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Fractional purification and kinetic parameters (K_m and V_{max}) of polyphenol oxidase extracted from three segments of *Solanummelongenas* and *Musa sapientum*fruits

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ABSTRACT

Polyphenol oxidase (PPO) was extracted from three segments of *Solanummelongenas* and *Musa sapientum*fruits and partially purified. The specific activity of PPO was measured at each purification step to ascertain level of enzyme purity. In all cases, PPO conformed to Michaelis-Menten kinetics, showing different values of kinetics parameters. Michaelis-Menten constant for PPO (PPO_{Km}) of *S. melongenas* mid-section and anterior segments showed no significant difference (p< 0.05), whereas the posterior gave PPO_{Km} = 4.6±0.49 mM (p > 0.05). Maximum PPO activity (PPO_{Vmax}) was highest in the posterior segment: PPO_{Vmax}= 0.602±0.09 U. Mid-section of *M. sapientum* exhibited the highest K_m value (PPO_{Km} = 5.8±0.69 mM) compared with the anterior (PPO_{Km} = 3.9±0.69 mM) (p> 0.05) and posterior PPO_{Km} = 4.9±0.11mM segments (p< 0.05). Overall, *M. sapientum*PPO_{Km} values were relatively higher than those of *S. melongenas.* Posterior *S. melongenas* exhibited the highest PPO_{Vmax} = 0.602±0.09 U, whereas the lowest value was registered in the anterior segment of *M. sapientum*PPO_{Vmax} = 0.234±0.09 U. Substrate specificity for PPO ($PPO_{Vmax/Km}$) extracted from various segments of *S. melongenas* was in the increasing order of Mid-section > Posterior > Anterior, whereas that of *M. sapientum* was Mid-section > Anterior > Posterior. $PPO_{Vmax/Km}$between the two fruits showed strong positive correlation (r = 0.862339). Catechol was a better substrate for $PPO_{S.\ melongenas}$ than $PPO_{M.\ sapientum.}$ The experimentally observed kinetic parameters of *S. melongenas* and *M. sapientum* signified the presence of PPO isoenzymes and non-uniform distribution of PPO in the two fruits.

KEYWORDS

Polyphenol oxidase;
Solanummelongenas;
Musa sapientum;
Kinetics parameters.

INTRODUCTION

Enzymatic browning describes the discoloration of fruits and vegetables, often facilitated by a collection of enzymes collectively called polyphenol oxidases (PPO)[1,2]. The enzyme action is initiated by disruption of cell integrity and when the content of plastid and vacuole are mixed caused by senescence, wounding, or other tissue damage[3,4]. In addition, Thipyapong *et al*[5], posited the connection between PPO activity and development of plant water stress and potential for photoinhibition and oxidative damage. Enzymatic browning reaction is initiated by interaction of phenolic compounds with PPO in the presence of molecular oxygen[6]. PPO catalyzes two reactions namely, hydroxylation of monophenols to give *o*-diphenol (monophenol oxidase, cresolase tyrosinase activity EC. 1.14.18.1)[7,8] and oxidation of *o*-diphenol to *o*-quinones (diphenol oxidase, catecholase activity EC.1.10.3.1)[9,10]. The *o*-quinones readily polymerize and/or react with endogenous amino acids and protein molecules and their derivatives to form complex brown or related pigments[3,7,11]. Some of PPO substrates that occur naturally in fruits and vegetables, very suitable to enzymatic browning are chlorogenic acid, catechin and epicatechin[12].

PPO is a copper (Cu^{2+}) containing metalloenzyme predominantly located in the chloroplast thylakoid membrane[13]. The two atoms of Cu^{2+} are tightly bound to three histidine residues of a polypeptide chain[7]. The enzyme exists in isoforms[3,4,15] and as zymogen[16,17]. PPO activation can be achieved by variety of treatments such as urea[18], polyamines[19], anionic detergents such as sodium dodecyl sulphate (SDS)[20] and trypsin or proteinase K[21,22]. PPO in plant tissues exist in two major states. These are 85% met-PPO and 10-15% oxy-PPO forms. PPO is often isolated in the met-PPO form[23].

The molecular weight of PPO extracted from different plant species have been reported by several studies. Probably due to partial proteolysis of the enzyme during its isolation, the molecular weight of plant PPO are very diverse and variable; *Eriobotrya japonica Lindl*; 59.2-61.2 kDa[17], *Brassica oleracea*; 39 kDa[24], *M. sapietum*; 62 kDa[25], *Phaseolus vulgaris* L; 120 kDa[26], *Malpighiaglabra* L; 52 and 38 kDa[27]and *Brassica rapa*; 65 kDa[28].

