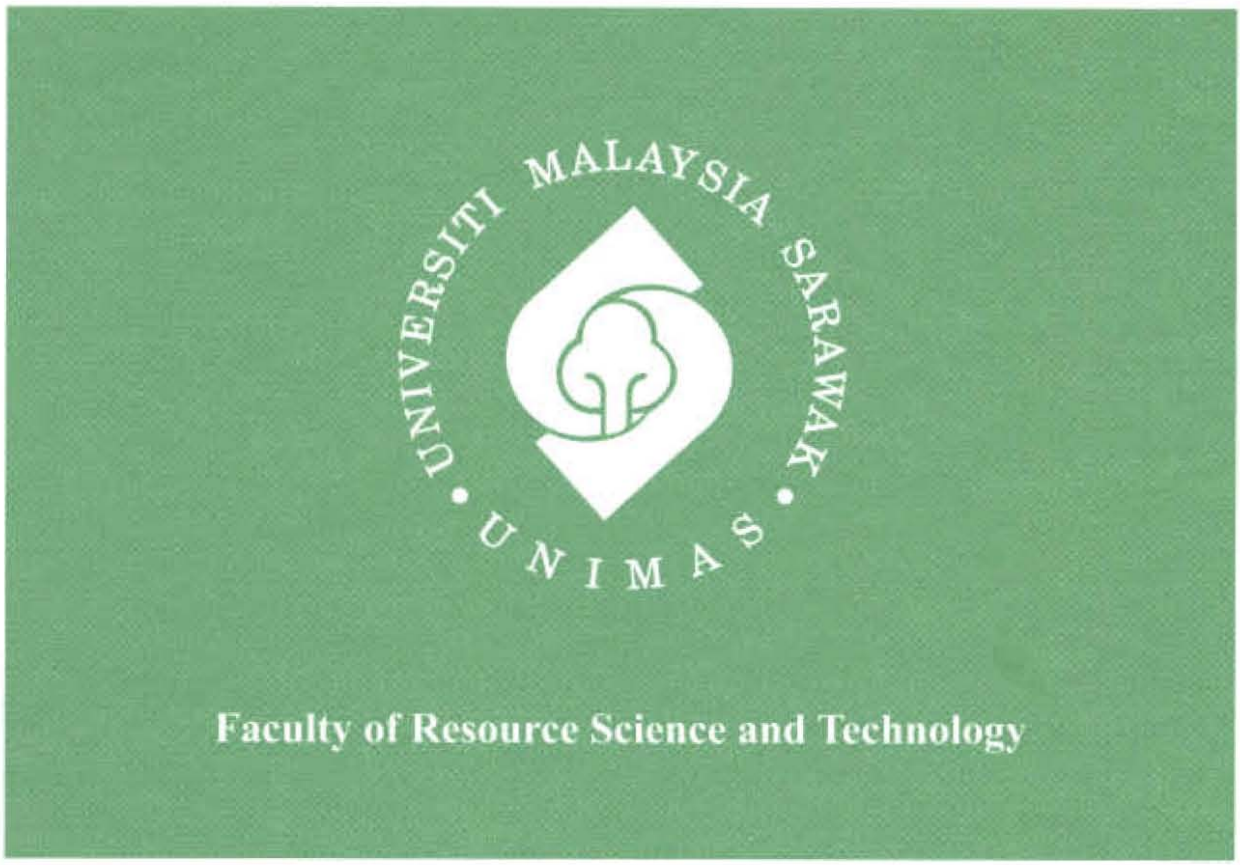

PROFILING OF PROTEIN INVOLVED IN TRUNKING AND NON-TRUNKING SAGO PALM

Nurhazlina Binti Hamdan

Bachelor of Science with Honours
(Resource Biotechnology)
2014

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Additionally I would also like to thank my family especially my mother, who have supported me throughout the entire process, both by keeping me calm and helping me putting all the pieces together.

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Table of Content

List of Abbreviations

%	*percent*
°C	*degree celsius*
2-D	*2-Dimentional*
DNA	*Deoxyribonucleic acid*
F (DFn,DFd)	*F (degrees of freedom numerator, degrees of freedom denominator)*
g	*gram*
IEF	*Isoelectric focusing*
M. sagu	*Metroxylon sagu*
min	*minute*
ml	*millilitre*
mRNA	*messenger Ribonucleic acid*
NaOH	*Sodium hydroxide*
nm	*nanometre*
RNA	*Ribonucleic acid*
rpm	*revolutions per minute*
SDS	*sodium dodecyl sulfate*
SDS-PAGE	*sodium dodecyl sulfate* polyacrylamide gel electrophoresis
TCA	*Trichloroacetic acid*
Tris-HCl	*Tris-Hydrochloride*
UV	*ultraviolet*
µg	*microgram*
µg ml	*microgram per millilitre*

List of Tables and Figure

Abstract

Metroxylon sagu is recognised as an important crop in this 21st century. Abnormalities of this crop growth contribute to the existence of a non-trunking *Metroxylon sagu*. This existence gives curiosity rise in the differences of genetic makeup in both trunking and non-trunking *Metroxylon sagu*. Thus, the objective of this study is to identify protein involved in both trunking and non-trunking sago palm by using different precipitation method screening on SDS-PAGE gel electrophoresis. Modified Trichloraocetic acid (TCA) in acetone precipitation method and Ethanol precipitation method were used for the precipitation followed by the SDS-PAGE electrophoresis analysis for protein separation comparison. The identified protein gained from SDS-PAGE was compared for protein separation differences. As the result, Ethanol precipitation shows better protein yield quantificationally but TCA in acetone precipitation give better image range in the protein analysis by SDS-PAGE gel electrophoresis. This shows that TCA in acetone as a better precipitation method for *Metroxylon sagu* extraction.

