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Differential latency toward (−)-epicatechin and catechol mediated by avocado mesocarp polyphenol oxidase (PPO)

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ABSTRACT

The polyphenol oxidase (PPO) metabolism of the endogenous substrate (−)-epicatechin has been correlated with browning in avocados and other fruit. Although total PPO activity declined during ripening in the avocado mesocarp, a selective retention of high electrophoretic mobility isoforms was observed during ripening. Avocado PPO was virtually inactive toward (−)-epicatechin without the addition of an activator such as sodium dodecyl sulfate. In contrast, PPO was active toward catechol, an exogenous substrate, in the absence of an activator. Low concentrations of the free fatty acids oleic acid, linoleic acid, and arachidonic acid were effective activators of (−)-epicatechin oxidation in vitro. The data suggest that these endogenous free fatty acids may play a role in the PPO-mediated browning of avocado fruit in vivo.

1. Introduction

Many factors have been implicated in the postharvest oxidative browning of fruits and vegetables, and among the most highly studied are a group of enzymes known as polyphenol oxidases (PPO). These are copper-containing enzymes found in all angiosperms that oxidize a variety of phenolic compounds and whose role in browning is well established (Mayer, 2006). The most widely studied PPOs are catechol oxidases that oxidize ortho-diphenols to highly reactive quinones. These quinones often continue reacting non-enzymatically, leading to the formation of melanin-like polymers and discolored tissue, which is one of the main causes of quality loss in fresh and processed fruits and vegetables (Mayer and Harel, 1991; Pourcel et al., 2007).

In the fruit of avocado (*Persea americana* Mill.), PPO activity has been found to correlate with the postharvest browning that frequently occurs after shipment and storage (Golan et al., 1977a; Van Lelyveld et al., 1984). Hershkovitz et al. (2005) identified a strong correlation between an increase in PPO activity and the mesocarp browning that resulted from chilling injury.

The mesocarp of avocado contains different forms of catechol oxidase with varying substrate specificities (Dizik and Knapp, 1970; Kahn, 1976; Van Lelyveld et al., 1984). In many plant tissues, catechol oxidase exists in a latent state and requires activation by anionic detergents, fatty acids, acid/base shock, or treatment with proteases to produce measurable activity (Mayer, 2006; Pourcel et al., 2007). Sodium dodecyl sulfate has been commonly used to activate avocado PPO (Kahn, 1977; Van Lelyveld et al., 1984).

Most of the research on avocado catechol oxidase has been conducted using 4-methylcatechol as a substrate. However, in vivo substrates for catechol oxidase are commonly the flavan-3-ols, particularly (+)-catechin and (−)-epicatechin. These phenolic compounds are present in plants both as monomers and as the structural units of proanthocyanidins (condensed tannins). The catechins are considered to be important contributors to the process of browning in fruit (Bajaj et al., 1997; Liu et al., 2010; Robards et al., 1999). The oxidation of (−)-epicatechin has been found to be a precursor to the browning of litchi fruit (Liu et al., 2010). The quinones produced by (−)-epicatechin oxidase accelerated the oxidation of other flavonols in the pericarp, thus bringing about postharvest browning in this fruit. In avocados, several researchers have shown that (−)-epicatechin is an important substrate for browning in this fruit as well (Prabha and Patwardhan, 1980; Van Lelyveld et al., 1984).

(−)-Epicatechin metabolism in avocado is of particular interest because of its association with resistance to postharvest decay by the avocado anthracnose fungus *Colletotrichum gloeosporoides* (Prusky and Lichter, 2007). A reduction in (−)-epicatechin levels of the fruit during ripening coincides with a concomitant increase in the susceptibility of the fruit to this ubiquitous pathogen. It has

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been proposed that (−)-epicatechin indirectly regulates levels of an antifungal compound that decreases during ripening. It is thought to do so by inhibiting the avocado lipoxygenase that degrades the antifungal compound. Thus, as (−)-epicatechin levels decrease during ripening, the lipoxygenase is able to detoxify the compound, and the fruit becomes more susceptible to decay.

A number of researchers had shown that that the specific activity of avocado PPO decreased significantly during ripening using 4-methylcatechol as a substrate (Golan et al., 1977a; Kahn, 1977; Prabha and Patwardhan, 1980). A decrease in (−)-epicatechin levels during ripening suggested that PPO activity could be metabolizing this flavonol and degrading it during the ripening process. Thus, we compared the activity of avocado fruit PPO toward (−)-epicatechin in unripe and ripe fruit to determine if there were changes in the metabolism of this compound during the ripening process.

(−)-Epicatechin metabolism has traditionally been considered to have been mediated by PPO (Nicolas et al., 1994). However, more recent work has shown that it can also be oxidized by *Arabidopsis thaliana* laccase (Pourcel et al., 2007) and by a fungal laccase from *C. gloeosporoides* in infected avocado fruit (Prusky and Lichter, 2007). In addition, horseradish peroxidase has also been shown to oxidize (−)-epicatechin and (+)-epicatechin (Nagarajan et al., 2008). Thus, another goal of our research was to determine if the oxidation of (−)-epicatechin in avocado fruit was being mediated by PPO rather than another type of oxidase.

