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ScienceDirect
Food Chemistry 108 (2008) 818–823
Food Chemistry
www.elsevier.com/locate/foodchem

A comparative analysis of property of lychee polyphenoloxidase using endogenous and exogenous substrates

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Received 29 August 2007; received in revised form 4 November 2007; accepted 15 November 2007

Abstract

Lychee polyphenoloxidase (PPO) was extracted and partially purified using ammonium sulphate precipitation and dialysis. The comparative analysis of PPO property was performed using its endogenous substrate (–)-epicatechin and exogenous substrate catechol. The pH optima for activity and activation temperature profiles of lychee PPO were very different when the enzyme reacted with endogenous and exogenous substrates. The addition of ethylenediaminetetraacetic acid disodium salt into the endogenous or exogenous substrate–enzyme system exhibited the same lowest inhibition of the PPO activity. However, L-cysteine was most effective in inhibiting enzymatic activity in the endogenous substrate–enzyme system while ascorbic acid was the best inhibitor in the exogenous substrate–enzyme system. Fe^{2+} greatly accelerated the enzymatic reaction between endogenous substrate and PPO, but Cu^{2+} exerted the same effect on the reaction between exogenous substrate and PPO. Based on the kinetic analysis, lychee PPO could strongly bind endogenous substrate but it possessed a higher catalytic efficiency to exogenous substrate.

Keywords: Endogenous substrate; Exogenous substrate; Lychee; Polyphenol oxidase

1. Introduction

Polyphenoloxidase (PPO) catalyzes the hydroxylation of monophenol into *o*-diphenol which is oxidized into the corresponding *o*-quinone and subsequently polymerized into brown, red or black pigments, depending on natural components present in a given plant material (Aydemir, 2004; Dincer, Colak, Aydin, Kadioglu, & Güner, 2002). PPO is widely distributed in fruits and vegetables, and leads to enzymatic browning and major losses in some fresh fruits and vegetables (Duangmal & Owusu Apenten, 1999). Lychee is a tropical and subtropical fruit with a high commercial value. The fruit has a white, translucent, sweet and juicy aril which is surrounded by a bright red peel (Jiang, Duan, Joyce, Zhang, & Li, 2004). There are many factors which result in browning of lychee pericarp. A particularly important factor is enzymatic browning of the fruit. After harvest, the membrane compartmentation of lychee pericarp cells is gradually damaged, and PPO is in contact with its substrates, which initiates browning reaction in the presence of oxygen (Nicolas, Richard-Forget, Goupy, Amiot, & Aubert, 1994). The enzymatic browning by PPO is still the major practical limitation to storage of harvested lychee fruits. The browning of lychee fruit was generally thought to be a rapid degradation of anthocyanins and oxidation of phenolics caused by PPO, producing brown by-products (Jiang, 2000; Lee & Wicker, 1991). However, in a previous study (Sun et al., 2006), (–)-epicatechin was identified as a lychee PPO endogenous substrate which reacted with PPO, resulting in pericarp browning. In other studies, pyrogallol, catechol and 4-methylcatechol, used as exogenous substrates of lychee PPO, were

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doi:10.1016/j.foodchem.2007.11.036

investigated (Jiang, Zauberman, & Fuchs, 1997), but these investigations did not precisely reveal the PPO property or enzyme-catalyzed browning mechanism in the fruit because these chemicals were not found in lychee pericarp tissues up to now. The objective of this study was to use endogenous and exogenous substrates to compare lychee PPO property, and then to determine the different effects of endogenous and exogenous substrates on the enzyme property. This study will provide a new reference on how to characterize an enzyme using its natural endogenous substrate, and to develop a method for inhibiting PPO activity and its related pericarp browning.

2. Materials and methods

2.1. Materials

Lychee (*Litchi chinensis* Sonn. cv. Guiwei) fruits used in this study were obtained from a commercial orchard in Guangzhou in July 2006. The bright red fruits were selected, and then unhealthy fruits were eliminated. They were peeled and the pericarp tissues were stored at −20 °C until further extraction and analysis.

