CHAPTER 3

Materials and Methods

Laboratory equipment and chemicals

Laboratory equipment

1. Precision balance (PA4102, Ohaus, USA)
2. Analytical balance (PA214C, Ohaus, USA)
3. Pocket refractometer (PAL-1, Atago, Japan)
4. Water bath (Eco Temp TW 20, Julabo, USA)
5. pH meter (Delta 320, Mettler Toledo, Switzerland)
6. Microcentrifuge (Biofuge Pico, Sorvall, Germany)
7. Colorimeter (MiniScan XE Plus, HunterLab, USA)
8. Thermocycler (T Professional Basic, Biometra, USA)
9. Firmness tester (Hunter Spring LKG-10, Ametek, USA)
10. Vortex-genie 2 mixers (G560E, Scientific Industries, USA)
11. Gel documentation systems (Gel Doc™ 2000, Bio-Rad, USA)
12. Refrigerated compact centrifuge (Himac CF7D2, Hitachi, Japan)
13. Visible spectrophotometer (Genesys 20, Thermo Scientific, USA)
14. UV/VIS spectrometer (Lambda25, Perkin Elmer Instruments, UK)
15. Conductivity meter (FiveEasy FE 30, Mettler Toledo, Switzerland)
16. Agarose gel electrophoresis systems (Sub-Cell® GT, Bio-Rad, USA)
17. Multi position hotplate magnetic stirrers (LD-846, Labinco, Netherland)
18. Refrigerated centrifuge (IEC Multi RF, Thermo Electron Corporation, USA)

Chemicals

1. Chemicals for mango immersion
   - Distilled water
   - Azoxystrobin (Syngenta, Thailand)

- Salicylic acid (SA) (Rankem, India)
- Methyl jasmonate (MJ) (Meiji, Japan)

2. Chemicals for analysis of fruit quality

- Citric acid (Ajax Finechem, Australia)
- Phenolphthalein (BDH Chemicals, England)
- Sodium hydroxide (NaOH) (Merck, Germany)

3. Chemicals for analysis of free radicals content

- Ether (Merck, Germany)
- Sodium nitrite (QRec, New Zealand)
- α-Naphthylamine (Sigma-Aldrich, USA)
- 2-Deoxy-D-ribose (Sigma-Aldrich, USA)
- Potassium iodide (KI) (Merck, Germany)
- Trichloroacetic acid (TCA) (Merck, Germany)
- 2-Thiobarbituric acid (TBA) (Merck, Germany)
- Potassium dihydrogen phosphate (Merck, Germany)
- Hydroxylammonium chloride (Loba Chemie, India)
- 4-Aminobenzenesulphonic acid (Loba Chemie, India)
- Di-potassium hydrogen phosphate (Merck, Germany)
- Hydrogen peroxide (H_2O_2) (Carlo Erba Reagent, Italy)
- Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, USA)

4. Chemicals for analysis of membrane damage

- Linoleic acid (Sigma-Aldrich, USA)
- Potassium tartate (J.T. Baker, USA)
- Sodium hydroxide (NaOH) (Merck, Germany)
- Sodium carbonate (Na_2CO_3) (Merck, Germany)
- Bovine serum albumin (BSA) (Merck, Germany)
- Folin-Ciocalteu's phenol reagent (Merck, Germany)
- Polyvinyl polypyrrolidone (PVPP) (Sigma Aldrich, USA)
- Copper(II) sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) (Ajax Finechem, Australia)

5. Chemicals for analysis of antioxidant enzyme activity

   - Xanthine (Sigma-Aldrich, USA)
   - Xanthine oxidase (Sigma-Aldrich, USA)
   - L-ascorbic acid (AsA) (Sigma-Aldrich, USA)
   - Cytochrome C from horse heart (Sigma-Aldrich, USA)

