CHAPTER 3

Materials and Methods

Laboratory equipment and chemicals

Laboratory equipment

1. Homogenizer (T25, IKA, USA)
2. Precision balance (PA4102, Ohaus, USA)
3. Analytical balance (PA214C, Ohaus, USA)
4. Water bath (Eco Temp TW 20, Julabo, USA)
5. pH meter (Delta 320, Mettler Toledo, Switzerland)
6. Colorimeter (MiniScan XE Plus, HunterLab, USA)
7. Vortex-genie 2 mixers (G560E, Scientific Industries, USA)
8. UV/VIS spectrophotometer (Lambda25, Perkin Elmer Instruments, UK)
9. Visible spectrophotometer (VIS) (Genesys 20, Thermo Scientific, USA)
10. Multi position hotplate magnetic stirrers (LD-846, Labinco, Netherland)
11. Refrigerated centrifuge (IEC Multi RF, Thermo Electron Corporation, USA)
12. High Performance Liquid Chromatography system (1200, Agilent, USA)

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Chemicals

1. Chemical used in fruit dipping

    - Adenosine triphosphate (Sigma Aldrich, USA)

2. Chemical used in fruit fumigation

    - Hydrochloric acid (Merck, Germany)
    - Sodium chlorite (Ajax Finechem, Australia)

3. Chemicals used in the analysis of total antioxidant capacity

    - Methanol (Merck, Germany)
    - Acetic acid (Merck, Germany)
    - Sodium acetate (Merck, Germany)
    - Potassium persulphate (Rankem, India)
    - Ferrous sulphate heptahydrate (QRëC, New zealand)
    - Ferric trichloride hexahydrate (QRëC, New zealand)
    - 2,4,6-Tri (2-pyridyl)-s-triazine (Sigma-Aldrich, USA)
    - 1,1-Diphenyl-2-picrylhydrazyl (Sigma-Aldrich, USA)
    - 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich, USA)
    - (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (Sigma-Aldrich, USA)

4. Chemicals used in the analysis of ATP, ADP and AMP contents

    - Acetonitrile (RCI-Labscan, Thailand)
    - Perchloric acid (Sigma-Aldrich, USA)
    - Potassium hydroxide (Merck, Germany)
    - Adenosine diphosphate (Sigma Aldrich, USA)
    - Adenosine monophosphate (Sigma Aldrich, USA)
    - Potassium dihydrogen phosphate (Merck, Germany)
    - Di-potassium hydrogen phosphate (Merck, Germany)
    - Ethylenediaminetetraacetic acid (Sigma-Aldrich, USA)

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

5. Chemicals used in the analysis of respiratory enzyme activity

    - Sodium azide (Loba, India)
    - Sodium succinate (Himedia, India)
    - Potassium tartrate (J.T. Baker, USA)
    - Sucrose (Ajax Finechem, Australia)
    - Mannite (Ajax Finechem, Australia)
    - Cytochrome *c* (Sigma Aldrich, USA)

- Sodium carbonate (Merck, Germany)
- Sodium hydroxide (Merck, Germany)
- Phenazine methosulphate (Sigma Aldrich, USA)
- Bovine serum albumin (Merck, Germany)
- Folin-Ciocalteu's phenol reagent (Merck, Germany)
- N,N-Dimethyl phenylene diamine (Sigma Aldrich, USA)
- Polyvinyl polypyrrolidone (Sigma Aldrich, USA)
- Tris (hydroxymethyl)-aminomethane-HCl (Merck, Germany)
- Copper (II) sulphate pentahydrate (Ajax Finechem, Australia)
- 2,6-Dichlorophenolindophenol (Sigma Aldrich, USA)
- Ethylenediaminetetraacetic acid (Sigma-Aldrich, USA)

6. Chemicals used in the analysis of pyridine nucleotide content

- Tetra-n-butylammonium hydrogen sulfate (Sigma-Aldrich, USA)
- Oxidized nicotinamide adenine dinucleotide (Sigma-Aldrich, USA)
- Reduced nicotinamide adenine dinucleotide (Sigma-Aldrich, USA)
- Oxidized nicotinamide adenine dinucleotide phosphate (Sigma-Aldrich, USA)
- Reduced nicotinamide adenine dinucleotide phosphate (Sigma-Aldrich, USA)

