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Temperature preconditioning affects ascorbate antioxidant system in chilled zucchini squash

Chien Yi Wang *

Horticultural Crops Quality Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705-2350, USA

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Abstract

Preconditioning of zucchini squash (*Cucurbita pepo* L., cv. Elite) at a temperature of 15°C for 2 days prior to storage at 5°C reduced the development of chilling injury symptoms and affected the activities of ascorbate related enzymes, ascorbate free radical reductase, ascorbate peroxidase, and dehydroascorbate reductase. Ascorbic acid content in control squash decreased after 2 days of exposure to 5°C. In squash treated with temperature preconditioning, ascorbic acid content also declined but to a lesser extent. There was no significant change in dehydroascorbic acid content in either control or treated squash during storage at 5°C. The activities of these enzymes in the ascorbate metabolic system increased initially for 4–8 days during storage, then declined thereafter. These enzyme activities remained higher in the temperature-conditioned squash than in the untreated squash. These results indicate that the enhancement of chilling resistance in squash by temperature preconditioning may also involve changes in the enzyme activities in the ascorbate antioxidant system.

Keywords: Chilling injury; Temperature preconditioning; Ascorbate; Zucchini squash; *Cucurbita pepo*

1. Introduction

Certain fruits and vegetables are injured by low but nonfreezing storage temperatures (Hardenburg et al., 1986). Several treatments have been reported to alleviate the injury caused by chilling temperatures (Wang, 1993). Our previous study showed that temperature preconditioning at 15°C for 2 days effectively delayed the onset of chilling injury in zucchini squash during subsequent storage at 5°C (Kramer and Wang, 1989). Temperature preconditioning also altered lipid composition (Wang et al., 1992) and enhanced polyamine levels (Kramer and Wang, 1989) in zucchini

* Fax: 301 504-5107.

squash. Higher activities of catalase and glutathione reductase and higher ratios of reduced to oxidized forms of glutathione were found in the temperature preconditioned squash fruit than in the control fruit (Wang, 1994, 1995). This suggests that the antioxidant mechanism may develop in temperature preconditioned squash and the treated squash may attain adaptive function by acquiring higher capacity to scavenge free radicals.

Ascorbic acid and its related enzymes also play a key role in detoxification of active oxygen. It reacts directly by reducing superoxide hydrogen peroxide and hydrogen radicals or by quenching singlet oxygen (Foyer et al., 1991). This could occur through a series of oxidation–reduction reactions involving ascorbate and glutathione (Dalton et al., 1987). Removal of free radicals and reduction of oxidative activities aid in alleviating injury caused by chilling. The objective of this study was to determine the effect of temperature preconditioning on the activities of ascorbate related enzymes in zucchini squash during storage at a chilling temperature.

2. Materials and methods

2.1. Plant materials and chilling injury evaluation

Zucchini squash (*Cucurbita pepo* L. cv. Elite) were obtained from a farm in Clinton, MD, USA. Methods of handling and chilling treatment and evaluation have been described previously (Wang, 1994). A group of squash was placed in storage at 5°C and served as control for the duration of the experiment. Another group of squash was preconditioned at 15°C for the first two days of storage and then transferred to 5°C for the remaining time in storage. The degree of chilling injury, as judged by the extent of surface pitting, was rated on a scale of 1 to 5, with 1 = no abnormality, 2 = trace, 3 = slight, 4 = moderate, and 5 = severe chilling injury. A 5.0 g sample of exocarp tissue was removed from each squash fruit and placed in liquid nitrogen then lyophilized. The freeze-dried samples were stored at –80°C prior to enzyme extraction and assay.