6. Chemicals for analysis of antioxidant enzyme gene expression

   - Ethanol (Merck, Germany)
   - Chloroform (Merck, Germany)
   - Isoamyl alcohol (Qrec, New Zealand**)**
   - β-Mercaptoethanol (Merck, Germany)
   - Quick Taq HS DyeMix (Toyobo, Japan)
   - Lithium chloride (LiCl) (Merck, Germany)
   - Sodium chloride (NaCl) (Merck, Germany)
   - Eight gene-specific primers (Bio Basic, Canada)
   - Polyvinylpyrrolidone (PVP) (Sigma-Aldrich, USA)
   - ReverTra Ace-α-® cDNA synthesis kit (Toyobo, Japan)
   - RNase-free water (Phyto Technology Laboratories, USA)
   - Tris(hydroxymethyl)-aminomethane]-HCl (Merck, Germany)
   - Hexadecyltrimethylammonium bromide (CTAB) (Amresco, USA)
   - HiYield™ Gel/PCR fragments extraction kit (Real Biotech Corporation, Taiwan)

7. Chemicals for analysis of non-enzymatic antioxidant contents

   - Gallic acid (GA) (Sigma-Aldrich, USA)
   - Metaphosphoric acid (Merck, Germany)
   - Glutathione reductase (GR) (Sigma-Aldrich, USA)
   - Reduced glutathione (GSH) (Acros Organics, Belgium)
   - 2,6-Dichloroindophenol (DPIP) (Sigma-Aldrich, USA)
   - 5, 5'-Dithiobis (2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich, USA)

- β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH) (Sigma-Aldrich, USA)

8. Chemicals for analysis of total antioxidant capacity

- Methanol (Merck, Germany)
- Acetic acid (Merck, Germany)
- Sodium acetate (Merck, Germany)
- Potassium persulphate (Rankem, India)
- 1,1-Diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA)
- 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich, USA)
- (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (Sigma-Aldrich, USA)

Mango fruits

Mango fruit cv. Nam Dok Mai No. 4 (Figure 3.1) at commercial maturity (110 days after full bloom) were obtained from a commercial orchard in Noen Maprang district, Phitsanulok province, Thailand and transported within 6 hours to the Postharvest Physiology and Technology Research Laboratory, Department of Biology at Chiang Mai University. Fruits were selected for uniform size, shape, maturity, color and absence of visual defects and then cleaned with tap water. To prevent postharvest infections, the fruits were dipped in 0.06 mM azoxystrobin solution for 30 seconds and air-dried at ambient temperature before use.

Figure 3.1 Mango fruits cv. Nam Dok Mai No. 4

Experimental design

The experiments were conducted with completely randomized designs and were divided into 3 experiments: Experiment 1 the effects of SA and MJ on chilling injury and quality of mango fruit cv. Nam Dok Mai No. 4 during low temperature storage, Experiment 2 the effects of SA and MJ on free radical and membrane damage of mango fruit cv. Nam Dok Mai No. 4 during low temperature storage and Experiment 3 the effects of SA and MJ on antioxidant defense system of mango fruit cv. Nam Dok Mai No. 4 during low temperature storage.

Experiment 1

Effects of SA and MJ on chilling injury and quality of mango fruit cv. Nam Dok Mai No. 4 during low temperature storage.

The effects of SA and MJ on chilling injury and quality of mango fruit cv. Nam Dok Mai No. 4 were investigated during low temperature storage at 5 °C and at ripening stage.

The fruits were randomly divided into five groups of 252 fruits, which were subdivided into 3 subgroups of 84 fruits each for three replications. Each group was dipped in aqueous solutions of 0.1 mM SA, 1 mM SA, 0.1 mM MJ, 1 mM MJ or distilled water (control) at 25±2 °C for 10 minutes. They were then air-dried at ambient temperature for 30 minutes, packed in corrugated cardboard boxes (12 fruits per box) (Figure 3.2a) and then stored in cold room at 5±1 °C with a relative humidity (RH) of 90±2% for 42 days (Figure 3.2b). One box from each subgroup of each treatment was randomly sampled every 7 days to determine the chilling injury and fruit quality by the following methods.

