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Research article

CHARACTERIZATION OF POLYPHENOL OXIDASE FROM CASSAVA LEAF (*MANIHOT ESCULENTA* CRANTZ)

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ABSTRACT: Fresh cassava (*Manihot esculenta* Crantz) leaves are unable to be stored for long as browning starts after harvesting from the plant. The purpose of this research is to determine the characteristics of polyphenol oxidase from the leaves of *Manihot esculenta* Crantz. Polyphenol oxidase (PPO) was extracted from cassava (*Manihot esculenta* Crantz) leaves and its characteristics were studied. The amount of acetone powder obtained from the extraction was 2.47 g ± 0.21 with a percentage yield of 4.12 % from 60 g of cassava leaves. The optimum pH was 7.5 and optimum temperature was found to be 20 °C. 95 % depreciation of PPO activity was observed after thermal inactivation at 90 °C for 40 minutes. Inactivation rate constant (k) values ranged between 0.025 and 0.043 min^{-1}. The half life ($t_{1/2}$) values of the enzyme ranged from 16.11 to 27.65 minute. The activation energy (E_a) and z-values were calculated to be 15.75 kJ mol^{-1} and 143.20 °C, respectively. K_m and V_{max} values were at 100 mM and 833.33 EU/min/mL, respectively. L-ascorbic acid exhibited the greatest efficiency in inhibiting cassava leaf PPO, with the lowest K_i value of 0.2 mM, followed by L-cysteine and citric acid. Studies showed that storage of cassava leaves increased the PPO activity. Characterization of the properties of cassava leaf PPO can serve as a guideline for predicting behavior of the enzyme to effectively prevent the browning of cassava leaves during processing and storage.
Keywords: Polyphenol oxidase, cassava leaf, characterization, kinetics, inhibitor

INTRODUCTION

Browning of raw fruit and vegetables is a major problem in the food industry as browning can cause deleterious changes in the appearance, flavour, hardness, nutritional properties and value of the food in the food market. Types of Injury includes cutting, freezing, bruising, or infection with diseases, leading to breakdown in cellular compartmentation of enzymes and substrates, which in turn causes a rapid oxidation of phenolics, producing brown by-products known as melanins [1]. These reactions are catalyzed by polyphenol oxidase, PPO (EC 1.14.18.1). PPO is a copper-containing enzyme that is widely distributed in the plant kingdom. It is also known as monooxygenase, catecholoxidase, and tyrosinase. PPO catalyses in two distinct reactions: a) o-hydroxylation of monophenol (cresolase activity) and b) oxidation of o-diphenol to o-quinones (Catecholase activity) in the presence of oxygen [2]. Control of enzymatic browning during processing and storage of fruit and vegetables is important to preserve the original appearance, thus this is becoming one of the main goals for food processors and researchers. According to Vámos-Vigyazo [3], enzymatic browning prevention is classified into two types, which are inhibition or inactivation of the enzyme and elimination or transformation of the substrate. Inactivation of the enzyme is performed by introducing high heat to denature the enzyme. Cassava (*Manihot esculenta* Crantz) is a staple crop for over 500 million people living throughout the tropics. It is a crop with great economic importance worldwide, yet its evolutionary and geographical origins have remain unresolved and controversial [4]. Cassava leaves are a good source of proteins, calcium, iron and vitamins and have the ability to provide a valuable supplement to predominantly starchy diets [5]. However, the fresh cassava leaves are unable to be stored for long as browning starts after harvesting from the plant. The leaves start to rot in a few days and an uncomfortable odour will be released.

Activity of PPO has been studied in butter lettuce [6], Thymus [2], wild mushroom species [7], Anamur banana [8], artichoke [9], Mulberry [10], coffee leaves [11], parsley [12] and mamey [13]. However, little is known about the PPO in cassava leaves. The aim of the present study was to characterize some of the properties of cassava leaf PPO in order to predict the behavior of the enzyme.

