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Role of peroxidase in anthocyanin degradation in litchi fruit pericarp

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Abstract

Postharvest browning of litchi fruit pericarp is a major problem, resulting in accelerated shelf life and reduced commercial value of the fruit. The browning was generally thought to be a rapid degradation of red pigments caused by polyphenol oxidase (PPO) and peroxidase (POD). This work is conducted to understand the role of POD in anthocyanin degradation in litchi pericarp, which is less understood than PPO. POD activity in the pericarp increased consistently with skin browning index during storage of litchi fruit, but was negatively associated with anthocyanin concentration. Although POD cannot directly oxidize litchi anthocyanin in the presence of H_2O_2 in vitro, the anthocyanin content decreased rapidly after the addition of guaiacol solution, which indicated that the anthocyanin degradation by POD exhibited a coupled oxidation mechanism. Furthermore, anthocyanidin, produced by hydrolysis of the purified anthocyanin, could act as a substrate of POD. Thus, enzymatic browning of litchi fruit pericarp caused by POD may involve an anthocyanase–anthocyanin–phenolic–H_2O_2 reaction.

Keywords: Anthocyanin; Browning; Degradation; Litchi; Peroxidase; Purification

1. Introduction

Litchi (*Litchi chinensis* Sonn.) is a subtropical fruit of high commercial value for its white, transulent aril and attractive red color. The major limitation in litchi marketing is the rapid lose of the red color after harvest, accompanied by pericarp browning (Holcroft & Mitcham, 1996; Nip, 1988). Postharvest browning of litchi fruit was generally thought to be a rapid degradation of anthocyanins caused by polyphenol oxidase (PPO) and peroxidase (POD), producing brown by-products (Akamine, 1960; Jiang, Zauberman, & Fuchs, 1997; Lee & Wicker, 1991; Zauberman et al., 1991). Jiang (2000) reported that litchi PPO cannot directly oxidize litchi anthocyanin, but the anthocyanin is degraded rapidly in an anthocyanin–PPO–phenol system. While PPO activity can be determined during storage of litchi fruit, its action relation to browning degree is inconsistent. Lin et al. (1988a) demonstrated a rapid increase in PPO activity during the first 48 h of storage, while Zauberman et al. (1991) found no significant change in PPO activity during the same period and Underhill and Critchley (1994) reported even a progressive reduction in PPO activity. The reason for the contradictory findings in different observations for litchi PPO activity needs further investigation.

The relative significance of PPO is further obscured by the fact that POD, a similar oxidative enzyme, is also present in the litchi pericarp. Lin et al. (1988b) and Underhill and Critchley (1995) have recorded an increased POD activity during pericarp browning. Gong and Tian (2002) reported that the POD purified partially from litchi fruit peel can rapidly oxidize 4-methylcatechol in the presence of H_2O_2, supporting the involvement of POD in litchi enzymatic browning. However, little is known about the role of POD in litchi anthocyanin degradation. The objectives of this study were to investigate changes in POD activity and anthocyanin concentration in pericarp tissue of litchi fruit after harvest, then to purify POD and anthocyanins, and finally to determine the role of the POD in anthocyanin degradation in vitro.

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2. Materials and methods

2.1. Plant materials

Fruit of litchi (*L. chinensis* Sonn.) cv. Huaizhi at 90% maturation were obtained from a commercial orchard in Guangzhou. Fruit (20 kg) was selected for uniformity of shape and colour and then divided into two groups. One group of the fruit was held in a controlled environment room at 28 °C and about 70% relative humidity (RH), while the other was peeled and the pericarp were collected and then stored at −20 °C.

2.2. Fruit browning assessment

Skin appearance was assessed by measuring the extent of the total browned area on each fruit pericarp of 30 fruit using the following scale: 1 = no browning (excellent quality); 2 = slight browning; 3 = < 1/4 browning; 4 = 1/4 −1/2 browning; 5 = > 1/2 browning (poor quality). The browning index was calculated as $\sum$(browning scale × percentage of corresponding fruit within each class). The subjective evaluation of skin browning index was well correlated with the objective determination of the value of absorbance at 410 nm of the skin extract (Jiang, 2000).

