Protein extraction method of Metroxylon sagu leaf for high resolution two dimensional gel electrophoresis and comparative proteomics

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Research note

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Abstract

Objective This study aimed to determine the best protein extraction method of Metroxylon sagu for the two-dimensional gel electrophoresis and the comparative analysis. Results To perform good proteome research, the most critical step is to establish a method that gives the best quality of extracted total proteins. To develop an optimized protein extraction protocol for two-dimensional polyacrylamide gel electrophoresis (2-DE) analysis of Metroxylon sagu, five protein extraction protocols were compared; polyethene glycol (PEG) fractionation method, SDS/phenol method, TCA/acetone method, combination SDS/phenol and TCA/acetone and imidazole method. The PEG fractionation method was found to give the most reproducible gels with the highest number of spots and highest protein concentration followed by SDS/Phenol method. The lowest number of spots were observed in Imidazole method. The PEG fractionation method provides improved resolution and reproducibility of two-dimensional polyacrylamide gel electrophoresis (2-DE) and reduces the time required to analyze samples. Partitioning rubisco by polyethene glycol (PEG) fractionation provides clearer detection of low-abundance protein. Hence the result from this study propose PEG fractionation as the effective protein extraction method for 2-DE proteomic studies of Metroxylon sagu.

Introduction

Sago palm is a starch producing plant and can accumulate high carbohydrate content in the trunk. Sago palm is socio-economically important for sustainable agriculture and considered as one approach for rural development in various areas in Sarawak, Malaysia. The sago palm (*Metroxylon sagu* Rottb.) is a versatile plant that can tolerate many biotic and abiotic stresses, during its growth stages. Sago palm is grown in the equator from southern Thailand to east and west of Malaysia, up to Papua Guinea and some southern region of the Philippines (Ehara, 2018).

Although this species is economically important for the country and were considered as crop par excellence for sustainable agriculture (Singhal et al., 2008), there is lack of scientific study related to this plant, including proteomics study. Proteomics is the most relevant technology to further investigation of highly complex and dynamic biological systems as it offers an accurate analysis of cellular state or system changes during growth, development, and response to environmental factors (Chen and Harmon, 2006). Extraction of proteins is the most crucial and essential step in the proteomic study due to various metabolites which could interfere in the protein separation and quantitation.

Selecting an efficient extraction method for a specific sample is utmost essential to yielding high quality and quantity proteins for two-dimensional gel electrophoresis (2-DE) to check the differential expression of proteins as well as other proteomic studies. Commonly used phenol and TCA-acetone methods remain popular despite the availability of some new techniques (Agrawal and Rakwal, 2011). Since no extraction method is universal for all kinds of samples which can capture the entire proteome, several protein extraction methods were used in this study to extract the protein from the leaf sample of *Metroxylon sagu*.

Materials And Methods

Protein Extraction Methods

Five different protein extraction methods were compared, i.e., TCA/Acetone method, phenol/SDS method, TCA-acetone and phenol/SDS method, Imidazole method and PEG fractionation method to determine the most suitable method for extraction protein from *Metroxylon sagu* for 2D analysis. Sago leaf samples used in this study were grounded in pre-chilled motor pestle in the presence of liquid nitrogen for all the five methods.

TCA/acetone method

Total proteins were extracted using the TCA/acetone method (Issacton *et al.,* 2006) with some modifications. A total of 1gram grounded leaf sample was suspended in 10 ml of 20% (w/v) TCA/acetone with 0.2% DTT and precipitated overnight at -20°C. Pellet was obtained by centrifugation the mixture at 10,000 x g for 20 mins 4°C, rinsed with 10 ml of 80% cold acetone (v/v), 0.2% DDT and incubated for 60 mins at -20°C. The pellet was collected by spinning 10,000 x g for 20 mins 4°C, washed twice with 80% ice-cold acetone, air-dried and kept at -20°C for further use.

Phenol/SDS method

Proteins were extracted according to Wang *et al.,* (2006) with some modifications. A total of 1 g of grounded leaf was suspended in 0.8ml phenol (Tris-saturated pH 8.0) and 0.8 ml of SDS buffer consisting of 0.1M Tris-HCl (pH 8.0), 2% SDS, 30% sucrose, 5% (v/v) B-mercaptoethanol and 1mM phenylmethylsulfonyl (PMSF). The mixture was vortexed and centrifuged at 10,000 x g for 20 mins 4°C. The aqueous phase was collected and reextracted with an equal volume of SDS buffer by centrifugation at 10,000g for 10 mins 4°C. The new aqueous layer containing proteins was precipitated with five volumes of 0.1M ammonium acetate in methanol at -20°C overnight. Precipitated proteins were recovered by centrifugation at 10,000 x g for 15 mins 4°C, washed and rinsed with 100% methanol containing 0.1% ammonium acetate and 80% acetone respectively, air-dried and stored at -20°C for further use.

