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Comparison on Characterization of Longan (*Dimocarpus longan* Lour.) Polyphenoloxidase Using Endogenous and Exogenous Substrates

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Longan polyphenoloxidase (PPO) was extracted and partially purified using ammonium sulfate precipitation and dialysis. The PPO characterizations were compared using endogenous substrate (−)-epicatechin and exogenous substrate catechol. The optimal pH and optimal temperature for the PPO activity were different when reacting with both substrates. The addition of ethylenediaminetetraacetic acid disodium salt into both substrate−enzyme systems exhibited the same lowest inhibition of the PPO activity. L-Ascorbic acid and L-cysteine were the best inhibitors to endogenous substrate−enzyme system, while L-ascorbic acid and glutathione were most effective inhibitors to exogenous substrate−enzyme system. Cupric (Cu^{2+}), ferric (Fe^{3+}), and ferrous (Fe^{2+}) ions accelerated the enzymatic-catalyzed reactions of both substrates. Kinetic analysis indicated that longan PPO strongly bound endogenous substrate but possessed a higher catalytic efficiency to exogenous substrate, and moreover, (−)-epicatechin was determined as the optimal substrate for longan PPO. This study is useful to exactly illuminate the enzymatic-catalyzed browning mechanism of postharvest longan fruit.

KEYWORDS: Longan (*Dimocarpus longan* Lour.); polyphenoloxidase; endogenous substrate; exogenous substrate; characterization

INTRODUCTION

Polyphenoloxidase (PPO) is a group of copper-containing enzymes. Most plants have multiple forms of PPO. Although the active site of PPO is conserved, the amino acid sequence shows very considerable variability among species. Expression of the genes coding for the enzyme is tissue specific and also developmentally controlled (*1*). PPO can catalyze the *o*-hydroxylation of monophenols into *o*-diphenols (EC 1.14.18.1, monophenol monooxygenase, cresolase) and the oxidiation of *o*-diphenols to *o*-quinones (EC 1.10.3.2, *o*-diphenoloxidase, catecholase), which rapidly polymerize into brown, red, or black pigments (*1−3*). PPO is widely distributed in fruits and vegetables, inducing tissue enzymatic browning after harvest or injury (*4*).

Longan (*Dimocarpus longan* Lour.), originating from South China, is a tropical and subtropical plant in the *Sapindaceae* family (*5−7*). Longan fruit is nonclimacteric type and will not continue to ripen once removed from trees (*8*). The fruit is small and round with a thin pericarp and a translucent, delicate, juicy, and sweet flesh that surrounds a black seed (*9*). Pericarp browning, short storage life, and susceptibility to diseases greatly influence the quality of postharvest longan fruit. Especially, the rapid pericarp browning after harvest has been one of the most important problems in marketing longan fruit (*10*). There are many factors causing longan pericarp browning. A particularly important one is the enzymatic-catalyzed oxidation of phenolic substrate by PPO in pericarp tissues (*11−13*). After harvest, the gradual breakdown of cellular ultrastructure results in loss of compartmentalization of PPO and its substrate. The contact between PPO and substrates initiates enzymatic-catalyzed reaction in the presence of oxygen and finally induces the formation of brown-colored byproduct (*14−16*).

Enzymatic browning by PPO is the major practical limitation to storage of postharvest longan fruit. In our previous research (*17*, *18*), an important endogenous substrate for longan PPO was identified as (−)-epicatechin,, which reacted with PPO and induced pericarp browning. It was further reported that this endogenous substrate possessed high antioxidant activity (*17*). Although other studies have investigated longan PPO by using exogenous substrates such as pyrogallol, catechol, and 4-methylcatechol (*12*), however, these exogenous substrates still are not found in longan pericarp tissues. It is necessary by using endogenous substrate to exactly elucidate longan PPO characterization. Unfortunately, up to now, there has no report on longan PPO characterization in endogenous substrate−enzyme systems. The further research is still needed. The objective of the present study was to compare longan PPO characterizations and then to determine the different effects of endogenous and exogenous

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substrates on the enzyme characterizations. This study provides a new reference on how to exactly characterize an enzyme by using its natural endogenous substrates. It is also helpful to develop appropriate methods for controlling longan pericarp browning through inhibiting reaction between PPO and its endogenous substrates.

