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Characterization of Polyphenol Oxidase from Litchi Pericarp Using (−)-Epicatechin as Substrate

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Polyphenol oxidase (PPO) from litchi (*Litchi chinensis* Sonn.) pericarp was characterized using (−)-epicatechin, which was the major endogenous polyphenol in litchi pericarp as a substrate. The optimum pH for PPO activity with (−)-epicatechin was 7.5, and the enzyme was unstable below pH 4.5 and stable in the pH range of 6.0−8.0. Residual activities of PPO were 86.25, 86.31, and 80.17% after 67 days of incubation at 4 °C at pH 6.0, 7.5, and 8.0, respectively. From thermostability studies, the K_i value increased with temperature and the results suggested that the enzyme was unstable above 45 °C. Moreover, the results also provided strong evidence that the denaturalization temperature of PPO was near 70 °C. The inhibition studies indicated that L-cysteine and glutathione were strong inhibitors even at low concentrations while NaF inhibited moderately. In addition, the results also indicated that the inhibition mechanisms of thiol groups were different from those of halide salts.

KEYWORDS: Polyphenol oxidase; litchi; (−)-epicatechin; characterization; inhibitor

INTRODUCTION

Polyphenol oxidase (PPO) is a widely distributed copper-containing enzyme that catalyzes the hydroxylation of monophenols to *o*-diphenols (EC 1.14.18.1, cresolase or monophenol monooxygenase) and the oxidation of *o*-diphenols to *o*-quinones (EC 1.10.3.2, diphenolase or catecholase). Enzymatic browning is caused by the oxidation of phenolic substrates by PPO to produce reactive quinones. These quinones are highly reactive species involved in different reaction pathways. They can be powerful electrophiles, which may suffer nucleophilic attack by other polyphenols (*1*), amino acids, and proteins to produce dark-brown or black pigments in senescent and postharvested fruits and vegetables (*2*−*5*). However, PPO is localized in plastids (*6*), while phenolic substrates are stored in the vacuole (*7*) in healthy tissues. Therefore, the browning is usually only initiated upon physiological or accidental tissue disruption.

Extraction, purification, and characterization of plant PPOs has been focused on fruits and vegetables because of the significance of enzymatic browning in the postharvest physiology and food technology. Litchi (*Litchi chinensis* Sonn.) is a subtropical fruit of high commercial value for its white, translucent aril and attractive red color. However, the fruit rapidly loses its bright red color and turns brown once harvested. Postharvest browning of litchi was thought to be caused by the rapid degradation of the red pigment and oxidation of phenolics by PPO, producing brown-colored products (*8*−*11*). The loss of cellular compartmentation after harvest is associated with the enhanced lipid peroxidation, reduced membrane fluidity, and increased membrane permeability (*12*−*14*). Deterioration in membrane function allows PPO and phenolic substrates to mix (*7*, *15*).

In a previous study, we found that (−)-epicatechin was the main endogenous substrate of PPO in litchi pericarp. Moreover, as far as we know, no kinetic property studies of PPO using (−)-epicatechin as a substrate have been carried out. Therefore, litchi pericarp PPO has been kinetically characterized using (−)-epicatechin as the substrate.

MATERIALS AND METHODS

Plant Material. Fruits of litchi (*L. chinensis* Sonn. cv. Nuomici) at commercial maturation were obtained from Guangdong. The fruit arrived at the laboratory within 24 h after harvest. Fruits were peeled, and the pericarp was stored at −30 °C until extraction.

Chemicals. Biochemicals were purchased from Sigma, and all other chemicals were of analytical grade.