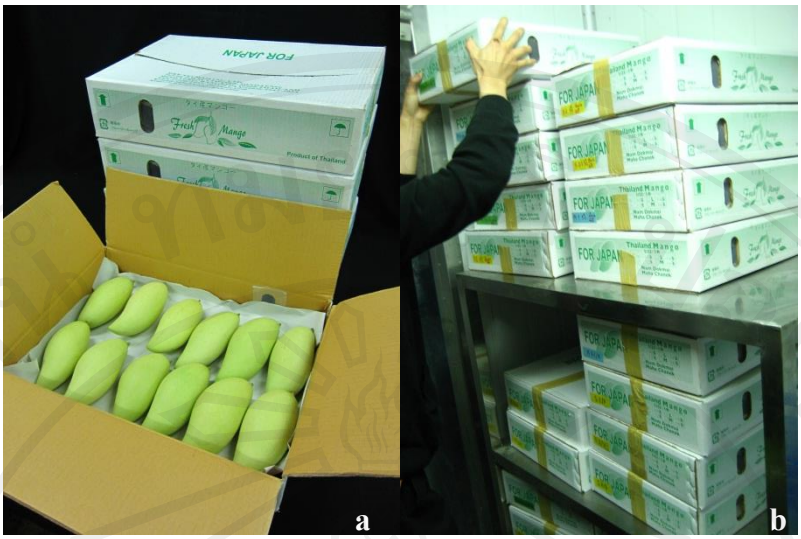

Figure 3.2 Nam Dok Mai No. 4 fruits packed in corrugated cardboard boxes (a) and then stored in a cold room (b).

1. Changes of chilling injury during low temperature storage

Visual chilling injury (CI) symptoms

The degree of CI was indicated using CI index, as described by Zhao *et al.* (2006) with some modifications. CI was scored by the percentage of surface area affected by exocarp browning compared with the total surface area (Figure 3.3).

Where 0 = no visible browning
1 = 1-10% browning
2 = 11-25% browning
3 = 26-50% browning
4 = 51-75% browning
5 = 76-100% browning

CI index was determined for each treatment by multiplying the number of fruits in each category with their CI score and then divided by the total number of fruits. Fruits having a CI index above 2 were considered as unacceptable for visual marketing quality.

0 1 2 3 4 5

Figure 3.3 CI score of Nam Dok Mai No. 4 fruits.

The other visible symptoms of CI in mango fruits such as mesocarp discoloration, pitting or sunken lesions on the exocarp and endocarp browning were also recorded.

2. Changes of fruit quality during low temperature storage

Exocarp and mesocarp color

The exocarp and mesocarp colors of mango fruits were determined with a colorimeter and reported according to CIE (Commission Internationale de l'Eclairage) L*, a*, b*, C* and hue angle (Minolta Camera Company Limited, 1990) are expressed as:

L* value is the darkness or brightness of exocarp and mesocarp colors which had value 0 to 100. A low L* value corresponded to a low brightness and a higher L* value indicated a brighter fruit.

a* value is the greenness and redness on a scale of -60 to +60. A negative a* value indicated a green color and a positive value of a* indicated red color.

b* value is the blueness and yellowness on a scale of -60 to +60. A negative b* value indicated a blue color and a positive value of b* indicated yellow color.

C* value is the saturation color.

Hue angle (h°) is the position around the color wheel or actual color.

Total soluble solids (TSS), titratable acidity (TA) and TSS/TA ratio

TSS is a measure of the sugar and organic acid contents of mango juice including other soluble components. TSS is determined by using a pocket refractometer scale 0-53%. TA measured the total available amount of hydrogen ions in the solution. Mango juice was titrated with 0.1 N NaOH according to AOAC (1990) procedures. TA was expressed as percentage of citric acid per volume of fruit juice. The TSS/TA ratio was obtained by dividing the total soluble solids by the titratable acidity.

Fruit firmness

Flesh firmness was determined using a firmness tester with a diameter tip of 6 mm and 11 mm, for unripe and ripe fruits, respectively. Fruit firmness was expressed as kg/cm^2.

Weight loss

Mango fruits were weighed at 7 day intervals and calculated for the percentage of weight loss as:

%weight loss = [(weight at day_0 – weight at day_x)/weight at day_0] ×100.

Disease index

The degree of disease in fruits was indicated using a disease index (DI) based on external damage on the exocarp, as described by Wang *et al*. (2008) with slight modifications. The DI score was assessed by the percentage of total surface area affected by disease (Figure 3.4).

