QUANTITATIVE PROTEIN EXPRESSIONOF MALAYSIAN PHALERIAMACROCARPA

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Herbal plants are the best alternative for synthetic medicines which are costly and proved to be double-edged source. P. macrocarpa is the traditional herb compatible in curing diseases like diabetes, inflammation, and even cancer. People have gained lots of benefits from most parts of the plant with largest usage of seeds. This study aimed to analyze protein expressions in three major parts of the plant; leaf, seed and fruit by using SDS PAGE and high-throughput two-dimensional gel electrophoresis as proteomics approached. There were 42 spots detected in a pH range of 3-10 with 3 spots have been analyzed by MALDI-TOF mass spectrometry. Most identified proteins were expressed in seeds with highest intensity of bands and spots. The identified spots are homologs to putative clathrin assembly protein and ferredoxin-NADP+ reductase which are characterized as 'housekeeping agent' in plant system.

Keywords - Protein expression, phaleriamacrocarpa, 2D-PAGE, clathrin, ferredoxin

I. INTRODUCTION

Diseases outbreaks nowadays demand less than hundred percent of reliance towards synthetic medicines and modern therapy due to its side effects and high prices. People of East Asian countries claim on the traditional ways of treating most commonly encountered ailments like diabetes, high blood pressure, inflammation and many more by consuming herbal plants [1]. There are incentives provided by the Malaysia government on research and development activities for herbal industry through agencies like FRIM and MARDI[2].

One of the challenges encountered when dealing with herbal plant is to produce the maximum yield of products with highest quality but at lowest possible expenditure. 'Mahkota Dewa' scientifically known as Phaleriamacrocarpa, is one of the most preferred herbal plants by medical practitioner since the last few decades. It is originated from Papua Island, Indonesia where a great story behind the use of this plant as a cure had begun. The species belongs to the SuperkingdomEukaryota, Kingdom Viridiplantae, Phylum Streptophyta, Class Eudicotyledons, Subclass Eurosids II, Order Malvales and Family of Thymelaceaea [3]. The ripen fruits are bright red in color while the dicotyledon seeds are inedible and white in color. The seeds cannot be consumed directly due to its poisonous properties [4]. The fruit tissue of P.macrocarpa is fibrous in texture and greenish white in color.

This study is to investigate protein profiling of P. macrocarpa, a non-model plant using two-dimensional electrophoresis (2DE). There were many research conducted on the secondary metabolites of the crude extracts which are the main source of the curing elements potentiallyoriginated from this plant. The fruit content of P.macrocarpathat are found to be rich in bioactive compounds do have healing properties such as anti-inflammatory, anti-oxidative and anticancer agent [5]. However, in depth study on the protein expression of the plant has lagged behind both in Malaysia and abroad. The significant of this study may lead to a new discovery on the potential of the protein to provide the healing effects of this well-known plant.

II. MATERIALS AND METHODOLOGY

2.1 Protein Extraction

Three 'Mahkota Dewa' plants were bought from a nursery (Exoticmatahari Trading, Bukit Rangin, Kuantan Pahang). The three replicates were of the same size and almost at the same stage of development. The methods for extraction were based on techniques proposed by Faurobert with slight modification [6]. The two samples; leaf and seeds, were grounded in liquid nitrogen using mortar and pestle. Meanwhile, the fruit was cut into pieces and ground directly with the extraction buffer. The samples were lysed with extraction buffer (0.5 M Tris-base, 0.05 M EDTA, 0.7 M sucrose and 0.1 M KCl in distilled water buffered to pH 8 with 6 M HCl) in 1 to 3 ratio. All samples were then vortexed and incubated by shaking on ice for 10 minutes. An equal volume of Tris-buffered phenol was added into the extracts and further incubated by shaking at room temperature for another 10 minutes. Upon centrifugation at 5500 g and 4°C for 20 minutes, the upper phenolic phase was recovered and transferred to a new falcon tube. The original tubes containing both pellets and extraction buffer were added again with equal volume of Tris-buffered phenol. The phenol phases obtained were pooled and proceeded for the back extraction with another 3 ml of extraction buffer. The centrifugation step was repeated to collect the phenolic phase and further concentrated it. The phenol collected was

precipitated by invert shaking with 0.1 M ammonium acetate in cold methanol and kept overnight at -20°C. Next, the extracts were centrifuged for 10 minutes at 5500 g in 4°C condition and the pellet was recovered with another volume of precipitation solution. This washing step was repeated twice and once with cold acetone. Centrifugation at 5500 g for 5 minutes took place in between of each washing step. The pellet obtained was finally dried under vacuum in the laminar hood. The dried pellets were then kept in -80°C until further uses.

