CHAPTER 3
MATERIALS AND METHODS

3.1 Anatomical comparison between normal and chilling injured longan fruit pericarps cv. Daw and Biew Kiew

3.1.1 Fruit materials

Longan fruits cv. Daw and Biew Kiew were harvested from commercial orchards in Chiang Mai and Lumphun provinces. The fruit maturity, checked by hand refractometer (Pocket Refractometer, PAL-1, Atago Co., Ltd., Japan), was 18-19% TSS. Uniform fruits were visually selected and the pedicles were cut into 0.5 cm in length. Two kilograms of fruits were packed in corrugated board box (29 × 41.8 × 9 cm; width × length × height) per replicate. Three replicates of each cultivar and temperature per sampling time were used. The boxes were stored at 5°C in an incubators (Low Temperature Incubator, MIR-553, Sanyo Electric Co., Ltd., Japan), and using a data logger (Temperature and Humidity Recorder, HiTemp 102 RH, Barnstead/ERTCO Thermometers Co., Inc., USA) to record temperature and relative humidity. Longan fruits were sampled every 2 days until complete chilling injury as a score of 4 was achieved.

3.1.2 Chilling injury evaluation

Chilling injury of fruit was monitored by observing the color of outer and inner pericarps. Appearance was estimated by measuring the extent of total browned and water soaked areas on each fruit pericarp on the following scales: **score 1** = no water soaking or browning (excellent quality); **score 2** = slight water soaking and browning ≤1/3; **score 3** = water soaking and browning >1/3 - 2/3 and **score 4** = water soaking and browning >2/3 (poor quality) (**Figure 3.1**). The chilling injury index was calculated from the following equation (Jiang and Li, 2001).

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

Score 1 **Score 2** **Score 3** **Score 4**

Figure 3.1 Chilling injury index criteria of inner surface pericarp.
Score 1 = no water soaking or browning (excellent quality), **Score 2** = ≤1/3, **Score 3** = >1/3 - 2/3, and **Score 4** = >2/3 of the area appeared water soaking and browning (poor quality).

3.1.3 Anatomical of longan fruit pericarps

Normal and chilling injured longan fruit pericarps cv. Daw and Biew Kiew were assessed for anatomical differences by using stereomicroscope, light microscope (LM), scanning electron microscope (SEM) and transmission electron microscope (TEM).

A. Preparation of longan pericarp for stereomicroscope

The longan pericarps were cut into 1 cm squares pieces and visible under a stereomicroscope to investigate the changes of longan pericarp during storage.

B. Preparation of longan pericarp for SEM, LM and TEM

The longan pericarps were cut into 5 mm squares for SEM and 2 × 5 mm (width × length) for LM and TEM. The pericarps 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.

C. Preparation of longan pericarp for electron microscope

The longan pericarp pieces from **B** were fixed in a fixative solution as described by Bozzola and Russell (1999) with slight modification for anatomical study. The pericarp 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 usually washed in the same buffer vehicle 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 scanning electron microscope (JEOL, JSM-5910LV, JEOL Ltd., Tokyo, Japan) at 15 kV.

The specimen for LM (Olympus AX70TF, Olympus Optical Co., Ltd., Japan) and TEM (ZEISS, EM10C/EM10CR, ZEISS Ltd., Germany) was embedded in a Spurr's resin low viscosity medium (Spurr, 1969). Cross-section specimens, approximately 1 micrometers (μm) in thickness, were cut with an ultramicrotome (Ultra Reichert-Jung, Germany) and then stained with 1% (w/v) toluidine blue O (O′Brien *et al*., 1964) for LM. The selected specimen was cut about 60-90 nm thick (ultrathin sections) with an ultramicrotome. The ultrathin sections were mounted on copper grids and post-stained first with 2% uranyl acetate for 10 minutes, then with lead citrate for 2 minutes. The sections were then viewed on a TEM at 60 kV.