Solanummelongenas commonly referred to as garden egg in Nigeria and banana (*Musa sapietum*) are fruits widely grown as cash and food crops in the Tropics. Browning reaction of fresh-cut fruits and vegetables is a crucial and limiting factor determining the shelf life and acceptability of these products. Understanding the biochemical properties and kinetics of PPO is an imperative for applying control measures to mitigate this undesirable reaction.

Previous reports on the kinetic properties of PPO involved the study of enzyme extracts obtained from whole fruits and vegetables[2,11-13,17,29,30,27]. Furthermore, there are reports on isoforms[3,31,32] and non-uniform distribution of PPO in plant systems[33,34]. Therefore, these earlier reported kinetic properties of PPO extracted from whole fruits and vegetables probably did not represent the true kinetic features of the various PPOs in those plant specimen. The present study seeks to measure two kinetic parameters, MichaelisMenten (K_m) and maximum velocity (V_{max}), of PPO extracted from the posterior, mid-section and anterior segments of *S.melongenas* and *M.sapietum*fruits. The study will give an insight into kinetic properties and, by extension, relative abundance/distribution of PPO in the three portions of the two fruits under investigation.

MATERIALS AND METHODS

Collection and preparation of fruit samples

Fresh and disease free fruits of*S. melongenas* and *M. sapietum* were harvested from a private botanical garden in Umuoziri-Inyishi, Imo State, Nigeria between 17th -30th of July, 2012. The fruits were identified and authenticated by Dr. F.N.Mbagwu at the Herbarium in the Department of Plant Science and Biotechnology, Imo State University, Owerri, Nigeria. The two fruits were washed under continuous current of distilled water for 5 min and air dried at room temperature. The stalk (*S.melongenas*) and rind (*M.sapietum*) were removed manually. The samples were cut into three distinct segments: anterior, mid-section and anterior, and stored at -4 °C until used for analyses.

Extraction and purification of PPO

Extraction and partial purification of PPO was according to the methods of Madani*et al*[10], with minor modifications. Ten grams (10 g) of the sample was homogenized in external ice bath, using Ultra-Turrax T25 (Janke and Kunkel, Staufen, Germany) homogenizer set in 80 mL of 0.1M phosphate buffer (pH 6.8) containing 10 mM ascorbic acid for 180 sec at intervals of 60 sec. The homogenate was quickly squeezed through two layers of clean cheese cloth into a beaker kept in ice. The crude extract samples were centrifuged at 32000 *g* for 20 min at 4 ºC. Solid ammonium sulphate $(NH_4)_2SO_4$ was added to the supernatant to obtain 80% $(NH_4)_2SO_4$ saturation and precipitated proteins were separated by centrifugation at 32000 *g* for 30 min at 4 ºC. The precipitate was re–dissolved in 10 mL distilled water and ultra-filtered in a Millipore stirred cell with a 10-kDa membrane (Millipore 8050, Milan, Italy). The filtrate was dialyzed at 4 ºC against distilled water for 24 h with 4 changes of the water during dialysis. The dialyzed sample constituted the partial purified PPO extract and was used as the enzyme source from the corresponding segments of the two fruits. Protein concentrations were determined by the method of Bradford[35], using bovine serum albumin as standard at λ_{max} = 595 nm. One unit of PPO activity was defined as the amount of enzyme that causes an increase in absorbance of 0.001 mL^{-1} min^{-1} under the condition of the assay[36]. The procedure and measure of PPO purification is summarized in TABLE 1.

Determination of PPO activity

PPO activity was measured immediately after the extract was partially purified. Enzyme assay was according to the methods of Qudsieh*et al*[33], with minor modifications[14]. Enzyme activity was determined by measuring the increase in absorbance at 540 nm using a spectrophotometer (U-2000 Hitachi, Japan) at 24 ºC. The reaction mixture contained 3.5 mL of 0.20 M phosphate buffer (pH = 6.8), 1 mL of each serial dilutions of 12 - 0.75 mM catechol, and 0.5 mL of enzyme solution ina final volume of 5 mL. The mixture was quickly transferred into a cuvette and the change in absorbance was monitored at λ_{max} = 540 nm at a regular interval of 30 sec. The rate of the reaction was calculated from the initial linear slope of activity curves.

Evaluation of kinetic constants

The K_m and V_{max} values of PPO were measured with the use of the Lineweaver– Burk (1/V_o versus 1/[S] values) graphs[37].

Statistical analysis

The experiments were designed in a completely randomized method and data collected were analyzed by the analysis of variance procedure while treatment means were separated by the least significance difference (LSD) incorporated in the statistical analysis system (SAS) package of 9.1 version, (2006). The correlation coefficients between the results were determined with Microsoft Office Excel, 2010 version.