2.2. Determination of ascorbic acid and dehydroascorbic acid

The lyophilized sample was pulverized with a cold mortar and pestle using 5% (w/v) trichloroacetic acid containing 80 mg polyclar AT and 400 mg sea sand. The ratio of the tissue to the homogenizing medium was 1:2 (w/v). The homogenate was filtered through 4 layers of Miracloth and centrifuged at 16,000 ×g for 10 min at 4°C. The supernatant was used for the ascorbate assay. Ascorbic and dehydroascorbic acid levels were determined using the method of Arakawa et al. (1981) and Nakagawara and Sagisaka (1984). This assay is based on the reduction of ferric ion to ferrous ion with ascorbic acid in acid solution followed by formation of the red chelate between ferrous ion and 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) that absorbs at 534 nm. Total ascorbic acid (including dehydroascorbic acid) was determined by the reduction of dehydroascorbic acid to ascorbic acid by dithiothreitol.

The ascorbic acid assay mixture contained 0.1 ml of the sample extract, 0.5 ml of absolute ethanol, 0.6 M trichloroacetic acid, 3 mM bathophenanthroline, 8 mM H_3PO_4 and 0.17 mM FeCl_3 . The final volume was 1.5 ml, and the solution was allowed to stand at 30°C for 90 min to allow the Fe^{2+} -bathophenanthroline complex to develop. The absorbance of the coloured solution was read at 534 nm. Total ascorbic acid assay mixture contained 0.1 ml of the sample solution, 0.15 ml of 3.89 mM dithiothreitol and 0.35 ml of absolute ethanol in a total volume of 0.6 ml. Then, the reaction mixture was left standing at room temperature for 10 min. After reduction of dehydroascorbic to ascorbic, 0.15 ml of 20% trichloroacetic acid was added. The colour was developed by adding several reagents in the following sequence: 0.15 ml of 0.4% (v/v) H_3PO_4 -ethanol, 0.3 ml of 0.5% (w/v) bathophenanthroline-ethanol and 0.15 ml of 0.03% (w/v) FeCl_3 -ethanol. The final volume was 1.5 ml, and after incubation at 30°C for 90 min, the absorbance at 534 nm was recorded. Dehydroascorbic concentrations were calculated from the difference of 'total ascorbic acid' and 'ascorbic acid' concentrations. A standard curve in the range 1–10 μmol ascorbic acid or dehydroascorbic acid was used.

2.3. *Extraction and assay of ascorbate free radical reductase, ascorbate peroxidase, and dehydroascorbate reductase*

The lyophilized tissue (400 mg) was homogenized in 12 ml of cold 50 mM Tris-HCl, pH 7.7, containing 2 mM dithiothreitol, 80 mg polyclar AT and 400 mg sea sand with chilled mortars and pestles. The homogenate was filtered through 4 layers of Miracloth and centrifuged at 16,000 $\times g$ for 10 min at 0°C. The supernatant was used for enzyme assays.

Ascorbate free radical reductase (AFR) activity was assayed according to the procedure of Arrigoni et al. (1981) by measuring the rate of NADH oxidation at 340 nm with a spectrophotometer (Shimadzu UV-160A, Columbia, MD). The reaction mixture contained 50 mM potassium phosphate, pH 7.3, 0.2 mM NADH, 1.0 mM ascorbate, 1.0 unit ascorbate oxidase, and 0.1 ml of crude enzyme preparation. The reaction was started by adding ascorbate oxidase.

The ascorbate peroxidase (ASPOD) activity was assayed according to the method of Shigeoka et al. (1980) by measuring the oxidation of ascorbate at 290 nm. The reaction mixture contained 25 mM potassium phosphate, pH 6.1, 0.2 mM ascorbate, 0.1 mM H_2O_2 , and 0.1 ml of crude enzyme. The reaction was started by adding H_2O_2 . The dehydroascorbate reductase (DHAR) activity was assayed by measuring the rate of NADPH oxidation at 340 nm (Shigeoka et al., 1980). The reaction mixture contained 50 mM potassium phosphate, pH 6.1, 0.2 mM NADPH, 2.5 mM dehydroascorbate, 2.5 mM glutathione, 0.6 unit glutathione reductase, and 0.1 ml of crude enzyme. The reaction was started by adding dehydroascorbate.