MATERIALS AND METHODS

Plant material and chemicals

The green leafy plant can be easily obtained from wide area in Malaysia as it undergoes vegetative reproduction just by cutting its stem and planting in wet soil. Mature leaves of *Manihot esculenta* Crantz were picked from a yard located in Kajang, Selangor, Malaysia. All chemicals were of analytical grade and used as obtained.

Enzyme extraction

Cassava leaves (60 g) were washed and chopped into small pieces. These leaves samples were then homogenized in 400 mL of prechilled (4°C) 0.1 M phosphate buffer, pH 6.8, using a Waring blender for 1 minute at maximum speed. The slurry was poured into 50 mL Falcon tubes and centrifuged at 4000 rpm for 30 minutes at 4°C. The supernatant was collected and filtered under vacuum using a Buchner funnel containing filter paper. The collected filtrate was pipetted drop by drop into 200 mL of cold acetone (-20°C) for the formation of precipitates. The precipitates obtained were centrifuged at 4000 rpm for 30 minutes at 4°C. The pellet was dried overnight at room temperature and stored at -20°C. The percentage yield was calculated using the following formula:

$$\text{Yield}(\%) = \frac{\text{Weight of extracts collected (g)}}{\text{Weight of samples for extraction (g)}} \times 100\%$$

To obtain the enzyme extract, 0.4 g of acetone powder was resuspended in 60 mL of prechilled 0.1 M phosphate buffer, pH 6.8 and stirred for 1 hour at 4°C. The suspension was then centrifuged at 4000 rpm for 40 minutes at 4 °C. The supernatant is the crude PPO extract.

Assay of PPO activity

PPO activity was determined by measuring the increase in absorbance at 410 nm at room temperature for catechol using a spectrophotometer. The reaction mixture contained 0.1 mL freshly prepared enzyme solution and 2.9 mL of 0.1 M catechol solution in 0.1 M phosphate buffer, pH 6.8, at room temperature. The blank consisted of 3 mL of substrate solution in 0.1 M phosphate buffer. The initial velocity was calculated from the slope of the absorbance vs. time curve. One unit of PPO activity was defined as the amount of enzyme that caused a 0.001 increase of absorbance per minute [14]. PPO activity was assayed in triplicate and the results expressed as means.

CHARACTERIZATION OF PPO

Optimum pH

The PPO activity was determined in a pH range of 5.0 – 9.0 in 0.1 M phosphate buffer using 0.1 M catechol. PPO activity was assayed by using the standard reaction mixture and changing the buffer. Calculated PPO activity was expressed in the form of relative activity (%) at the optimum pH. The optimum pH obtained for this enzyme was used in all other experiments.

Optimum temperature

The optimum temperature was determined by measuring PPO activity at different temperatures ranging from 5°C to 90 °C. Enzyme was added after equilibration of the standard reaction mixture at the selected temperatures and PPO activity was measured. Calculated PPO activity was expressed in the form of relative activity (%) at the optimum temperature.

Enzyme kinetics and substrate specificity

The substrate specificity was investigated for four different commercial grade substrates, catechol, 4-methylcatechol, pyrogallol and catechin at various concentrations (10 mM to 100 mM). The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values of the enzyme were determined from a Lineweaver-Burk plot and substrate specificity (K_m/V_{max}) was calculated from this data.

Thermal inactivation

Thermal inactivation was studied at 50°C, 60°C, 70°C, 80°C and 90°C. 1 mL of enzyme solution in a test tube was incubated at the selected temperature for 10, 20, 30 and 40 minutes. At the end of the required time interval, the enzyme samples were removed from the water bath and were immediately transferred into an ice bath to stop further thermal inactivation. The enzyme was brought to room temperature and added to the reaction mixture, which consisted of 2.9 mL of substrate in 0.1 M phosphate buffer, pH 7.5. The residual PPO activity (A) was determined spectrophotometrically at 410 nm using catechol. The stability of the enzyme was expressed as relative activity (%) and was calculated by comparison with the non-heated enzyme sample (A_0). The first order inactivation rate constant, k (min^{-1}) was calculated by linear regression according to $\log\left(\frac{A}{A_0}\right) = -\left(\frac{k}{2.303}\right) \times t$ [15]. A_0 is the initial enzyme activity and A is the enzyme activity after heating for a specific time, t.