3.2 Physico-chemical changes between normal and chilling injured longan fruit pericarps

3.2.1 Measurement of longan fruit pericarp color

Longan fruit pericarp color both outer and inner sides were measured with a colorimeter (ColorQuest XE, Hunter Associates Laboratory, Inc., New York, USA). Three replicates of 20 fruits were used for each treatment. Longan color was recorded using the CIELAB (L^*, a^* and b^*). The L^* indicates lightness, ranged from black = 0 to white = 100. The a^* indicates chromaticity on a green (-) to red (+) axis, and b^* chromaticity on a blue (-) to yellow axis. Numerical values of a^* and b^* were calculated into chroma (C^*) [chroma = $(a^{*2} + b^{*2})^{1/2}$] and hue angle (h°), an index color saturation or intensity [hue angle = tangent $^{-1}$ (b^*/a^*)]. The value of chroma C^* is 0 at the center and increases according to the distance from the center. The hue angle is expressed in degrees from 0° to 360° of the color wheel; 0° = red, 90° = yellow, 180° = green and 270° = blue (McGuire, 1992).

3.2.2 Determination of moisture content of longan fruit pericarp

The moisture content was determined by the hot air-oven (Air Oven, Venticell 111, MMM Medcenter Einrichtungen GmbH, Germany) at 105°C for 72 hours (Hall, 1980). Three grams of longan pericarp were randomly selected and placed in an individual moisture can and its weight was recorded. At the end of the heating period the moisture cans were removed to a desiccator until cooled and weighed. Moisture content (wet weight basis) was calculated as following.

$$\% \text{ Moisture content (wet basis)} = \frac{\text{Initial weight - Final weight}}{\text{Initial weight}} \times 100$$

3.2.3 Determination of electrolyte leakage

Electrolyte leakage was determined by the method of King and Ludford (1983). Two pericarp discs (1 cm diameter) were punched from both sides of each

fruit pericarp with a cork borer. Fifteen discs weighing about 1 g, were rinsed 3 times in deionized water, dried with tissue paper and placed in 25 mL of 0.4 M mannitol (Ajax Finechem, Seven Hills, Australia) solution at room temperature. Electrical conductivity reading of the solutions (electrolyte leakage from the discs) was measured after 3 hours with a conductivity meter (Sartorius Professional Meter PP-20, Sartorius AG, Germany). 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.

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

3.3 The relationship between polyphenol oxidase activity and electrolyte leakage of longan fruit pericarp during chilling injured of longan cv. Daw

The objective of this study was to monitor the polyphenol oxidase activity and electrolyte leakage during chilling injury development in longan pericarp tissues.

A. Plant materials

The longan fruit only cv. Daw was prepared the same as **3.1.1**. Fruits were stored at 5°C, 90-95% RH and sampled every 2 days until completely chilling injury. Measuring the electrolyte leakage, color of outer and inner surface pericarps and polyphenol oxidase activity of inner and outer pericarps (**Figure 3.2**).

B. Extraction of polyphenol oxidase

The polyphenol oxidase activity was determined according to method of Jiang (1999). The longan pericarp was ground with liquid nitrogen in mortar. One gram of sample was homogenized with 10 mL of 0.1 M sodium phosphate buffer pH 6.4

containing 1% polyvinylpyrrolidone at 4°C for 6 hours. Then, extracted pericarp solution was centrifuged at 10,000 x g for 20 minutes. The supernatant was collected and used as a crude enzyme.

C. Enzyme assay and protein determination

PPO activity was assayed with catechol (Sigma, Sigma-Aldrich Chemie, Gmbh, Steinheim, Germany) as substrate by a spectrophotometric procedure. The assay was performed using a 3 mL of 50 mM catechol in 0.1 M sodium phosphate buffer (pH 6.4) and 100 μL of the crude enzyme. The increased in absorbance at 400 nm at 25°C was record automatically for 3 minutes. One unit of enzyme activity was defined as the amount of the enzyme, which caused a change of 0.001 in absorbance per minute. Protein content was determined according to the dye-binding method of Bradford (1976) with bovine serum albumin (BSA) as the standard (Jiang, 1999).

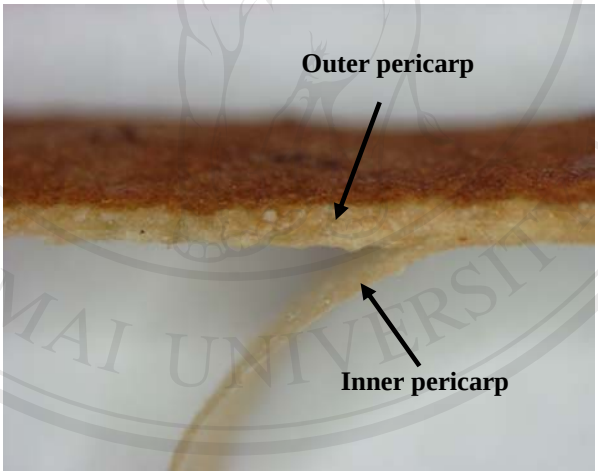

Figure 3.2 Stereomicrograph of whole pericarp was separated into 2 parts, the inner and outer pericarps.