2.3. POD activity assay and protein determination

Litchi POD was extracted by homogenising the pericarp (2 g) from 10 fruit with 10 ml of 0.05 M phosphate buffer (pH 7.0) and Polyclar AT (insoluble polyvinylpyrrolidone; 10% of peel by weight). The homogenate was centrifuged for 20 min at 16,000*g* and 4 °C and then the supernatant was collected as the crude enzyme extract. POD activity, with guaiacol as a substrate, was assayed by the method of Lin et al. (1988b) in a reaction mixture (3 ml), containing 25 l enzyme extract, 2.73 ml 0.05 M phosphate buffer (pH 7.0), 0.1 ml of 1% H_2O_2 and 0.15 ml of 4% guaiacol. The increase in absorbance at 470 nm due to the guaiacol oxidation was recorded for 2 min. One unit of enzyme activity was defined as the amount of the enzyme which caused a change of 0.001 in absorbance per minute.

Protein content was determined according to the dye-binding method of Bradford (1976).

2.4. Measurement of anthocyanin concentration

Litchi pericarp (10 g) from 10 fruit during storage was blanched with 200 ml of 0.1 M HCl. Anthocyanin concentration of the extraction solution was determined using a pH-differential method (Wrolstad, Culbertson, Cornwell, & Mattick, 1982). The extract (5 ml) was diluted in 25 ml of 0.1 M KCl–HCl buffer (pH 1.0) solution and 25 ml of 0.1 M citric acid–Na_2HPO_4 buffer (pH 4.5) solution, respectively. A spectrophotometer (Shimadu 300 UV), with 1 cm pathlength cells, was used for optical measurements at 510 nm. Anthocyanin concentration was calculated as cyanidin-3-glucoside by the method of Wrolstad et al. (1982).

2.5. Extraction and purification of POD

According to the method of (Sciancalepore, Longone, & Alviti, 1985), frozen litchi pericarp (100 g) was homogenized with 200 ml of 0.05 M phosphate buffer (pH 7.0) and 20 g Polyclar AT (insoluble polyvinylpyrrolidone, 10% of peel by weight) at 4 °C. After filtration of the homogenate through four-layer of muslin, the filtrate was centrifuged at 16,000*g* for 20 min and the supernatant was collected. The enzyme extract was fractionated with 30–70% saturation of solid ammonium sulfate and the precipitate was collected by centrifugation at 16,000*g* for 20 min. The precipitate was resuspended in 0.05 M phosphate buffers (pH 7.0) and dialyzed overnight against the same buffer. The dialyzed solution was incubated for 20 min at 50 °C and clarified by centrifugation at 8000*g* for 20 min. The supernatant was collected and applied to a DEAE–Sepharose (Amphamicia) column (1.5 × 50 cm), previously equilibrated with 0.05 M phosphate buffer (pH 7.0). The POD was eluted with a NaCl linear gradient (0–0.5 M) in the same buffer at a flow rate of 0.5 ml/min. Fractions (5 ml/fraction) were collected and assayed for POD activity and absorbance value at 280 nm. Two major peaks with POD activity were observed (Fig. 2) and the fraction with the higher enzymatic activity, appearing firstly, was pooled. The pooled fraction was further loaded onto a Sephadex G-75 column (1.5 × 50 cm), preequilibrated with 0.05 M phosphate buffer (pH 7.0), and eluted with 0.05 M phosphate buffer (pH 7.0) at a flow rate of 0.2 ml/min. Fractions (5 ml/fraction) were collected and assayed for POD activity and absorbance value at 280 nm, respectively, and the fraction with the highest activity was collected and pooled.

2.6. Extraction and purification of anthocyanins

Anthocyanins were extracted by 0.5 M HCl, purified to be homogeneous by Amberlite XAD-7 and Sephadex LH-20 and identified as cyanidin-3-rutinoside, as described by Zhang, Pang, Yang, Ji, and Jiang (2004).

2.7. Purification of anthocyanidin

The acidic hydrolysis procedure, previously described by Hong and Wrolstad (1986), was used for purification of anthocyanidin. The purified litchi anthocyanin solution (10 ml) was incubated with 50 ml of 2 M HCl for 60 min in a boiling water bath. To remove HCl and concentrate anthocyanidin, the hydrolysate was purified

using an Amberlite XAD-7 column by the method of Baublis, Spomer, and Berber-Jimenez (1994).

