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KINETICS AND ACTIVITY OF POLYPHENOL OXIDASE EXTRACTED FROM TAMARIND PULP (*Tamarindus Indica* L.)

ELJACK ND*, ALKAMEL GA AND ALOTAIBI MT

Department of Chemistry, Turabah University College, Taif University, KSA

***Corresponding Author: Nasma D. Eljack: E Mail: nasma_74@hotmail.com**

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ABSTRACT

Polyphenol oxidase (PPO) is a biological catalyst responsible for the oxidation of phenolic compounds in some plants, seafood, causing enzymatic browning and melanin formation in the skin.

In this study, PPO was extracted from the pulp of tamarind (*Tamarindus indica* L.) and partially purified by an ammonium sulphate precipitation method. The crude PPO was characterized in terms of, temperature, pH, and kinetics. The results show that PPO exhibits maximum activity at pH = 7.0 and temperature 30°C in the presence of catechol as substrate. The kinetics of the enzymatic reaction was followed and determined spectrophotometrically. The specificity of tamarind PPO towards different substrates was studied using catechol, gallic acid, and hydroquinone as sources of phenolic compounds. The results showed that the enzyme has higher affinity and specificity for catechol followed by hydroquinone and gallic acid. Using Michelis – Menten model, the kinetic parameters, (K_m and V_{max}) were determined, and the values of these parameters were as follow: 12.33 mM and 0.125 au/min for catechol, 33.23 mM and 0.121 au/min for hydroquinone 23.37 mM and 0.115 au/min for gallic acid.

The results showed that ascorbic acid suppressed PPO activity more than EDTA and the percentage inhibition was calculated as 70% for ascorbic acid and 45.5% for EDTA. On the other hand, metal ions were found to increase the enzyme activity.

Regarding enzyme stability of tamarind PPO, it was clear that the PPO activity decreased with increasing temperature, as the protein denatured and thus lost its catalytic property.

Keywords: Polyphenol oxidase, Oxidation, Substrate, Inhibitor

INTRODUCTION

Polyphenol oxidase (PPO) is an enzyme responsible for oxidizing phenolic compounds and producing dark color. However, the typical color characteristic of enzymatic browning appears to result from non-enzymatic secondary reactions.

The food industry is facing a serious problem with brown discoloration associated with processing and storing foods, especially fruits, vegetables, and meat. Browning reduces the nutritional value, sensory quality, and taste of food **[1]**.

It is important to note that enzyme-induced browning reduces the shelf life of some foods and affects the preservation of fruits and vegetables **[2]**.

PPO is a copper-enzyme with two binding sites, one site binds aromatic phenolic compounds like catechol, while the other site binds oxygen and metal-substances, such as cyanide.

The mechanism of these reactions is complex, since PPO is capable of catalyzing the oxidation of a wide range of phenolic compounds (monophenols and/or diphenols) **[3]**.

In the presence of oxygen, monophenols are hydroxylated into o-diphenols followed by the oxidation of o-diphenols into o-quinones **[4]**.

However, the exact mechanism of phenol oxidation by PPO is unknown.

Understanding this mechanism may improve the control or prevention of browning **[5]**.

Food browning in some cases is a desire property, it is an important reaction in the fermentation of tea and cocoa and in the production of date, raisin, and prune products.

It protects higher plants from insects and microorganisms and forms an impermeable scab of melanin when injured to protect them from further micro-organism attacks and forms a melanin scab when they are wounded **[6]**.

PPO also finds uses in industrial applications such as the development of biosensors for immunoassays, the detection of phenolic compounds in foods and beverages, and the detection of morphine, codeine, and catecholamines. In cosmetics, some hair dyes contain PPO **[7]**.

PPO has been isolated and characterized from the product of many commercially important plants (fruits and vegetables) such as strawberries **[8]**, lettuce **[9]**, cocoa beans **[10]**, tea leaves **[11]** and peppers **[12]**.

