Effects of Ascorbic Acid on Peroxidase and Polyphenoloxidase Activities in Fresh-Cut Cantaloupe Melon

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ABSTRACT: Peroxidase (POD) and polyphenol oxidase (PPO) in minimally processed cantaloupe melon (*Cucumis melo* L. var. *reticulatus* Naud) were assayed and the effect of ascorbic acid on their respective activities during storage at 4 °C was determined. POD activity decreased with storage and was inhibited by ascorbate treatment. Two native electrophoretic POD bands with estimated molecular weights 240 and 170 kDa respectively were obtained. Isoelectric focusing separated six acidic POD isozymes with isoelectric points between 5.1 and 6.0, possibly subunits of the POD isozymes. PPO activity in the cantaloupe melon extract was very weak and the fruit's content of oxidizable phenolic compounds was negligible.

Keywords: shelf life, fruit, quality, browning, antioxidant

Introduction

THE ACTIONS OF PEROXIDASE (POD) AND POLYPHENOL OXIDASE (PPO) enzymes primarily affect the ability of fresh and processed fruits and vegetables to retain their characteristic flavor and color (Burnette 1977; Vamos-Vigyazo 1981). POD activity may result in oxidative actions that involve hydrogen donors in foods (Kahn and others 1995; Padiglia and others 1995a). The antioxidative and free radical scavenging activities of some POD enzymes such as ascorbate POD are also well known (Chen and Asada 1992; Wang 1995). Discoloration and oxidative changes caused by PPO result from their creolase and catecholase activities (Vamos-Vigyazo 1981; Mayer and Harel 1991).

Fresh-cut processing is accompanied by the disruption of surface cells and injury stress of underlying tissues. Enzymatic activities increase as a consequence of the increased permeability that results from tissue disruption and mixing of enzymes and substrates that are otherwise sequestered within vacuoles. In light of the increased enzymatic activity in fresh-cut products relative to the intact produce, the properties of enzymes such as POD and PPO that affect fruit flavor and texture would significantly affect storage qualities of fresh-cut products. Recently we demonstrated that cantaloupe melon POD activity appears to be consistent with that of ascorbate peroxidase (Lamikanra and Watson 2000) and that POD activity in minimally processed cantaloupe melons could be the result of a preservative response to increased oxidative stress in the cut fruit. The correlation was based on the relative affinity of cantaloupe POD for ascorbic acid (AA) over guaiacol, and the ability of AA in dip solutions to prevent the oxidation of carotenoids in cut cantaloupe melon pieces stored at 4 °C for 25 d. The proposed mechanism for the preservative action is one that proceeds by way of an ascorbate-peroxidase complex and trace metal ion cofactors. Temperature and pH optima for cantaloupe melon POD activity were 50 to 55 °C and 5.5 to 7.5, respectively.

The potential use of AA to control PPO induced browning reactions in minimally processed fruits and vegetables has been reported (Sapers and others 1995; Kim and Klieber 1997; Gamage and others 1997; Gil and others 1998). Browning reactions, however, do not appear to take place in minimally processed cantaloupe during storage at 4 and 20 °C (Lamikanra and others 2000), in spite of the indication of some amount of PPO in the fruit (Pratt 1971). This apparent lack of PPO activity could have resulted from low levels of the enzymes and/or oxidizable phenolic compounds in the fruit.

This study was undertaken to determine the effect of AA on POD and PPO activity under conditions similar to those encountered in the processing and storage of fresh-cut cantaloupe. The possible involvement of such ascorbic acid-enzyme interaction in the preservative effect of this additive (Lamikanra and Watson 2000) was also determined. Phenolic compounds and, sometimes, some functional groups of proteins serve as substrates for both POD and PPO (Matheis and Whitaker 1984). The effects of storage and ascorbic acid on the contents of these substrates and PPO in minimally processed cantaloupe were also determined.

