MATERIALS AND METHODS

PART I: Cell Wall Metabolism during Fruit Dehiscence

Plant Materials

Durian fruits cv. Monthong at about 106 days after anthesis, which is the minimum maturity stage were purchased directly from orchards in Chantaburi and Trat provinces in the eastern part of Thailand during 2002 and 2003, respectively, for duplicate experiments. Fruit samples were transported by a refrigerated truck to the Postharvest Research Unit, Central Laboratory and Greenhouse Complex, Kasetsart University, Kamphaeng Saen Campus within 12 hours after harvest.

A total of 120 fruits from each year were stored at room temperature (28±3°C and 80±5% relative humidity) and evaluated for pulp firmness and dehiscence scores. Two types of tissues were collected from each fruit by long sectioning; the husk tissue, approximately 2 cm apart from the dehiscence zone (DZ), and the DZ tissue. The sizes of both tissues were about 5 mm wide, 15 cm long and 5 mm thick. All tissue samples were cut into small pieces, frozen with liquid N_2 and kept in PE plastic bags at -70°C until used. Every 2 days up to 10 days after harvest, pectin and hemicellulose fractions, as well as enzyme activities of the two tissues were extracted and determined. The following parameters were recorded:

1. Firmness

Pulp firmness was determined using an Effegi firmness tester with a 5 mm plunger head on both sides of the durian aril to a depth of 0.5 cm. The unit of force was recorded in kilograms and then converted to Newtons (multiply by 9.807).

2. Degree of Fruit Dehiscence

Every two days, twenty fruits were sampled, divided into 4 groups of replications and scored for their degree of fruit dehiscence. A score of 0 was given when there was no dehiscence. Scores 1, 2 and 3 for fruit dehisced at ¼, ½ and ¾ of locules length and a score of 4 for full length dehiscence. Each durian fruit had 5 locules. Thus, the maximum possible score was 20 for each fruit.

3. Extraction and Analysis for Pectin and Hemicellulose Fractions

Alcohol Insoluble Solid (AIS) was prepared by immersing 20 g of frozen durian husk and husk DZ tissue in 95% alcohol and boiling for 25 minutes. The mixture was blended to disrupt and solubilized most of the low molecular weight compounds. The residue was then thoroughly excessively washed with 400 ml of cooled acetone and then filtered through Whatman#1 paper. The residues were air dried overnight and the weight of the resulting AIS powder was recorded.

An AIS sample of about 50 mg was sequentially extracted for pectin fractions. The cell wall material was first extracted 3 times with 20 ml of distilled water by shaking at 150 rpm for 2 hours. The supernatant was collected by centrifugation at 15,000 rpm for 30 minutes and designated as water soluble pectin (WSP). The water insoluble pellet was suspended 3 times in 20 ml of 50 mM tran-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) pH 6.5, in a 1 M imidazole buffer. The supernatant was collected in similar way as described above, and designated as the chelator-soluble pectin (CSP). The pellet extraction was done 3 times with 20 ml of 50 mM Na_2CO_3 containing 20 mM $NaBH_4$. The supernatant was collected as above and designated as the Na_2CO_3 soluble pectin.

The hemicellulose fractions (1 and 4 M KOH fractions) were obtained by sequentially extracting the residues from pectin fractions as described by Maclachlan and Brady (1994). The pellet from the Na_2CO_3 soluble fraction was extracted twice with 20 ml of 1 M KOH (4%) for 2 hours. The supernatant was collected by centrifugation at 15,000 rpm for 30 minutes and designated as 1 M KOH soluble fraction or loosely bound matrix glycan fraction. The pellet was then extracted 2 times with 20 ml of 4 M KOH (24%) for 2 hours. The supernatant was collected as above and designated as 4 M KOH soluble fraction or tightly bound matrix glycan fraction. The supernatant from hemicellulose fractions was neutralized using concentrated HCl before dialysis.

The supernatant of each pectin and hemicellulose fraction was then dialyzed with cool distilled water overnight at 4°C with two changes of water (12 hours each). The total volume of each dialyzed fraction was recorded. An aliquot (20 ml) of each dialyzed fraction was taken for uronic acid and total sugars determination. The pectin content in each fraction was determined by measuring the uronic acid content according to the colorimetric method at 520 nm of Blumenkrantz and Asboe-Hansen (1973). Galacturonic acid was used as a standard. The amount of hemicellulose fractions content was determined by measuring the total sugars content by the anthrone assay (Dische, 1962) at 620 nm. Glucose was used as a standard.

