Changes in Hydrogen Peroxide Content and Antioxidative Enzyme Activities during the Maturation of Sweet Pepper (*Capsicum annuum* L.) Fruit

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Summary

The chlorophyll content during the maturation of the sweet pepper fruit decreased steadily from the Green to the Yellow/Green stage; subsequently, there was a significant decrease to the Yellow stage. Conversely, carotenoid content increased steadily. Hydrogen peroxide increased 2.5 fold between the Green and the Green/Yellow stage; it then declined slowly from the Yellow/Green to the Yellow stages. Yet the final level was higher than that in the Green stage. Superoxide dismutase (SOD) activity increased from the Green to Yellow/Green stage, as did the ascorbate peroxidase (APX) activity. The trends in SOD and APX activities during the maturation of the sweet pepper fruit paralleled that of hydrogen peroxide. Glutathione reductase activity also increased from the Green to the Green/Yellow stage. However, catalase activity remained nearly constant during the maturation. These results indicate that the activity of antioxidative enzymes could be enhanced because of the accumulation of hydrogen peroxide during the maturation of the sweet pepper fruit.

Key Words: antioxidative enzyme, carotenoid, hydrogen peroxide, maturation, sweet pepper fruit.

Introduction

Maturation is a readily recognized phenomenon in a fruit by virtue of a series of transformations, including changes in the background color, development of flavor and aroma, and fruit softening (Leshem et al., 1986). In addition, fruit maturation can be considered as a specialized stage of plant senescence. Plant senescence is a genetically regulated, oxidative process that involves a general degradation of the cellular structures and enzymes and the mobilization of products of degradation to other parts of the plant (del Río et al., 1998). Furthermore, the phenomenon is characterized by a cessation of photosynthesis, disintegration of organelle structures, intensive losses of chlorophyll and proteins, and dramatic increases in lipid peroxidation and membrane leakiness. These changes are mainly the results of strong enhancement in the generation of activated oxygen species that takes place in plant tissues during the senescence process (Thompson et al., 1987). The oxidative damage in plant tissues is especially important during senescence and is characterized by a notable increase in the metabolism of activated oxygen species (Jiménez et al., 1997). Processes that enhance the formation of oxyradicals and initiate lipid breakdown are stimulated in the senescing plant tissue, and the increase in hydrogen peroxide or hydrogen peroxide-generating compounds promotes senescence (Bowler et al., 1992).

We reported in a previous paper that ascorbate content and the activities of the ascorbate-glutathione cycle enzymes in the sweet pepper fruit increased during maturation, and that these processes were correlated (Imahori et al., 1998). The ascorbate-glutathione cycle is implicated in the scavenging of activated oxygen species and free radicals. This cycle is an efficient way for plant cells to dispose of hydrogen peroxide in certain cellular compartments where this metabolite is produced, and it makes use of the nonenzymic antioxidants, ascorbate and glutathione, in a series of reactions catalyzed by four antioxidative enzymes (del Río et al., 1998).

Our objective in this study is to characterize the antioxidant system that is stimulated during maturation of the sweet pepper fruit by determining the levels of hydrogen peroxide and the activities of antioxidative enzymes.

Materials and Methods

Plant materials and the stage of maturity

Sweet pepper (*Capsicum annuum* L. cv. Golden Bell) fruit was grown on an experimental farm of Osaka Prefecture University in May 1997. The fruit was harvested and prepared for analysis as described previously (Imahori et al., 1998).

The process of fruit maturity was divided into four stages (Imahori et al., 1998): stage I, the Green stage, the developed fruit with a fully enlarged pod exhibiting no

yellow pigment 50 days after anthesis. The fruit is mature enough for harvest. At stage II, the Green/Yellow stage, the fruit has 10–20% yellow surface 53 days after anthesis, whereas in stage III, the Yellow/Green stage, the fruit has 50–60% yellow surface at about 57 days after anthesis. In stage IV, the Yellow stage, the fruit is completely yellow 63 days after anthesis.