2.8. Determination of anthocyanin degradation by POD in absence or presence of guaiacol

The purified anthocyanin (0.5 ml) was diluted to 10 ml with 0.1 mM citric acid–Na_2HPO_4 buffer (pH 3.5) according to the method of Miller and Schreier (1985). The reaction mixture contained 2.95 ml of the diluted anthocyanidin solution, 50 μl of POD solution (0.526 U/μl) and/or 10 μl of 1% H_2O_2 in the absence or presence of 50 μl of 0.6 M guaiacol (Fig. 4). The anthocyanin degradation was initiated by the addition of the POD solution, following incubation of 5 and 15 min at 25 °C. The anthocyanin concentrations of the reaction mixtures were measured by the pH differential method (Wrolstad et al., 1982). In addition, the absorbance of the mixtures was automatically scanned at a range of 350–600 nm using a spectrophotometer (Shimadu 300 UV) after 5 and 15 min of incubation.

2.9. Determination of anthocyanidin degradation by POD

The purified anthocyanidin (0.5 ml) was diluted to 10 ml with 0.1 mM citric acid–Na_2HPO_4 buffer (pH 3.5). The reaction mixture contained 2.95 ml of the diluted anthocyanidin solution, 50 μl of POD solution (0.526 U/μl with 0.1 μg protein) and/or 10 μl of 1% H_2O_2 (Fig. 5). The reaction was initiated by addition of the purified POD solution, following a 3 min incubation at 25 °C. The visible spectra of the reaction mixtures were recorded at 350–600 nm.

3. Results and discussion

3.1. The changes in skin browning index, anthocyanin concentration and POD activity

The browning index of litchi fruit increased during storage at ambient temperature, while anthocyanin concentration decreased markedly (Fig. 1). Increased browning index of litchi fruit pericarp was associated with reduced anthocyanin concentration.

POD can catalyze oxidation of many kinds of phenols in the presence of peroxides and result in enzymatic browning of harvested fruits, such as pear (Richard & Gauillard, 1997), pineapple (Selvarajah, Herath, & Bandara, 1998) and peach (Stutte, 1989). In the present study, there was high POD activity measured in litchi fruit pericarp, and the activity increased with storage time (Fig. 1). Similar results were observed by Zauberman et al. (1991) and Underhill and Critchley (1995). As anthocyanin is the major phenol in litchi pericarp (Zhang, Grigor, & Quantick, 2000), the results showed

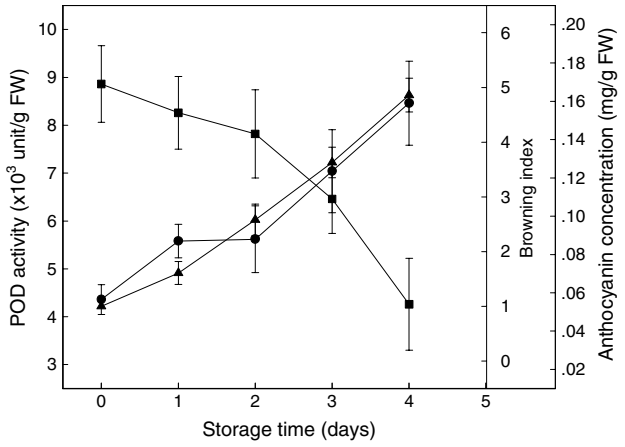

Fig. 1. Changes in POD activity (●), browning index (▲) and anthocyanin concentration (■) of litchi fruit during storage at 28 °C.

that POD was involved in anthocyanin degradation in litchi pericarp, in terms of the increased POD activity and browning index, and reduced anthocyanin concentration.

3.2. Extraction and purification of POD

A 12.5-fold purification of POD relative to protein was achieved (Table 1), using $(NH_4)_2SO_4$, DEAE–Sepharose and Sephadex G-75. The elution profiles of the POD on DEAE–Sepharose and Sephadex G-75 are shown in Figs. 2 and 3, respectively. In the study, no activity of PPO was detected in the purified enzyme solution.

3.3. Degradation of anthocyanin by POD in the presence of guaiacol

In the presence of H_2O_2 and guaiacol, the purified litchi anthocyanin was rapidly degraded by POD (Table 2). As the reaction progressed, anthocyanin concentration decreased, which was associated with appearance of brown colour. The results indicated that POD accelerated anthocyanin degradation in the POD–H_2O_2–anthocyanin–guaiacol system. The visible spectra of the POD–H_2O_2–anthocyanin–guaiacol mixtures incubated for 5 or 15 min further confirmed a significant decrease in absorbance at 510 nm (Fig. 4), which was the characteristic absorbance of the anthocyanin.