2. Materials and methods

2.1. Chemicals and experimental material

Chemicals were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Mature avocado fruit (cv. Hass) for the comparison of ripe and unripe enzyme preparations were obtained from a local packinghouse (Carpinteria, Calif.) during October 2004. The fruit were peeled, and the mesocarp was de-seeded, cut into chunks, flash-frozen in liquid nitrogen, and stored at −70 °C. Unripe fruit were frozen immediately. The progression of ripening was monitored for each fruit, which was kept in an individual jar at 25 °C in a flow-through system. Ethylene production was monitored by gas chromatography (Hach Carle, series 400 AGC, Loveland, Colorado, U.S.A) as a measure of the climacteric ripening stage (Christoffersen et al., 1982). In this study, ripe edible fruit were frozen at one day past their peak of ethylene production.

2.2. PPO extraction

Tissue from each fruit was randomized by being ground in liquid nitrogen in a Waring blender (New Hartford, Conn., U.S.A) with a metal blender cup. This step was taken to prevent sampling bias, since PPO levels have been shown to vary between proximal and distal ends of the avocado fruit (Golan et al., 1977b) and between the seed base and the outer pulp (Hershkovitz et al., 2009).

Frozen powdered tissue (4 g) was homogenized with a Tissumizer (Tekmar Inc., Cincinnati, Ohio, U.S.A) on ice for four 30 s intervals with 30 s breaks in 20 mL of chilled 0.1 M sodium phosphate buffer, pH 6.8, containing 4 g polyvinylpolypyrrolidine (PVPP) and 0.5 mM phenylmethanesulfonylfluoride (PMSF). The PMSF was added immediately before use from a 0.1 M stock dissolved in isopropanol. The homogenate was centrifuged at 10,000 × *g* for 30 min at 4 °C, then the supernatant was filtered through four layers of cheesecloth. PPO was concentrated by 40–90% ammonium sulfate precipitation at 4 °C and re-suspended in 1 ml of chilled 0.1 M sodium phosphate buffer, pH 6.8 (assay buffer). Enzyme samples were stored at −70 °C in aliquots.

2.3. SDS and native PAGE

Protein samples of 10 μg or less were subjected to non-denaturing 12% PAGE in a Mini-Protean II cell (Bio Rad Laboratories Inc., Hercules, California, U.S.A) at 10 mA with cooling (Laemmli, 1970). The gel contained SDS, although β-mercaptoethanol was omitted from the loading buffer. The samples were not boiled before being loaded on the gels to preserve enzyme activity. Immediately after electrophoresis, the gels were immersed in 4 mM (−)-epicatechin or 30 mM catechol in assay buffer at room temperature. Gels were photographed as the reactions proceeded, generally in 1–10 min. For the analysis of latency, SDS was omitted from native PAGE gels and individual lanes were incubated with and without SDS. The concentrations of SDS used were 3.5 mM for (−)-epicatechin and 0.7 mM for catechol. For each experiment, three different extracts from each fruit were analyzed twice.

2.4. Quantitative measurement of PPO activity and protein

Enzyme diluted to 200 μL was added to 800 μL of substrate to give a final reaction volume of 1 mL, and the rate was measured at 5–10 s intervals for 30 s to 2 min at 410 nm for catechol (Kahn, 1976) and 440 nm for (−)-epicatechin (Liu et al., 2007). The standard PPO assay used a concentration of (−)-epicatechin of 1 mM to avoid substrate inhibition, while the concentration of catechol was 30 mM. When SDS was included in the assays, it was added in water to the substrate to a final concentration of 3.5 mM for (−)-epicatechin and 0.7 mM for catechol analyses. Rates were measured from the linear portion of the velocity plots.

Latency was measured as the difference in the rate between the reaction with and without activator. Quantitative PPO assays for (−)-epicatechin oxidase comparing degrees of latency were performed in a Beckman DU40 spectrophotometer (Beckman Coulter, Inc., Brea, California, U.S.A.) containing a water-jacketed cuvette set to 25 °C. Other experiments utilizing (−)-epicatechin and all experiments involving catechol were performed in a Beckman DU64 spectrophotometer at 21 °C.

Units of specific activity are given as $\Delta A\,\mathrm{min}^{-1}\,\mathrm{mg}^{-1}$ (Kahn, 1976). Assays to determine the amount of activity and latency during ripening were conducted in triplicate on each of three extractions of enzyme from each fruit sampled. Assays for (−)-epicatechin oxidase activity utilized 1–10 μg and 3.2–27 μg protein from unripe and ripe extracts, respectively. The catechol oxidase assays utilized 67–480 ng and 320–750 ng protein from unripe and ripe extracts, respectively.

Apparent K_m values toward (−)-epicatechin were determined by assaying a range of concentrations from 0.05 mM to 5 mM in the presence of 3.5 mM SDS. Each fruit extract was assayed, with each concentration being assayed in triplicate.

While all of the extracts were tested for their ability to undergo activation by SDS, fatty acid activation profile studies were conducted solely on unripe preparations. The studies to examine activation of (−)-epicatechin oxidase by fatty acids and methyl linoleate were performed with 50 μM of each compound using 5% ethanol (v/v) in the reaction volume. Triton X-100 is water soluble and was added at a final concentration of 0.5% (v/v).

The inhibitors tropolone and salicylhydroxamic acid (SHAM) were tested for their effect on the oxidation of (−)-epicatechin and catechol. Each compound was tested against PPO extracts from unripe and ripe fruit. A 100 μM concentration of compound was tested in a final concentration of 1% ethanol (v/v). Three replicates were performed on an extract from three unripe and ripe fruit.

To study the effects of pH on latency, the activity of PPO toward (−)-epicatechin and catechol was measured at a range of pH values with or without 3.5 mM SDS for (−)-epicatechin and 0.7 mM SDS for catechol. Both substrates were assayed in triplicate with 0.1 M