

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

Mehvish Nisar

Universiti Malaysia Sarawak Fakulti Sains dan Teknologi Sumber

Hasnain Hussain (✉ hhasnain@unimas.my)

Universiti Malaysia Sarawak <https://orcid.org/0000-0002-2630-450X>

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; polyethylene 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 polyethylene 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 (*Metroxylon sagu* Rottb.) is a starch producing plant and 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. This versatile plant can tolerate many biotic and abiotic stresses during its growth stages. Sago palm is grown in the equator from southern Thailand, east and west of Malaysia up to Papua Guinea and some southern region of the Philippines [1].

Although this species is economically important for the country and were considered as crop par excellence for sustainable agriculture [2], there is lack of scientific study on 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 [3]. 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 yield high quality and quantity proteins for 2-DE, to check the differential expression of proteins and other proteomic studies. Commonly used phenol and TCA-acetone methods remain popular despite the availability of some new techniques [4]. 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 to determine the most suitable method for extraction protein from *Metroxylon sago* for 2D analysis. Sago leaf samples used in this study were ground in pre-chilled motor pestle in the presence of liquid nitrogen for all five methods.

TCA/acetone method

Based on Isaacson et al. (2006) with some modifications [5], 1 gram ground leaf sample was suspended in 10 ml of 20% (w/v) TCA/acetone, 0.2% DTT and precipitated overnight at -20°C . Pellet was obtained by centrifugation at 10,000g for 20 min, 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 after centrifugation at 10,000g 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

Based on Wang *et al.* (2006) with some modifications [6], 1 g of ground leaf was suspended in 0.8 ml 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) β -mercaptoethanol and 1mM PMSF. The mixture was centrifuged at 10,000g for 20 min 4°C . The aqueous phase was collected and reextracted with an equal volume of SDS buffer by centrifugation at 10,000g for 10 min 4°C . The proteins aqueous layer 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 [6]. One gram of ground leaf sample was incubated in 10 ml 20% (w/v) TCA/acetone for 1–2 hours, -20°C . The pellet was obtained by centrifugation at 10,000x g for 20 min, 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 then added. The mixture was vortexed for 5 min, centrifuged at 10,000 x g for 15 mins, 4°C . The clear phase was collected and mixed with four volumes of methanol containing 0.1 M ammonium acetate, incubated at -20°C overnight. Precipitated proteins were recovered by centrifugation at 10,000x g for 20 mins, 4°C , rinsed with 80% cold acetone twice, dried and stored at -20°C .

Imidazole method

Based on Nakamura et al. (2012) with some modifications [7], 2 grams of ground leaf sample was incubated in 8ml of extraction buffer consisting of 50mM imidazole-HCl (pH 7.4), 8mM MgCl₂, 12% glycerol, 2.5% (v/v) β-mercaptoethanol and 1mM PMSF. The sample was vortexed for 5 mins, centrifuged at 9000x g for 15 min, 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 centrifugation at 9000 x g for 15 mins, 4°C, washed thrice with 100% acetone, air dried and stored until further use.

PEG Fractionation Method.

Based on Alam et al. (2013) with some modifications [8], 1 gram of ground 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 MgCl₂, 2% β-mercaptoethanol and 1mM PMSF. After 2 min vortex, the sample was centrifuged at 10,000 x g for 15 min, 4°C. The supernatant was subjected to PEG fractionation by adjusting the final concentration of 15% (w/v) of the sample, by adding 50% stock solution (w/v) of PEG, then incubated on ice for 30 mins. After centrifugation, the supernatant was precipitated with four volumes of cold 100% acetone at –20°C for 3 hours, centrifuged at 10,000 x g for 15 min 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). 0.7 M sucrose was added, vortexed and centrifuged at 10,000 x g for 15 min, 4°C. The upper phase proteins were precipitated with four volumes of 100% methanol containing 0.1M ammonium acetate at –20°C overnight. The pellet was recovered by centrifugation at 10,000g 15 mins at 4°C followed by washing thrice with same methanol-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 [9]. The total protein concentration was determined in triplicates [10].