4. Size Exclusion Chromatography and Analysis of Cell Wall Fractions

A 50 ml portion of each fraction was freeze-dried at below -40°C under pressure below 100 mmHg, using a Freeze dryer system, LABCONO for about 24 hours each to obtain the dry weight of the fraction. The lyophilizate was then kept at -20°C until use.

The lyophilizate samples approximately 5 mg each of water, chelator, Na_2CO_3, 1 and 4 M KOH-soluble fractions were chromatographed on a size exclusion column (2.5 × 35 cm) of Sepharose CL-4B (Pharmacia), and eluted with 50 mM sodium acetate pH 5.0. The sample fraction (2.0 ml) was collected at the a flow rate of 17 ml/h and the sample from water, chelator and Na_2CO_3-soluble pectins were assayed for uronic acid content by the m-hydroxydiphenyl method (Blumenkratz and Asboe-Hansen, 1973) while the samples from the 1 and 4 M KOH-soluble fractions were assayed for total neutral sugars content by the anthrone assay method (Dische, 1962).

5. Determination of Cell Wall Hydrolases

5.1 Polygalacturonase (PG) extraction and determination was conducted as described by Yoshida *et al*. (1984).

Frozen durian tissue (10g) was homogenized for 5 minutes in 20 ml cooled distilled water. The homogenate was centrifuged at 12,000 rpm at 4°C for 20 minutes and the supernatant was discarded. The pellet was re-suspended in 50 ml of 0.2 M Tris-HCl buffer (pH 9.0) containing 5% NaCl and stirred for 2 hours at 4°C. The slurry was centrifuged as previously described. An ammonium sulfate (0.526 g/ml) was added to the supernatant and then stirred for 20 minutes. The slurry was centrifuged as above and the supernatant dialyzed against 20 mM sodium acetate buffer pH 6.0 overnight at 4°C. The supernatant was collected and referred as crude enzyme.

PG activity was assayed by measuring the formation of reducing groups in a reaction mixture containing 0.25 ml of 0.5% polygalacturonic acid, 0.25 ml of 0.4 M NaCl, 0.25 ml of 0.4 M sodium acetate buffer (pH 4.5) and 0.25 ml of the enzyme solution. The reaction was incubated at 37°C for 2 hours, and then terminated by adding 0.5 ml of 30% K_2CO_3 solution containing 5% $Na_2S_2O_3$. A solution (0.5 ml) of 0.3% 3,6-dinitrophthalic acid monopyridinium salt was added to the reaction mixture and boiled for 10 minutes. After cooling, 8 ml of cooled distilled water was added and the enzyme activity was measured by spectrophotometer at 450 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 nmol of reducing groups per min per mg protein under the above conditions.

5.2 Pectin methylesterase (PME) extraction and determination was conducted as described by Hangerman and Austin (1987).

Ten grams of frozen durian tissue were homogenized in 40 ml of distilled water at 4°C. The homogenate was centrifuged at 15,000 rpm for 30 minutes and the supernatant was discarded. The pellet was added with 50 ml of 1 M NaCl and 1% PVP (w/v), and stirred at 4°C for 2 hours. The supernatants were collected by centrifugation at 15,000 rpm for 30 minutes and adjusted to pH 7.5 with 0.1-2 N NaOH.

PME activity was assayed by preparing the reaction mixture containing 2 ml of 0.5% pectin, 0.2 ml of 0.01% bromthylmol blue in 0.003 M, potassium phosphate buffer pH 7.5 and 0.7 ml of distilled water, then incubated at 25°C. The reaction was started by adding 100 µl of crude enzyme and the rate of decreased in absorbance at 620 nm was recorded after 1 minute as nmol acetic acid released per mg protein.

5.3 β-galactosidase (β-gal) extraction and determination was conducted as described by Ross *et al*. (1993).

Ten grams of frozen durian tissue were homogenized in 40 ml of 25 mM Na-acetate buffer, pH 4.5 (containing 10 mM sodium tetratrithionate; NaTT) and 2% (w/w) PVP. The homogenate was centrifuged at 15,000 rpm for 30 minutes and the supernatant was discarded. The pellet was washed with 5 mM NaTT and dissolved with 30 ml of 25 mM Na-acetate buffer pH 4.5 (containing 10 mM NaCl and 2 mM NaTT). The reaction mixture was stirred at 4°C for 1 hour, before the supernatant was collected by centrifugation at 15,000 rpm for 30 minutes and designed as crude enzyme.

β-gal activity determination was assayed by preparing the reaction mixture containing 0.8 ml of enzyme solution, 0.4 ml of 25 mM sodium acetate buffer pH 4.0, 0.4 ml of 0.5% β-mercaptoethanol and 0.4 ml of 2 mM nitrophenyl-β-D-galactopyranoside and 0.8 ml of crude enzyme solution. The reaction mixture was then incubated at 30°C for 1 hour, and terminated by adding 2 ml of 200 mM Na_2CO_3. The activity was recorded as change in absorbance at 410 nm and converted to nmol ρ-nitrophenol released per min per mg protein.

