CHAPTER III

MATERIALS AND METHODS

3.1. Plant materials

P. mirifica leaves and tubers of PM-III, PM-IV and PM-V (Cherdshewasart & Sriwatcharakul 2007, Cherdshewasart et al 2008) were employed in this study (Figure 12). Fresh leaves and tubers were harvested in Banpong District, Ratchburi Province (E99°52°/N13°37°) and cleaned since March 2007 until February 2008. The mature fresh leaves, weight at least 3 kg were randomly harvested from 3 areas in each plant species since March 2007 to February 2008 (Figure 13). The at least 3-year-old tubers of 3 cultivars of PM-III, PM-IV and PM-V were freshly harvested in seasonally term (Figure 14). The monthly record of daily mean temperatures and rainfall in the Ratchaburi Province was provided by the Meteorological Department, Ministry of Information and Communication Technology, Thailand.

3.1.1. Metabolomic study (major isoflavonoid contents)

The harvested leaves (monthly harvested since March 2007 to February 2008) and tubers (seasonally harvested in summer (April 2007), rainy season (August 2007) and winter (December 2007) were cleaned and dried in hot air oven at 80°C for 72 hours and subsequently powdered at a size of 120 μm. The leaf and tuber powder samples were stored in the dark room temperature with humidity control.

3.1.2. Proteomics study

The leaves and tubers were freshly harvested during summer (April 2007) rainy season (August 2007) and winter (December 2007) were cleaned and stored at -80°C.

a)

b)

c)

Figure 12 The plant trial field of PM; a) PM-III, b) PM-IV and c) PM-V

a)

b)

c)

Figure 13 The trifoliate leaves of *P. mirifica*; a) PM-III, b) PM-IV and c) PM-V

