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The effect of different temperatures on browning incidence and phenol compound metabolism in fresh-cut lotus (*Nelumbo nucifera* G.) root

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ABSTRACT

Lotus root (*Nelumbo nucifera* G.) is an important aquatic vegetable in China. Browning easily during the processing and storage reduces the value of lotus root. Therefore, browning is an important issue for the lotus root industry. *PAL*, *PPO* and *POD* genes were reported as being involved in fruit and vegetable tissue browning, and PAL, PPO and POD enzymes have long been associated with lotus root browning. However, the molecular mechanism of *PAL*, *PPO* and *POD* involved in fresh-cut lotus root browning is poorly understood. This paper analyses the effect of different temperatures on the phenolic content, browning, PPO, PAL and POD enzyme activity and the expression pattern of *PPO*, *PAL* and *POD* genes in fresh-cut lotus root. The results show that the change of PAL, PPO and POD enzymatic activity occurs in parallel with the increase in browning degree in storage at different temperatures. Furthermore, the total phenol content and PAL enzyme activity changes are basically identical, suggesting that PAL might be the key genes involved in the synthesis of total phenol content. Two *NnPAL*, 2 *NnPPO* and 7 *NnPOD* genes were isolated using RNA-seq, and the upregulation of *NnPAL1*, *NnPPOA*, *NnPOD1*, *NnPOD2*, *NnPOD3*, *NnPOD4*, *NnPOD5* and *NnPOD6* by high temperature coincided with the increase in related enzyme activities and the browning degree of fresh-cut lotus root, which should be considered the most important candidates for fresh-cut lotus root browning.

1. Introduction

Fresh-cut fruits and vegetables have been in high demand worldwide in recent years because of their convenient and highly edible (Shah and Nirankar, 2006). As a kind of aquatic and rhizome vegetable, recently, fresh-cut lotus root slices have risen in popularity and received increasing attention as a novel minimally processed vegetable (Zhang et al., 2013). Lotus root (*Nelumbo nucifera* G.) is widely planted in China and contains a lot of nutrition such as starch, sugars, proteins (arginine, tyrosine and another 17 kinds of amino acids), fat, lecithin, alkaloids, flavonoids, carotene, riboflavin (vitamin B2), niacin, vitamin C, vitamin B6, thiamine, copper, manganese, titanium, phosphorus, chlorogenic acid and other phenolic compounds (Chiang and Luo, 2007). However, it is well known that the processing of fresh-cut fruits and vegetables promotes a faster physiological deterioration, biochemical changes and microbial degradation of the product, which may result in degradation of the colour, texture and flavour (Toivonen and Brummell, 2008). Browning is the most important problem for fresh-cut lotus root during storage and processing, and it will limit the development of the lotus root industry (Jiang et al., 2014).

Fruit and vegetable browning is the process of coloured quinine formation, in which the polyphenols are oxidized by polyphenol oxidase (PPO) and peroxidase (POD) (Toivonen and Brummell, 2008; Artés et al., 2007). *PAL*, *PPO* and *POD* were reported as multi-gene families in previous research, and they were involved in the tissue browning in many species, such as apples (Murata et al., 2001), pineapples (Zhou et al., 2003), sweet potatoes (Liao et al., 2006), pears (Yan et al., 2013; Cheng et al., 2015) and cabbage (Banerjee et al., 2015).

PAL, PPO and POD enzymes have been associated with lotus root browning. Slicing induces higher PAL activity and parallel more total phenol accumulation after 7 d' storage compared to control (Hu et al., 2014). Similar changes in POD activities have also been observed during lotus root cv. 3537 browning, and browning

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degree was significantly positively correlated with POD activity during storage (Jiang et al., 2014). And PPO and POD activities in fresh-cut lotus root slices were also significantly inhibited by H_2S treatment inhibiting the browning of fresh-cut lotus root slices (Sun et al., 2015). However, only 2 papers have reported on PPO gene cloning and expression analysis in lotus root (Dong et al., 2006; Zhang et al., 2011). However, the molecular mechanism of *PAL*, *PPO* and *POD* involved in fresh-cut lotus root browning is poorly understood.

The primary purpose of this study is to analyze the effect of different temperatures on the phenolic content, browning, PPO, PAL and POD enzyme activity and the expression pattern of *PPO*, *PAL* and *POD* genes in fresh-cut lotus root and further to clarify the biochemical and molecular mechanism of fresh-cut lotus root browning during different temperatures storage.

2. Materials and methods

Lotus roots (E Lian 6) were harvested form a commercial pond (Caidian, Wuhan) in 2015 and then transported to the laboratory. They were selected for their uniformity of size and ground colour, as well as freedom from defects and mechanical damage. Before treatment, the lotus roots were stored at 4 °C for 24 h. They were washed with tap water to remove soil and peeled. Then they were cut into 5 mm-thick slices along the cross section of the lotus root using a sharp stainless steel knife. After being transferred to clean water, and soaked for two minutes, these fresh-cut lotus root slices were dried with filter paper and packed in fruit and vegetable boxes (18.8 cm * 12 cm * 1.5 cm) (MS-08, China Shanghai), with two pieces in each box and transferred to the refrigerator (4 °C, 10 °C, 15 °C, 20 °C, respectively) with 60%–80% relative humidity.