Keywords: *Metrxylon sagu*, trunkng, non-trunking, protein extraction, method comparison

Abstrak

Metroxylon sagu diiktiraf sebagai tanaman yang penting pada abad ke-21 disebabkan oleh kepentingan tanaman tersebut. Pertumbuhan tanaman ini secara abnormal menyumbang kepada kewujudan Metroxylon sagu tiada-batang. Kewujudan ini menimbulkan rasa ingin tahu dalam perbezaan genetik dalam kedua-dua Metroxylon sagu berbatang dan tiada-batang Oleh itu, objektif kajian ini adalah untuk mengenal pasti protein yang terlibat dalam kedua-dua Metroxylon sagu berbatang dan tiada-batang dengan menggunakan kaedah mendakan yang berbeza melalui saringan SDS-PAGE gel elektroforesis. Kaedah mendakan asid trikloroasetik (TCA)dalam aseton dan kaedah pemendakan etanol yang diubah suai telah digunakan untuk pemendakan protein yang diikuti dengan analisis SDS-PAGE gel elektroforesis untuk perbandingan pemisahan protein. Protein yang dikenal pasti yang diperoleh daripada SDS-PAGE telah dibandingkan dari segi perbezaan pemisahan protein. Hasilnya, kaedah pemendakan Etanol menunjukkan hasil yang lebih baik dalam hasil kuantifikasi walau bagaimanapun kaedah pemendakan TCA memberi julat imej yang lebih baik dalam analisis protein menggunakan SDS-PAGE gel elektroforesis. Ini menunjukkan bahawa TCA dalam aseton sebagai kaedah mendakan yang lebih baik untuk pengekstrakan Metroxylon Sagu.

***Kata kunci**: Metroxylon sagu, berbatang, tiada-batang, pemendakan protein, perbandingan kaedah*

Introduction

Metroxylon sagu or commonly known as sago palm is cultivated for it rich starch trunk (Avé, 1977). This sago starch can be produced into variety of production. Thus, the trunk of the sago palm is the most precious part. Unfortunately, the stunted growth sago palm affected the starch cultivation as the stunted growth parts are generally occurred in the trunk of the palm. This atypical growth of sago becomes the main focus for many researchers that are interested in the differences of physical and biological growth of the sago. Roughly, these differences may be varied because of the presences or absences of some proteins or may be in the genetic material. Thus, the identification of protein involved in both trunking and non-trunking may verify this statement.

For the records, most research involving *Metroxylon sagu* are all about the starch capacity. Most of the research are focused on starch debranching, sugar developing from starch, sago hampas and even the transplant of sago before the trunking periods. This shows less information about the genetic material of *Metroxylon sagu*. Fortunately, there is a study focused on the salt mechanism that resulted on the effect of salt stress on the sago growth ability (Ehara et al., 2013). This research progressively helps in the study of the stunted growth sago palm. Impressively, the very utmost research of the sago palm is the bioethanol production from sago hampas (Awg-Adeni et al., 2012). This discovery helps much in green campaign as the discovery are one of the new renewable energy.

In this study, one of the focuses is in extracting the protein from the leaves. Proteins are more preferable in this identification study because protein establish particular biological activity. From this protein extraction and purification, valuable things could be recognized. Apart from that, proteins are the key component that determines gene expression and mRNA translations machinery (Ehle et al., 1989). This helps in the leads of the differences between

trunking and non-trunking sago palm. From this step, the study can be further analysed by identifying the specific protein structure and even metabolism pathway that causes the differences in the growth metabolism. However, before the study can further be continued, the basic step of protein extraction plays the crucial role in the protein preparation. As we know, plants cell wall thickness range from 5 – 80 nm because plants cell needs to protect their cell from dryness and infections (Cosgrove, 2005). This thickness became one of the boundaries in protein extraction. Thus, the right protein extraction method should be used in overcoming this obstacle.

The second focus is in protein precipitate formation. This formation occurs in a chronicle process. This process consist of (1) mixing phase for precipitant and protein collision, (2) nucleation phase for protein aggregation generation and lastly (3) aging phase where protein particles stabilize to withstand fluid shearing (Zellner et al., 2005). The first phase involved only the introduction of precipitant to the protein solution for the present of collision between both particles. This collision continues to nucleation phase where protein masses are generated through flocculation. This phase take more time since it has slower rate. While the final phase involve breakup of the particle to reach stable state where the protein particle can resist the high fluid shear force. The protein precipitation formation followed by salting out for protein-protein interaction. This step causes the protein to aggregate, precipitate and normalize the protein.

