e.g. benzoic and cinnamic acids) quinones couplers and various substrate binding compounds (Sapers,1993; Lambrecht,1995; Vamos-Vigyaz,1980; Walker,1995; Walker,1976;Manson and Peterson,1965;Richard-Forget *et al*,1992,Hsu,*et al*,1988 and Sapers,*et al*,1989).

Enzymatic browning cannot occur unless there is cellular damage on the fruit or vegetable e.g. when kolanut is broken or chewed out. Therefore, cellular integrity is necessary to set up control mechanisms which prevent polyphenol oxidase mediated browning reactions. Such control mechanisms exist in the form of enzyme and substrate compartmentalize. Both come into contact when the separating cell membranes are punctured as the nut is cut and chewed out. Mayer and Harel (1979), have postulated that at least two or three distinct reactions may be involved leading to enzymatic browning. These include increase in oxygen tension at the enzyme active site, sequestration of phenolic compounds to increase their accessibility to enzyme active site and enzyme activation by limited proteolysis, aggregation reaction or conformational changes.

This present study is intended to investigate and ascertain some kinetic parameters Vmax and Km values, of polyphenol oxidase extracted from three plant species. It is believed these values may give a preliminary insight into the physicochemical nature and relative abundance of the enzyme in the three plant specimen. Furthermore, the result may attempt to show and establish interspecies variations with respect to the biochemical nature of PPO in plant tissues.

Material and Methods

Collection of Samples: The two species of kolanuts (*C. nitida* and *C. acuminata)* and cocoa (*T.cacao*) were purchased at Eke Ukwu Owerri, located along Douglas Road in Owerri Municipal Local Government Area, Imo State, Nigeria on 8th October,2005.The plant specimens were identified and authenticated by Dr. F.N Mbagwu of Plant and Biotechnology Department Imo State University, Owerri, Nigeria.

Preparation of Crude Extract of Polyphenol Oxidase: Ten grams of the plant samples were washed and cut into a 10g/l ice cold sodium sulphite solution and allowed to stand for 20 minutes. After soaking, the sodium sulphite solution was decanted and the cut sample

subsequently washed thoroughly with distilled water. The samples were blended in 20ml phosphate buffer (pH = 7) for 3 minutes. The resulting homogenate was quickly squeezed through two layers of clean cheese cloth into a beaker kept in ice. The crude extract was filtered through Whatman No 1 filter paper. The separated filtrate was subsequently centrifuged at 1000g for 10 minutes. The supernatant constituted the crude enzyme extract of polyphenol oxidase.

Determination of Polyphenol Oxidase Activity: The activity of polyphenol oxidase was determined based on the methods of Ensiminger and Vamos – Vigyazo, (1995). Serial dilutions of 0.012M, 0.006M 0.003M, 0.0015M and 0.00075M were prepared from a stock solution of 0.024M of catechol. To each test tube corresponding concentrations of 1ml of catechol solution was added. It was followed by the addition of 1ml of 0.1M phosphate buffer (pH = 7.0). A 3ml distilled water was added and the enzyme reaction started by the introduction of 0.5ml of the enzyme extract of polyphenol oxidase. The mixture was quickly transferred into a cuvette and the change in absorbance was monitored spectrophotometrically at λmax = 540nm at a regular interval of 30 seconds.

Results

The increase in absorbance (Optical Density (O.D)) of the reaction mixture with time intervals of 30 seconds which reflects polyphenol oxidase activity is presented in tables 1, 2 and 3 for enzyme extract of PPO available in *C. nitida, C. acuminata* and *T.cacao* respectively.

Table 1: Change in Absorbance during the Determination of Polyphenol Oxidase Activity in *C.nitida*

Times (seconds)	Concentrations of Catechol				
	0.012M	0.006M	0.003M	0.0015M	0.00075M
30	0.03	0.04	0.02	0.02	0.02
60	0.06	0.08	0.04	0.04	0.04
90	0.09	0.11	0.06	0.06	0.05
120	0.12	0.15	0.07	0.07	0.06
150	0.16	0.19	0.09	0.08	0.08
180	0.18	0.23	0.10	0.10	0.09
V_1 (O.D/Sec)	$1.0X10^{-3}$	$1.3X10^{-3}$	$7.0X10^{-4}$	$7.0X10^{-4}$	$5.0X10^{-4}$
$^1/_{V1}$ (O.D/Sec^{-1})	$1.0X10^{3}$	$7.29X10^{2}$	$1.429X10^{3}$	$1.429X10^{3}$	$2.0X10^{3}$
$^1/_{[S]}M^{-1}$	$8.3X10^{1}$	$1.67X10^{2}$	$3.33X10^{2}$	$6.67X10^{2}$	$1.333X10^{3}$

