1.0 INTRODUCTION

1.1 General

Persicaria minus (formerly *Polygonum minus*) or commonly known as *kesum* belongs to the family Polygonaceae. Kesum is also known as daun laksa among Malays and the other synonyms currently used for *P. minus* are Vietnamese coriander or Vietnamese mint. Kesum is widely grown throughout Southeast Asia and it can be found in tropical and subtropical zones in warm and damp conditions. The plant can grow up to 35 cm tall and it has pointed leaves that are usually 6-15 cm in length (Figure 1.1). Kesum leaves have a strong coriander and lemony type of smell attributed to the presence of aldehydes such as dodecanal and decanal. The green olfactory notes are attributed to (Z)-3-hexenal, (E)-2-hexenal, and (Z)-3-hexen-1-ol whereas the spicy odours are attributed to eugenol and terpenes.

Figure 1.1: Kesum or *Persicaria minus* at four weeks old

Kesum is traditionally known for its flavour and fragrance. Among the local community, kesum leaves is used as an ingredient in a local cooking, *laksa*. Kesum or *P. minus* has been identified as one of the crops having market opportunities in essential oil. The use of kesum

as a herb in cooking is of advantage as it has high content of phenolic compound and reducing antioxidant power (Huda-Faujan, Noriham, Norrikiah, & Babji, 2007). Additionally, kesum oil as compared to others such as parsley (*Petroselinum crispum*) and fennel (*Foeniculum vulgare*), has a complex of long-chain aldehydes and 1-alcohols created in nature and the presence of 1-alcohols and other constituents enhance the oil's olfactory profile. Besides, kesum oil may be a potential natural source of ingredient for the production of flavour and fragrance to replace the synthetic aliphatic aldehydes which are currently highly expensive, and have limited availability.

1.2 Plant proteomics

Proteomics is a high-throughput approach to address gene function that cannot be offered by genome sequences (2-D Electrophoresis Principles and Methods, Amersham Pharmacia Biotech). It is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique sorts protein in two steps: the first-dimension step is isoelectric focusing (IEF) which separates proteins according to their isoelectric points (pI); the second-dimension step is SDS-polyacrylamide gel electrophoresis (SDS-PAGE) which separates proteins according to their molecular weight (MW). After second-dimensional separation, each spot represents a single protein species from a sample. Thousands of different proteins can be separated, and information such as pI, molecular weight and amount of each protein can be obtained (Jorrin-Novo, Maldonado, Echevarria-Zomeno *et al.*, 2009).

Proteomic analysis of various plants have been published which focused on the protein profile of different parts of a plant. Some of the studies focused on an organelle or subcellular proteomes such as chloroplast (Lonosky, Zhang, Honavar *et al.*, 2004), the mitochondria

(Kruft, Eubel, Jansch, Werhahn, & Braun, 2001) or ribosome (Yamaguchi, Prieto, Beligni *et al.*, 2002; Yamaguchi & Subramanian, 2000). Other studies focused on specific tissues such as soybean seed (Mooney, Krishnan, & Thelen, 2004), *Arabidopsis* seed (Gallardo, Job, Groot *et al.*, 2001) and maize root (Shoresh & Harman, 2008) (Chang, Huang, Shen *et al.*, 2000). Proteomics studies on leaves such as rice leaf (Kim *et al.*, 2001), maize leaf (Porubleva, Vander Velden, Kothari, Oliver, & Chitnis, 2001), soybean leaf (Xu, Garrett, Sullivan, Caperna, & Natarajan, 2006) and tomato leaf (Ahsan, Lee, Lee *et al.*, 2007) have also been reported, and these studies focused particularly on the identification of low-abundance proteins.

1.3 Leaves Senescence

Plants are always exposed to stress which are generally defined as external factors that may affect growth, development, or productivity (Mahajan & Tuteja, 2005). Any unpleasant condition or substance that affects or blocks a plant's metabolism, growth or development can be categorized as stress. These stresses may play a role in leaf senescence, as if affects the plant metabolism and protein degradation. To date, there are several studies on leaf senescence induced by external factors such as drought (Hernandez, Alegre, & Munne-Bosch, 2004), light stress (Murchie, Chen, Hubbart, Peng, & Horton, 1999) and water stress (Kao, 1981).

Leaf senescence is an inbuilt programme in plant development which involves the breakdown of proteins, decrease in chlorophyll content, changes in RNA levels, and alterations of membranes and organelles. Nutrients are accumulated and transported to other parts of the plant during senescence. These macromolecular degradation and transportation during senescence are reported to be strictly controlled by certain genes, which are often referred to

as senescence associated genes (SAGs). These genes are also reported to be actively involved in energy metabolism and in supplying the metabolic energy for the transportation of nutrients. Besides, these genes also assist in ensuring the effectiveness of transcription and translation of plant proteins. The genes were identified to be up-regulated as leaf senescence progresses (Lim, Woo, & Nam, 2003).

Leaf senescence can also be caused by internal factors such as age-dependant, reproductive development and phytohormone levels (Gan & Amasino, 1997). Leaf senescence is also triggered by the level of plant regulators such as hormones and reactive organ species, shading, extreme temperatures, solar radiation, drought, excessive soil salinity, nutrient deficiency, wounding, phototoxic compounds such as ozone and pathogen attack (Gan & Amasino, 1997; Hernandez *et al.*, 2004).

Several studies reported that when plants are in stress, secondary metabolite production may increase because growth is inhibited more than photosynthesis, therefore the carbon allocated to growth will then be allocated to secondary metabolites (Gera M. Jocum, 2007). It has also been reported that secondary metabolites increases in response to elevated temperatures, however there are studies that reported otherwise. In general, plants respond towards abiotic stress stimuli by regulating signalling cascade followed by modulating gene expression machinery which could lead to the synthesis of stress responsive protein or valuable bioactive compounds.

