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Antioxidant activities and contents of polyphenol oxidase substrates from pericarp tissues of litchi fruit

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ABSTRACT

The experiments were performed to extract and purify substrates for polyphenol oxidase (PPO) from pericarp tissue of postharvest litchi fruit. Two purified PPO substrates were identified as (−)-epicatechin and procyanidin A2. The antioxidant properties of two PPO substrates were further evaluated in the present study. Variation in the content of the major substrate (−)-epicatechin of litchi fruit during storage at 25 °C was analysed using the HPLC-UV method. The results showed that (−)-epicatechin exhibited stronger antioxidant capability than procyanidin A2, in terms of reducing power and scavenging activities of DPPH radical, hydroxyl radical and superoxide radical. Furthermore, (−)-epicatechin content in pericarp tissue tended to decrease with increasing skin browning index of litchi fruit during storage at 25 °C. Thus, these two compounds can be used as potential antioxidants in litchi waste and the fresh pericarp tissue of litchi fruit exhibited a better utilisation value.

1. Introduction

Oxidation reaction involves the production of free radicals. Antioxidants are molecules that mainly decelerate or prevent the oxidation reaction *in vitro* and *in vivo* by terminating the oxidation chain reaction (Yanishlieva, Marinova, & Pokorny, 2006). In biological system, the oxidative stress has been associated with the pathogenesis of many diseases (Aktan, Taysi, Gumustekin, Bakan, & Sutbeyaz, 2003; Karp & Koch, 2006; Mariani, Polidori, Cherubini, & Mecocci, 2005). The application of antioxidants in pharmacology is valuable to improve current treatments for diseases. There are numerous antioxidants with varied antioxidant activity, such as glutathiones, ascorbic acid, polyphenols, carotenoids and many other phytochemicals, which contribute to maintenance of the homeostasis of redox state (Dimitrios, 2006; Seifried, 2007). Among these compounds, vitamin C was a common antioxidant present in most of fruits and vegetables, while polyphenols are an important group of antioxidants present in plants because of their diversity and extensive distribution. They possess the ability to scavenge both active oxygen species and electrophiles (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999). Recent investigations have showed that many plant polyphenols, including flavonoids, tannins and phenolic acids, exhibit a strong antioxidant activity (Heim, Tagliaferro, & Bobilya, 2002; Rakić, Povrenović, Tešević, Simić, & Maletić, 2006). Polyphenolic substances exist generally in lots of fruits (Balasundram, Sundram, & Samman, 2006), which act naturally as antioxidants and possess antioxygenic property with the advantage of low toxicity (Weiss & Landauer, 2003). Litchi pericarp tissue contains great amount of polyphenols with a strong antioxidant activity (Duan, Wu, & Jiang, 2007; Liu, Xie, et al., 2007). In previous study, the PPO substrates from pericarp tissues of litchi fruit were identified as the polyphenolic compounds (Liu, Cao, et al., 2007; Sun et al., 2006, 2007). They are usually regarded as one of the inducements of pericarp browning of litchi fruit after harvest (Jiang, Duan, Joyce, Zhang, & Li, 2004). At present, most researches mainly focus on how to prevent pericarp browning using physical or chemical methods to inhibit these phenolic substrates being catalysed into brown-coloured by-products. These studies concerning pericarp browning are necessary to extend shelf life of harvested litchi fruit. However, it would be another advantageous aspect in litchi industry to obtain the substrates with antioxidant activities from pericarp tissues of litchi fruit and transform them from browning-induced wastes into health-beneficial bioactive compounds. The objective of the present study was to evaluate the difference in antioxidant activity and content of PPO substrates from pericarp tissues of litchi fruit during storage, and then if the substrates can be used as potential antioxidants in litchi wastes.

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2. Materials and methods

2.1. Plant materials

Fruits of litchi (*Litchi chinensis* Sonn.) cv. Huaizhi, a common cultivar in markets of South China, at a mature stage were obtained from a commercial orchard in Guangzhou. The bright red fruits without disease symptoms were selected and then peeled. The fresh pericarp tissues were collected, then lyophilised in liquid nitrogen and finally stored at −20 °C until further extraction and analysis.