MATERIALS AND METHODS

Plant Materials. Longan (*Dimocarpus longan* Lour. cv. Shixia) fruits were obtained from Nanning, Guangxi province of China, and transported into Guangxi Crop Genetic Improvement and Biotechnology Laboratory on the same day in August 2009. The healthy fruits at the mature stage were selected, cleaned, and then peeled. The fresh pericarp tissues were lyophilized in liquid nitrogen and stored at −20 °C until further extraction and analysis.

Isolation and Identification of Endogenous Substrate. The extraction, isolation, and identification of endogenous substrate for longan PPO was performed according to our previously reported methods (*17*, *18*). In brief, lyophilized pericarp tissues (600 g) were extracted using 2000 mL of methanol−acetone−water (4.5:4.5:1, v/v/v) containing 6 g of ascorbic acid at 4 °C. The substrate extracts were filtered through vacuum filter, and the filtrate was centrifuged for 15 min at 7000*g*. The supernatant was extracted twice with petroleum ether (2:1, v/v). The aqueous phase was collected, concentrated, and then dried under vacuum at 50 °C as crude substrate extracts. The crude substrate extracts (6 g) were further isolated using polyamide column (450 mm × 25 mm, 60−80 mesh, Taizhou Luqiao Biochemical Corp., Taizhou, China) with water−methanol mixture (at a line gradient of 100, 80, 60, 40, 20 and 0, v/v) as an eluent, Sephadex LH-20 column (1500 mm × 15 mm, Amersham Biosciences, Uppsala, Sweden) with methanol as an eluent, and silica gel column (400 m × 6 mm, 200−300 mesh, Qingdao Marine Chemical Factory, Qingdao, China) with chloroform/methanol/formic acid (90:10:0.4, v/v/v) as an eluent, respectively. The purified endogenous substrate (0.052 g) was selected by 0.5% $FeCl_3$ solution and enzymatic reaction with longan PPO. Its chemical structure was identified using UV (UV-2802 Spectrophotometer, Unic, Shanghai, China), ^{1}H and ^{13}C NMR (Bruker DRX-400 NMR Spectrometer, Bruker Co., Rheinstetten, Germany), and ESI-MS (PE Sciex API 2000 LC/MS/MS System, ABI, Foster City, CA) spectroscopy methods.

Partial Purification and Protein Determination of PPO. PPO was extracted according to the modified methods of Jiang (*12*) and Kavrayan and Aydemir (*19*). All steps were carried out at 4 °C. Longan pericarp tissues (30 g) were homogenized for 1.5 h by continual stirring with a glass rod in 200 mL of 0.1 M sodium phosphate buffer (PB) (pH 6.8) containing 1.0% (w/v) polyvinyl pyrrolidone and 1.0% (v/v) Triton X-100. The extracts were centrifuged at 9690*g* for 15 min, and then the supernatant was collected. The enzyme solution was fractionated with solid ammonium sulfate (30−80% saturation). The precipitate was collected by centrifugation at 9690*g* for 15 min and then was further redissolved in small volume of 10 mM PB (pH 6.8). After overnight dialysis against the same buffer, the dialyzed solution was collected as the partially purified PPO, which could be used to analyze well the enzymatic characterization. PPO activity was assayed with endogenous substrate (−)-epicatechin and exogenous substrate catechol (the most common substrate to assay activity of plant polyphenoloxidases) through a spectrophotometric procedure (*12*). The enzyme solution (0.2 mL) was rapidly added into 2.8 mL of 10 mM (−)-epicatechin and catechol solutions (prepared in 10 mM PB with pH 6.8), respectively. The increase in absorbance at 440 nm (the absorption peak of the enzymatic-catalyzed product when (−)-epicatechin reacting with longan PPO) and 400 nm (the absorption peak of the enzymatic-catalyzed product when catechol reacting with longan PPO) at 25 °C was recorded automatically for 3 min using a UV−visible spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). One unit of PPO activity was defined as the amount of the enzyme that causes a change of 0.001 in absorbance per minute at the absorption peaks of enzymatic-catalyzed products. Relative activity described enzymatic activity as the percentage of the activity expressed as $(A/A_{max}) \times 100$, where A indicates the increase in optical density per minute (*20*). Protein content was determined by the dye-binding method of Bradford (*21*) with bovine serum protein as the standard.