Extraction of PPO from Litchi Pericarp. A 100 g amount of litchi pericarp was homogenized with 500 mL of acetone (−20 °C) for 1 min, and the mixture was refrigerated for 2 h and then filtered. The residue was rinsed with 200 mL of acetone (−20 °C) to eliminate phenolic compounds, and the PPO was in the residue. The residue was laid in the fume hood to remove the residual acetone, and acetone powder was obtained. Acetone powder of litchi was homogenized in 400 mL of 0.1 M phosphate buffer (pH 7.5) for 20 min, and then, the homogenate was centrifuged at 8000*g* for 5 min. Solid ammonium sulfate was added to the supernatant, and the precipitate obtained between 80 and 100% saturation was collected by centrifugation at 8000*g* for 10 min; then, the precipitate was dissolved in 0.1 M phosphate buffer (pH 7.5). The enzyme extract was dialyzed against the same buffer at 4 °C overnight.

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Enzyme Assay. The PPO activity was assayed spectrophotometrically at 15 °C using (−)-epicatechin as a substrate by monitoring at 440 nm. The reaction medium (3 mL) contained 1 mL of 3 mM (−)-epicatechin, 1.98 mL of 50 mM of phosphate buffer (pH 7.5), and 0.02 mL of the enzyme solution. One unit of enzyme was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min at 15 °C.

Enzyme Kinetics. For determination of the Michaelis constant (K_m) and maximum velocity (V_{max}) of the enzyme, PPO activities were measured with (−)-epicatechin as a substrate at various concentrations. K_m and V_{max} values of PPO were calculated from a plot of the reciprocal of initial velocity ($1/V$) vs the reciprocal of substrate concentration ($1/[S]$) according to the method of Lineweaver and Burk.

Effect of pH on PPO Activity and Stability. The optimum pH for litchi pericarp PPO activity was determined in the pH range of 3.5−8.0 by using 50 mM citrate (pH 3.5−5.0) and 50 mM phosphate (pH 5.5−8.0) buffer at 15 °C. The reaction medium (3 mL) contained 1 mL of 3 mM (−)-epicatechin, 1.98 mL of 50 mM of phosphate buffer (pH 7.5), and 0.02 mL of the enzyme solution. To determine the effect of pH on PPO stability, 0.2 mL of enzyme solution was incubated in 0.8 mL of various buffer solutions ranging from pH 3.1 to 8.0 at 4 °C. The residual PPO activity was determined at pH 7.5 at 15 °C at given time intervals.

Optimum Temperature and Thermostability. For determining the optimum temperature value of the enzyme, the PPO activity was measured at different temperatures, in the range of 15−75 °C. The optimum temperature of PPO was tested by heating the buffer solution (pH 7.5) to corresponding temperatures. Once temperature equilibrium was reached, (−)-epicatechin and enzyme were added, respectively, and spectrophotometric measurements were taken for 3 min. To analyze the thermostability of PPO, the enzyme was incubated at various temperatures between 15 and 75 °C, and the residual activity was determined at pH 7.5 at 15 °C at given time intervals.

Effect of Inhibitors on PPO Activity. Inhibition properties of L-glutathione, L-cysteine, ascorbic acid, and NaF were tested. The PPO activity was measured in phosphate buffer (pH 7.5), with (−)-epicatechin as the substrate and different inhibitor concentrations.

Statistical Analysis. For all enzyme activity measurements in the study, each sample was assayed in triplicate. Means were compared using Tukey's test with a significance level at $P < 0.05$.

RESULTS AND DISCUSSION

Enzyme Kinetics. The Michaelis−Menten constant (K_m) and maximum velocity (V_{max}) of PPO with (−)-epicatechin were 0.65 mM and 337.80 U/min, respectively. This K_m is much smaller than the value reported by using catechol or 4-methylcatechol as the substrate (*9*, *11*), indicating that litchi PPO had a much higher affinity for (−)-epicatechin, one of the major polyphenols in litchi pericarps.

Effect of pH. The activity of litchi pericarp PPO was measured at different pH values using (−)-epicatechin as the substrate. As seen in **Figure 1**, the activity of the PPO was highly pH-dependent. It was found that the optimum pH of the PPO was 7.5. Alyward and Haisman (*16*) reported that differences in optimum pH for PPO activity depended on plant sources, extraction methods, and purities of enzyme, buffers, and substrates. In general, most plants show maximum PPO activity near neutral pH values (*11*, *17*−*19*).

