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Short Communication

Degradation of anthocyanin from litchi fruit pericarp by H_2O_2 and hydroxyl radical

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ABSTRACT

Litchi (*Litchi chinensis* Sonn.) is one of the most desirable subtropical fruits with high commercial values, which is significantly affected by its skin colour due to the embedded anthocyanin pigments. This work was conducted with a view to explaining the unexpected observation that litchi polyphenol oxidase (PPO) did not oxidise directly anthocyanins. Litchi fruit were stored for 4 days at 25 °C and 80–90% relative humidity. Browning index and H_2O_2 and ·OH contents of pericarp tissues of litchi fruit during storage were determined. The browning index of litchi fruit rapidly increased while H_2O_2 and ·OH contents decreased and then increased markedly, as storage time progressed. The obvious pericarp browning was associated with the rapid increases in H_2O_2 and ·OH contents of litchi fruit after 4 days of storage. Furthermore, litchi anthocyanins were purified by column chromatography and then H_2O_2 and hydroxyl radical were used to examine their degradation roles in the purified anthocyanin. It was found that the purified litchi anthocyanin was degraded markedly in the presence of H_2O_2 or hydroxyl radical. Increasing concentration of H_2O_2 or hydroxyl radical enhanced the anthocyanin degradation, of which the latter exhibited a greater effect on the anthocyanin degradation although no peak of litchi anthocyanin appeared after the treatment with 0.1% H_2O_2 for 10 min. This study can account for the pericarp browning of postharvest litchi fruit during storage based on the oxidative degradation of anthocyanin caused by PPO.

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1. Introduction

Reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and superoxide anion can play an important role in the degradation of plant pigments (Merzlyak & Hendry, 1994; Reszka, O'Malley, McCormick, Denning, & Britigan, 2004). Among these ROS, hydroxyl radical is able to cause oxidative decomposition of anthocyanins (Özyürek, Bektaşoğlu, Güçlü, & Apak, 2008) while other ROS might affect the anthocyanin degradation (Matta, Hanna, & Chiron, 2008; Nerud, Baldrian, Gabriel, & Ogbeifun, 2001). Reszka et al. (2004) confirmed the oxidation of pyocyanin by H_2O_2 in the presence of peroxidase as evidenced by loss of the characteristic absorption spectrum of the pigment while Lopez-Serrano and Barcelo (1999) suggested that peroxidase in the absence of H_2O_2 may play an important role in anthocyanin degradation of strawberry slice. Although oxidative reaction of plant pigments is well-known, information on the role of ROS in the anthocyanin degradation of harvested fruits during storage is relatively limited and, thus, it needs further investigation.

Litchi (*Litchi chinensis* Sonn.) is one of the most desirable subtropical fruits with high commercial values, which, however, is significantly affected by red skin colour due to the embedded anthocyanin pigments (Jiang, Duan, Joyce, Zhang, & Li, 2004; Zhang, Pang, Duan, Ji, & Jiang, 2005). The major identified litchi anthocyanins include cyanidin-3-glucoside (Zhang, Pang, Yang, Ji, & Jiang, 2004) and malvidin (Lee & Wicker, 1991). After the harvest, litchi fruit easily loses its red colour once harvested, resulting in reduced market value (Huang & Scott, 1985). The anthocyanin degradation caused by polyphenol oxidase (PPO) or peroxidase results in enzymatic browning reaction of harvested strawberry and grapefruit (Cheynier, Osse, & Rigaud, 1988; Cheynier, Souquet, Kontek, & Moutounet, 1994; Lopez-Serrano & Barcelo, 1999) but litchi PPO did not oxidise directly litchi anthocyanin (Jiang, 2000), which is not in agreement with most reports in the literature. Our previous study indicated that the oxidation of endogenous or exogenous phenolics by PPO can degrade rapidly litchi anthocyanin (Ruenroengklin, Sun, Shi, Xue, & Jiang, 2009) and, thus, we hypothesise that pericarp browning of harvested litchi fruit during storage could be the involvement of ROS caused by the oxidation of phenolics by PPO. In this study, litchi fruit were stored for 4 days at 25 °C and 8–90% relative humidity, and then browning index and H_2O_2 and ·OH contents of pericarp tissues of the fruit during storage were measured. Furthermore, litchi anthocyanin was purified and H_2O_2 and hydroxyl radical were then used to examine their degradation roles in the purified anthocyanin. The objective of this study was to

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elucidate well the pericarp browning of postharvest litchi fruit during storage.

