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Effects of a phospholipase D inhibitor on postharvest enzymatic browning and oxidative stress of litchi fruit

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ABSTRACT

Membrane lipid degradation catalyzed by phospholipase D (PLD) results in postharvest browning and senescence of litchi fruit. The effects of *n*-butanol, a specific PLD inhibitor, on enzymatic browning and oxidative stress during storage of litchi fruit at room temperature were evaluated. *n*-Butanol-treated fruit had a lower browning index and disease index than untreated fruit. *n*-Butanol treatment also decreased PLD activity. As a result, the decompartmentalization of litchi polyphenoloxidase and substrates was reduced. The conversion of substrates (−)-epicatechin and procyanidin A_2 into quinones was slowed down and enzymatic browning of litchi pericarp tissues was lower after 6 d storage. Additionally, *n*-butanol-treated fruit possessed significantly lower malondialdehyde contents than untreated fruit after 4 d storage. Analysis of antioxidative enzyme activities showed that *n*-butanol treatment inhibited oxidative stress mainly by maintaining high catalase activity in litchi pericarp tissues. Consequently, senescence of litchi fruit during storage was moderated.

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1. Introduction

Litchi (*Litchi chinensis* Sonn.) is a non-climacteric tropical and subtropical fruit. It is widely consumed but has a very short shelf-life after harvest because of browning and senescence, which are related to enzymatic reactions of polyphenols and oxidative stress from reactive oxygen species (ROS) in pericarp tissues (Sun et al., 2010). Postharvest browning and senescence significantly reduces the commercial value of litchi fruit.

Many factors induce postharvest browning and senescence of fruit. Membrane disruption in cells has been proposed to be a key event, and deterioration of membrane integrity accelerates enzymatic browning and ROS over-production in fruit. Preservation of membrane structure is helpful in maintaining fruit quality and lengthening shelf-life. Phospholipase D (PLD) has been suggested as a key enzyme in mediating the membrane phospholipid degradation that is a rapid and early event in postharvest senescing tissues (Li et al., 2009). PLD and its catalyzed products, linolenic acid and phosphatidic acid, initiate the oxylipin pathway and cellular signal transduction, which may be involved in producing the wound signal responsible for increased wound-induced activity, accumulation of phenolics and enzymatic browning in plant tissues (Choi et al., 2005; Bargmann and Munnik, 2006). Thus the regulation of PLD activity may have an important impact in maintaining postharvest fruit quality. Chemicals such as *n*-butanol, N-acylethanolamines, lysophosphatidylethanolamine, 2,3-diphosphoglycerate and wortmannin have been reported to be PLD inhibitors (Kanaho et al., 1993; Motes et al., 2005; Peters et al., 2007). Amongst them, *n*-butanol is a specific inhibitor of PLD-dependent production of the signalling molecule phosphatidic acid. A recent study has shown that *n*-butanol, serving as a substrate for the transphosphatidylation reaction, may protect membrane integrity by inhibiting phosphatidic acid production and membrane lipid degradation (Motes et al., 2005). It is worthwhile therefore to investigate PLD inhibitors for modulating postharvest browning and senescence of litchi fruit. However, there are no reports on the effects of PLD inhibitors on harvested litchi fruit. The objective of the present study was to determine the effects of PLD inhibitor *n*-butanol on enzymatic browning and oxidative stress of harvested litchi fruit. The results provide information useful for maintaining postharvest quality and extending storage life of litchi fruit.

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2. Materials and methods

2.1. Plant materials and treatments

Fruit of litchi (*Litchi chinensis* Sonn.) cv. Feizixiao, a common cultivar in South China, were harvested at commercial maturity from an orchard in Qinzhou, Guangxi province, during June 2009. The healthy fruit of uniform size and color were selected, washed, and then dipped in 0.1% (v/v) aqueous solution of *n*-butanol (Kelong Chemical Reagent Factory, Chengdu, China) for 10 min at room temperature (25 °C). Fruit dipped in water at 25 °C were used as controls. After air-drying, triplicate samples of 20 fruit were packed into polyethylene bags (0.03 mm thick) and stored at 25 °C for 0, 2, 4 and 6 d. Litchi pericarp tissues were collected every 2 d, lyophilized in liquid nitrogen and then stored at −20 °C until further analysis.

2.2. Browning index and disease index

A pericarp browning index was determined as follows (Jiang, 2000): 1, no browning (excellent quality); 2, slight browning; 3, <1/4 browning; 4, 1/4–1/2 browning and 5, >1/2 browning (poor quality). The browning index was calculated as Σ(browning scale × percentage of the corresponding fruit within each class). Fruit evaluated at a higher index than 3.0 was considered to be unacceptable for marketing (Jiang et al., 2004). Three replicates were carried out in this study.

The development of disease resulting from natural infection was assessed by observing visible fungal growth or bacterial lesion on the fruit surface, according to the following scale (Wu et al., 2006): 0, no lesion; 1, slight lesion; 2, lesion area < 1/4; 3, lesion area 1/4−1/2 and 4, lesion area > 1/2. The disease index was assessed by measuring lesion area on each fruit pericarp. The disease index was calculated as Σ(disease scale × number of fruit within the corresponding scale)/(total number of fruit × maximum severity scale) × 100%.

