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Identification and characterization of serotonin as an anti-browning compound of apple and pear

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ABSTRACT

Serotonin (5-hydroxytryptamine) is an indoleamine with important physiological functions in plants, including developmental processes and stress responses. In this study, a simple and rapid bioassay was developed to investigate the inhibition of the fruit browning enzyme, polyphenol oxidase (PPO) via serotonin, employing cut apple discs as well as apple tissue homogenate treated with serotonin. The present study demonstrated that serotonin is significantly effective in inhibiting browning compared to ascorbic acid. Serotonin reduced fruit browning in tissues as well as suppressed the activity of PPO enzyme in the browning model solutions containing epicatechin (Epi) and caffeoyl tartrate (CT) as polyphenol substrates. The determination of enzyme kinetic parameters also suggested that serotonin may act as a putative, non-competitive inhibitor of PPO. Apart from its anti-browning effect, the application of serotonin significantly increased total phenolics content and antioxidant capacity when applied to the apple homogenate. Furthermore, the analysis of *MdPPOs* showed that five (*MdPPO2, MdPPO4, MdPPO6, MdPPO8 and MdPPO10*) out of the nine *MdPPOs* expressed in apple fruits were down-regulated in apple tissues treated with serotonin. To the best of our knowledge, this is the first report documenting a role for serotonin in the biochemical and transcriptional regulation of PPO enzymes and a simple system to study the biochemical control of browning in pome fruits.

1. Introduction

Enzymatic browning in fruits and vegetables causes significant losses due to its negative effects on color, taste, flavor, and nutritional value (Take et al., 2012; Ioannou and Ghoul, 2013). Although phenolic compounds are involved in the development of color, taste and flavour of many fruits and beverages, the polyphenols such chlorogenic acid, caffeic acid and epicatechin (Epi) are oxidized to quinones by polyphenol oxidase (PPO). These quinones react with each other and surrounding proteins leading to the formation of melanin, a black pigment which results in fruit browning. Thus, inactivating the PPO enzyme through suppression of oxidation reaction offers an opportunity to assess potential application of compounds with antioxidant properties in reducing browning of tissues (He et al., 2008). In intact plant cells, oxidation of polyphenols does not take place because phenolics and PPOs are located in different cellular compartments, but in damaged or cut fruits, PPO and phenolics come into contact with each other resulting in browning (Vaughn and Duke, 1984). PPO catalyses two basic reactions viz., hydroxylation and oxidation as both reactions utilize molecular oxygen (air) as a co-substrate (Toivonen and Brummell, 2008). Therefore, ascorbic acid is widely used as a commercial anti-browning compound because of its anti-oxidation activity (Rupasinghe et al., 2005).

Although functions of *PPO* genes are mostly associated with defense against herbivore feeding or pathogen attack, expression profiles of *PPO* genes in tomato and poplar have showed that most *PPO* genes are developmentally regulated (Constabel and Ryan, 1998; Thipyapong et al., 1997; Tran and Constabel, 2011). These report that molecules and/or pathways with dual role in stress-alleviation and development might be involved in the regulation of *PPO* genes. Serotonin has been implicated in diverse physiological functions in plants (Ramakrishna et al., 2011). Serotonin acts as a growth regulator in a range of responses including organogenesis

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and stimulation of root growth (Murch et al., 2001; Csaba and Pal, 1982; Pelagio-Flores et al., 2011), pollen growth (Roshchina and Melnikova, 1998) and seed germination (Roshchina, 2001), flowering and senescence (Kang et al., 2009).

Recently, Takahashi and Miyazawa (2011) identified antioxidant and tyrosinase inhibition activities of serotonin derivatives. Endogenous presence of serotonin has been reported in several different plants and its cyto-protective role due to its anti-oxidant ability has been suggested (Mukherjee et al., 2014; Ramakrishna et al., 2012; Kang et al., 2007; Murch et al., 2009; Hotta et al., 2002; Tan et al., 2007). However, the use of serotonin as an effective inhibitor of fruit browning has not been examined. In the present study, we investigated the role of serotonin application in reducing pome fruit browning, and inhibiting PPO enzymes activity biochemically and transcriptionally. The findings of the present study demonstrate the novel role of serotonin as an anti-browning agent reducing browning in apple and pear fruits using an efficient method to determine PPO enzyme inhibition.

2. Materials and methods

2.1. Preparation and treatment of apple and pear discs

Peeled and sliced (2 cm diameter with 3–5 mm thickness) apple cv. 'Gala' and 'Red Delicious', and pear cv. 'Bosc', were prepared using a cork borer and immersed immediately in 100 ml of one of the following freshly prepared solutions: serotonin (0, 100, 500, 1000 μM), ascorbic acid (0, 100, 500, 1000 μM) or water (control) for 30 min at room temperature. After the treatment, the discs were placed on paper towels and kept at room temperature exposed to air for evaluating fruit browning upon exposure to air. Browning of the exposed discs was measured using CR-400Chroma meter (Konica Minolta Sensing Americas Inc., NJ, USA) at every 30 min interval for 2 h. The changes in tissue lightness, ($\Delta L = L_{initial} - L_{at30,60,90or120min}$) was used to quantify the browning of fruit discs. The experiment was setup using five fruits of each cultivar of apple and pear. For each treatment, two discs from each of the five fruits were used (i.e. a total of 10 discs per treatment with 2 discs per treatment per fruit from each of the five fruits of each cultivar).