SDS-PAGE

The quality of the proteins obtained was evaluated using SDS polyacrylamide gel electrophoresis [11]. 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 min. The denatured proteins were centrifuged for 5 min 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 CBB G–250.

2-Dimensional Polyacrylamide Gel Electrophoresis

Dry IPG strips (7cm strip, pH 3–9 non-linear) (Bio-Rad) were rehydrated with 125 µl of protein solution containing 250 µg/ml of proteins in an IPG re-swelling tray with 2 ml of mineral oil for 14 hours. IEF was performed using the PROTEAN i12 IEF system using these parameters: 250V, 20 mins (linear); 4000V, 2 hours (linear), 4000–10000VHours (rapid). Then, the strips were incubated twice for 10 mins each with gentle shaking in equilibration buffer. The second-dimension gel electrophoresis was performed by insertion of equilibrated strips on 12% SDS-PAGE gels [11]. The strips were sealed with 0.5% agarose before running on 100 V for 2 hours. The gels were then fixed using deionized water for 10 mins and stained with for 12–14 hours with CBB G–250. Images of stained gels were captured using a Bio-Rad gel doc.

Results

Determination of Protein Concentration from the Five Extraction Methods

Evaluation of protein reproducibility was done based on the amount of proteins extracted from 2g of frozen leaf samples. 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

Evaluation of Proteins from Different Extraction Methods using SDS-PAGE

The proteins were separated using SDS-PAGE (Fig 1) and were resolved between 10 and 245 KDa. The highest number of bands were resolved using PEG 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.

Fig 1. SDS-PAGE of *Metroxylon sagu*. (M1) TCA/acetone method, (M2) combination method, (M3) imidazole method, (M4) SDS/phenol method, (M5) PEG fractionation method

Comparison of Protein Using 2-D PAGE

The proteins were subjected to 2-DE separation. Because the imidazole method resolves the lowest number of bands, it was not subjected to 2-DE analysis. Gels stained with CBB G–250 were shown in Fig 2. The protein extraction from all 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, exhibited the highest number of spots (509), followed by SDS/phenol and combined methods (310 and 278 spots, respectively). The lowest number of spots were shown by TCA/acetone method (197).

Fig 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.

Discussion

The most crucial step to obtain high-quality protein is sample preparation and extraction, the interfering compounds present in abundance in green tissues can strongly hinder in extraction and separation on 2 DE [12]. Various studies about the TCA-acetone precipitation as best extraction method was reported in Brassica sp, rice [13], date palm [14, 15]. In present study, the TCA-acetone extraction method resulted in the lowest concentration and fewer spots detected in 2-DE separation.

There are various findings of the phenol/SDS method producing interference-free high quality and quantity protein from diverse plants e.g. potato [16], apple and banana [6]. In this study, the phenol/SDS method and combination of phenol/SDS and TCA method gave a high protein yield than TCA/ acetone method alone. The phenol-SDS method is not suitable for detecting low-abundance proteins in the leaf sample because of approximately 50% of the soluble proteins are ribulose–1,5-bisphosphate carboxylase oxygenase (rubisco), mask them. The partitioning of the rubisco by PEG method resolved many abundance proteins from leaves of rice [17, 18], sunflower [19]. PEG fractionation method resolves more proteins on SDS PAGE with the partitioning of the rubisco (Fig 1), and more spots were seen on the 2D gel with no vertical or horizontal streaking (Fig 2). Thus, PEG fractionation method provides 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 [20]. To overcome this, the TCA-acetone precipitate was re-extracted with phenol; which efficiently remove interfering substances, that results in optimal electric conductivity, less time during IEF.