Different Characterizations of PPO Using Endogenous and Exogenous Substrates. *Effects of pH*. PPO activity was assayed in the pH range of 4.0−5.5 in 50 mM citric acid−sodium citrate buffer and 6.0−8.0 in 50 mM sodium phosphate buffer using (−)-epicatechin and catechol as substrates. To determine the optimal pH values of the enzyme, 1 mL of 10 mM (−)-epicatechin or catechol solution was added into 1.9 mL of buffer solution and then mixed at 25 °C prior to the addition of 0.1 mL of crude enzyme solution. The relative activities were compared using the method described above.

Effects of Temperature. The PPO activity was measured at different temperatures in a range of 20−60 °C using a water bath. A total of 2.9 mL of 10 mM (−)-epicatechin and catechol solutions was prewarmed to the corresponding temperatures and incubated for 3 min. Then, 0.1 mL of crude enzyme was added into the substrate solutions. To determine the effects of high temperature, the enzyme solutions were incubated in a water bath at 70 and 80 °C for up to 5 min. Every 1 min, 0.1 mL of enzyme solution was transferred into 2.9 mL of 10 mM (−)-epicatechin and catechol solutions to assay the enzyme stability.

Effects of Inhibitors. To determine the effects of inhibitors (ethylenediaminetetraacetic acid disodium salt (EDTA-2Na), L-ascorbic acid, sodium metabisulphite ($Na_2S_2O_5$), glutathione, L-cysteine, and 1,4-dithio threitol) on PPO activity, 0.9 mL of 10 mM (−)-epicatechin or catechol solution was added into 1 mL of 10 mM PB (pH 6.8), and then 0.55 mL of various inhibitor solutions at 1 mM were mixed immediately at 25 °C prior to the addition of 0.05 mL of enzyme solution. The control was the substrate−enzyme reaction system without any inhibitors.

Effects of Metal Ions. A total of 0.9 mL of 10 mM (−)-epicatechin or catechol solution was added into 1 mL of 10 mM PB (pH 6.8), and then 0.55 mL of various metal ionic solutions (NaCl, KCl, $CaCl_2$, $MgCl_2 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, and $CuSO_4 \cdot 5H_2O$) at 1 mM were mixed immediately at 25 °C before the addition of 0.05 mL of enzyme solution. The control was the substrate−enzyme reaction system without any metal ionic compounds.

Determination of Kinetic Parameters. The enzyme kinetic parameters (*22*, *23*), Michaelis−Menten constant, and maximum rate values for longan PPO were determined at 25 °C when using (−)-epicatechin and catechol as substrates. The assay cuvette (3 mL) contained 2.95 mL of (−)-epicatechin or catechol solution with gradient concentration (from 0.5 to 10 mM) and 0.05 mL of the enzyme solution. The kinetic parameters were estimated by linear regression analysis using the following equation.

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} \quad (1)$$

where [S] corresponds to the substrate concentration, while K_m is the Michaelis−Menten constant and V_{max} is the apparent maximum rate for the enzymatic reaction. For each substrate, data were plotted as $1/v$ vs $1/[S]$. K_m and V_{max} were determined as the reciprocal absolute values of the intercepts on the x- and y-axis of the regression curve (*24*).

Data Analysis. The enzymatic reaction was carried out in triplicate. The results represented mean ± standard error (SE) of three replicate determinations.

RESULTS AND DISCUSSION

Determination of Endogenous Substrate and Partial Purification of Longan PPO. The spectral data (*17*, *18*) of endogenous substrate, $UV_{\lambda max}$ 209 and 278 nm, ESI-MS m/z 289.2 $[M - H]^-$, and the signals of 9 hydrogens and 15 carbons in ^{1}H and ^{13}C NMR spectra, were consistent with those reported in previous literatures for (−)-epicatechin (*25*−*27*).