It was found that the ripe pulp of tamarind turns from white color to brownish red. During storage, the brown pulp slowly turns to deep brown and finally becomes black, so evaluating the presence of PPO in the pulp of tamarind and its activity and kinetics has been studied. There is also no previous study on the kinetics and activity of PPO of this fruit. Therefore, it is necessary to observe the color changes in tamarind pulp during storage and consequently to understand the mode of these changes. Thus, general objective of this study is to extract and partially purify PPO in tamarind pulp and kinetically study the enzymatic reaction using spectrophotometric method.

MATERIALS AND METHODS

Collection of samples

The tamarind fruits used in this study were purchased from a local market in Khartoum, Sudan and dried at room temperature.

Chemicals and instruments

Polyvinylpyrimidine (PVP) from Acros Organics, ammonium sulfate, ascorbic acid, sodium monohydrogen phosphate, sodium dihydrogen phosphate, sodium acetate, acetic acid, copper sulfate, catechol, hydroquinone, gallic acid, benzoic acid and EDTA.

All these chemicals were of analytical grade and were used without further purification. Distilled water was used for the preparation of all solutions.

The instruments and apparatus used in this study are: UV spectrophotometer (UV-1800 SHMADZU), pH meter (Hannah), magnetic stirrer, centrifuges (Shimadzu), ice bath and water bath.

Extraction and partial purification of Tamarind PPO

All steps of enzyme extraction and purification were carried out at 4°C.

Twenty g of the homogenized brown pulp of tamarind was extracted with 0.1 M phosphate buffer (pH=7.0) containing 0.1 g polyvinylpyrrolidone (PVP), and the mixture was stirred for 15 min with a magnetic stirrer. The supernatant was filtered through Whatman No.42 filter paper and collected as an enzyme extract and precooled at 4° C. The enzyme extract was then removed. The homogenate was centrifuged at 12,000 rpm (1000 centrifuge) for 30 minutes. The supernatant was leached at 4°C for

about 3 hours. Then it was centrifuged again for 20 minutes. Finally, the precipitate was discarded and the supernatant was collected as enzyme extract for analysis. It is worth mentioning that the insoluble polymer polyvinylpyrrolidone (PVP) was used for the extraction of soluble enzymes from plant tissues.

PVP is very effective in removing the phenolic compounds **[13]**. PVP was used to extract soluble enzymes from plant tissues.

Determination of tamarind PPO activity

In order to determine the tamarind PPO activity, a UV-Vis spectrophotometer was used to measure the rates of quinone formation, as indicated by an increase in absorbance at 400 nm.

To evaluate the optimal concentration of enzyme preparation, activity was determined in a volume of 3.0 mL containing 2.0 mL of substrate (200 mM) and various amounts (0.1-1.0 mL) of enzyme preparation. The mixture was made up to 3.0 mL with phosphate buffer (pH 7.0) in a quartz cuvette with a layer thickness of 1 cm. The blank sample consisted of 3.0 mL of 0.1 M phosphate buffer (pH 7.0). The reaction was carried out at 30° C.

From the slope of the linear part of the absorption-time curve, the initial rate of the oxidation reaction was calculated.

PPO activity is defined as the amount of enzyme required to catalyze the conversion of 1/µmol of substrate per minute under the experimental conditions.

Effect of pH on tamarind PPO activity

The activity of PPO was determined at different pH values ranging from (3.0-9.0) using 0.2 M acetate buffer (pH 3.0-5.0) and 0.1 M phosphate buffer (pH 6.0-9.0). The optimum pH of PPO activity was determined using catechol as substrate by the spectrophotometric method described above.

Effect of temperature on PPO activity of tamarind

To determine the optimum temperature for PPO, the activity of the enzyme was measured at different temperatures (10-60°C) in the presence of catechol. The reaction mixture (buffer and substrate) was heated to the appropriate temperatures for 15 minutes. Once thermal equilibrium was reached, the unheated enzyme was added and the

reaction was followed spectrophotometrically.

