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A Simple, Rapid and Effective Protocol for Extraction of Total Plant Proteins from Cotton Leaf

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A B S T R A C T

An attempt was made to conceive a simple, rapid and effective method to extract total plant proteins from cotton leaf samples for proteomic analysis; particularly SDS PAGE. The protocol combines TCA/acetone precipitation, methanol washes and phenol extraction as reported by Wang *et al.*, (2006) with few modifications to separate the proteins from several interfering compounds like lipids, polysaccharides, phenolics, terpenes, pigments, organic acids, nucleic acids, free ions, proteolytic and oxidative enzymes; and precipitate high-quality protein from a very low amount of starting material. The key components of this protocol were water soluble PVP and DTT (those prevent oxidation of phenolic compounds); and PMSF which acted as a protease inhibitor and the key step in this protocol was the phenol extraction step. The overall quality of the protein was good with less vertical streaking and smearing in the SDS PAGE gel and the average protein yield obtained was approximately 510 µg/g fresh weight of cotton leaf.

Introduction

Extraction of quality total plant protein from cotton leaf is a cumbersome process due to the presence of several interfering compounds those include lipids, polysaccharides, phenolics, terpenes, pigments, organic acids, nucleic acids, free ions, proteolytic and oxidative enzymes. Phenolics can build irreversible complexes with proteins, forming very strong hydrogen bonds with the oxygen atoms of peptide bonds or condensing with -SH (sulfhydryl) and -NH_2 (amino) groups. The oxidation of phenolics by phenoloxidases and peroxidases results in streaking in the SDS PAGE (Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis) gel. Lipids bind proteins via hydrophobic interactions, affecting their charge and relative molecular weight. In many cases the lipid–protein complex is insoluble in aqueous solution. Polysaccharides can interfere with SDS PAGE by obstructing gel pores. Nucleic acids can bind proteins through electrostatic interactions, preventing fluent gel run. Higher molecular weight nucleic acids can additionally clog the pores of the acrylamide matrix. Other substances, such as endogenous ions, nucleotide metabolites and phospholipids, which are present in the cell lysates, are often negatively charged those may interfere with the symmetrical charge distribution created by SDS (Sodium dodecyl sulfate) (Wu *et al.*, 2014). Thus elimination of

phenolics and other interfering compounds is a prerequisite for satisfactory protein extraction from plant tissues. In broad sense there are two different methods for total plant protein extraction; 1) Supernatant method (O'Farrell *et al.,* 1975) and 2) precipitation method (Hurkman *et al.,* 1986; Granier, 1988; Wang *et al.,* 2003, 2006). In the classical supernatant method an aqueous extraction buffer is used for solublizing the total plant protein. Even though a buffer is used which solublizes all possible water soluble proteins there are heavy chances that some amount of proteins may get trapped with the debris pellet during centrifugation which reduces the yield of the proteins obtained.

The supernatant may also contain lower molecular weight interfering compounds those do not settle down by centrifugation along with trace amounts of proteolytic and oxidative compounds those may degrade the proteins. Unlike the precipitation method where the proteins are pelleted and rest other cell components are discarded with the supernatant; in the supernatant method the vice-versa is done which leads the protein to get more diluted than necessary. Thus, the precipitation method is best for extracting total plant proteins from cotton leaves. Although there are few papers published on the extraction of total proteins from plant tissues (Wu *et al.,* 2014); no simple and exclusive protocol for the extraction of total plant proteins from cotton leaves are available. In the present study the protein extraction was done from cotton leaf using a protocol that combined TCA/Acetone precipitation, methanol washes and phenol extraction as reported by Wang *et al.,* (2006) with few modifications introduced after referring two more papers published by Lin *et al.,* (2014) and Du *et al.,* (2014) independently. SDS PAGE was conducted for the analysis of the protein profile.