Table 2: Change in Absorbance During the Determination of Polyphenol Oxidase Activity in *C.acuminata*

Times (seconds)	Concentrations of Catechol				
	0.012M	0.006M	0.003M	0.0015M	0.00075
30	0.003	0.003	0.015	0.015	0.003
60	0.007	0.008	0.015	0.015	0.003
90	0.007	0.013	0.016	0.025	0.008
120	0.012	0.013	0.016	0.025	0.013
150	0.012	0.018	0.017	0.026	0.013
180	0.017	0.023	0.017	0.026	0.018
V_1 (O.D/Sec)	**$9.3X10^{-5}$**	**$1.3X10^{-4}$**	**$1.1X10^{-4}$**	**$1.7X10^{-4}$**	**$6.7X10^{-5}$**
$^1/_{V1}$ (O.D/Sec^{-1})	**$1.1111X10^4$**	**$7.692X10^3$**	**$9.520X10^3$**	**$5.882X10^3$**	**$1.4925X10^4$**
$^1/_{[S]}M^{-1}$	**$8.3X10^1$**	**$1.67X10^2$**	**$3.33X10^2$**	**$6.67X10^2$**	**$1.333X10^3$**

Table 3: Change in Absorbance During the Determination of Polyphenol Oxidase Activity in *T.cacao*

Times (seconds)	Concentrations of Catechol				
	0.012M	0.006M	0.003M	0.0015M	0.00075M
30	0.06	0.05	0.02	0.03	0.02
60	0.22	0.14	0.05	0.04	0.03
90	0.31	0.21	0.08	0.05	0.03
120	0.37	0.27	0.09	0.05	0.04
150	0.41	0.31	0.14	0.06	0.04
180	0.43	0.35	0.15	0.07	0.04
V_1 (O.D/Sec	**$3.0x10^{-3}$**	**$2.0x10^{-3}$**	**$9.0x10^{-4}$**	**$4.0x10^{-4}$**	**$3.0x10^{-4}$**
$^1/_{V1}$ (O.D/Sec^{-1})	**$3.33x10^2$**	**$5.0x10^{-3}$**	**$1.111x10^3$**	**$2.500x10^3$**	**$3.333x10^3$**
$^1/_{[S]}M^{-1}$	**$8.3x10^1$**	**$1.67x10^2$**	**$3.33x10^2$**	**$6.67x10^2$**	**$1.333x10^3$**

Evaluation of Kinetic Constants, Km and Vmax

The kinetic constants, (Km and Vmax values) were evaluated with the aid of the Lineweaver – Burk plot i.e. the double reciprocal plot of initial velocity (1/v) versus substrate concentration ($^1/_{[S]}$).

$$^1/_v = {}^{Km[s]}/_{Vmax} + {}^1/_{[s]}$$

The plots are illustrated in figure 1,2 and 3 for *C.nitida. C. acuminata* and *T.cacao* respectively to show Vmax and Km values