Enzyme kinetics

The enzymatic oxidation was studied spectrophotometrically to determine the kinetic parameters, Michaelis constant (K_m) and maximum velocity (V_{max}). The initial velocities (V) were measured at different substrate concentrations [S]. From the Line Weaver-Burk graphs (1/V versus 1/[S]), the values of K_m and V_{max} were determined for each substrate. The results are shown in **Table 2 and Figure 5 and 6**.

The K_m and V_{max} values of Tamarind PPO for catechol were calculated from the Line Weaver-Burk graph using the Michaelis-Menten equation, which describes the kinetic behavior of many enzymes

$$V - V_{max}[S] / K_M + [S]$$

The double reciprocal was obtained from Michaelis-Menten equation for a straight-line.

$$\frac{1}{V} = \left(\frac{K_M}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

$$\text{Intercept} = \frac{1}{V_{max}}$$

$$V_{max} = 1/\text{intercept}$$

Thus, the slope of the regression line, is equal to $\frac{K_M}{V_{max}}$,

K_M = slope /intercept

Substrate Specificity of Tamarind PPO

An attempt was made to select the best substrate corresponding to the highest enzyme activity. Tamarind-PPO activity was determined using three different substrates: Catechol, hydroquinone and gallic acid. The concentrations of the substrates were varied in the range of (20-153.3) mM under optimal conditions.

Effect of inhibitors and activator on PPO activity of tamarind

The effects of two inhibitors (ascorbic acid and ethylenediaminetetraacetic acid sodium salt) and copper sulphate as activator on tamarind PPO activity were studied using 0.3 mL of enzyme preparation, 1.0 mL of 200 mM catechol and different concentrations of inhibitor or activator at fixed [catechol] = 200 mM.

Analysis of the experimental data from the regression line was carried out using Orgin7.0 computer program.

RESULTS AND DISCUSSION

Optimization of the experimental conditions

Effect of pH on tamarind PPO activity

Determination of the activity of PPO over the pH range 3.0-9.0, where below pH 6.0 acetate was replaced by phosphate buffer. Tamarind PPO has a fairly broad pH profile with an optimum of 7.0 when catalyzing the oxidation of catechol **(Figure 1)**.

The optimum pH for tamarind PPO is similar to that reported for tea leaves **[11]**, Amasya apple **[15]** and sweet potato **[16]**. In general, most plants show maximum PPO activity at or near neutral pH values. However, the enzyme has a lower or higher pH optimum in some species, such as strawberries (pH 4.5) **[8]** and apples (pH 9.0) **[14]**, (pH 6.0) in DeChaunac grapes **[17]** and 8.5 in dog roses **[18]** when catechol is used as substrate.

The optimum pH for PPO activity ranged from 4.0 to 7.0 in most plants **[19]**, depending on the purity of the enzyme, the type of buffer used and the substrates used for the assay. In addition, the type of phenolic substrates and extraction methods can also affect PPO activity. The pH was increased from acidic to neutral media and then started to decrease after pH 7.0.

Effect of temperature on PPO activity of tamarind.

Temperature is another important factor that significantly affects the catalytic activity of PPO, as it affects the solubility of oxygen and can lead to denaturation of the enzyme. Knowledge in this area is of practical importance as discoloration of processed fruits and vegetables is often prevented by thermal inactivation of PPO. The effects of different temperatures on PPO activity were studied in a temperature range of (10 - 60)°C at optimum pH using 200 mM catechol and 0.3 mL PPO extract. The results are shown in (Figure 2). The results show that the tamarind PPO activity increased with increasing temperature up to 30 °C and decreased at higher temperatures. The decrease in activity at high temperatures may be due to thermal denaturation of PPO, as the intermolecular bonds holding the structure of PPO in place were broken by heat. As a result, the specificity of the active site of the enzyme was lost and the enzyme was denatured.

It has been reported that the optimal temperature of PPO varies depending on species and habitat temperature **[20, 21]**. Most enzymes have an optimal temperature in the range of 30°C to 40°C. The optimum temperature of PPO varies for different plant sources and also for different parts of the same plants. Reportedly, the optimum temperature is 25°C for dog rose **[17]**, 30°C for Rooster potato (*Solanum tuberosum* cv. Rooster) **[21]** and 53°C for Jerusalem artichoke (*Helianthus tuberosus* L.) **[22]** using catechol as substrate.