During extraction of longan PPO, a phenol-binding agent, polyvinyl pyrrolidone (PVP), was used to bind phenols which could deactivate PPO (*28*, *29*). In addition, Triton X-100 is nonionic surfactant which could extract plasma membrane proteins in the absence of extensive cellular destruction (*30*, *31*). In this study, the membrane-bound PPO was fully extracted by the use of Triton X-100. Furthermore, ammonium sulfate was removed by dialysis to avoid interfering with the determination of protein concentration. After partial purification of longan PPO, the protein content was about 122.72 μg protein/mL, with a total activity of 228×10^3 units when (−)-epicatechin as a

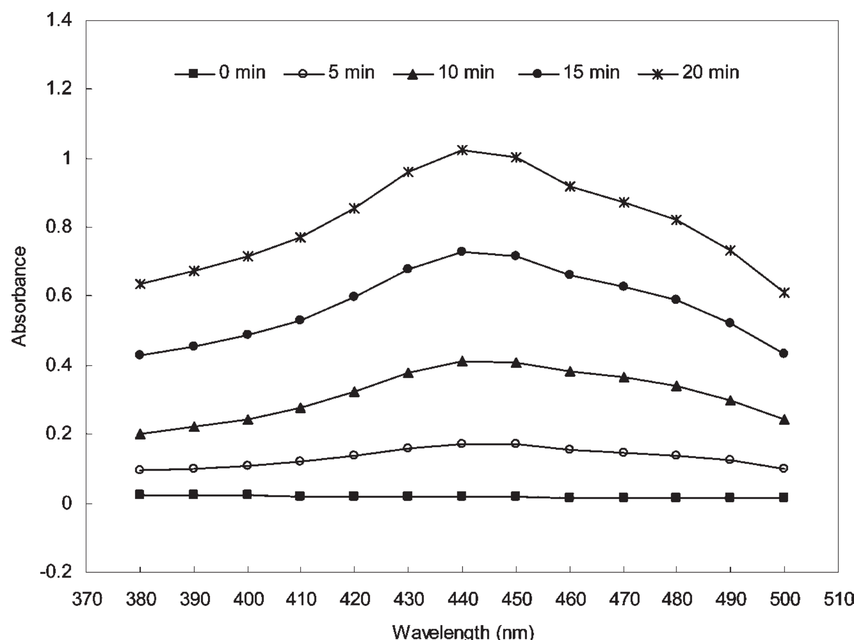

Figure 1. Absorption spectra of endogenous substrate (−)-epicatechin catalyzed by longan PPO. The absorption spectrum at 0, 5, 10, 15, and 20 min of enzymatic reaction was scanned and absorbencies were recorded every 10 nm. Reacting system: 1 mL of 10 mM (−)-epicatechin solution +1.5 mL of 10 mM sodium phosphate buffer (pH 6.8) + 0.05 mL PPO solution.

substrate and 111×10^3 units when catechol as one. The enzymatic reaction between the partially purified PPO and its endogenous substrate (−)-epicatechin is shown in **Figure 1**. The new absorption peak presented around 440 nm after PPO catalyzed the oxidation of (−)-epicatechin, and the absorbance at 440 nm continuously increased within 20 min, indicating that, in current endogenous the substrate−enzyme system, 440 nm was the absorption maximum of enzymatic-catalyzed product, and the product gradually accumulated within 20 min (this absorption maximum still increased after 20 min). During this 20 min reaction time, the reacting solution gradually turned brown because of the accumulation of byproduct and a distinct browning occurred after 20 min. (−)-Epicatechin, as the endogenous substrate of PPO, was also found in other plant tissues, e.g. litchi pericarp (*32*), apple (*33*), and tea germplasm (*34*). In the following experiments, longan PPO activity was detected at 440 nm to endogenous substrate−enzyme system and at 400 nm to exogenous substrate−enzyme system.

Difference of Optimal pH. The pH profile for the oxidation of endogenous substrate (−)-epicatechin and exogenous substrate catechol by longan PPO is shown in **Figure 2**. At 25 °C, two curves exhibited the similar trends and the PPO had the maximum activities around neutral pH values in endogenous or exogenous substrate−enzyme system. However, there were a few differences about the enzymatic activities in both systems. A peak for longan PPO activity appeared at pH 6.5 when using (−)-epicatechin as a substrate (i.e., the optimal pH for the PPO activity was at pH 6.5 in endogenous substrate−enzyme system), whereas a peak for the PPO activity appeared at pH 7.0 when using catechol as a substrate (i.e., the optimal pH for the PPO activity was at pH 7.0 in exogenous substrate−enzyme system). The optimal pH for longan PPO was just contrary to that for lychee (another fruit in the *Sapindaceae* family) PPO. Sun et al. (*35*) reported that the optimal pH for lychee PPO activity was at pH 7.0 in endogenous substrate−enzyme system, while the optimal pH for the PPO activity was at pH 6.5 in exogenous substrate−enzyme system.

