CHAPTER 3

MATERIALS AND METHODS

3.1 Experiment I Effect of UV-B irradiation on chlorophyll-degrading enzyme activities and postharvest quality in stored lime (*Citrus latifolia* Tan.) fruit

Plant materials and UV-B treatment

Mature green Tahitian lime (*C. latifolia* Tan.) fruit were purchased from Tokio-Fukuoka Co., Ltd., Japan. After transportation to the laboratory, fruit were selected for uniformity in maturity, size, shape, peel color and lack of defects. Fruit were irradiated with UV-B (spectral peak value: 280-315 nm, MK Scientific, Inc.). Each fruit was placed under UV-B lamps at a distance of 15 cm, resulting in UV-B energy doses of 0 (0min; control) and 19.0 (20 min) kJm^{-2}. A layer of aluminum film was placed under the fruit to ensure that UV-B would be irradiated to the bottom of the fruit. After irradiation, the fruit were kept in polyethylene film bags (0.03mm in thickness) with the top folded over and stored at 25 °C in the dark. The samples (3 replications) were removed at 5 d intervals, and the peel tissues were sampled and used for analysis. The fresh weight of each lime fruit was monitored every 5 d and data were expressed as percent of weight loss.

Surface colour and chlorophyll assays

The surface color of lime fruit was determined by measuring the hue angle with a colorimeter (Nippon Denshoku NF 777). Chl content was determined using *N,N*-dimethylformamide (Moran, 1982).

Preparation of substrates

1. Chlorophyll a

Spinach leaves were homogenised for 3 min in cold acetone (-20 °C). The homogenate was filtrated through two layers of Miracloth (Calbiochem, USA). The filtrates were treated with dioxane and distilled water and then kept for 1 h on ice. The filtrates were centrifuged at 10,000g for 15 min at 4 °C. After centrifugation, the pellets were treated again with acetone, dioxane and distilled water, and then kept for 1 h on ice. Afterwards, the soluble pellets were centrifuged at 10,000g for 15 min at 4 °C and where subsequently dissolved in petroleum ether. Soluble chlorophyll in petroleum ether was stored at 20 °C until the individual pigments were separated using sugar powder column chromatography (Perkins and Roberts, 1962). Finally, five hundred µg/ml of Chl *a* was prepared in acetone.

2. Chlorophyllide a

Chlide *a* was prepared from a Chl *a* acetone solution (500 µg/ml) with 0.765 mg protein of partial purified Chlase (20–40% of $(NH_4)_2SO_4$) from green citrus fruits. The reaction mixture was incubated at 25 °C for 40 min. The reaction was stopped using acetone and the remaining Chl *a* was separated by hexane. The lower part of the reaction mixture was used as the Chlide *a*.

3. Pheophytin a

Phein *a* was prepared by adding one drop of 0.1 N HCl into the Chl *a* acetone solution (500 μg/ml). After 2 min, 0.1 N NaOH was added to neutralize the solution, which was then employed as substrate in the assays.

Analyses of chlorophyll-degrading enzyme activities

An acetone powder (500 mg) of peel tissues was suspended in 15 ml 10 mM phosphate buffer (pH 7.0) containing 0.6% CHAPS for Chlase. For MDS, an acetone powder (500 mg) of peel tissues was suspended in 15 ml 50 mM phosphate buffer (pH 7.0) containing 50 mM KCl and 0.24% Triton-X 100, or in 15 ml 10 mM phosphate buffer (pH 7.0) for Chl-POX. For PPH, an acetone powder (500 mg) was suspended in 15 ml 50 mM Tris-HCl buffer (pH 8.0). The crude enzyme was stirred for 1 h at 0 °C and the mixture was filtered with two layers of Miracloth. The filtrate was then centrifuged at 16,000g at 4 °C for 15 min. The supernatant was used as the crude enzyme extract. The enzyme protein contents were determined based on Bradford's method (1976).