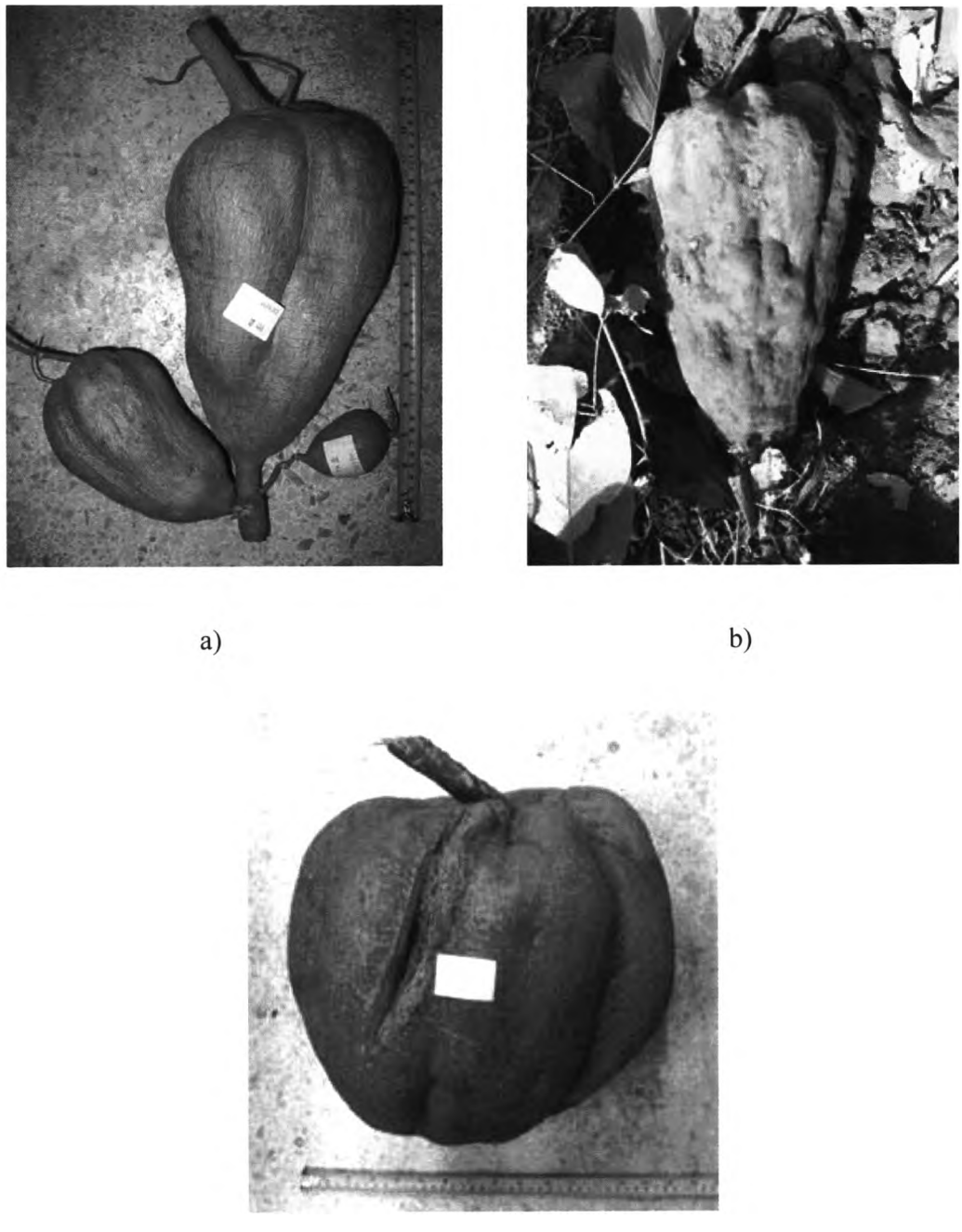

Figure 14 The storage tubers of *P. mirifica*; a) PM-III, b) PM-IV and c) PM-V

3.2. Analysis of the major isoflavonoids contents of *P. mirifica* tubers and leaves

3.2.1. Plant crude extraction method

The 50 g of samples (both leaf and tuber) powder were extracted with 500 ml methanol overnight for 3 times. The pellet was discarded and the supernatant was filtrated (Whatman filter paper No.4, Whatman, USA) and evaporated with rotary evaporator in vacuo (Buchi, Germany) at 37°C until completely dried. The plant crude extract was dissolved in distilled water and partitioned in chloroform (1:1 (v/v)) until no green color appear in the distilled water part to get rid of chlorophyll. The obtained water part was partitioned in butanol (1:1 (v/v)) for 3 times and the butanol phase was subsequently evaporated in vacuo at 45°C until completely dried. The obtained crude extract was stored at 15°C.

3.2.2. Chemicals and equipments

Isoflavonoid standards, including puerarin, genistin, daidzein and genistein were purchased from Sigma, St. Louis, MO, U.S.A., daidzin was purchased from Fluka Biochemika (Buchs, Switzerland). The organic solvents for chromatography (HPLC grade) were purchased from Merck, Germany. The water of over 16 MΩ/cm for a component of the mobile phase of HPLC was prepared by Maxima Ultrapure Water Systems (ELGA). HPLC system control and data processing were carried out by a Waters™ apparatus (Waters-717 plus Autosampler, Waters-600 Controller, Waters-2996 Photodiode Array Detector). The reversed phase C18 column (250 x 4.6 mm) was filled with 5 μm ODS2 (Waters Spherisorb, Ireland), pre-filtered with Waters Spherisorb S5 ODS2 (4.6x10 mm) guard cartridge. The filter set was Millipore membrane at 0.45 μm pore size with 13 mm diameter for the sample and 47 mm diameter for the mobile phase, of HA type for aqueous solution and HV type for organic solvent. The chromatography manager software Empower™ was operated on a personal computer.

3.2.3. HPLC sample preparation

The 1 mg of sample crude extract was extracted at room temperature with 1 ml methanol (Merck, HPLC grade) with the aid of sonication for 30 min. The supernatant was filtrated with a 0.45 μm pore size, 13 mm diameter membrane.

3.2.4. Quantitative RP-HPLC

Methods for major isoflavonoids analysis were modified from those previously described (Cherdshewasart *et al.*, 2007[a]) by setting the linear gradient system for 50 min from 100: 0 to 55: 45 with 0.1 % acetic acid: acetonitrile, with a flow rate of 1 ml/min and analyzed at the wavelength of 254 nm. The standard isoflavonoids were serially diluted from 1:1 to 1:16 with methanol to establish the concentrations of 1/4, 1/8, 1/16, 1/32 and 1/64 mg/ml, to generate a five point calibration curve. Calibration curves were obtained for all isoflavonoids by plotting the standard concentration as a function of peak area from HPLC analysis of a 10 µl injection volume. The concentrations of standard were chosen to cover the range of isoflavonoid concentrations in the samples. The analyses of the samples were run in triplicate and identified by comparing the retention times and quantified for the amount using standard curves of peak area of the isoflavonoid standards.

3.2.5. Statistical analysis

The mean ± S.E.M. of isoflavonoid contents from the tuberous root and leaf samples of *P. mirifica* were analyzed for statistical significant by the un-paired T-test and analysis of variance (ANOVA) followed by Duncan's multiple range test at the significance level of $P < 0.05$.