Determination of chlorophyll and carotenoid

Chlorophyll was determined by the method of Inskeep and Bloom (1985) by extracting one gram of frozen pericarp tissues in an Erlenmeyer flask, containing 10 ml of N, N-dimethylformamide and 40 μ l of 20% sodium carbonate. After 24 hr in dark at 8 °C, the chlorophyll concentration was assayed spectrophotometrically at 647 and 664.5 nm.

Carotenoid was determined by the method of Hamazu et al. (1994) by homogenizing 5 grams of frozen pericarp tissues with 5 ml of 40% methanol, 0.1 g butyl hydroxytoluene, 0.2 g magnesium carbonate, and a small amount of quartz sand with a mortar and pestle. The mixture was filtered through a glass filter with 1 cm Celite 545 layer, and the solid residue was washed with 40% methanol. The filtrate was discarded; the residue was extracted with acetone until the resulting filtrate became colorless. The acetone extracts were combined and transferred to diethylether; the combined ether extract was washed with saturated NaCl solution until the alkali could not be detected, and then partitioned with water. The ether solution was dehydrated by anhydrous Na_2SO_4 for 1 hr and the mixture filtered; the residue was washed with additional ether. The absorption of the ether extract was recorded at 451 nm by a spectrophotometer, and the total carotenoid content was calculated as β -carotene.

Extraction and measurement of hydrogen peroxide

Hydrogen peroxide was assayed by the method of Okuda et al. (1991) by grinding two grams of frozen pericarp tissues in 20 ml of 0.2 N HClO_4 and 1 g quartz sand with a mortar and pestle at 0 °C. The slurry was centrifuged at $20,000 \times g$ for 5 min. The separated supernatant was neutralized to pH 7.5 with 4 N KOH to remove HClO_4 and the solution centrifuged at $1,000 \times g$ for 1 min. An aliquot (200 μ l) of supernatant was applied to a 1 ml column of AG-1 (0.8 cm i.d. $\times$ 2 cm; Bio-Rad), and the column was washed with 800 μ l of distilled water. The eluate was assayed for hydrogen peroxide by mixing 1 ml of the eluate, and 400 μ l of 12.5 mM 3-dimethylammonobenzoic acid in 0.375 M phosphate buffer (pH 6.5), 80 μ l of 1.3 mM 3-methyl-2-benzo-thiazolinone hydrazone and 20 μ l (0.25 units) horseradish peroxidase (Oriental Yeast Co., Ltd. Japan) for a total volume of 1.5 ml. The reaction was initiated by the addition of peroxidase at 25 °C, and the increase in absorbance at 590 nm was monitored and compared with increases elicited by standard samples of hydrogen

peroxide.

Extraction and assay of SOD

Five grams of frozen pericarp tissues were homogenized in a cooled mortar and pestle with 30 ml of ice-cold 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 5% polyvinylpyrrolidone. The homogenate was filtered through two layers of miracloth and the filtrate centrifuged at $15,000 \times g$ for 20 min at 4 °C. The supernatant was dialyzed overnight against 5 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA; an aliquot of dialyzed solution was assayed for superoxide dismutase (SOD) according to Crapo et al. (1978). The amount of SOD required to inhibit the rate of reduction of cytochrome C by 50% was defined as one unit of activity.

Extraction and assay of APX, CAT and GR

Ten grams of frozen pericarp tissues were homogenized in a cooled mortar and pestle with 50 ml of ice-cold 50 mM potassium phosphate buffer (pH 7.0), containing 1 mM ascorbate, 1 mM EDTA, and 5% polyvinylpyrrolidone. The homogenate was filtered through two layers of miracloth, and the filtrate was centrifuged at $10,000 \times g$ for 10 min at 4 °C. An aliquot of the supernatant was assayed for ascorbate peroxidase (APX) activity, according to Nakano and Asada (1981). In assays of activity, the oxidation of ascorbate was monitored by the decrease in absorbance at 290 nm at 25 °C by a spectrophotometer; an absorption coefficient of $2.8 \cdot \text{mM}^{-1} \cdot \text{cm}^{-1}$ was used for calculating APX activity.