1. Chlorophyllase activity

The reaction mixture contained 0.5 ml 0.1 mM phosphate buffer (pH 7.5), 0.2 ml 500 μg/ml Chl *a* acetone solution (100 μg/ml) and 0.5 ml enzyme solution. The reaction mixture was incubated in a water bath at 25 °C for 40 min, and the enzyme reaction was stopped by adding 4 ml of acetone. Chlide *a* was separated by adding 4 ml of hexane. The upper phase contained the remaining Chl *a* while the lower phase contained the Chlide *a*. The activity was spectrophotometrically detected by Chlide *a* formation at 667 nm per unit per mg protein.

2. Chlorophyll-degrading peroxidase activity

Chl-POX was determined as previously described (Yamauchi et al., 1997). The reaction mixture contained 50 µl of enzyme solution, 100 µl 1.0% Triton-X 100, 100 µl 5 mM *p*-coumaric acid, 100 µl of 500 µg/ml Chl *a* acetone solution, 500 µl 0.2 mM phosphate buffer (pH 5.5) and 50 µl 0.3% hydrogen peroxide. Activity was determined spectrophotometrically by measuring the decrease of Chl *a* at 668 nm per unit per mg protein at 25 °C.

3. Mg-dechelating substance activity

MDS using Chlide *a* substrate was determined by the method of Suzuki and Shioi (2002) with slight modification. The activity of Mg-dechelating substance was measured with Pheide *a* formation, the reaction mixture contained 0.75 ml 10 mM phosphate buffer (pH 7.5), 0.25 ml Chlide *a* (8.80 µg) and 0.2 ml of enzyme solution.

4. Pheophytinase activity

PPH was modified by the method of Schelbert et al. (2009). The reaction mixture contained 0.5 ml 50 mM Tris-HCl buffer (pH 8.0), 0.2 ml Phein *a* solution (22.40 µg) and 0.5 ml enzyme solution. The reaction mixture was incubated in a water bath at 25 °C for 40 min, and the enzyme reaction was stopped by adding 2 ml of acetone. After that, sample was analyzed by HPLC using a Hitachi L-7100 pump with an automated gradient controller and a Hitachi L-2450 diode array detector or a Hitachi L-7420 UV-Visible spectrophotometer. The absorption spectrum of the product as Pheide *a* was recorded at 665 nm.

5. Protein content

Protein content of broccoli floret were determined based on the Bradford method (Bradford, 1976) with bovine serum albumin as a standard by using 100 µl aliquots of crude extracts per 5 ml of a Coomassie protein assay reagent.

Postharvest quality parameters

The organic acids and sugars from the fruit juice (15 ml) were extracted with hot ethanol (final concentration 70% ethanol) for 15 min. A 1 ml aliquot of 70% ethanol extracts was vacuum-evaporated to dryness and redissolved in 1 ml of Milli-Q water. The citric and malic contents were determined by HPLC using Hitachi L-7420 UV-Visible spectrophotometer. Sample were separated on a Mightysil RP-18 column, 4x250 mm, using a solvent: water: methanol:50 mM phosphoric acid (69:1:30). The absorption spectrum of the citric and malic acid was recorded at 210 nm. The flow rate was 1 $ml \cdot min^{-1}$, and the injection volume was 100 µl. The sugar extract was filtered by passing through Sep-pak C18 and analyzed by HPLC using Lichrocart column with a solvent: acetonitrile: water (80:20) according to determined glucose, fructose and sucrose contents.

Stomatal apertures

Stomatal of lime fruit was observed by Suzuki's Universal Micro-Printing (SUMP) method using SUMP liquid and SUMP plate (SUMP Laboratory, Tokyo). The fruit peel was pressed onto 10 µl of SUMP liquid placed on a cover glass until liquid become solid. The copies SUMP resin images were then observed by light microscopy.

3.2 Experiment II Hot water treatment delays chlorophyll degradation and postharvest quality in lime (*Citrus aurantifolia* Swingle cv. Paan) fruit

Plant materials and heat treatment

The lime fruit were held in hot water (50°C) was circulated for 3 or 5 min and dried at ambient temperature. After drying, five fruit per perfolated polyethylene-film bag (20 x 14 cm, 0.04 mm thick, with two 6 mm holes) and stored at 13°C in the dark for 35 day. Three bags of each treatment were removed at scheduled intervals during the 35 d period could be analyzed.