The temperature dependence of the inactivation rate constant, k can be described by the Arrhenius equation [16] which is $\ln(k) = \frac{E_a}{RT}$. The k values obtained were plotted in an Arrhenius plot to determine the activation energy, E_a (kJ/mol) by obtaining the slope and times with R. R in the equation is the gas constant (8.314 J/mol/K) and T is the temperature in unit K.

The half-life of the enzyme ($t_{1/2}$) was calculated using $t_{1/2} = \frac{\ln 2}{k}$. The D-value is defined as the decimal reduction time or time required to pre-incubate the enzyme at a given temperature to maintain 10% residual activity and was calculated using $D = \frac{\ln 10}{k}$ [17]. The z-value is defined as the temperature rise necessary to reduce the D-value by one logarithmic cycle and was obtained from the beginning of the slope of the graph of $\log_{10}$ D versus T (°C) and calculated using $\text{Slope} = -\frac{1}{z}$.

Effect of inhibitors

Ascorbic acid, L-cysteine and citric acid were used as PPO inhibitors. The PPO activity was determined without inhibitor, and in the presence of inhibitors, at three different concentrations (1, 3, and 5 mM) by using catechol as the substrate. Percentage inhibition was calculated using the following formula:

$\text{Inhibition}\,(\%) = \frac{A_0 - A_i}{A_0} \times 100\%$, where A_0 was the initial PPO activity (without inhibitor) and A_i was the PPO activity with inhibitor.

Percent activity graphs were plotted from these results to determine the I_{50} values. Then, PPO activities were determined at fixed inhibitor concentrations using the inhibitors indicated above using five different concentrations (10 mM to 100 mM) of catechol. Lineweaver-Burk plots of these data were used to determine the dissociation constant (K_i) and inhibition type for each inhibitor.

Effect of storage

The fresh picked cassava leaves was cleaned under running tap water and the excessive water was drained off. After that, the cassava leaves were washed again with distilled water for two times. The cassava leaves were carefully wrapped in separate plastic bags and stored in the chiller (4°C) for two and four weeks. The methodology for extraction of PPO from the stored cassava leaves was similar to the enzyme extraction mentioned previously. The PPO activity from the stored cassava leaves was assayed at the optimum temperature and pH according to the substrate used. The optical density was measured at absorbance 410 nm for catechol as substrate for 15 seconds intervals up to 360 seconds. PPO activity was calculated.

Statistical analysis

Statistical analysis of all the experimental data was performed with Microsoft Office Excel 2007 data analysis software. All the characterizations of polyphenol oxidase (PPO) from cassava (*Manihot esculenta* Crantz) leaves were assayed in triplicates. The data collected were presented as means ± standard deviations and also relative activity in percentage (%).

RESULTS AND DISCUSSION

PPO extraction and activity

Cold acetone played an important role in the process of enzyme precipitation. 2.47 g ± 0.21 of acetone powder was obtained from 60 g of cassava leaves. The yield of PPO extracted from sweet potato was 18761.19 EU/g ± 0.34 of acetone powder.

Characterization of PPO

Optimum pH

The effect of pH on the activity of cassava leaf PPO was investigated in the range of 5.0-9.0. As seen from Figure 1, the optimum pH of the enzyme was found to be 7.5. The enzyme activity decreased rapidly at pHs below or above the optimum (7.5). PPO optimum pH varies from pH 4.0-8.5 as reported in the literature, depending on the origin of the material, the purity of enzyme, extraction method, the type of buffer used and substrate [18-21]. In general, fruits and vegetables show maximum activity at or near neutral pH values.

