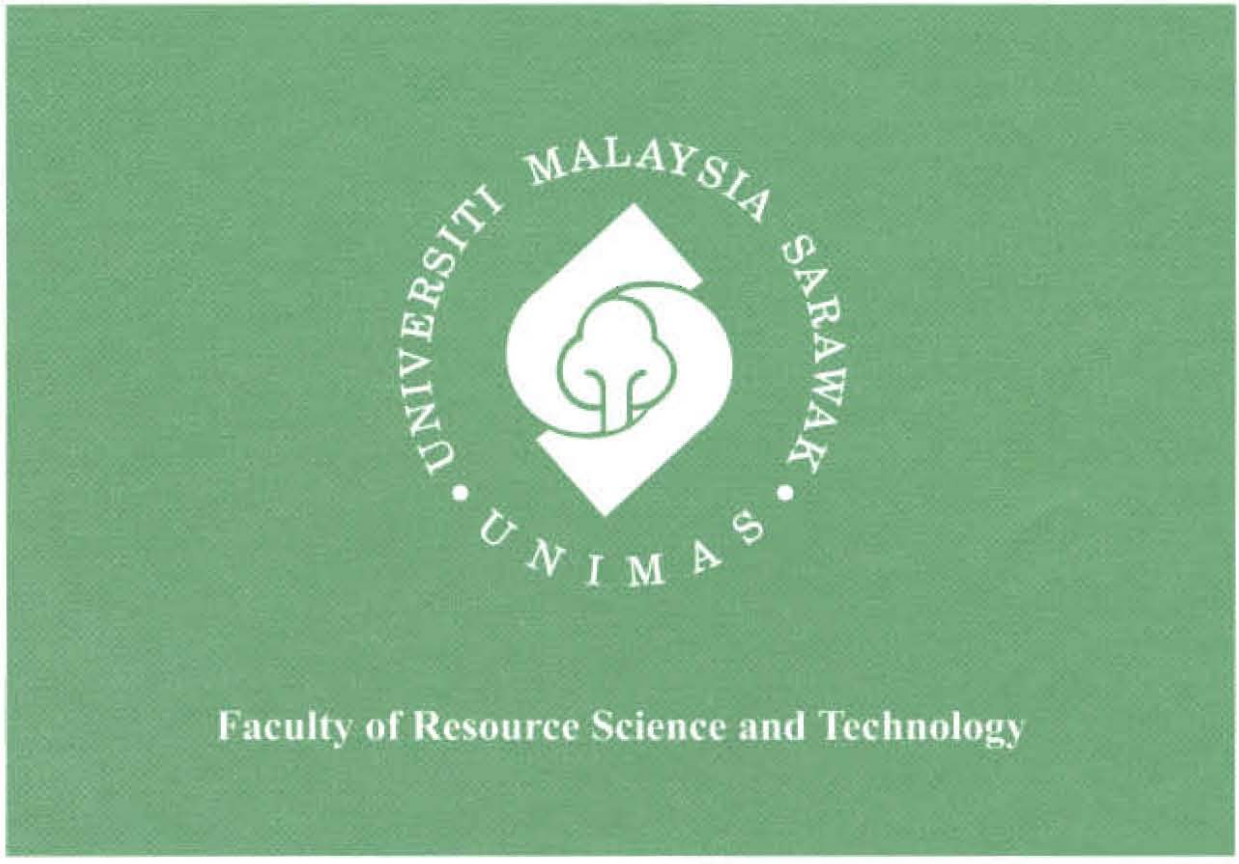

PROFILING OF PROTEINS INVOLVED TRUNKING AND NON-TRUNKING SAGO PALM

Zulaikha Binti Pol Ong

Bachelor of Science with Honours
(Resource Biotechnology)
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List of Abbreviations

°C	Degree Celsius
g	Gram
M	Molar
mM	Milimolar
µl	Microliter
µg/ml	Microgram per mililiter
rpm	Round per minute
mm	Millimeter
min	Minute
ml	Milliliter
kDa	Kilo Dalton
HCl	Hydrochloric acid
EDTA	Ethelynediaminetetraacetic acid
KCl	Potassium chloride
BSA	Bovine serum albumin
DTT	Dithiothreitol