Catalase (CAT) was determined according to Klapheck et al. (1990) by recording the consumption of hydrogen peroxide at absorbance at 240 nm at 25 °C by a spectrophotometer; an absorption coefficient of $39.4 \cdot \text{M}^{-1} \cdot \text{cm}^{-1}$ was used for calculating CAT activity.

Glutathione reductase (GR) was determined according to Klapheck et al. (1990) by monitoring the oxidation of NADPH at 334 nm at 25 °C by a spectrophotometer; an absorption coefficient of $6.2 \cdot \text{M}^{-1} \cdot \text{cm}^{-1}$ was used for calculating GR activity.

Determination of protein

Protein in extracts was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Results and Discussion

Changes in chlorophyll and carotenoid content during maturation

In the Green stage of sweet pepper fruit, chlorophyll content was approximately 8 mg/100 g FW. It decreased steadily to the Yellow/Green stage as the fruit matured to 75% and 50% of the original level at the Green/Yellow and the Yellow/Green stages, respec-

Fig. 1. Changes in chlorophyll and carotenoid contents in sweet pepper fruits harvested at different stages of maturity. The colors of the fruits at different stages are: Green=100%, Green/Yellow=10% to 20% yellow, Yellow/Green=50% to 60% yellow, Yellow=100% yellow. Vertical bars indicate standard errors of means ($n=3$).

□ chlorophyll ■ carotenoid

tively. A significant decrease in chlorophyll content occurred at the Yellow stage (Fig. 1). In contrast, the carotenoid content of sweet pepper fruit increased steadily during maturation. From approximately 2 mg/100 gFW in the Green stage, it increased by 1.9, 2.4, and 3.0 fold in the Green/Yellow, Yellow/Green to the Yellow stages, respectively (Fig. 1). Carotenoid acts as an antioxidant to protect cells and organisms from oxidative damage. This protection is attributed to their ability to quench active oxygen species (Gross, 1991). In addition, carotenoid is an essential component of thylakoid membranes which can quench singlet oxygen extremely rapidly. During the maturation of the sweet pepper fruit, the ratio of carotenoid to chlorophyll increased with the chlorophyll degradation, and provided an additional means of scavenging active oxygen species (Fig. 1).

Changes in hydrogen peroxide content during maturation

Hydrogen peroxide content which was approximately $0.9 \mu\text{mol}/100 \text{ g FW}$ in the Green stage of sweet pepper fruit increased by 2.5 fold in the Green/Yellow stage. It then slowly declined in the Yellow/Green stage to the Yellow stage. The final level was still higher than that in the Green stage (Fig. 2).

Much of the hydrogen peroxide present in plant cells is usually formed by the action of SOD, which is principally located in chloroplast. Hydrogen peroxide is a strong oxidant that initiates localized oxidative damage that leads to disruption of metabolic function and loss of cellular integrity at sites where it accumulates (Foyer et al., 1997). Hydrogen peroxide also causes DNA

Fig. 2. Changes in hydrogen peroxide content in sweet pepper fruits harvested at different stages of maturity. Colors of maturing fruits are the same as in Fig. 1. Vertical bars indicate standard errors of means ($n=3$).

breakage and inactivates thiol-containing enzymes, such as thioredoxin-modulated enzymes of the chloroplast stroma (Alscher et al., 1997).

Frenkel (1978) reported that the onset of maturation in the Bartlett pear was accompanied by an increase in hydrogen peroxide levels, and that the maturation process could actually be delayed by inhibiting the formation of hydrogen peroxide. Thompson et al. (1987) summarized that pear and banana fruits contained fluorescent products of lipid peroxidation that increased during natural or ethylene-induced maturation, and that mature fruits acquired enhanced levels of hydrogen peroxide and lipid hydroperoxides. In addition, the accumulation of hydrogen peroxide signals oxidative stress in the compartments where it originates and leads to an appropriate response in the cellular defense systems (Foyer et al., 1997). This oxidative function is intimately associated with the redox balance within plant cells, to which hydrogen peroxide plays an important role. The redox state is implicated in the activation of genes that leads to acclimation, stress tolerance, and other defense responses (Foyer et al., 1997). Therefore, hydrogen peroxide has many important metabolic roles during the maturation of the sweet pepper fruit.