Optimum temperature

The relative activity of the PPO cassava leaves in a range of temperature was shown in Figure 2. It was found that cassava leaf PPO had an optimum temperature of 20°C. It has been reported that the optimum temperatures for PPO of peach is 20°C [22]. At 60°C, approximately 80 % of PPO activity was lost but the enzyme was not completely inactivated. It appears that the crude PPO is sensitive to the increase in assay temperature.

Enzyme kinetics and substrate specificity

The substrate specificity of the PPOs varies widely depending on the source and purity of the enzyme, where it has often been suggested that the preferred substrates is considered as the most abundant phenolic compound [23]. The K_m and V_{max} values for the cassava leaf PPO are summarized in Table 1. Pyrogallol exhibited the lowest K_m value among the four substrates, which was 10.64 mM and the highest V_{max} value of 12500.00 EU/min/mL with the highest catalytic efficiency of 1174.81 min^{-1}. Pyrogallol fulfilled the criterion of a low K_m value and high V_{max}, which indicates the best substrate for an enzyme. The cassava leaf PPO showed a decrease in catalytic efficiency from trihydroxyphenols (pyrogallol) to dihydroxyphenols (catechol, 4-methylcatechol and (+)-catechin).

Thermal inactivation

The thermal inactivation profile of cassava leaves PPO is shown in Figure 3. PPO activity was reduced by approximately 95 % at 90 °C with an incubation time of 40 minutes. Decrease in the percentage residual activity at a higher temperature is due to the unfolding of the tertiary structure [14].

Thermal inactivation parameters are summarized in Table 2. The first order inactivation rate constant ranged between 0.025 min^{-1} and 0.043 min^{-1}. It is clear that the PPO from cassava leaves was less thermostable at a higher temperature because of the higher rate constant [24].

The half life ($t_{1/2}$) is another important parameter used in the characterization of enzyme stability. Increasing the temperatures from 50 to 90 ºC resulted in a decrease in $t_{1/2}$ value (Table 2). The $t_{1/2}$ values ranged from 16.11 to 27.65 minutes. The D- values ranged between 53.52 to 91.85 minutes. Cassava leaf PPO exhibited high z-values, 143.26°C, which correlated with the low E_a, 15.75 kJ mol^{-1} (r^2=0.8205). These results suggested that the cassava leaf PPO was more heat resistant and a longer heating period was necessary to inactivate the enzyme completely [25].

Table 1. K_m and V_{max} values with different substrates for *M. esculenta* leaves PPO

Substrates	K_m(mM)	V_{max}(EU/min/mL)	V_{max}/K_m(min^{-1})
4-methylcatechol	100.00	1000.00	10.00
Catechol	100.00	833.33	8.33
Pyrogallol	10.64	12500.00	1174.81
(+)-Catechin	22.73	35.71	1.57

Table 2. Thermal inactivation parameters of *M. esculenta* leaves PPO

Temperature(°C)	k(min^{-1})	$t_{1/2}$(min)	D(min)	Z(°C)	E_a(kJ mol^{-1})
50	0.02507	27.65	91.85	-	-
60	0.02473	28.03	93.11	-	-
70	0.03984	17.40	57.80	-	-
80	0.04190	16.54	54.95	-	-
90	0.04302	16.11	53.52	-	-
				143.26	15.75

Effect of Inhibitors

From Table 3, it can be seen that the percentage of inhibition increased with an increased of inhibitor concentrations (1.0 – 5.0 mM). L-ascorbic acid was the most effective inhibitor among the three inhibitors, being able to inhibit the PPO activity up to 70 % at a low concentration (1.0 mM). However, citric acid showed a minimum inhibition, even at the highest concentration (5.0 mM), for both assays, indicating that citric acid was not a good inhibitor of PPO from cassava leaves. Furthermore, L-ascorbic acid has the lowest I_{50} and K_i values among all the inhibitors, which were 0.45 mM and 0.20 mM, further indicating that L-ascorbic acid is a good inhibitor. From the Lineweaver-Burk plot (Figure 4), it was concluded that L-ascorbic acid was a competitive inhibitor.