Enzyme Kinetics

Assaying the activity of Tamarind PPO

In order to determine the best concentration of enzyme preparation corresponding to the

highest enzyme activity various amounts of PPO in the range of (0.1-1.0) mL was assayed in the presence of catechol in the pH and temperature optima.

From the slope of the linear part of the absorbance-time curves, the activity of PPO enzyme was calculated. The activity of tamarind PPO of different enzyme preparation were tabulated in **Table (1)**.

It's clear that an enzyme preparation in in the range 0.1- 0.5 mL exhibits higher activity and it decreased with increasing enzyme amount, 0.3 ml was used as the optimum volume in all other experiments.

Substrate Specificity

A number of compounds were used as substrates for PPO in the literature as the affinities of enzyme changing depend on the substrates under study. In this study catechol, hydroquinone, and gallic acid were chosen as substrates for the enzymatic reaction.

The substrate specificity for PPO was evaluated using different substrate concentrations in the range of (20.0 - 153.3) mM. The initial rate was assayed with the same spectrophotometric procedure mention above.

It's clear from **(Figure 3)**. that in the earlier stage of the oxidation, the rate increased with increasing the concentrations of substrate, and the reaction reached saturation state at higher substrates concentrations, which indicates an excellent performing of Michaelis - mentin kinetic model.

From line weaver-Burk plots in (Figure 4), Michaelis constants (K_m) and maximum reaction velocities (V_{max}) of the tamarind–PPO were obtained for each substrate at optimum conditions (pH 7.0 and 30°C) and were summarized in **Table (1)**.

It's clear that the affinities of the enzyme changing depend on the type of substrate under study. The findings reveal that, Tamarind PPO had higher affinity and specificity towards catechol followed by hydroquinone then gallic acid. Catechol was considered the most common compound used as a substrate for PPO enzyme **[9]**.

R^2 indicate that the data follows the equation for a straight line within Origin7 parameters of linear regression.

K_m value is a measure of enzyme affinity to the substrate. The higher the K_m value the smaller the affinity of the enzyme towards the substrate and vice versa, The value of (V_{max}/K_m) reflects the specificity of enzyme for substrates. Thus, catechol is the best substrate for tamarind PPO, followed by hydroquinone and gallic acid. There have been many studies reported the kinetics of this enzyme in different species of plant,

using catechol as a substrate. K_m values were found to vary in dill (Anethum graveolens) **[23]**, pursalane **[24]** and Hibiscus PPO **[25]** as 2.17 mM, 4.40 mM and 19.23 mM respectively when using catechol as the substrate for PPO.

Effect of inhibitor and activator on tamrind PPO activity

Various techniques have been developed for inhibiting and suppressing the activity of PPO over the years to control the undesirable enzyme activity. These methods eliminate one or more of the essential components (oxygen, enzyme, copper, or substrate) required for PPO reaction.

There are several inhibitors, such as ascorbic acid used by researchers to inhibit enzymatic browning of plants. In this work, ascorbic acid and EDTA were used to inhibit the activity of PPO.

The effect of these inhibitors on Tamarind–PPO activity was studied by varying inhibitor concentrations in the range (13.33 to 113.33) mM at fixed enzyme and catechol concentrations, the results were shown in Table (1), and from the double reciprocal plot the kinetic parameters were obtained as well as the mode of inhibition was investigated.