5.4 Endo-(1,4)β-glucanase (EGase) extraction and determination was conducted as described by Abeles and Takeda (1990).

Ten grams of frozen durian tissue was homogenized with 20 ml of 0.1 M potassium phosphate buffer pH 6.8 for about 2 minutes. The slurry was placed on a pre-moistened Mira Cloth and a portion of the filtered homogenate was collected. The supernatant was collected by centrifugation at 15,000 rpm for 30 minutes and designed as crude enzyme extracted.

Enzyme activity in the supernatant after centrifugation at 15,000 rpm for 30 minutes was determined. The reaction mixture contained 1 ml of crude enzyme solution and 2 ml of 1.5% high viscosity sodium salt carboxyl methyl cellulose (CMC). The reaction was incubated at 37°C for 18 hours. The sample (1 ml) was measured for its viscosity by using a micro viscometer model LVT Wells-Brookfield. The drainage times were recorded and subtracted from blank (without enzyme), then calculated for the percentage of reaction. One unit of enzyme was defined as one percentage of reaction per hour per mg protein.

6. Protein Content

Protein content was determined according to the method of Bradford (1976). The reaction containing 4 ml of Coomassie blue and 1 ml of crude enzyme solution was determined at absorbance 595 nm, and its protein concentration was calculated. Bovine serum albumin (BSA) was used to generate a standard curve.

PART II

Effect of Gibberellic Acid (GA_3) on Durian Fruit Dehiscence

1. Sample Preparation

Durian fruit cv. Monthong from Trat Province was harvested at 106 days after anthesis. Fruit samples were transported to the laboratory within 12 hours. Fruit samples were stored according to uniformity of shape and size, and only fruit which consisted of at least four locules were selected. Each fruit was sprayed with 50 ppm GA_3 on two locules whereas the other two locules were not sprayed. All samples were stored at room temperature (27±3°C) for ripening. Every 2 days up to 8 days after harvest, twenty fruits were sampled and divided into 4 groups of replications. Dehiscence score, percentage of weight loss and peel color (L, a, b scale) were recorded. The tissues along the dehiscence zone (DZ) from control and GA_3 treated fruits were collected. Tissues samples were cut into small pieces, frozen with liquid N_2 and kept at -70°C until use.

2. Preparation of Alcohol Insoluble Solids (AIS)

Frozen durian tissues (10 g) were boiled with 100 ml of 95% ethanol at 70°C for 30 minutes. The sample was then homogenized in 100 ml boiled ethanol for 5 minutes. The homogenate was filtered through Whatman#1 paper, before washing it with 200 ml of 95% ethanol. The materials were then washed successively with a solution of methanol and chloroform at a 3:1 ratio, followed by acetone. The residues were dried overnight then weighted and referred to as the amount of AIS. The sample of cell wall materials were kept in desiccators until use.

3. Sequential Extraction for Pectin Fractions

AIS (100 mg) were first extracted with 30 ml of water and shaken for 5 hours at 25°C. The sample was centrifuged at 10,000 rpm and the supernatant was collected. The extraction was repeated overnight with two changes of water and the supernatants were pooled together. The residue was then similarly extracted with 30 ml of 50 mM CDTA (in 1 M imidazol), and then with 50 ml of 50 mM Na_2CO_3 (containing 20 mM $NaBH_4$). The supernatant of each extraction was dialyzed against 2 liters of distilled water at 4°C overnight. The content of uronic acid of each fraction was determined. D-galacturonic acid was used as a standard (Blumenkrantz and Asboe-Hensen, 1973).

4. Sequential Extraction for Hemicellulose Fractions

The residues from previous pectin fractions were used for sequentially extraction with 1 and 4 M KOH for loosely-and tightly-bound matrix glycan fractions, respectively and referred to as the hemicellulose fractions. The residues were first extracted with 50 ml of 1 M KOH containing 20 mM $NaBH_4$ for 2 hours. The supernatant was collected after centrifugation at 12,000 rpm for 20 minutes. The residues were re-extracted with the same amount of 1 M KOH for 5 hours and the

supernatants were pooled and designated as 1 M KOH-soluble fraction. The residues were then similarly extracted with the same amount of 4 M KOH. Using similar steps as described previously, the supernatant was collected on extraction with 1 M KOH. The supernatant from hemicellulose fractions was neutralized using concentrated HCl before dialysis. The supernatants from both 1 and 4 M KOH were determined for the content of total neutral sugars by anthrone assay and the absorbance at 620 nm was measured, with D-glucose used as a standard (Dische, 1962).