RESULTS

The specific activity of PPO extracts, which was a measure of level of enzyme purity, is summarized in TABLE 1. A cursory look at TABLE 1 showed increasing level of PPO specific activity with the progression of each purification step.

TABLE 1: Specific activity of PPO extracted from three segments of *S. melongenas* and *M. sapietum*fruits at various purification steps.

Purification step	Specific activity (U/mg protein)					
	S. melongenas M. sapietum					
	A	**MS**	**P**	**A**	**MS**	**P**
Centrifuged at 32000 *g* for 20 min at 4 ºC	0.68±0.87	0.78±0.67	2.08±0.87	0.58±0.99	1.98±0.67	1..28±0.95
80% $(NH_4)_2SO_4$	2.72±1.07	2.48±0.37	5.14±0.36	1.98±0.90	3.72±0.81	2.08±1.27
Ultra-filtration	5.18±0.65	5.88±0.91	7.77±0.15	5.91±1.27	6.88±1.07	4.78±1.05
Dialyzed at 4ºC	6.78±0.77	7.18±0.27	8.07±0.47	7.78±1.03	7.85±0.77	6.98±0.93
%yield	11.3±0.78	11.9±0.9.1	12.8±0.71	10.0±0.97	12.1±0.59	10.3±0.87
[protein] mg mL^{-1}	0.012±0.7	0.017±0.9	0.025±0.7	0.010±0.9	0.018±0.5	0.011±0.8

A : Anterior; MS: Mid-section; P: Posterior. The results are means (*X*) ± S.D of three (*n* = 3) determinations.

The kinetic parameters of PPO extracted from three segments of the two fruits are presented in TABLE 2. The K_m values of PPO (PPO_{Km}) extracted from the three segments of *S. melongenas*was in the range of 1.5±0.09-4.6±0.49 mM. Furthermore, PPO_{Km} of mid-section and anterior segments showed no significant difference (p< 0.05), whereas the posterior gave PPO_{Km} = 4.6±0.49 mM; $p > 0.05$. Overall, PPO_{Km} of the three segments of *S. melongenas*was in the order: Mid-Section, Anterior < Posterior. PPO maximum activity (PPO_{Vmax}) was highest in the posterior segment (PPO_{Vmax}= 0.602±0.09 U) compared with the other two segments: mid-section

TABLE 2 : Kinetic constants (K_m and V_{max}) of PPO extracted from three segments of *S. melongenas* and *M. sapietum* fruits.

Fruit segment	*S. melongenas*		*M. sapietum*	
	$K_{m\ (mM)}$	$V_{max\ (U)}$	$K_{m\ (mM)}$	$V_{max\ (U)}$
Anterior	2.0±0.99[a]	0.251±0.04[a]	3.9±0.69[a]	0.234±0.09[a]
Midsection	1.5±0.09[a]	0.393±0.60[b]	5.8±0.69[b]	0.420±0.06[b]
Posterior	4.6±0.49[b]	0.602±0.09[c]	4.9±0.11[b,c]	0.241±0.05[a,c]

The results are means (X) ± S.D of three (n = 3) determinations. Means in the columns with the same letter are not significantly different at $p < 0.05$ according to LSD.

PPO_{Vmax} = 0.393±0.60 U; $p > 0.05$ and anterior PPO_{Vmax} = 0.251±0.04 U; $p > 0.05$.

PPO extracted from the mid-section of *M. sapietum* exhibited the highest K_m value (PPO_{Km} = 5.8±0.69 mM) compared with the anterior (PPO_{Km} = 3.9±0.69 mM) ($p > 0.05$) and posterior (PPO_{Km} = 4.9±0.11mM segments) ($p < 0.05$). *M. sapietum* anterior PPO_{Vmax} was not significantly different ($p < 0.05$) from posterior PPO_{Vmax}. *M. sapietum*mid-section PPO_{Vmax} was highest compare to other two segments ($p > 0.05$). An overview of TABLE 2 showed that *M. sapietum*PPO_{Km} values were relatively higher than those of *S. melongenas*. Posterior *S. melongenas* exhibited the highest PPO_{Vmax} = 0.602±0.09 U, whereas the lowest value was registered in the anterior segment of *M. sapietum*PPO_{Vmax} = 0.234±0.09 U.

TABLE 3 : Catechol specificity for PPO extracted from three segments of *S. melongenas* and *M. sapietum*fruits.