7. Chemicals used in the analysis of ubiquinone content

- Ethanol (Merck, Germany)
- Ubiquinone (Sigma-Aldrich, USA)
- Petroleum ether (RCI-Labscan, Thailand)
- Sodium borohydride (Sigma-Aldrich, USA)

8. Chemicals used in the analysis of ascorbate and glutathione contents

- Ascorbic acid (Sigma-Aldrich, USA)
- 2,2'-Dipyridyl (Sigma-Aldrich, USA)
- 2-Vinylpyridine (Sigma-Aldrich, USA)

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

- Dehydroascorbate (Sigma-Aldrich, USA)
- n-Ethylmaleimide (Sigma-Aldrich, USA)
- *ortho*-Phosphoric acid (Merck, Germany)
- Reduced glutathione (Sigma-Aldrich, USA)
- Dithiothreitol (Sigma-Aldrich, USA)
- Oxidized glutathione (Sigma-Aldrich, USA)
- Trichloroacetic acid (Sigma-Aldrich, USA)
- Glutathione reductase (Sigma-Aldrich, USA)
- 5, 5'-Dithiobis (2-nitrobenzoic acid) (Sigma-Aldrich, USA)

9. Chemicals used in the analysis of ascorbate-glutathione cycle enzyme activity

- Sodium bicarbonate (Merck, Germany)
- Ascorbate oxidase (Sigma-Aldrich, USA)
- Hydrogen peroxide (Carlo Erba Reagent, Italy)

10. Chemicals used in the analysis of NADPH recycling enzyme activity

- Glycerol (Merck, Germany)
- Triton X-100 (Merck, Germany)
- 6-Phosphogluconate (Sigma-Aldrich, USA)
- Glucose-6-phosphate (Sigma-Aldrich, USA)
- Magnesium chloride (Ajax Finechem, Australia)
- 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (Sigma-Aldrich, USA)

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

11. Chemicals used in the analysis of H_2O_2 content

- Potassium iodide (Merck, Germany)

Plant materials

Longan fruits (*Dimocarpus longan* Lour. cv. Daw) at the harvesting stage (180 days after full bloom) were obtained from a commercial orchard in Lamphun province, Thailand and transported within 2 hours to the Postharvest Physiology and Technology Research Laboratory at Chiang Mai University. The fruits were individually selected for uniformity in shape, color, size and defect.

Experimental design

The experiments were conducted in a completely randomized fashion. There were 2 experiments composing of Experiment 1; Effects of gaseous ClO_2 fumigation on mitochondrial energy levels and redox status of harvested 'Daw' longan pericarp during storage and Experiment 2; Effects of gaseous ClO_2 fumigation on redox status in free radical scavenging of harvested 'Daw' longan pericarp during storage.

Experiment 1

Effects of gaseous ClO_2 fumigation on mitochondrial energy levels and redox status of harvested 'Daw' longan pericarp during storage

1.1 Effects of exogenous ATP on antioxidant capacity and fruit quality of harvested 'Daw' longan during storage

To investigate the importance of energy in correlation to antioxidant and senescence, longan fruits were divided into 12 groups of 240 fruits each. A set of 3 groups was submerged in 0 (control), 0.5, 1 and 2 mM ATP solution for 10 minutes at 25 °C and air-dried for 30 minutes. The submerged fruits in each group were further divided into 8 subgroups of 30 fruits each and stored in cardboard boxes (25 cm × 17 cm × 9 cm). The boxes were kept in a storage room at 25±1°C with a relative humidity of 82±5% for 7 days. Everyday from the beginning of the experiment, the fruits from each treatment were tested for total antioxidant capacity and fruit quality by the following methods.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

1. Determination of total antioxidant capacity (TAC)

Longan pericarp (1 g) was sliced and homogenized in 10 mL of 80% methanol for 30 seconds at 4°C. The homogenate was centrifuged at 16,000 × *g* for 20 minutes at 4 °C. The supernatant was collected as a sample solution to determine TAC by using ABTS and DPPH radicals scavenging and FRAP assays.

ABTS radical scavenging activity was determined as described by Huang *et al.* (2005) with some modifications. $ABTS^{\bullet+}$ solution was prepared by adding 2 mL of 7 mM $ABTS^{\bullet+}$ to 3 mL of 2.45 mM of potassium persulfate and kept in the dark at 5°C for 16 hours before being diluted with 80% ethanol until its absorbency at 734 nm was 7.0. Sample solution (0.02 mL) was mixed with 2 mL of the $ABTS^{\bullet+}$ solution. The mixture was placed at room temperature for 10 minutes. The absorbency at 734 nm of the mixture was measured by the VIS spectrophotometer. TAC was determined from the linear equation of a standard curve prepared with Trolox (Appendix: Figure 1) and expressed as µmol Trolox g^{-1} FW.