SDS/phenol and TCA/acetone method

Proteins were extracted by combining TCA/acetone and phenol/SDS method with ammonium acetate in methanol precipitation (Wang *et al.,* 2006). 1gram of grounded leaf sample was incubated in 10 ml of 20% (w/v) TCA/acetone for 1-2 hours at -20^{o}C. The pellet was obtained by centrifugation at 10,000xg for 20 mins at 4°C and rinsed with 10 ml 80% cold acetone(v/v). SDS buffer and tris-saturated phenol (pH 8.0)in the ratio of 1:1 was added to it. The mixture was vortexed for 5 mins, followed by centrifugation at 10,000 x g for 15 mins 4°C. The clear phase collected was mixed with four volumes of methanol containing 0.1 M ammonium acetate and incubated at -20°C overnight. Precipitated proteins were recovered by centrifugation at 10,000 x g for 20 mins 4°C, rinsed with 80% cold acetone twice, dried and stored at -20°C.

Imidazole method

This method is based on Nakamura et al. (2012) with some modifications. Two grams of grounded leaf sample was incubated in 8ml of extraction buffer consisting of 50 mM Imidazole-HCl (pH 7.4), 8mM $MgCl_2$, 12% glycerol, 2.5% (v/v) beta-mercaptoethanol and 1Mm PMSF. The sample was vortexed for 5 mins, followed by centrifugation at 9000 x g for 15 mins at 4°C. The supernatant was collected using Mira cloth, an equal volume of 20% TCA/ acetone was added and incubated 1 hour at -20°C. Precipitated proteins were recovered by centrifuging the mixture at 9000 x g for 15 mins 4°C, washed thrice with 100% acetone, air-dried and stored until further use.

PEG Fractionation Method.

This method is based on Alam et al. (2012) with some modifications. 1gram of grounded leaf sample was suspended in 10 ml of Mg/Triton-X extraction buffer consisting of 0.5M Tris-HCl (pH 8.3), 2% Triton X, 20mM $MgCl_2$, 2% β-mercaptoethanol and 1mM PMSF. After vortexing for 2 mins, the sample was centrifuged at 10,000 x g for 15 mins 4°C. The supernatant containing proteins were subjected to PEG fractionation, by adjusting the final concentration of 15% (w/v) of the sample by adding 50% stock solution (w/v) of Polyethylgyycol (PEG), then was incubated on ice for 30 mins. The supernatant collected after centrifugation was precipitated with four volumes of cold 100% acetone at -20°C for 3 hours, followed by centrifugation at 10,000 x g for 15 mins at 4°C. The recovered pellet was dissolved in 10ml of Mg/Triton-X extraction buffer, vortexed for 2 mins and mixed with an equal volume of Tris-saturated phenol (pH 8.0). Sucrose (0.7 M) was added, mixed well and the mixture was centrifuged at 10,000 x g for 15 mins at 4°C. The upper phase was collected carefully, and proteins were precipitated by adding four volumes of 100% methanol containing 0.1M ammonium acetate at -20°C overnight. The pellet was recovered by centrifugation the mixture at 10,000 x g 15 mins at 4°C followed by washing thrice with 100% methanol containing 0.1M ammonium acetate and stored in 80% acetone at -20°C until use.

Protein Quantification Using Bradford Assay

Prior to further analyses, the dried protein pellets were solubilized for 1 hour in protein lysis buffer. The Bradford assay was carried out to determine the concentration of solubilized protein using Bradford reagent (Bradford, 1976). The standard protein graph of bovine serum albumin (BSA) was prepared in seven dilutions of 100 ug/ml of BSA. Samples with unknown protein concentration were prepared with different dilution factors. Absorbance was measured at 595 nm after 5 mins of adding 1 ml of Bradford reagent. The total protein concentration was determined in triplicates (Kruger, 2002).