The percent inhibition for each inhibitor was calculated as in the following equation;

$$\text{Inhibition (\%)} = [(Ao - AI)/[Ao]x100$$

where Ao is initial PPO activity (without inhibitor); AI is PPO activity with inhibitor

$$\text{Inhibition of ascorbic acid (\%)} = [(0.1265 - 0.0368)/0.1265]x100 = 70.1\ \%$$

$$\text{Inhibition of EDTA(\%)} = [(0.12650 - 0.0690)\ /\ 0.12650] \times 100 = 45.5\%$$

PPO from different plant tissues shows different degrees of inhibition. Ascorbic acid that acts as antioxidants reduce the quinonoid products to their initial phenolic form, thus avoiding secondary reactions to form brown polymers and causing apparently a strong inhibition. Based upon the results in **(Figure 5)**, it seems that both ascorbic acid and EDTA are uncompetitive inhibitors. Since uncompetitive inhibition occurs as a result of the inhibitor interacting with a site other than the enzyme's active site, competing with the substrate.

The effect of activator on the enzyme activity was examined using copper sulphate as an activator at fixed reaction conditions. It's clear that the value of V_{max} has increased markedly and the results were shown in **Table (1) and (Figure 6)**.

Table 1: Kinetic parameters for tamarind-PPO reaction at pH = 7.0 and T= 30°C

Amount of the enzyme preparation (mg/mL)	0.1	0.3	0.5	0.7	1.0
PPO Activity (U/min. mL)	213.4	215.7	206.5	146.9	119.2
Substrate	V_{max} (au/mL/min)			K_m (mM)	V_{max}/K_m
Catechol	0.125			12.33	0.010
Hydroquinone	0.115			23.37	0.005
Gallic acid	0.121			33.23	0.004
Inhibitor					
Ascorbic acid	0.068			21.5	0.003
EDTA	0.035			17.32	0.002
Activator					
Copper sulphate	0.35			21.8	0.016