1.4 Secondary metabolites

Secondary metabolites are low-molecular weight organic compounds with unique and complex structures that are not directly involved in the normal growth (Goossens, Hakkinen,

Laakso *et al.*, 2003). The term "secondary" was designated by a plant physiologist Albrecht Kossel and it refers to non-functional compounds (Hartman, 2008). Originally, waste and detoxification products were classified as secondary metabolites, but recently many complex biological functions discovered by ecologist and pharmacologist have been included. Besides the term "secondary", the term "byways of metabolism", "special metabolism", "idiolytes" (Demain, 1996), "allelochemicals", and "dispensable metabolites" (OkazakiFurunoKasukawa *et al.*, 2002) have been suggested. The term "secondary metabolism" has been established by its repeated use in books, particularly relating to plants starting in the 1950s.

Plants have the ability to produce a vast variety of secondary metabolites naturally. Secondary metabolites play a role in plants adaptation towards their environment such as biotic and abiotic stress, and also serve as a major source for pharmaceutical products (Mulabagal & Tsay, 2004). The function or importance of these compounds to plants are usually of an ecological nature as they are used as defences against predators, parasites and diseases, for interspecies competition, and to facilitate the reproductive processes, such as colouring agents and attractive smells.

1.5 Bioapplication

Plant natural products, particularly secondary metabolites have been exploited by human for centuries for use as medicines, dyes and to improve food quality. The two largest industries that rely on plant extracts are the pharmaceuticals and consumers' products industries.

Secondary metabolites are also referred to as natural products and presently, there are at least 100,000 of these secondary metabolites which have been isolated from higher plants (Goossens *et al.*, 2003). These natural products may have important functions in the plants

such as for adaptation to specific ecological niches or as responses to biotic and abiotic stresses (Terryn, Montagu, Inze, & Goossens, 2006).

In the pharmaceutical industry, secondary metabolites are beneficial for humans as pharmaceuticals and many are used as a source for drugs, dyes, flavours and fragrances. There are at least 119 pure chemical substances extracted from higher plants that are being used worldwide today as medicine. This is supported by the World Health Organization (WHO) report that at least 80% of the people in developing countries rely on traditional medicine, and plant extracts are applied in most of the treatments (Farnsworth, Akerele, Bingel, Soejarto, & Guo, 1985). To date, the application and use of these secondary metabolites are increasing, including for their broad range of antimicrobial activity as well as their use as essential oil for physical and psychological therapeutic benefits.

1.6 Biosynthetic pathway

Plant natural products are important for its medicinal, flavour and fragrance compounds. Metabolites have complex structures and cannot be produced economically through total chemical synthesis, but have to rely upon extraction from plant materials and the development of engineered production hosts.

Despite the importance of secondary metabolites to humans as well as for the plant itself, the pathways that produce these secondary compounds remain poorly characterized (Pichersky & Gang, 2000). Plant genomes are estimated to contain 20,000-60,000 genes, but the genetic maps of biosynthetic pathways are still far from complete and the knowledge on the regulation of these pathways is practically non-existent.

Plants that are exploited by harsh environmental conditions would activate the expression of certain abiotic stress related genes which are involved in the biosynthesis pathway of plant secondary metabolites (Timperio, Egidi, & Zolla, 2008). Therefore, it becomes a crucial need to identify the stress responsive genes and study their signal transduction pathways, not only for a better understanding of plant response and adaptation towards abiotic stress, but also for further development of strategies for commercial production of valuable compounds by manipulating certain metabolic pathways based on gene expression (Gor, Ismail, Mustapha *et al.*, 2010). A number of studies have been done to study the application of elicitation to plant cultures, either to enhance secondary metabolites production or to discover novel compounds. Many of these studies showed positive results, but the stress responsive genes which are involved in the reactions of defence-related pathway and secondary metabolites biosynthesis pathway remain largely unexploited.

To date, there are a few studies that have been published on plant secondary metabolism using two dimensional gel electrophoresis-based proteomics (Goossens *et al.*, 2003). More recently, the use of recombinant microorganisms to elucidate plant biosynthetic pathways and characterization of the individual biosynthetic enzymes has been reported. As more pathways are being characterized, the focus now is towards the use of microorganisms together with construction of short biosynthetic pathways to target overproduction of these compounds.

1.7 Objective of study

The main objectives of this study is to establish the kesum leaves proteome at different age and to identify proteins involved in kesum leaves biosynthetic pathway.

The specific aims are:

1) To prepare proteins from kesum leaves at different age
2) To establish the kesum leaves proteome map at various age stage
3) To analyse the proteome maps and identify potential proteins involved in the biosynthetic pathways of kesum leaves

2.0 MATERIALS AND METHODS

2.1 Plant sample

Kesum that is being used was obtained in Genting Highlands, Pahang which is located approximately 1650m above sea level. The collected kesum was then grown in different conditions from its natural environment such as temperature and soil. For this study, kesum was grown at lower latitude in University Kebangsaan Malaysia (UKM), Bangi, Selangor. In this study, the biological sample was pulled from approximately six kesum plants at each age.

2.2 Sample preparation

2.2.1 Sample harvesting

The leaves from the kesum plants grown in UKM were harvested at different ages over a period of time which is at four weeks, six weeks and eight weeks old. These time frames were choosen based on the phenotypic, which means the observable physical characteristics of the plant. In this study, the phenotypic characteristic of the kesum leaves are size and color. At four weeks old, the leaves were plucked at the second and third nodes (Figure 2.1a). Following this, a red string was tied in between the second and the third nodes as a marker. At six weeks old, the leaves that were initially at the second and the third nodes became the fourth and the fifth nodes (Figure 2.1b), and these leaves were harvested. At the end of the harvesting period, which is at eight weeks old, the leaves were already at the seventh and eighth nodes (Figure 2.1c), and were harvested. All kesum leaves that were plucked were frozen in liquid nitrogen and subsequently transferred into -80°C freezer for storage before the samples were processed.