To investigate the pH stability of litchi pericarp PPO, the enzyme was incubated in various buffer solutions ranging from pH 3.1 to 8.0 at 4 °C, and the residual PPO activity was determined at given time intervals (**Figure 2**). Incubation of the enzyme at pH 3.1 for 1 day caused 49.50% loss in PPO activity, and only 2.43% of the activity remained after 12 days of incubation, indicating that litchi pericarp PPO was very unstable at pH 3.1. The PPO activity decayed more moderately when incubated at pH 4.5 than when incubated at pH 3.1, and incubation of PPO at pH 4.5 for 15 days caused 41.70% loss in

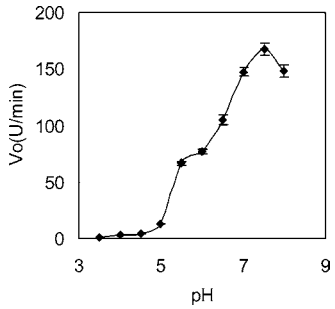

Figure 1. Effect of pH on initial velocity of the oxidation of (–)-epicatechin by litchi pericarp PPO. The initial velocity was determined at 385 (pH 3.5–5.0) or 440 nm (pH 5.5–8.0). The assay medium contained 1 mL of 3 mM (–)-epicatechin, 1.98 mL of 50 mM citrate (pH 3.5–5.0) or phosphate (pH 5.5–8.0) buffer, and 0.02 mL of the enzyme solution. Vertical bars indicate standard deviations of the means.

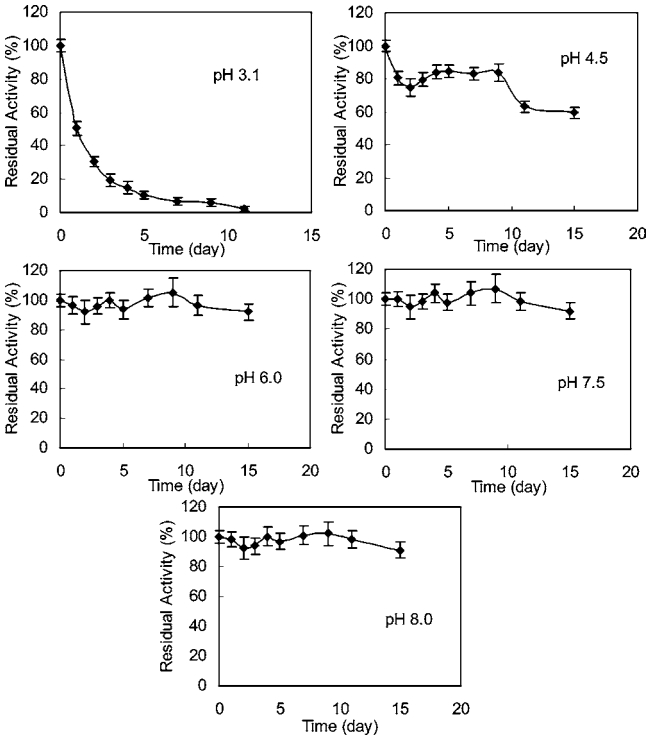

Figure 2. pH stability of litchi pericarp PPO with (–)-epicatechin as a substrate. A 0.2 mL amount of enzyme solution was incubated in 0.8 mL of various buffer solutions ranging from pH 3.1 to pH 8.0 at 4 °C. The residual PPO activity was determined at pH 7.5 at 15 °C at given time intervals. Vertical bars indicate standard deviations of the means.

PPO activity. Tukey's test showed no significant change in the enzyme activity during the 15 days of storage at pH 6.0, pH 7.5, and pH 8.0, respectively ($P > 0.05$), concluding that PPO was stable in the pH range of 6.0−8.0. For instance, litchi pericarp PPO lost only 7.88, 7.80, and 8.96% after 15 days of incubation at 4 °C at pH 6.0, 7.5, and 8.0, respectively. Moreover, after 67 days of incubation at 4 °C, residual activities of PPO were 86.25, 86.31, and 80.17% at pH 6.0, 7.5, and 8.0, respectively.