The type of inhibition exhibited by L-ascorbic acid explained the high V_{max} value of the cassava leaf PPO. A competitive inhibitor is able to diminish the rate of catalysis by reducing the proportion of enzyme molecules bound to a substrate. However, competitive inhibition can be relieved by increasing substrate concentration, thus it exhibits a higher V_{max} compared to the other inhibitors [26]. In the presence of PPO substrates, L-ascorbic acid reduces the PPO oxidized reaction product which resulted in a lag period [27]. L-cysteine and citric acid were non-competitive inhibitors (Figures 5 and 6), which cannot be overcome by increasing the substrate concentration. A similar result was obtained from apple using L-cysteine as an inhibitors, indicating that the PPO inhibition by L-cysteine was non-competitive [28]. Gawlik-Dzik *et al* [6]. Mentioned that L-ascorbic acid may act more as an antioxidant than as an enzyme inhibitor due to the ability of reducing the initial o-quinone formed by the enzyme to the original diphenol before it undergoes the secondary reactions leading to browning. However, browning might still occur after the ascorbic acid is exhausted as the L-ascorbic acid is a competitive inhibitor with a reversible reaction [29,30]. L-ascorbic acid is commonly used as the reductant for anti-browning in fruit juices as well as canned vegetables and fruits and functions as a PPO inhibitor through its site-directed reactivity to the histidine residues [31].

Table 3. Effect of inhibitors on PPO activity of *M. esculenta* leaves

Inhibitor	[I] (mM)	Inhibition (%)	I_{50} (mM)	V_{max} (EU/min/ml)	K_i (mM)
L-ascorbic acid	1.0	70.51		333.33	0.20
	3.0	78.21	0.45	400.00	0.43
	5.0	79.48		400.00	0.43
L-cysteine	1.0	60.69		200.00	1.00
	3.0	76.92	0.65	111.11	1.15
	5.0	81.03		64.52	0.96
Citric acid	1.0	14.62		333.33	4.99
	3.0	29.92	5.25	333.33	15.00
	5.0	36.74		250.00	8.33

