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Effect of temperature preconditioning on catalase, peroxidase, and superoxide dismutase in chilled zucchini squash

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Abstract

The development of chilling injury symptoms in zucchini squash (*Cucurbita pepo* L., cv. 'Elite') stored at 5°C was delayed by preconditioning the fruit at a temperature of 15°C for two days. This temperature preconditioning treatment suppressed the increase in peroxidase activity and reduced the decline of catalase activity in squash during subsequent storage at 5°C. The superoxide dismutase activity remained higher in temperature-conditioned squash than in untreated squash throughout storage. These results indicate that acclimation to chilling temperature in squash may also involve modifications in the activities of catalase, peroxidase, and superoxide dismutase.

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

1. Introduction

Chilling injury induces degradative processes in membranes and disrupts normal metabolism in plants (Raison and Orr, 1990). Even though the fundamental mechanism which causes chilling injury is not clear, the involvement of membrane damage is apparent. In a previous study (Kramer and Wang, 1989), we found that exposing zucchini squash to chilling temperature resulted in an increase in chloroform-soluble fluorescent products. Since the accumulation of these fluorescent products has been shown to be correlated with oxidative damage (Tappel, 1975), these results may indicate that chilling injury enhances lipid peroxidation in the membranes. In fact, chilling has been found to induce lipid degradation in cucumber fruit (Parkin

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and Kuo, 1989), cucumber seedlings (Whitaker and Wang, 1987), and tomato pericarp (Nguyen and Mazliak, 1990). Oxidative damage is considered to be an early response of sensitive tissues to chilling (Hariyadi and Parkin, 1991). Many free radicals are highly reactive chemically and can induce the oxidative breakdown of double bonds in the fatty acids of membrane lipids. Several enzymes are involved in the production and scavenging of free radicals in plant systems. Catalase can enhance the removal of H_2O_2 without producing dangerously-active free radicals (Burris, 1960). Peroxidase also catalyzes the decomposition of H_2O_2, but the mode of action of peroxidase differs from catalase action in that peroxidase liberates free radicals instead of oxygen (Burris, 1960). These free radicals are highly phytotoxic. Increased production of superoxide radicals is also related to injury induced by chilling (Hodgson and Raison, 1991a). The damaging effects of superoxide can be modulated if the radicals are scavenged by reacting with superoxide dismutase (SOD) (Clare et al., 1984). SOD occurs in three molecular forms; with Cu–Zn, Mn, or Fe as prosthetic metals, and is widely distributed in prokaryotic and eukaryotic organisms (Fridovich, 1986; Bannister et al., 1987). SOD catalyzes the dismutation of the superoxide free radical to H_2O_2 and O_2, removes singlet oxygen, and prevents formation of OH^-. The combined action of SOD and catalase converts the potentially dangerous superoxide radical and hydrogen peroxide to molecular oxygen and water, thus averting cellular damage (Scandalios, 1993). Plants with different chilling tolerances have different capabilities to reduce the deleterious effects of free radicals generated during chilling and rewarming. Several postharvest techniques have shown promise in increasing the tolerance of fruits and vegetables to chilling injury (Wang, 1993). In the temperature preconditioning treatment, a concomitant reduction of fluorescent products is associated with the increase of chilling tolerance in zucchini squash (Kramer and Wang, 1989). Temperature preconditioning treatment also protects membranes from chilling-induced degradation of phospholipids and galactolipids in zucchini squash (Wang et al., 1992). The present study was undertaken to determine the effect of temperature preconditioning on the activities of catalase, peroxidase, and SOD in zucchini squash during storage at chilling temperature.

2. Materials and methods

Plant materials and chilling injury evaluation

Zucchini squash (*Cucurbita pepo* L., cv. 'Elite') were freshly harvested from a local farm near Beltsville, Md. Samples were selected for their uniformity of size (16–22 cm long). After equilibration to room temperature (ca. 25°C), the squashes were randomly divided into two lots. The first group was placed in storage at 5°C and served as control for the duration of the experiment. The second group was preconditioned at 15°C for the first two days of storage and then transferred to 5°C for the remainder of the study.