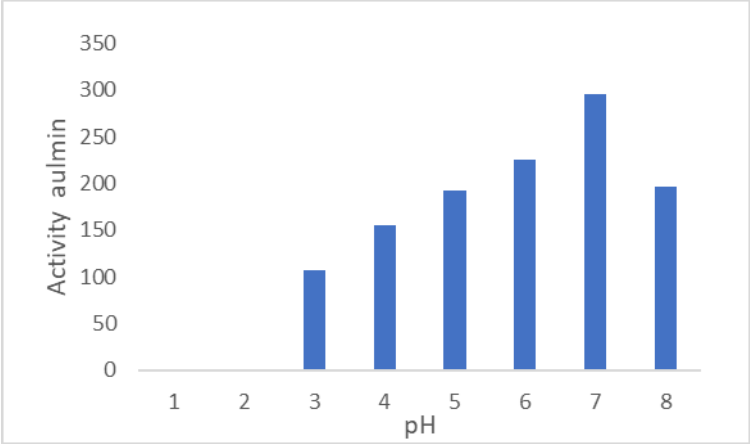

Figure 1: Effect of pH on enzyme activity at PPO = 0.3ml and T= 30°C

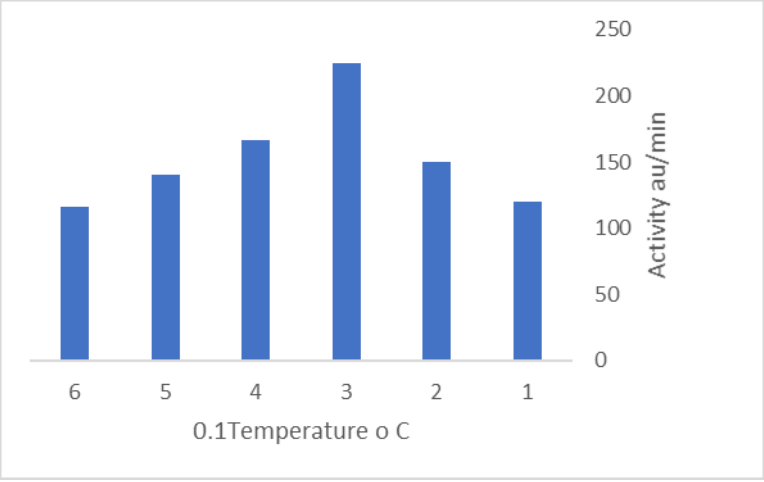

Figure 2: Effect of temperature on enzyme activity at PPO = 0.3ml and pH=7.0

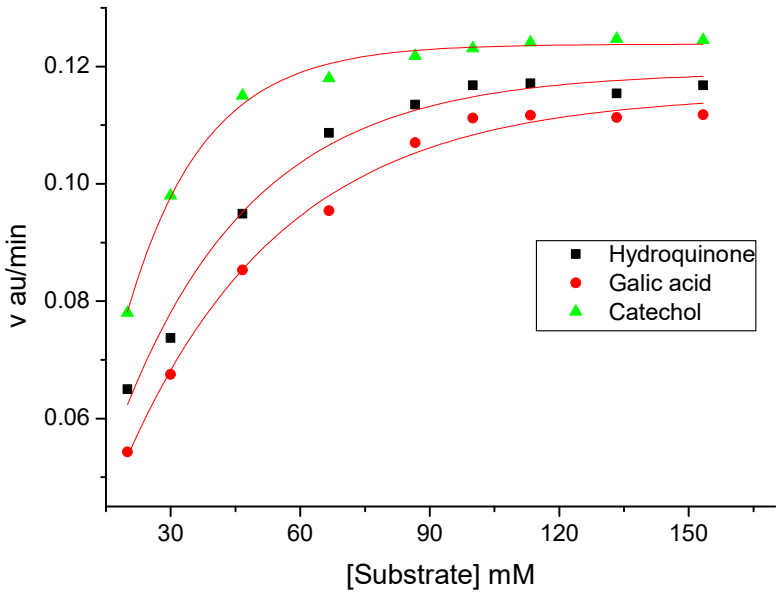

Figure 3: Michaelis-Menten model (velocity (v) au/min vs [substrates] mM) for the substrates under study at PPO = 0.3ml, pH = 7.0 and T= 30°C

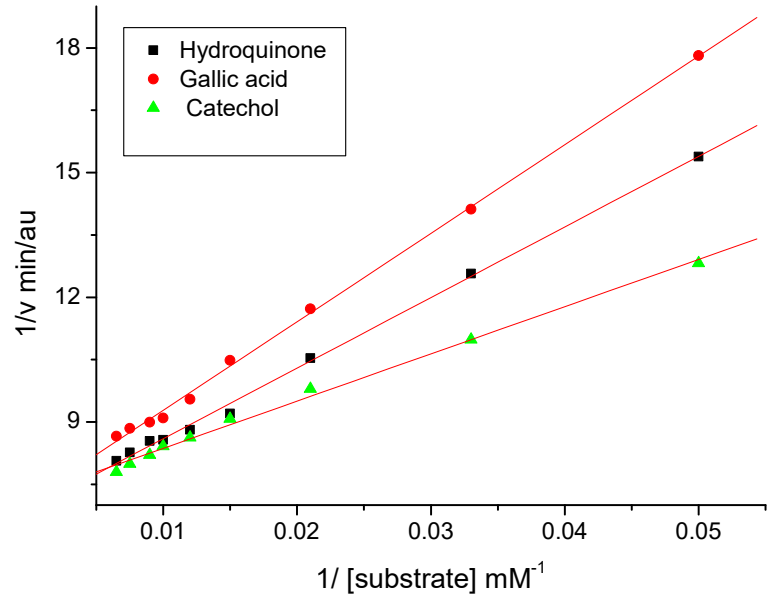

Figure 4: Line weaver-Burke plot for the substrates under study at PPO = 0.3ml, pH = 7.0 and T= 30°C