The enzyme activities were expressed in terms of nmol of NADH per milligram protein per min for AFR and as nmol of NADPH per milligram protein per min for DHAR. The ascorbate peroxidase activity was expressed as nmol ascorbate per milligram protein per minute.

3. Results

3.1. Severity of chilling injury

Surface pitting, a symptom of chilling injury, was detected on the skin of control fruit after 4 days of exposure to 5°C (Fig. 1). The extent of the injury intensified rapidly with time in storage. By the tenth day of storage, most squash had developed moderate or severe pitting. In severe cases, the entire fruit was covered with numerous sunken areas. In temperature preconditioned squash, surface pitting did not develop on the skin until after 8 days of exposure to 5°C (Fig. 1), and only slight to moderate degree of pitting was detected on these squash after 14 days of storage at the chilling temperature. Therefore, the onset and the development of chilling injury symptoms were delayed by the temperature preconditioning treatment.

3.2. Ascorbic acid and dehydroascorbic acid contents

Ascorbic acid content in control squash decreased after 2 days of exposure to 5°C (Fig. 2). The ascorbic acid content in these samples continued to decline during further storage at 5°C. The amount of dehydroascorbic acid, the oxidized form of ascorbate, did not change significantly throughout storage period at 5°C. Therefore, the total ascorbic acid pool decreased and the ascorbateic acid/dehydroascorbic ratio also declined. In squash treated with temperature preconditioning, ascorbic acid content also decreased at 5°C but to a lesser extent compared to the untreated chilled squash.

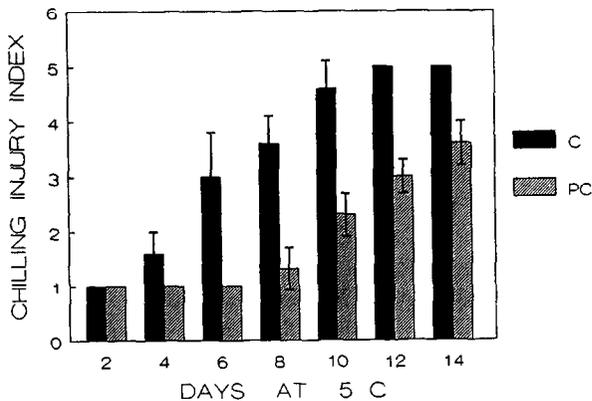


Fig. 1. Development of chilling injury in zucchini squash with time of storage at 5°C. The preconditioned (PC) squash were exposed to 15°C for 2 days before the 5°C storage while the control (C) squash were held continuously at 5°C. Chilling injury index: 1 = no abnormality, 2 = trace, 3 = slight, 4 = moderate, and 5 = severe. Vertical bars represent the standard errors.

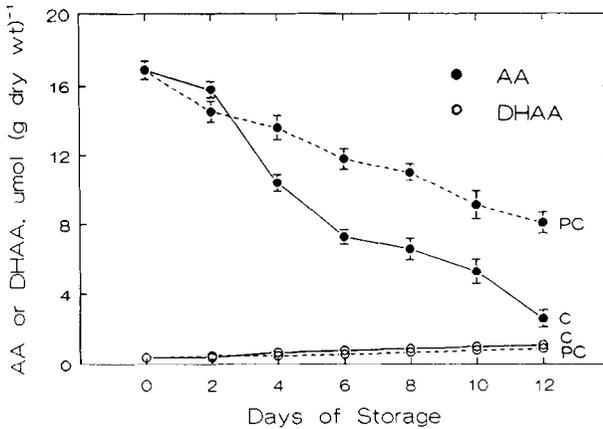


Fig. 2. Effect of temperature preconditioning (PC) on ascorbic acid (AA) and dehydroascorbic acid (DHAA) contents in exocarp of zucchini squash. The preconditioned squash were stored at 15°C for the first 2 days then transferred to 5°C. The control (C) samples were kept at 5°C throughout the storage period. Vertical bars represent the standard errors.