BB	Bromophenol blue
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid

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ABSTRACT

Metroxylon sagu Rottb is one of the sago palm species that produce sago starch in its trunk. This sago palm may exist in normal form where it has trunk and the abnormal form which it has stunted growth of trunk. The existence of these features can be closely related to the improper management of sago plantation, deficiency of some of the nutrients and the protein composition between these two types of sago plant. Study was performed at proteomic level by extracting and comparing the proteins in leaf part between trunking and non-trunking sago plant using two different protein extraction methods which are acetone precipitation and phenol extraction. Among those two procedures assessed, phenol extraction was shown to be the best extraction method of protein. The results were further characterized by using one dimensional gel electrophoresis which were sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) followed by quantification through Bradford assay. These results lead to conclusion that there was some protein bands visualized on gel but both trunking and non-trunking sago palm protein does not have any differences at protein level.

Keywords: *sago palm, trunking, non-trunking, protein extraction*

ABSTRAK

Metroxylon sagu Rottb merupakan salah satu spesis pokok sagu yang biasanya diproses dalam penghasilan kanji sagu yang terkumpul di dalam batang pokok ini. Pokok sagu ini boleh wujud sama ada dalam bentuk yang biasa didapati iaitu pokok sagu yang mempunyai batang dan juga boleh didapati dalam pertumbuhan yang terbantut iaitu tidak mempunyai batang. Pembentukkan batang pokok sagu yang berlainan ini boleh dikaitkan dengan beberapa aspek seperti pengurusan yang tidak teratur sepanjang proses penanaman pokok sagu, kekurangan nutrien yang diperlukan dan juga mempunyai komposisi protein yang berlainan antara dua jenis pokok sagu ini. Satu kajian telah dijalankan dari segi proteomik dengan mengekstrak dan membandingkan protein yang terdapat dalam bahagian daun di antara pokok sagu yang berbatang dan tidak berbatang dengan menggunakan dua kaedah yang berlainan iaitu pemendakan aseton dan pengekstrakan fenol. Antara dua kaedah yang telah dijalankan, pengekstrakan fenol telah menunjukkan hasil yang terbaik dalam kaedah pengekstrakan protein. Protein yang terhasil diteruskan dengan menggunakan gel elektroforesis satu dimensi atau SDS PAGE diikuti dengan dikuantifikasikan melalui kaedah Bradford. Keputusannya, beberapa jalur protein telah kelihatan pada gel tetapi jalur tersebut tidak mempunyai sebarang perbezaan yang ketara antara protein pokok sagu yang berbatang dengan yang tidak mempunyai batang.

Kata kunci: pokok sagu, berbatang, tidak berbatang, pengekstrakan protein

1. Introduction

Metroxylonsagu is the true sago palm which can be found at swamp area in southeastern Asia. This *Metroxylon* species is a medium to tall palm tree stand around 9 and 33m which is 30-108 ft in height (McClatchey et al., 2006). *M. sagu* has long, flattened and thin leaves with single to multiple spines grow to 46 cm in length (McClatchey et al., 2006). Particularly, the leaves can grow longer as it emerged higher on the stem. The leaves are unfolded which visible in the crown when it starts developing (Flach, 1997). Sago is a slow growth plant to mature and it is a hapaxanthic palm because each stem can only flowering once throughout of its lifetime(Jamel et al., 2011).The leaves will senesce after fruiting and it dies completely. The species grown in flooded environment that tolerate salinity and acidity of the wet soil(McClatchey et al., 2006).Thus, the pneumatophores, the air roots are present for this plant.

In Sarawak, sago palm at rural area treated as dietary starch sources among communities. The starch can be obtained from the pith of trunk and the leaves were left for thatch. This sago starch can be used for several purposes such as an ingredient in white bread production which usually used among communities in Indonesia, the production of high-fructose syrup and many other products (Flach, 1997). Besides, floor material also can be made by the bark of the sago palm or used as a fuel other than using starch as the main purpose on planting sago palm (Flach, 1997).It also can he used for the production of animal food and the production of ethanol as a source of fuel (McClatchey et al., 2006).

This species of sago palm also could exist either in the form of trunking or non-trunking sago palm. The abnormality of sago palm which either it has trunks or not is depend on the proper management of plantation. Its growth may be disturbed by the lack of nutrients or the presence

of some stress factors which affect into stunted growth of trunks or failure to form boles(Edward A. S., 2009). The stunted growth of sago palm will contribute to low production of starch content as it was accumulated mostly in the trunk. Some of the proteins might be up or down regulated which results to non-trunking features. This non-trunking feature has been attracted by other researchers by studying it into proteomics level because most of other parts of sago palm including the gene and starch have already been studied. Thus, a further research needs to be approached to overcome these three main objectives which are to:

- extract proteins from the leaf of trunking and non-trunking sago palm.
- compare protein results on quantification of Bradford assay and visualization on gel for both trunking and non-trunking sago palm.
- compare the proteins based on different methods used between the trunking and non-trunking sago palm.