3.3. Proteomics study of *P. mirifica*

3.3.1. Verification of protein extraction method

3.3.1.1. Acetone precipitation

The 1 g of *P. mirifica* tissue (leaf or tuber) was pulverized to fine powder with liquid nitrogen in a mortar and pestle. The powdered samples were extracted with 5 ml acetone containing 10% TCA (Trichloroacetic acid) (w/v) and 1% DTT (Dithiothreitol) (w/v). The samples were kept at -20°C for 2 h (overnight, if necessary) and centrifuged at 4,000×g_{max} at 4°C for 20 min to collect the precipitated protein, decant supernatant. The pellets were washed twice with acetone containing 1% DTT, incubated at -20°C for 2 h and centrifuged at 4,000×g_{max} at 4°C for 20 min. The pellet was dried by inverting on Kimwipe for 15 min at room temperature and stored at -80°C.

3.3.1.2. Tris-HCl extraction

The 1 g of *P. mirifica* tissue (leaf or tuber) was pulverized to fine powder with liquid nitrogen in a mortar and pestle. The amount of 5 ml of extraction media (0.175 M Tris-HCl, pH 8.8, 5% SDS, 15% glycerol, 0.3M DTT) was directly added to the mortar and ground for 30 sec. The sample tissues were filtrated through the two layers of Miracloth and collected in a 50 ml Falcon tube. The amount of 4 volumes of ice cold 100% acetone was immediately added to the filtrated and mixed with the aid of a vortex. The proteins were precipitated overnight at -20°C. The samples were centrifuged at 4,000×g_{max} for 20 min to collect the precipitated protein. The pellet was washed twice in 15-20 ml of cold 80% acetone. The protein was collected by centrifugation at 4,000×g_{max} for 20 min and dried by inverting on Kimwipe for 15 min and stored at -80°C.

3.3.1.3. Phenol extraction method A and B

Extraction buffer of method A comprises 0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 0.1 KCl, 2% β-mercaptoethanol, whereas, in the extraction buffer of method A, 2 mM PMSF (phenylmethylsulfonyl fluoride) and 50 mM EDTA were added.

The 1 g of fresh *P. mirifica* tissue was pulverized to fine powder with liquid nitrogen in a mortar and pestle. The amount of 5 ml of extraction buffer was added and vortexed then kept at 4°C at least 30 min and centrifuged at 4,000×g_{max}, 4°C for 10 min. The supernatant was transferred to a new tube, kept at 4°C. The pellet was resuspended and repeated for extraction. The amount of one volume of water-saturated phenol was added and mixed vigorously and kept on ice for 1 h. The solution was centrifuged at 4,000×g_{max}, 4°C for 10 min. The upper phenol phase was transferred to a new tube and did a back-extract phase with 1:1 (v/v) of extraction media and phenol by vortex. The solution was centrifuged at 8,000×g_{max}, 4°C for 10 min and added to the previous extraction. The proteins were precipitated by adding 5 volumes of 0.1 M ammonium acetate in 100% methanol to the phenol phase, stored at –20°C overnight. The solution was centrifuged at 4,000×g_{max} at 4°C for 10 min. The pellet was dissolved immediately with 1 volume of cold water and subsequently sonicated for 3 min. The nine volumes of cold acetone was added, kept at –20°C at least 4 hours and centrifuged at 4,000×g_{max}, 4°C for 10 min to recover the precipitate protein. The solution was kept at -80°C until use.

3.3.2. Protein Quantification

The standard curve of bovine serum albumin (BSA) was established at the concentrations of 0, 0.2, 0.3, 0.5, 0.7, and 1.0 mg/ml. The amount of 0.8 ml of BSA sample was mixed with 0.2 ml Bradford reagent. The solutions were incubated for 10 min at room temperature. The absorbencies were measured at 595 nm using the spectrophotometer against a blank of 0 mg/ml BSA. The experiment was triplicate repeated. The average values of the absorbency were plotted to set up a standard curve. The slope and R^2 values were determined. The amount of 0.8 ml diluted extracted protein of *P. mirifica* samples were mixed with 0.2 ml Bradford reagent in the test tubes and submitted to the same measurement.

3.3.3. Protein Separation (by using 2-Dimensional Polyacrylamide Gel Electrophoresis, 2D-PAGE)

3.3.3.1. First dimension (Isoelectric focusing)

The amount of 50 μg for tuber and 80 μg for leave of each protein sample was dissolved in lysis buffer was mixed with 75 μl rehydration buffer and subsequently loaded onto IPG strips of linear pH gradient 3–10. Rehydration and subsequent isoelectric focusing were conducted using the Ettan IPGphor III Isoelectric Focusing System (Amersham Biosciences, USA). Rehydration was performed overnight in the strip holder with Drystrip Cover Fluid (GE health care). The isoelectric focusing for first dimension was run in the following steps: 1. Step and hold 300V 0.2kVh, 2. Gradient 1,000V 0.3kVh, 3. Gradient 5,000V 4.5kVh, 4. Step and hold 5,000V 2.0kVh.