DPPH radical scavenging activity was determined according to Mun'im *et al.* (2003) with some modifications. A 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. The mixture was placed in a dark room at 25±1°C for 30 minutes. The absorbency at 517 nm of the mixture was measured by VIS spectrophotometer. DPPH radical scavenging activity was determined from the linear equation of a standard curve prepared with Trolox (Appendix: Figure 2) and expressed as µmol Trolox g^{-1} FW.

The FRAP assay was determined as described by Benzie and Strain (1999) with some modifications. FRAP solution was prepared just before the experiment by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) at the ratio of 10:1:1 and kept in the dark at room temperature (25±1 °C) for 30 minutes. Sample solution (0.3 ml) was mixed with 2.7 mL of FRAP solution before determining its absorbency at 593 nm. FRAP was determined from the linear equation of a standard curve prepared with ferrous sulphate ($FeSO_4$) (Appendix: Figure 3) and expressed as µmol Fe^{2+} g^{-1} FW.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

2. Storage fruit quality

Browning index

Pericarp browning of longan fruit was estimated using the browning index (BI), as described by Jiang and Li (2001). The BI was assessed visually by measuring the extent of the total brown area on the surface of each fruit using the following scales (Figure 3.1).

Browning score	Browning area
1	no browning (excellent quality)
2	slight browning, 1-10% browning
3	11-24% browning (fair, acceptable)
4	25-50% browning
5	more than 50% browning (poor quality)

Brown area	0%	1-10%	11-24%	25-50%	> 50%
Browning score	1	2	3	4	5

Figure 3.1 BI score of longan pericarp

BI was calculated using the following formula:

$$BI = \frac{\sum (\text{browning score} \times \text{number of fruit within each score})}{\text{total number of fruit}}$$

Fruits having BI above 3.0 were considered unacceptable for marketing quality.

Color measurement

The color of pericarp was determined with the colorimeter and enumerated in CIELAB scale. The degree of browning was expressed as L* value, which indicates lightness of the pericarp, ranging from black (0) to white (100) (Apai, 2010).

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Disease index

Disease index (DI) was visually assessed by the lesions or rot on the fruit surface (Thavong *et al*., 2010). The severity of fungal development on the surface was examined using the following scales (Figure 3.2).

Disease score	Disease area
0	no visual evidence of fungi (excellent quality)
1	slight area affected with fungus, 1-10% disease
2	11-30% affected with fungus (fair, acceptable)
3	31-70% affected with fungus
4	more than 70% affected with fungus (poor quality)

Disease area	0%	1-10%	11-30%	31-70%	> 70%
Disease score	0	1	2	3	4

Figure 3.2 DI score of longan fruit

DI was calculated using the following formula:

$$DI = \frac{\sum (\text{disease score} \times \text{number of fruit within each score})}{\text{total number of fruit}}$$

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Overall quality acceptance

Overall quality acceptance was evaluated by a semi-trained panel of six graduate students from Postharvest Physiology Laboratory at the Department of Biology, Chiang Mai University. Overall quality acceptance of fruit was scored based on a modification of the 9-point hedonic scale (Wright and Kader, 1997; Apai, 2010).

Score	Hedonic scale
9	like extremely, excellent quality, extremely fresh
8	like very much
7	like moderately, very good quality, marketable
6	like slightly
5	neither like nor dislike, limit of marketability
4	dislike slightly
3	dislike moderately, fair quality, limit of usability
2	dislike very much
1	dislike extremely, poor quality, unusable

Fruit having an overall quality score below 5.0 were considered unacceptable for marketing.