In order to study protein, the protein must be separated to recognize the broad range of the protein according to their sizes. This help in the initial step of the protein study. Appropriate protein detection method is highly essential because the protein study approach measures the quantitative changes in the expression levels in the biological samples. For the basic instrument, SDS-PAGE electrophoresis is used. This technique is used because the

Literature Review

Metroxylon sagu

Metroxylon sagu or commonly known as sago palm, originated from Javanese, sago means palm pith that contains starch. *Metroxylon sagu* belongs to *Arecales Nakai* order in *Palmae Jussieu* family in *Calamoideae Griffith* subfamily in *Metroxylinae Blume* sub-tribe in *Metroxylon Rottboell* genus (Heywood, 1993). The generic name for *Metroxylon* is a Greek that means the pith of the tree wood (Orwa et al., 2009). Different to the other *Metroxylon* species, *Metroxylon sagu* has 18 scales rows on the fruits and massive robust size of the inflouresent branches (McClatchey et al., 2006). Briefly, *Metroxylon sagu* is a true sago palm without leaf sheaths that have a trunk in the size of 30-60 cm diameters and a height of 6-16 m. Sago palm is a pinnate-leaved tree and also hapaxanthic (Orwa et al., 2009). There is four growing stage for *Metroxylon sagu*; rosette stage, bole formation stage, inflorescence stage, and fruit ripening stage (Flach, 1997). After 4-6 years, the seedling of *Metroxylon sagu* grows into the rosette stage where the leaves and trunks are formed (Jong, 1995). In bole formation stage, the trunk grows to the length of 6-14 m and reach an optimum crown leaves of 24 (Flach, 1997). Formation of inflorescence stage (after 4-14 years of trunk formation) is the stage where the store starch is used (Jong, 1995). When mature (where the starch fills the trunk to the crown), the primary axis divide the secondary and tertiary axes (Karim et al., 2008). According to Flach (1977), the fruiting occur at the tertiary axes. This development last for about 2 years. Compare to the other *Metroxylon, Metroxylon sagu* reproduced by vegetative sucker and often by stolon (McClatchey et al., 2006).

Trunking and Non-Trunking Metroxylon sagu

A normal growth sago palm is a palm with starch full trunk when reach the maturity stage that is also known as trunking *Metroxylon sagu* (sago palm). While, an abnormal growth sago palm is a palm that have stunted growth and development that is also known as non-trunking *Metroxylon sagu* (sago palm). This trunk formation are influenced by the peat depth (Fong et al., 2005). A shallow peat area is the best condition for trunking sago palm. Whereas, the non-trunking sago palm are usually found in the deep soil area. Trunking sago palm needs extra moist soil to fulfil the nutrient content and to have a better compactness. But, a less humidified soil causes stunted growth of the sago palm that eventually deprived the capability to develop bole part (Fong *et al.*, 2005). This poor nutrient growth medium causes the slow growth in palm with premature branch desiccation, slim crowns, minor trunks and very little starch yield.

Protein from the leaves

Protein located from the leaves of the plants is easier to extracted compare to the trunk and the root part. Marsoni *et al.* (2005) stated that the leaves part is the easiest part to grind and this part give higher extraction yield compare to the roots. While, compare the leaves and the trunk part, protein extraction from the leaves is easier to extract compare to the trunk because of the high level of starch in the trunk. For *Metroxylon sagu* in both trunking and non-trunking sago palm, the expression of protein may be differ. Thus, the proteomic analysis is done to identify the differences. As stated by Hussain *et al.* (2012), several level of protein has been detected and successfully differentiated from both trunking and non-trunking by using 2 different method and analyse by using 2D-gel electrophoresis. The protein extracted from the leave can also be first detected using SDS-PAGE electrophoresis before proceeding to 2D-gel electrophoresis.

Protein extraction and precipitation

Briefly, there are several techniques for protein extraction. Many different techniques have been developed because every protein is different. Different techniques had been used to achieve the best possible yield and purity from different type of species of an organism, different sample types and even different target molecule. The basic method of extraction is by using mortar and pestle. With the assist from liquid nitrogen, this extraction became easier. Liquid nitrogen acts as freeze medium to ease the cell disruption. The liquid nitrogen also can be replaced with detergent-based lysis buffer. This cell disruption step act as the first step in organelle isolation, cell fractionation, protein isolation and purification (Moré et al., 1994).

Followed by the cell disruption for protein is protein precipitation. This step is to concentrate or to remove interferences before doing electropboresis or even protein determination. This is the principle step in proteomics (Jiang et al., 2004).