Surface color

The surface color of broccoli florets was determined by measuring the hue angle with a colorimeter (Nippon Denshoku NF 777). Hue angle of 0, 90, 180 and 270 degree showed red-purple, yellow, bluish-green and blue color, respectively.

Chlorophyll contents

The Chl content was made on broccoli florets based on the method of Moran (1982). The broccoli florets (0.5 g) were extracted in 20 ml of *N,N*-dimethylformamide and keep overnight at 4°C in the dark. An absorbance was read spectrophotometrically at 647 and 664 nm. Chls *a* and *b* were calculated with the following equations.

Chl *a* (μg/ml) = 12.64 OD_{664} - 2.99 OD_{647}

Chl *b* (μg/ml) = -5.6 OD_{664} + 23.26 OD_{647}

The unit of μg/ml is converted into mg/100 FW of broccoli floret using the following equations:

$$\text{Chl } a \text{ (mg/100 gFW)} = (\text{Chl } a \text{ (μg/ml)}) \times 20.5 \times \frac{100}{0.5} \times \frac{1}{1000}$$

$$\text{Chl } b \text{ (mg/100 gFW)} = (\text{Chl } b \text{ (μg/ml)}) \times 20.5 \times \frac{100}{0.5} \times \frac{1}{1000}$$

Analyses of chlorophyll-degrading enzyme activities

An acetone powder (500 mg) of peel tissues was suspended in 15 ml 10 mM phosphate buffer (pH 7.0) containing 0.6% CHAPS for Chlase. For MDS, an acetone powder (500 mg) of peel tissues was suspended in 15 ml 50 mM phosphate buffer (pH 7.0) containing 50 mM KCl and 0.24% Triton-X 100, or in 15 ml 10 mM phosphate buffer (pH 7.0) for Chl-POX. For PPH, an acetone powder (500 mg) was suspended in 15 ml 50 mM Tris-HCl buffer (pH 8.0). The crude enzyme was stirred for 1 h at 0 °C and the mixture was filtered with two layers of Miracloth. The filtrate was then centrifuged at 16,000g at 4 °C for 15 min. The supernatant was used as the crude enzyme extract. The enzyme protein contents were determined based on Bradford's method (1976).

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5. Protein content

Protein content of broccoli floret were determined based on the Bradford method (Bradford, 1976) with bovine serum albumin as a standard by using 100 µl aliquots of crude extracts per 5 ml of a Coomassie protein assay reagent.

Postharvest quality parameters

Total titratable acidity (TA) was measured by titration 1 mL of extracted juice diluted with 9 mL of distilled water with 0.1 M NaOH to an endpoint of pH 8.1, using an automatic titrator (AUT-501, DKK-TOA Corporation, Tokyo, Japan). Total titratable acidity was expressed as percentage of citric acid. Total soluble solid (TSS) from fruit juice was measured by a digital refractometer (PAL-1, Atago, Tokyo, Japan). The units of TSS were expressed as the percentage.

Postharvest physiology parameters

Lime fruit from each treatment was weighed individually before and during storage period, and the percentage of weight loss was calculated using the equation below.

$$\text{Total weight loss (\%)} = \frac{\text{initial weight of fruit} - \text{final weight of fruit}}{\text{initial weight of fruit}} \times 100$$

Respiration rates were determined by gas chromatography (GC) (Chromatopac, CR 8A, Shimadsu Co., Kyoto, Japan), with a thermal conductivity detector (TCD) fitted with a 80/100 mesh Porapack Q column using helium as the carrier gas. The

ethylene production rates were measured by gas chromatography (Chromatopac, CR 14A, Shimadsu Co., Kyoto, Japan), with a frame ionization detector (FID) equipped with a 80/100 mesh Porapack Q column using nitrogen as the carrier gas. Five fruit were sealed in a plastic container (610 mL) then incubated at room temperature for 3 hours. One mL of gas sample was withdrawn from the headspace with a gas syringe type and then injected into the gas chromatography.