Where 0 = no visible diseased area
1 = 1-25% diseased area
2 = 26-50% diseased area
3 = 51-75% diseased area
4 = 76-100% diseased area

0 **1** **2** **3** **4**

Figure 3.4 DI score of Nam Dok Mai No. 4 fruits.

The DI index was determined for each treatment by multiplying the number of fruits in each category with their score and then divided by total number of the fruits assessed.

Fruit quality at ripening stage

The quality of ripe mango fruits was investigated after the fruits were transferred from each period of cold storage to room temperature (25±2 °C, 70±2% RH) and allowed to ripen. The number of days required to ripen was counted when fruits were transferred from 5 °C storage room to room temperature (25±2 °C, 70±2% RH). Fruit quality was also determined in ripe fruits using the methods mentioned above. Sensory evaluation was carried out using 5 trained panelists. Sensory attributes assessed were appearance, taste, aroma, texture and overall acceptability of ripe mangoes. A nine-point hedonic scale in which 1 was the lowest acceptance and 9 was the highest, was used for the assessment (Wang *et al*., 2008). An average score of six or more than six was considered as acceptable for consumption. CI index was also determined in ripe mango fruits.

Experiment 2

Effects of SA and MJ on free radical and oxidative membrane damage of mango fruit cv. Nam Dok Mai No. 4 during low temperature storage.

Mango fruits were randomly divided into five groups of 252 fruits, which were subdivided into 3 subgroups of 84 fruits each for three replications. Each group was dipped in aqueous solutions of 0.1 mM SA, 1 mM SA, 0.1 mM MJ, 1 mM MJ or distilled water (control) at 25±2 °C for 10 minutes. After dipping, fruits were air-dried at ambient temperature for 30 minutes, packed in corrugated cardboard boxes (12 fruits per box) and then stored in cold room at 5±1 °C with a relative humidity (RH) of 90±2% for 42 days. One box from each subgroup of each treatment was randomly sampled every 7 days to determine free radical content and oxidative membrane damage in both exocarp and mesocarp by the following methods.

1. Changes of free radical content

Superoxide radical ($O_2^{\bullet-}$) content

$O_2^{\bullet-}$ content was determined using the protocol in Jiang *et al*. (2010) with some modifications. Exocarp and mesocarp tissues (1 g) were homogenized with a mortar and pestle at 4 °C in 6 ml of 65 mM potassium phosphate buffer pH 7.8. Then 2 ml of 10 mM hydroxylammonium chloride and 2 ml of 0.1 M EDTA were added. The homogenate was centrifuged at 12,000 × g, 4 °C for 15 minutes. Then 2 ml of the supernatant with 2 ml of 17 mM 4-aminobenzenesulphonic acid were added to 2 ml of 7 mM α-naphthylamine. The mixture was placed in a water bath for 15 minutes at 40 °C before being mixed with 2 ml of ether. After being centrifuged at 3,000 × g for 15 minutes, the absorbance of water phase was measured with a visible spectrophotometer at 530 nm. The standard curve was constructed with different concentrations of sodium nitrite (Appendix: Figure 1). The $O_2^{\bullet-}$ content was expressed as nmol g^{-1} FW.

Hydrogen peroxide (H_2O_2) content

H_2O_2 content was determined using the method of Velikova *et al*. (2000) with some modifications. Exocarp and mesocarp tissues (1 g) were homogenized with a mortar and pestle in 5 ml of 1% (w/v) TCA at 4 °C. The homogenate was centrifuged at 20,000 × g at 4 °C for 20 minutes and 0.5 ml of the supernatant was added to 2.4 ml of 10 mM potassium phosphate buffer pH 7.0 and 0.1 ml of 1 M KI. The absorbance of mixture was measured with a visible spectrophotometer at 390 nm and the standard curve was constructed with different concentrations of H_2O_2 (Appendix: Figure 2). The H_2O_2 content was expressed as µmol g^{-1} FW.