Materials and Methods

Fruit treatments

Cantaloupe melon (*Cucumis melo* L. var. *reticulatus* Naud) was minimally processed into slices (approximately 2 cm^3) as previously described (Lamikanra and Watson 2000). Cut fruit pieces (200 g) were transferred into polypropylene baskets and dipped in cold water (4 °C) and other solutions containing the following additives: ascorbic acid (1.25 mM), ascorbic acid (2.5 mM), ascorbic acid (2.2 mM) plus EDTA (10 mM), and ascorbic acid (2.5 mM) plus $MnCl_2$ (2.5 mM) in separate treatments for 1 min. L-Ascorbic acid (sodium salt) and the other chemicals were obtained from Sigma-Aldrich Co. (St. Louis, M.O.). After the fruits were dipped in the respective solutions, they were allowed to drain for about 1 min before being transferred into sealed plastic containers. A set of fruit pieces not dipped in water was used as control. Treated fruits were stored at 4 °C. Acetone powders of 3 replicates of each treatment were prepared on the day the fruits were processed (d 0) and 3 other days (d 2, 6, and 15). The acetone powders were kept at −20 °C until enzyme extraction. Enzyme extraction from acetone powders was carried

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out by homogenizing the powder (1 g) in a mixture of phosphate buffer (0.05 M; pH 7.0; 30 mL) and Triton X-100 (0.1%). The homogenate was centrifuged at 12,000 × g at 4 °C for 20 min. and the supernatant was used for analyses.

Determination of total proteins

Protein content of extracts was determined using bovine serum albumin standards and the Bio-Rad assay dye reagent. The diluted reagent in deionized water (20%; 5 mL) was added to 1 mL of standard solutions (0-1.5 mg/mL) and mixed thoroughly. Absorbance at 595 nm of the solutions was read after 15 min. Protein content of extracts was determined from a standard curve after they were reacted with the dye in a similar procedure.

POD assay procedure

POD activity in enzyme extracts was assayed by monitoring changes in absorbance at 470 nm in mixtures consisting of 0.02 M Na_2HPO_4 and 0.08 M NaH_2PO_4, 20 mM guaiacol, 4 mM H_20_2, enzyme extract (10 μL), pH 6, in a total volume of 3 mL (Civello and others 1995). Enzyme activity unit represents the amount of enzyme that caused an increase in absorbance of 0.001 unit/min at 50 °C. The effects of β-mercaptoethanol, L-cysteine and *p*-chloromercuribenzoate (5 mM) on POD activity were determined at pH 8 by adding the compounds to the buffer prior to preparation of reaction mixtures and enzyme activity assays.

PPO assay procedure

The enzyme extracts were diluted (1:1) in sodium phosphate buffer (0.05 M, pH 7). Assays for PPO activity were carried out on the diluted extract (1 mL) by measuring changes in absorbance at 420 nm when added to catechol (0.5 M; 2.5 mL) dissolved in the same buffer (Lamikanra 1995). The effect of temperature on PPO activity was determined by incubating the reaction mixtures at temperatures ranging from 20 to 80 °C. PPO activity as a function of pH was determined by adjusting pH of the buffer using appropriate amounts of 1.0 M HCl or 1.0 M NaOH. PPO activity of lettuce (*Lactuca sativa L.*) and Red Delicious apples (*Malus domestica* Borkh) were compared with that of cantaloupe melon using the same procedure.

Determination of total phenols and flavonoids

Total phenol and flavonoid contents of fresh-cut and treated cantaloupe were determined by a modification of the method of Amerine and Ough (1980). Cut cantaloupe pieces (50 g) were frozen in liquid nitrogen and lyophilized. The powder (0.5 g) obtained after grinding the lyophilized fruit was added to methanolic HCl (0.1% HCl in methanol; 4 mL), vortexed for 30 s and then kept overnight at 4 °C. After a second agitation, the mixture was centrifuged at 4 °C and 10,000 × g for 20 min. The supernatants obtained were used for phenol and flavonoid analyses.

The phenol extract (100 μL) was added to a mixture of water (6.9 mL) and Folin-Ciocalteu reagent (Sigma-Aldrich Co., St. Louis, Mo. U.S.A.); 500 μL). After at least 30 s but less than 8 min, Na_2CO_3 solution (20%; 1.5 mL) was added to the mixture, vortexed and kept at ambient temperature for 2 h. Total phenol was determined at 765 nm from a standard curve in which gallic acid solutions were used in place of the phenol extracts in a similar procedure.

To determine the total flavonoid in phenol extracts, HCl (20 %; 1 mL) was added to the extract (1 mL) followed by formaldehyde (0.5 mL). The mixture was left for 24 h. after which it was filtered. Total phenol in the filtrate (100 μL) was determined as previously described. Total flavonoid was calculated as the difference between this value and that obtained for the total phenol content of the same phenol extract without the added HCl and formaldehyde.