2.2.2 Protein extraction from the kesum leaves

Protein sample was extracted from kesum leaves of the kesum plants using phenol extraction method. The frozen plant tissues were ground into fine powder using pestle and mortar. The ground kesum leaves were weighed and the powder was divided into aliquots of a known size, which is about 100 mg to 120 mg. These ground kesum leaves were processed immediately or stored at -80°C. The ground plant tissues were suspended in 1 ml of cold phenol Tri-ReagentTM (MRC Inc, Ohio USA). The tubes were then incubated at room temperature for 5 minutes. Subsequently, 200 µl of chloroform (Merck, Germany) was added into each tube to separate the homogenized sample into aqueous and organic phases. The tubes were shaken vigorously for 15 seconds and incubated for 15 minutes at room temperature. Centrifugation of the tubes was performed at 12,000 xg for 15 minutes at 4°C and the supernatant was discarded. To the remaining solution in the tube which has the DNA and protein, the DNA was removed by precipitation using 300 µl of 100% (v/v) ethanol. The tubes were then incubated at room temperature for 3 minutes and followed by centrifugation at 2,000 xg for 5 minutes at 4°C. The supernatant which now only has the protein was transferred into new tubes. Next, three times volume of acetone were added to the supernatant and the samples were mixed by inversion for 15 seconds. The tubes were then incubated at room temperature for 10 minutes. Next, the tubes were centrifuged at 12,000 xg for 10 minutes at 4°C. The supernatant was removed and the protein pellet was washed three times in washing solution containing 0.3M guanidine hydrochloride in 95% (v/v) ethanol and 2.5% (v/v) glycerol. At least, 1 ml of the washing solution was added per 0.5 ml of Tri-Reagent^{TM} used for the initial homogenization. For each wash step, the samples were incubated in the washing solution for 10 minutes at room temperature, vortexed and centrifuged at 8,000 xg for 5 minutes at room temperature. This was followed by a final wash step where the protein

a)

b)

c)

Figure 2.1 Marking of kesum plant nodes for harvesting of the leaves. A red string was tied in between the nodes as a marker. At four weeks old the leaves were harvested at the second and the third nodes (a). At six weeks old, the leaves were harvested at the fourth and the fifth nodes (b) and lastly at eight weeks old, the leaves were harvested at the seven and the eight nodes (c).

pellet was washed with 1 ml of 2.5% (v/v) glycerol in 100% (v/v) ethanol, vortexed, incubated for 10 minutes at room temperature and centrifuged at 8,000 xg for 5 minutes at 4°C. Finally, the ethanol wash was removed and the protein pellet was air dried for approximately 20 minutes at room temperature. The protein pellet was dissolved in ReadyPrep Sequential Extraction Kit Reagent 2 (R2) containing 8M urea, 4% CHAPS, 40 mM Tris and 0.2% Bio-Lyte 3/10 ampholyte. Approximately 60 µl of R2 was added into each tube to dissolve the protein pellet. Next, centrifugation was performed at 12,000 xg for 45 minutes at 4°C. The supernatant containing the dissolved protein was kept, while the remaining pellet was discarded. Lastly, tributylphosphine (TBP) was added to a final concentration of 200 mM. The protein samples were immediately stored in -80°C.

2.2.3 Determination of protein concentration

The concentration of the protein extracted from the kesum leaves sample was determined using the Micro BCA^{TM} Assay System (Pierce Biotech, USA). The protein samples were diluted to 50x, 100x, 250x and 500x in Homogenization Buffer (2.0 M Tris-HCl pH 7.5, 1.5 M KCl, 0.2 M $MgCl_2$, 0.2 M $CaCl_2$ and distilled water). A total of 150 µl of protein sample each was pipetted into a 96-well round-bottom microplate and Homogenization Buffer was used as the blank. The working reagent was prepared according to the manufacturer's protocol and 150 µl was added into each well containing the protein sample. The mixture was mixed well and incubated at 37°C in a moist chamber for 2 hours. After the incubation period, the plate was read using a microplate reader at 570 nm with 655 nm as reference wavelength. The concentration was calculated using a standard curve prepared before the quantification.

2.3 Electrophoresis

2.3.1 Preparation of SDS-PAGE gel

A 12.5% (w/v) SDS-PAGE of 0.75 mm thickness was prepared. Separating gel was prepared by adding the following components: distilled water, 30% (w/v) bis-acrylamide, 1.5 M Tris-HCl pH 8.0, 10% (w/v) SDS, 10% (v/v) APS and TEMED. The gel solution was poured into the gel casting apparatus (Bio-Rad, USA) until it reaches a predetermined level. Subsequently, this gel solution was overlaid with distilled water to even-out the polymerizing gel surface. Upon complete polymerization of the separating gel, the distilled water was poured away and the gel was blotted-dry with paper towels. A 4.5% stacking gel (30% bis-acrylamide, 0.5 M Tris-HCl pH 6.8, 10% SDS,10% APS and TEMED) was then prepared. A 10-well comb was inserted into position for formation of the wells after the stacking gel was added onto the polymerized separating gel. The stacking gel was then allowed to polymerize.

2.3.2 One-dimensional SDS-PAGE separation

A total of 15 µg of each kesum leaves protein sample prepared in step 2.2.2 was mixed with 3x SDS sample buffer (New England, Biolabs Inc, USA), followed by heating at 95°C for 5 minutes in a block heater. The protein samples were then loaded into the wells of the SDS-PAGE. The dual color protein maker (Bio-Rad, USA) was used and it was loaded into the side well. The gel was then electrophoresed at a constant voltage of 100 V for one hour in fresh Tris-glycine buffer (Tris-base, glycine, SDS and distilled water). The gel was then silver stained.

2.3.3 Two-dimensional SDS-PAGE separation

2.3.3.1 First dimension protein separation

2.3.3.1 (a) Rehydration

Isoelectric focusing (IEF) was performed on the kesum leaves protein using immobilized pH-gradient (IPG) strips [pH 3-10, 7 cm; pH 4-7, 7 cm and pH 4-7, 18 cm (Bio-Rad Lab, USA)] with Protean IEF Cell (Bio-Rad Lab, USA). The amount of protein loaded for 18 cm IPG strips was 150 µg of protein. From the determined concentration, the amount of sample to be loaded was calculated. The appropriate amount of rehydration buffer (8 M urea, 2% CHAPS, 0.001% of bromophenol blue, 18 mM dithioethreitol, 0.2% ampholytes of pH 3-10 and top up with distilled water) for 18 cm strip is 350 µl and was added to the sample. The sample solution was incubated for an hour at 4°C in rehydration buffer prior to rehydration. After an hour, the sample solution was filled into the centre of the rehydration tray lanes. The strips' cover sheets were removed and the strips were placed with the gel side facing down, making contact with the solution. The strips were then covered with mineral oil to ensure that the heat is distributed evenly. Active rehydration was performed for 12 hours at 20°C with constant voltage of 50 V.

