# CHAPTER 3
# MATERIALS AND METHODS

## 3.1 Effect of hot water treatment (HWT) on green mold infection in tangerine fruit cv. Sai Num Pung

### 3.1.1 Effect of HWT on spore germination of *Penicillium digitatum in vitro*

#### *3.1.1.1 Preparation of spore suspension*

The identified *P. digitatum* obtained from Department of Biology, Faculty of Science, Chiang Mai University. This fungus was proved by highly specific method called Koch's postulates to confirm that it can be an actual cause of green mold rot disease. Then *P. digitatum* culture was maintained on potato dextrose agar (PDA) and incubated at 24±2°C for 10 days. After that conidia were washed from the Petri dishes, using sterile distilled water and filtered through four layers of sterile cheesecloth. The concentration of conidia was measured with a haemacytometer and adjusted with sterile distilled water to $2 \times 10^5$ conidia $ml^{-1}$.

#### *3.1.1.2 Hot water treatments*

Tests on conidial viability after exposure to heat treatments were carried out. Glass tubes containing 1.2 ml of distilled water were placed in water bath at 45, 50 and 55°C. When water in the tubes reached the temperature (measured by thermometer), 0.8 ml of a concentrated *P. digitatum* spore suspension was added to the tubes to achieve a final concentration of $2 \times 10^5$ conidia $ml^{-1}$. After 0.5, 1, 2 and 3 minutes, the tubes were immediately cooled in ice water. The control tubes were placed in water bath at 20°C for 1 minute. Aliquots (50µl) of the spore suspensions were transferred to glass tubes containing 450 µl of 10% potato dextrose broth. Samples of these solutions (30 µl drops) were placed on ethanol-washed microscope

slides (three drops per slide), kept in Petri dishes padded with moistened filter paper and incubated for 24 and 48 hours at 25±2°C in darkness. Spore germination was measured in three microscope fields, each containing 180-200 spores, under a light microscope; LM (Olympus AX70TF, Olympus Optical Co., Ltd., Japan) and the experiment was repeated twice with similar procedures.

### 3.1.2 Effect of HWT on infection of *Penicillium digitatum* in tangerine fruit cv. Sai Num Pung

#### *3.1.2.1 Fruit preparation*

Tangerine fruit cv. 'Sai Num Pung' obtained from a commercial orchard in Amphoe Fang, Chiang Mai province, Thailand. The fruit were harvested in November, 2006 at the age of 9 months after full bloom to reach commercially mature. After being harvested, the fruit were selected at random to have equal size from plastic containers before any commercial postharvest treatment was imposed. The fruit were transported to Department of Plant Science and Natural Resource, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand on the day of harvest and stored at room temperature (20±3°C) overnight before receiving their respective treatments the following day. Samples of blemish-free and uniform fruit were washed with water at room temperature (23±2°C), air-dried and placed at random in plastic baskets.

#### *3.1.2.2 Postharvest HWT and storage conditions*

The fruit samples of each treatment were dipped in hot water at 45±2, 50±2 and 55±2°C for 0.5, 1, 2 and 3 minutes. The fruit were then incubated at 24±2°C and 90±5% relative humidity (RH) for 5 days. Each treatment comprised of three replicated boxes, each box containing 10 fruit.

#### *3.1.2.3 Inoculum preparation*

The fungal pathogen mentioned in 3.1.1.2 was cultured on PDA and incubated at 24±2°C for 10 days. The conidia were washed off PDA surface, using sterile distilled water containing 10 drops $l^{-1}$ of Tween 20 and filtered through four layers of sterile cheesecloth. The concentration of conidia was measured with a haemacytometer and adjusted with sterile distilled water to $4\times10^5$ conidia $ml^{-1}$.