The results also suggest that POD had a low affinity for anthocyanins (Table 2, Fig. 4). Thus, POD can exhibit a coupled oxidation mechanism in the anthocyanin degradation. Namely, in the sequential reactions of the POD–phenol–H_2O_2–anthocyanin system, POD catalyzed oxidation of guaiacol by H_2O_2, resulting in formation of quinone which was very reactive, followed by anthcyanin degradation. In intact litchi pericarp, the accumulation of active oxygen, including H_2O_2, was observed (Lin et al., 1988b), while phenols, such as

Table 1
Purification of POD from litchi fruit pericarp

Step	Volume (ml)	Total activity (U/min)	Protein (μg)	Specific activity (U/μg)	Yield (%)	Purification (fold)
Crude extract	400	538,400	25,300	21.28	100	1
$(NH_4)_2SO_4$	24	129,312	3867	33.44	24.0	1.6
DEAE–Sepharose	23	40,020	345	116	7.4	5.5
Sephadex G-75	19	9994	38	263	1.9	12.5

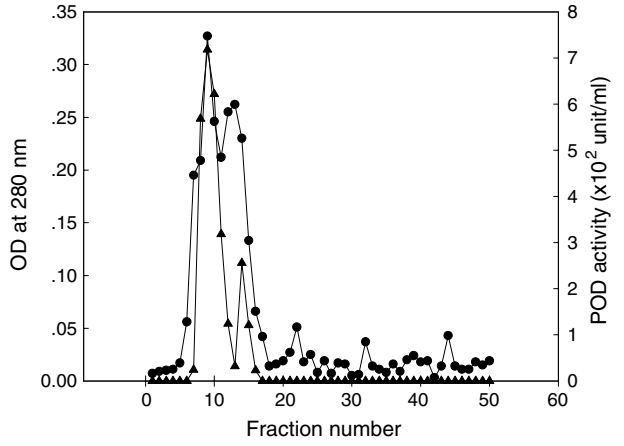

Fig. 2. Elution profiles of POD activity (▲) and OD value (●) at 280 nm purified by SEDE–Sepharose.

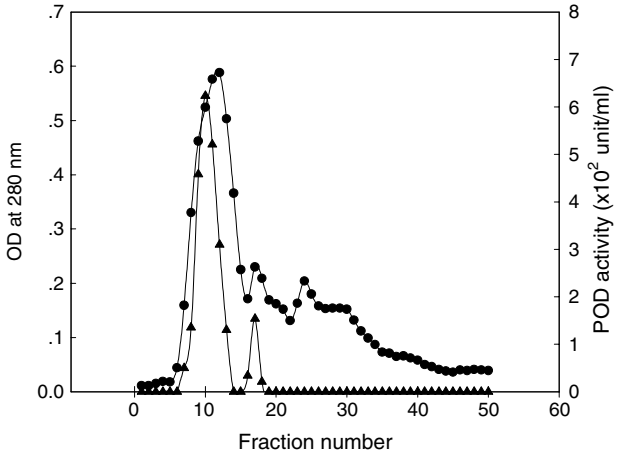

Fig. 3. Elution profiles of POD activity (▲) and OD value (●) at 280 nm purified by Sephadex G-75.

Table 2
Effects of POD on anthocyanin concentration (μg/ml) in the presence of guaiacol

	Reaction time (min)	
	5	10
Anthocyanin	1.02 ± 0.030	1.02 ± 0.030
Anthocyanin + H_2O_2	0.984 ± 0.032	1.032 ± 0.047
Anthocyanin + H_2O_2 + POD	1.01 ± 0.063	1.01 ± 0.052
Anthocyanin + H_2O_2 + POD + guaiacol	0.531 ± 0.018	0.501 ± 0.011

Values are means ± standard errors for three replicates.