2.2. Extraction and partial purification of PPO

PPO was extracted by the methods of Jiang, Giora, Yoram, and Fu (1998) and Kavrayan and Aydemir (2001) with some modifications. All steps were carried out at 4 °C. Thirty grams of lychee pericarp tissues were homogenized by continual stirring with a glass rod in 200 mL of 0.1 M sodium phosphate buffer (pH 6.8) containing 1.0% (w/v) polyvinyl pyrrolidone and 1.0% (v/v) Triton X-100. After filtration of the homogenate through muslin, the filtrate was centrifuged at 8500*g* for 15 min and then the supernatant was collected. The enzyme solution was fractionated with solid ammonium sulphate (30–80% saturation) and the precipitate was collected by centrifugation at 8500*g* for 15 min, which was further re-dissolved in small volume of 10 mM sodium phosphate buffer (pH 6.8). After overnight dialysis against the same buffer, the dialyzed solution was collected as the partially purified PPO. The partially purified enzyme can be used to analyze well the PPO property (Jiang, 1999). In this study, polyvinyl pyrrolidone was used to bind phenols which could inactivate PPO activity during extraction (Erat, Sakiroglu, & Kufrevioglu, 2006) while Triton X-100 is nonionic surfactant which could extract plasma membrane proteins in the absence of extensive cellular destruction (Li, Cai, & Zeng, 2000). Furthermore, as the interfere of ammonium sulphate with the determination of protein concentration, it needs to be removed by the dialysis method.

2.3. Measurement of PPO activity and protein determination

PPO activity was assayed with endogenous substrate (–)-epicatechin and exogenous substrate catechol, according to a spectrophotometric procedure (Jiang, 1999). The enzyme solution (0.2 mL) was rapidly added into 2.8 mL of 20 mM (–)-epicatechin and catechol (prepared in 10 mM sodium phosphate buffer, pH 6.8) solutions, respectively. The increase in absorbance at 435 nm [the absorption peak of the enzymatic-catalyzed product after the reaction of (–)-epicatechin with lychee PPO] and 400 nm [the absorption peak of the enzymatic-catalyzed product after the reaction of catechol with lychee PPO] at 25 °C was recorded automatically for 3 min using a UV-2802 spectrophotometer (Unic, Shanghai, China). 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 the enzymatic-catalyzed products. All the determinations were performed in triplicate and corresponding means were plotted. 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 (Montero, Ávalos, & Pérez-Mateos, 2001).

Protein content was determined by the dye-binding method of Bradford (1976) with bovine serum protein as the standard.

2.4. The different properties of PPO using endogenous and exogenous substrates

2.4.1. Effects of pH

PPO activity was assayed in the pH range of 4.0–5.5 in 50 mM citric acid-phosphate buffer and 6.0–8.0 in 50 mM sodium phosphate buffer using (–)-epicatechin and catechol as substrates, respectively. To determine the optimum pH values of the enzyme, 0.5 mL of 10 mM (–)-epicatechin or catechol were added into to 2.95 mL of buffer solution and then mixed at 25 °C, prior to the addition of 0.05 mL of crude enzyme solution [the protein content was about 26.4 μg protein/mL, with a total activity of 157 units/mL when (–)-epicatechin as a substrate and 116 units/mL when catechol as one]. The relative activities were compared using the method described above.

2.4.2. Effects of temperature

The PPO activity was measured at different temperatures in a range of 20–55 °C using a water bath. A total of 2.9 mL of 10 mM (–)-epicatechin and catechol (prepared in 10 mM sodium phosphate buffer, pH 6.8) solutions were pre-warmed to the corresponding temperatures, respectively. 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, 0.05 mL of enzyme solution was respectively transferred into 2.95 mL of 10 mM (–)-epicatechin and catechol (prepared in 10 mM sodium phosphate buffer, pH 6.8) solutions to assay the enzyme stability.

2.4.3. Effects of inhibitors

To determine the effects of inhibitors [EDTA-$(Na)_2$, ascorbic acid, $Na_2S_2O_3 \cdot 5H_2O$, Na_2SO_3 and L-cysteine] on PPO activity, 2.9 mL of 10 mM (–)-epicatechin or catechol (prepared in 10 mM sodium phosphate buffer, pH 6.8) solution and 25 μL of various inhibitor solutions at 0.1 M were mixed immediately at 25 °C prior to the addition of 75 μL of enzyme solution.

2.4.4. Effects of metal ions

A total of 1.95 mL of 10 mM (–)-epicatechin or catechol (prepared in 10 mM sodium phosphate buffer, pH 6.8) solutions and 1 mL of various metal ionic compounds (NaCl, KCl, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$ and $FeCl_3 \cdot 6H_2O$) at 1.5 mg/mL were mixed immediately before the addition of 0.05 mL of enzyme solution. The control was the substrate–enzyme reaction system without any metal ionic compounds.