To further test whether the role of serotonin in reducing browning is due to its color reducing properties, six concentrations of serotonin (188–6000 μM) were applied to the apple homogenate of 'Gala' before (pre-treatment) the color development and 2 h after the color development. The results were compared to control (no serotonin added) after the storage time of 2 h at room temperature (Fig. S1).

2.2. Preparation and treatment of apple juice

To measure the browning of fruit homogenate after different treatments, small pieces of peeled apple (cv. Gala) were ground in a blender at a slow speed for 1 min. The homogenate was filtered through 100 micron nylon mesh and 800 μL of this filtrate was transferred immediately into a 2 ml microfuge tube containing 200 μL of treatment solution. The treatment solutions included aqueous solutions of serotonin and ascorbic acid each at the final concentrations of 0 (water control), 188, 375, 750, 1500, 3000 and 6000 μM. Samples were then incubated at room temperature for 1 h.

Subsequently, one ml of 70% Methanol was added to each tube and the tubes were centrifuged at 10,000 g for 5 min. Browning of the treated juice was evaluated by measuring the absorbance of the supernatant at 420 nm (three technical replicates, of 200 μL each) using a flat bottom microplate (Costar 9017, Corning Inc., NY) and a microplate reader (Synergy H1 hybrid reader, BioTek Inc. VT, USA). Browning units were calculated for each sample according to the method of Chen et al. (2000), where 1 browning unit is equivalent to the difference of 0.01 absorbance unit from control per gram homogenate. Browning index for each sample was calculated by dividing absolute value with 0.01.

2.3. Model solutions to test inhibitory effects of serotonin on PPO activity

The model solutions to test the inhibitory effects of serotonin on browning were prepared as described by Oszmianskii and Lee (1990). Briefly, eight different concentrations of serotonin (0, 200, 390, 780, 1560, 3130, 6250, 12,500) in 2.5 g L^{-1} potassium bitartrate solution (pH 3.65), were added to 1 ml of 2 mM caffeoyl tartrate (CT) or Epi in a potassium bitartrate solution (pH 3.65), followed by the addition of 50 μL of PPO (0.5 mg/mL) to this solution. The rate of browning was also measured in triplicate at 420 nm using a microplate reader (Synergy H1 hybrid reader, BioTek Inc., VT, USA).

The percentage inhibition of PPO activity by treatment from control was calculated using the formula

$$\%\ \text{Inhibitation} = \frac{A\ 420\ \text{nm Control} - A\ 420\ \text{nm Treatment}}{A\ 420\ \text{nm Control}} \times 100$$

2.4. Kinetics of inhibitory effect of serotonin on PPO–Epi system

Two levels of serotonin (0 and 700 μM) were added to each of five different concentrations of Epi (375, 750, 1250, 2500 and 5000 μM) in potassium bitartrate solution. The rate of browning was measured at 420 nm using a microplate reader for 200 s after initiating the reaction with the addition of 50 μL of PPO enzyme. The results are expressed as V_{max} (AU/min), K_m (μM) and their ratio (V_{max}/K_m).

2.5. Total phenol content

Total phenol content was estimated using a modified fast blue BB method (Medina, 2011). Briefly, 20 μL of 0.1% fast blue BB was added to 200 μL of sample or gallic acid standard in each well of a flat bottom microplate (Costar 9017) and mixed manually for a minute. Ten microliter of 5% NaOH was added in each well of the microplate to initiate the reaction. The reaction ran for 10 min at room temperature and the absorbance at 420 nm was measured for each sample using a microplate reader. Gallic acid standards (0, 10, 20, 40, 80, 160 and 320 μg/ml) were analyzed in triplicate on each microplate along with the samples (1:25, v/v) in triplicate. Calculation of the total phenolic contents was made from gallic acid standard curves ($R^2 > 0.98$). Results are expressed as gallic acid equivalents (GAE mg g^{-1}) and presented as means ± standard error of three replications.

2.6. Antioxidant potential by DPPH assay

The average antioxidant potential of 'Gala' apple homogenate extract treated with 5 mM solutions of serotonin and ascorbic acid was estimated using a 96-well plate assay similar to the method previously proposed (Fukumoto and Mazza, 2000; Herald et al., 2012). Briefly, 25 μL of the extract sample or standard was transferred to individual wells of a flat bottom microplate (Costar 9017) to which 200 μL of 150 μM 2,2-diphenyl-1-picrylhydrazyl (DPPH) in 80% Methanol were added. The microplate was incubated in the dark for 60 min at room temperature followed by measuring the absorbance at 517 nm using a microplate reader. Trolox standards (0, 62.5, 125, 250, 500 μM) were assayed on each microplate along with the samples in triplicate. The absorbance of each sample (25 μL), with 200 μL of methanol without DPPH, was recorded in triplicate as sample blanks to eliminate interference