2.2 Protein Quantification

The amounts of proteins for each sample were quantified using Bradford assay with lysis buffer as the solvent for Bovine Serum Albumin (BSA) [7]. The frozen pellets were resuspended in 200 µl IEF buffer (7 M urea, 2 M thiourea, 40 mMTris-base (pH 8.8) and 2 % (w/v) CHAPS). BSA standard was prepared from 0.5 mg/ml stock solution dissolved in IEF buffer with 6 dilutions from 0 to 500 µg/ml. Both standards and samples were added with 10 µl of 0.1 N HCl after being adjusted to 100 µl with distilled water.

2.3 One-dimensional Protein Separation by SDS-PAGE

The Acrylamide:Bisacrylamidepercentages used for resolving and stacking gel were 12% and 4% formulation respectively. The amount of protein loaded into each well was 120 µg for each sample. These samples were denatured at 90°C for 5 minutes. Gel electrophoresis was carried out at 200 V for approximately 45 minutes until the bromophenol blue dye migrates out of the gel entirely. The gel was stained with Coomasie Blue R250 dissolved in acetic acid solution for 1 hour. Destaining of the gel was carried out twice with first destaining (30% acetic acid; 40% methanol) for30 minutes followed by second destaining step (7% acetic acid; 5% methanol) for overnight. The gel was viewed using GS-8000 densitometer (Bio-Rad).

2.4 Two-dimensional Protein Separation using IEF and SDS PAGE

For two-dimensional analysis, the IPG strip used was 7 cm with pH range from 3 to 10. The amount of sample required was increased up to 250 µg for leaf and fruit tissue extracts while the amount of seed taken was 200 µg. All samples were rehydrated in 125 µl of IEF buffer. Sample incubation took place overnight at 18°C. The strips were then isoelectric focused following the program conditions; 20 minutes at 100 V, 1 h at 350 V, 2 h at 3500 V, 10000 V per hour and hold at 100 V as according to Görg (2004) with slight modification [8]. The strips were then equilibrated in buffer (6 M Urea, 20% glycerol, 2% SDS, 1.5 M Tris pH 8.8 and 1% (w/v) DTT) for 20 minutes. 5 µl of protein marker were loaded and electrophoresis was carried out at 200 V for 45 minutes. The process of staining and destaining were completed using the similar methods previously used for SDS-PAGE. After an overnight destaining, the gels were scanned with densitometer and analyzed usingPDQuest software (Bio-Rad).

2.5 Protein Identification

The spots of interest were excised and kept in microcentrifuge containing 5 µl distilled water. The spots were then digested with trypsin upon screening with MALDI-TOF mass spectrometry. Dehydration of the spots took place in 100 µl of 100% acetonitrile (ACN) for 5 minutes at room temperature. The ACN solution was removed via drying in Speed Vac for 10 to 15 minutes at room temperature. The trypsin solution was prepared by resuspending the Trypsin Gold(Promega, USA) at 1 µg/µl in 50 mM acetic acid and further diluted in 40 mM NH_4HCO_3 /10% ACN to 20 µg/ml. The spots were preincubated in that solution in a minimal volume of 10 µl at room temperature. Digestion buffer (40 mM NH_4HCO_3/10% ACN) was added covering up the gel spots and incubation took place overnight at 37˚C.

The gel spots were incubated in 150 µl of nanopure water for 10 minutes, with frequent vortexing. The liquid obtained were removed and kept in a new microcentrifuge tube. Extraction of the spots was carried out twice with 50 µl of 50% ACN/ 5% TFA followed by mixing for 60 minutes each time at room temperature. All extracts were pooled and kept dry in a Speed Vac at room temperature for an average of 4 h. Pipette tips were used to purify and concentrate the extracted peptides (Millipore Corporation). Peptide sequencing was done by using MALDI-TOF and the data obtained from MS analysis were then identified and characterized using MASCOT Search Database from http://www.matrixscience.com. The search was performed based on peptide mass fingerprint of the proteins digested earlier.

III. RESULTS AND DISCUSSION

3.1 Protein Content

The total protein content is the highest in seeds with an average of 92.1 µg/ml concentration recorded. Meanwhile the average amounts for fruit tissue and leaf protein are 8.9 µg/ml and 18.4 µg/ml respectively.