All of the treatments were performed with three replicates. The samples were frozen in liquid nitrogen and stored at −80 °C for further use.

2.1. Browning degree

The extraction and determination of degree of browning were carried out according to the Hu et al. (2014), with some modifications. Flesh tissue of 3.0 g was homogenized with 30 ml distilled water at 4 °C, then centrifuged at 10,000*g* for five minutes at 4 °C. The supernatant was collected and incubated in a 25 °C water bath for five minutes. The absorbance was measured using a spectrophotometer (GL-20G-II, Shanghai Anting) at 410 nm and browning degree was expressed as A410 × 10.

2.2. Total phenolic content

The total phenolic content was measured according to the Folin-Ciocalteu procedure (Guo et al., 2012). A 3.0 g fresh sample of flesh tissues was homogenized with 30 mL of 60% ethanol, and centrifuged at 10,000 r/min for 5 min at 4 °C (GL-20G-II, Shanghai Anting). The supernatants (10 mL) were diluted with 40 mL of 60% ethanol for the next measurement. 0.125 mL of the solution and 0.625 mL distilled H_2O were mixed, then 0.125 mL Folin-Ciocalteu reagent added. After thoroughly mixing, the mixture was held for 3 min at room temperature, and then made up with 1.25 mL 7% $NaCO_3$ and 1.0 mL distilled H_2O. After incubation for 90 min at room temperature in the dark, the solution was measured using a spectrometer (V-1100D, Shanghai, MAPADA) by reading absorbance at 760 nm. A standard curve for gallic acid was used to quantify the total phenolic content. The results were expressed as gallic acid equivalents per kg of fresh weight (mg kg^{-1}), and all treatments were performed with three biological replicates.

2.3. PAL activity

The extraction and assay of PAL activity were performed as described using a Phenylalnine Ammonialyase Kit (Nan jing jiancheng Bioengineering Institute). 0.1 g of flesh tissue was homogenized in 1 mL of extracting solution in ice mortar, and the solution was then centrifuged at 10,000*g*/min for 10 min at 4 °C. The supernatant was collected as a crude PAL extract. The reaction mixture consisted of 20 ul crude PAL extract, 780 ul reagent 1 (the blank group for 800 ul) and 200 ul reagent 2, Afterwards, the mixture was incubated at 30 °C for 30 min, and 40 ul reagent 3 was added into the above mixture and left to stand for 10 mins. The absorbance value of the sample at 290 nm was measured. An enzyme activity unit (U) was defined spectrophotometrically as a change of 0.1 in absorbance per minute per gram fresh weight.

2.4. PPO activity

The extraction and assay of PPO activity were performed as described by Serradell et al. (2000). 3.0 g of flesh tissue was homogenized in 50 mL of phosphate buffer (PBS 0.05 mol/L, pH 7.0) in ice mortar, and the solution was then centrifuged at 6000 r/min for 15 min at 4 °C. The supernatant was collected as a crude PPO extract. The reaction mixture consisted of 1.0 mL 0.1 mol/L catechol and 1.5 mL 0.05 mol/L phosphate buffer (pH 7.0). Afterwards the mixture was incubated at 35 °C for 5 min, 1.0 mL crude PPO was added into the above mixture, and the mixture of changes in the absorbance at 420 nm was measured. One unit (U) of PPO activity was defined as a change of 0.001 at 420 nm in the absorbance per min.

2.5. POD activity

POD activity was extracted and assayed spectrophotometrically according to the modified method based on Wang et al. (2009). 5.0 g mortar of fresh-cut lotus root was homogenized with 5.0 mL of 0.2 mol/L sodium phosphate buffer (pH 7.0, 1 mmol/L PEG, 4% (W/V) PVPP, 1%(W/V)Triton X-100)in an ice bath. The homogenates were then centrifuged at 12,000 r/min for 30 min at 4 °C. The supernatant was collected for the POD activity assay.

The reaction cuvette contained 3.0 mL 25 mmol/L guaiacol, 0.5 mL of the enzyme solution (the blank group for sodium phosphate buffer) and 0.2 mL 5 mol/L dissolved hydrogen peroxide. The change of the mixtion in absorbance at 470 nm was recorded once every 10 s. An enzyme activity unit (U) was defined spectrophotometrically as an increase of 0.01 in absorbance per minute per gram fresh weight.

2.6. RNA extraction and cDNA synthesis

Total RNA was prepared according to the method used previously (Min et al., 2015). Traces of contaminating genomic DNA in total RNA was removed with TURBO Dnase (Ambion). 1.0 μg DNA-free RNA was used for cDNA synthesis using an iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocol. For each sampling point, three biological replicates were used for RNA extraction.

2.7. Gene isolation and sequence analysis

The *PAL*, *PPO* and *POD* genes were isolated based on RNA-Seq and NCBI (http://www.ncbi.nlm.nih.gov/). RNA-Seq was performed by the Beijing Genome Institute (BGI) (Shenzhen, China) according to our previous paper (Min et al., 2014). The PAL, PPO and POD genes sequence found in the lotus root database available in NCBI were referenced using for primer design for the gene isolation