### *3.1.2.4 Hot water treatments*

Tangerine fruit were wound-inoculated with a sterile dissecting needle (2 mm long and 1 mm diameter) that had been dipped into a spore suspension ($4\times10^5$ conidia $ml^{-1}$) of *P. digitatum*. Using the needle to pierce at opposite of the fruit about the midway of the stem and stylar end to make two tiny holes (2×1 mm) per one fruit. The inoculated fruit were incubated for 3 hours after inoculation at room temperature (23±2°C). After 3 hours, the fruit of each treatment were dipped in the water bath at 45±2, 50±2 and 55±2°C for 0.5, 1, 2 and 3 minutes, respectively. Afterwards, the fruit were removed from the water bath and cooled down with tap water (25±2°C), air-dried and placed in plastic trays before loading in corrugated board box (29×41.8×9 cm; width×length×height) 3 boxes per treatment. A second batch of fruit was dipped in hot water at the temperature and time mentioned above and left for 45 minutes before inoculation. The control treatment was divided into 2 groups, one of which was inoculated with the pathogen another was inoculated with sterile water, both without hot-water treatment (untreated and uninoculated fruit). Disease index, severity (lesion diameter) and sporulation index, as well as external disease appearance, were recorded daily after inoculation at 24±2°C and 90±5% RH for 5 days. The experiment was conducted in completely randomized design (CRD) with 26 treatments. Each treatment comprised of three replicated boxes, each containing 10 fruit (total of 60 wounds/treatment), and the experiment was repeated twice with similar procedures.

The disease index described the percentage of the peel surface infected with *P. digitatum* on the following scales: **score 0** = no discolored spot and watery; **score 1** = 1-20% discolored spot and watery; **score 2** = 21-40% discolored spot, watery and white mycelium appearing on the peel surface; **score 3** = 41-60% white mycelium and green mold spores appearing on the peel surface; **score 4** = 61-80% white mycelium and green mold spores appearing on the peel surface and **score 5** = 81-100% white mycelium and green mold spores appearing on the peel surface (Figure 3.1). Disease index was calculated using the following formula.

$$\text{Disease index} = \frac{\text{Sum of disease level score}}{\text{Number of diseased fruit}} \times \frac{100}{\text{Height of disease level score}}$$

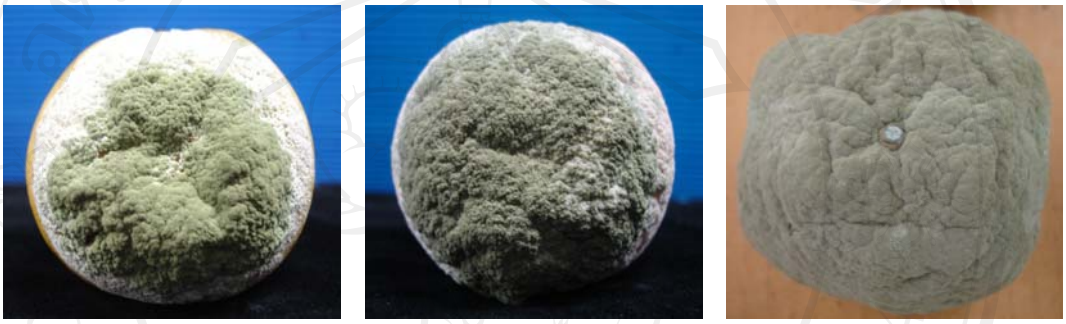
**Score 0** **Score 1** **Score 2**

**Score 3** **Score 4** **Score 5**

**Figure 3.1 Scores of the fruit surface infected with *Penicillium digitatum***
**Score 0 = no discolored spot and watery, Score 1 = 1-20%, Score 2 = 21-40%, Score 3 = 41-60%, Score 4 = 61-80% and Score 5 = 81-100% of the area appeared discolored spot, watery, white mycelium and green mold spores**

Sporulation index described the percentage of the fruit surface covered with green mold spores where **Score 0** = no sporulation on the surface of the fruit; **Score 1** = 1-20%; **Score 2** = 21-40%; **Score 3** = 41-60%; **Score 4** = 61-80% and **Score 5** > 80% of sporulation on the surface of the fruit (Figure 3.2).