Changes in antioxidative enzymes activities

Senescence causes dramatic increases in lipid peroxidation and membrane leakiness which are primarily the results of a strong enhancement in the generation of activated oxygen species that occur during the senescence process (del Río et al., 1998). These harmful reactions are minimized by enzymatic and nonenzymatic antioxidative defense systems. To resist this oxidative stress, SOD and the hydrogen peroxide-decomposing system detoxify activated oxygen species. SOD defends against oxyradical-mediated injury (Alscher et al., 1997), and catalyzes the dismutation of superoxide

Fig. 3. Changes in superoxide dismutase (SOD) activity in sweet pepper fruits harvested at different stages of maturity. Colors of maturing fruits are the same as in Fig. 1. One unit was defined as the amount of enzyme required to inhibit the reduction rate of cytochrome C by 50%. Vertical bars indicate standard errors of means ($n=3$).

radicals to molecular oxygen and hydrogen peroxide. Superoxide radicals are also strong oxidants which can cause irreparable metabolic dysfunction and cell death (del Río et al., 1998).

SOD activity increased 1.8 fold from the Green to the Green/Yellow stage and then 2 fold to the Yellow/Green stage. The activity declined slightly to the Yellow stage (Fig. 3). The changes in SOD activity during the maturation of sweet pepper fruit tended to parallel those of the hydrogen peroxide content. These results imply that the activities of the superoxide radical-detoxifying enzyme keep abreast of the formation of the radicals during the maturation of the sweet pepper fruit. Furthermore, SOD activities correlate well with carotenoid synthesis. Rabinowitch et al. (1982) found that susceptibility of tomato, cucumber, and pepper fruit to sunscald is related to photoinhibition caused by a combination of light and heat. Hence SOD, which scavenges the superoxide, appears to supplement the protective action of carotenoid against photooxidative injury because the enzyme is intertwined with other enzymes and antioxidants in what is likely a highly optimized balance that reduces the risk of hydroxyl radical formation.

The seasonal trend of APX activity during maturation closely paralleled that of SOD activity and hydrogen peroxide content (Fig. 2, 3, and 4). The ascorbate-glutathione cycle is an efficient system to metabolize hydrogen peroxide in certain cellular compartments because the substrates, ascorbate and glutathione, are catalyzed by four antioxidative enzymes (del Río et al., 1998). We recently reported that the ascorbate content and activities of the ascorbate-glutathione cycle enzymes in sweet pepper fruit increased during maturation (Imahori et al., 1998). GR activity during the

Fig. 4. Changes in ascorbate peroxidase (APX) activity in sweet pepper fruits harvested at different stages of maturity. Colors of maturing fruits are the same as in Fig. 1. Vertical bars indicate standard errors of means ($n=3$).

Fig. 5. Changes in glutathione reductase (GR) activity in sweet pepper fruits harvested at different stages of maturity. Colors of maturing fruits are the same as in Fig. 1. Vertical bars indicate standard errors of means ($n=3$).

maturation of the sweet pepper fruit also increased from the Green to the Green/Yellow stage by 2.1 fold, and remained high to the Yellow stage (Fig. 5). GR maintains the glutathiones in its reduced form (Foyer et al., 1997), which has a pivotal role in antioxidant defense, particularly as a reductant for dehydroascorbate. It also functions as an antioxidant, intimately involved with the redox balance of the cell. Römer et al. (1992) reported an increased GR activity and a concurrent accumulation of glutathione during the maturation of the sweet pepper fruit.