In addition, at the acidic pHs (such as pH 4.0, 4.5, and 5.0), longan PPO activity in the endogenous substrate−enzyme system was lower than that in exogenous substrate−enzyme system, which was due to the slower enzymatic reaction between the PPO and endogenous substrate under acidic conditions. However, at the alkali pHs (such as pH 7.5 and 8.0), the PPO activity in endogenous substrate−enzyme system was higher than that in exogenous substrate−enzyme system, which was due to the rapider enzymatic reaction between the PPO and endogenous substrate under alkali conditions. From above results, it could be confirmed that the enzymatic activities and optimal pHs were somewhat different when longan PPO reacted with endogenous and exogenous substrates at a pH range of 4.0−8.0.

Differences of Optimal Temperature and Heat Stability. When using endogenous and exogenous substrates, the optimal temperature for longan PPO activity is shown in **Figure 3A**. The temperature has a two-sided influence on enzymatic activity, i.e. increasing incubation temperature enhances enzymatic reaction velocity but simultaneously leads to denaturation of enzyme. The optimal temperature is dependent on the effect of the two-sided equilibrium. From **Figure 3A**, in a temperature range of 20−60 °C, longan PPO had the maximum activities at 55 and 35 °C when (−)-epicatechin and catechol were used as substrates (i.e., the optimal temperatures for longan PPO activities were at 55 °C in the endogenous substrate−enzyme system and at 35 °C in the exogenous substrate−enzyme system). It was apparent in **Figure 3A** that the PPO activity exhibited obvious fluctuation in the temperature range of 20−60 °C and a small enzymatic activity peak presented at 25 °C when using endogenous substrate (−)-epicatechin. However, there was a completely different trend in the activation temperature profile and only slight fluctuation was observed for the enzymatic activity when using exogenous substrate catechol. A possible explanation for these results were that the enzymatic reaction velocity for endogenous substrate−enzyme system was more strongly associated to temperature variation, i.e. the incubation temperature significantly affected the enzymatic reaction between (−)-epicatechin and longan PPO.

The effect of high temperatures on longan PPO activities is shown in **Figure 3B** by using endogenous and exogenous substrates. Longan PPO was relatively unstable at high temperatures (70 and 80 °C), which was similar to some reports concerning the

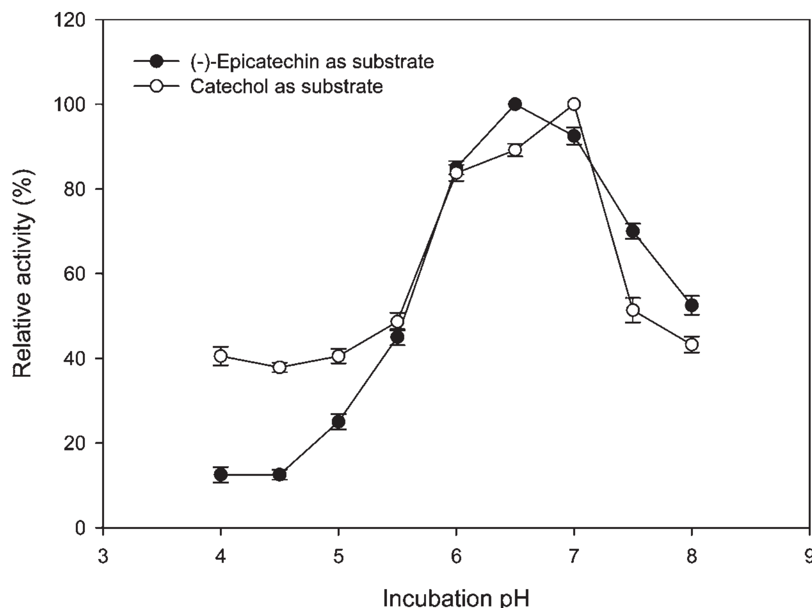

Figure 2. Effects of various pH values on longan PPO activities using endogenous and exogenous substrates.