3.2 SDS PAGE

The proteins were resolved for all three samples into distinct bands in the range of 20 to 260 kDa (Figure 1). Proteins extracted from leaf showed higher intensity at 60 kDa while the seed proteins were highly resolved between the sizes of 40 to 50 kDa. There were three bands produced at less than 30 kDa size for the seed extracts.

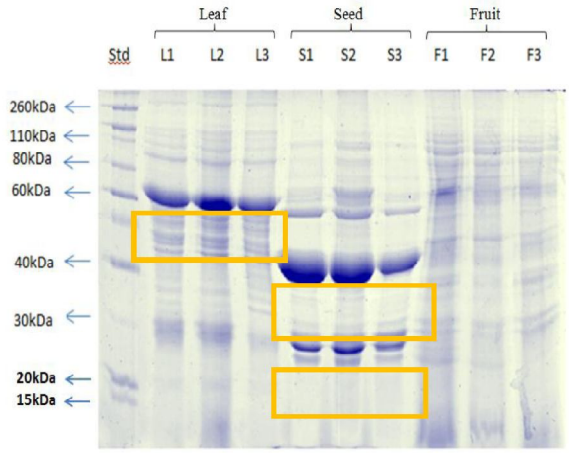

Figure 1
Gel image of one dimensional gel separation of the three samples from different parts of P. macrocarpa. Bands with high intensity were highlighted in the box.Std, Marker; L, leaf; S, seed; F, fruit; 1-3 represent replicates.

3.3 Two-dimensional electrophoresis of P. macrocarpa extracts

Protein profiles from three different parts of P. macrocarpawere compared in order to identify the different protein expressions by using 2DE approach (Figure 2). Approximately there are 13, 22 and 7 spots reproducibly observed in leaf, seed and fruit sample respectively. The spots were determined based on faint, cluster and small features observed on the master gel. Three spots have been selected for further identification using MALDI-TOF (Figure 2). The differences in the intensity of protein spots between leaf, seed and fruit tissue samples were compared using PDQuest software. The results from the analysis showed 8 matched spots between leaf and seed by using quantitative set analysis (Figure 3). No matched spot was found in fruit samples. The histogram represented the intensity of each spot and it can be interpreted that most spots are highly intensified in seed compared to leaf.

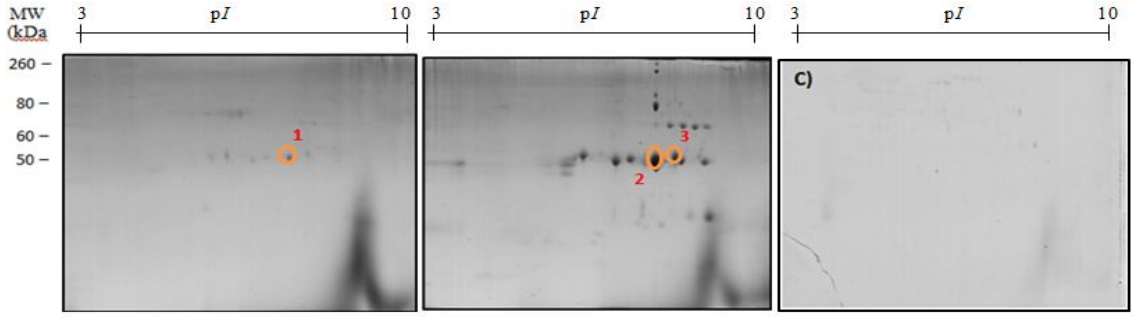

Figure 2
2DE images of the three samples; A) leaf, B) seed and C) fruit tissues.
Three selected spots for MALDI-TOF analysis were numbered in the diagram

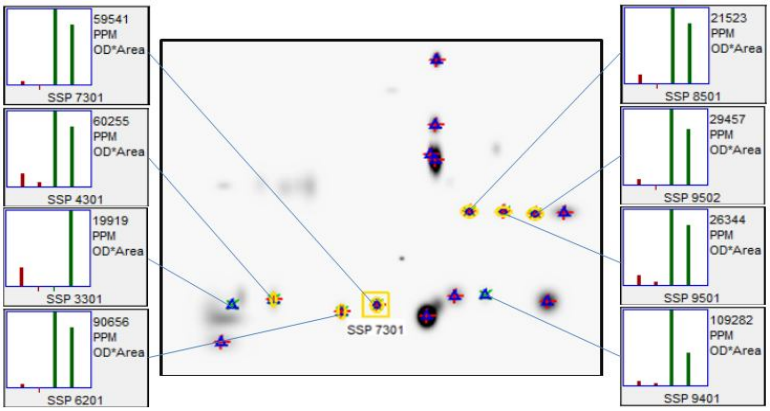

Figure 3
The protein matched spots between leaf and seed were shown on the master gel. The protein intensities graph showed green bar corresponds the seed while red bar represents the leaf.