Hydroxyl radical ($OH^{\bullet}$) content

$OH^{\bullet}$ content was determined using the protocol of Yang *et al*. (2008) with some modifications. The exocarp and mesocarp tissues (1 g) were homogenized in 15 ml of 20 mM potassium phosphate buffer pH 6.0 by using a mortar and pestle at 4 °C. The homogenate was centrifuged at 3,000 × g at 4 °C for 20 minutes and 1 ml of the supernatant was mixed with 1.5 ml of 20 mM potassium phosphate buffer pH 6.0 containing 20 mM 2-deoxy-D-ribose for 30 minutes. Then 1 ml of 0.5% (w/v) TBA containing 1.4% (w/v) TCA was added. The mixture was heated in boiling water for 10 minutes and then cooled to room temperature. The absorbance of the mixture was measured at 532 and 553 nm. The $OH^{\bullet}$ content was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} FW.

2. Changes of oxidative membrane damage

Lipoxygenase (LOX) activity

Enzyme extraction and activities of LOX were determined according to the methods of Sovrano *et al.* (2006) and Wongsheree *et al.* (2009) with some modifications. Exocarp and mesocarp tissues (1 g) were ground in liquid nitrogen and homogenized using a mortar and pestle at 4 °C in 10 ml of 0.1 M potassium phosphate buffer pH 6.8 containing 1.5% PVPP. The homogenate was centrifuged at 12,000 × g at 4 °C for 20 minutes and the supernatant was used as an enzyme extract for LOX activity assays.

LOX activity was assayed in a 2 ml reaction mixture containing 1.85 ml of 0.1 M phosphate buffer pH 6.8, 0.1 ml of 10 mM linoleic acid and 0.05 ml of enzyme extract. Increase in absorbance due to the formation of conjugated diene fatty acid hydroperoxide was observed with a UV/VIS spectrometer at 234 nm ($E = 25\ mM^{-1}\ cm^{-1}$). The LOX activity was expressed as nmol mg^{-1} protein min^{-1}.

The activity of LOX enzyme was expressed on a protein basis. Protein content was determined according to the method of Lowry *et al*. (1951) with some modifications. The enzyme extract was mixed with 2.5 ml of an alkaline copper solution, containing 4% Na_2CO_3: 0.2 N NaOH: 1% $CuSO_4 \cdot 5H_2O$: 2% potassium tartate which had a ratio of 5: 5: 0.1: 0.1 for 15 minutes at room temperature. Then, the solution was added to 0.25 ml of 50% Folin-Ciocalteu's phenol reagent for 30 minutes. The absorbance was measured with a visible spectrophotometer at 750 nm. The standard curve was constructed with different concentrations of BSA (Appendix: Figure 3). The protein content was expressed as mg g^{-1} FW.

Malondialdehyde (MDA) content

The MDA content was determined according to the method of Hodges *et al.* (1999) with some modifications. Exocarp and mesocarp tissues (1 g) were ground in liquid nitrogen and homogenized using a mortar and pestle in 5 ml of 0.1% (w/v) TCA. The homogenate was centrifuged at 10,000 × g for 20 minutes. Then, 0.5 ml of 20% (w/v) TCA containing 0.5% (w/v) TBA was mixed with 0.5 ml of supernatant. The mixture was heated in boiling water for 10 minutes, cooled to room temperature, and centrifuged at 10,000 × g for 5 minutes. The absorbance of the supernatant was measured with a visible spectrophotometer at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The amount of MDA was calculated from the extinction coefficient of 155 $mM^{-1}\ cm^{-1}$ and expressed as nmol g^{-1} FW.

Electrolyte leakage (EL)

EL was measured according to the method of Chan *et al*. (1985), with some modifications. Ten discs, taken with a cork borer (10 mm diameter) from exocarp and mesocarp tissues, were placed in an Erlenmeyer flask with 25 ml deionised water. After

30 minutes at 25 °C, the conductivity was measured with a conductivity meter. The Erlenmeyer flask was heated at 98 °C for 15 minutes. The conductivity was re-measured after cooling. Membrane permeability units were calculated as:

EL (%) = (ion leakage at 25 °C/ion leakage at 98 °C) ×100.

Experiment 3

Effects of SA and MJ on antioxidant defense system of mango fruit cv. Nam Dok Mai No. 4 during low temperature storage.