2.2. PPO preparation and substrate identification

The preparation of litchi PPO was performed at 4 °C. The lyophilised pericarp tissue (30 g) was ground in liquid nitrogen with a mortar and pestle. The ground powder was homogenised 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 the supernatant was collected. The enzyme solution was fractionated with sold ammonium sulphate (30–80% saturation) and the precipitate was then collected by centrifugation at 8500*g* for 15 min. The precipitate was re-dissolved in small volume of 0.01 M sodium phosphate buffer (pH 6.8). After overnight dialysis against the same buffer, the dialysed solution was collected as the crude PPO. Isolation and identification of PPO substrates were performed according to our previous method (Sun et al., 2006, 2007).

2.3. Skin browning assessment

The skin browning was assessed by measuring the extent of the total browned area on each fruit pericarp as litchi fruit were stored for 0, 2, 4, 6 and 8 days at room temperature (25 °C), using following scale (Zheng & Tian, 2006): 1 = no browning; 2 = slight browning; 3 = <1/4 browning; 4 = 1/4–1/2 browning; 5 = >1/2 browning. The browning index was calculated using the formula: Σ(browning scale × percentage of corresponding fruit within each class). Fruit evaluated at a higher index than 3.0 was considered to be unacceptable for marketing (Jiang, Li, & Li, 2004). Three replicates (20 fruits per replicate) were carried out in this study.

2.4. Variation in contents of PPO substrates

2.4.1. Sample preparation

Pericarps tissues (10 g) of litchi fruit after storage of 0, 2, 4, 6 and 8 days at 25 °C were ground into powder in liquid nitrogen, respectively. Then 50 mL of methanol containing 0.2% ascorbic acid was added and extracted for 20 min at 4 °C with continuous stirring. The extract was centrifuged for 10 min at 7000*g*, and the supernatant was then filtered quickly through a 0.45 μm membrane filter (Millex-HV, Millipore Co., Billerica, MA, USA). The filtered sample was loaded to HPLC. Each determination was carried out in triplicate.

2.4.2. HPLC analysis

The substrate was separated on a Waters 2695 HPLC system (Waters Co., Milford, MA, USA) equipped with a Waters 2487 dual wavelength absorbance detector (Waters Co.) and a Pinnacle II C_{18} column (250 × 4.6 mm, 5 μm, Restek Co., Bellafonte, PA, USA) maintained at 25 °C. The mobile phase consisted of water (solvent A), acetonitrile (solvent B) and acetic acid (solvent C). The linear gradient program was used. The elution was allowed to run for 4 min with 97% A, 1% B and 2% C, and then solvent A decreased from 97% to 48% while solvent B increased from 1% to 50% for 30 min. Solvent A further decreased from 48% to 18% while solvent B increased from 50% to 80% for 4 min. Finally, the elution system returned to 97% A, 1% B and 2% C within 3 min. The column was equilibrated prior to injection of next sample with the mobile phase containing 97% A, 1% B and 2% C for 7 min. The flow rate was 1 mL/min and the injection volume was 10 μL, while the monitoring wavelength was 280 nm. The identification of the substrate was based on a combination of retention time and spectral matching with standard compound from Sigma–Aldrich Company. The quantification was made via a calibration with standard using the external standard method. Results were expressed as mg/mL crude substrate extracts.

2.5. Assay for antioxidant activities

2.5.1. Reducing power

Reducing power was determined according to the method of Oyaizu (1986). Purified PPO substrates were prepared into solutions at 50, 100 or 250 mg/mL in ethanol. A 0.5-mL aliquot of the substrate solutions was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was then incubated at 50 °C for 20 min. After 2.5 mL of 10% trichloroacetic acid (w/v) was added into each sample, the mixture was centrifuged at 650*g* for 10 min. A 5-mL aliquot of the upper layer was mixed with 5 mL of distilled water and 1 mL of 0.1% ferric chloride. The absorbance at 700 nm was measured using UV-2802 spectrophotometer. A higher absorbance indicates a stronger reducing power. The vitamin C was used as a positive control.