2.3.3.1 (b) Isoelectric focusing (IEF)

After 12 hours of rehydration, IEF was performed using the following parameters. The IEF parameters for 7 cm IPG strips, pH 3-10 and 18 cm IPG strips, pH 4-7 are similar for step 1: 200 V for 200 V/hour, step 2: 500 V for 500 V/hour and step 3: 1,000 V for 1,000 V/hour. In step 4, the parameters for 7 cm, pH 3-10 and 18 cm, pH 4-7 IPG strips was 4,000 V for 16,000 V/hour and 8,000 V for 32,000 V/hour respectively. Paper or electrode wicks were

placed on each end of the strips during IEF to ensure that ionic contaminants present in the sample were absorbed and removed. At the end of the focusing, the IPG strips were removed from the tray and kept at -70°C until needed.

2.3.3.2 Second dimension protein separation

2.3.3.2 (a) Equilibration

The focused strip was equilibrated before second-dimension separation as equilibration was essential to saturate the strip with SDS buffer system of the second-dimension separation. Two equilibration buffers were prepared with the following constituent: 6 M urea, 2% SDS, 0.375 M Tris-HCL pH 6.8 and 2% glycerol. In Equilibration Buffer I, 130 mM dithiothreitol (DTT) (GIBCO BRL, Life Technologies Inc., USA) was added to the solution and 135 mM iodoacetamide (Sigma, USA) was added to Equilibration Buffer II. The strip was equilibrated for 15 minutes in Equilibration Buffer I and subsequently equilibrated in Equilibration Buffer II for the same period of time. The strip was then rinsed briefly with distilled water and then soaked in fresh Tris-glycine buffer for a few seconds. Finally the equilibrated strip was positioned on the surface of a prepared 12% SDS-PAGE gel.

2.3.3.2 (b) Vertical SDS-PAGE separation

For SDS-PAGE separation, 12% SDS-PAGE gel of 1.0 mm thickness gels was prepared as stated in section 2.3.1 but without the stacking gel. The focused and equilibrated IPG strip was transferred onto the gel surface. The broad-range dual color protein marker (Bio-Rad Lab, USA) was pipetted onto a paper wick and this paper wick was placed beside the IPG strip. After ensuring that no air bubbles were trapped between the IPG strip edge and the gel

surface, 0.5% of warm agarose was used to seal the IPG strip to the surface of the SDS-PAGE gel. The agarose was then allowed to solidify. The second dimension separation for the 7 cm IPG strip was performed at 80 V in fresh Tris-glycine buffer until the front dye had migrated to the end of the gel. Second dimension separation for an 18 cm IPG strip was performed at a constant current of 15 mA for 1 hour, 17.5 mA for 1 hour and finally 20 mA for 5 hours per gel. The gels were then stained with Colloidal Coomassie Brilliant Blue.

2.4 Staining of SDS-PAGE gels

2.4.1 Silver staining

The gels were fixed in fixative solution [40% (v/v) methanol, 10% (v/v) acetic acid and distilled water] for at least one hour and then washed with 30% (v/v) methanol for 20 minutes. A second wash was performed with water for another 20 minutes. After the washing, the gels were incubated in enhancing solution containing sodium thiosulphate (0.8 mM) for 20 minutes. The gels were then briefly rinsed with water three times before incubating with silver nitrate solution (10 mM silver nitrate, 0.028% formaldehyde) for 20 minutes. At the end of the incubation, the gels were briefly rinsed with water three times. The gels were developed in developing solution (566 mM sodium carbonate, 0.16 mM sodium thiosulphate, 0.0185% formaldehyde) until the protein bands were visible. The gels were briefly rinsed with water three times and followed by incubation with stopping solution (1.46% EDTA) for 10 minutes. Finally, the gels were washed in water for three times, each time for 10 minutes. All the incubation steps were performed with constant agitation at room temperature.

2.4.2 Colloidal Coomassie Brilliant Blue staining

Firstly, the gel was fixed in fixative solution [40% (v/v) methanol, 10% (v/v) acetic acid and distilled water] overnight. Following fixation, the gel was washed in distilled water two times, 10 minutes each washing. After washing, the gel was stained using Colloidal Coomassie solution [20% (v/v) methanol, 8% (w/v) ammonium sulphate, 0.96% (v/v) phosphoric acid, 0.08% (w/v) Coomassie Brilliant Blue G250 (Bio-Rad Laboratories)] for at least one day, after which the dye was poured away and the gel was destained with distilled water several times. The distilled water was replaced every 30 minutes until the background colour fades and the bands or protein spots become a satisfactory shade of blue.

2.5 Computational analysis of the established protein profiles

The stained gels were scanned with an Epson Expression 1,600 Pro scanner (Epson, Japan). The images for triplicate gels of each sample were analysed using The Discovery Series PDQUEST^{TM} two-dimensional analysis software version 7.2.0 (Bio-Rad Lab, USA). In the analysis, the gel images were filtered to remove background noise prior to protein spot detection. The gels were then aligned, normalized and matched. Protein spots were analysed quantitatively for differentially expressed proteins. Up-regulation was defined as 5 X significant increase in spot density and down-regulation was defined as 5 X significant decrease in spot density.