3.3. Activities of ascorbate peroxidase, ascorbate free radical reductase, and dehydroascorbate reductase

The activities of ASPOD, AFR, and DHAR increased initially for 4–8 days during storage then declined thereafter (Figs. 3 and 4). In both control and temperature preconditioned squash, the activities of ASPOD reached their peak on the 6th day of storage. However, the preconditioned squash maintained higher ASPOD activity

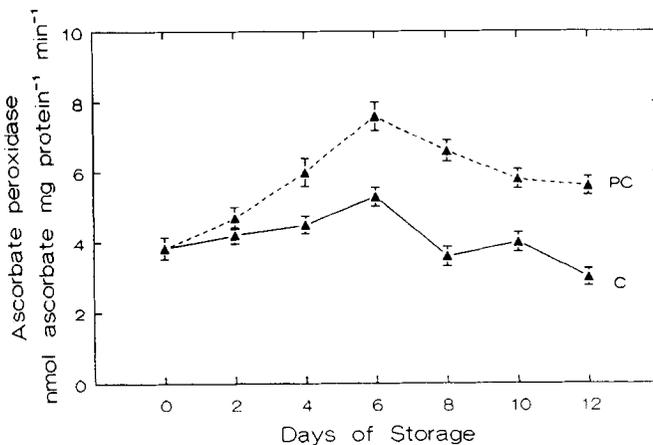


Fig. 3. Effect of temperature preconditioning (PC) on activities of ascorbate peroxidase in exocarp of zucchini squash. The preconditioned squash were stored at 15°C for the first 2 days then transferred to 5°C. The control (C) samples were kept at 5°C throughout the storage period. Vertical bars represent the standard errors.

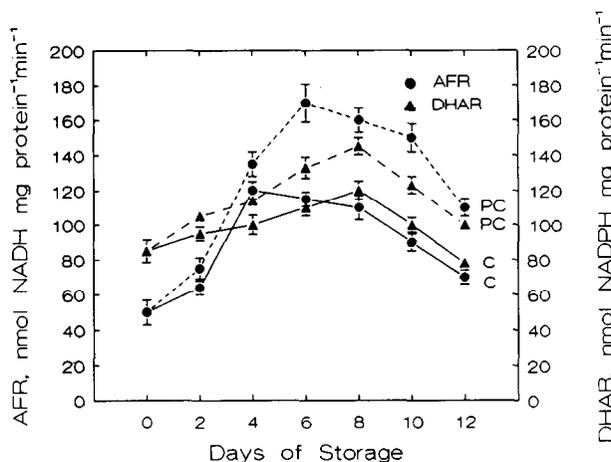


Fig. 4. Effect of temperature preconditioning (PC) on activities of ascorbate free radical reductase (AFR) and dehydroascorbate reductase (DHAR) in exocarp of zucchini squash. The preconditioned squash were stored at 15°C for the first 2 days then transferred to 5°C. The control (C) samples were kept at 5°C throughout the storage period. Vertical bars represent the standard errors.

than the control squash throughout the storage at 5°C (Fig. 3). The activity of AFR increased upon exposure to 5°C for 4 days in the control samples, then declined gradually thereafter (Fig. 4). A similar increase in AFR activity was detected after 2 days of preconditioning treatment and after transfer of squash from 15°C to 5°C. The AFR activity in the treated samples continued to increase until 6 days of storage. A significant difference in AFR activity between the treated and the control squash was observed on the 6th day of storage and this difference was maintained for the remainder of the storage. The DHAR activity in the control samples increased slightly for the first 8 days at 5°C and then declined steadily thereafter (Fig. 4). In preconditioned squash, DHAR activity increased even more during and after the preconditioning treatment. The activity decreased after the 8th day but maintained at higher levels than the control samples throughout the storage period.