3.4 Identification the main classes of phenolic compounds and other components of normal and chilling injured longan fruit pericarps

3.4.1 Identification of phenolic compounds in longan fruit pericarp

A. Sample extraction

Freeze-dried pericarp powder samples (10 g) of normal and chilling-injured longan fruits (cv. Daw and Biew Kiew) were refluxed in 200 mL 80% aqueous methanol for 24 hours (**Figure 3.3 A**). The extracts were concentrated to 15 mL with a rotary evaporator at 40°C under reduced pressure.

B. Size-exclusion P2 chromatography

Pericarp extracted solution (15 mL) was dried by SpeedVac Concentrator (Savant, SVC 200H, Farmingdale, New York, USA). The dried extract was dissolved in 20% aqueous ethanol, filtered through a 0.45 μm syringe filter, and applied to a Biogel P2 (BioRad, Hercules, California, USA) size-exclusion column (30 x 2.5 cm i.d.). The phenolic compounds were eluted with 20% aqueous ethanol at 0.5 mL/minute. Elutions of the phenolic compounds were monitored at 280 nm, and phenolic content was monitored by HPLC-PDA-MS in every third tube.

C. Acid hydrolysis

Acid hydrolysis was performed according to Mabry *et al*. (1970) with slight modifications. P2 column fractions (5 mL) were added to 5 mL of 2 N HCl, heated at 100°C for 1 hour, cooled, and neutralized with 2.5 N KOH. The hydrolyzed samples were concentrated by a SpeedVac Concentrator for 1 hour, and passed through a C18 Sep-Pak (Waters Corporation, Milford, Massachusetts, USA) cartridge pre-washed with methanol and deionized water. Phenolic compounds bound to the C18 Sep-Pak were eluted with 80% methanol and analyzed by HPLC-PDA-MS.

D. High performance liquid chromatography-mass spectrometry (HPLC-MS)

An Alliance 2695 (Waters Corporation, Milford, Massachusetts, USA) liquid chromatography system was used with a Waters 996 Photo diode array (PDA) detector. Spectra were scanned between 600 and 200 nm. Chromatograms were monitored at both 330 nm and 280 nm. A Waters ZQ single quadrupole mass spectrometer with an electrospray interface was used in conjunction with the above HPLC system. Interface parameters were: source temperature 100°C, desolvation temperature 225°C, capillary voltage 3.33 kV, cone voltage 20 V, extractor voltage 2 V, RF lens voltage 0.1 V, desolvation gas flow 465 L/hour, and cone gas flow 70 L/hour (Manthey and Busling, 2003).

Chromatographic separations were obtained with a C16 Discovery RP Amide column (25 cm x 4.6 mm, 5 μm) (Supelco, St. Louis, Missouri, USA). Initial solvent conditions were water/acetonitrile/2% formic acid (85/10/5, v/v/v). Linear gradients were subsequently run to (81/14/5), (77/18/5), (70/25/5), (40/55/5) and (0/95/5) at 15, 20, 30, 55 and 67 minutes, respectively, at a flow rate of 0.75 mL/minute. Chromatograms were monitored at 330 nm. Data acquisition was done with MassLynx Version 3.5 software (Waters Micromass, Manchester, UK).

E. Isolation of phenolic unknown

P2 size-exclusion column fractions that contained a set of 4 unknown phenolic compounds that exhibited similar UV and MS properties were combined and dried by rotary evaporation under vacuum. The dried fraction was dissolved in methanol, and loaded onto tapered silica gel GF preparative TLC plates with fluorescence indicator (Alltech, Newark, Delaware, USA). Plates were developed twice in butanol/acetic acid/water (65/15/20, v/v/v). This solvent system provided a broad separation of the compounds in these P2 column fractions. Lanes detected with 365 nm UV irradiation were scraped, extracted with methanol, and analyzed by HPLC-PDA-MS. The targeted unknown phenolic compounds were identified in a single lane, and were further isolated by preparative HPLC. Chromatographic separations were obtained with a Waters Delta 600 preparative HPLC system, using a C18 Atlantis column (10 cm × 19 mm, 5 μm) (Waters, Milford, Massachusetts, USA). Initial solvent

conditions were water/acetonitrile/2% formic acid (85/10/5, v/v/v). Linear gradients were subsequently run to (81/14/5), (77/18/5), (70/25/5), (40/55/5) and (0/95/5) at 15, 20, 30, 55 and 67 minutes, respectively, at a flow rate of 5.0 mL/minute. Chromatograms were monitored with a Waters 996 PDA detector at 330 nm. Phenolic unknowns (4 separate peaks) were collected, dried, and analyzed for purity on analytical silica gel TLC using butanol/acetic acid/water (65/15/20, v/v/v).