2. Materials and methods

2.1. Fruit materials

Fresh fruit of litchi (*L. chinensis* Sonn.) cv. Huaizhi at the commercial mature stage were obtained from an orchard in Guangzhou, China. Fruit were chosen for uniformity in shape and colour. The fruit were washed carefully by tap water, then air-dried at 25 °C for 2 h, and finally divided into two groups. The first group of the fruit were placed in punnets (18 fruits/punnet), then stored for up 4 days at 25 °C and 80–90% relative humidity and finally used for fruit browning evaluation and analyses of H_2O_2 and ·OH contents. The fruit of the second group were manually peeled, and then the peel tissues were collected, frozen at −20 °C and used for extraction and purification of litchi anthocyanin in relation to the anthocyanin degradation caused by H_2O_2 and hydroxyl radical.

2.2. Fruit browning evaluation

Fruit browning was assessed according to the method of Jiang (2000) by measuring the extent of the total browned area of 60 individual fruits, using the following visual appearance scale: 0 = no browning (excellent quality); 1 = <1/8 browning; 2 = 1/8–1/4 browning; 3 = 1/4–1/2 browning and 4 = >1/2 browning (poor quality). The browning index was calculated as the following formula, $\sum$ (browning rating × percentage of fruit within each class)/4.

2.3. Analyses of H_2O_2 and ·OH contents

H_2O_2 and ·OH contents were determined by the fluorescent methods of Schopfer, Plachy, and Frahry (2001), and Halliwell, Grootveld, and Gutteridge (1988), respectively. Pericarp tissues (5 g) of litchi fruit stored for 0, 2 and 4 days were homogenised for 30 min with 15 ml of 20 mM phosphate buffer (pH 6.0). The homogenised solution was centrifuged at 2500*g* for 10 min and the supernatant was then collected for the analyses of H_2O_2 and ·OH contents. For the measurement of H_2O_2 content, an aliquot (1 ml) of the supernatant was incubated for 5 min with 3 ml of 20 mM M phosphate buffer (pH 6.0) containing 5 μM scopoletin (Sigma Chemical Corporation, St. Louis, MO, USA) and 0.3% (w/v) horseradish peroxidase (Boehringer Mannheim Corporation, Indianapolis, IN, USA) in darkness at 25 °C using a shaker. The decrease in fluorescence at the excitation wavelength of 346 nm and the emission wavelength of 455 nm) of each sample was measured against the reagent blank solution. H_2O_2 contents of litchi fruit after 0, 2 and 4 days of storage were expressed as the relative values against fruit at harvest based the fluorescent value. For the analysis of ·OH content, an aliquot (1 ml) of the supernatant was incubated for 30 min in 1.5 ml of 20 mM phosphate buffer (pH 6.0) containing 20 mM 2-deoxy-D-Rib (Sigma Chemical Co.) at 25 °C, then mixed with 0.5 ml of 1% (w/v) 2-thiobarbituric acid and 0.5 ml of 2.8% (w/v) trichloroacetic acid, and finally the fluorescent intensity was measured at the excitation wavelength of 532 nm and the emission wavelength of 553 nm against the reagent blank solution. ·OH contents of litchi fruit after 0, 2 and 4 days of storage were expressed as the relative values against fruit at harvest based the fluorescent value.

2.4. Extraction and purification of anthocyanin

Litchi anthocyanin was extracted according to method of Zhang et al. (2005). In brief, frozen peel tissues (50 g) were homogenised with liquid nitrogen in a blender before 250 ml of 1.5 M HCl in 95% ethanol were added. The slurry was kept at 4 °C overnight. The extract was filtered and concentrated using a rotary evaporator (RE-52A, Shanghai Woshi Co., Shanghai, China) under vacuum at 40 °C. Amberite XAD-7 resin (Sigma, St. Louis, MO, USA) column (2.0 × 50 cm) was used for purification of litchi anthocyanins by the method of Duan, Jiang, Su, Zhang, and Shi (2007). The concentrated anthocyanin solution was loaded onto the column and then eluted with 0.1% HCl in ethanol at 0.4 ml/min, using a fraction collector. The fraction with the highest absorbance value at 510 nm was collected and then concentrated using a rotary evaporator under vacuum at 40 °C. The concentrated litchi anthocyanin was filtered through a 0.45 μm Nylon syringe filter prior to high-performance liquid chromatography (HPLC) analysis.