1.2 Effects of ClO_2 on mitochondrial energy levels and redox status of 'Daw' longan pericarp during storage

Freshly picked longan fruit were divided into 12 groups of 240 fruits each and exposed to ClO_2 (0, 5, 10 and 25 mg L^{-1}) for 10 minutes in a fumigation chamber. Each concentration was done in triplicate. After fumigation, the chamber was ventilated for 30 minutes to remove any residual ClO_2. Each group was collected and stored in a cardboard box as described in the ATP treatment. From the beginning until the end of the experiment, samples from each replication of the treatments were taken daily to determine energy status, energy producing enzyme activities, redox status and storage fruit quality by the following methods.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

1. Determination of energy status

ATP, ADP and AMP were extracted and evaluated as described by Liu *et al*. (2006) with some modifications. Longan pericarp (2 g) was sliced and homogenized in 10 mL of 0.6 mM perchloric acid at 4°C for 1 minute. The homogenate was centrifuged at 6,000 × *g* for 10 minutes at 4°C. The supernatant (5 mL) was taken and its pH immediately adjusted to 6.5-6.8 with 1 M KOH. The neutralized supernatant was kept

for 30 minutes in an ice bath before filtration with Whatman® No.1 filter (Whatman, England) followed by 0.45 µm filter (MS® Nylon Syringe Filter, USA).

ATP, ADP and AMP were separated and measured with Agilent 1200 HPLC system (Agilent Corporation, USA) using a reserved phase Eclipse XDB-C18 column (5 µm, 4.6×150 mm) and a diode array detector (model G1314B) at 254 nm. Mobile phase A consisted of 50 mM phosphate buffer (pH 7.0). Mobile phase B was pure acetonitrile. HPLC separation was achieved using continuous gradient elution as follows: 0 min 100% A, 0% B; 2 min 95% A, 5% B; 4 min 80% A, 20% B; 5.3 min 75% A, 25% B and 6 min 100% A, 0% B. The flow rate was 1.2 mL minute^{-1}, while the injection volume was 20 µL. The total retention time was about 5 minutes and the gradient was run for 6 minutes to ensure full separation. ATP, ADP and AMP presented in the samples were identified by comparison with the retention times of standards, while their concentrations were determined using the external standard method. EC was calculated using the following formula:

$$EC = \frac{ATP + 0.5ADP}{ATP + ADP + AMP}$$

2. Energy producing enzyme activities

Crude mitochondria were extracted from longan pericarp by the method of Jin *et al*. (2013) with the following modifications. Longan pericarp (1 g) was sliced and homogenized in 10 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose, 0.3 M mannite, 1 mM EDTA and 0.5 g L^{-1} polyvinyl pyrrolidone at 4°C for 1 minute. The homogenate was then centrifuged at 4,000 × *g* for 10 minutes at 4°C. The supernatant was collected and centrifuged at 20,000 × *g* for 20 minutes at 4°C. The pellet was washed twice with washing buffer (10 mM Tris-HCl buffer (pH 7.2), containing 0.25 M sucrose, 0.3 M mannite and 1 mM EDTA. The washed pellet was suspended with the washing buffer to yield crude mitochondrial extract.

Crude mitochondrial extract (0.3 mL) was mixed with 3 mL of 0.2 mM potassium phosphate buffer (pH 7.4), 1 mL of 0.2 mM sodium succinate, 0.1 mL of 10 mM sodium azide, 0.1 mL of 10 mM phenazine methosulphate and 0.1 mL of 1 mM

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

DCPIP. The mixture was incubated at 30°C for 5 minutes. The SDH activity was determined by measuring the decrease in absorption at 600 nm. One unit of SDH activity was defined as a decrease of 0.01 in absorbency per minute at 600 nm under the assay conditions (Popov *et al*., 2010).

The activity of CCO was determined using a modified method of Jin *et al*. (2013). Crude mitochondrial extract (0.2 mL) was incubated with 0.3 mM cytochrome *c* solution and 20 mM dimethyl phenylene diamine at 37°C for 5 minutes. One unit of CCO activity was defined as an increase of 0.01 in absorbance per minute at 510 nm under the assay conditions.

The protein content was determined by Lowry method (Lowry *et al*., 1951) using BSA as standard.

3. Determination of redox status in energy production

NAD^+ and NADH contents

A modified method of Brugidou *et al*. (1991) was utilized to determine NAD^+ and NADH contents. Acid and alkaline extraction was used to extract NAD^+ and NADH, respectively. Longan pericarp (1 g) was sliced and ground in liquid nitrogen. The powder was homogenized in either 10 mL of 0.5 M perchloric acid in 10% methanol (v/v) (acid extraction) or 0.5 M NaOH in 10% methanol (v/v) (alkaline extraction) at 4°C for 1 minute. The homogenate was centrifuged at 5,000 × *g* for 15 minutes at 4°C. In case of acid extraction, the supernatant was adjusted to pH 5.0 with 6 M KOH while it was adjusted to pH 8.0 with 1 M HCl for alkaline extraction. The supernatant was filtered through a 0.45 µm filter (MS® Nylon Syringe Filter, USA). Twenty microliter of the supernatant was used for HPLC analysis.