Three squashes were taken daily from each group throughout the storage period for evaluation of chilling injury and for enzyme assay. The degree of chilling injury, as judged by the extent of surface pitting, was evaluated one day after the transfer

of squash from storage chambers to room temperature by rating on a scale of 1 to 5, with 1 = no abnormality; 2 = trace; 3 = slight; 4 = moderate; and 5 = severe chilling injury. After evaluation of injury, a 5.0 g sample of exocarp tissue was removed from various locations on each fruit and lyophilized. The freeze-dried samples were stored at −80°C prior to enzyme extraction and assay.

Extraction and assay of catalase

The lyophilized tissue (100 mg) was pulverized in a cold mortar and pestle with 3 ml Tris-HCl buffer (pH 8.5) containing 5 mM EDTA-Na, 5 mM DTT, 10% (w/v) insoluble pyrrolidone (PVP) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was strained through four layers of miracloth and centrifuged at 20,000 *g* for 30 min at 4°C. The supernatant was used for assays of catalase activity. The floating disc method (Nir et al., 1986) was used to determine catalase activity. Discs of 6 mm diameter were cut from a Whatman 3 MM chromatographic paper. 10 μl of crude enzyme extract was applied to each disc and placed in a vial containing 5 ml of 30 mM H_2O_2 at 20°C. The elapsed time for the discs to float was recorded. Ten replicates of individual discs were used for each crude extract. The activity of catalase in zucchini squash exocarp extracts was calculated according to the activity of bovine-liver catalase (σ = 11,000 units/mg protein). The level of catalase activity was expressed as units per milligram of protein.

Extraction and assay of peroxidase

The lyophilized tissue (100 mg) was homogenized in 12 ml cold 0.1 M phosphate buffer (pH 6.1) containing 90 mg of insoluble PVP and 30 mg sodium ascorbate in chilled mortars and pestles. The homogenate was filtered through four layers of miracloth and centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was used for the peroxidase assay. The assay mixture contained 0.1 M phosphate buffer (pH 6.1), 4 mM guaiacol as hydrogen donor, 3 mM H_2O_2 as substrate and 0.4 ml crude enzyme extract. The total reaction volume was 1.2 ml. The rate of change in absorbance (OD) at 420 nm was measured by spectrophotometer (Shimadzu UV-160A). The levels of enzyme activity were expressed as OD difference per minute per milligram of protein.

Extraction and assay of superoxide dismutase

The lyophilized tissue (100 mg) was pulverized in a cold mortar and pestle with 3 ml of 0.1 M potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA-Na and 5% (w/v) insoluble PVP. The homogenate was strained through four layers of miracloth and centrifuged at 15,000 *g* for 20 min at 0°C. The supernatant was purified by gel filtration using Sephadex G-25 (Pharmacia, Piscataway, N.J.), which had been equilibrated with the extraction buffer. The extracts were then passed through molecular filter Centricon™ 10 (Amicon, Danvers, Mass.) to remove naturally occurring reductants or antioxidants with low molecular weight before assay of SOD enzyme activity.

Total SOD activity was assayed photochemically based on the photoreduction of nitro blue tetrazolium (NBT) by light in the presence of riboflavin and methionine

(Monk et al., 1987). NBT is reduced to blue diformazan, which has a strong absorbance at 560 nm wavelength. Under aerobic assay conditions, SOD inhibits the formation of blue diformazan. The reaction mixture (1 ml) contained 100 μM dicoumarol, 1.3 μM riboflavin, 13 mM methionine, 0.05 M Na_2CO_3, 0.01 M K-phosphate buffer (pH 7.8) and 0.1 ml of the enzyme extract. NBT (63 μM) was added after 3 min. The mixtures were illuminated by fluorescent lamp (170 μE m^{-2} s^{-1}) for 3 min and the absorbance was then determined at 560 nm. Identical solutions held in the dark served as blanks.

Cu, Zn-SOD activity is inhibited by cyanide, but Fe- and Mn-SOD activities are not. Thus, Cu, Zn-SOD was estimated as total SOD activity in the absence of KCN minus activity in the presence of 1 mM KCN. Fe-SOD activity is inactivated by H_2O_2 while Mn-SOD is not. Therefore, Mn-SOD was estimated as total Fe- and Mn-SOD activity minus activity following incubation of the extract with 2 mM H_2O_2 and 1 mM KCN. Fe-SOD activity was calculated as the difference between the CN-resistant and Mn-SOD activities. Incubations were carried out at 25°C for 10 min. Protein was determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