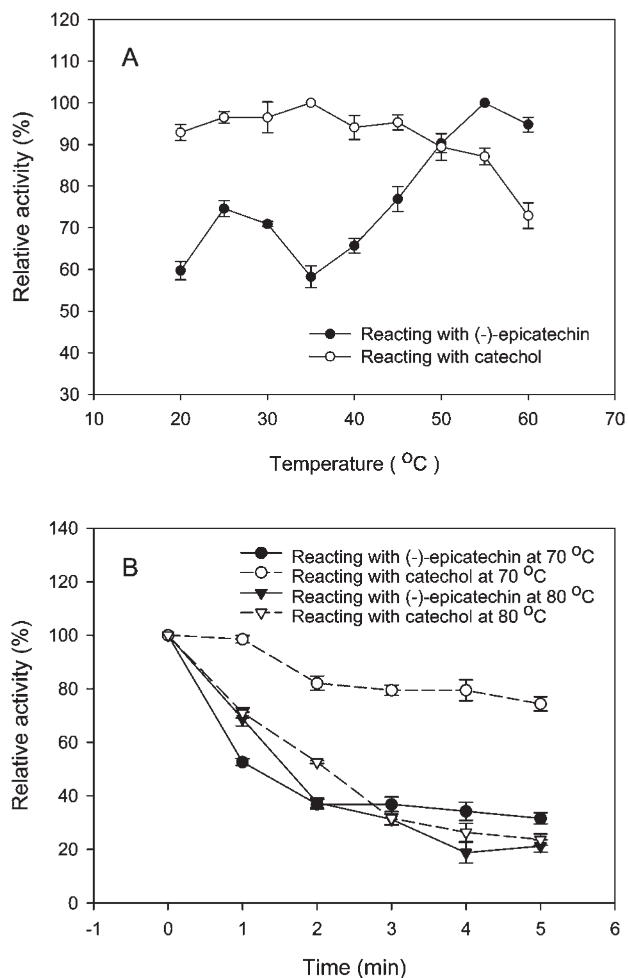

Figure 3. Effects of various temperatures on longan PPO activities using endogenous and exogenous substrates. (**A**) the optimum temperature of PPO activity, and (**B**) the heat stability of PPO activity.

thermal stability of PPO in other plants. Dincer et al. (*2*) confirmed that the medlar PPO was very heat-labile and the heat denaturation occurred after 10 min of incubation above 60 °C.

Table 1. Effects of Various Inhibitors and Metalline Compounds on Longan PPO Activities Using Endogenous Substrate (−)-Epicatechin and Exogenous Substrate Catechol[a]

compounds	relative activity (%)	
	(−)-epicatechin	catechol
	Inhibitors	
control	100 ± 0.0	100 ± 0.0
EDTA-2Na	83.7 ± 1.4	46.3 ± 0.9
L-ascorbic acid	0 ± 0.0	0 ± 0.0
$Na_2S_2O_5$	19.9 ± 1.5	2.4 ± 0. 6
glutathione	12.0 ± 1.1	0 ± 0.0
L-cysteine	0 ± 0.0	2.4 ± 1.4
1,4-dithio threitol	4.0 ± 0.2	12.2 ± 1.8
	Metalline Compounds	
control	12.7 ± 1.9	39.5 ± 1.6
NaCl	14.3 ± 1.8	43.2 ± 1.4
KCl	12.0 ± 1.4	38.2 ± 2.0
$CaCl_2$	13.5 ± 1.9	52.6 ± 1.9
$MgCl_2 \cdot 6H_2O$	11.6 ± 1.5	40.8 ± 1.8
$FeSO_4 \cdot 7H_2O$	15.5 ± 1.8	48.7 ± 1.6
$FeCl_3 \cdot 6H_2O$	15.9 ± 1.1	50.5 ± 2.3
$CuSO_4 \cdot 5H_2O$	100 ± 0.0	100 ± 0.0

[a] After the addition of Fe^{2+}, Fe^{3+}, and Cu^{2+} into endogenous and exogenous substrate−enzyme systems, the color changes of reaction solutions were as follows: adding Fe^{2+} and Fe^{3+} into both systems, substrate−enzyme solutions turn purple, while adding Cu^{2+} into endogenous and exogenous substrate−enzyme systems, substrate−enzyme solutions exhibited yellow and gray, respectively.