**Score 0** **Score 1** **Score 2**



**Score 3** **Score 4** **Score 5**

**Figure 3.2 Sporulation index of the fruit surface covered with green mold spores**
**Score 0 = no sporulation on the surface of the fruit, Score 1 = 1-20%, Score 2 = 21-40%, Score 3 = 41-60%, Score 4 = 61-80% and Score 5 > 80% of the area covered with green mold spores**

## 3.2 Effect of HWT on anatomy and chemical component changes in tangerine fruit during infection of *Penicillium digitatum* and chilling injury under low-temperature storage

### 3.2.1 Effect of HWT on infection of *Penicillium digitatum* in tangerine fruit under low-temperature storage

#### *3.2.1.1 Fruit preparation*

Tangerine fruit cv. 'Sai Num Pung' obtained from a commercial orchard in Amphoe Fang, Chiang Mai province, Thailand. The fruit were harvested in January, 2007 at the age of 10 months after full bloom to reach commercially mature. After being harvested, the fruit were selected at random to have equal size from plastic containers before any commercial postharvest treatment was imposed. The fruit were transported to Department of Plant Science and Natural Resource, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand on the day of harvest and stored at room temperature (20±5°C) overnight and heat treatment was done on the next day. Samples of blemish-free and uniform fruit were washed with water at room temperature (24±2°C), air-dried and placed at random in plastic baskets.

#### *3.2.1.2 Postharvest HWT and storage conditions*

The fruit samples of each treatment were dipped in hot water at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes (result from section **3.1**). The fruit were then incubated at 4±2°C and 90±5**%** RH for 35 days. Each treatment comprised of three replicated boxes, each box containing 10 fruit.

#### *3.2.1.3 Inoculum preparation*

Spore suspension was prepared as described in section **3.1.2.3**.

#### *3.2.1.4 Hot water treatments*

The treatments were carried out similarly to **3.1.2.4** except that the fruit samples were dipped in the water bath at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes. Disease index, severity and sporulation index were recorded at every 5

days after inoculation at 4±2°C and 90±5% RH for 35 days. The experiment was conducted in CRD with 5 treatments. Each treatment comprised of three replicated boxes, each containing 10 fruit (total of 60 wounds/treatment), and the experiment was repeated twice with similar procedures.

### 3.2.2 Effect of HWT on chemical component changes and chilling injury in tangerine fruit under low-temperature storage

#### *3.2.2.1 Fruit preparation*

Tangerine fruit cv. 'Sai Num Pung' were prepared by the same method as section **3.2.1.1**. Fruit were dipped in the water bath at 50±2°C for 3 minutes and 55±2°C for 2 and 3 minutes compared with control. Afterwards, fruit were removed from the water bath and cooled down with tap water (25±2°C), air-dried and placed in plastic trays before loading in corrugated board boxes. After treatments, the fruit were incubated at 2±2°C and 90±5% RH for 30 days. The chilling injury index, electrolyte leakage and SSC were measured at every 5 days. The experiment was conducted in CRD with 4 treatments. Each treatment comprised of three replicated boxes, each box containing 10 fruit, and the experiment was repeated twice with similar procedures.

#### *3.2.2.2 Chilling injury evaluation*

Chilling injury of tangerine fruit was monitored by observing the rind pitting, sunken areas and watery breakdown symptoms on the peel surface. Appearance was estimated by measuring the extent of total rind pitting and/or discoloured patches and/or watery breakdown areas on each fruit peel on the following scales: **score 0** = no pitting and discoloured patches (excellent quality); **score 1** = 1-25% pitting and/or discoloured patches; **score 2** = 26-50% pitting and/or discoloured patches; **score 3** = 51-75% pitting and/or discoloured patches and **score 4** = 76-100% pitting and/or discoloured patches and/or watery breakdown (poor quality). A rating scale from 1 to 5, based on necrotic surface and intensity of browning, was used to evaluate chilling injury and the average chilling injury index determined as indicated in the following formula.