During senescence, while a strong oxidative damage takes place, the ascorbate-glutathione cycle could inhibit any enhancement of the activated oxygen species (Jiménez et al., 1997). Pastori and del Río (1997) recently demonstrated that peroxisomal membranes of senescent leaves increased the NADH-dependent generation of superoxide radicals and hydrogen peroxide

concentration significantly. Therefore, the diffusion of these superoxide radicals into the cytosol which accompanies senescence could react with excess hydrogen peroxide that easily leaks out of peroxisomes. The hydrogen peroxide might act in the cytosol as a second messenger in cellular signal transduction pathways that lead to specific gene expression (del Río et al., 1998). But, such signaling is intimately associated with the redox balance within plant cells and modification of the balance via changes in oxidant production may lead to more specific changes than those elicited by hydrogen peroxide per se (Foyer et al., 1997). Jiménez et al. (1997) postulated that during senescence, oxidative injuries could be accelerated in the mitochondria because of peroxisomes that depress the antioxidant system; the result is an enhanced hydrogen peroxide production and membrane damage. They surmised that finally, a buildup of hydrogen peroxide and a decrease of APX and monodehydroascorbate reductase activities enhances this potentially serious situation for all cellular compartments because of the formation of strongly oxidizing $\cdot\text{OH}$ radicals that are produced by the reaction between hydrogen peroxide and superoxide radicals.

CAT activity remained approximately constant during the maturation (Fig. 6). Rajatsubhra and Krishanu (1992) reported that in primary leaves of rice, APX increased but CAT activity remained at a steady level during development. Consequently, the inhibition of CAT activity elicited a restrictive and/or adaptive response whereby APX and monodehydroascorbate reductase activities were enhanced. However, Pastori and del Río (1997) found that during senescence, there is an enhancement in the activity of activated oxygen-producing enzymes in leaf peroxisomes, whereas CAT activity decreases rapidly, implying that an accumulation of toxic oxygen species could take place in

peroxisomes.

We determined that an increase in hydrogen peroxide levels and an activation of antioxidative enzymes occur during the maturation of the sweet pepper fruit. The elevation of the hydrogen peroxide level initiates localized oxidative damage that lead to disruption of metabolic function and losses of cellular integrity, which in turn, change the redox status of surrounding cells and tissues where at relatively low concentrations hydrogen peroxide initiates an antioxidative response. Our data confirms the belief of Durnger et al. (1997) that hydrogen peroxide plays key roles in the defense responses by serving as a second messenger for the activation of defense gene expression during the maturation of sweet pepper fruit.

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Fig. 6. Changes in catalase (CAT) activity in sweet pepper fruits harvested at different stages of maturity. Colors of maturing fruits are the same as in Fig. 1. Vertical bars indicate standard errors of means (n=3).

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ピーマン果実の成熟に伴う過酸化水素含量と抗酸化酵素の活性の変化

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摘 要

ピーマン果実の成熟に伴いクロロフィル含量は Green から Yellow/Green の熟度段階において徐々に減少し、Yellow の熟度段階で著しく減少した。対照的に、カロテノイド含量は熟度の進展に伴って徐々に増加した。ピーマン果実の成熟に伴う過酸化水素含量は Green/Yellow の熟度段階で著しく増加し、その増加量は Green の熟度段階の 2.5 倍であり、Yellow/Green の熟度段階と Yellow の熟度段階でやや減少したが、それら両方のレベルは Green の熟度段階より高かった。ピーマン果実の成熟に伴うスーパーオキシドジスムターゼ (SOD) の活性は Green から Yellow/Green の熟度段階で増加

し、アスコルビン酸ペルオキシダーゼ (APX) の活性も Green から Yellow/Green の熟度段階で増加した。ピーマン果実の成熟に伴う SOD と APX の活性変化の傾向は、過酸化水素含量の変化の傾向と同じであった。ピーマン果実の成熟に伴うグルタチオン還元酵素 (GR) の活性は Green から Green/Yellow の熟度段階で増加した。しかしながら、ピーマン果実のカタラーゼ (CAT) における活性の変化は、成熟中ほぼ一定であった。これらの結果はピーマン果実の成熟に伴い過酸化水素が蓄積し、そのために抗酸化酵素の活性が高められたことを示している。