3.4.2 Quantification of phenolic compounds in longan fruit pericarp

A. Sample extraction

Freeze-dried pericarp powder samples (10 g) of normal and chilling injured longan fruits (cv. Daw and Biew Kiew) were refluxed in 200 mL 80% aqueous methanol for 24 hours. The extracts were concentrated to 15 mL with a rotary evaporator at 40°C under reduced pressure.

B. ***Acid hydrolysis***

Acid hydrolysis of methanolic extracts was determined using the method by Mahattanatawee *et al*. (2006) which was originally optimized by Mabry *et al*. (1970). Methanolic extracts of freeze-dried pericarp (15 mL) were hydrolyzed by refluxing for 1 hour of 2 N HCl, heated at 100°C. The solutions were cooled and extracted with chloroform (**Figure 3.3 B**) to recover the hydrolyzed flavonoid aglycons. The chloroform extracts were concentrated by rotary evaporator, re-dissolved in 10 mL acetone, filtered through a 0.45 μm nylon membrane for analysis by HPLC-MS (**Figure 3.3 C**).

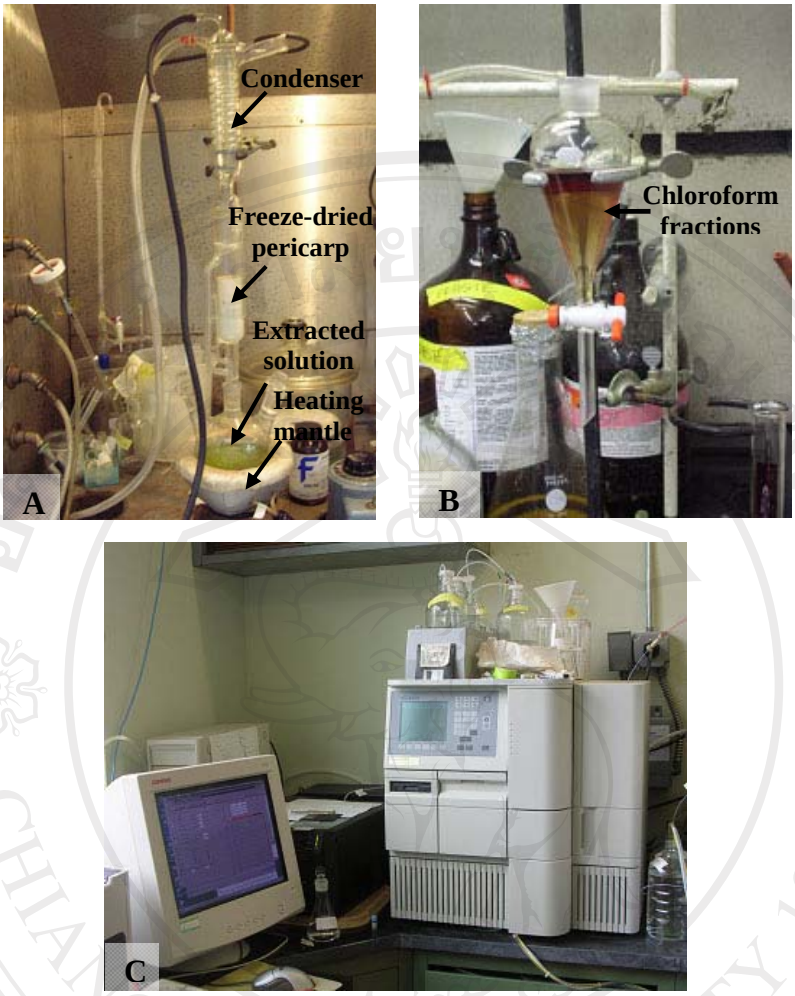

Figure 3.3 The equipment for phenolic compound analysis.