2. Literature review

2.1. Sago Palm (*Metroxylon sagu*)

Metroxylon sagu is the scientific name of sago palm which can be found in Southeast Asia countries such as Malaysia, Indonesia, Philippines and Papua New Guinea. *Metroxylon* species stand on salinity, prolonged flooding, acidic and wet soil (McClatchey et al., 2006). It also is the only sago palm species which are hapaxanthic (one-flowering) and soboliferous (suckering) plant (Flach, 1997). Sago palm contains plenty of starch that stored in its trunks which act as one of the food sources among the communities in Southeast Asia. Some sago plant has a stunted growth of trunks for 10-14 years that affect on low starch contents (Hussain, 2012). This is known as non-trunking sago palm. Research on trunking and non-trunking sago palm has been carried out previously byEdward (2009) about the differential expression of gene profiling but not yet have been done more about sago palm proteins.

2.2. Phenol extraction

Phenol extraction method is one of the common procedures in extracting out the proteins from leaves samples. There are many other procedure involve in protein extraction such as trichloroacetic acid (TCA) precipitation, acetone precipitation, precipitation with TCA in acetone and ammonium sulfate precipitation (Li et al., 2012). In this experiment, phenol extraction was used to test on the leaves of trunking sago palm. After the protein was successfully extracted from phenol extraction method, the sample was extracted by using acetone precipitation procedure as to make sure which method was the best used to extract proteins from leaves sample.

2.3. Bradford assay

Bradford assay are used to quantify the protein sample as to make sure all the protein concentration are the same so that it can be visualized on the gel. Bradford assay procedure was done based on Clare (2008) procedure. The protein concentration between trunking and non-trunking sago palm were compared whether it gives the same results as have been visualized with the same protein concentration on SDS gel.

2.4. SDS PAGE

Sodium dodecyl sulfate of polyacrylamide gel electrophoresis (SDS PAGE) are the analysis which can be used to analyze protein samples other than 2-D gel electrophoresis. Many bands will results at the end of electrophoresis when using SDS PAGE in analyzing the proteins. For 2-D gel electrophoresis, several blots of proteins might be appearing on the 2D gel. This experiment was conducted using SDS PAGE since there are lacked of some equipment to proceed with 2-D gel electrophoresis.

2.5. Proteomics of Sago Palm

Proteomics of sago palm was the study of molecular level of protein in both trunking and non-trunking sago palm. Since many of previous research focused on genes and starch of sago palm, a new study on proteins between trunking and non-trunking sago palm was done to differentiate at proteomic level. In a study done by Hussain et al. (2012), several proteins which show differences in protein expression have heen identified on the 2D gel blots. 10 proteins have been successfully spotted on the gel blots by using two different protein extraction methods. Thus, in this experiment, other proteins were compared by using anothertwo different methods from previous research and visualized with SDS PAGE.

3. Materials and methods

3.1. Plant materials

The mature leaves were collected as samples from cultivated trunking and non-trunking sago palms in Mukah, Sarawak. The samples were kept cool on ice immediately after collection process to maintain the freshness when work in the laboratory. The leaves samples were cut into small pieces and dry crushed into powder in a mortar of food grinder by liquid nitrogen.

3.2. Acetone precipitation

0.35 g of the leaf powder was resuspended in 1.2 ml of ice-cold Tris-HCl buffer (pH 8) containing 500 mM guanidine-HCl, 100 mM sucrose, 500 mM ascorbic acid and 50 mM polyvinylpyrrolidone. The cell debris was removed after centrifugation at 13200 rpm at 4°C for 15 min. The supernatant at the upper layer was transferred into new microcentrifuge tube and it was centrifuged again in 13200 rpm at 4°C for 60 min. Two volume of ice-cold acetone was added to precipitate the proteins for overnight at -20°C. After centrifugation at 13200 rpm at 4°C for 10 min, the pellet left was washed twice with acetone and centrifuged again. Then, acetone was discarded and the pellets were dried at room temperature. The pellets then were solubilized with 0.05 M of Tris-HCl buffer and stored at 4°C for further use.