$$\text{Chilling injury index} = \frac{\Sigma\ (\text{injury level score} \times \text{number of injured fruit})}{\text{Total numbers of fruit}}$$

### *3.2.2.3 Determination of electrolyte leakage*

Electrolyte leakage of flavedo tissue was determined following the procedure of McCollum and McDonald (1991) with slight modification. Discs of peel tissue were taken from the equator of each fruit using a 1 cm diameter stainless steel cork borer. Ten discs weighing about 1 g, were rinsed 3 times in deionized distilled water, dried with tissue paper and placed in 30 ml of 0.4 M mannitol solution and shaken at 100 cycles per minute. Initial electrolyte leakage was determined following incubation at room temperature for 3 hours of constant shaking. Electrical conductivity reading of the solutions (electrolyte leakage from the discs) was measured using a conductivity meter (Hanna EC 214, HANNA instruments, Protugal). The total conductivity reading was measured after completely damaged the tissue in autoclave (Speedy Autoclave Vertical Type, HL- 341, Gemmy Industrial Corp., Taiwan) at 121°C, 15 psi for 30 minutes and cooled to room temperature. The percentage of electrolyte leakage was calculated as the ratio of the initial reading to the final reading.

$$\text{Electrolyte leakage (\%)} = \frac{\text{Initial conductivity reading}}{\text{Total conductivity reading}} \times 100$$

### *3.2.2.4 Determination of soluble solid content (SSC)*

The tangerine fruit were cut into halves along the equator and juice was extracted from individual fruit using a hand juice extractor. Percentage of SSC was determined with a digital refractometer (PR-101, ATAGO Co., Ltd., Japan).

### 3.2.3 Effect of HWT on anatomy changes of tangerine fruit peel during infection of *Penicillium digitatum* under low-temperature storage

Tangerine fruit cv. 'Sai Num Pung' obtained from section **3.2.1**. Anatomical characteristics on artificially-inoculated tangerine fruit were assessed using scanning electron microscope (SEM).

#### *3.2.3.1 Preparation of tangerine fruit peel for SEM*

The tangerine peels were cut into 5 mm squares for SEM. The peels were cut in a dish of 0.1 M phosphate buffer pH 7.3. The pieces were transferred immediately after they were cut into a primary fixative. The tangerine peel pieces were fixed in a fixative solution as described by Bozzola and Russell (1999) with slight modification for anatomical study. The peel specimens were fixed with a primary fixative containing 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3 at 4°C for 2 hours. After that the tissue was washed in the same buffer solution used in the glutaraldehyde fixation step. Washing is extremely important because it eliminates any free unreacted glutaraldehyde that remains within the tissue. If aldehydes remaining from the primary fixation are oxidized by osmium tetroxide they may generate a "peppery" spot background and interfere in the specimens. Next, the specimens were post-fixed in 1% osmium tetroxide in the same buffer for 2 hours. Then, the specimens were dehydrated stepwise by exposure to ethanol-buffer mixture (30, 50, 70, 80, 90, and 100%) allowing 15 minutes in each, and critical point drying with liquid $CO_2$. This is a critical drying technique, as it achieves a phase change from liquid to dry gas without the effects of surface tension and is, therefore, suitable for delicate biological specimens for removal of water from the specimens. For SEM, the dried specimen was mounted on specimen studs and sputter coated with gold. Coated samples were stored in desiccator until assessed. Finally, the specimens were viewed with a SEM (JEOL, JSM-5910LV, JEOL Ltd., Tokyo, Japan) at 15 kV.

The tangerine fruit from section **3.2.1** and **3.2.2** were peeled and flavedo tissues were frozen in liquid nitrogen. Then sample tissues were stored at -80°C for determination of biochemical changes in section **3.3**.