Quantification method

Quantification method or known as protein assay method are divided into copper-based protein assay and dye-based protein assay. Copper-based protein assay involve protein-copper chelation in copper reduction and dye-based protein assay involve the binding of color change bounded dye (Bradford, 1976; Seevaratnam et al., 2009). Example for copper-based protein assay are; Peptides and the Biuret Reaction, Bicinchoninic Acid (BCA) protein assays and Lowry protein assays. While, dye-based protein assay are Coomassie Dye (Bradford) protein assays and Pierce 660 nm protein assay. Each assay has its own benefits and limitation. Thus, selecting appropriate method is crucial to a specific sample and for precise estimation results.

Gel staining

Proteins extracted are separated using electrophoresis. This protein bands can be visualized by using different staining methods for detection. This staining technique improved in term of sensitivity over decades. Staining method is listed as Coomassie Dye staining, Silver staining, Zinc staining, Fluorescent Dye staining and Functional Group-Specific staining. Each of this staining method has its own particular advantages and disadvantages. But, the common of all of this staining method are the incubation steps. All of the staining method must have this four basic step; (1) water-wash step that roles as the remover of electrophoresis buffers from the gel matrix, (2) an acid- or alcohol-wash step for gel fixing that will limit the diffusion of protein bands, (3) staining step to allow the dye or chemical substance to diffuse into the gel and bind with the proteins and (4) de-staining step to remove excess dye from the background gel matrix (Hayworth, 2009).

Materials and Methods

Sample preparation

The leaves sample of *Metroxylon sagu* was readily prepared. Both the trunking and non-trunking leaves are originated from Dalat, Sarawak. These leaves are stored at -20°C to maintain their freshness. Both of these leaves were cut into smaller pieces with removed midriff. Using cold mortar and pestle with added liquid nitrogen, these leaves were grind into fine tissues powder.

Trichloraocetic acid (TCA) in acetone precipitation

This was a modified publish TCA protocol (Caruso et al., 2009). Tissue powder (0.5 g) was dissolved in 1 ml of ice-cold extraction buffer (0.175 M Tris-HCl; pH 8.8, 0.173 M SDS, 15% glycerol, 0.07% 2-mercaptoethanol). The cell debris was removed by centrifuging at 13,600 rpm at 4 C for 15 min. The supernatant was transferred into a new tube. Four times volume of ice-cold precipitation buffer (acetone containing 10% Trichloroacetic acid and 0.07% 2-mercaptoethanol) was added. The mixtures were stored at -20 C overnight. The pellets were obtained by centrifugation at 13,600 rpm at 4 C for 20 min. Then washed with 20% acetone with 0.07% 2-mercaptoethanol followed by centrifugation at 13,600 rpm at 4 C for 5 min. The supernatant were discarded and the pellets were air dried.

Ethanol precipitation

This was modified Ethanol protocol (Lebendiker, 2002). Tissue powder (0.5 g) was dissolved in 1 ml of ice-cold extraction buffer (0.175 M Tris-HCl; pH 8.8, 0.173 M SDS, 15% glycerol, 0.07% 2-mercaptoethanol). The cell debris was removed by centrifuging at 13,600 rpm at 4 C for 15 min. The supernatant was transferred into a new tube. Four times volume of ice-cold 99.98% ethanol with 0.07% 2-mercaptoethanol was added for protein precipitation. The mixture were incubated overnight at -20°C The pellets were obtained by centrifugation at 13,600 rpm at 4 C for 20 min. Then the pellets were washed with 99 98% ethanol followed by centrifugation at 13,600 rpm at 4 C for 5 min. The supernatant were discarded and the pellets were air dried.

Protein quantification

Protein concentration was quantified by using two techniques; Bradford protein assay and BCA protein assay (Bradford, 1976). Both methods used BSA as a standard with six different dilutions and quantified by using UV spectrophotometer (UVmini-1240).

1 ml of Bradford reagent (Coomassie Brilliant Blue G-250, methanol, phosphoric acid) was added to the sample and incubated for 5 min. The mixture was measured at the absorbance of 595 nm.

1 ml of BCA reagent (Reagent A; Bicinchoninic acid, Sodium Carbonate, Sodium Tartrate, Sodium Bicarbonate in NaOH and Reagent B; Copper (II) Sulphate penta-hydrate) was added to the sample and incubated at 60 C for 15 min The mixture was measured at the absorbance of 562 nm.

Protein separation and staining

SDS-PAGE electrophoresis was performed according to Laemmli (1970). The separating gel was prepared in 12% of polyacrylamide content and stacking gel with 5% of polyacrylamide content. The protein ladder used was in the range of 7 – 175 kDa. Each gel stack was stained using two different staining method; Coomassie Briliant Blue R-250 staining and Silver staining (Kawasaki et al., 1990; Wray et al., 1981).

For Coomassie Briliant Blue R-250 staining, the gel was soaked with staining solution (Coomassie R-250, Methanol, Acetic acid) overnight. The gel was then de-stained with de-staining solution (Methanol, Acetic acid) twice for 30 min and 10 min respectively.