3.3. Phenol extraction

0.35g of leaf powder was resuspended in extraction buffer which containing 500 mM Tris (pH = 8.0), 50 mMEDTA, 700 mM sucrose, 100 mM KCl and2-mercaptoethanol (0.07 %). The samples were vortexed and incubated with shaking for 10 min on ice. Then, it was added with equal volume of tris-buffered phenol at pH 8 and it was incubated on shaker for 10 min at room temperature. After centrifugation at 13200 rpm in 4°C for 10 min, the upper phenolic phase was transferred and back extracted with 1 ml extraction buffer. It was incubated again

on shaker for 3 min, vortexed and undergoes centrifugation for 20 min. The phenolic phase that transferred into another tube were mixed with four volumes of precipitation buffer (100 mM ammonium acetate in ice-cold methanol) by inversion and incubate for overnight at -20°C. After centrifugation for 15 min, the pellets will be washed with ice-cold precipitation buffer and then with ice-cold acetone. All the samples were centrifuged again for 5 min and pellets were dried at room temperature. The pellets then were solubilized with 0.05 M of Tris-HCl buffer and stored at 4°C for further use.

3.4. Protein quantification

The protein samples were quantified by using Bradford assay. Bradford reagent was prepared with the mixture of 0.025 g Coomassie Brilliant Blue G-250, 100% methanol and 85% phosphoric acid. The reagent then was diluted into one time Bradford reagent by adding 250 ml of distilled water and finally it was filtered and the reagent were ready to use.

A calibration curve was created using 1 mg/ml of BSA standard with series of dilution of 0, 4, 8, 12, 16, 20µl in each tube. The reading was taken using spectrophotometer at 595 nm of absorbance. All the absorbance values of protein samples were recorded as shown in results.

3.5. SDS PAGE

SDS PAGE analysis was conducted because lacks of the equipments for 2-D gel electrophoresis. Sample buffer (SDS reducing buffer) was prepared as ingredients shown below which was stored at room temperature.

Deionized water	3.8 ml
0.5 M Tris-HCl, pH 6.8	1.0 ml
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml
2-mercaptoethanol	0.4 ml
1% (w/v) bromophenol blue	0.4 ml
	8.0 ml

10% of separating gel was prepared referring the ingredients as below:

Deionized water	3.35 ml
1.5 M Tris-HCl< pH 8.8	2.5 ml
10% (w/v) SDS stock (store at room temperature)	100 µl
Acrylamide/Bis (30% stock)	4 ml
10% ammonium persulfate (fresh daily)	50 µl
TEMED	5 µl
TOTAL MONOMER+	10 ml

All the ingredients were mixed in a small beaker and the Biorad Protean II system was assemble using 0.7 mm spacers. The separating gel solution was swirled gently but thoroughly and was then pipetted carefully into the gap between the glass plates. The top of the separating gel was filled with water until overflow to make the gel be horizontal. The separating gel was allowed to polymerize for 30 min.

After 30 min, the water on top of the separating gel was discarded. Stacking gel solution was prepared as below:

Deionized water	3.05 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
10% (w/v) SDS	50 ml
Acrylamide/Bis (30% stock)	0.665 ml
10% ammonium persulfate (fresh daily)	25 µl
TEMED	5µl
TOTAL STOCK MONOMER	5 ml

The stacking gel solution was pipetted until overflow. The well-forming comb was inserted without trapping air under the teeth. The gel was waited for 30 min to polymerize. After 30 min, the comb was taken out. The wells were rinsed with 1x Tris-glycine gel buffer (0.125 M trizma, 0.125 M glycine, 10% SDS, 100 ml water) to remove any un-polymerised gel.

The protein samples were adjusted to a concentration of 3 µg and a volume of 10 µl using protein extraction buffer (+DTT/+BB). It was vortexed briefly and heated at 95°C for 4 min. The protein samples were prepared with no dilution and 2x dilution before loading into the wells. The upper chamber was filled with 1x tris-glycine buffer. The entire sample was loaded into well carefully using a 10 µl pipette. 10 µl of Benchmark Prestained Ladder was loaded in one lane. The lower chamber was filled again with 1x tris-glycine buffer. The electrophoresis was run at 120 V for 45 min.