Duangmal and Owusu Apenten (*4*) found that both taro PPO and potato PPO were irreversibly inactivated by 10 min heating at 70 °C. Zhou et al. (*30*) reported that Monroe apple PPO was heat stable up to 40 °C but rapidly inactivated above 50 °C. From **Figure 3B**, longan PPO activities gradually decreased with heating time because of the denaturation of enzyme. There were some differences as to the PPO activities at high temperatures in both substrate−enzyme systems. After heating for 5 min at 70 °C, the relative PPO activities were about 31.6% when using (−)-epicatechin as substrate and 74.3% when using catechol as substrate. While at 80 °C, the relative PPO activities only remained about

21.3% when using (−)-epicatechin as substrate and 23.6% when using catechol as substrate. Above results indicated that the decline in longan PPO activity at high temperature was more obvious using endogenous substrate (−)-epicatechin, suggesting that the enzymatic reaction between (−)-epicatechin and longan PPO was more strongly affected by high temperature.

Different Effects by Enzymatic Inhibitors and Metal Ions. *Difference of Effects of Enzymatic Inhibitors*. The effects of inhibitors on longan PPO activity are listed in **Table 1**. In comparison with control, the addition of inhibitors into the endogenous or exogenous substrate−enzyme system reduced the enzymatic activity to some extent. Comparing six inhibitors, the addition of EDTA-2Na exhibited the same lowest inhibiting effect on the PPO activity in both substrate−enzyme systems. L-Ascorbic acid and L-cysteine were the most effective inhibitors for the PPO activity when using (−)-epicatechin as substrate, while L-ascorbic acid and glutathione were most effective for inhibiting this enzymatic activity when using catechol as substrate. Moreover, the addition of $Na_2S_2O_5$ into endogenous substrate−enzyme system could partially inhibit the PPO activity, but the inhibition effect of this chemical was very strong in exogenous substrate−enzyme system. From **Table 1**, L-ascorbic acid, $Na_2S_2O_5$, glutathione, L-cysteine, and 1,4-dithio threitol were all good inhibitors of longan PPO activity for endogenous or exogenous substrate−enzyme systems. Their inhibiting capabilities were as follows: L-ascorbic acid = L-cysteine > 1,4-dithio threitol > glutathione > $Na_2S_2O_5$ to endogenous the substrate−enzyme system, and L-ascorbic acid = glutathione > $Na_2S_2O_5$ = L-cysteine > 1,4-dithio threitol to the exogenous substrate−enzyme system.

Table 2. Kinetic Parameters of Longan PPO in Endogenous and Exogenous Substrate−Enzyme Systems

	substrates	
parameters	(−)-epicatechin	catechol
assay wavelength (mm)	440	400
K_m (mM)	0.8	1313.0
V_{max} (units/mL min)	48.0	10537.4
V_{max}/K_m	60.0	8.0

The inhibitor reaction mechanism differs, depending on the reducing agents employed. Ascorbic acid acts more as an antioxidant than as an enzyme inhibitor because it reduces the initial quinone formed by PPO to the original diphenol before it undergoes browning reaction (*3*). Above results have confirmed that ascorbic acid is one of the optimal inhibitors for both substrate-enzyme systems, indicating that antioxidant effect is especially important factor for inhibiting longan PPO activity. Inhibition by thiol compounds is attributed to either the stable colorless products formed by an addition reaction with *o*-quinones (*36*) or binding to the active center of PPO, like $Na_2S_2O_5$ (*37*). Glutathione or L-cysteine can easily form colorless complexes with *o*-quinones and PPO is inhibited by the formation of stable adducts (*38*, *39*). L-cysteine serving as optimal inhibitor to endogenous substrate-enzyme system while glutathione serving as optimal inhibitor to exogenous substrate-enzyme system is probably due to the following reasons: L-cysteine is easier to form the stable adduct with *o*-quinone derived from longan PPO reacting with (−)-epicatechin, while glutathione is easier to form the stable adduct with *o*-quinone derived from the PPO reacting with catechol. 1,4-Dithio threitol is an effective PPO inhibitor because of its capability to bind to copper at the active site. Presently, considerable efforts, such as blanching and sulfur fumigation, have been devoted to enzymatic browning control of postharvest longan fruits. These treatments sometimes cause undesirable quality decline or sulfur dioxide residues. Enzymatic inhibitors, e.g. ascorbic acid, glutathione and L-cysteine, have no toxicity and exhibit good capability to inhibit enzymatic reaction in longan pericarps. Therefore, it is beneficial to utilize these compounds to prevent browning of longan fruits.