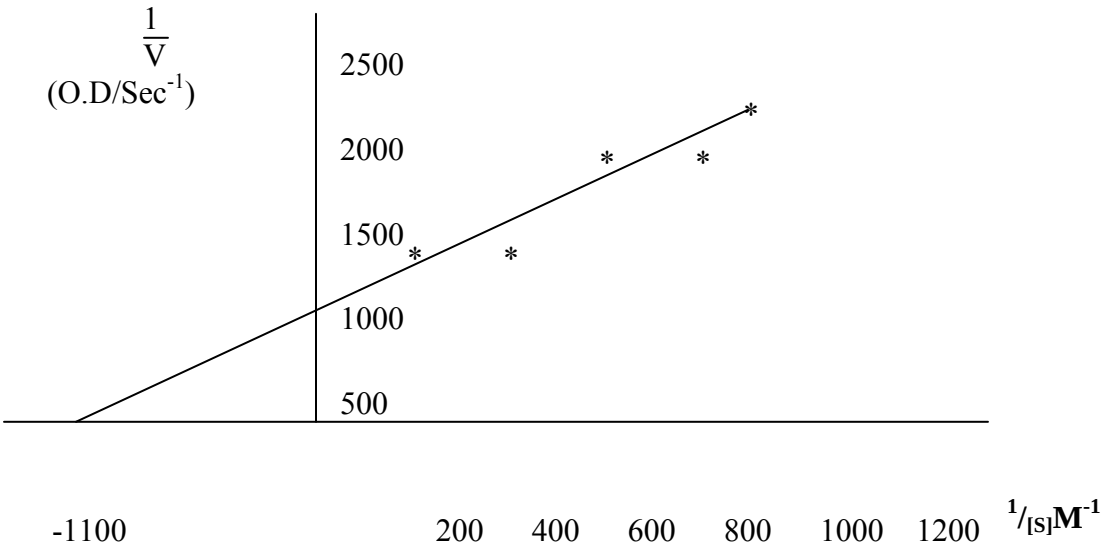

Figure 1: Double reciprocal plot for enzyme extract of *C.nitida*

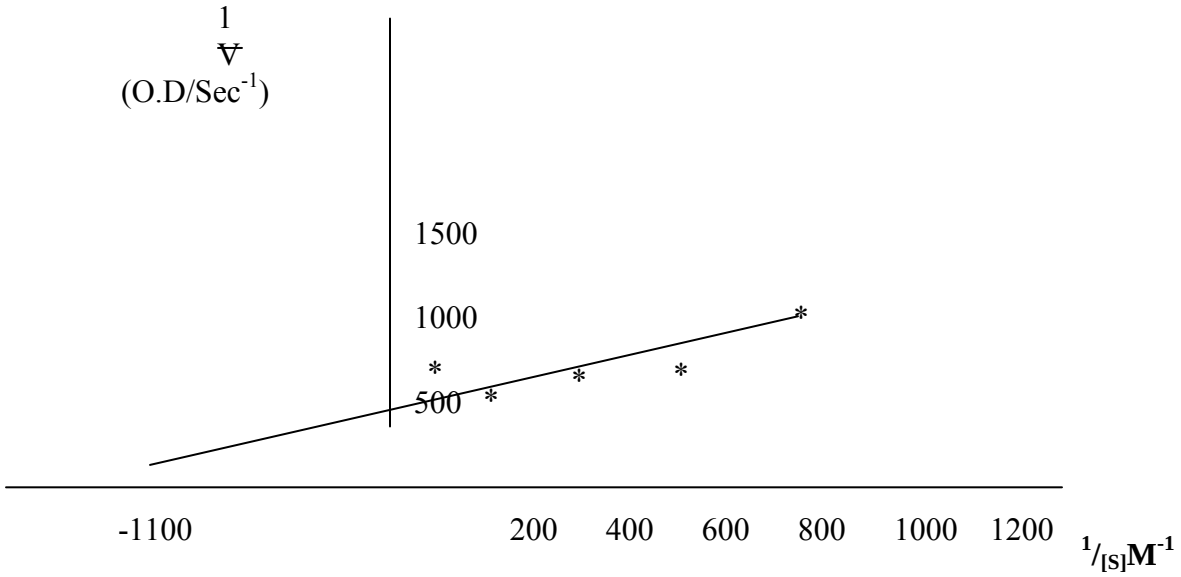

Figure 2: Double reciprocal plot for enzyme extract of *C.acuminata*

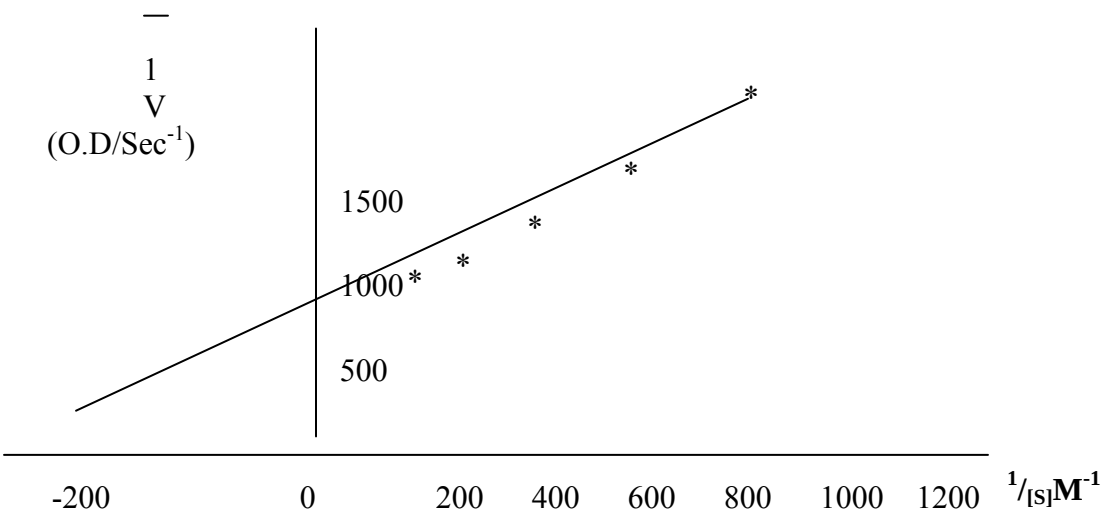

Figure 3: Double reciprocal plot for enzyme extract of *T. cacao*

Values of Km and Vmax for *C.nitida, C.acuminata* and *T.cacao* are as follows:

C.nitida: Km = 9. 1 x 10^{-4}M
Vmax = 1.1 x 10^{-3} O.D/Sec.