For Silver staining, the gel was fixed with fixing solution (Methanol, Formaldehyde, Sodium Thiosulfate) overnight. The gel was then sensitized with sensitizing solution (Sodium Thiosulfate) to increase the stain sensitivity and contrast followed by staining with 0.1% Silver Nitrate solution. The band was then visualized after soaking in the developing solution (Sodium Carbonate, Sodium Thiosulfate, Formaldehyde) for several of seconds.

Results

In this study, two different protein extraction methods were valued for this protein study of *Metroxylon sagu*. The main highlight was set on each extraction method, the gel background, and protein band visualize in term of smearing and separation (Table 1, Table 2). For precipitation using TCA in acetone precipitation method, an average protein yield for trunking *M. sagu* of 0.633 µg/ml and non-trunking *M. sagu* of 0.624 µg/ml were obtained from Bradford protein assay. Whereas, precipitation using Ethanol precipitation method, an average protein yield for trunking *M. sagu* of 1.213 µg/ml and non-trunking *M. sagu* of 1.225 µg/ml were obtained also using Bradford protein assay. These protein yields comparisons showed that Ethanol precipitation method gave greatest yield for the trunking and non-trunking *M. sagu* among the two precipitation method using Bradford protein assay. While in BCA protein assay, precipitation using TCA in acetone precipitation method gave an average protein yield for trunking *M. sagu* of 3.062 µg/ml and non-trunking *M. sagu* of 2.632 µg/ml were obtained. Whereas, precipitation using Ethanol precipitation method, an average protein yield for trunking *M. sagu* of 6.911 µg/ml and non-trunking *M. sagu* of 6.923 µg/ml were obtained. These protein yields comparisons also showed that Ethanol precipitation method gave greatest yield for the trunking and non-trunking *M. sagu* among the two precipitation method using BCA protein assay.

Table 1: Comparison of two precipitation method with Coomassie Brilliant Blue R-250 staining referring to Figure 1A. Protein yield using Bradford protein assay.

	TCA Trunking	TCA Non-trunking	Ethanol Trunking	Ethanol Non-trunking
Protein yield	0.633	0.624	1.213	1.225
Smearing	None	None	None	Low smear
Separation	Good	Good	Poor	Poor

Table 2: Comparison of two precipitation method with Silver staining referring to Figure 1B. Protein yield using Bradford protein assay.

	TCA Trunking	TCA Non-trunking	Ethanol Trunking	Ethanol Non-trunking
Protein yield	0.633	0.624	1.213	1.225
Smearing	Low smear	Low smear	Big smear	Big smear
Separation	Good	Good	Poor	Poor

SDS-PAGE protein profiles with two different staining methods were used to compare both of the precipitation method. Using standard Coomassie Brilliant Blue R-250 staining method, precipitation using TCA in acetone precipitation method produced better resolution of protein separation, lower smearing and clearer background (Figure 1A, 2A, 2B, 2C). While precipitation using Ethanol precipitation method also gives protein separation but poorer in resolution and it also gives average to no smearing (Figure 1A2A, 2B. 2C). Meanwhile staining using Silver staining method showed that both methods produced average protein separation. The precipitation using TCA in acetone precipitation method produced uniform protein separation compare to the Ethanol precipitation method (Figure 1B). In term of smearing, TCA in acetone precipitation method gives lesser smear compare to Ethanol precipitation method. From these results, precipitation with TCA in acetone precipitation method shows the most efficient protein precipitation method compare to Ethanol precipitation for *M. sagu* protein extraction in the application of proteomic studies.