SDS-PAGE

The quality of the proteins obtained was evaluated according to the molecular weight using one dimensional SDS Polyacrylamide gel electrophoresis. (Laemmli, 1970). A 20µl of protein sample (20 mg/mL) was mixed with 5µl of 5x sample loading dye (1:4 ratio), and proteins were denatured by heating

at 95°C for 5 mins. The denatured proteins were centrifuged for 5 mins at 16,000 x g and run through 4 % stacking gel followed by 12 % resolving gel at 120 V for 1 hour. The gels were stained with Coomassie Brilliant Blue (CBB) G-250.

2-Dimensional Polyacrylamide Gel Electrophoresis

The extracted proteins were separated according to their isoelectric point and molecular weight by subjected to 2-DE. Dry IPG strips (7cm strip, pH 3-9 non-linear) (Bio-Rad) were rehydrated with 125 µl of protein solution containing 250 ug/ml of proteins in an IPG re-swelling tray with 2 ml of mineral oil for 14 hours. Isoelectric focusing (IEF) was performed using the PROTEAN i12 IEF system according to the following parameters: 250 V for 20 mins, 4000 V for 2 hours, 4000 V for 10,000VHours. Prior to electrophoresis in the second dimension, the strips were incubated twice for 10 mins each with gentle shaking in equilibration buffer. The second-dimension gel electrophoresis was performed by inserted equilibrated strips 12% SDS-PAGE gels (Laemmli,1970). The strips were sealed with 0.5% agarose before running on 100 V for 2 hours. Following electrophoresis, the gels were fixed using deionized water for 10 mins and stained with for 12-14 hours with Coomassie Brilliant Blue (CBB) G-250 stain with gentle shaking. Images of the stained gels were captured using a Bio-Rad gel doc.

Results

Determination of Protein Concentration from the Five Extraction Methods

Elevation of protein reproducibility was done based on the amount of protein extracted from 2g of frozen leaf sample. The higher protein concentration of 8.9 µg/µl and 4.6 µg/µl were obtained from PEG fractionation and Phenol/SDS method respectively(Table 1), followed by phenol/SDS combination method 3.9µg/µl and TCA/acetone method 2.5µg/µl. Imidazole method gives the lowest protein yield of 1.2µg/µl.

Table 1. Protein concentration was determined after dissolving the pellets in the re-swelling buffer; parameters are presented as the mean ±for triplicates

Extraction method	Number of spots	Protein concentration mg/ml
PEG fractionation method	750	8.9± 0.07
SDS/ phenol method	525	4.6± 0.08
TCA/Acetone method	197	2.5±0.005
SDS-phenol/TCA/acetone method	278	3.9± 0.003
Imidazole method	- not detected -	1.6±0.02

Evaluation of Proteins from Different Extraction Methods using SDS-PAGE

The proteins extracted were separated using one-dimensional SDS-PAGE (Fig 1) and were resolved between 10 and 245 kDa. The highest number of bands were resolved using PEG with the partitioning of the rubisco, and phenol/SDS methods. However, most of the bands were common in all the four protocols except Imidazole method, which gives the lowest number of bands.

Comparison of Protein Using 2-D PAGE

The extracted proteins were subjected to 2-DE separation. Imidazole method resolved the lowest number of bands. Thus, proteins extracted using this method was not subjected to 2-DE analysis. After staining the gels with CBB G-250, images representing proteins were shown in Fig 2. The protein extraction from all the protocols resulted in higher protein spots abundance on the acidic side. The PEG fractionation method, which showed the highest protein concentration and highest bands in SDS- PAGE, showed the highest number of spots at 750, followed by SDS/Phenol and combined methods with the number of spots at 525 and 278 respectively. The lowest number of spots were shown by TCA/Acetone method at 197.

Discussion

The most crucial step to obtain high-quality protein is sample preparation and extraction. However, the interfering compounds present in abundance in green tissues can strongly hinder in extraction and separation on 2-DE (Matsui *et al.,* 1995). The purpose of this study was to compare five different protein extraction methods for use in high-resolution 2D PAGE of *Metroxylon* sagu. Results showed that PEG fractionation method is the best in terms of reproducibility, yield, high protein definition in SDS-PAGE and good compatibility to IEF (Fig 1). Results showing the PEG method is the best for protein extraction from sago leaves, both in number and resolution of the spots, were also observed using the 2D PAGE (Fig 2). Similar to what was observed in sago palm leaves, the partitioning of the rubisco by PEG method resolved many abundance proteins from leaves of rice (Kim *et al.,* 2001; Lee *et al.,* 2007) and sunflower (Walliwalagedara *et al.,* 2010).