Fruit Segment	V_{max}/K_m (U/mM)	
	S. melongenas	*M. sapietum*
Anterior	0.126	0.060
Mid-section	0.262	0.072
Posterior	0.131	0.049

Substrate specificity for PPO ($PPO_{Vmax/Km}$) extracted from various segments of the two fruits was in the range of 0.049-0.262 U/mM. For *S. melongenas* enzyme extract, the increasing order of $PPO_{Vmax/Km}$was Mid-Section > Posterior > Anterior, whereas that of *M. sapietum* was Mid-Section > Anterior > Posterior. $PPO_{Vmax/Km}$between the two fruits showed strong positive correlation (r = 0.862339).

DISCUSSION

The enzyme extracts from the three segments of *S. melongenas* and *M. sapietum* exhibited PPO activity, which was in conformity with previous reports elsewhere[12,38,39]. The present kinetic study showed that in all cases, $PPO_{S.\ melongenas}$ and $PPO_{M.\ sapietum}$ conformed to Michaelis-Menten kinetics, exhibiting different values of kinetics parameters. In concord with the present findings, Rocha *et al*[40], had earlier noted that PPO isolated from higher plants oxidized a wide range of monophenols and *o*-diphenols with highly variable V_{max} and K_m values.

A measure of affinity of the enzyme for its substrate is defined by the K_m value. Overall, PPO extracts from *S.melongenas* exhibited higher affinity for the experimental substrate (catechol) than those extracted from *M. sapietum*. The affinity of plant PPO for the phenolic substrates was generally low (high K_m values, 2±6 mM) according to Nicolas *et al*[41]. Likewise, the results presented here showed that PPO_{Km} extracted from the three segments of *S. melongenas*and *M. sapietum* was in the range of 1.5±0.09-5.8±0.69 mM (TABLE 2). The variability of PPO_{Km} in the three segments of the two fruits confirmed differences in affinity of the enzymes for phenolic substrates. According to Altunkaya and Gökmen[32], the variability in PPO_{Km} is diagnostic of isoenzymic forms of PPO in *Lactuca sativa*. They noted that substrate specificity in terms of V_{max}/K_m values of two fractions of PPO extracts ($PPO_1$and PPO_4) was different and therefore, order of affinity of the isoenzymes for various substrates varied. Furthermore, Marshall *et al*[42], averred that variations in K_m values of *Mangiferaindica* fruit extracts with concomitant difference in affinity between mono- and polyphenol substrates for the enzyme was an indication of the presence of isoenzyme in *M. indica* fruits. In another study, Cornish-Bowden and Cardenas[43], in their research paper showed that variability in kinetic parameters of non-Michaelis-Menten enzymes provided necessary information for analyzing metabolic pathways associated with isoenzymes. Values of PPO_{Km} of the mid-section and posterior segments of *S.melongenas* showed significant difference ($p > 0.05$) whereas, the difference in K_m values of *M.sapietum* enzyme extract between the mid-section and posterior segments was not significant ($p < 0.05$) (TABLE 2). These observations indicated the presence of isoenzymic forms of PPO in the corresponding segments of the two fruits as reported elsewhere[3,14,32,31]. In another perspective, the K_m values could also give an insight into the physiologic concentrations of the PPO substrates in the three portions of the two fruits under investigation. More than four decades ago, Sheen[44], posited that there is correlation between phenolic quantity and oxidase activity, which varied depending upon the organs and tissues. However, it is worthy to note here that the experimentally observed K_m value is a function of pH and ionic strength of the enzyme assay solution[41,45-47]. The variability of PPO_{Vmax} in the various segments of the two fruit enzyme extracts was a pointer to the fact that differences exist in the relative abundance and distribution of PPO in biologic tissues and systems[4,33,34,48].

PPO V_{max}/K_m defines the suitability of the experimental substrate (catechol) for PPO extracted from the two fruits. An overview of TABLE 3 showed that catechol exhibited relatively low specificity for $PPO_{M. sapietum}$ compared to $PPO_{S. melongenas}$. Previous reports have established that certain categories of phenolic compound are poor substrate to PPO by virtue of their specificity ratio V_{max}/K_m [40,49]. For instance, monophenol (tyrosine) was found to be a poor substrate for the apple PPO[40,41]. Richard-Forget *et al*[50]. showed that several compounds such as chlorogenic acid and catechins appeared to be better substrates than 4-methylcatechol for PPO extracted from Red Delicious apples. The kinetic parameters (TABLES 2 and 3) indicated that catechol was a better substrate for $PPO_{S. melongenas}$ than $PPO_{M. sapietum.}$ Furthermore, the experimentally observed kinetic parameters of *S.melongenas* and *M.sapietum* signified the presence of PPO isoenzyme and non-uniform distribution of PPO in the two fruits.

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