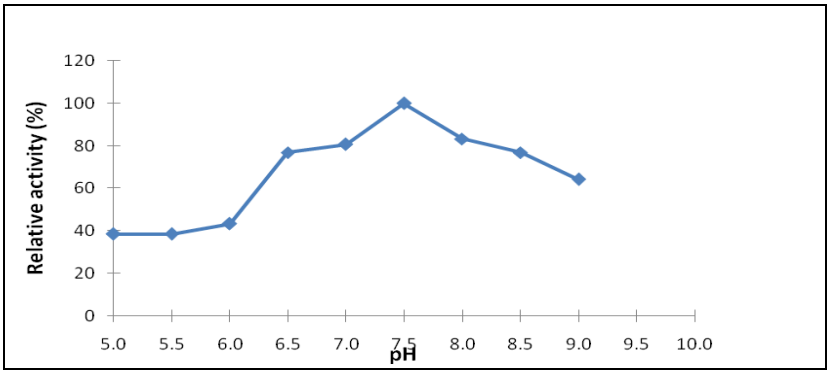

Figure 1. Effect of pH on PPO activity of *M. esculenta* leaves

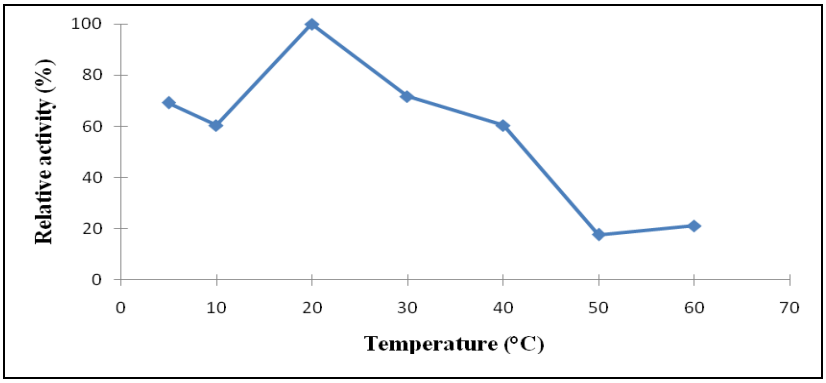

Figure 2. Effect of temperature on PPO activity of *M. esculenta* leaves

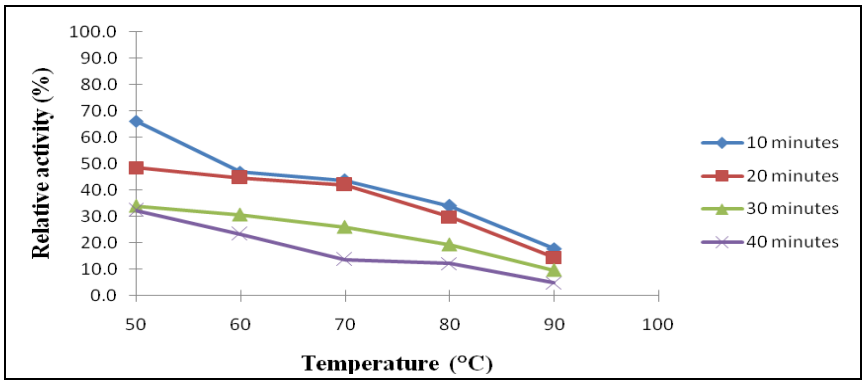

Figure 3. Thermal inactivation profiles of *M. esculenta* PPO

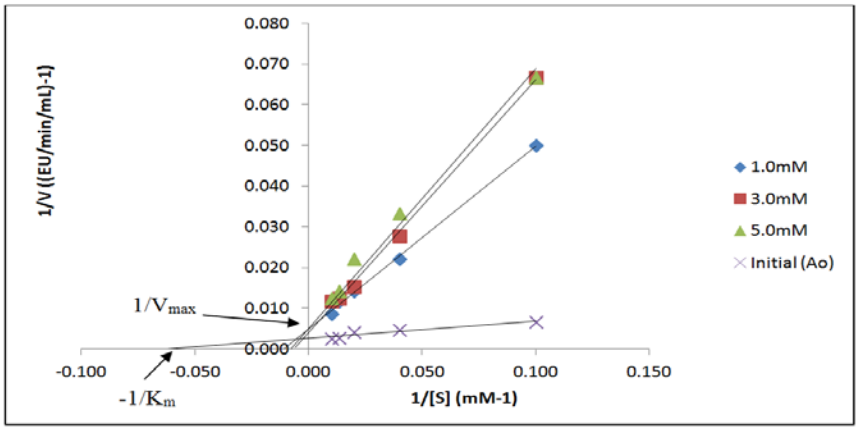

Figure 4. Effect of 1.0 mM, 3.0 mM and 5.0 mM of L-ascorbic acid on *M. esculenta* PPO

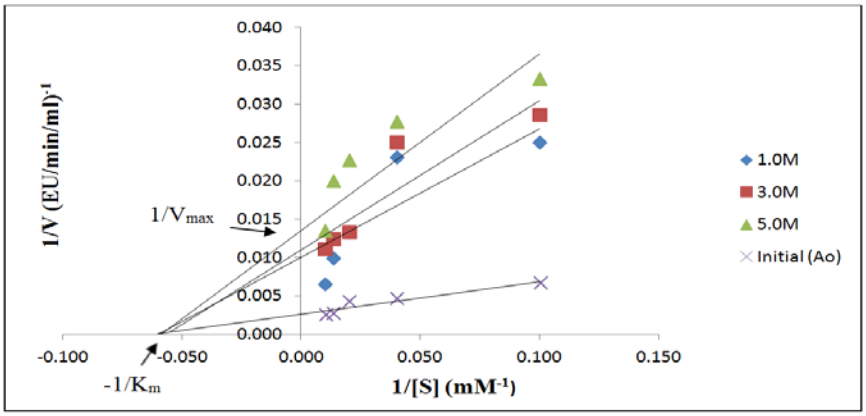

Figure 5. Effect of 1.0 mM, 3.0 mM and 5.0 mM of L-cysteine *on M. esculenta* PPO