NAD^+ and NADH were eluted with 0.1 M KH_2PO_4, adjusted to pH 6.0 with 0.1 M Tris-base, as a mobile phase with a flow rate of 1.3 mL min^{-1} and detected at 254 nm. NAD^+ and NADH in the samples were identified by comparison with the retention time of standards, while the concentrations of NAD^+ and NADH were calculated from the

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

area under the peak of external standards. The concentrations of NAD^+ and NADH were expressed as μg g^{-1} FW.

Q and QH_2 contents

The concentration of Q and QH_2 were determined using the procedure of Wagner and Wagner (1995) with some modifications. Longan pericarp (1 g) was sliced and ground in liquid nitrogen and the powder was then homogenized in 3 mL of 0.2 M perchloric acid in 100% methanol at 4°C for 1 minute. The mixture was immediately transferred to a tube containing 3 mL of petroleum ether, mixed and centrifuged at 1,500 × *g* for 2 minutes. The lower phase was reextracted with 3 mL of petroleum ether. The upper petroleum ether phases from both extractions were pooled and evaporated to dryness under a flow of nitrogen. Immediately before HPLC analysis, the extracted Q and QH_2 were resuspended in 100 μL of ethanol. Q and QH_2 were eluted with ethanol:methanol (9:1, v/v) with a flow rate of 1 mL $minute^{-1}$ and detected at 275 nm. Respective quantity of Q and QH_2 detected at 275 nm was calculated based on the peak areas comparing with quantified external standards. Q was reduced to QH_2 by the addition of sodium borohydride.

4. Storage fruit quality

The quality of longan fruit including BI, color of pericarp, DI and overall quality acceptance, was assayed as Experiment 1.1.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

Experiment 2

Effects of gaseous ClO_2 fumigation on redox status in free radical scavenging of harvested 'Daw' longan pericarp during storage

Longan fruits were divided into 12 groups of 240 fruit each as Experiment 1.2. Each group was exposed to ClO_2 (0, 5, 10 and 25 mg L^{-1}) in a fumigation chamber for 10 minutes. Each concentration was done in triplicate. After fumigation, the chamber was ventilated for 30 minutes to remove any residual ClO_2. The fumigated fruit in each group were further divided into 8 subgroups of 30 fruits each and stored in cardboard boxes. The boxes were stored in a storage room at 25±1°C with a relative humidity of

82±5% for 7 days. From the beginning until the end of the experiment, samples from each replication of the treatments were taken every day to determine redox status of ASA, GSH and NADPH, ASA-GSH cycle enzymes activities, NADPH recycling enzyme activities and H_2O_2 content.

1. Determination of redox status in free radical scavenging

ASA and DHA contents

ASA and DHA contents were assayed by the procedure of Kampfenkel *et al*. (1995) with some modifications. Longan pericarp (1 g) was sliced and homogenized in 5 mL of 6% trichloroacetic acid (TCA) (w/v) at 4°C for 1 minute. The homogenate was then centrifuged at 15,600 × *g* for 5 minutes at 4°C and the supernatant was immediately assayed for ASA and DHA content.

Determination was based on the reduction of Fe^{3+} to Fe^{2+} by ASA, and the complex of Fe^{2+} and 2,2-dipyridyl was detected at 525 nm. For ASA content, 0.2 mL of the supernatant was added into a 3.8 mL of reaction mixture containing 0.2 M potassium phosphate buffer (pH 7.4), 10% TCA (w/v), 42% *ortho*-phosphoric acid (v/v), 4% 2,2'-dipyridyl (w/v) and 3% $FeCl_3$ (w/v). The mixed solution was placed at 42°C for 40 min. The absorbance was measured at 525 nm using a spectrophotometer (Thermo Spectronic model Helios Epsilon, USA). Total ASA content was determined by the reduction of DHA into ASA using dithiothreitol (DTT) as reducing agent. Briefly, 0.2 mL of supernatant was added to 0.8 mL of 10 mM DTT (w/v). The reaction was allowed to proceed for 15 minutes at 42°C and stopped by adding 0.2 mL of 0.5% n-ethylmaleimide (w/v). The reduced samples were then assayed for total ASA under similar conditions as for ASA. ASA and total ASA contents were determined from the linear equation of a standard curve prepared with ASA (Appendix: Figure 4). For each sample, the concentration of DHA was obtained by subtracting ASA from the total ASA. The concentrations of ASA and DHA were expressed as mg g^{-1} FW.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