3. Results

Reduction of chilling injury by temperature preconditioning

The control squash which were stored at 5°C from the outset of the experiment started to develop chilling injury symptoms such as surface pitting by the fourth day of storage (Fig. 1). These symptoms became even more evident after the squash were transferred to room temperature. The chilling injury symptoms in the control samples rapidly became more pronounced with increasing length of storage at

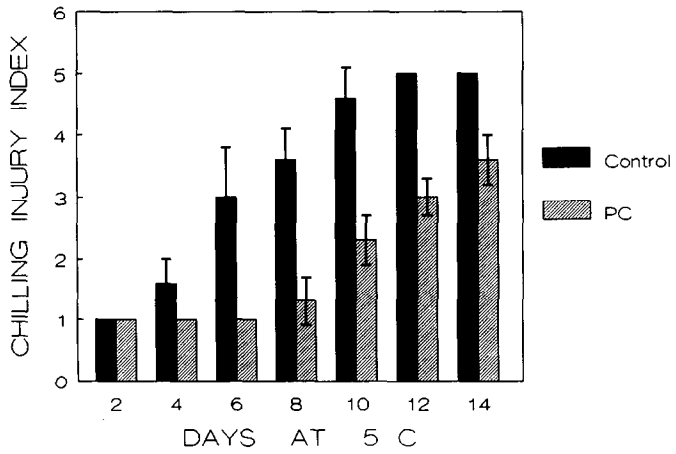

Fig. 1. Effect of temperature preconditioning (PC) on chilling injury of zucchini squash. Chilling injury index: 1 = no abnormality; 2 = trace; 3 = slight; 4 = moderate; and 5 = severe. Vertical bars represent SE.

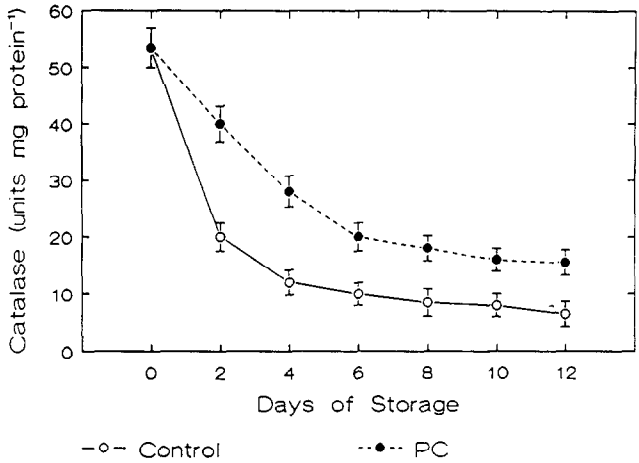

Fig. 2. Effect of temperature preconditioning (PC) on catalase activity in zucchini squash. The preconditioned squash were stored at 15°C for the first two days and then transferred to 5°C. Vertical bars represent SE.

5°C. By the tenth day, the control squash had either moderate or severe pitting. Temperature-preconditioned squash did not develop chilling injury symptoms until after eight days of storage at 5°C. Therefore, the onset of chilling injury was delayed by temperature preconditioning treatment.

Activities of catalase and peroxidase

Catalase activity in the control samples showed a marked decrease after two days of exposure to 5°C (Fig. 2). The activity continued to decline in these samples during storage at 5°C. In squash treated with temperature preconditioning, catalase activity also declined but to a lesser extent. A difference between the two treatments was apparent after only two days of storage at 5°C. This difference persisted throughout the storage period. Peroxidase activity increased steadily in the control samples during storage at 5°C (Fig. 3). After twelve days of chilling exposure at 5°C, peroxidase activity increased more than 3-fold in the control samples. A significant difference in peroxidase activity was found between temperature preconditioned squash and the control samples. Peroxidase activity also increased in temperature-preconditioned squash during storage. However, the rate of increase was much less in the treated squash than in the control samples.