2.5. HPLC analysis

Litchi anthocyanin was quantified by HPLC according to the method of Zhang, Quantick, and Grigor (2000). LC-20AT HPLC (Shimadzu, Kyoto, Japan) consists of a SPD-20A UV/Vis detector. Separation was performed using Shim-Pack VP-ODS column (250 × 4.6 mm, Shimadzu Corporation, Kyoto, Japan). The mobile phase consisted of 2% acetic acid (solvent A) and acetonitrile-methanol (10:15, v/v) (solvent B), with the following elution conditions: at 0 min 90% A and 10% B, at 10 min 80% A and 20% B, at 15 min 70% A and 30% B, at 25 min 60% A and 40% B, at 30 min 50% A and 50% B, and at 40 min 50% A and 50% B at a flow rate of 10 μl min^{-1}. All analyses were made by injecting 20 μl aliquots into HPLC and then their chromatographs were recorded at 510 nm.

2.6. Anthocyanin degradation in the presence of H_2O_2

The reaction mixture consisted of 1.7 ml of the extraction solution, 0.2 ml of 0.5 μM anthocyanin solution and 0.1 ml of H_2O_2 at 0 (control), 0.1 or and 1%. After 5 or 10 min of incubation, the reaction mixture was scanned immediately over range 300–650 nm by a spectrophotometer (Unic UV-2802, Shanghai, China).

2.7. Anthocyanin degradation in the presence of hydroxyl radical

The effect of $FeSO_4/H_2O_2$ (Fenton's reagent) on the litchi anthocyanin degradation was examined at 23 °C. The reaction mixture consisted of 1.7 ml of the extraction solution, 0.2 mL of 0.5 M anthocyanin solution, 0.1 ml of $FeSO_4/H_2O_2$ at 0 (control), 2, 5, 10, 20, 40 or 60 mM. After 5 and 10 min of incubation at 23 °C, the absorbance or chromatograph at 510 nm of the reaction mixture was measured by a spectrophotometer or analysed by HPLC, as described above.

2.8. Statistical analysis

Experiments were carried out in triplicates. The Duncan's Multiple Range Test (SAS Version 6.12, SAS Institute Inc., Cary, NC, USA) was used to determine significant differences. Differences between the means at the 5% level were considered to be significant.

3. Results and discussion

3.1. Fruit browning and H_2O_2 and ·OH contents

Browning index of litchi fruit rapidly increased while H_2O_2 and ·OH contents decreased and then increased markedly, as storage time progressed. The obvious pericarp browning was associated with the rapid increases in H_2O_2 and ·OH contents of litchi fruit after 4 days of storage (Table 1). Similar results were obtained in

Table 1
Browning index and H_2O_2 and ·OH contents of litchi fruit during storage[a].

Storage day	Browning index	H_2O_2 content	·OH content
0	0.1c	100b	100b
2	1.6b	81c	87c
4	2.8a	112a	123a

[a] Data were presented as the means ± standard errors (SE) of three replicate determinations. The mean values within a column followed by the same letter are not significantly different at the 5% level.

banana fruit during storage by Yang et al. (2008), who reported that the increased H_2O_2 and ·OH contents were related to the initiation of fruit aging. Thus, it was suggested that enhanced production of H_2O_2 and ·OH could participate in the formation of the brown-coloured by-products of litchi fruit during storage.

3.2. Purification and HPLC analysis of anthocyanin

Amberlite XAD-7 column or Sephadex LH-20 column chromatography is commonly used for anthocyanin purification (Li & Jiang, 2007). The absorbance at 510 nm was well associated with litchi anthocyanin content (Fig. 1). In this study, a major peak of litchi anthocyanins was obtained at the retention time of about 15 min (Fig. 3) and identified as cyanindin-3-rutinoside based on our previous study (Zhang et al., 2004). Thus, the HPLC analysis established can be used further to investigate the anthocyanin degradation caused by H_2O_2 and hydroxyl radical.