Phenol/SDS method and combination of phenol/SDS and TCA method resulted in a high protein yield compared to the TCA/ acetone method alone, which resulted in the lowest concentration and fewer spots in 2-DE. The phenol/SDS method is not suitable for detecting low-abundance proteins in the leaf sample, because ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), approximately 50% of the soluble proteins mask them. Various studies about the TCA/ Acetone precipitation as best extraction method was reported in Brassica sp, rice (Shen *et al.,* 2003) and date palm (Devouge *et al.,* 2007; Gomez *et al.,* 2008). Other studies showed that phenol/SDS method provided an interference-free, high quality and quantity protein from diverse plants species such as potato (Carpentier *et al.,* 2005), apple and banana (Wang *et al.,* 2013).

Thus, comparing various methods described here, the PEG fractionation method also showed that it can provide a more detailed proteome of *Metroxylon sagu*, where rubisco is prevalent. In PEG fractionation method, the interfering substances present in many low-abundance proteins may cause poor electrophoretic separation on IPG strip during IEF as seen in sunflower leaf proteome (Walliwalagedra et al., 2012). To overcome this problem, the TCA-acetone precipitate was re-extracted with phenol; which efficiently remove interfering substances, that resulted in optimal electric conductivity and less time during IEF. The pre-fractionation of protein samples using PEG before the 2-DE can assist proteomic studies in general, because of the detection of low-abundance proteins. This method can be applied to the leaf tissues of varieties of species; those contain high levels of secondary metabolites and high starch content.

Limitations

The limitation of this study is that the analysis is done up to 2D-PAGE only. Further study will use MALDI-TOF/TOF MS for the identification of proteins of interest.

Abbreviations

2-DE: two-dimensional gel electrophoresis; 2D-PAGE: two-dimensional polyacrylamide gel electrophoresis; BSA: bovine serum albumin; IEF: Isoelectric focusing; IPG: immobilized pH gradient; PAGE: polyacrylamide gel electrophoresis; PEG: polyethene glycol; PMSF: phenylmethylsulfonyl fluoride; SDS: sodium dodecyl sulfate; TCA: trichloroacetic acid.

Declarations

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Authors contribution

MN wrote the proposal, participated in sample and data collections, analyzed the data and drafted the paper, HH approved the proposal, participated in sample collection, data collection and revised subsequent drafts of the paper. Both the authors read and approved the final manuscript.

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Availability of data and material

The dataset supporting the conclusions of this study and the information about the materials used is included within the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests:

The authors declare that there are no competing interests.

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Figures

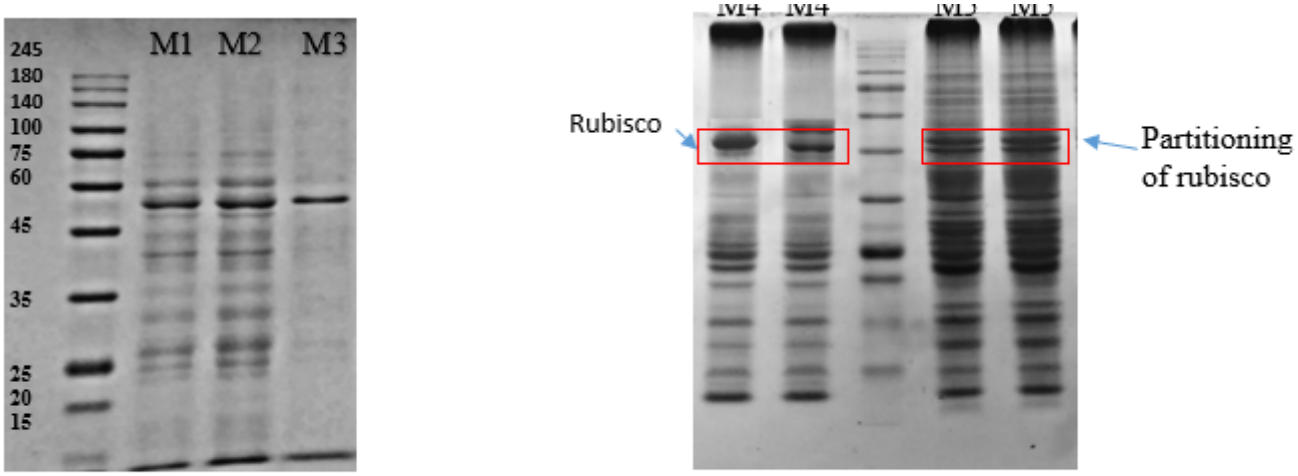

Figure 1

SDS-PAGE of Metroxylon sagu. (M1) TCA/acetone method, (M2) combination method, (M3) Imidazole method, (M4) SDS/phenol method, (M5) PEG fractionation method