Activities of superoxide dismutases

Zucchini squash contained three distinct forms of SOD: Cu, Zn-SOD; Mn-SOD; and Fe-SOD (Figs. 4 and 5). Most of the SOD activity in zucchini squash was attributed to the Cu, Zn-SOD, followed by Mn-SOD. Small amounts of Fe-SOD were also found. Total SOD; Cu, Zn-SOD; and Fe-SOD increased at the beginning of storage but declined thereafter. Mn-SOD also increased initially, but then stayed mostly at a steady level (Fig. 5). In squash treated with temperature

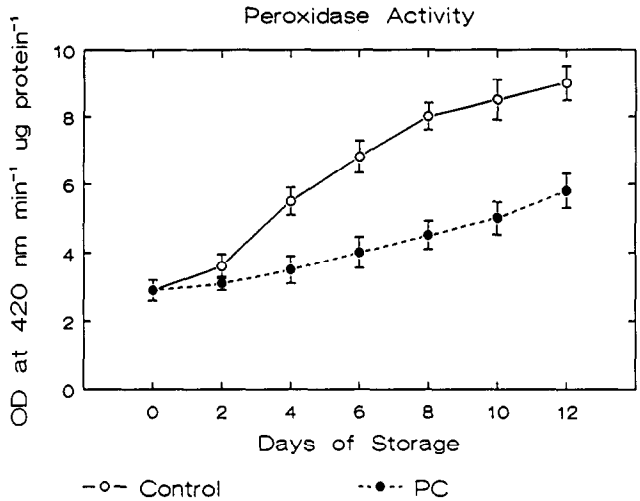

Fig. 3. Effect of temperature preconditioning (PC) on peroxidase activity in zucchini squash. The preconditioned squash were stored at 15°C for the first two days and then transferred to 5°C. Vertical bars represent SE.

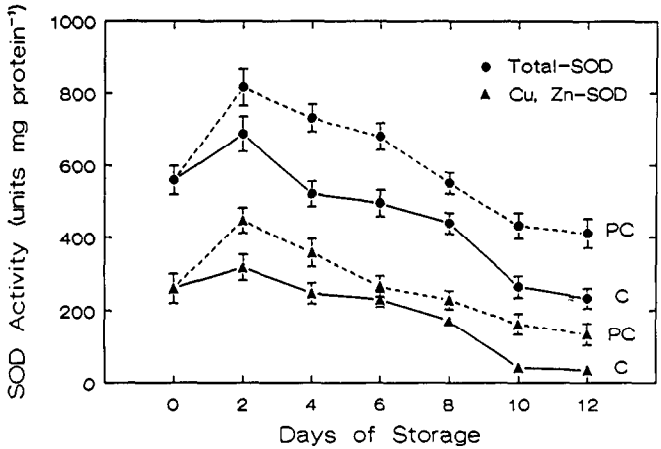

Fig. 4. Effect of temperature preconditioning (PC) on total and Cu, Zn-SOD activities in zucchini squash. The preconditioned squash were stored at 15°C for the first two days and then transferred to 5°C. Vertical bars represent SE.

preconditioning, the activities of all the SOD isoenzymes were higher than those of the control squash. The rate of decline of SOD activity including total SOD; Cu, Zn-SOD; and Fe-SOD during storage at 5°C was also slower in the treated samples compared to the control.

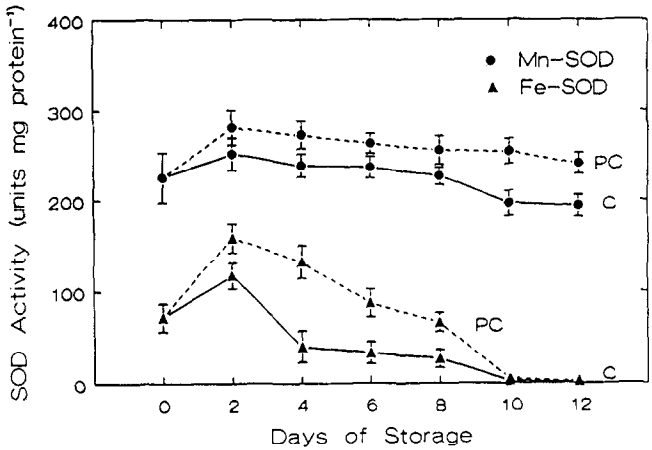

Fig. 5. Effect of temperature preconditioning (PC) on Mn- and Fe-SOD activities in zucchini squash. The preconditioned squash were stored at 15°C for the first two days and then transferred to 5°C. Vertical bars represent SE.

4. Discussion

The onset of chilling injury in zucchini squash stored at 5°C was delayed by temperature-preconditioning treatment (Fig. 1). While the use of temperature preconditioning to reduce chilling injury has been previously reported for a number of other fruits and vegetables, the underlying mechanism of this technique is still not fully understood (Wang, 1993). Zucchini squash conditioned at 15°C for two days were found to maintain higher levels of the phospholipids phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and phophatidylinositol; and the galactolipids monogalactosyldiacylglycerol and digalactosyldiacylglycerol during chilling at 5°C (Wang et al., 1992). Increases in phosphatidylcholine and phosphatidylethanolamine during the temperature conditioning of cucumber plants were also associated with a greater tolerance to chilling (Horvath et al., 1983).