3.3. The degradation of litchi anthocyanin by H_2O_2

Anthocyanins are relatively unstable due to their degradation or the formation of condensed polymers (Brownmiller, Howard, & Prior, 2008; Harbourne, Jacquier, Morgan, & Lyng, 2008). Fruit browning was associated with changes in pigment concentrations (Felicetti & Schrader, 2009; Zhang et al., 2004). The participation of ROS such as H_2O_2 involved in the anthocyanin degradation of various plant tissues was reported by Nerud et al. (2001), Matta et al. (2008) and Reszka et al. (2004). Merzlyak and Hendry (1994) determined the oxidation of pyocyanin by H_2O_2, as evidenced by loss of the characteristic absorption spectrum. Furthermore, Es-Safi et al. (2008) found the formation of two new colourless phenolic compounds from malvidin 3-O-glucoside. They proposed that the formation of the two new compounds could involve in the passage through the chalcone form of the anthocyanin and an oxidation process. In this study, the absorbance of litchi anthocyanin at 530 nm decreased markedly with increasing H_2O_2 concentration.

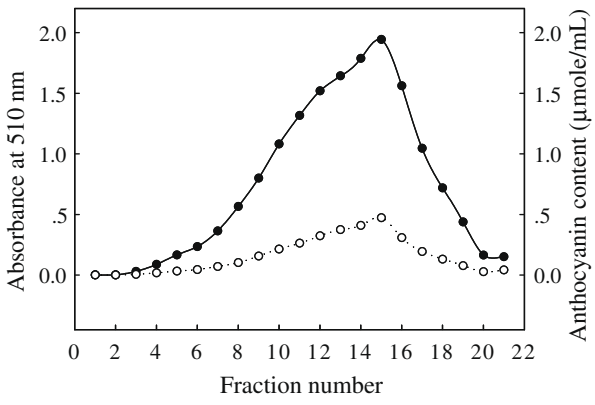

Fig. 1. Elution profile of absorbance value (●) and anthocyanin content (○) purified by Amberite XAD-7.

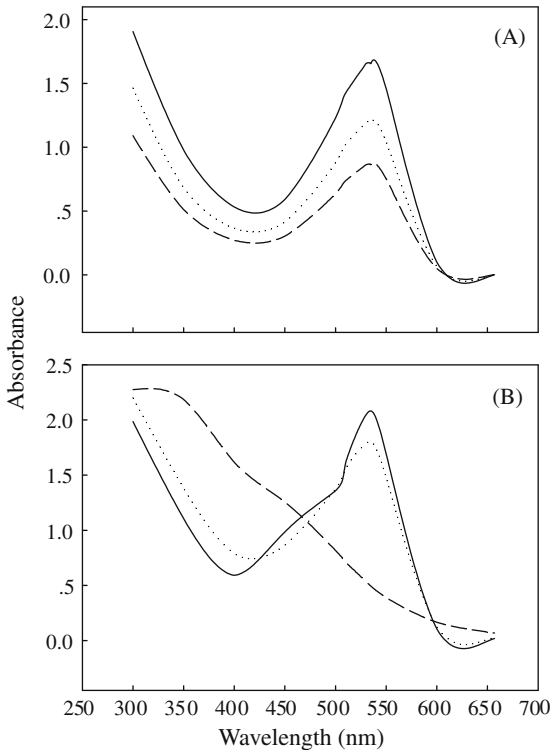

Fig. 2. The litchi anthocyanin degradation in the presence of H_2O_2 at 0 (control) (—), 0.01 (···) and 0.1% (–) after 5 (A) and 10 (B) min of incubation.