2.6 Protein identification by MALDI TOF/TOF mass spectrometry

2.6.1 Peptide mass fingerprinting (PMF)

Colloidal Coomassie blue-stained protein spots were excised from gels and placed in a 96-well plate. The gel plugs were destained in 50% (v/v) methanol containing ammonium bicarbonate (50mM) for 30 minutes and this step was repeated thrice. Following washing, the gel plugs were dried for 15 minutes and trypsin digestion was performed by adding trypsin (10 µl of 0.02 µg/µL of Sequencing Grade Modified Trypsin, Promega, USA) diluted in ammonium bicarbonate (20 mM) onto each gel plug. The gel plugs were digested for two hours at 37°C. After the incubation, peptide extraction from the gel plugs was performed using solvent solution (60 µL per sample well) consisting 0.1% (v/v) trifluoroacetic acid (TFA) and 50% (v/v) acetonitrile (ACN). The extracted peptides were then transferred into new wells, respectively. The extraction step was repeated using the same solvent solution (40 µL per sample well). The extracted peptides were transferred into the same new wells respectively. The solvent was evaporated at 37°C and the dried peptides were reconstituted with another solvent solution consisting of 0.5% (v/v) TFA and 50% (v/v) ACN (2.2 µL). The peptide solution (0.7 µL) was manually spotted onto the sample slides, followed by 0.4 µL of saturated matrix [α-cyano-4-hydroxy cinnamic acid (LaserBio labs, France) prepared in 0.5% (v/v) TFA and 50% (v/v) ACN]. The peptide-matrix mixture was then air dried and peptide sample and saturated matrix spotting was repeated again on the same sample spot. The sample slides were then analyzed using the ABI 4800 Plus MALDI TOF/TOF™ Analyzer (Ab Sciex, MA, USA).

2.6.2 Mass spectrometry (MS) data analysis

The calibration mix (Mass Standards Kit for Calibration of AB SCIEX TOF/TOF, Ab Sciex, MA, USA) was used as internal calibrants. Peptide mass spectra generated were analysed using the web engine, MASCOT; http://www.matrixscience.com/search. The parameters used for searching were as follows: at least one missed cleavage, specific modifications of oxidized methionine, variable modification of carbamidomethyl (C), charge state of +1 and mass tolerance of 50 ppm. Protein candidates were then examined based on the highest MOWSE score, sequence coverage and the number of peptides matched.

2.7 Chemicals and reagents

All reagents, chemicals and apparatus for one and two-dimensional electrophoresis were supplied by Bio-Rad Lab, USA. All other chemicals, unless stated were purchased from Sigma Aldrich, USA. All organic solvents or solutions were purchased from BDH, UK.

3.0 RESULTS

3.1 Establishment of the protein profile of kesum leaves from different groups

Total protein from kesum leaves of three different age groups: four weeks, six weeks and eight weeks were extracted using phenol extraction. The extracted protein was first analysed using one-dimensional electrophoresis (1-DE) for protein integrity. Multiple protein bands ranging from 250 kDa to 15 kDa were observed for three different age group samples of kesum leaves (Figure 3.1). In the four weeks old kesum leaves protein, protein bands up to 150 kDa were detected, while in the six weeks old sample, lesser high molecular weight bands were visible. The density of higher molecular weight bands was the lowest in the eight weeks old sample. As 1-DE analysis only separates proteins according to their molecular mass and each of the protein bands may represent one protein species or a mixture protein species, therefore, to further evaluate whether the band is a complex protein mixture, the protein samples were analysed using two-dimensional electrophoresis (2-DE).

To improve the separation and resolution of the kesum leave proteins, the complex protein mixtures were separated according to their pI values in the first dimension and then separated according to their molecular mass in the second dimension. A broad IPG strip of pH 3-10, 7 cm was used for the purpose of screening the suitability of the protein samples for IEF and also the IEF parameters. At least one hundred protein spots were detected on each of the gels for all three samples. The complex mixture of proteins was successfully separated and protein spots were noted to be in abundance in the range of pH 4-7. Therefore, a narrower range IPG strip pH 4-7, 7 cm was used for further separation of the protein spots and this resulted in better resolution of the protein spots.

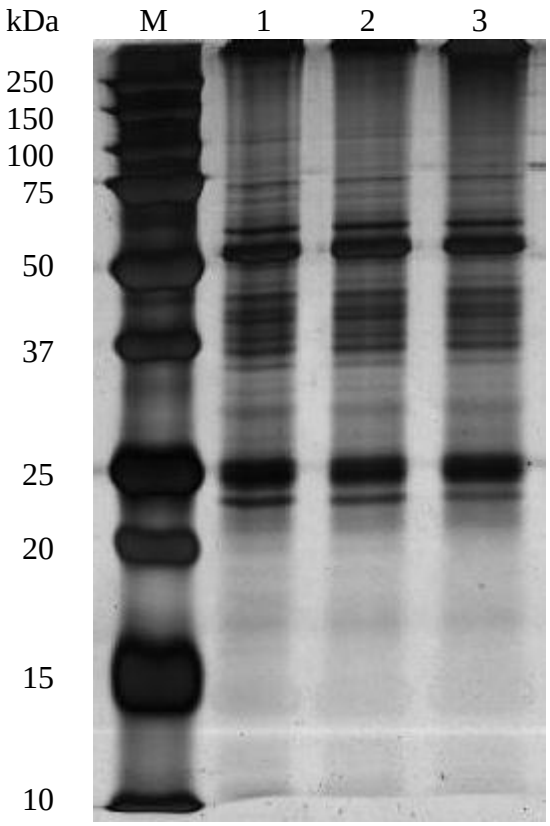


Figure 3.1 One dimensional-polyacrylamide gel electrophoresis (1D-PAGE) separation of kesum leaves protein at different age four weeks, six weeks and eight weeks-old on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The extracted proteins were electrophoresed on the 12% SDS-PAGE, and silver stained. Lane 1 indicates the protein sample extracted from kesum leaves at four weeks old, Lane 2 the protein sample extracted from kesum leaves at six weeks old and Lane 3 the protein sample extracted from kesum leaves at eight weeks old. Lane M indicates the protein marker.

Improved protein spots separation was further achieved using longer IPG strips (18 cm) and larger PAGE (18 cm). More than 200 individual protein spots were detected in four weeks old and six weeks old kesum leaves protein and about 100 protein spots were detected in eight weeks old kesum leaves protein.