C.acuminata: Km = 9. 1 x 10^{-4}M
Vmax = 1.5 x 10^{-4} O.D/Sec.

T.cacao: Km = 5. 0 x 10^{-3}M
Vmax = 2.5 x 10^{-3} O.D/Sec.

Discussion

The crude enzyme extracts of the three plant species, *C.nitida, C.acuminata* and *T.cacao* exhibited polyphenol oxidase activity. These findings conformed to earlier reports by Feinberg (1987), Coseteng and Lee, (1987); Cornwell and Wrolstad (1981), and Sapers (1993) who noted the activity of this enzyme in apple, potato and mushroom respectively. The occurrence and wide distribution of the 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 (polyphenol oxidase) in plants (Mayer and Harel, 1979; Vamos-Vigyazo, 1981). The activity of the enzyme present in the three plant extracts showed a degree of interspecies variability. The maximum enzyme activity (Vmax) varied in the three plant

species in the order *C.nitida> T.cacao> C.acuminata.* The variability of Vmax values amongst the three plant species was related to the absolute concentration of polyphenol oxidase present in the respective plant specimen, since absolute concentration of enzyme [E] shows direct proportionality with maximum enzyme activity (Vmax): [E] ∝ Vmax (Uboh, 2004). This result conformed to previous reports by Chen *et al* 1991, which noted significant differences and marked variations in polyphenol oxidase kinetic parameters between enzyme extract of Florida spiny lobster and Western Australian lobster. The level of polyphenol oxidase activity of a particular plant species is inextricably connected to the physiological needs of the plant. Blick, (2005) and Connie and Gina (1996) reported that plants which posses relatively high levels of polyphenol oxidase activity are less susceptible to fungi and bacterial infections. This is obviously connected to the bacteriostatic properties of the brown products/pigments (melanin) of the enzyme action.

The Km values of the enzyme extract of *T.cacao* varied markedly from the two kolanut species, *C-nitida* and *C. acuminata* ($Km_{T.cacao}$ = 5.0 x 10^{-3}M, $Km_{c.nitida}$ and $Km_{C.acuminta}$ = 9.1 x 10^{-4}M). Many enzyme posses Km values that is approximately equal to the physiologic concentration of their substrate (Rodwell, 1983). Therefore, it could be inferred that the difference in the Km values between the two genera is an obvious reflection of relatively higher concentrations of mono- and polyphenolic compounds in cocoa than kolanut. Polyphenol oxidase is a generic term that defines any protein molecule capable of oxidizing polyphenolic compounds to their equivalent quinone derivatives. This study has shown that the catalytic protein extracted from *T.cacao* and the two kolanut species (*C.nitida* and *C.acuminata*) differ in their respective affinity for the same substrate (catechol) - which is a reflection and measure of the Km values. Therefore, this is an indication that the catalytic protein molecules extracted from the two genera are functionally different with respect to their physical and chemical properties. However, the catalytic protein molecules extracted from the two genera belong to the same catalytic entity-polyphenol oxidase. Furthermore, it is worthwhile to note that the affinity between an enzyme and its corresponding substrate is a cumulative function of the chemical and physical properties of the catalytic protein molecule. Marshall, e*t al* (2000) had earlier reported difference in the Km values of enzyme extract from mango fruit. They postulated that the relative affinities of the enzyme for both mono – and polyphenolic compounds were a reflection of the presence of isoenzymic forms of polyphenol oxidase. Furthermore, polyphenol oxidase isoenzyme isolated from mushroom showed variation with respect to their respective chemical, physical and kinetic properties (Fugimoto, *et al* (1972). The subunit differences were believed to be responsible for relative variations in affinities of the enzymes for both mono- and polyphenolic substances (Goy, *et al* 1992).

This research work suggests interspecies variability in the absolute quantity of polyphenol oxidase and the presence of isoenzymes between two genera – *Cola spp* and *Theobromine spp.*

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