3.3.3.2. Second dimension (SDS-PAGE)

Each strip was equilibrated with 1.5 ml equilibration buffer containing 50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT and trace amount of bromophenol blue for 20 min. The strip was placed in fresh 1.5 ml equilibration buffer containing 2.5% iodoacetamide (instead of DTT) for another 20 min. The equilibrated strips were loaded on 14.0% SDS-polyacrylamide gels electrophoresis second dimension and separated using PROTEAN II XI multi-cell (Bio- Rad, USA). Three replicated gels were run for each *P. mirifica* sample. The analyzed gels were stained with Colloidal Coomassie Brilliant Blue G-250 (CCB).

3.3.4. Image analysis

The analyzed gels were scanned using Labscan 5.0. Image Master 2D Platinum 6.0 and analyzed by computer. The 2-DE was independently performed and compared in triplicate and the consistent spots were reported for statistical analysis. The unpaired *t*-test was performed for comparison of derived data during season. Every spots will be subjected to perform in MS analysis.

3.3.5. In-gel digestion with trypsin

The protein spots on the gel was cut and washed twice with 100 µl distilled water. The amount of 50 µl of 0.1 M NH_4HCO_3/ 50% ACN was added, vortex and incubated at 30°C for 20 min to destain the dye. The gel was dried using the speed-vac and added with 50 µl 0.1 M NH_4HCO_3/ 10 mM DTT/ 1 mM EDTA. The gel was incubated for 45 min at 60°C. The solution was discarded. The amount of 50 µl 100 mM IAA/ NH_4HCO_3 was added to promote alkylation and incubated for 30 min in the dark. The solution was discarded. The gel was washed 3 times with 0.05 M Tris-HCl, pH8.5/ 50% ACN and dried using the speed-vac. The digestion buffer was prepared (1 part trypsin solution: 90 µl 0.05 M Tris-HCl pH 8.5/ 10% ACN/ 1 mM $CaCl_2$). The amount of 30 µl digestion buffer was added and incubated at 37°C overnight. The solution from the micro-centrifuge tube was passed to a new tube (master tube), The amount of 20 µl 2% TFA add was added to the previous tube and incubated at 60°C for 30 min to stop the reaction. The solution was removed and added to the master tube. The amount of 40 µl 0.05 M Tris-HCl pH 8.5/ 1 mM $CaCl_2$ was added to the previous tube, incubated at 30°C for 10 min. The solution was sonicated for 5 min and 40 µl ACN was added to the gel in the previous tube, incubated at 30°C for 10 min. The mixture was sonicated for 5 min, the supernatant was collected and added to the master tube. The amount of 40 µl 5% formic acid/ 100% ACN were added to the previous tube, incubated at 30°C for 10 min and sonicated for 5 min. The supernatant was collected and added to the master tube. The solution in the master tube was subsequently dried using speed-vac for the mass spectrometry preparation.

3.3.6. Mass spectrometry and protein identification

The LC-MS/MS analysis was performed by using a capillary LC system (Waters) coupled to a Q-TOF mass spectrometer (Micromass, UK) equipped with Z-spray ion-

source working in nanoelectrospray mode. The Glu-fibrinopeptide was used to calibrate the instrument in MS/MS mode. The tryptic peptides were concentrated and desalted on a 75 μm id × 150 mm C_{18} PepMap column (LC Packing, Netherland). Eluents A and B were 0.1% formic acid in 97% water, 3% ACN and 0.1% formic acid in 97% ACN, 3% water respectively. The plant sample (6 μl) was injected into the nano-LC and separation was performed using the gradient: 0 min 7% B, 35 min 50% B, 45 min 89% B, 49 min 80% B, 50 min 7% B and 60 min 7% B. For ESI-Q-TOF analysis, automatic scan rate was 1.0 s with interscan delay of 0.1 s. The parent mass peaks within the range of 400-1,600 *m/z* were selected for MS/MS analysis. The collision energy was fixed at 38 eV. The MS/MS data were processed using MassLynx 4.0 software (Micromass) and converted to pkl files by the ProteinLynx 2.2 software (Waters), which were analyzed using the MASCOT search engine (http://www.matrixscience.com). The search parameters were defined as follows: Database, NCBIr (for comprehensive, non-identical protein database); taxonomy, *Viriplantae* (Green Plants); enzyme, trypsin; one missed cleavage allowed; Variable modifications are Carbamidomethyl (C) and Oxidation (M); Peptide and fragment mass tolerance were set at 1.2 and 0.2 Da; Peptide charge +1,+2 and +3; Instrument ESI-QUAD, respectively. Proteins with molecular weight and p*I* consistent with gel region, with at least one peptide exceeding score threshold ($P < 0.05$), were considered positively identified.