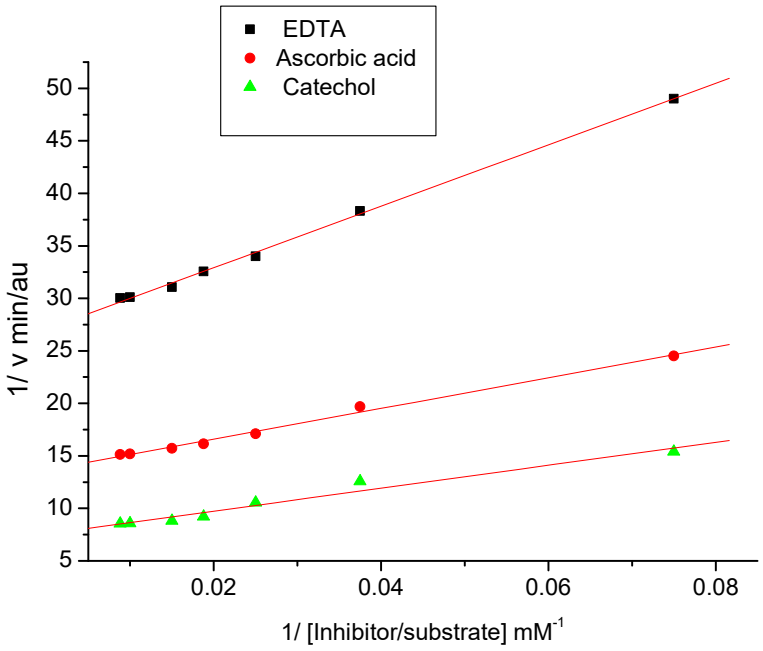

Figure 5: Line weaver-Burke plot showing the effect of inhibitors on the PPO activity, in the presence of catechol at PPO = 0.3ml, pH = 7.0 and T= 30°C

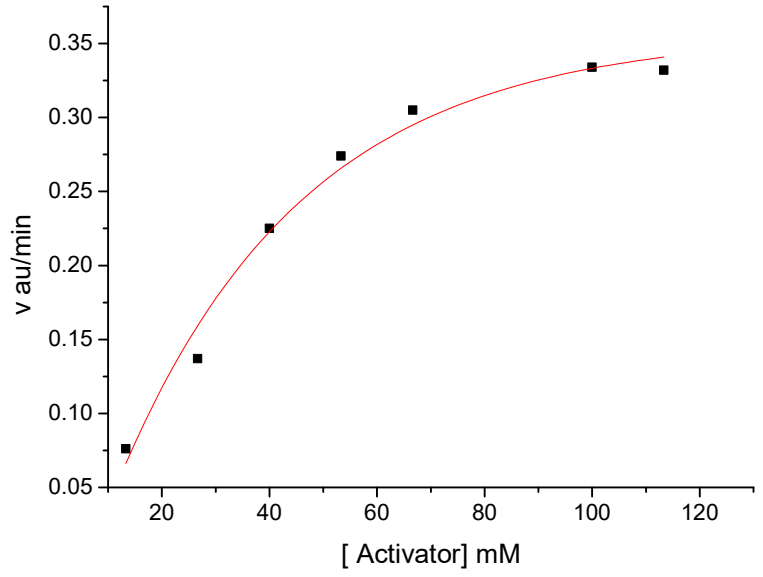

Figure 6: Michaelis-Menten model of the effect of copper sulphate on Tamarind PPO activity in the presence of catechol at PPO = 0.3ml, pH = 7.0 and T= 30°C

CONCLUSION

In this study the characterization of PPO extracted from Tamarind (*Tamarindus Indica* L.) has been reported. Enzyme kinetic parameters such as (K_m, V_{max} and V_{max}/K_m) were evaluated for the three substrates. It was found that the best conditions for measuring the enzyme activity were at pH 7.0 and temperature 30°C. For the substrate specificity, it was found that Tamarind PPO was very active towards catechol, followed by hydroquinone and gallic acid. Besides, Tamarind PPO activity was reduced in the presence of some inhibitors such as ascorbic acid and EDTA. Ascorbic acid was capable of inhibiting the enzyme activity more than EDTA and the mode of inhibition is uncompetitive one. Regarding thermal inactivation the enzyme activity decreased as the temperature increased and this was most properly due to heat denaturation of the enzyme. These findings evidenced the hypothesis that PPO was responsible for brown discoloration of the plant tissue followed cell damaged when exposed to oxygen throughout storage and processing.

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