## 3.3 Effect of HWT on biochemical changes in tangerine fruit peel during infection of *Penicillium digitatum* and chilling injury under low-temperature storage

### 3.3.1 Effect of HWT on activities of the defensive enzymes in tangerine fruit peel during infection of *Penicillium digitatum* under low-temperature storage

Chitinase, β-1,3-glucanase and peroxidase activities were assayed from flavedo tissues of tangerine fruit treated with HWT, then challenge-inoculated with *P. digitatum*, and compared with activities in the untreated and uninoculated fruit. Fruit samples in each treatment were obtained from section **3.2.1** and repeated likewise.

#### *3.3.1.1 Extraction of the defensive enzymes*

Chitinase and β-1,3-glucanase were extracted according to Cao and Jiang (2006). The flavedo tissues of tangerine fruit were dipped in liquid nitrogen and ground in blender. Five grams of the sampled tissue was well homogenized with 10 ml of 100 mM sodium acetate buffer, pH 5.5, containing 5 mM β-mercaptoethanol and 1 mM ethylene diaminetetraacetic acid (EDTA), and centrifuged at 9,000 × g at 4°C for 20 minutes. The supernatant was collected for the enzymatic assay.

Peroxidase was extracted according to Flurkey and Jen (1978). One gram of the sampled tissue was well homogenized with 10 ml of 50 mM sodium phosphate buffer, pH 6.2, containing 100 mM sodium chloride, and centrifuged at 9,000 × g at 4°C for 20 minutes. The supernatant was used for the enzymatic assay.

#### *3.3.1.2 Enzyme assay and protein determination*

Chitinase activity was assayed using dye-labeled carboxymethylchitin according to the method of Ippolito *et al*. (2000), with slight modification. Chitinase activity was measured by mixing 100 µl of crude enzyme solution with 300 µl of 33.33% dye-labeled carboxymethylchitin in 100 mM sodium acetate buffer pH 5.5. After 1 hour of incubation at 37°C, the reaction was stopped by adding 100 µl of 1.0 M hydrochloric acid (HCl), then cooled the reaction mixture and finally centrifuged at

6,000 x g for 10 minutes. The absorbance of the supernatant was measured at 550 nm using a spectrophotometer (SPECORD 40 analytikjena, Thermo Spectronic, UK). Chitinase activity was expressed in international units $mg^{-1}$ protein. 1 unit was defined as the amount of enzyme required to catalyze the formation of 10 mmol $min^{-1}$ of product.

β-1,3-Glucanase was assayed by measuring the amount of reducing sugar released from the substrate by the dinitrosalicitate method (Cao and Jiang, 2006). A 50 µl of the enzymatic extract plus 50 µl of 4 % (w/v) laminarin were incubated at 37°C for 30 minutes. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The reaction was stopped by adding 400 µl of 3,5-dinitrosalicilate and boiling for 5 minutes in a water bath. After cooled, absorbance of the reaction at 500 nm was measured using a spectrophotometer (SPECORD 40 analytikjena, Thermo Spectronic, UK). The enzyme activity was expressed as unit $mg^{-1}$ protein, where 1 unit was defined as the reducing sugar equivalent to 10 mmol of glucose produced per minute.

Peroxidase activity was determined using guaiacol as substrate (Flurkey and Jen, 1978). The reaction mixture consisted of 0.1 ml of crude extract, 2.4 ml of guaiacol substrate (4 mM guaiacol and 98 mM hydrogen peroxide ($H_2O_2$) of substrate in 10 mM sodium acetate pH 6.0). Specific activity was expressed as the change in absorbance at 470 nm unit $mg^{-1}$ protein. 1 unit was defined as an increase in A470 of 10 mmol $min^{-1}$ of product.

Soluble protein content was detected by calculating specific enzyme activity using the method described by Bradford (1976). The samples were measured at 595 nm using a spectrophotometer (SPECORD 40 analytikjena, Thermo Spectronic, UK) and protein concentration was determined for each sample with a bovine serum albumin (BSA) standard curve.