A = extraction set

B = chloroform fractions in acid hydrolysis sample

C = Alliance 695 LC-MS

3.5 Quantification of other components of normal and chilling injured longan fruit pericarps cv. Daw and Biew Kiew

3.5.1 Plant materials

The longan fruit pericarp of normal and chilling injured longan fruit pericarps cv. Daw and Biew Kiew submerged in liquid nitrogen and stored at -80°C (Deep

Freezer -86°C, MDF-U71V, Sanyo Co. Ltd., Tokyo, Japan) until used. Frozen pericarps were freeze-dried by lyophilizer (Flexi-Dry MP, FTS System, Stone Ridge, New York, USA) for 24 hours. The freeze-dried longan pericarps were ground with a sample mill (Pertern Mill 3303, Perten Instruments AB, Finland). Dried pericarp powder samples were kept in auto-desiccator (Auto-C-3W, Sanplatec Corp., Osaka, Japan) until chemical component analysis was performed.

3.5.2 Quantification of pectin and lignin

Pectin and lignin (Klason lignin) were determined according to the method of Theander (1995).

A. Principle

Sample was dissolved in acetate buffer. Starch was removed by using thermostable α-amylase and amyloglucosidase. Soluble polymers were precipitated with 80% ethanol. Precipitate and insoluble polysaccharides was hydrolyzed with concentrated sulfuric acid (H_2SO_4, 96.2% Baker Analyzed 9681-33, J. T. Baker, Inc., USA). Galacturonic acid in hydrolyzed sample solutions were determined by colorimetry and also Klason lignin was determined gravimetrically as acid-insoluble residue. Total dietary fiber was defined as amylase-resistant polysaccharides plus Klason lignin.

B. Sample preparation for pectin analysis

The sample preparation method was according to Theander (1995) with slight modifications. Triplicate 100 mg of the dried pericarp powder was weighed into a 50 mL polypropylene centrifuge tubes (Beckman Instruments, Inc., California, USA). Acetate buffer (5 mL) was added at pH 5.0 along with 40 μL of α-amylase solution (heat stable α-amylase from *Bacillus amyloliquefaciens*, Sigma-Aldrich Chemie, Gmbh, Steinheim, Germany). The solution was mixed, placed in a boiling water bath for 1 hour, and cooled to 40°C. The enzyme amyloglucosidase pellet (from *Aspergillus niger*, 126.9 U/mg, Fluka Biochemika, Switzerland) was dissolved in acetate buffer to obtain 140 U/mL and prepared just before analysis. A 500 μL

amyloglucosidase solution was added. Tubes were incubated overnight in a 60°C water bath equipped with shaker (Shaker Water Bath, Gallenkamp BKS-350, Illinois, USA). Tubes were again cooled, 21 mL of absolute ethanol was added, and the tubes were mixed using a Vortex mixer. Tubes were refrigerated for 1 hour at 4°C and then centrifuged at 5,000 rpm for 10 minutes (Avanti J-E Centrifuge, Beckman Coulter, Inc., California, USA) or until a clear supernatant was obtained. The supernatant was discarded, and the pellet was washed twice by suspending and re-centrifuging with 20 mL of 80% ethanol and then twice with 15 mL acetone. The resulting residue was allowed to dry overnight in a 40°C oven and then was dispersed with 3 mL of 12 M sulfuric acid solution. The mixture was incubated in a 30°C water bath for 1 hour and stirred occasionally. The content was transferred quantitatively with 74 mL of deionized water into a 250 mL beaker and covered with a glass plate. The solution was hydrolyzed in an autoclave for 1 hour and 15 minutes at 121°C. The hydrolyzed solution was filtered while still warm through a glass-fritted crucible (Robu®, porosity No. 2, 40-60 μm, Glasfilter-Geräte GmbH, Germany) into 100 mL volumetric flask. The residue was washed with 10 mL deionized water. Filtrates were brought to a volume of 100 mL with deionized water at room temperature. The hydrolyzed solution was used for determination of galacturonic acid analysis. The residues were used for Klason lignin analysis.