Difference of Effects of Metal Ions. Effects of various metal ionic compounds on longan PPO activity are also summarized in **Table 1**. Compared with control, the relative activities of longan PPO were highest when adding Cu^{2+} into endogenous or exogenous substrate−enzyme system, indicating that Cu^{2+} greatly accelerated enzymatic-catalyzed reactions of both substrates and the PPO. In addition, Fe^{3+} and Fe^{2+} also partly improved the enzymatic-catalyzed reactions of both substrates and PPO. Similar records on Cu^{2+} and Fe^{3+} ions (at 1 mM) activating the oxidation of PPO from other biological materials had been reported by Aydemir (*3*), Simpson et al. (*40*), and Leoni et al. (*41*), etc.

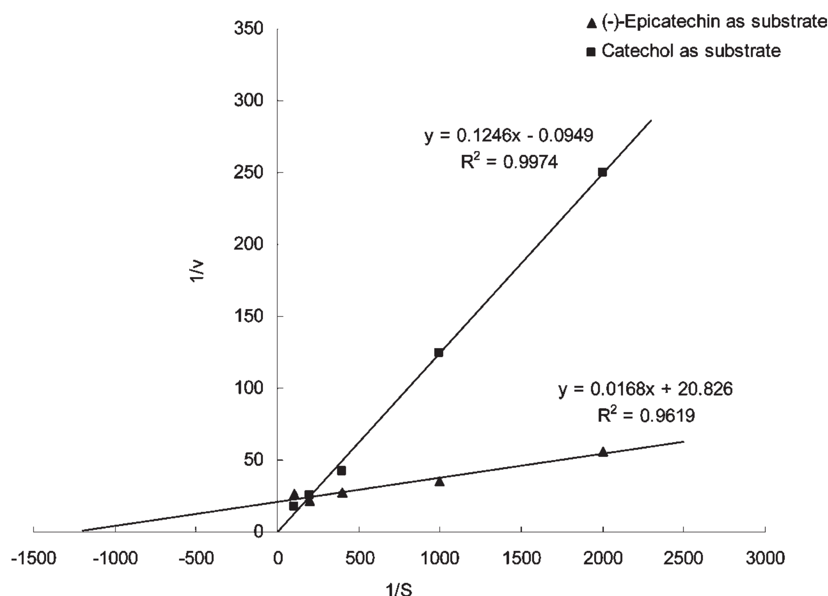

Figure 4. Lineweaver−Burk double reciprocal plots of longan PPO. The endogenous and exogenous substrate concentrations were 0.5, 1.0, 2.5, 5.0, and 10 mM, respectively.

The addition of Ca^{2+} into endogenous substrate−enzyme system exhibited weaker effect to longan PPO activity, while this ion obviously improved the enzymatic activity in exogenous substrate−enzyme system. Among all the metal ions tested, Na^{+}, K^{+}, and Mg^{2+} had little effect on longan PPO activity in both substrate−enzyme systems.

Kinetic Study. The kinetic analysis of longan PPO activity at 25 °C is shown in **Table 2**. Michaelis constant (K_m) and maximum reaction velocity (V_{max}) values were calculated from the Lineweaver−Burk double reciprocal plots (**Figure 4**) for (−)-epicatechin and catechol. The criterion for evaluation of the optimal substrate, the V_{max}/K_m ratio, was used (*42*). From **Table 2**, in comparison with catechol, longan PPO had a lower K_m and V_{max} value for (−)-epicatechin as a substrate, indicating that the enzyme was strongly bound with endogenous substrate while possessed a high catalytic efficiency to exogenous substrate. In addition, the V_{max}/K_m ratio for the endogenous substrate−enzyme system was higher than that of exogenous substrate−enzyme system, indicating that (−)-epicatechin was the optimal substrate for longan PPO in the present study. Although longan PPO possessed a higher catalytic efficiency to catechol than to (−)-epicatechin in this study, catechol has not still been identified from longan fruit tissues up to now. Therefore, it is more precise and authentic to investigate longan PPO characterizations using endogenous substrate (−)-epicatechin, which would illuminate the exact mechanism of PPO catalyzing endogenous substrate into browning product.

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