### 3.3.2 Effect of HWT on protein patterns in tangerine fruit peel during infection of *Penicillium digitatum* under low-temperature storage

Protein patterns were assayed from flavedo tissues of tangerine fruit treated with HWT, then challenge-inoculated with *P. digitatum*, and compared with the untreated and uninoculated fruit. The samples in each treatment were taken from section **3.2.1** and extracted protein the same as section **3.3.1.1**. Soluble protein content was determined according to the dye-binding method of Bradford (1976) with BSA as the standard. Then ten micrograms of proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electroproresis (SDS-PAGE), using a Hoefer$^{TM}$ mini VE vertical electrophoresis system (Amersham Biosciences Limited, Buckinghamshire, England) according to Laemmli's method (1970).

## SDS-PAGE (SDS-Polyacrylamide gel electroproresis) of protein

#### *3.3.2.1 Preparation of separating gel*

Gel concentration of 10% in 1.5 M Tris-HCl pH 8.8 was prepared following these processes. First, set up gel apparatus and then prepared separating gel monomer. After that added TEMED just prior to pouring gel by a precision microliter pipette (Adjustable air-displacement pipette, GILSON, France). Finally, allowed to polymerize before adding stacking gel by overlaying gently with alcohol.

**Separating gels, in 0.375 M Tris pH 8.8**

| | | |
|---|---|---|
| Distilled water | 9.70 | ml |
| 1.5 M Tris-HCl, pH 8.8 | 5.00 | ml |
| 10% (w/v) SDS | 0.20 | ml |
| 40% Acrylamide/Bis solutions | 5.00 | ml |
| 10% (w/v) ammonium persulfate | 0.10 | ml |
| TEMED | 0.01 | ml |
| Total monomer | 20.01 | ml |

String the mixer of the ingredients shown above gently, to avoid occurrence of air bubbles. The mixer was poured into a space between the two glass plates that had been prepared earlier. Using concentrated ethanol which helped to overlay the mixer

that later became gelatinous with flat surface without air bubbles. Rinsed off with deionized water after gel became solid.

***3.3.2.2 Preparation of stacking gel***

After the separating gel had polymerized, decanted the overlay, prepared the stacking monomer, added the TEMED, and pored. Inserted the comb and allowed to polymerize.

**Gel concentration of 4% in 0.125 M Tris-HCl pH 6.8**

| | | |
|---|---|---|
| Distilled water | 3.18 | ml |
| 0.5 M Tris-HCl, pH 6.8 | 1.26 | ml |
| 10% (w/v) SDS | 0.05 | ml |
| 40% Acrylamide/Bis solutions | 0.50 | ml |
| 10% (w/v) ammonium persulfate | 0.025 | ml |
| TEMED | 0.005 | ml |
| Total stack monomer | 5.02 | ml |

The stacking gel solution was poured on the top of the separating gel, inserted comb, allowed to set, removed comb, filled with electrophoresis buffer.

***3.3.2.3 Running the gel***

The running buffer composition was 192 mM glycine, 0.1% SDS, 8.3 mM Tris-HCl pH 8.3, made by diluting a 5× stock solution to 1× before using.

***3.3.2.4 Sample preparation***

The samples were diluted with sample buffer containing 0.5 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.05% bromophenol blue at the rate of 1:1. Before loading the samples on stacking gel, the samples were heated at 95°C for 5 minutes and transfer the samples to the buffer on the stacking gel. The volume loaded per well was 20 µl (for 7 lane comb). The concentration of protein was about 10 µg per well. Molecular mass markers were run on border of the gel. Connected up the apparatus and ran gel at a constant voltage of 120 volt until the dye migrated toward the end of the gel. The gels were then removed from the glass plates.