C. Determination of galacturonic acid by colorimetry

The determination of galacturonic acid in the hydrolyzed samples was optimized from the original method of Scott (1979) by Luzio (2004) using a microplate reader (**Figure 3.4**) (Power Wave 340 Microplate Reader with KC4 version 3.01 software, Bio Tek Instruments, Inc., Minnesota, USA). The brown pigment formed after hydrolysis interfered with the galacturonic acid assay. Therefore, the hydrolyzed solution (from **3.5.2 B**) was passed through a Sep-Pak C-18 (Waters Corporation, Milford, Massachusetts, USA) to remove the brown pigments (Mahattanatawee *et al.*, 2006) and stored at 4°C until galacturonic acid analysis. The determination was performed in triplicate for each of the hydrolyzed samples. In separate test tube 300 μL of sample was added with deionized water to bring the

volume up to 700 μL. Three milliliters of concentrated sulfuric acid containing 0.1% NaCl solution was added to each tube and immediately vortexed for 15 seconds. Each tube was then immediately placed on ice and allowed to cool before transferring to a solution basin. From each solution basin, 240 μL was pipetted into individual wells of a microplate (**Figure 3.4 A**), which had been preheated to 75°C in the block heater (VWR Standard Heat Block 13259-032, California, USA). The plate was then heated for 20 minutes at 75°C, removed, cooled in a water bath at room temperature for 20 minutes, and then read at 450 nm using a microplate reader for background absorbance. Forty microliters of 3,5-dimethylphenol (DMP) solution [0.2 g of DMP; (Sigma-Aldrich Chemie, Gmbh, Steinheim, Germany) in 100 mL of glacial acetic acid (Fisher Chemicals, Thermo Fisher Scientific Inc, Massachusetts, USA)] was added to each well (**Figure 3.4 B**). The microplate was shaken for 35 seconds on a microplate shaker (Orbis Plus, shaker with timer model 51513-10, Cole-Parmer International, USA). The wells were read at 450 nm using microplate reader (**Figure 3.4 C**).

Galacturonic acid standard curve. The determination was performed in duplicate. To separate test tubes 0, 100, 200, 300, 400 and 500 μL of a 0.02% galacturonic acid (D-(+)-galacturonic acid, 48280, Fluka BioChemika GmbH, Switzerland) solution was added along with deionized water to bring the volume up to 700 μL in each tube. The assay was performed, and when the pectin content was determined on the basis of calibration with galacturonic acid, an adjustment factor of 0.81 was used in the calculation (Theander, 1995).