2.5.2. DPPH radical-scavenging activity

The DPPH-scavenging activity was determined according to the modified method of Duan, Jiang, Su, Zhang, and Shi (2007). A 0.2-mL aliquot of the substrate solutions was added into 2.8 mL of 0.1 mM DPPH solution in ethanol. The absorbance at 517 nm of samples was measured after 30 min of incubation at 25 °C. Percent scavenging activity was expressed as $[1 - (A_i - A_j)/A_c] \times 100$, where A_i was the absorbance measured with different substrates with a free radical source, A_j was the absorbance measured with different substrates without a free radical source and A_c was the absorbance measured with different solvent solution (without any substrate).

2.5.3. Hydroxyl radical-scavenging activity

The hydroxyl radical-scavenging activity was evaluated by the modified method of Lee, Kim, Kim, and Jang (2002). A 0.3-mL aliquot of the substrate solutions was mixed with 1 mL of reaction buffer containing 100 μM $FeCl_3$, 104 μM EDTA, 2.5 mM H_2O_2, 2.5 mM deoxyribose, and 100 μM ascorbic acid. The reaction solution was incubated for 1 h at 37 °C before 1 mL of 0.5% thiobarbituric acid dissolved in 25 mM NaOH and 1 mL of 2.8% trichloroacetic acid were added into the mixture. The mixture was incubated for 30 min at 80 °C and cooled rapidly in an ice bath. The absorbance of sample was measured at 532 nm and then percent scavenging activity was calculated by the method of Lee et al. (2002).

2.5.4. Superoxide anion radical-scavenging activity

The superoxide anion-scavenging activity was measured according to the modified method of Siddhurajua, Mohanb, and Beckera (2002). A 0.2-mL aliquot of the substrate solutions was mixed with 3 mL of reaction solution containing 1.3 μM riboflavin, 13 mM methionine, 63 μM NBT, 100 μM EDTA, and 0.05 M sodium phosphate buffer (pH 7.8). The reaction solutions were incubated for 30 min under the 4000 lux illumination. The absorbance of sample was measured at 560 nm and the scavenging activity was

then calculated and expressed a percentage by the method of Siddhurajua et al. (2002).

2.6. Data analysis

All experiments were performed in triplicate ($n = 3$). Data were presented as the mean ± standard error (SE) of three replicate determinations. An ANOVA test (using SPSS 13.0 statistical software, SPSS Inc., Chicago, USA) was used. Significant differences between the means of parameters were determined using the LSD test at 5% level.

3. Results and discussion

3.1. Identification of PPO substrates

Two PPO substrates from litchi pericarp tissues according to Sun et al. (2006, 2007) were isolated and selected using 0.5% $FeCl_3$ solution and the crude PPO solution. The two substrates were snuff-coloured amorphous powder and were identified as (−)-epicatechin (S1) and procyanidin A2 (S2) by nuclear magnetic resonance (NMR) and electrospray ionisation mass spectrometry (ESI-MS) described previously in details (Sun et al., 2006, 2007). The chemical structures of S1 and S2 are shown in Fig. 1A and B.

3.2. Variations in contents of substrates from pericarp tissue of litchi fruit during storage

To quantify (−)-epicatechin (S1) present in litchi pericarp tissues, the standard (−)-epicatechin (Sigma–Aldrich Ltd., Oakville, ON, Canada) was used in the study. Under the gradient chromatographic condition, the standard of (−)-epicatechin was eluted in approximately 17.1–17.2 min. The calibration curve was linear in the range of 0.05–0.8 mg/mL, with $R^2 > 0.99$ and the calibration curve equation of $Y = 8007217.5\ X + 18722.0$. The retention time of (−)-epicatechin obtained from litchi pericarp tissue at different skin browning indexes was around 17.1 min.