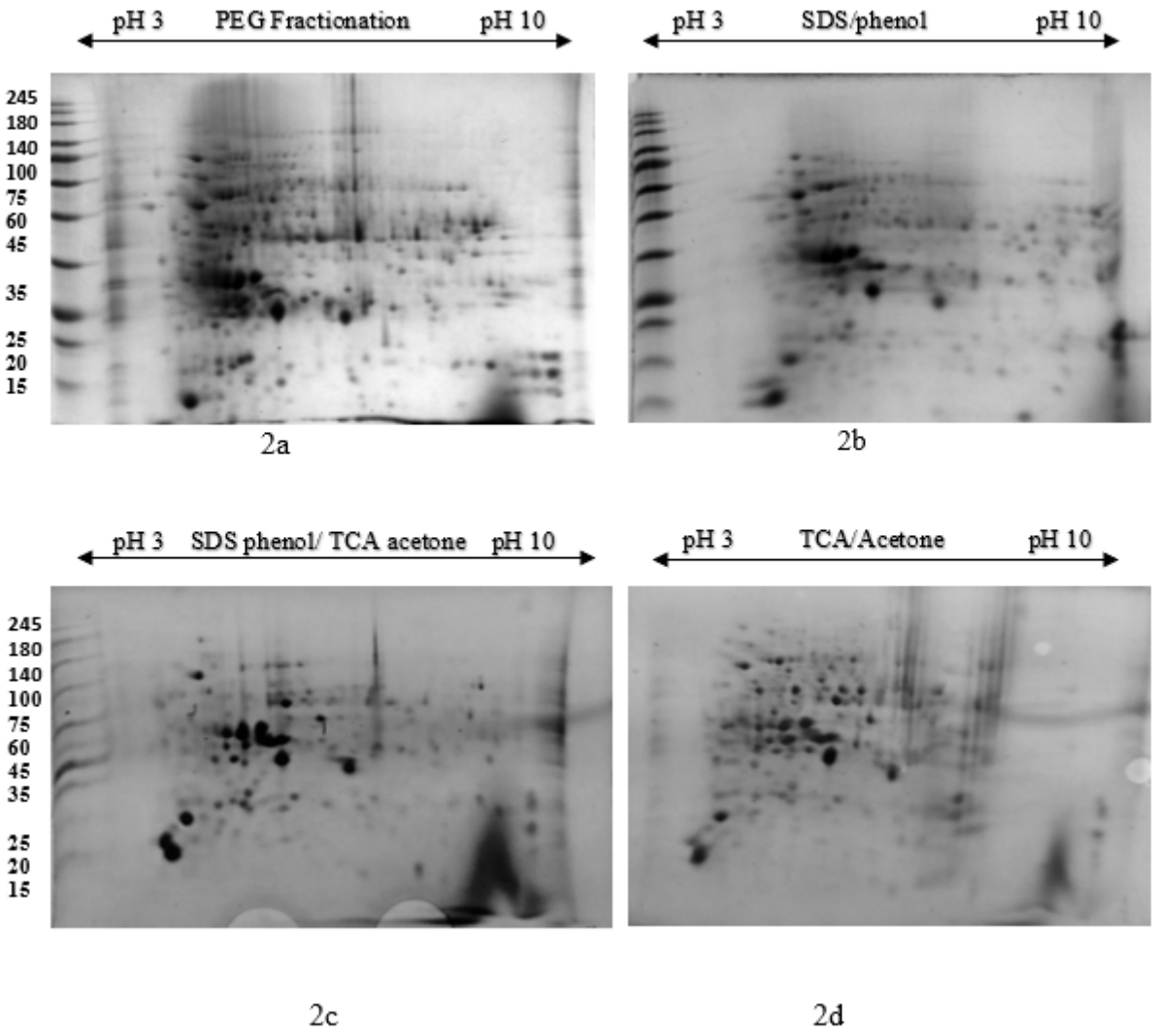

Figure 2

Comparison of the 2-DE representative gels obtained from Metroxylon sagu using four different methods. 125 µg protein samples were separated on 7cm pH3-10 non-linear IPG strip.