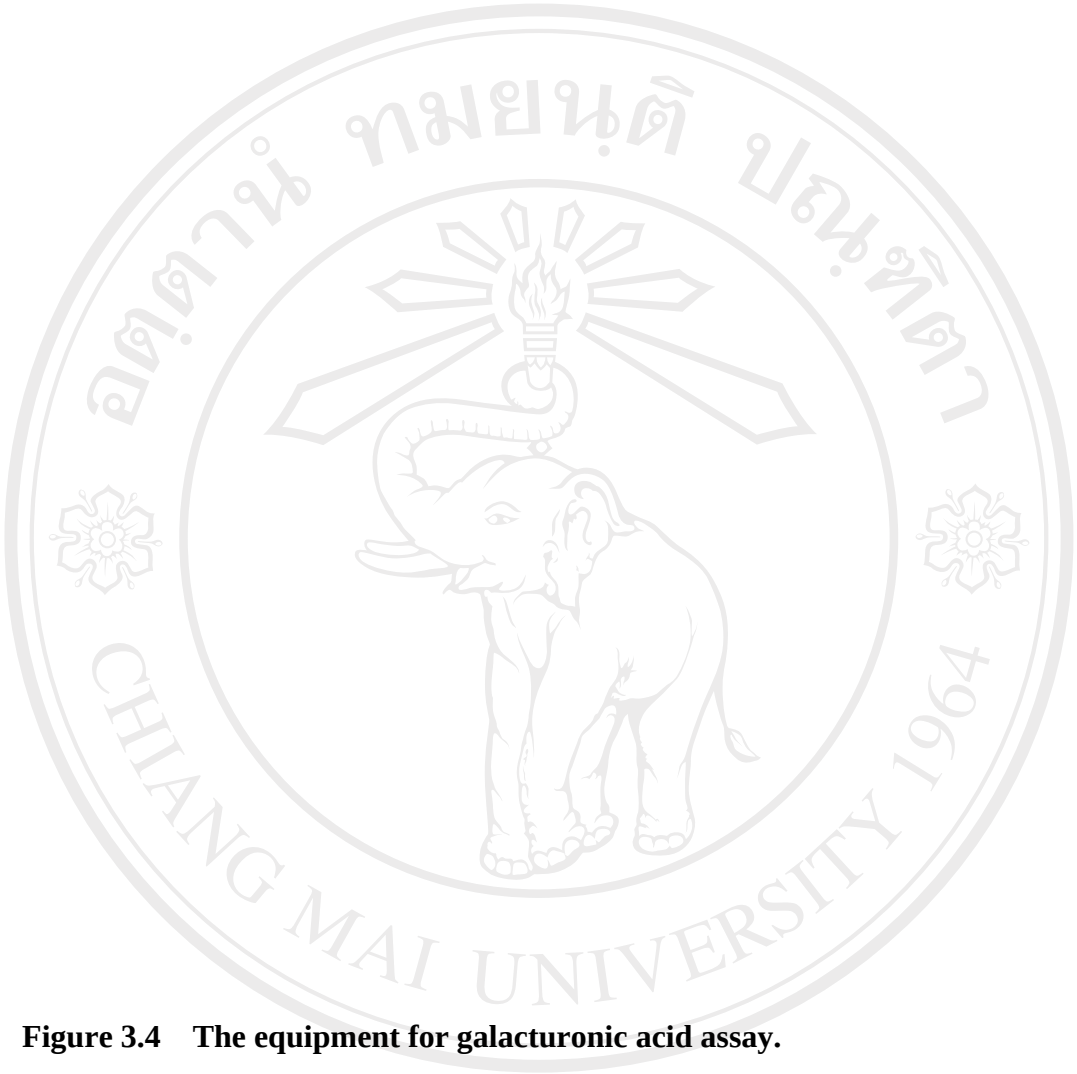

Figure 3.4 The equipment for galacturonic acid assay.

A = microtiter plate wells

B = the addition of DMP solution

C = microtiter plate reader on computer

D. Lignin analysis

The residues (from **3.5.2 B**) were washed on glass-fritted crucible carefully with 50 mL deionized water and then dried 24 hours in 105°C hot air-oven. Filter crucible and residue (W_1) were weighed after cooling in a desiccator. The crucibles were then removed from the desiccator and placed in a muffle furnace (CWF 1100, Carbolite, Sheffield, England) for 1 hour at 500°C to ash. Each crucible was then weighed for ash (W_2) after cooling in a desiccator. Loss in weight (W_1-W_2) after ashing is Klason lignin.

Calculated content (%) of Klason lignin (K) in a sample was calculated as follows:

$$K = (L_w \times 100)/S$$

where L_w = weight loss (mg) on ashing of dried insoluble residue obtained in hydrolysis step

S = weight (dry matter, mg) of original sample

3.5.3 Quantification of total dietary fiber (TDF)

A. Principle

TDF was determined according to AOAC Method 985.29 (AOAC, 1997) using the total dietary fiber assay kit (TDF-100A) (Sigma-Aldrich Chemie, Gmbh, Steinheim, Germany). The freeze-dried longan pericarp powder was suspended in phosphate buffer pH 6.0 and enzymatically digested with heat-stable α-amylase and then with protease (protease from *Bacillus licheniformis*, lyophilized) and amyloglucosidase (amyloglucosidase from *Aspergillus niger*, solution) to remove starch and protein present in the samples. Four volumes of 95% ethanol were added to precipitate soluble dietary fiber. Total residue was filtered and washed with 78% ethanol, 95% ethanol and acetone. After drying, the residue was weighed. Duplicate samples were used to analyze protein content and ash by incineration at 525°C.

Total dietary fiber = weight residue - weight (protein + ash)

B. Crucible preparation

The crucibles were washed thoroughly with 95% ethanol and then with deionized water. Then, the crucibles were heated for 1 hour at 525°C and cooled in desiccator. After that, crucibles were rinsed and soaked in water and air-dried. The celite (Celite, acid-washed, Sigma-Aldrich Chemie, Gmbh, Steinheim, Germany) of 0.5 g was added to each crucible and dried at 130°C to constant weight (one hour or more). The crucibles were cooled in a desiccator and weighed to the nearest 0.1 mg. This was recorded as "celite + crucible weight" (W_1). The crucibles were stored in desiccator until used.