5. Crude Enzymes Extraction and Analysis

5.1 PG and EGase activities were determined as described by Abu-Bakr *et al*. (2003).

Frozen durian tissue (10g) was homogenized for 5 minutes in 50 ml cooled distilled water. The homogenate was centrifuged at 12,000 rpm at 4°C for 20 minutes and the supernatant was discarded. The pellet was re-suspended in 50 ml of 0.2 M Tris-HCl buffer (pH 9.0) containing 5% NaCl and stirred for 2 hours at 4°C. The slurry was centrifuged as previously described before the supernatant was then added to an ammonium sulfate (0.526 g/ml) and stirred for 20 minutes. The slurry was centrifuged as above, and the supernatant was dialyzed overnight at 4°C against a 20 mM sodium acetate buffer pH 6.0. The supernatant was collected and referred to as crude enzyme extract.

The reaction mixture containing 0.5 ml of 0.5% polygalacturonic acid (PGA) for PG assay or 0.1% carboxyl methylcellulose (CMC) for EGase assay, and 0.5 ml of 20 mM sodium acetate buffer pH 6 and 0.5 ml of crude enzyme, was incubated at 37°C for 2 hours. The activities of PG and EGase were assayed by measuring the formation of the released reducing groups from PGA and CMC, respectively. The absorbance at 260 nm was measured. D-galacturonic acid and D-glucose were used as standards for PG and EGase assays, respectively.

5.2 PME activity was determined as described by Hangerman and Austin (1986).

Durian tissues from the control and the GA_3 treatment (20 g) were homogenized for 2 minutes in 20 ml of 1 M NaCl in a homogenizer. 2% (w/v) of PVPP was added to the homogenate and then stirred for 2 hours at 4°C. The mixture was filtrated through two layers of cheesecloth and then centrifuged at 15000 rpm for 20 minutes at 4°C. The supernatant was adjusted to pH 7.5 with NaOH and then used for enzyme assay and protein content measurement.

The reaction mixture containing 2.0 ml of 0.5% (w/v) pectin (pH 7.5), 0.2 ml of 0.01% bromthymol blue (in a 0.003 M potassium phosphate buffer pH 7.5), and 0.7 ml of distilled water (pH 7.5), was incubated at 25°C. The reaction was started after adding 100 ml of enzyme solution, and the rate of decrease in absorbance 260 nm was recorded after 1 minute using distilled water as the blank.

5.3 β-gal activity was determined as described by Ross et al.(1986).

Twenty grams of durian tissues from the control and GA_3 treatment were mixed with 2% (w/v) PVPP and homogenized for 2 minutes in a homogenizer in 20 ml of 25 mM sodium acetate buffer containing 10 mM sodium tetrathionate (NaTT). The homogenate was centrifuged at 15,000 rpm for 20 minutes at 4°C and the supernatant was discarded. The pellet was washed twice with 5 mM NaTT. The pellet was resuspended in 6 ml of 25 mM sodium acetate, 100 mM NaCl, 2 mM NaTT, pH 4.5. The mixture was stirred for 1 hour at 4°C and centrifuged at 15,000 rpm for 20 minutes at 4°C. The supernatant was collected for enzyme assay and protein content measurement.

Samples to be assayed for enzyme activity were incubated in test tubes for 60 minutes at 30°C. The 2 ml mixture contained 0.4 ml of 25 mM sodium acetate pH 4.0, 0.4 ml of 0.3% β-mercaptoethanol, 0.4 ml of 2 mM ρ–nitrophenyl-β-D-galactopyranoside and 0.8 ml of enzyme solution. The reaction was then terminated by the addition of 2 ml of 200 mM Na_2CO_3. The activity of β-gal was assayed by measuring the ρ-nitrophenol released at the absorbance at 410 nm.

6. Protein Content

Protein content was determined according to the method of Bradford (1976). Four ml of Coomassie blue were added to 1 ml of crude enzyme solution. The concentration was determined at absorbance 595 nm, and bovine serum albumin (BSA) was used to generate a standard curve.

7. Statistical Design

The statistical was designed as a Completely Randomized Design (CRD). The experimental data are means. Data analysis using *t*-test was performed to determine differences between means of the treatments at $p \leq 0.05$.

8. The Experimental Locations

1. Durian orchards in Chantaburi and Trat Provinces

2. Postharvest Research Unit, Central Laboratory and Greenhouse Complex, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom

3. Department of Pomology, University of California at Davis, California, United States of America