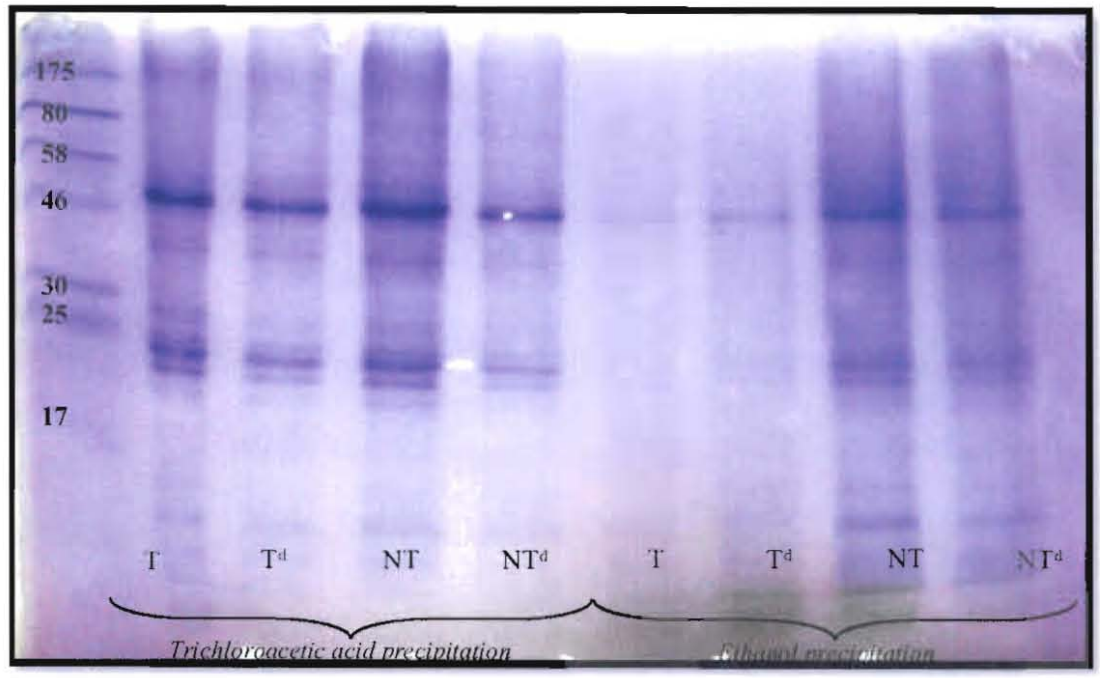

Figure 1A: SDS-PAGE run on 12 % gels of protein samples extracted using different methods. Equal amount of protein (10 µg) was loaded in each lane. Proteins were visualized with Coomassie Brilliant Blue R-250 staining.
(T: Trunking; T^d: Trunking with two time dilution; NT: Non-Trunking; NT^d: Non-Trunking with two time dilution.)

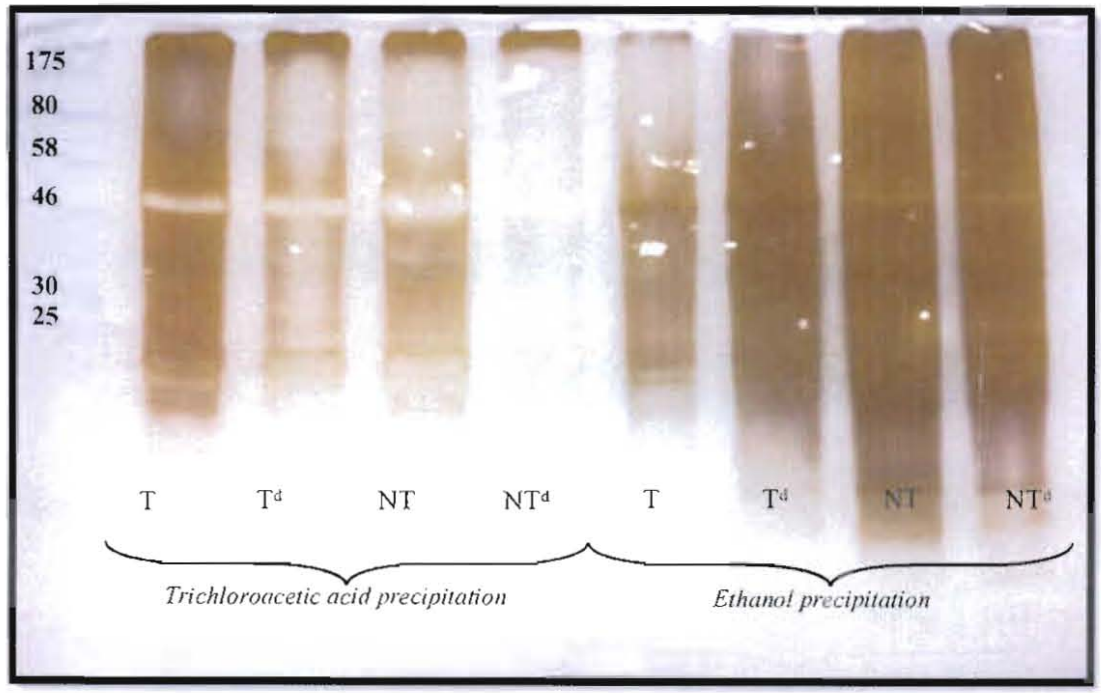

Figure 1B: SDS-PAGE run on 12 % gels of protein samples extracted using different methods. Equal amount of protein (10 µg) was loaded in each lane. Proteins were visualized with Silver staining.
(T: Trunking; T^d: Trunking with two time dilution; NT: Non-Trunking; NT^d: Non-Trunking with two time dilution.)