***3.3.2.5 Staining of gels with Coomassie Brilliant Blue (CBB) R-250***

Preparing stain 0.1% CBB R-250 in methanol and acetic acid (methanol 50 ml, water 40 ml, acetic acid 10 ml). Pouring the staining solution to the gel in the

plastic box, the solution was agitated on the shaker to allow the stain to cover the gel evenly and left overnight. The gel was then destained with methanol and acetic acid (methanol 25 ml, water 68 ml, acetic acid 7 ml).

#### *3.3.2.6 Rf Value of protein and marker dye*

The Rf value of protein and marker dye was calculated by following formula:

$$\text{Mobility (Rf)} = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

The Rf values were plotted with standard molecular weight and expressed on a semi-logarithmic scale. The standard proteins used in the present study were Full-Range Rainbow Molecular Weight Markers 12-225 kDa (GE Healthcare Bio-Sciences).

### 3.3.3 Effect of HWT on lipid peroxidation in tangerine fruit peel under low-temperature storage

Malondialdehyde concentration and lypoxygenase activity, based on lipid peroxidation, were assayed from flavedo tissues of tangerine fruit treated with HWT and compared with control fruit. Fruit samples in each treatment were obtained from section **3.2.2** and repeated likewise.

#### *3.3.3.1 Determination of malondialdehyde (MDA) concentration*

MDA concentration was measured according to Mao *et al*. (2007) with modification. The flavedo tissues of tangerine fruit were dipped in liquid nitrogen and ground in blender. Two grams of sample was homogenized in 5 ml of 0.1% (w/v) trichloroacetic acid. The homogenate was spun at 6,000 x g for 10 minutes. The supernatant was collected and 1 ml was mixed with 4 ml of 20% (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. The mixture was heated at 95°C for 15 minutes, then cooled quickly and finally centrifuged at 6,000 x g for 10 minutes to clarify the solution. The supernatant was used to measure the absorbance at 450, 532 and 600 nm, respectively. The MDA concentration was calculated according to the formula:

$$\text{MDA concentration } (\mu\text{mol g}^{-1}\text{ FW}) = \frac{[6.45\,(OD_{532}-OD_{600}) - 0.56\,(OD_{450})]}{8}$$

### *3.3.3.2 Extraction of lipoxygenase*

The LOX activity was determined according to method of Lara *et al*. (2003) with modification. The flavedo tissues of tangerine fruit were dipped in liquid nitrogen and ground in blender. Two grams of sample was homogenized with 6 ml of 0.1 M potassium phosphate buffer pH 7.5 containing 2 mM DL-Dithiothreitol (DTT), 1 mM EDTA, 0.1% (v/v) Triton X-100 and 1% (w/v) polyvinylpyrrolidone (PVPP). The homogenate was centrifuged at 15,000 x g at 4°C for 20 minutes. The supernatant was collected and used as a crude enzyme.

### *3.3.3.3 Enzyme assay and protein determination*

LOX activity was assayed with linoleic acid as substrate by a spectrophotometric procedure (SPECORD 40 analytikjena, Thermo Spectronic, UK). The assay was performed by mixing to 2.5 ml of 0.1 M potassium phosphate buffer pH 8.0 in a cuvette and added 400 µl of substrate solution containing 8.6 mM linoleic acid, 0.25% (v/v) Tween-20, 10 mM sodium hydroxide (NaOH) in 0.1 M potassium phosphate buffer pH 8.0 and 100 µl of the crude enzyme. The increase in absorbance at 234 nm at 25°C was record automatically for 3 minutes, due to the formation of hydroxyperoxides from linoleic acid during the catalytic reaction. One unit of enzyme activity was defined as the amount of the enzyme, which caused a change of 0.001 in absorbance per minute. Soluble protein content was determined as described in section **3.3.1.2**.

## 3.4 Statistical data analysis

All statistical analyses were performed using SPSS version 6.0. The data were analyzed by one-way analysis of variance (ANOVA). Mean separation within each inspection time was calculated where applicable, using the least-significant difference (LSD) test together with Duncan's multiple range test. Differences at $P$=0.05 were considered as significant.