2.3. PLD activity

PLD was extracted according to the modified methods of Yi et al. (2008). At 4 °C, litchi pericarp tissues were ground into powder in liquid nitrogen using a mortar and pestle. The powder (0.5 g) was extracted for 30 min with 2.5 mL of 0.1 M Tris–HCl buffer (pH 7.0). The extracts were centrifuged at 13,000 × *g* for 15 min. The supernatant was used as a crude enzyme for PLD activity measurement. PLD activity was assayed according to the modified methods of Suttle and Kende (1980). The reaction substrate was prepared by adding 0.05 g of phosphatidylcholine (lecithin) into 3 mL of chloroform and 3 mL of distilled water. Then the solution was evaporated to dryness in a N-1000 rotary evaporator (EYELA, Tokyo, Japan) at 40 °C and again dissolved in 250 mL of 100 mM acetate acid buffer (pH 5.5, containing 5 mM dithiothreitol and 25 mM $CaCl_2$). For PLD assay, 1 mL of the crude enzyme extract and 3 mL of the reaction substrate was vigorously shaken for 1 h at 28 °C and then washed with petroleum ether. The water layer was collected and 2 g of Reinecke salt (ammonium tetrarhodanatodiammonchromate) in 100 mL of methanol was added into the reaction solution to obtain a precipitate. The precipitate was collected by centrifugation at 26,000 × *g* and re-dissolved in 3 mL of acetone. The absorbance of the clear supernatant was recorded at 520 nm using a UV–visible spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). A standard curve for choline was made by diluting 20 mg of choline chloride in 100 mL of 100 mM acetate buffer (pH 5.6). One unit of PLD activity was defined as micromoles of choline per minute on fresh weight (FW) basis.

2.4. Effects on enzymatic browning

2.4.1. Activities of browning-related enzymes

Polyphenoloxidase (PPO) and phenylalanine ammonialyase (PAL) are crucial enzymes closely related to enzymatic browning of postharvest litchi fruit. PPO was extracted according to the modified methods of Jiang (2000). At 4 °C, litchi pericarp powder (0.5 g) was extracted for 10 min with 2.5 mL of 0.1 M sodium phosphate buffer (pH 6.8) containing 0.1% (w/v) polyvinyl pyrrolidone. The extract solution was centrifuged for 15 min at 12,000 × *g*. The supernatant was collected for the determination of PPO activity. PPO activity was assayed with catechol as a substrate according to a spectrophotometric procedure. The enzyme solution (0.1 mL) was rapidly added to 2.9 mL of 10 mM catechol prepared in 0.01 M sodium phosphate buffer (pH 6.8). The increase in absorbance at 400 nm was recorded for 3 min at 25 °C. One unit of PPO activity was defined as the amount of the enzyme that causes a change of 0.001 in absorbance per minute. PAL was extracted according to the modified methods of Lister et al. (1996). At 4 °C, litchi pericarp powder (0.5 g) was extracted for 10 min with 2.5 mL of 0.05 M sodium borate buffer (pH 8.8) containing 5 mM mercaptoethanol and 0.05 g of polyvinyl pyrrolidone. The enzyme extract was centrifuged at 12,000 × *g* for 15 min. The supernatant was collected and used for the determination of PAL activity. The reaction mixture consisted of 1.9 mL of 0.1 M sodium borate buffer (pH 8.8), 1 mL of 20 mM L-phenylalanine and 0.1 mL of enzyme solution, with a final volume of 3 mL. Enzyme samples were incubated for 1 h at 37 °C. In the control sample, the enzymatic extract was replaced by 1 mL of borate buffer. The reaction was stopped by the addition of 0.2 mL of 6 M trichloroacetic acid. One unit of PAL activity was defined as the amount of the enzyme that caused a change of 0.01 in absorbance at 290 nm per hour.

2.4.2. Contents of total phenolics and browning substrates

Litchi pericarp powder (0.5 g) was extracted for 30 min at 4 °C with 2.5 mL of methanol/acetone/water (3.5:3.5:3, v/v/v) containing 1% (v/v) formic acid in a Thermo Forma Orbital shaker (Thermo Fisher Scientific Inc., Waltham, MA, USA). The extract was centrifuged for 15 min at 2,000 × *g*. The supernatant was collected and filtered through the 0.45 μm membrane filter (Millex-HV, Millipore Co., Billerica, MA, USA). The filtrate was used as sample to determine contents of total phenolics and browning substrates.

Total phenolics content was measured using the Folin–Ciocalteau method described by Singleton and Rossi (1965) with some modifications. At 25 °C, a 10-fold diluted sample (1 mL) was thoroughly mixed with 1 mL of Folin–Ciocalteau reagent (Sigma–Aldrich Co., St. Louis, MO, USA) and set for 3 min. Then 3 mL of sodium carbonate (75 g L^{-1}) was added into the mixture and allowed to stand for 1 h. Sample aliquots were centrifuged for 10 min at 10,000 × *g* and the supernatant was collected for the determination of total phenolics content using a spectrophotometer, at 760 nm. Total phenolics content was standardized against gallic acid and expressed as microgram per millilitre of gallic acid equivalents (GAE) on FW basis.

The contents of enzymatic browning substrates were analyzed according to the modified method of Sun et al. (2009). The high-performance liquid chromatography (HPLC) analysis was performed on the Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA, USA) equipped with a Venus C_{18} column (250 × 4.6 mm, 5 μm particle size; Dalian Create Science and Technology, Dalian, China) and a UV detector (Dionex). Chromeleon chromatography management software (Dionex) was used for data recording and processing. The mobile phase was (A) acidic water (2% acetic acid, v/v) and (B) acetonitrile-methanol (10:15, v/v). The gradient elution was as follows: at 0 min 90% A, at 10 min 80% A, at 15 min 70% A, at 25 min 60% A, at 30 min 50% A, at 40 min 50% A, at 45 min 90% A

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