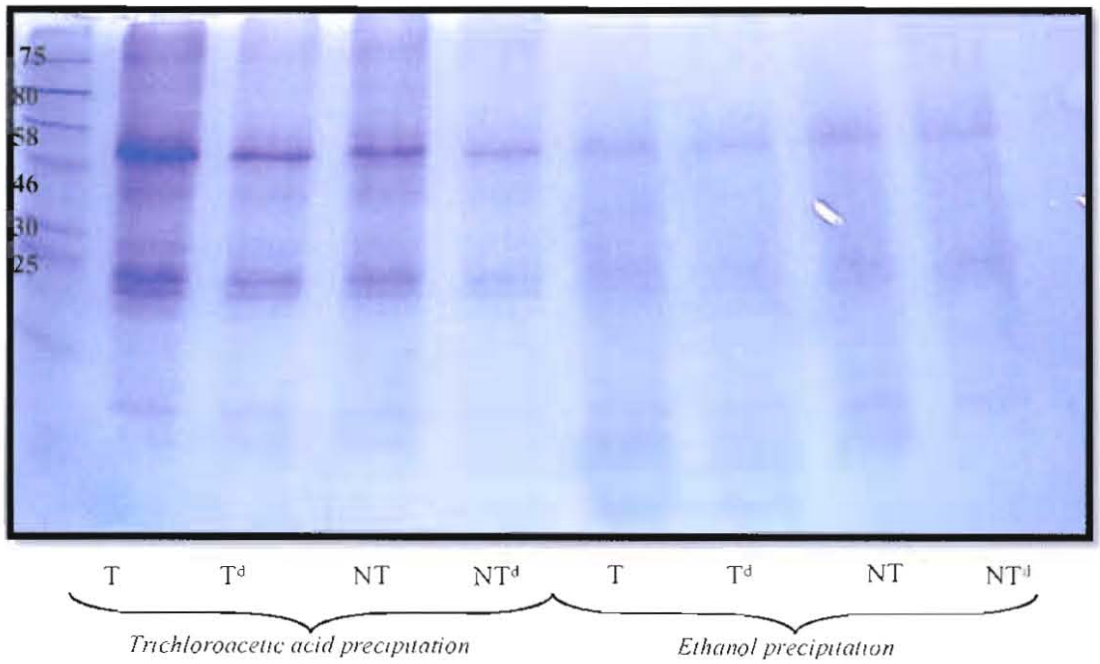

Figure 2A: SDS-PAGE run on 12 % gels of protein samples extracted using different methods. Equal amount of protein (10 µg) was loaded in each lane. Proteins were visualized with Coomassie Brilliant Blue R-250 staining. (T: Trunking; T^d: Trunking with two time dilution; NT: Non-Trunking; NT^d: Non-Trunking with two time dilution.)

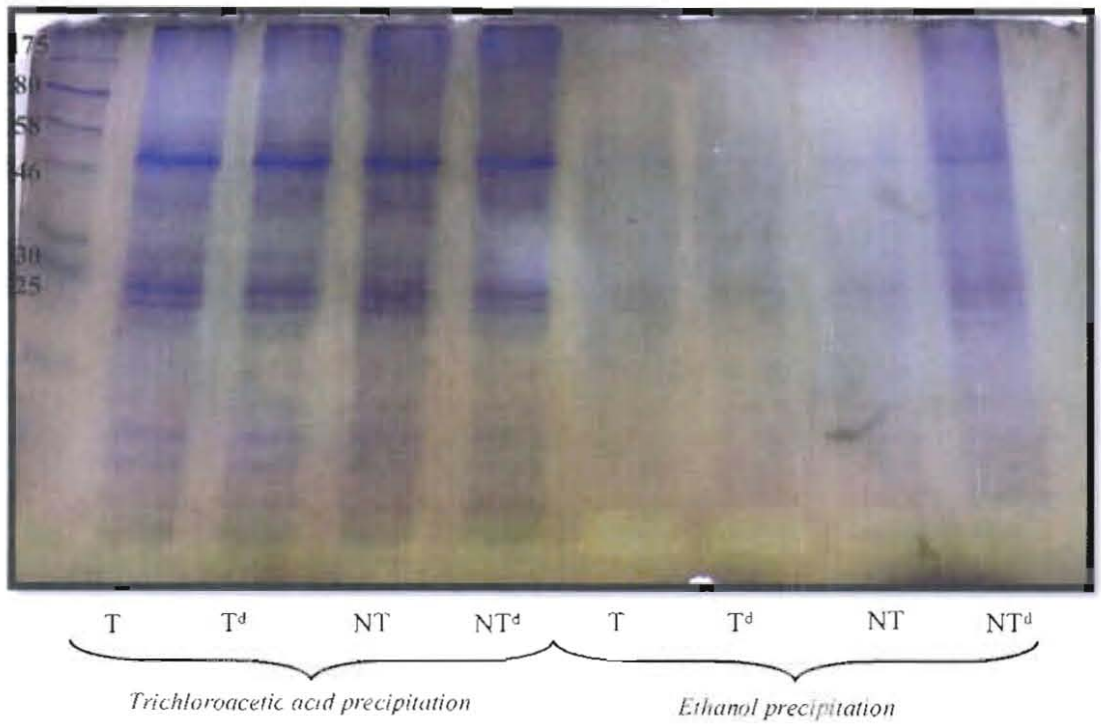

Figure 2B: SDS-PAGE run on 12 % gels of protein samples extracted using different methods. Equal amount of protein (10 µg) was loaded in each lane. Proteins were visualized with Coomassie Brilliant Blue R-250 staining. (T: Trunking; T^d: Trunking with two time dilution; NT: Non-Trunking; NT^d: Non-Trunking with two time dilution.)