After done with electrophoresis, the glass plates was removed out from the Biorad Protean II system and immersed in the running water to take out the gel from the glass plates. This step was handled carefully because the gel can easily break. The stacking gel at the top was removed out from separating gel. Then, the gel left was immersed with Coomassie Blue R-250 in another container for staining the gel. The gel was left for overnight at room temperature.

The gel that left for overnight was washed with running water to remove the blue dye. It was handled with carefully and it was immersed in destaining solution two times for 30 and 10 min for clear view of protein band. The gel then was placed on the white tile so that the protein band can be visualized clearly.

4. Results

In this study, two different protein extraction methods were evaluated between the leaves sample of trunking and non-trunking sago palm. The absorbance value recorded by mass spectrometry was fitted into the equation of linear regression from standard calibration curve to obtain protein concentration. The protein samples were visualized on SDS gel enables the comparison of protein bands in each sample.

4.1. Acetone precipitation

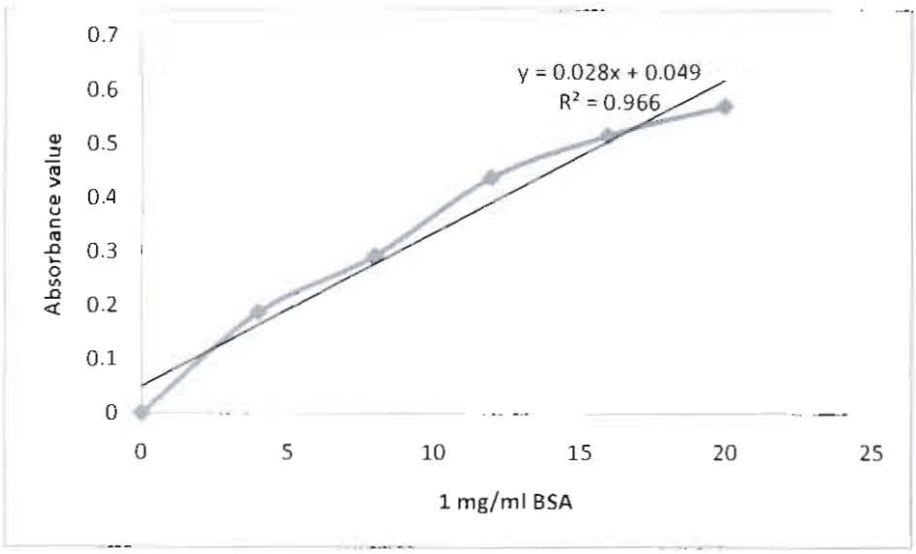

Figure 1: Standard calibration curve for acetone precipitation

Figure 1 above shows the standard calibration curve on acetone precipitation method. Based on the equation of protein standard calibration curve above, the protein concentration for trunking sago palm was 0.06 µg/ml. For non-trunking sago palm, the protein concentration was 0.077 µg/ml as shown in table 1 below.

Table 1: Comparison of protein yield from different samples following different methods

Samples	Protein concentration (µg/ml)	
	Acetone	Phenol
Trunking	0.060	2.750
Non-trunking	0.077	4.432

4.2. Phenol extraction

Figure 2 below shows the standard calibration curve on phenol extraction method. Based on the equation of protein standard calibration curve above, the protein concentration for trunking sago palm was 2.75 µg/ml whereas for non-trunking sago palm, the protein concentration was 4.432 µg/ml as in table 1.