Optimum Temperature and Thermostability. The effects of temperatures between 15 and 85 °C at pH 7.5 were assayed using (−)-epicatechin as a substrate. **Figure 3** showed that the optimum temperature was 45 °C. Above 45 °C, the PPO activity decreased with increasing temperature.

To study the thermostability, the enzyme was incubated in a water bath at different temperatures. **Figure 4** showed the

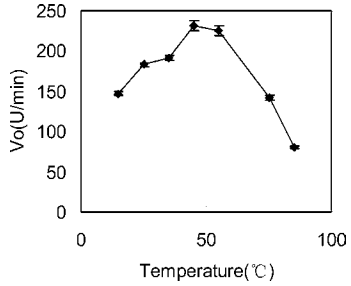

Figure 3. Optimum temperature of litchi pericarp PPO activity with (–)-epicatechin as a substrate. The assay medium at various temperatures contained 0.2 mL of 15 mM (–)-epicatechin, 2.78 mL of 50 mM phosphate buffer (pH 7.5), and 0.02 mL of the enzyme solution. Vertical bars indicate standard deviations of the means.

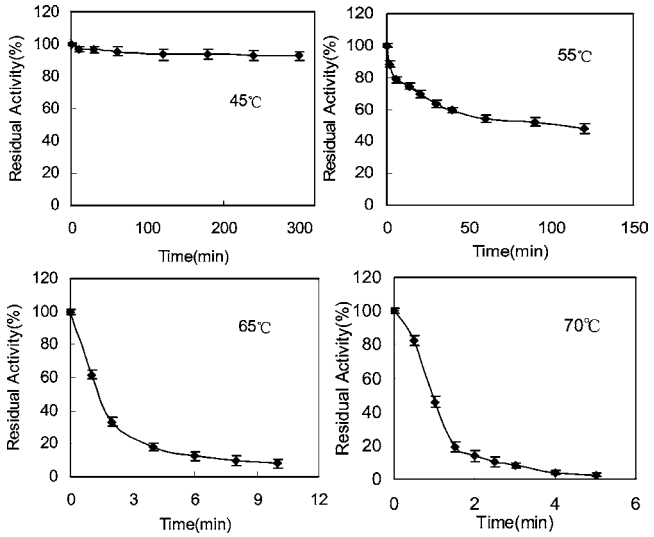

Figure 4. Thermostability of litchi pericarp PPO with (–)-epicatechin as a substrate. The enzyme was incubated at various temperatures between 45 and 75 °C, and the residual activity was determined at 15 °C at given time intervals. Vertical bars indicate standard deviations of the means.

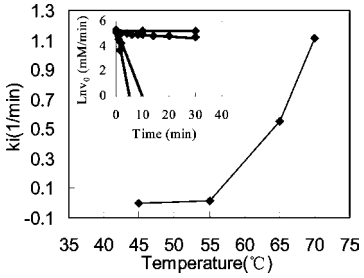

Figure 5. First-order rate constant for thermal inactivation of litchi pericarp PPO. Inset: semilogarithmic plots of PPO reaction rate vs incubation time at various temperatures: 45 (▲), 55 (■), 65 (◆), and 70 °C (●), respectively.