The variation of (−)-epicatechin content related to skin browning index is shown in Fig. 2. On the whole, (−)-epicatechin content in pericarp tissues of litchi fruit tended to decrease during storage at room temperature. However, after the first 2 days of storage, the PPO substrate content slightly increased, which could be due to the induced synthesis of more secondary metabolites such as (−)-epicatechin when fruit detached from trees soon. On the other hand, skin browning index of litchi fruit increased gradually during storage (Fig. 2) and the fruit was unacceptable for marketing after 4 days of storage. Jiang, Duan, et al. (2004) reported that disappearance of the initial red colour and occurrence of pericarp browning of litchi fruit after harvest were mainly due to the catalysed oxidation of phenolic substrates into *o*-quinones by PPO and the further reaction of *o*-quinones with other compounds to form brown-coloured by-products. In the present study, PPO continuously catalysed oxidation of (−)-epicatechin present into *o*-quinone in litchi fruit during storage, resulting in a continual decline in (−)-epicatechin content. The decreasing trend of (−)-epicatechin content after litchi fruit browned during storage was confirmed further by the quantitative HPLC analysis (Fig. 2). Meanwhile, the continuously accumulated *o*-quinone in pericarp tissues of litchi fruit could further convert into brown-coloured by-products. As a result, (−)-epicatechin content decreased gradually as litchi fruit browned and the content of (−)-epicatechin presented a negative correlation with skin browning index.

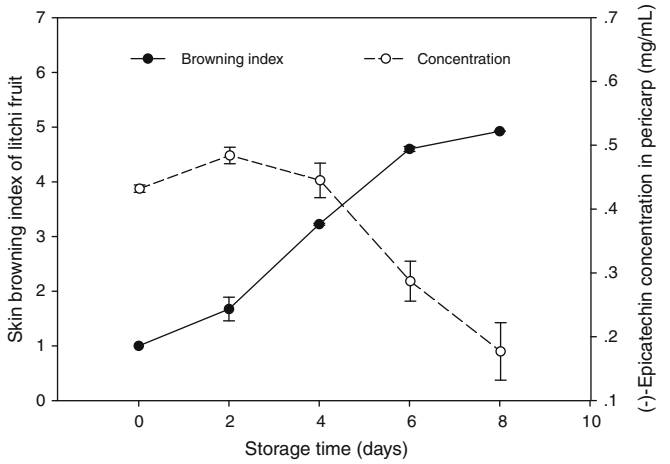

Fig. 2. Variation in content of (−)-epicatechin at different skin browning indexes of pericarp tissue of litchi fruit stored for 0, 2, 4, 6 or 8 days at 25 °C.

In litchi pericarp tissue, (−)-epicatechin was identified as the major substrate for PPO. Furthermore, two molecule of (−)-epicatechin can further polymerise into procyanidin A2 (S2) by a double

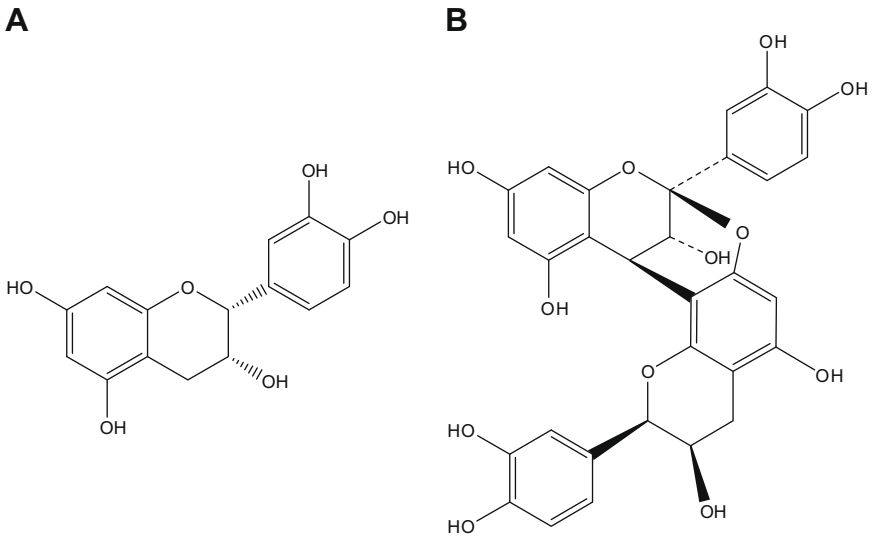

Fig. 1. Chemical structures of S1 (−)-epicatechin (A) and S2 procyanidin A2 (B).

linkage (C_2–O–C_7 and C_4–C_8) (Le Roux, Doco, Sarni-Manchado, Lozano, & Cheynier, 1998). The content of procyanidin A2 was positively related to that of (−)-epicatechin. Thus, it could be estimated that the content of procyanidin A2 in pericarp tissues of litchi fruit also exhibited a decreasing trend as storage time extended.