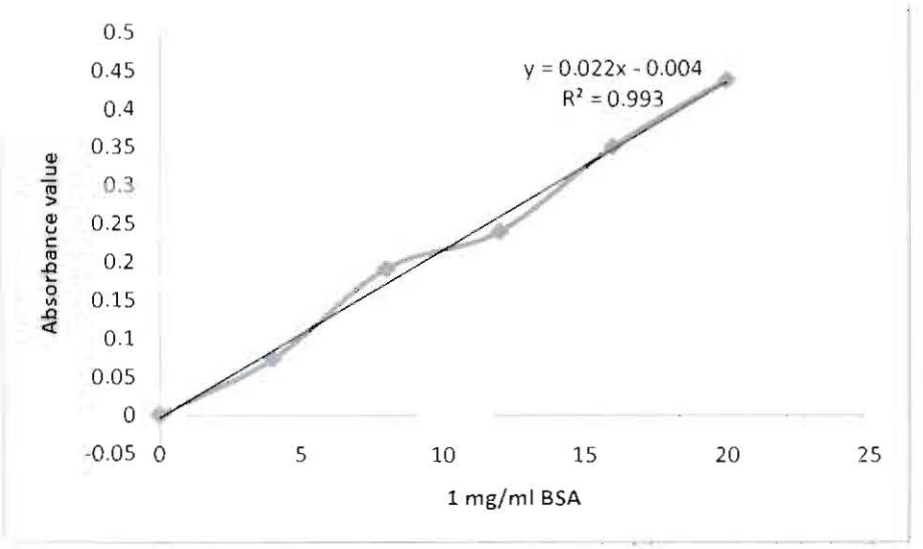

Figure 2: Standard calibration curve for phenol extraction

4.3. SDS PAGE

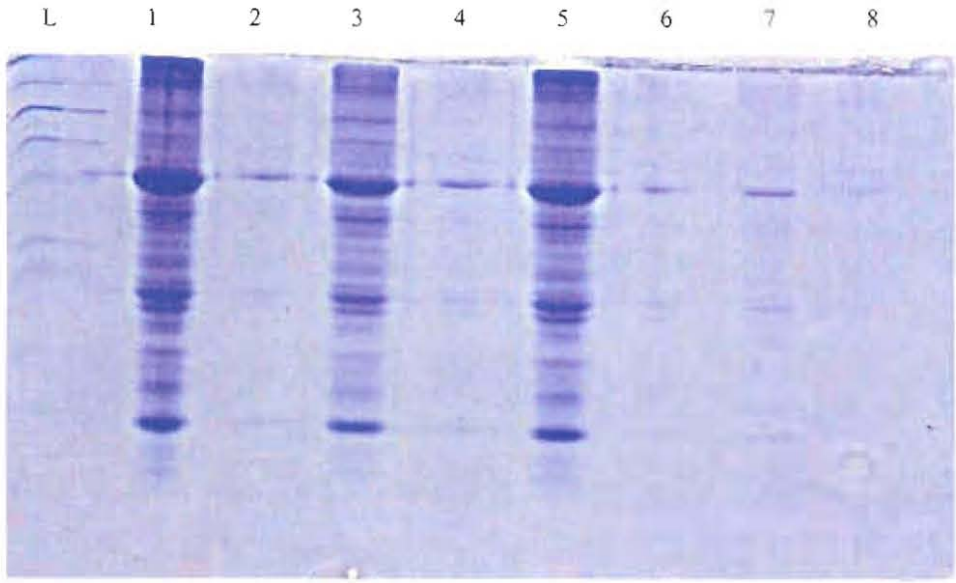

Figure 3: Protein profile on 12% SDS PAGE by phenol extraction and acetone precipitation. L is the protein marker used as a ladder. Lane 1 and 3 are protein samples from trunking sago palm by phenol extraction. Lane 5 and 7 are non-trunking sample from the same method. Lane 2 and 4 are trunking sample from acetone precipitation whereas lane 6 and 8 are non-trunking samples.

Figure 3 above shows a results of protein samples which visualized on 12% SDS PAGE by phenol extraction and acetone precipitation. L was protein ladder used as prestained protein marker in the range of 7-175 kDa. All of the protein samples were loaded in the same concentration of protein.