Mango fruits were randomly divided into five groups of 252 fruits, which were subdivided into 3 subgroups of 84 fruits each for three replications. Each group was dipped in aqueous solutions of 0.1 mM SA, 1 mM SA, 0.1 mM MJ, 1 mM MJ or distilled water (control) at 25±2 °C for 10 minutes. After dipping, the fruits were air-dried at ambient temperature for 30 minutes, packed in corrugated cardboard boxes (12 fruits per box), and then stored in cold room at 5±1 °C with a relative humidity (RH) of 90±2% for 42 days. One box from each subgroup of each treatment was randomly sampled every 7 days to determine for enzymatic and non-enzymatic antioxidants and total antioxidant capacity in both exocarp and mesocarp by the following methods. For antioxidant enzymes gene expression analysis, the exocarp was removed from each fruit and immediately frozen in liquid nitrogen before storage at -70 °C until used.

1. Enzymatic antioxidants

Activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX)

Enzyme extraction and activity assays were determined using the method of Sunohara and Matsumoto (2004) with some modifications.

Enzyme extraction

Exocarp and mesocarp tissues (1 g) were ground in liquid nitrogen and homogenized using a mortar and pestle at 4 °C in 10 ml of 25 mM potassium phosphate buffer pH 7.8 containing 0.4 mM EDTA, 1mM ascorbic acid and 2% PVPP. The

homogenate was centrifuged at 15,000 × g for 20 minutes at 4 °C and the supernatant was filtered through filter paper (Whatman® No. 1, England). The filtrate was used as an enzyme extract for CAT (EC 1.11.1.6) and APX (EC 1.11.1.11) assays. For SOD (EC 1.15.1.1) assay, the enzyme extract was dialyzed against regenerated cellulose tubular membrane (Cellu•Sep®, USA) overnight with 10 mM potassium phosphate buffer pH 7.8 at 4 °C. The dialyzed extract was centrifuged at 15,000 × g for 20 minutes at 4 °C. The supernatant was used as an enzyme extract for SOD assay.

Enzyme assays

CAT activity was assayed in a 2 ml reaction mixture containing 1.9 ml of 50 mM potassium phosphate buffer pH 7.0 containing 25 mM H_2O_2 and 0.1 ml of enzyme extract. The subsequent decomposition of H_2O_2 was observed with a UV/VIS spectrometer at 240 nm (E = 0.0394 mM^{-1} cm^{-1}). The CAT activity was expressed as µmol H_2O_2 decomposed mg^{-1} protein min^{-1}.

APX activity was assayed in a 2 ml reaction mixture containing 0.5 ml of 100 mM potassium phosphate buffer pH 7.0, 0.5 ml of 1 mM AsA, 0.5 ml of 0.4 mM EDTA, 0.02 ml of 10 mM H_2O_2, 0.38 ml of distilled water and 0.1 ml of enzyme extract. The subsequent decrease in ascorbic acid was observed with a UV/VIS spectrometer at 290 nm (E = 2.8 mM^{-1} cm^{-1}). The APX activity was expressed as µmol AsA decomposed mg^{-1} protein min^{-1}.

SOD activity was assayed in a 2 ml reaction mixture containing 0.2 ml of 500 mM potassium phosphate buffer pH 7.8, 0.2 ml of 0.1 mM cytochrome C from horse heart, 0.2 ml of 1 mM xanthine dissolved in 10 mM NaOH, 0.04 ml of xanthine oxidase, 1.32 ml of distilled water and 0.04 ml of enzyme extract. The rate of reduction of cytochrome C was measured with a UV/VIS spectrometer by the initial rate of increase in absorbance at 550 nm. The SOD activity was expressed as Unit mg^{-1} protein.

The activity of each enzyme was expressed on a protein basis. Protein content was determined according to the method of Lowry *et al*. (1951) with some modifications as mentioned above.

Expression of *SOD, CAT,* and *APX* genes

Expression pattern of *SOD, CAT,* and *APX* genes were studied in the exocarp of mango fruits between control group and 1 mM SA treated group (the best treatment for enhancing SOD, CAT and APX activities).