GSH and GSSG contents

GSH and GSSG contents were assayed by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)-glutathione reductase (GR) recycling method as described by Hodges and Forney (2000) with some modifications. Longan pericarp (1 g) was sliced and homogenized in 15 mL of 5% TCA (w/v) at 4°C for 1 minute. The homogenate was then centrifuged at 12,000 × *g* for 15 minutes at 4°C and the supernatant was immediately assayed for GSH and GSSG content.

For determination of total glutathione (GSH + GSSG), the supernatant (0.2 mL) was neutralized with 5 mL of 0.5 M potassium phosphate buffer (pH 7.0). 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. The NADPH used was previously dissolved 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 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 (pH7.5) and 0.2 mL of diluted supernatant. The change in absorbance of the mixture was measured by spectrophotometer at 412 nm for 5 minutes.

For determination of GSSG, an aliquot (0.2 mL) of the supernatant was neutralized with 0.5 M potassium phosphate buffer (pH 6.5) leading to 1:25 dilution. GSH was first sequestered by incubating 5 mL of the diluted and neutralized extract with 0.1 mL of 2-vinylpyridine at 25°C for 1 hour, and then subjected to a similar reaction as described above for total glutathione. Total glutathione and GSSG contents were determined from the linear equation of a standard curve prepared with GSH (Appendix: Figure 5). For each sample, GSH concentration was obtained by subtracting GSSG from total glutathione. The concentrations of GSH and GSSG were expressed as nmol g^{-1} FW.

$NADP^+$ and NADPH contents

$NADP^+$ and NADPH contents were assayed by the procedure of Brugidou *et al*. (1991) with some modifications. Acid extraction was used for $NADP^+$ whereas alkaline

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

extraction was used for NADPH. Longan pericarp (1 g) was sliced and ground in liquid nitrogen and the powder was then homogenized in 10 mL of 0.5 M perchloric acid in 10% methanol (v/v) for acid extraction, or 0.5 M NaOH in 10% methanol (v/v) for alkaline extraction at 4°C for 1 minute. The homogenate was then centrifuged at 5,000 × *g* at 4°C for 15 minutes. The supernatant was adjusted to either pH 5.0 with 6 M KOH for the acid extraction or to pH 8.0 with 1 M HCl for the alkaline extraction. After centrifugation (5,000 × *g*) at 4°C for 10 minutes, the supernatant was filtered through a 0.45 µm filter (MS® Nylon Syringe Filter, USA). Twenty microliter of the supernatant was used for HPLC (Agilent Corporation, USA) analysis.

$NADP^+$ and NADPH were eluted with 0.1 M KH_2PO_4, adjusted to pH 6.0 with 0.1 M Tris-base as a mobile phase with a flow rate of 1.3 mL $minute^{-1}$ and detected at 254 nm. $NADP^+$ and NADPH in the samples were identified by comparison with retention time of standards, while the concentrations of $NADP^+$ and NADPH were determined using the external standard method. The concentrations of $NADP^+$ and NADPH were expressed as µg g^{-1} FW.

2. ASA-GSH cycle enzyme activities

Activities of APX, DHAR, MDHAR and GR were assayed by the procedure of Hodges and Forney (2000) with some modifications. Longan pericarp (1 g) was sliced and ground in liquid nitrogen and the powder was then homogenized in 10 mL of extraction buffer (100 mM potassium phosphate buffer (pH 7.0), 1 mM ascorbate, 1 mM EDTA and 2.5% PVPP) at 4°C for 1 minute. The homogenate was centrifuged at 15,000 × *g* for 25 minutes at 4°C and the supernatant was filtered through Whatman® No.1filter paper (Whatman, England) immediately before assayed for APX, DHAR, MDHAR and GR activities.