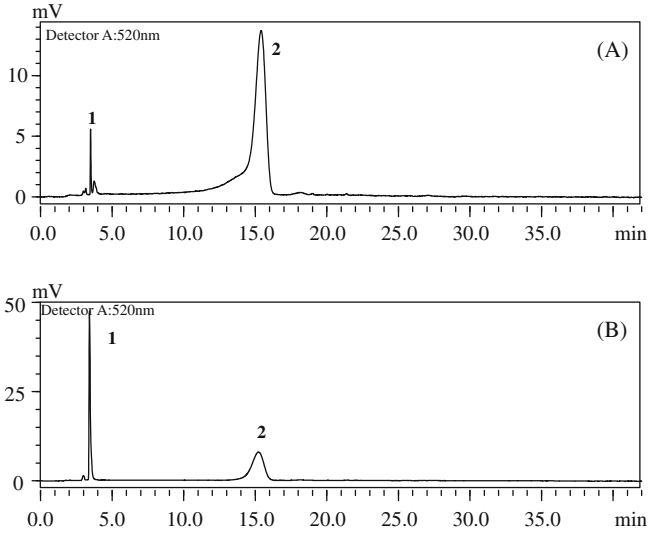

Fig. 3. HPLC chromatogram of litchi anthocyanin after 10 min of incubation. (A) litchi anthocyanin and (B) anthocyanin plus the Fenton's reagent.

It was observed that no peak of litchi anthocyanin appeared in the presence of 0.1% H_2O_2 when the incubation was extended for 10 min (Fig. 2), suggesting the structural modification and the subsequent formation of the brown-coloured by-products of the anthocyanin of litchi fruit during storage, which requires to be investigated further.

3.4. The degradation of litchi anthocyanin by hydroxyl radical

Ferrous iron in the Fenton's reagent was usually used to provide metal catalyst to produce hydroxyl radical (Pimentel, Oturan,

Table 2
The litchi anthocyanin degradation in the presence of Fe^{2+}/H_2O_2 at various concentrations after 5 or 10 min of incubation. The relative amount of litchi anthocyanin was expressed as an absorbance at 510 nm[a].

Fe^{2+}/H_2O_2 concentration (mM)	5 min	10 min
0	0.13a	0.13a
2	0.125b	0.117b
5	0.112c	0.085c
10	0.110c	0.071d
20	0.089d	0.051e
40	0.074e	0.044f
60	0.064f	0.042f

[a] Data were presented as the means ± standard errors (SE) of three replicate determinations. The mean values within a column followed by the same letter are not significantly different at the 5% level.

Dezotti, & Oturan, 2008). As shown in Fig. 2, an accelerated degradation of the litchi anthocyanin was observed in the presence of Fe^{2+}/H_2O_2. Furthermore, the litchi anthocyanin degradation by hydroxyl radical enhanced significantly at the 5% level with increasing Fe^{2+}/H_2O_2 concentration used in this study (Table 2). The reduced amount of litchi anthocyanin was also associated with increasing incubation time. This study indicated evidently the involvement of Fe^{2+}/H_2O_2 into the litchi anthocyanin degradation.

Reszka et al. (2004) reported that the increase in the production of oxygen radical results from involvements of phytohormone metabolism, oxidative modification and subsequent proteolytic degradation in aging and senescing leaves while the degradation of fruit pigments could depend largely on their structural modifications caused by ROS (Perez-Galvez & Minguez-Mosquera, 2001). Merzlyak and Hendry (1994) found that the oxidation of pyocyanin as one of anthocyanins involved an extensive modification of the pigment's phenazine chromophore. Es-Safi et al. (2008) reported the formation of new compounds obtained by oxidation of malvidin 3-O-glucoside by hydroxyl radical. This study suggested that the degradation of litchi anthocyanin by hydroxyl radical which could from the oxidation of endogenous phenolics by PPO could participate in enzymatic browning reaction of harvested litchi fruit during storage. Further investigations into the structural modification of litchi anthocyanin caused by hydroxyl radical in relation to the oxidation of endogenous phenolics by PPO are needed to elucidate well the pericarp browning of the fruit.

4. Conclusions

The obvious pericarp browning was associated with the rapid increases in H_2O_2 and ·OH contents of litchi fruit after 4 days of storage. Litchi anthocyanin can be degraded markedly in the presence of ROS, particularly hydroxyl radical. HPLC analysis further confirmed the involvement of hydroxyl radical into the litchi anthocyanin degradation. This study suggested that the degradation of litchi anthocyanin by ROS which could from the oxidation of endogenous phenolics by PPO could participate in the enzymatic browning reaction of harvested litchi fruit during storage but it requires to be investigated further.

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