2.4.5. Determination of kinetic parameters

The enzyme kinetic parameters (Dincer et al., 2002; Doğan & Doğan, 2004), Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) for lychee PPO were determined at 25 °C when using (–)-epicatechin and catechol as substrates, respectively. The assay cuvette (3 mL) contained 2.95 mL of (–)-epicatechin or catechol (prepared in 10 mM sodium phosphate buffer, pH 6.8) solution with gradient concentration and 0.05 mL of the enzyme solution, respectively. Data were plotted by the method of Lineweaver and Burk (1934).

3. Results and discussion

3.1. The difference of optimum pH

The pH profile for the oxidation of endogenous substrate (–)-epicatechin and exogenous substrate catechol by lychee PPO is shown in Fig. 1. At 25 °C, three peaks for lychee PPO activity were observed when (–)-epicatechin was used as a substrate, and the optimum pH for the enzyme activity was determined to be pH 7.0. However, only two peaks for PPO activity were found when using catechol as a substrate, and the optimum pH for the enzyme activity was at pH 6.5. These results indicated that the reaction of lychee PPO with endogenous and exogenous substrates at a pH range of 4.0–8.0 showed different enzymatic activities and optimum pHs. Furthermore, the small peaks at pH 4.5 and 6.0 using (–)-epicatechin as a substrate and at pH 5.0 using catechol as a substrate suggest that lychee PPO isoenzymes might exist, and there were different optimum pHs to these isoenzyme activities for endogenous and exogenous substrate–enzyme systems. In Fig. 1, a similar trend of the PPO activity is exhibited when using (–)-epicatechin and catechol as substrates. At pH 4.0 and 8.0, the lychee PPO activities were 60.3 and 87.3% of the maximum activity for (–)-epicatechin, and 38.1 and 57.1% for catechol, respectively.

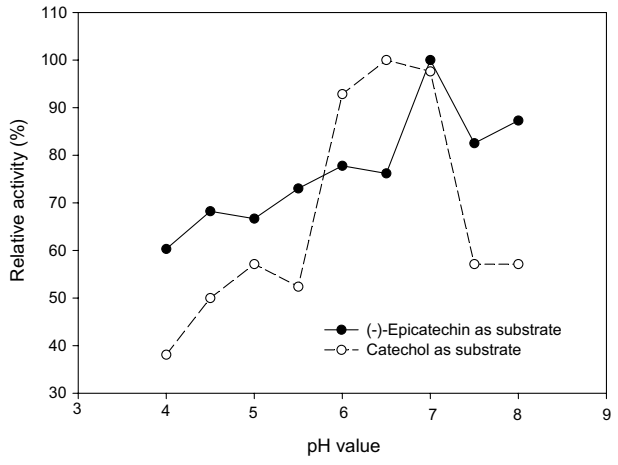

Fig. 1. Effects of various pH values on lychee PPO activities using the endogenous and exogenous substrates.

3.2. The differences of optimum temperature and heat stability

When using endogenous and exogenous substrates, the optimum temperature for lychee PPO activity is shown in Fig. 2A. The temperature had a two-sided influence on enzymatic activity, i.e. increasing incubation temperature enhanced enzymatic reaction velocity but simultaneously led to denaturation of enzyme. The optimum temperature was dependent on the effect of the two-sided equilibrium. From Fig. 2A, in a temperature range of 20–55 °C, it is apparent that the PPO activity changed slightly and then began to decrease above 50 °C when using endogenous substrate (–)-epicatechin. However, when using exogenous substrate catechol, there was a completely different trend in the activation temperature profile, i.e. the PPO activity increased linearly from 20 to 30 °C and the optimum temperature for the enzyme activity was at 35 °C. From 35 to 60 °C, the PPO activity gradually decreased because of the denaturation of enzyme.

The effects of high temperatures (70 and 80 °C) on lychee PPO activities are shown in Fig. 2B by using endogenous and exogenous substrates. The lychee PPO was relatively stable at 70 °C but unstable at 80 °C. After heating for 5 min at 70 °C, the relative PPO activities were 67.5% when using (–)-epicatechin and at 76.9% when using catechol as a substrate while at 80 °C, the relative PPO activities only remained 7.6% when using (–)-epicatechin and 36.4% when using catechol as a substrate. The above results indicated that the decrease in lychee PPO activity at high temperature was more obvious when the endogenous substrate (–)-epicatechin was used.