thermal stability of the enzyme at 45, 55, 65, and 70 °C, respectively. It was found that the thermal inactivation had two periods: The first period was called the steady-state period in which the activity decreased rapidly and linearly; after this period, an equilibrium-state period existed in which the activity decreased slightly as compared with the steady-state period. The inset of **Figure 5** shows the time-dependent activity decay in a semilogarithmic plot for litchi pericarp PPO. The first-order rate constant for the thermal inactivation, K_i, was determined from the slope at each incubation temperature and is plotted in **Figure 5**. The K_i value at 45 °C was approximately zero, indicating that the enzyme was stable at this temperature. The higher K_i value at 55 °C indicated that the enzyme was less stable at 55 °C as compared with 45 °C. These results could also be obtained from **Figure 4**, in which the residual activity of the enzyme decreased significantly ($P < 0.05$) at 55 °C and 59.64% of the activity remained after 40 min of incubation, while there was no significant loss in activity after 300 min of incubation at 45 °C ($P > 0.05$). The enzyme was very unstable at 65 and 70 °C. The activity of the enzyme decreased significantly ($P < 0.01$), and only 17.93% of the activity remained after 4 min of incubation at 65 °C. Similarly, the activity of the enzyme decreased significantly ($P < 0.01$), and only 19.12% of the activity remained after 1.5 min of incubation at 70 °C. Furthermore, the residual activity was 2.40% after incubation at 70 °C for 5 min. This result indicated that the denaturalization temperature was near 70°C.

Table 1. Effect of Different Inhibitors on Litchi Pericarp PPO Activity with (–)-Epicatechin as the Substrate[a]

inhibitor	[I] (mM)	residual activity (%)	I_{50} (mM)	lag period (min)
glutathione	0.2	49.36	0.19	5.5
	0.15	58.08		4.5
	0.1	68.65		2.5
	0.04	87.33		1
L-cysteine	0.2	36.96	0.13	7
	0.15	44.67		5.5
	0.1	55.03		3
	0.04	70.01		1
NaF	100	24.26	16.84	NF
	50	38.39		NF
	18	52.04		NF
	9	58.58		NF
	3	69.39		NF

[a] NF, not found.

These results were similar to the results obtained by using catechol as a substrate (*9*). However, our results were different from the results obtained by Jiang et al. (*11*), who used 4-methylcatechol as a substrate. They reported that litchi pericarp PPO had an optimum temperature at 70 °C and lost 50% of activity after 8.6 min of incubation at 90 °C. The difference was probably due to the difference in substrates, cultivars, and assaying methods.

Inhibition Studies. Table 1 shows the effect of different inhibitors at different concentrations on litchi pericarp PPO with (−)-epicatechin as the substrate. The results showed that the PPO activity was strongly inhibited by L-cysteine and glutathione. The ability of thiol groups to inhibit the PPO activity has been extensively described (*11*, *20*−*23*). Jiang et al. (*11*) investigated the effects of various inhibitors on the activity of litchi pericarp PPO using 4-methylcatechol as the substrate and found that glutathione was the best inhibitor. However, it was not the case with (−)-epicatechin as the substrate. In our study, the best inhibitor was L-cysteine. I_{50} values (inhibitor concentration at which 50% of PPO activity is inhibited) of L-cysteine were 0.13 mM, while glutathione was 0.19 mM. In addition, with either L-cysteine or glutathione, a lag period existed, in which the absorbance at 440 nm did not have any change or did not increase linearly. The higher concentrations produced longer lag periods (**Table 1**). As compared with the strong inhibition by L-cysteine and glutathione, sodium fluoride was inhibited moderately and the I_{50} value of sodium fluoride was 16.84 mM. Moreover, no lag period existed when sodium fluoride was used as the inhibitor. The higher concentration of sodium fluoride only produced a lower rate of oxidation. These

results could indicate that the inhibition mechanisms of thiol groups were different from halide salts.

In conclusion, the optimum pH for PPO activity with (−)-epicatechin was 7.5, and the enzyme was unstable below pH 4.5 and stable in the pH range of 6.0−8.0. Residual activities of PPO were 86.25, 86.31, and 80.17% after 67 days of incubation at 4 °C at pH 6.0, 7.5, and 8.0, respectively. From thermostability studies, the K_i value increased with temperature and the results suggested that the enzyme was unstable above 45 °C. A very low residual activity could be determined after incubation at 70 °C for 5 min, which provided strong evidence that the denaturalization temperature of PPO was near 70 °C. The inhibition studies indicated that L-cysteine and glutathione were strong inhibitors even at low concentrations while NaF was moderately inhibited. In addition, the existence of a lag period indicated that the inhibition mechanisms of thiol groups were different from those of halide salts.

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