Gel electrophoresis

Gel electrophoretic separations were carried out using Xcell II Mini-Cell on 8cm × 8cm × 1mm, 10-well polyacrylamide pre-cast gels. Novex Tris-glycine buffer systems were used for SDS and native gel electrophoresis. IEF (pH 3-10) buffers were used with IEF gels for isoelectric focusing. Protein separations were performed according to the manufacturer's instructions (Novel Experimental Technology, San Diego, Calif., U.S.A.). Gels were stained for proteins using GelCode blue stain reagent (Pierce Chemical Co, Rockford, Ill., U.S.A.). POD assay was done by immersing gels in acidic benzidine (100 mL; 0.5% w/v benzidine and 4.5% w/v glacial acetic acid) for 5 min after which H_2O_2 (3 mL of 30% v/v H_2O_2) was added. Gels were photographed immediately. PPO activity was detected by immersing the gels for 10 min in sodium acetate buffer (0.1 M; pH 5.0) containing 20 mM catechol and 0.05% *p*-phenylenediamine.

Results and Discussion

UNLIKE GUAIACOL POD, WHICH IS CHARACTERIZED BY BROAD specificity with respect to electron donors, and which participates in many physiological processes, such as the biosynthesis of lignin and ethylene, ascorbate POD exhibits high sensitivity for ascorbate as the electron donor (Amako and others 1994), and in its physiological role it scavenges potentially harmful H_2O_2 (Dalton and others 1998) and free radicals (Wang 1995). The sensitivity of ascorbate POD to thiol reagents (Chen and Asada 1992) and *p*-chloromercuribenzoate (Amako and others 1994) distinguishes it from guaiacol POD. The ascorbate-specific nature of cantaloupe melon POD is further indicated in this study by the complete inhibition of color development by b-mercaptoethanol and L-cysteine. *p*-Chloromercuribenzoate reduced the reaction rate, as measured at 470 nm, by 80 %, but a purple coloration was observed instead of the dark brown color that develops in its absence.

POD activity, particularly ascorbate POD, could be indicative of oxidative stress in plant tissues (Kampfenkel and others 1995; Gueta-Dahan and others 1997). In light of the ascorbate nature of cantaloupe POD, an increase in enzyme activity as a result of fresh-cut processing and storage should be a manifestation of the stress level. POD under such condition would act to reduce potential oxidative damage to the fruit. The presence of AA effectively reduced POD activity in the cantaloupe melon pieces (Figure 1). POD activity in fruit dipped in 1.25 and 2.5 mM AA solutions were reduced by over 60% at the time of processing. Addition of Mn^{2+} and EDTA to the dip solutions inhibited the capability of AA to reduce POD activity. This result demonstrates the need for the presence of trace metal ions in the ascorbate interaction with POD, and that the presence of significant amounts of metal ions has a reverse effect. At the higher metal ion concentration, the ability of the ions to initiate free radical and oxidative reactions (American Health Foundation 1993) apparently predominates over their role as cofactors in ascorbate POD activity. The results are also consistent with the conclusion that the use of AA as a processing aid in minimally processed cantaloupe melon preserves the fruit by way of an enhanced antioxidative action of the enzyme-hydrogen

donor complex and trace metal ion cofactors (Lamikanra and Watson 2000). The reduced POD activity in the ascorbate-treated fruit could be the result of a lower oxidative stress on the fruit surface, because of the antioxidant nature of molecular AA, or the utilization of POD enzyme-hydrogen donor complex formation. The requirement of trace metal ions for the AA-induced POD reduction, however, is in favor of the latter. POD activity in fruit dipped in 1.25 mM AA increased considerably after 2 d of storage, unlike those dipped in the 2.5 mM solution. Such regeneration of POD appears to be the result of a depletion of residual AA by direct oxidation. AA retention is lower in non-acidic fruits such as cantaloupe, than in the more acidic fruits (Albrecht and others 1991). With the exception of the increased POD observed in fruit dipped in AA (1.25 mM) after 2 d of storage, the overall trend was a decrease in POD activity with storage time. Exudates from cut cucumber aid the formation of protective sclerified materials on the cut fruit surface (Walter and others 1990). Formation of similar materials on cut cantaloupe will help reduce moisture loss from the fruit and provide a surface protective layer against atmospheric oxygen, thereby reducing oxidative degradation rate with storage time, and consequently, the need for POD. AA POD also appears to be less stable than some types of POD (Zapata and others 1998), and the decrease in enzymatic activity in the stored fruit with time might be the result of POD degradation.