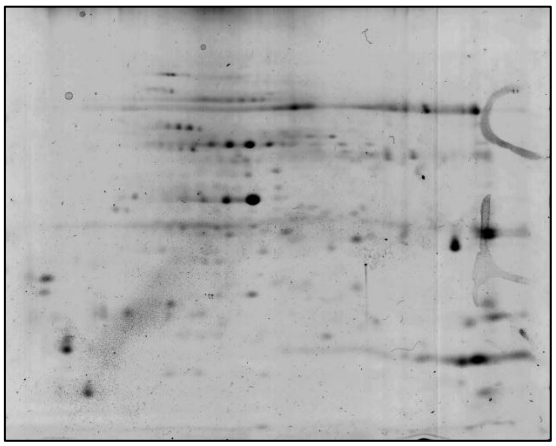
3.2 Computational analysis of the differentially expressed proteins in kesum leaves

A step-by-step comparison using PDQUEST^{TM} computational analysis was performed on the four weeks old and six weeks old kesum leaves protein. A similar comparison was made between the six weeks old and eight weeks old kesum leaves protein profiles established on pH 4-7 large format gels. A total of 223 protein spots were visualized in the four weeks old kesum leaves protein profile (Figure 3.2a). In the six weeks old protein profile (Figure 3.2b), 200 protein spots were detected and there were 193 protein spots in the eight weeks old protein profile (Figure 3.2c).

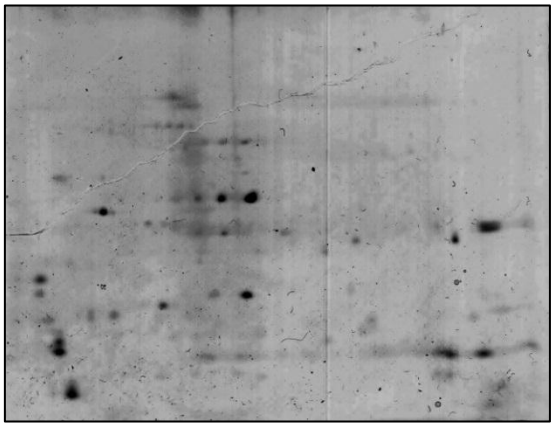
A standard reference gel image was constructed from the 2-DE of the four weeks old kesum leaves protein profile. Quantitative analysis was then performed by comparing the occurrence of every spot among the two sets of protein profiles (four weeks old and six weeks old kesum leaves protein, and six weeks old and eight weeks old kesum leaves protein consisting of three gels in each set). Several differentially expressed proteins either up-regulated or down-regulated of expressed protein were detected.

At least nine protein spots detected in the six weeks old kesum leaves protein profile were up-regulated in comparison to the four weeks old kesum leaves protein profile, while four protein spots were up-regulated in the eight weeks old kesum leaves protein profile in comparison to the six weeks old kesum leaves protein profile (Figure 3.3). A total of eight protein spots were down-regulated in the six weeks old kesum leaves protein profile in comparison to the four weeks old kesum leaves protein profile, while comparison between six weeks old and eight weeks old kesum leaves protein profile revealed that six protein spots were down-regulated in the eight weeks old kesum leaves protein profile (Figure 3.4).

(a)



(b)



(c)

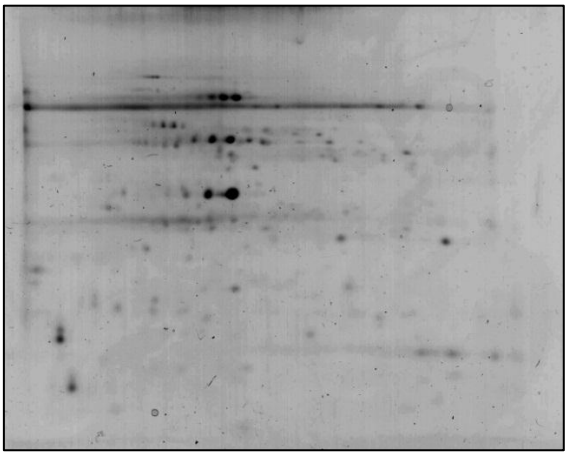


Figure 3.2 Coomassie blue stained two-dimensional-polyacrylamide gel electrophoresis (2D PAGE) of kesum leaves proteins on broad range pH 4-7, 18 cm gel. The protein samples from kesum leaves at four weeks old (a), six weeks old (b) and eight weeks old (c) were focused with the following parameters: active rehydration at 50 V for 12 hours; 200 V for 200 V/hour at gradient mode; 500 V for 500 V/hour at gradient mode; 1,000 V for 1,000 V/hour at gradient mode and final focusing at 8,000 V for 36,000 V/hour at step and hold mode. The proteins were then separated on 12% SDS-PAGE in the second dimension.