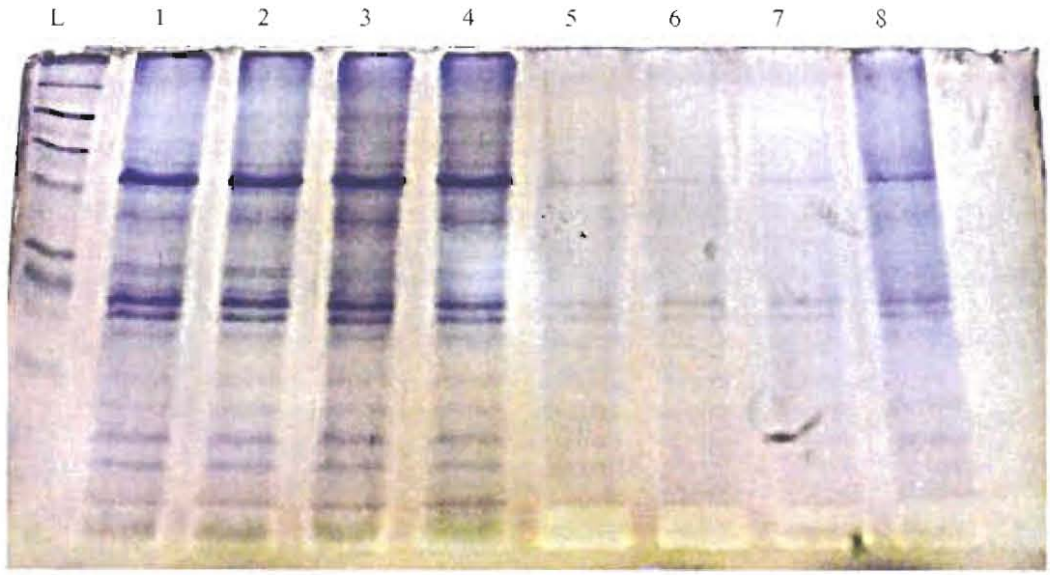

Figure 4: Protein profile on 12% SDS PAGE by TCA precipitation and ethanol precipitation. L is the protein marker used as a ladder. Lane 1 and 2 are protein samples from trunking sago palm by TCA in acetone precipitation. Lane 3 and 4 are non-trunking sample from the same method. Lane 5 and 6 are trunking sample from ethanol precipitation whereas lane 7 and 8 are non-trunking samples.

Figure 4 above shows results from another study done by my laboratory mate, Nurhazlina Binti Hamdan. This results was used to compare with Figure 3 results by comparing between the resolution of protein visualized based on several different method used. From this results also the protein bands present can be compared between trunking and non-trunking sago palm protein.

5. Discussion

In this study, the protein samples based on two different methods have been successfully obtained. The results on protein concentration as have been shown previously indicated that for phenol extraction method; trunking sago palm has less amount of protein concentration compared to non-trunking sago palm which was 2.75 µg/ml and 4.432 µg/ml. These results were the average value taken from triplicate samples. From this observation, non-trunking sago palm has more amount of protein than trunking sago palm quantitatively measured using mass spectrophotometer. In acetone precipitation method, the protein concentration for trunking sago palm was 0.06 µg/ml. It has the same results as phenol extraction method because the protein concentration for trunking was lesser than the protein concentration for non-trunking sago palm which was 0.077 µg/ml. These show that non-trunking sago palm has some extra protein from trunking sago palm.

When compared with phenol extraction and acetone precipitation methods, the value of protein concentration shows a higher value in phenol extraction method rather than acetone precipitation method. A statistical analysis has been conducted to compare two factors which were between samples and methods. The null hypothesis stated that there is no significant differences between these two factors. The alternative hypothesis supported when there is a significant differences between these factors. The statistical analysis results shows that there is no significant differences between trunking and non-trunking sago palm whereas between acetone and phenol method, there is a significant differences.

Figure 3 shows the results which were visualized on 10% SDS gel. When comparing the protein band between lane 1 and lane 2, lane 1 gave higher resolution on gel compared to lane 2 since lane 1 was the protein sample on phenol extraction method and lane 2 was the protein

sample from acetone method. Through this observation, clearly the two different method used can be distinguished based on the present of proteins band on one dimensional gel. In phenol extraction, by comparing the protein of trunking and non-trunking sago palm which were lane 1 and lane 5, approximately both have the same band. Both of the protein bands were visible on gel but hardly to recognize whether some of protein bands can be distinguished from one to another. All lane 2, 4, 7 and 8 which resembled acetone precipitation results in low resolution of protein bands on SDS gel. This might be the cause of using guanidine-HCl instead of cysteine-HCl in extraction buffer for acetone precipitation. Cysteine-HCl may give better results than guanidine-HCl as it functions in refolding the protein so that more protein present on SDS gel (Raman et al., 1996).

For non-trunking protein, lane 5 and lane 7 supposed have the same resolution of protein bands on the gel but the results show a slightly different. This could be related on several errors on handling the protein samples such as pipetting error before loaded the samples into the wells. Other possibilities also might be resulted which was due to several loss of protein sample during washing the protein precipitate. Besides, there were high possibilities that wrong sample was taken such as the protein sample between phenol extraction and acetone precipitation was swapped since the results shown in figure 3 was not more or less has the same results as acetone precipitation in lane 6 and lane 8.

Another results of trunking and non-trunking sago palm which also have been done by my laboratory mate, Nurhazlina Binti Hamdan which using another two different methods; TCA precipitation and ethanol precipitation. As shown in figure 4, TCA precipitation method gave better resolution than ethanol precipitation. All of the protein concentration for both methods