We were able to generate 2-DE separation of *Metroxylon sagu* leaf protein with better resolution. Most of the spots separated by other methods were poorly separated, smeary or streaky (Fig 2b, 2c, 2d), whereas 95% of the spots detected by PEG extraction method were well resolved, clear and focused (Fig 2a)..The pre-fractionation of protein samples using PEG before 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; CBB: Coomassie Brilliant Blue; PAGE: polyacrylamide gel electrophoresis; SDS: sodium dodecyl sulfate; PEG: polyethylene glycol; PMSF: phenylmethylsulfonyl fluoride; IEF: Isoelectric focusing; IPG: immobilized pH gradient; BSA: bovine serum albumin; 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 they have no competing interests.

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Table

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

Extraction method	Number of spots	Protein concentration mg/ml
PEG fractionation method	509	8.9+ 0.07
SDS/ phenol method	310	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

Figures

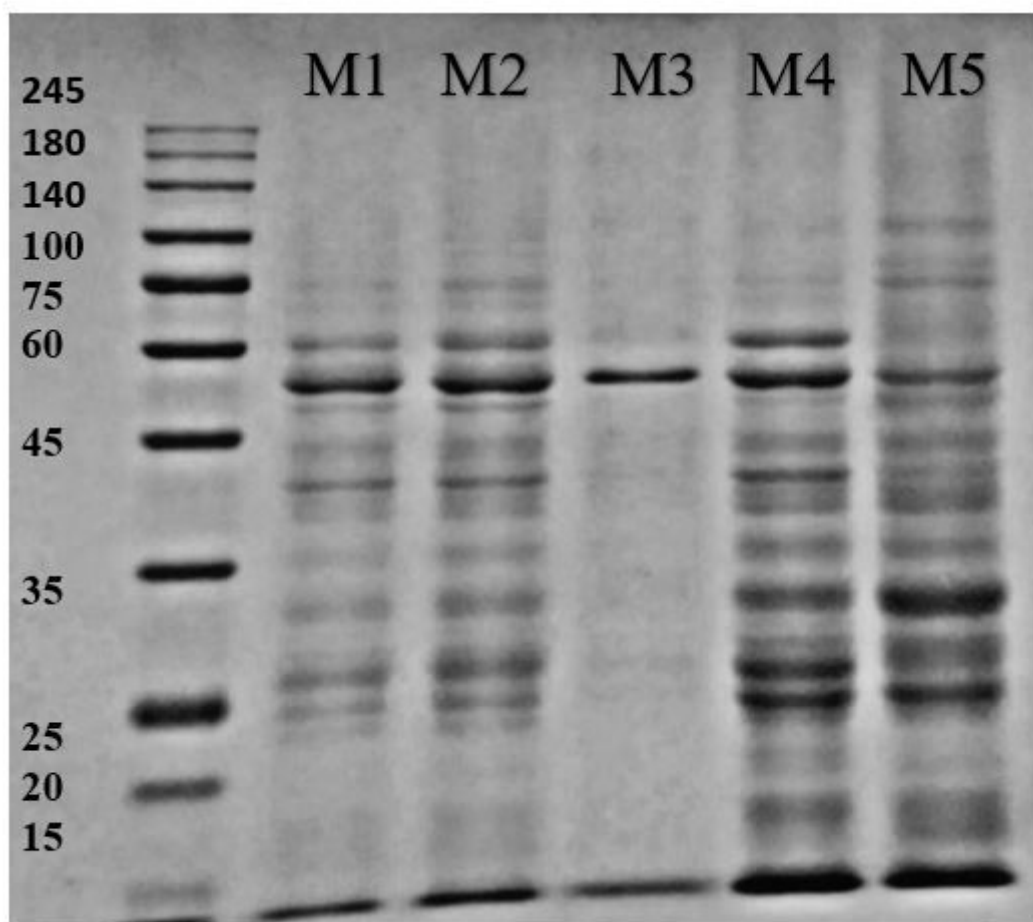


Figure 1

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

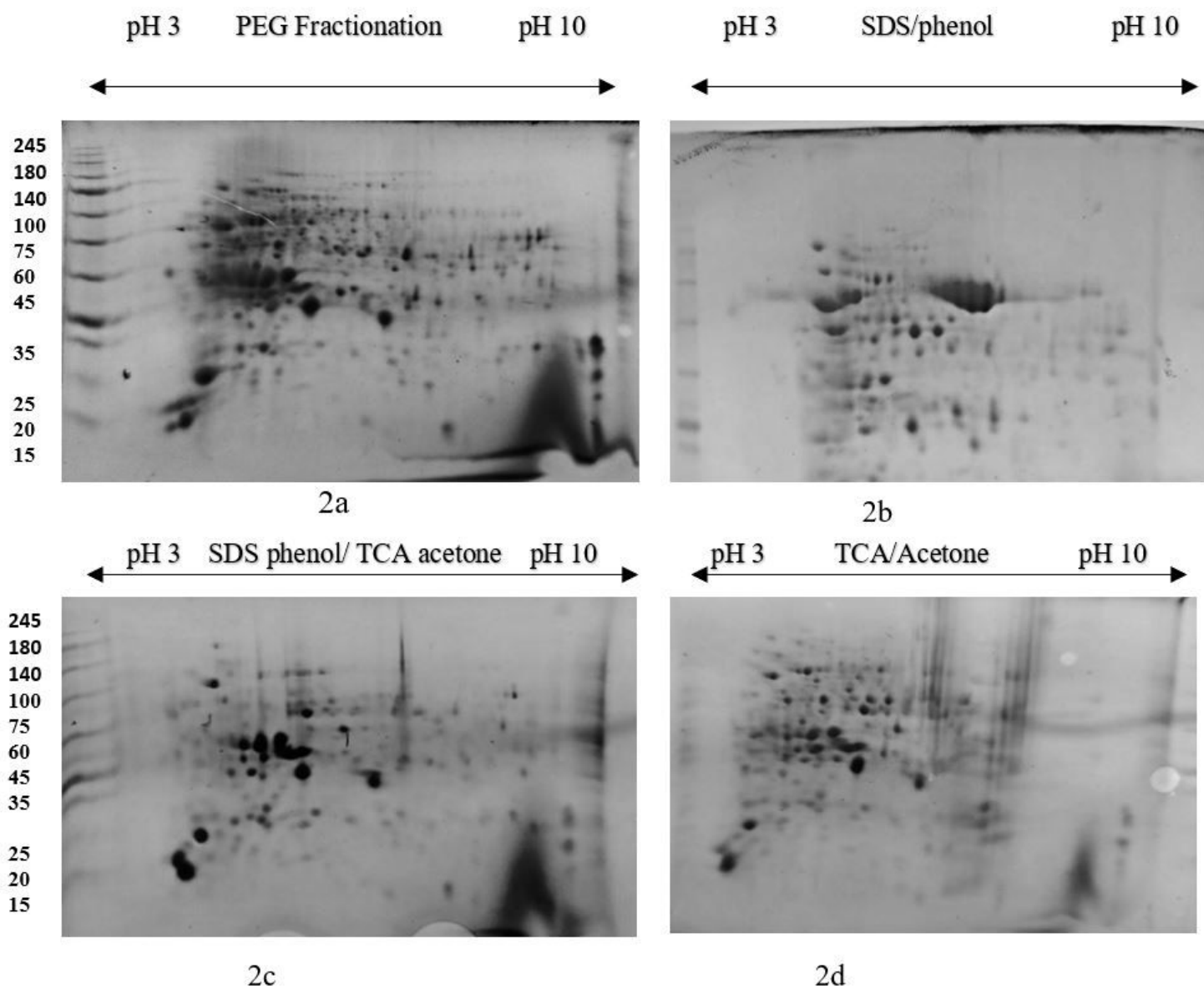


Figure 2

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.