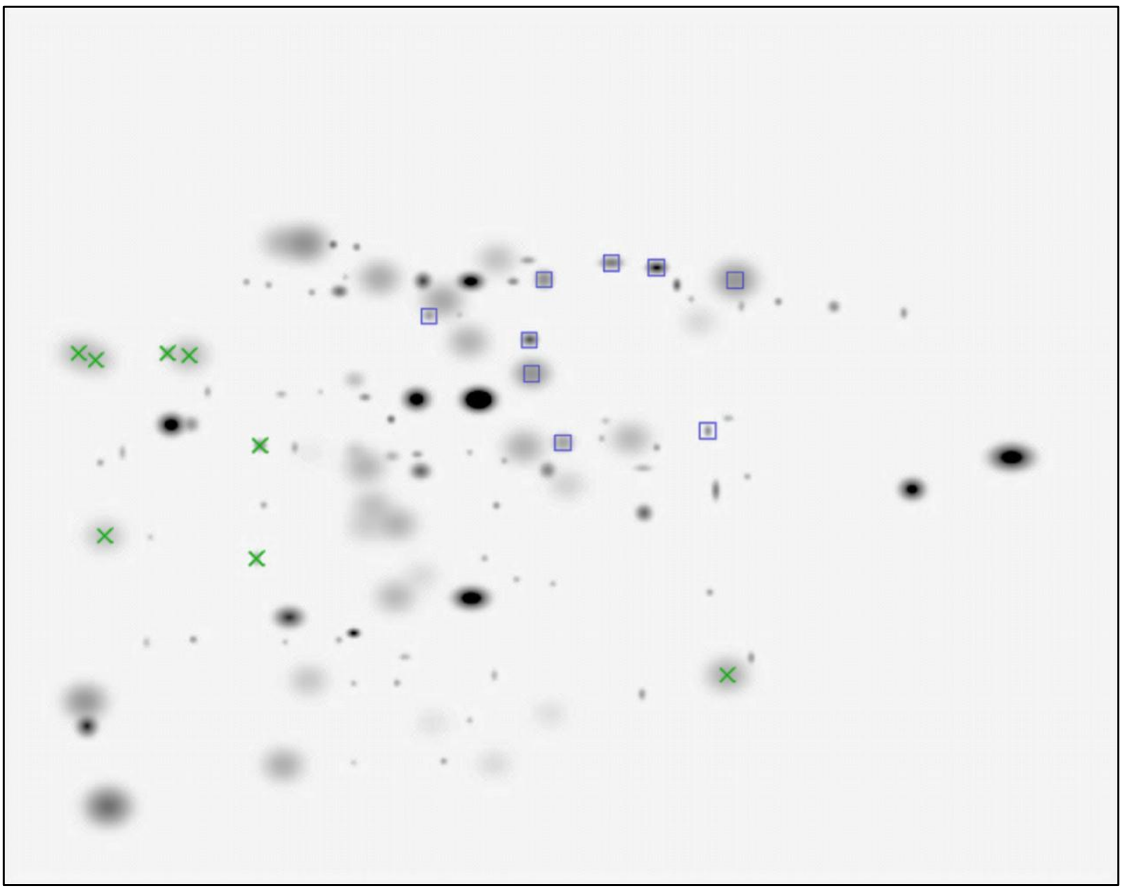


Figure 3.3 Composite gel image of the two-dimensional PAGE protein profiles of kesum leaves using PDQUEST^{TM} software (Bio-Rad, USA) for the quantitative analysis between four weeks old and six weeks old. In this standard reference image, the up-regulated proteins were marked in blue (□) and the down-regulated proteins were marked in green (x).

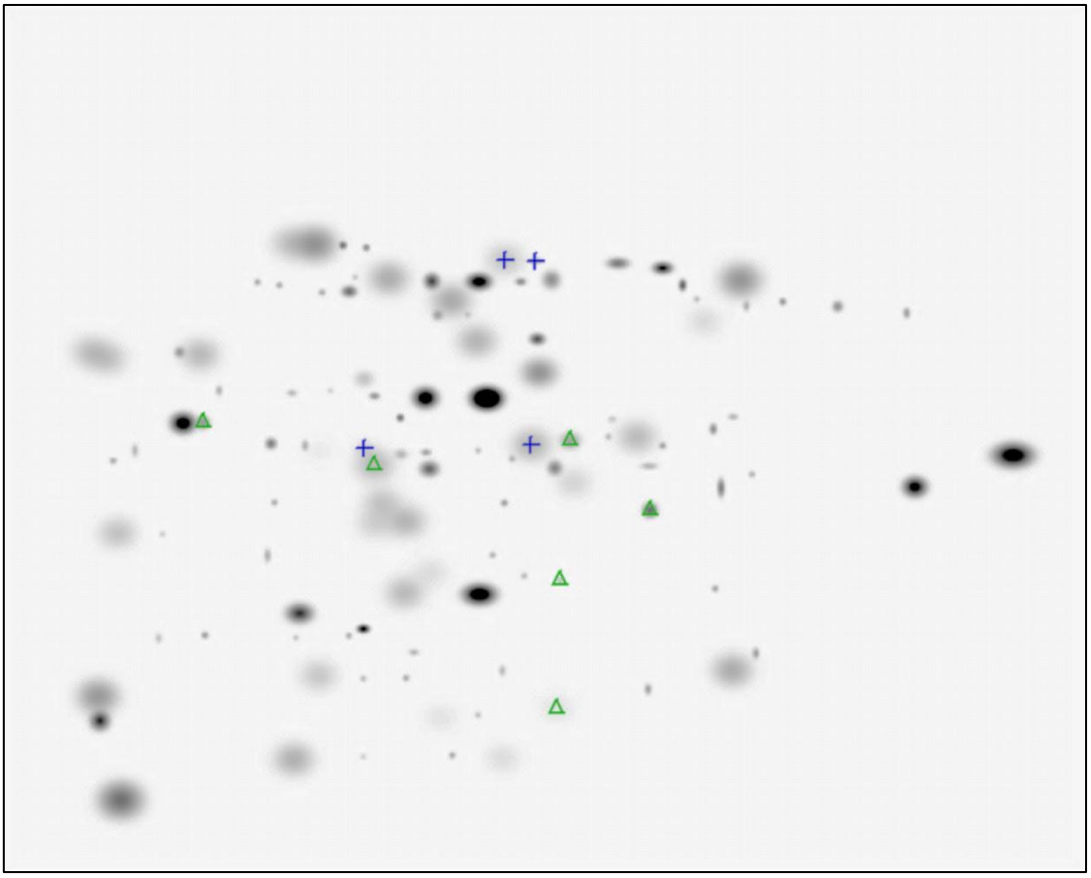


Figure 3.4 Composite gel image of the two-dimensional PAGE protein profiles of kesum leaves using PDQUEST^{TM} software (Bio-Rad, USA) for the quantitative analysis between six weeks old and eight weeks old. In this standard reference image, the up-regulated proteins were marked in blue (+) and the down-regulated proteins were marked in green (Δ).

3.3 Analyses of proteins using mass spectrometry (MALDI-TOF)

In this study, the up-regulated and down-regulated protein spots detected from the computational analyses were excised and destained. The gel plugs were reduced and alkylated, digested with trypsin and the digested peptides were identified using MALDI-TOF MS. At least five proteins were successfully identified using MALDI-TOF MS and these proteins are ATP synthase, oxygen evolving enhancer (OEE), Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase and malate dehydrogenase.