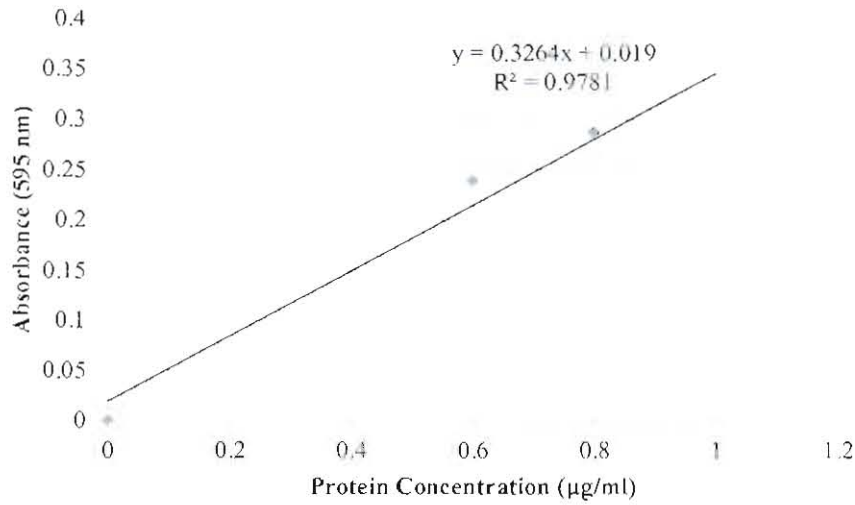

Figure 3A: Standard curve for Bradford protein assay quantification.

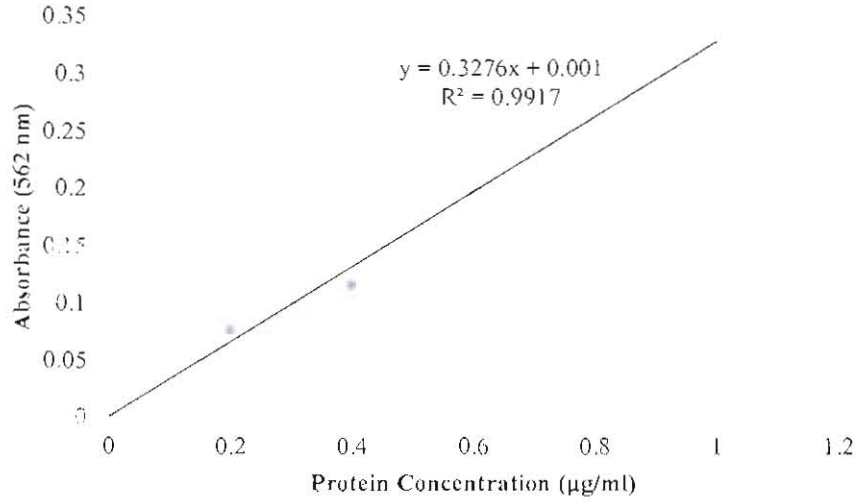

Figure 3B: Standard curve for Bicinchoninic Acid (BCA) protein assay.

Table 3A: Average protein quantification using Bradford protein assay.

	TCA	Ethanol
Trunking	0.633	1.213
Non-trunking	0.624	1.125

Table 3B: Average protein quantification using BCA protein assay.

	TCA	Ethanol
Trunking	3.063	6.911
Non-trunking	2.363	6.923

Table 4A: Anova results between TCA in acetone and ethanol precipitation method using Bradford protein assay.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Type of Sago palm (a)	7.04E-06	1	7.04E-06	0.0008	0.978	5.318
Precipitation method (b)	1.047	1	1.047	118.370	4.51E-06	5.318
Interaction (a x b)	0.00034	1	0.00034	0.039	0.848	5.318
Within	0.071	8	0.00885			

Table 4B: Anova results between TCA in acetone and ethanol precipitation method using BCA protein assay.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Type of Sago palm (a)	0.355	1	0.355	0.727	0.419	5.318
Precipitation method (b)	53.029	1	53.029	108.654	6.22E-06	5.318
Interaction (a x b)	0.380	1	0.380	0.780	0.403	5.318
Within	3.904	8	0.488			

Table 4C: Anova results between quantification method (Bradford protein assay and BCA protein assay) for TCA in acetone precipitation method.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Quantification method (a)	13.030	1	13.030	254.421	2.39E-07	5.318
Type of Sago palm (b)	0 377	1	0.377	7.366	0.026	5.318
Interaction (a xb)	0 358	1	0 358	6.989	0.030	5.318
Within	0 410	8	0.051			

Table 4D: Anova results between quantification method (Bradford protein assay and BCA protein assay) for Ethanol precipitation method.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Quantification method (a)	97.388	1	97.388	218 516	4.32E-07	5.318
Type of Sago palm (b)	0.000	1	0.0004	0.001	0.975	5.318
Interaction (a xb)	1.51E-09	1	1.51E-09	3.39E-09	1.000	5.318
Within	3.565	8	0.446			

Figure 4: SDS-PAGE run on 12 % gels of protein samples extracted using different methods. Equal amount of protein (10 µg) was loaded in each lane. Proteins were visualized with Coomassie Brilliant Blue R-250 staining. Lane 1, 3, 5 and 7 (counted after the protein ladder) was protein extracted using Phenol method. Lane 2, 4, 6 and 8 was protein extracted using acetone method.

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