3.3. Antioxidant activities of PPO substrates

3.3.1. Reducing power

Reducing power may serve as a significant reflection of antioxidant activity and high reducing power indicates strong antioxidant activity (Duan, Jiang, et al., 2007). Fig. 3A shows that the reducing power of (−)-epicatechin and procyanidin A2, with linear relationships [for (−)-epicatechin, $Y = 0.0032\ X + 0.0574$, $R^2 = 0.989$; and for procyanidin A2, $Y = 0.0011\ X + 0.0431$, $R^2 = 0.9811$)] at 0–250 μg/mL. Compared with the positive control vitamin C, the two substrates possessed lower reducing power. However, reducing power of (−)-epicatechin was significantly higher than that of procyanidin A2 at the same concentration, suggesting that (−)-epicatechin exhibited a stronger electron-donating capacity than procyanidin A2.

3.3.2. DPPH radical-scavenging activity

Free radical-scavenging is one of the known mechanisms by which antioxidants inhibit lipid peroxidation (Bloknina, Virolainen, & Fagerstedt, 2003). As shown in Fig. 3B, (−)-epicatechin exhibits a stronger but procyanidin A2 presents a lower DPPH-scavenging activity than that of vitamin C. Statistical analysis further confirmed that (−)-epicatechin possessed a significantly ($P < 0.05$) higher DPPH-scavenging activity than procyanidin A2. In addition, at the range of 0–250 μg/mL, DPPH-scavenging activity was positively correlated with the substrate concentration. The corresponding correlation coefficients were determined to be 0.9042 and 0.9968 for (−)-epicatechin and procyanidin A2, respectively.

3.3.3. Hydroxyl radical-scavenging activity

Hydroxyl radical can be formed by the Fenton reaction in the presence of reduced transition metals (such as Fe^{2+}) and H_2O_2, which is known to be the most reactive of all the reduced forms of dioxygen and is thought to initiate cell damage *in vivo* (Duan, Jiang, et al., 2007; Rollet-Labelle et al., 1998). In comparison with vitamin C, (−)-epicatechin significantly ($P < 0.05$) inhibited hydroxyl radical production, but procyanidin A2 had no significantly higher ($P > 0.05$) hydroxyl radical-scavenging activity. The linear increase of the hydroxyl radical-scavenging activity with the sub-