RNA isolation

Total RNA was isolated from exocarp according to the method of Yu and Goh (2000) with some modifications. Frozen exocarp tissue (100-200 mg) was ground in liquid nitrogen and extracted with 1.5 ml of extraction buffer containing 2% (v/v) β-mercaptoethanol, 2% (w/v) CTAB, 100 mM Tris-HCl pH 7.5, 20 mM EDTA, 2 M NaCl and 1% (w/v) PVP. The homogenate was incubated in a water bath at 65 °C for 15 minutes with occasional shaking. After centrifugation at 12,000 × g at 4 °C for 15 minutes, the aqueous phase was extracted at least twice with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). Total RNA was precipitated by adding 0.25 volumes of 10 M lithium chloride to RNA solution, and kept overnight on ice. The pellet was washed twice with 70% ethanol, dried and dissolved in RNase-free water. RNA purity and concentration were determined spectrophotometrically.

Analysis of gene expression using RT-PCR technique

First-strand cDNA was synthesized with ReverTra Ace-α-® cDNA synthesis kit according to the manufacturer's instructions and used as a template in subsequent PCR reactions.

Eight gene-specific primers (Table 3.1) were designed and used for amplification of the *actin, SOD, CAT* and *APX* genes. Quick Taq HS DyeMix was used to amplify specific genes according to the manufacturer's instructions. PCR amplification was carried out by 35 three-step cycles of a 30 seconds denaturation at 94 °C, 30 seconds annealing at 50-57 °C and 50 seconds elongation at 72 °C and finally 5 minutes elongation at 68 °C in a thermal cycler. The actin gene was amplified as the control.

Table 3.1 Details of specific primers used in the analysis of gene expression.

Primers	Nucleotide sequence (5'-3')	Annealing temperature (°C)	Product size (bp)
SOD (F)	GCGATCAGTGGCGAGATCATGC	50	468
SOD (R)	CTGCAGGTAGTATGCATGCTCCC		
CAT (F)	GAGGTGCCAGTGCCAAGGG	56	602
CAT (R)	CCACTCGGGGTAGTTTCCAGCAG		
APX (F)	CCTATTATGCTTCGTCTTGCATGGC	50	594
APX (R)	GAAGGCATCTTCATCCGCAGC		
Actin (F)	GGAACTGGTATGGTCAAGGC	57	775
Actin (R)	AGTCTCATGGATACCCGCAG		

The semi-quantitative RT-PCR intensity value of a gene of interest in each sample was transformed to be a relative expression of the gene compared with actin expression by dividing it with an *actin* intensity value in each sample. Comparison of relative expressions of *SOD*, *CAT* and *APX* with *actin* in the exocarp of control and SA treated fruits during storage time (Days 0, 7, 14, 21, 28, 35 and 42) were analyzed using the Scion Image 4.03 software.

The PCR products of *SOD*, *CAT* and *APX* genes were purified using HiYield™ Gel/PCR fragments extraction kit and sequenced by Macrogen, Korea. Identification of nucleotide sequences from RT-PCR was established using the NCBI Blast program [http://www.ncbi.nlm.nih.gov/BLAST]. Alignment and comparison of sequence were made using the ClustalW program [http://www.ebi.ac.uk/clustalw].

2. Non-enzymatic antioxidants

Total phenolic content

Total phenolic content was determined by the Folin-Ciocalteu method according to the procedure of Singleton and Rossi (1965) with some modifications. The exocarp and mesocarp tissues (1 g) were homogenized in 10 ml of 80% ethanol using a mortar

and pestle at 4 °C. The homogenate was centrifuged at 16,000 × g for 20 minutes at 4 °C and 0.4 ml of the supernatant was mixed with 2 ml of 10% Folin-Ciocalteu's phenol reagent for 8 minutes. Then 1.6 ml of 7.5% Na_2CO_3 was added. The mixture was placed at room temperature for 2 hours. The absorbance was measured with a visible spectrophotometer at 765 nm. A standard curve was constructed with different concentrations of GA (Appendix: Figure 4). The total phenolic content was expressed as mg GA g^{-1} FW.