PPO activity was unaffected by AA in similar treatments and was very weak in cantaloupe melon relative to apple and lettuce (Table 1). Changes in enzyme activity in cantaloupe as a result of the presence of AA might thus be too low for detection. The low PPO activity indicates that enzymatic browning reactions are unlikely to contribute significantly to cantaloupe melon product deterioration, and that there is no need for the use of a treatment aimed at controlling PPO activity in the fruit.

PO activity is not necessarily related to the total phenol content of fruits and vegetables, and only a relatively small part of food phenolic compounds can serve as substrates for PPO. Common fruit PPO substrates are flavonoid and related compounds such as catechins, cinnamic acid, leucoanthocyanidins and flavonols (Vamos-Vigyazo 1981). The total phenol content of cantaloupe melon determined was 5.16 mg/100 g of fresh fruit. The total phenol content of untreated fruit pieces and the dipped fruit did not change with storage time. These phenolic compounds were all nonflavonoid compounds. The absence of oxidizable phenolic compounds, coupled with the relatively low PPO activity in the fruit, thus accounts for the lack of browning reactions in minimally processed cantaloupe melon (Lamikanra and others 2000).

Table 1—Relative polyphenol oxidase (PPO) activity (%) per gram of fresh weight for apple, cantaloupe melon, and lettuce

	Relative PPO Activity (%)	
Commodity	pH 6.5	pH 4.5
Apple	20	100
Cantaloupe	2	0
Lettuce	35	60

The protein content of cantaloupe was determined to be 30.8mg/100 g of fresh fruit and did not change significantly with storage time. Fruit pieces dipped in AA solutions also maintained the same protein level with storage. Twelve proteins were separated by native gel electrophoresis, with estimated molecular weights ranging from 30 to 650 kDa (data not shown). The banding pattern remained essentially the same with storage time. There was no indication that the separated proteins were involved as substrates in the activities of the POD and PPO. Isoelectric focusing indicated that cantaloupe melon proteins are predominantly acidic with isoelectric pH values ranging from pH 4.5 to 6.0. Two peroxidase bands of approximate molecular weights of 240 and 165 kDa, respectively, were obtained when the native-PAGE gel was stained with acidic benzidine and H_2O_2 (Figure 2). These molecular sizes are considerably higher than the 30 kDa reported for peroxidase extracted from muskmelon leaves

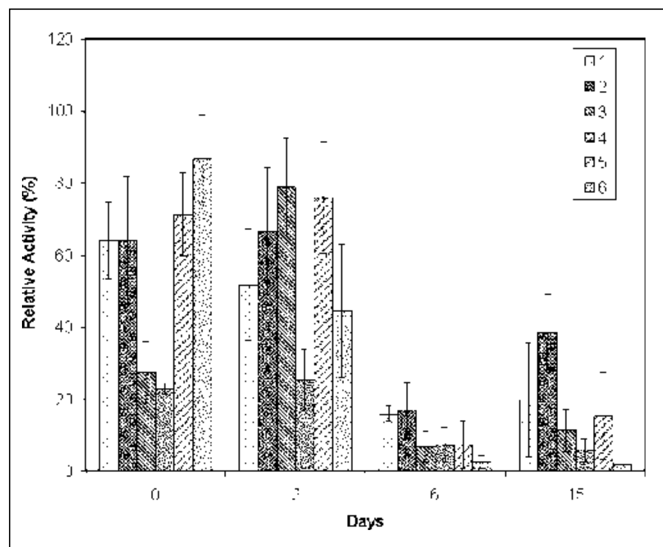

Figure 1—Effect of storage time at 4 °C on peroxidase activity of cut cantaloupe melon subjected to the following treatments: 1 = control (no dipping), 2 = dipped in water, 3 =dipped in ascorbic acid solution (1.25 mM), 4 =dipped in ascorbic acid (2.5 mM), 5 =dipped in ascorbic acid (2.5 mM) plus EDTA (10 mM), and 6 = dipped in ascorbic acid (2.5 mM) plus Mn C:$^{2+}$ (2.5 mM).