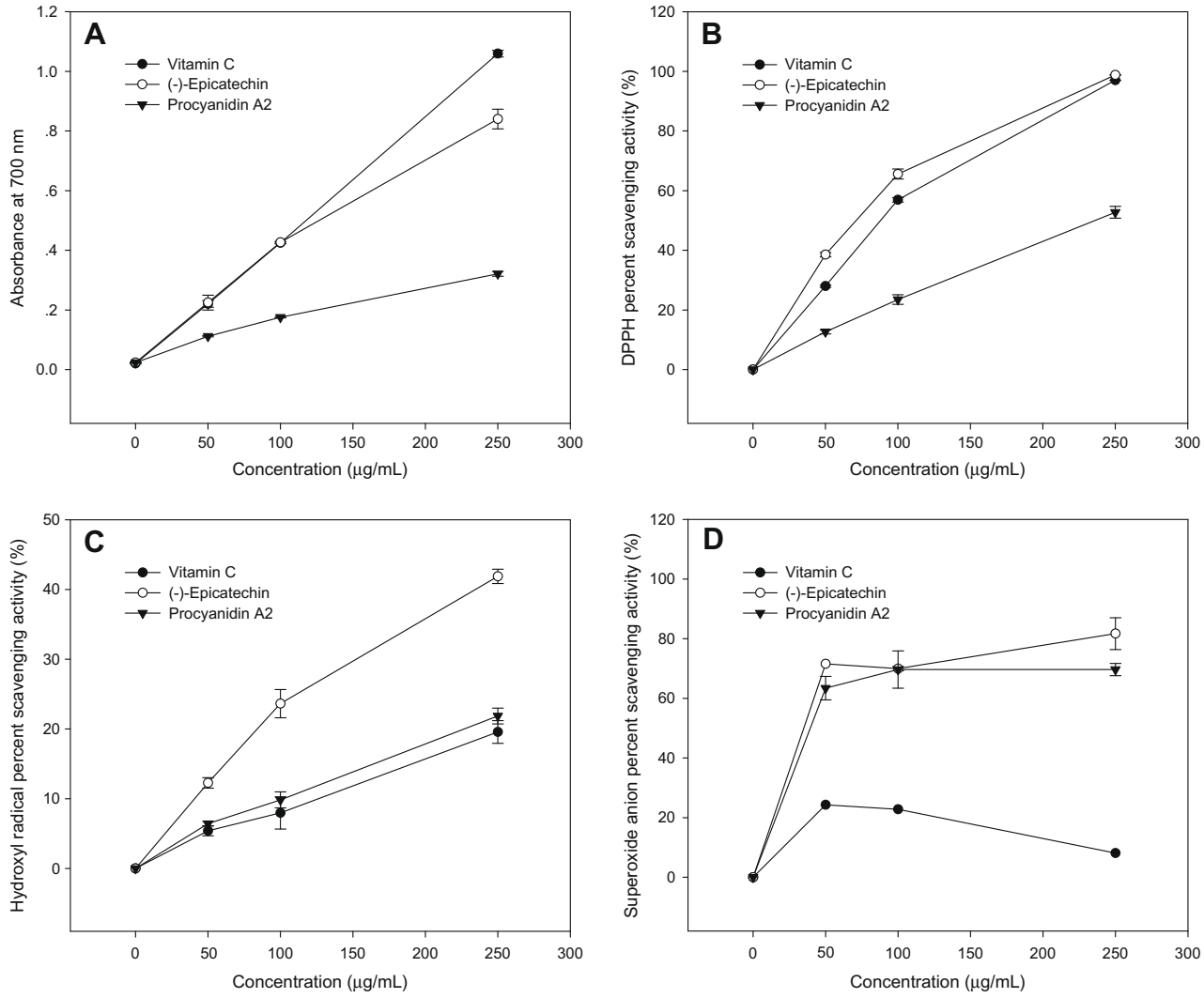

Fig. 3. Comparisons of reducing power (A), percent DPPH-scavenging activities (B), percent hydroxyl radical-scavenging activities (C) and percent superoxide anion scavenging activities (D) of S1 and S2.

strate concentration at 0–250 μg/mL is shown in Fig. 3C, with the correlation coefficients being 0.9659 and 0.9894 for (−)-epicatechin and procyanidin A2, respectively.

3.3.4. Superoxide anion radical-scavenging activity

Superoxide anion is important radical which is involved in the formation of other cell-damaging free radicals (Bloknina et al., 2003). As shown in Fig. 3D, the superoxide radical-scavenging activities of the two substrates are significantly ($P < 0.05$) higher than that of vitamin C, and (−)-epicatechin presents a better superoxide anion radical-scavenging activity than procyanidin A2. Unlike DPPH radical and hydroxyl radical-scavenging activities, the superoxide radical-scavenging activities of the two substrates had no linear increase at a concentration range of 0–250 μg/mL.

4. Conclusions

The PPO substrates present in pericarp tissue of harvested litchi fruit were selected using 0.5% $FeCl_3$ solution and crude PPO solution. The substrates were identified as (−)-epicatechin and procyanidin A2. The content of (−)-epicatechin in pericarp tissue presented a negative correlation with skin browning index after litchi fruit browned during storage at room temperature. The comparative analyses of reducing power and scavenging activities of DPPH radical, hydroxyl radical and superoxide radical showed that both litchi PPO substrates possessed a good antioxidant activity but (−)-epicatechin exhibited stronger reducing power and radical-scavenging activity than procyanidin A2. Thus, these two PPO substrates can be used as potential antioxidants in litchi waste and the fresh pericarp tissue of litchi fruit exhibited a better utilisation value.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2009.07.025.

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