Free-radical processes are involved in several membrane-associated disorders including chilling injury (Purvis and Shewfelt, 1993). Superoxide and hydrogen peroxide produced in plant tissues exposed to low temperature may cause the peroxidation of membrane lipids. Chilling cucumber leaf tissue caused higher production of superoxide radicals which subsequently leads to the peroxidation of membrane lipids (Hodgson and Raison, 1991b). Hariyadi and Parkin (1993) found that lipid fluorescent pigments accumulated in both the phospholipid and glycolipid fractions of thylakoid lipids extracted from rewarmed cucumber seedlings after only one or two days of chilling at 4°C, indicating that peroxidation of thylakoid lipids was induced during the rewarming period. Kramer and Wang (1989) also showed that the levels of chloroform-soluble fluorescent products in the skin of zucchini squash increased after chilling and these levels were reduced by temperature conditioning. These results suggest that chilling injury is related to lipid peroxidation caused by

accumulation of free radicals and that temperature preconditioning may reduce chilling injury by protecting membrane lipids from peroxidation.

Several active free-radical scavenging enzyme systems exist in plant tissues as defenses against free-radical attack. Catalase uses hydrogen peroxide both as a donor of hydrogen and as a substrate in the catalytic decomposition of hydrogen peroxide to form oxygen and water (Burris, 1960). However, catalase activity was found to decrease in zucchini squash tissue during chilling (Fig. 2). Decrease in catalase activity during chilling has also been found in avocado fruit (Sharon and Kahn, 1979), cucumber leaves (Omran, 1980), and maize leaves (Taylor et al., 1974). On the other hand, peroxidase activity increased steadily in zucchini squash during storage at 5°C (Fig. 3). Chilling temperatures also enhanced peroxidase activity in mango fruit (Zauberman et al., 1988) and avocado fruit under restricted ventilation (Van Lelyveld and Bower, 1984). Peroxidases are ubiquitous enzymes that have diverse biochemical functions in higher plants and are involved in the response of plants to stress (Gaspar et al., 1981). Severe mechanical stress has been reported to stimulate peroxidase activity after 24 h of storage in cucumber fruit (Miller and Kelley, 1989). Peroxidase has also been found to be a major protein in cucumber, enhanced by ethylene which was induced by stress (Abeles et al., 1990). Decreasing catalase activity and increasing peroxidase activity could lead to the slow removal of H_2O_2 in the tissue. Accumulation of H_2O_2 may aggravate oxidative damage, such as the oxidation of the sulfhydryl group, and intensify chilling damage in tissues. Temperature-preconditioning treatment of zucchini squash reduced the decline of catalase activity and suppressed the increase of peroxidase activity (Figs. 2 and 3). These effects may contribute to the increased tolerance of tissues to chilling injury.

Upon chilling, the cell surface in the periplasmic space of peel can result in the generation of superoxide anions. These anions are potential intermediates in the generation of hydroxyl radicals which freely attack membrane lipids and proteins (Wolf et al., 1986). SOD are a group of metalloenzymes which catalyze the dismutation of the superoxide anions to H_2O_2 and O_2. The induction of SOD is an important mechanism of cellular protection under chilling stress. For example, the chilling-resistant strain of *Chlorella ellipsoidea* contains more SOD than the chilling-sensitive strain (Clare et al., 1984). Zucchini squash contained three distinct forms of SOD: Cu, Zn-SOD; Mn-SOD; and Fe-SOD (Figs. 4 and 5). Most of the SOD activity in zucchini squash was attributed to the Cu, Zn-SOD, followed by Mn-SOD. Small amounts of Fe-SOD were also found in zucchini squash. Temperature-preconditioned zucchini squash consistently had higher SOD activities during storage than the control fruit (Figs. 4 and 5). This relationship indicates that SOD from temperature-preconditioned fruit may play a protective role by detoxifying free radicals. The superoxide radicals produced during chilling stress might be more efficiently dismutated to H_2O_2 and molecular oxygen in the preconditioned squash, rendering the fruit less susceptible to chilling injury.

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