3.3. Different effects by enzymatic inhibitors

The effect of inhibitors on lychee PPO activity is listed in Table 1. Compared with control, the added inhibitors in

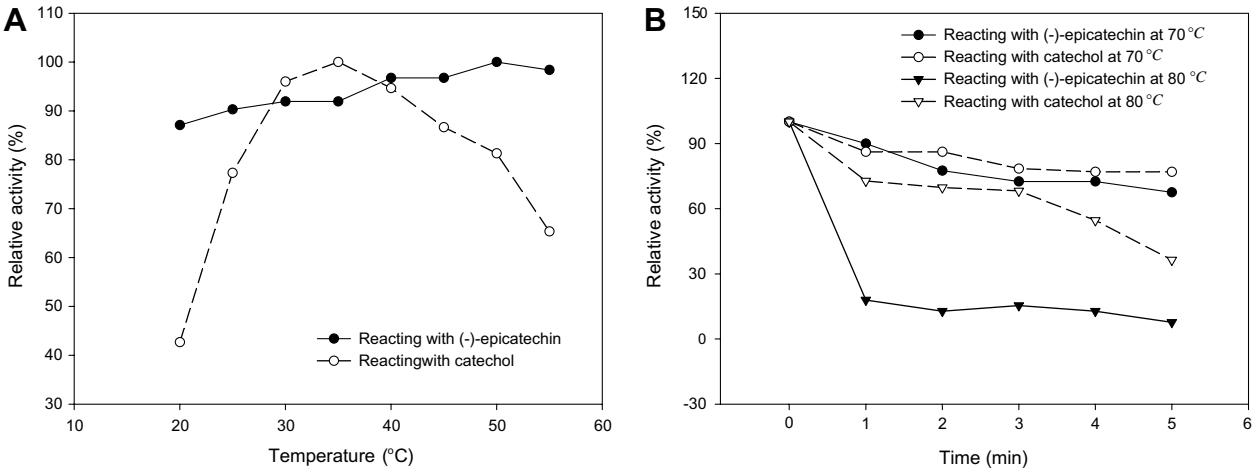

Fig. 2. Effects of various temperatures on lychee PPO activities by using the endogenous and exogenous substrates. (A) The optimum temperature of PPO activity and (B) the heat stability of PPO activity.

Table 1
Effects of various inhibitors on lychee PPO activities by using endogenous substrate (–)-epicatechin and exogenous substrate catechol

Inhibitors	Relative activity (%)	
	(–)-Epicatechin as substrate	Catechol as substrate
Control	100	100
EDTA-(Na)2	83.5	98.1
Ascorbic acid	2.1	1.9
$Na_2S_2O_3{\cdot}5H_2O$	27.8	6.7
Na_2SO_3	4.1	2.9
L-Cysteine	0	9.5

*Enzyme activity was assayed by using 2.9 mL of 10 mM (–)-epicatechin and catechol (prepared in 10 mM sodium phosphate buffer, pH 6.8), 25 μL of various inhibitors at 0.1 M and 75 μL of crude enzyme solution. The control was substrate–enzyme mixture without any inhibitor.

the substrate–enzyme system reduced the enzymatic activity to some extent. Comparing these five inhibitors, EDTA-$(Na)_2$ added into the endogenous or exogenous substrate–enzyme systems exhibited the lowest inhibiting effect on PPO activity. L-Cysteine was the most effective inhibitor for PPO activity when (–)-epicatechin was used as a substrate. Ascorbic acid was an effective inhibitor when catechol was used as a substrate. Furthermore, the addition of $Na_2S_2O_3 \cdot 5H_2O$ into the endogenous substrate–enzyme system could partially inhibit PPO activity, but the inhibition by this chemical was very strong in the exogenous substrate–enzyme system. From Table 1, ascorbic acid, Na_2SO_3 and L-cysteine were all good inhibitors of lychee PPO activity using an endogenous or exogenous substrate. Ascorbic acid could act more as an antioxidant than as an enzyme inhibitor because it reduced the initial quinone formed by PPO to the original diphenol before it underwent browning reaction (Aydemir, 2004). Na_2SO_3 is a good reducing agent for *o*-quinone and this is attributed to reduction of *o*-quinone and formation of colourless complexes with *o*-quinone. L-cysteine can easily form colourless complexes with *o*-quinones and PPO is inhibited by the formation of stable adducts (Janovitz-Klapp, Richard, & Nicolas, 1990; Sanada, Suzue, Nakashima, & Kawada, 1972).