4. Discussion

Temperature preconditioned squash retained more ascorbic acid than the control samples during storage at 5°C (Fig. 2). Ascorbic acid is an essential compound in plant tissues and plays an important role in detoxification of activated oxygen (Foyer et al., 1991). It can react directly and remove harmful radicals by reducing superoxide, hydrogen peroxide, and hydroxyl radicals, or quenching singlet oxygen. It also participates in the removal of H₂O₂ as the donor of electrons to ASPOD (Foyer et al., 1991). Higher ascorbic acid content in the preconditioned samples indicates a greater antioxidant capacity in these tissues.

Ascorbic acid undergoes continuous oxidation and reduction. Oxidation products are ascorbic free radical and dehydroascorbic acid. These products can be converted

back to ascorbic acid by AFR and DHAR (Yamaguchi and Joslin, 1951). Higher levels of AFR and DHAR were found in the preconditioned squash than in the control fruit (Fig. 4). Therefore, regeneration of ascorbic acid is more likely to occur in the preconditioned samples than in the control. AFR is a ubiquitous enzyme and is essential in maintaining the ascorbic acid system at the reduced state (Arrigoni et al., 1981). AFR catalyses the regeneration of ascorbate from ascorbate free radicals, which is produced by univalent oxidation of ascorbic in both enzymic and nonenzymic reactions (Arrigoni et al., 1981). DHAR plays a minor role in maintaining the ascorbic acid system at the reduced state by a reaction where the reduced form of glutathione serves as a substrate (Borraccino et al., 1989; Hossain and Asada, 1984; Kosower and Kosower, 1978). It seems that AFR and DHAR may contribute to the maintenance of a reduced state of ascorbate system in the cells under low temperature stress.

The ASPOD is a hydrogen peroxide-scavenging enzyme in plants, but loses its activity if the electron donor is absent (Asada, 1992). Ascorbic acid is the most effective electron donor for ASPOD. Ascorbate peroxidase is needed in the scavenging reaction to protect chloroplasts and other cell constituents from damage by hydrogen peroxide and hydroxyl radicals (Asada, 1992). The activity of ASPOD is higher in temperature preconditioned squash than in control squash (Fig. 3). ASPOD is involved in the formation of dehydroascorbic acid. However, dehydroascorbic acid in temperature preconditioned squash did not accumulate and remained low and virtually unchanged (Fig. 2). This is probably because the treated tissue also contained high activity of AFR which continuously converted dehydroascorbic acid back to ascorbic acid. Since the preconditioned samples also contained higher level of ascorbate, the scavenging capacity of these tissues for free radicals and the resistance to the deleterious degradative reactions associated with activated oxygen should also be greater than the control tissues.

A decrease in the ability to remove peroxide occurred in zucchini squash during storage at chilling temperature, especially in the control samples with resultant inactivation or reduction of some enzymes. When the rate of production of reducing power, such as that of NADH, NADPH, and ascorbate, becomes insufficient to handle active oxygen species, cells suffer oxidative damage and will no longer be able to survive and the squash will show symptoms of chilling injury. Both NADH and NADPH can be utilized in the reaction catalyzed by the enzymes in the peroxide-scavenging systems of ascorbate metabolism. The removal of H_2O_2 is dependent on the activities of AFR, DHAR, and ASPOD (Nakagawara and Sagisaka, 1984). The results in this study suggest that temperature preconditioning treatment increased the activities of the peroxide-scavenging systems of ascorbate metabolism in zucchini squash fruit. These data indicated that the ascorbate antioxidant system was modified by temperature preconditioning treatment and this modification may have contributed to the reduction of chilling injury in the treated tissue. These results are also consistent with the dynamic models developed by Tijskens et al. (1994). According to these models, the radical-scavenging activity during prestorage temperature conditioning period is so high that it substantially reduces the radical concentrations in the tissues and delays the onset of chilling injury.

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