5.0 DISCUSSION

Plant proteomics is still in its infancy and lags several years as compared with proteomics of unicellular prokaryotes and eukaryotes. However, with the complete genome sequence of Arabidopsis and other plant genomes, and EST sequence data, plant proteomics will become a very active field.

For proteomics methodologies, protein extraction and sample preparation are important and critical steps to ensure optimal results. Protein extraction from plant tissues represents a great challenge. In addition to protein extraction, 2-DE is often challenged by complications of protein sample preparation due to the differences among plant species and tissues. Several protein extraction methods have been previously improved to obtain well-resolved 2-DE maps for plants. The present study uses a protein extraction method that has been optimized for establishment of protein profiles from plant samples.

Plant tissues contain a wide range of proteins, which vary greatly in their properties, and require specific conditions for their extraction and purification. Firstly, protein extraction and sample preparation requires non-covalently bound protein complexes to be disrupted and all interfering compounds such as salts, polysaccharides and phenolic compounds to be removed. This was achieved using the phenol extraction method used in this study to extract protein from *P. minus* leaves. As a result, protein binding profile that was obtained was excellent with minimal streaking and smearing. The second step involved solubilization of the proteins before electrophoresis. Sample preparation is the most crucial step since any impurities will interfere the IEF process and SDS-PAGE separation. Presence of salts in sample will lead to horizontal streaking and unremoved impurities will cause gap in the gel. The extraction method has been optimized to obtain the maximum protein concentration, to

prevent protein modification and to maintain minimum salt level in the sample. Based on the 2-DE gels, it was observed that adequate amount of proteins were obtained. However, the salt level could still be considered as high as indicated by the presence of streaking.

From the quantitation of the extracted protein, the results showed that the concentration of the protein was not very high although the optimization has been performed. In general, protein concentration in plant materials is lower compared to those from microorganisms and animal cells. This may be due to the presence of polysaccharide and that of vacuoles which occupy a large portion of plant cells (Jacobs *et al*., 2000).

Conventional SDS-PAGE separation was performed to screen the proteins extract. From the electrophoresis result, the decrement of high molecular mass proteins was observed. These maybe due to metabolization as the leaves grow older. However, these bands were too complex for the identification of single species protein. Therefore 2-DE was performed to establish the proteome maps of kesum leaves protein obtained at different ages in order to analyse its protein expression profile. Firstly, 2-DE was performed on small format 7 cm immobilized IPG strip with pH range of 3-10 for screening and optimization purposes. This was performed to ensure that good protein samples obtained using phenol extraction protocol was suitable for 2-DE analysis and also to include highly acidic and basic protein. The spots were successfully separated and evenly distributed in the range of pH 4-7. Since the broad range pH 3-10 was inappropriate for analysis because the spots were not well resolved, a narrow pH range pH 4-7 IPG strip was used to further separate and resolve the protein spots.

For analytical analysis, the proteins were subjected to longer IPG strips (18 cm) and narrower pH range of pH 4-7. Analysis of the proteome maps obtained was performed based on the

mathematical parameters using PDQuest. The results of the analysis revealed that a total number of 223 protein spots were detected in the four weeks old sample gel, 200 protein spots were detected in the six weeks old sample gel, and approximately 100 protein spots were detected in the eight weeks old sample gel. Quantitative analysis was performed and compared with the standard reference gel image, constructed from the 2-DE gel of the four weeks old sample since it exhibited the most number of protein spots. Up-regulated proteins as determined by 5 X increase of spot density and down-regulation which was determined as 5 X decrease was performed. Based on the analyses, at least nine protein spots in the six weeks old sample were up-regulated in the comparison with the four weeks old sample. Quantitative analyses also showed that only four spots in the eight weeks old sample were up-regulated in comparison between the six weeks old sample. The numbers of up-regulated proteins decreased as the leaves grew older as this may be due to the mobilization of the proteins. Analyses on the down-regulate ion of proteins revealed that eight spots were down-regulated in the six weeks old sample in comparison of the four weeks old sample. Comparison between the six weeks old sample and the eight weeks old sample revealed that six protein spots were down-regulated in the eight weeks old sample. These down-regulated proteins were perhaps metabolized as the leaves grow older.

As the identification of proteins using 2-DE are based on the pI, molecular weight and relative abundance of proteins. It does not provide direct information on the identity of the protein or its biological function. Mass spectrometry provides an accurate mass measurement, and the identification and characterization of proteins. In the present study, the protein spots of interest were identified using MALDI-TOF MS. The proteins identified include the ATP synthase, oxygen evolving enhancer (OEE) and Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO). Another protein identified is the 4-hydroxy-3-methylbut-2-en-1-yl

diphosphate synthase which is involved in the terpene biosynthesis pathways and non-mevalonate pathways. This enzyme participates in the biosynthesis of steroids and is a reasonable evidence to serve as a good drug target. The other protein that was detected is the malate dehydrogenase which is involved in synthesizing malate. Malate is a key metabolite in plants which is involved in C4 and Crassulacean acid metabolism photosynthesis, stomatal and pulvinual movement, as well as nutrient uptake, respiration, nitrogen assimitalation and fatty acid oxidation.

With the identification of these different protein expression patterns in different ages of kesum leaves, more studies can be performed to investigate the exact role and function of these proteins in the biosynthetic pathways of kesum leaves. Besides, post-source decay (PSD) investigation will further confirm the identity of the protein spots.

Research limitation

To establish a reliable proteome map, multiple gels (10 to20 gels) were scanned and the collated images will be used as a master gel. This master gel will be the representative map of the proteins in the sample. However, due to time constraints, it was not possible to attempt such large number of gels for the present study. To identify the protein spots in the proteome maps, the gel plugs have to be processed manually where the gel plug was subjected to digestion, alkylation and reduction processes, followed by MALDI-TOF MS analysis. Due to time and financial constraints, the identification of all protein spots in the kesum leaves proteome maps is not in our primary aim.

Thus, the findings from this study are only preliminary and perhaps by extension of research fund and time, further characterization of the protein spots on the kesum leaves proteome maps can be performed and analysed.

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