3.4. Effects of different metal ions

Effects of various metal ionic compounds on lychee PPO activity are summarized in Table 2. The results showed that metal ions had different influences on the two substrate–enzyme systems. Compared with control, the relative activity of lychee PPO was the highest when Fe^{2+} was added to the endogenous substrate–enzyme system, which indicated that Fe^{2+} greatly accelerated the enzymatic-catalyzed reaction of substrate (–)-epicatechin with PPO, while the addition of Cu^{2+} into the exogenous substrate–enzyme system showed the greatest accelerated effect on the reaction between substrate catechol and PPO. In addition, Fe^{2+} also partly increased the enzymatically-catalyzed reaction between catechol and PPO. The addition of Na^+ into the endogenous or exogenous substrate–enzyme system weakly inhibited lychee PPO activity. The weak inhibition by Na^+

Table 2
Effects of various metal ionic compounds on lychee PPO activities by using endogenous substrate (–)-epicatechin and exogenous substrate catechol

Compound	Relative activity (%)	
	(–)-Epicatechin as substrate	Catechol as substrate
Control	23.1	33.8
NaCl	16.4	31.0
KCl	21.6	35.2
$CuSO_4 \cdot 5H_2O$	20.1	100
$FeSO_4 \cdot 7H_2O$	100	54.2
$FeCl_3 \cdot 6H_2O$	23.9	43.5

*Enzyme activity was assayed by using 1.95 mL of 10 mM (–)-epicatechin and catechol (prepared in 10 mM sodium phosphate buffer, pH 6.8), 1 mL of metal ionic compounds at 1.5 mg/mL and 50 μL of crude enzyme. The control was the substrate–enzyme mixture without any metal ionic compound.

Table 3
Kinetic parameters of lychee PPO in endogenous and exogenous substrate–enzyme systems

Parameters	Substrates	
	(–)-Epicatechin	Catechol
Wavelength (λ)	435	400
K_m (mM)	46.3	66.7
V_{max} (units/mL min)	78.2	142.2
V_{max}/K_m	1.7	2.1

on PPO activity has been observed in several other plant tissues (Kavrayan & Aydemir, 2001; Yang, Fujita, Ashrafuzzaman, Nakamura, & Hayashi, 2000). In the endogenous substrate–enzyme system, other metal ions, such as K^+, Cu^{2+} and Fe^{3+}, exhibited little effect on lychee PPO activity, but in the exogenous substrate–enzyme system, they increased the activity of this enzyme.

3.5. Kinetic study

The kinetic analysis of the lychee PPO activity at 25 °C is shown in Table 3. Michaelis constant (K_m) and maximum reaction velocity (V_{max}) values were calculated from the Lineweaver-Burk plot for (–)-epicatechin and catechol, respectively. The criterion for the evaluation of the optimum substrate, the V_{max}/K_m ratio, was used (Palmer, 1995). From Table 3, compared with catechol, lychee PPO had a lower K_m and V_{max} value for (–)-epicatechin as a substrate, indicating that the enzyme was strongly bound with the endogenous substrate and possessed a high catalytic efficiency to the exogenous substrate. The V_{max}/K_m ratio for (–)-epicatechin and catechol had a similar value, but the ratio of catechol was a little higher than that of (–)-epicatechin, which indicated that catechol was the optimum substrate for lychee PPO, but this compound was not found in lychee fruits.

4. Conclusions

By using endogenous substrate (–)-epicatechin and exogenous substrate catechol to compare lychee PPO properties, it was found that the optimum pH of PPO activity and the effect of temperature on the activity of the enzyme were greatly different when PPO reacted with both substrates. L-cysteine and ascorbic acid were the best inhibitors for the endogenous substrate–enzyme system and exogenous substrate–enzyme system, respectively. Fe^{2+} and Cu^{2+} accelerated the enzymatically-catalyzed reactions of endogenous and exogenous substrates by lychee PPO, respectively. In this study, although lychee PPO possessed a higher catalytic efficiency to catechol than to (–)-epicatechin, catechol has not been identified from lychee fruit pericarp tissues and, thus, it is better to investigate lychee PPO properties using endogenous substrate (–)-epicatechin.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (Grant Nos. 30425040 and 39900102) and the International Foundation for Science (Grant No. E2265/3F).

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