APX activity was assayed in a 1.9 mL-reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 1 mM ascorbate, 0.1 mM EDTA and 1 mM hydrogen peroxide (H_2O_2) and 0.1 mL of enzyme extract. The decrease in ascorbic acid was observed at 290 nm (E = 2.8 mM^{-1} cm^{-1}). The APX activity was expressed as µmol ASA decomposition mg^{-1} protein $minute^{-1}$.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

DHAR activity was assayed in a 1.9 mL-reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 5 mM GSH, 0.1 mM EDTA and 0.2 mM DHA and 0.1 mL of enzyme extract. The subsequent reduction of DHA was observed at 265 nm (E = 14.7 mM^{-1} cm^{-1}). The DHAR activity was expressed as µmol DHA decomposition mg^{-1} protein $minute^{-1}$.

MDHAR activity was assayed in a 1.9 mL-reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 0.25 unit ascorbate oxidase, 0.1 mM EDTA, 0.2 mM NADH and 2.5 mM ASA and 0.1 mL of enzyme extract. The subsequent decrease in NADH was observed at 340 nm (E = 6.2 mM^{-1} cm^{-1}). The MDHAR activity was expressed as µmol NADH decomposition mg^{-1} protein $minute^{-1}$.

GR activity was assayed in a 1.9 mL-reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 2.5 mM GSSG, 0.1 mM EDTA and 0.5 mM NADPH in 1% $NaHCO_3$ and 0.1 mL of enzyme extract. The subsequent decrease in NADPH was observed at 340 nm (E = 6.2 mM^{-1} cm^{-1}). The GR activity was expressed as µmol NADPH decomposition mg^{-1} protein $minute^{-1}$.

3. NADPH recycling enzyme activities

Activities of G6PDH and 6PGDH were assayed by the modified procedure of Valderrama *et al*. (2006). Longan pericarp (1 g) was sliced and ground in liquid nitrogen. The ground pericarp was then homogenized in 10 mL of extraction buffer (50 mM Tris-HCl buffer (pH 7.8), 0.1 mM EDTA, 0.2% (v/v) TritonX-100 and 10% (v/v) glycerol) at 4°C for 1 minute. The homogenate was then centrifuged at 20,000 × *g* for 20 minute at 4°C and the supernatant was immediately assayed for G6PDH and 6PGDH activities.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved

G6PDH activity was assayed in a 1.9 mL-reaction mixture containing 50 mM HEPES (pH 7.6), 2 mM $MgCl_2$, 0.8 mM $NADP^+$ and 5 mM glucose-6-phosphate and 0.1 mL of enzyme extract. The subsequent increase in NADPH was observed at 340 nm (E = 6.2 mM^{-1} cm^{-1}). The G6PDH activity was expressed as µmol NADPH mg^{-1} protein $minute^{-1}$.

6PGDH activity was assayed in a 1.9 mL-reaction mixture containing 50 mM HEPES (pH 7.6), 2 mM $MgCl_2$, 0.8 mM $NADP^+$ and 5 mM 6-phosphogluconate and 0.1 mL of enzyme extract. The subsequent increase in NADPH was observed at 340 nm (E = 6.2 mM^{-1} cm^{-1}). The 6PGDH activity was expressed as μmol NADPH mg^{-1} protein $minute^{-1}$.

4. Hydrogen peroxide content

H_2O_2 content was determined according to the method of Velikova *et al*. (2000) with some modifications. Longan pericarp (1 g) was sliced and homogenized at 4 °C in 5 ml of 1% (w/v) TCA for 30 seconds. The homogenate was centrifuged at 20,000 × *g* for 20 minutes at 4 °C. Then, 0.5 ml of the supernatant was mixed with 2.4 ml 10 mM potassium phosphate buffer (pH 7.0) and 0.1 ml of 1 M KI. The absorbance of the mixture was measured with a VIS spectrophotometer at 390 nm. The H_2O_2 content was determined from the linear equation of a standard curve prepared with H_2O_2 (Appendix: Figure 6) and expressed as μmol g^{-1} FW.

Statistical analysis

All statistical analyses were performed using Statistical Packages for the Social Science (SPSS) version 15.0. Data were presented as mean ± standard deviation. Data were tested by one-way analysis of variance (ANOVA). Least significant differences (LSD) were calculated to compare significant effects at the $P = 0.05$ level.

Correlation analysis between antioxidant capacity and fruit quality was determined using Pearson correlation coefficients.

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่
Copyright© by Chiang Mai University
All rights reserved