C. Sample preparation

Extraction. One gram of the freeze-dried longan pericarp powder was weighed into a 400 mL beaker, and then 50 mL phosphate buffer pH 6.0 and 100 μL α-amylase were added and mixed well. The beakers were covered with a glass plate and incubated in a boiling water bath for 15 minutes after the internal temperature of the beakers had reached 95°C and allowed to cool to room temperature. Then, the cool sample solution was adjusted to the pH 7.5±0.2 by adding 20 mL of 0.275 N NaOH solution (the pH, adjusted if necessary with either 0.275 N NaOH or 0.325 M HCl). Next, 100 μL of protease (50 mg/mL of protease in phosphate buffer pH 6.0) was added to the sample solution and mixed well. The beakers were covered with a glass plate and placed in 60°C water bath, incubated for 30 minutes after the internal temperature of the beaker had reached 60°C and allowed to cool to room temperature. The sample solution was adjusted to a pH between 4.0 and 4.6 by adding 20 mL of 0.325 M HCl. Then, the 100 μL of an amyloglucosidase solution was added, mixed well and placed in 60°C water bath. The sample solution was incubated for 30 minutes after the internal temperature of the beaker had reached 60°C. When it was cooled, soluble dietary fiber was precipitated in the sample solution by adding 4 volumes of 95% ethanol. The solutions were allowed to sit overnight at room temperature to complete precipitation.

Filtration. The crucibles were redistributed on the bed of celite using 78% ethanol and gentle suction was applied to draw the celite into the glass-filter crucible. Then, the precipitate and suspended materials were quantitatively transferred from

each beaker to the crucible with gentle suction. The residues were washed with three 20 mL portions of 78% ethanol, two 10 mL portions of 95% ethanol, and two 10 mL portions of acetone. After being washed with acetone, the crucibles were dried overnight at 105°C in an hot air-oven, cooled in a desiccator, weighed to the nearest 0.1 mg, and the weight recorded as "residue + celite + crucible weight" (W_2). Duplicate residues were analyzed for protein by Kjeldahl nitrogen analysis. The others were incinerated at 525°C to ash, cooled in a desiccator, weighed to the nearest 0.1 mg and the weight was recorded as "ash + celite + crucible weight" (W_3).

D. Protein determination

The protein (total nitrogen content) was determined by protein analyzer (Kjeldatherm-Automatic, KBL 20S, Gerhardt, Germany) according to AOAC Method 978.04 (AOAC, 2000) with some modifications. One gram of residue (from **3.5.3 C**) was digested with 20 mL concentrated sulfuric acid and 10 g of catalyst (K_2SO_4 : $CuSO_4$, 10:1) at 400°C for 1.5 hours in Kjeldalift-Digestion Unit (KBL20S, Gerhardt, Germany). The nitrogen compounds in longan pericarp were decomposed by concentrated sulfuric acid and the product is an ammonium sulfate solution. The digested samples were cooled for 1 hour and then analyzed using a rapid distillation system (Vapodest 30, Gerhardt, Germany) in excess 32% NaOH solution (w/v) to convert ammonium ion to ammonia gas. The ammonia gas was collected in 50 mL of 4% boric acid solution. The ammonium borate was formed and titrated back to boric acid with 0.1 N H_2SO_4 using autotitrator (Autotitrator, Titroline Easy, Schott, Germany) until reached the end point at pH 3.8. Protein was calculated from percentage of nitrogen multiplied by 6.25.

$$\text{Nitrogen (\%)} = \frac{(\text{mL } 0.1 \text{ N } H_2SO_4 - \text{mL blank}) \times \text{normality of } H_2SO_4 \times 1.4007}{\text{weight of sample (g)}}$$

$$\text{Protein (\%)} = \text{\% Nitrogen} \times 6.25$$

$$\text{Milliequivalent weight of nitrogen} \times 100 = 1.4007$$

E. Calculation of total dietary fiber

$$TDF\ (mg) = [(R_{SAMPLE} - P_{SAMPLE} - A_{SAMPLE} - B)/ SW]$$

$$B = R_{BLANK} - P_{BLANK} - A_{BLANK}$$

Where: TDF = total dietary fiber (mg)

R = residue weight (mg) = $W_2 - W_1$

P = protein weight (mg)

A = ash weight (mg) = $W_3 - W_1$

SW = sample weight (mg)

3.6 Statistical data analysis

All data were analyzed by Analysis of Variance (ANOVA) using SX version 8.0 and the differences between treatments were analyzed by a Least Significant Difference (LSD) comparison. Level of significant difference was indicated with the following: $^{*}P = 0.05$ for all comparisons.