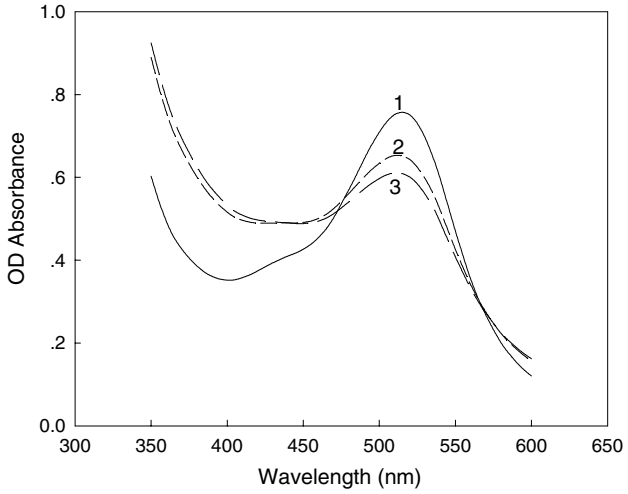

Fig. 4. Reaction of litchi anthocyanin with POD: (1) anthocyanin + POD + H_2O_2 (after 5 min of reaction); (2) anthocyanin + POD + H_2O_2 + guaiacol (after 5 min of reaction); and (3) anthocyanin + POD + H_2O_2 + guaiacol (after 15 min of reaction).

catechin and gallocatechin, were detected in litchi pericarp (Zhang et al., 2000) and POD was reported to show different activities towards various phenols, such as catechol and pyrogallol (Miller & Schreier, 1985). Based on the high POD activity and phenols existing in litchi pericarp, it was therefore suggested that POD can play an important role in anthocyanin degradation associated with increased pericarp browning of litchi fruit.

3.4. Degradation of anthocyanidin by POD

The sugar moiety of anthocyanins was thought to be the steric hindrance against PPO attack and, thus, there was a low affinity for PPO (Zhang, Pang, Ji, & Jiang, 2001). Zapata, Calderon, and Ros Barcelq (1995) suggested that removal of the sugar moiety from anthocyanins, to produce phenolic aglycones, was an important step before POD acted on the anthocyanins as substrates. In the present study, Fig. 5 shows that the peak at 510 nm disappeared after 3 min of incubation in POD–H_2O_2–anthocyanidin reaction system. Furthermore, there was a lower absorbance value at 510 nm in the mixture of the H_2O_2–anthocyanidin system than in the anthocyanidin alone, but a higher absorbance value than in POD–H_2O_2–anthocyanidin reaction system (Fig. 5), corroborating that anthocyanidin is more vulnerable to H_2O_2 than anthocyanin (Zhang et al., 2001).

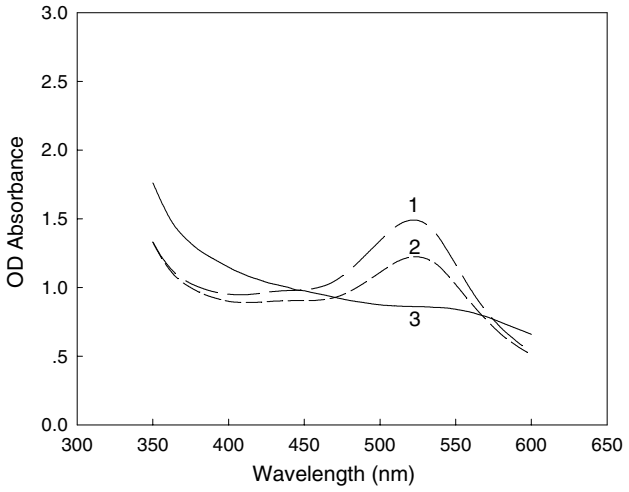

Fig. 5. Reaction of litchi anthocyanidin with POD: (1) anthocyanidin (3 min of reaction); (2) anthocyanidin + H_2O_2 (after 3 min of reaction); and (3) anthocyanidin + POD + H_2O_2 (after 3 min of reaction).

The results indicated that POD could catalyze the degradation of the anthocyanidin as a substrate in the presence of H_2O_2. As a high activity of anthocyaninase was measured in litchi pericarp (Zhang et al., 2001), it was suggested that the anthocyanase could first remove the sugar moiety from litchi anthocyanins, producing anthocyanidin, and finally POD caused the degradation of the anthocyanidin in the presence of H_2O_2.

4. Conclusion

A high activity of POD in litchi fruit pericarp was measured. POD activity increased consistently with browning index, but negatively with anthocyanin concentration during storage of litchi fruit. Although POD could not directly catalyze anthocyanin degradation in the presence of H_2O_2, the anthocyanin could be rapidly degraded by POD when both H_2O_2 and simple phenols, such as guaiacol, were in present. The anthocyanidin, resulting from the hydrolysis of the anthocyanin, could act as a substrate for POD. Thus, litchi enzymatic browning caused by POD may involve an anthocyanase–anthocyanin–phenolic–H_2O_2 reaction.

Acknowledgements

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