Matching set analysis used in this experiment includes four different categories; quantitative, T-test, union and intersection of all gels. The details on the spots analyzed using these four categories are summarized in Table 1.

Table 1
Summary of analysis from PDQuest software

Analysis	Type	Spot count
Quantitative	Quantitative	8
Student's	Statistical	18
t-test	-	-
Union	Boolean	20
Intersection	Boolean	6

Student's t-test revealed significant differences in mean values and standard deviations for 18 spots. Meanwhile the union of all spots between the two different gels yielded total of 20 spots and the intersection between two gels produced 6 spots altogether.

The summary analysis of the identified spots from the MASCOT database search engine was presented in Table 2. The comparison made between tissue versusseed and tissue versusleaf yielded no matched spots. The seven spots observed in fruit samples were not able to be identified as

there is no match found with protein database. Two identified spots in seed are known as ferrodoxin NADP+ reductase involves in glucose synthesis and putative clathrin assembly protein for growth regulation mechanism. Meanwhile the third protein is associated with uncharacterized protein discovered in leaf of the plant that indirectly participates in homeostasis.

Table 2
Putative proteins identified from SWISS-PROT and NCBI databases

Spot no	Protein Name	Accession No	Mw (kDa) (predicted)	pI (calculated)	Matched (%)
1	Uncharacterized protein	Y2433_ARATH	46182	7.71	20
2	Putative clathrin assembly protein	CAP12_ARATH	43813	N/A	19
3	Ferredoxin-NADP+ reductase	gi|5730139	40481	8.66	23

Ferredoxin_NADP+ reductase

Ferredoxin-NADP+ reductase (FNR) is the concurring enzyme in the starch synthesis whereby this enzyme catalyzes the reduction of ferredoxin from metabolically generated NADPH molecule via oxidative pentose pathway [9]. The transfer of electrons from ferrodoxin protein molecule commonly takes place in chloroplast where light is the primary inducer. However, the protein homolog was found in the seed of P. macrocarpa where plastids are the organelle present instead of chloroplast. This is possible as previous study has successfully resolved FNR from the proteomic profiling of wheat endosperm [10]. A study on pea root plastid showed positive results on the expression of FNR and ferrodoxin molecule by using the plant leaf counterpart antibody [11]. They suggested that the induction of the protein was nitrate-dependent based on the up-regulation of the proteins when the plant was irrigated with nitrate-rich water supply.

Uncharacterized protein

One of the spots identified has the highest similarity with uncharacterized protein AT2G24330 from Arabidopsis that encodes predicted integral membrane metal-binding protein (DUF2296) family member. The protein encoded is involved in transporting metal ions across plant membranes and present in different range and localization depending on the requirement of the plant [12]. Heavy metals are one type of micronutrients acquired for normal growth of plants. However, the excessive uptake of these metal ions may lead to intoxication within the plant thus requires the host to regulate specific mechanism in homeostasis. The three typesof membrane transporters in higher plants include natural resistance-associated macrophage protein (Nramp), ATPases and cation diffusion facilitator (CDF) proteins [12]. Hence, the uncharacterized protein found in the leaf of P. macrocarpa may functional towards metal ions homeostasis of the plant.

Putative clathrin assembly protein

Clathrin is a component of coating molecules associated with assembly protein in the process of presence of clathrin is frequent in mammals yet disputable for plants due the presence of cell wall as well as the exertion of tugor pressure of that area[15]. An article explained in details the clathrin mediate endocytosis (CME) where generally it helps in plant growth regulation [16]. There are few important roles played by CME which include plant defense regulation in tobacco cells [17], modulation of potential accumulated toxic metal ions [18] and at least partly involves in cell plate formation [19].

CONCLUSION

In conclusion, the protein profiling of P. macrocarpa resulted in three housekeeping proteins which involved indirectly to the normal growth and development of the plant. The identified proteins are working together in the plant system to ensure the optimum growth of the plant.

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