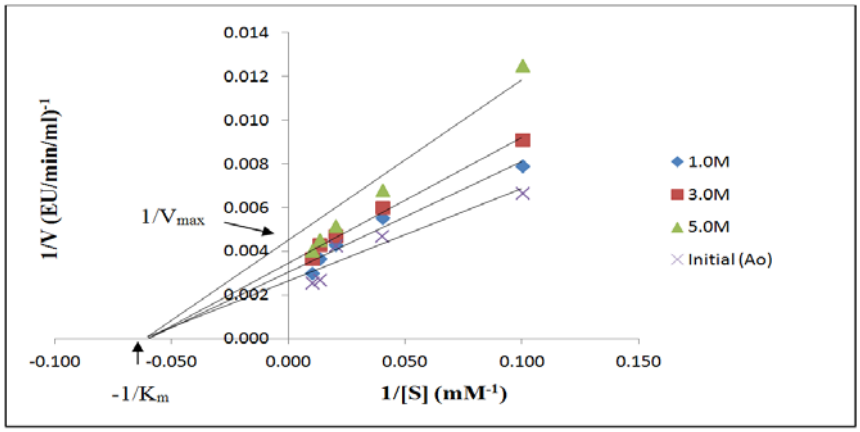

Figure 6. Effect of 1.0 mM, 3.0 mM and 5.0 mM of citric acid on *M. esculenta* PPO

Effect of storage

The enzyme activity for cassava leaves that stored for 2 weeks was 480.00 EU/min/mL. The enzyme activity increased by approximately 16% after stored for 2 weeks compared with the enzyme activity of the fresh cassava leaves (413.30 EU/min/mL). As for the cassava leaves that stored for 4 weeks, the enzyme activity was 550.00 EU/min/mL, which was increased by approximately 33%. The PPO activity in stored cassava leaves was time-dependent, as the activity increased when the length of storage increased.

Harvested plants proceeded with quality loss due to the respiration, progression of ripening, water loss, enzymatic discolouration of the cut surfaces and decay by microbial activity. All these consequences caused the increase of PPO level in the plant as PPO involves in plant defense system [32] and accumulating towards time. This makes the storage of the fruits and vegetables important to make sure that the rate of enzyme activity was lowered down. However, the PPO enzyme activity increased although cassava leaves was stored at low temperature. One of the reason was the temperature was not low enough to inhibit the activity of the enzyme to prolong the shelf-life, as the optimum temperature of PPO in cassava leaves was relatively low (20°C) using pyrocatechol as substrate. Therefore, proper temperature management during the storage in refrigerator is essential for maintaining the quality of the fruits and vegetables [33].

Although storage at low temperature is a common practice to retard the PPO enzyme activity, chilling injury might occurred if the storage temperature is too low and caused reverse reaction. The free phenolics are present mainly in the vacuole of the plant cells, and they are synthesized in the cytoplasm or deposited in cell walls. If low-temperature storage treatments induced membrane damage in vacuoles, vacuolar phenolics came into contact with PPO thus causing the browning reaction in plant materials [34].

CONCLUSION

This study concluded that PPO prepared from cassava leaves possess diphenolase activity and has the greatest substrate specificity towards pyrogallol among the substrates tested. The pH optimum for the enzyme was 7.5. In addition, the cassava PPO activity was sensitive to some general inhibitors, especially to L-ascorbic acid and L-cysteine.

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