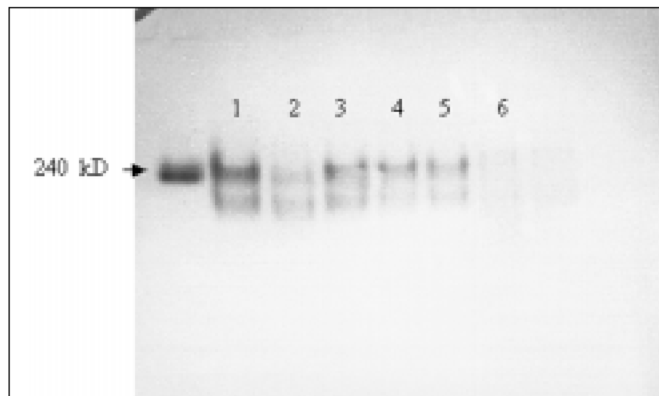

Figure 2—Native polyacryamide gel electrophoresis of cantaloupe melon peroxidase. 1 = fresh-cut fruit immediately after processing, 2 = the same cut fruit after 6 d storage at 4 °C, 3 = fruit dipped in ascorbic acid (1.25 mM after 2 d storage, 4 = fruit dipped in ascorbic acid immediately after fresh cut processing, 5 =cut fruit after 15 d storage and 6 = fruit dipped in ascorbic acid (2.5 mM) after 15 d storage.

(Smith and Hammerschmidt 1988) and those of most fruits and vegetables (Vamos-Vigyazo 1981; Prestamo 1989; Padiglia and others 1995b; Civello and others 1995). A similar stain for the IEF gel, however, indicates 6 acidic peroxidase isozymes (Figure 3). The major band in the native electrophoresis thus appears to be comprised of 6 subunits that were unseparated, but pulled apart by the higher electrical charge in the IEF electrophoretic run. The 2nd native protein band (MW. = 165 kDa) also appears to correlate to the first band (MW. = 240 kDa) that lost 2 of the protein subunits either endogenously or during the electrophoretic analysis. Isoelectric points for the isozymes were between pH 5.1 and 6.0. Peroxidase extracted from muskmelon leaves were also reported to be acidic and are associated with induced resistance in the plant (Smith and Hammerschmidt 1988; Madi and Katan 1998). The presence of POD isozymes with different isoelectric points is consistent with the broad optimum activity pH of cantaloupe melon POD (Lamikanra and Watson 2000). PPO activity was too weak to be observed on gels stained with catechol and *p*-phenylenediamine.

Conclusions

CANTALOUPE MELON POD EXHIBITS PROPERTIES OF ASCORBATE POD. POD activity was highest on the processing date, and there was a gradual decrease in enzyme activity with storage time at 4 °C. AA reduce the amount of POD detected during fresh-cut processing. The ability of AA to reduce ascorbate POD, an oxidative stress indicator, appears to be related to its preservative action on fresh-cut cantaloupe. The presence of trace metal ions such as Mn^{2+} is essential for ascorbate reduction of POD. However, exposure of the cut fruit to an increased amount of metal ions inhibits this effect. Trace metal ions such as Mn^{2+} thus appear to serve as cofactors in an antioxidative action that proceeds by way of the enzyme-hydrogen donor complex. PPO activity is weak in cantaloupe melon, and is rapidly deactivated by an increase in temperature. The fruit is also deficient in oxidizable phenols that could serve as PPO and POD substrates. There should be no need for the use of a treatment, such as AA, for the purpose of inhibiting PPO activity in the minimally processed fruit, or other processed products of cantaloupe melon. Functional groups of proteins do not appear to serve as substrates to POD and PPO. Cantaloupe melon POD is comprised of 6 acidic isozyme units that appear to combine to form a large molecule.

Figure 3—Isoelectric focusing of cantaloupe melon peroxidase. 1 = fresh-cut fruit immediately after processing, 2 = the same fruit after 6 d storage at 4 °C, 3 = fruit dipped in ascorbic acid (1.25 mM) after 2 d storage, 4 = fruit dipped in ascorbic acid immediately after fresh cut processing, 5 = fruit dipped in ascorbic acid (1.25 mM) immediately after fresh cut processing, 6 = fruit dipped in ascorbic acid (2.5 mM) after 2 d, 7 = fruit dipped in ascorbic acid (2.5 mM) after 6 d, 8 = cut fruit after 15 d storage and 9 = fruit dipped in ascorbic acid (2.5 mM) after 15 d storage.

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