Ascorbic acid content

Ascorbic acid content was determined according to Deepa *et al*. (2006) and AOAC (1990) with some modifications. Exocarp and mesocarp tissues (2 g) were homogenized in 10 ml of 3% (w/v) metaphosphoric acid using a mortar and pestle at 4 °C. The homogenate was centrifuged at 3,000 × g at 4 °C for 20 minutes and 2 ml of the supernatant was added to 5 ml of 3% metaphosphoric acid and then titrated with 0.1 mM DPIP for end point. The ascorbic acid content was expressed as mg AsA g^{-1} FW.

Total glutathione content

Total glutathione content was determined according to Gronwald *et al.* (1987) with some modifications. Exocarp and mesocarp tissues (1 g) were homogenized in 12 ml of 5% (w/v) TCA using a mortar and pestle at 4 °C. The homogenate was centrifuged at 12,000 × g, 4 °C for 10 minutes. The supernatant was diluted 1:1 with 0.5 M potassium phosphate buffer pH 8.0 and this dilution was further diluted 1:9 in 0.1 M potassium phosphate buffer pH 8.0. The reaction medium consisted of 2.2 ml of 0.1 M potassium phosphate buffer pH 7.5 containing 5 mM EDTA and 0.2 ml of 1 mM NADPH prepared in 0.1 M potassium phosphate buffer pH 7.5 containing 5 mM EDTA and 0.2 ml (1 unit) of GR prepared in 0.1 M potassium phosphate buffer pH 7.5. These components were equilibrated in a test tube at 25 °C for 2 minutes. The reaction was initiated by adding 0.2 ml of 6 mM DTNB prepared in 0.1 M potassium phosphate buffer pH 7.5 and 0.2 ml of diluted supernatant. The change in absorbance of the mixture was measured by a UV/VIS spectrometer every 1 minute for 5 minutes at 412 nm. The rate of change in absorbance was used to calculate total glutathione

content. A standard curve was constructed with different concentrations of GSH (Appendix: Figure 5). The total glutathione content was expressed as µg GSH g^{-1} FW.

3. Total antioxidant capacity (TAC)

Crude extraction

Exocarp and mesocarp tissues (1 g) were homogenized in 10 ml of 80% ethanol using a mortar and pestle at 4 °C. The homogenate was centrifuged at 16,000 × g for 20 minutes at 4 °C and the supernatant was collected as a sample solution to determine the total antioxidant capacities by using DPPH and ABTS radical scavenging methods.

DPPH radical scavenging activity

The DPPH radical scavenging activity was determined according to the method of Mun'im *et al*. (2003) with some modifications. The volume of 0.1 ml of sample solution was mixed with 0.4 ml of 0.3 M acetate buffer pH 5.5, and 2.5 ml of 0.12 mM $DPPH^{\bullet}$ in 100% methanol was added. The mixture was placed in the dark at room temperature (25±2 °C) for 30 minutes. The absorbance was measured with a visible spectrophotometer at 517 nm. Trolox was used as a standard antioxidant and analyzed at the same time (Appendix: Figure 6). The final results were calculated and expressed as µmol Trolox g^{-1} FW.

ABTS radical scavenging activity

The ABTS radical scavenging activity was determined according to the method of Huang *et al*. (2005) with some modifications. The volume of 0.02 ml of sample solution was mixed with 2 ml of $ABTS^{\bullet+}$ solution prepared from 2 ml of 7 mM ABTS added to 3 ml of 2.45 mM of potassium persulphate. The mixture was kept in the dark at 5 °C for 16 hours to give a dark blue solution before being diluted with 80% ethanol until the absorbance at 734 nm was 0.7. The mixture was placed at room temperature for 10 minutes. The absorbance was measured with a visible spectrophotometer at 734 nm. Trolox was used as standard antioxidant and analyzed at the same time (Appendix: Figure 7). The final results were calculated and expressed as µmol Trolox g^{-1} FW.

Statistical analysis

All statistical analyses were performed using SPSS 17.0 statistical software (SPSS Incorporation Chicago, IL, USA). Data were presented as mean ± standard error and analyzed by one-way analysis of variance (ANOVA). Mean separations were performed by Tukey's multiple range tests. Differences at $P < 0.05$ were considered as significant. The correlation among variables was calculated by the Pearson Coefficient.