Materials and Methods

The present study was conducted in the Department of Biotechnology, College of Agriculture, University of Agricultural Sciences, Dharwad, Karnataka, India in the year 2017. A schematic representation of the devised protocol is given in (Figure 1). The material used in the present study to extract total plant proteins from cotton (*Gossypium hirsutum* var. Bikaneri Nerma) leaf and perform SDS PAGE (Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis) are 10 % TCA/Acetone (Wash solution I), PVP (Poly-vinyl pyrrolidone), 50 mM PMSF (Phenyl methane sulfonyl fluoride), 40 mM DTT (Dithiothritol), 80 % Methanol plus 0.1 M Ammonium acetate (Wash solution II), 80 % Acetone (Wash solution III), 2X SDS PAGE sample buffer (pH 6.8) (Table 1), 10 % Water saturated phenol (pH 8.0)/ SDS PAGE sample buffer (1:1), 100 % Methanol (Wash solution IV), Tris-HCl (pH 6.8) (Resuspension buffer), Sample loading dye (Table 2), 1 M DTT (Dithiothritol), 30% Acrylamide mix, 1.5 M Tris-HCl (pH 6.8, pH 8.8), 10% SDS (Sodium dodecyl sulfate), 10% APS (Ammonium per sulfate) and TEMED (Tetra-methyl ethylenediamine).

The protein extraction was done at a low temperature of 22 ± 3^{o}C with pre-chilled mortar and pestle in an ice bath.

Collection of leaf samples

The *Gossypium hirsutum* var. Bikaneri Nerma plants were grown in a ventilated green house in earthen pots and the fourth and/or older leaf samples were collected from the plants at 45 days after showing for total plant protein extraction. The leaves of the plants were first sprayed with distilled water and then wiped with a kim wipe to remove dust from the surface of the leaves. Each individual sample leaf was then plucked from the plant, folded

and wrapped in a small piece of aluminium foil and immediately dipped into liquid nitrogen for snap freezing. The protein extraction can be carried out immediately or the samples dipped in the liquid nitrogen flask can be stored at -80°C for protein extraction later.

Pre-chilling of reagents and crushing of samples

The wash solutions namely 10 % TCA/Acetone (Wash solution I), 80 % Methanol plus 0.1 M Ammonium acetate (Wash solution II), 80 % Acetone (Wash solution III) and 100 % Methanol (Wash solution IV) were pre-chilled for at least 1 hour at -20 ° C or for at least 15 minutes at -80 ° C. The other reagents were aqueous solutions and were stored at 4 ° C and used directly from the refrigerator without pre-chilling. The 50 mM PMSF (Phenyl methane sulfonyl fluoride) had to be prepared fresh with chilled isopropanol, ethanol or methanol for use. The mortar and pestle were also pre-chilled before use.

For crushing each individual leaf sample was removed from the wrapped aluminium foil in the liquid nitrogen flask and then put in the pre-chilled mortar and again crushed in liquid nitrogen for at least 10 to 15 minutes to make fine powder of the leaf sample.

TCA/Acetone wash

A very small amount i.e. 0.3 to 0.5 g of individual crushed leaf sample powder was weighed and 2 ml of pre-chilled 10 % TCA/Acetone (Wash solution I), 10 mg of PVP (Poly-vinyl pyrrolidone), 8 µl of 50 mM PMSF (Phenyl methane sulfonyl fluoride) and 40 µl of 40 mM DTT (Dithiothritol) was added and crushed in a pre-chilled mortar and pestle rigorously to form a fine solution which was then transferred into a 2 ml tube. Vortexing was done in an ice bath for not more than 2 minutes followed by centrifugation at 16000 xg for 3 minutes at a temperature of 4°C in a cooling centrifuge.

Methanol wash

After removal of the supernatant in the TCA/Acetone wash a decolorized white pellet was obtained in the 2 ml tube of each individual leaf sample. Each 2 ml tube was then filled with pre-chilled wash solution II; namely 80 % Methanol plus 0.1 M Ammonium acetate for further washing of the pellet. Vortexing was done in an ice bath for not more than 2 minutes followed by centrifugation at 16000 xg for 3 minutes at a temperature of 4°C in a cooling centrifuge as was done in the TCA/Acetone wash step.

Acetone wash and air drying

In this step 80 % Acetone (Wash solution III) was used to wash the pellet obtained after the methanol wash step. 1 ml of 80 % Acetone (Wash solution III) was added to the pellet and then it was gently dispersed in the solution by using a micro-pestle. Then 1 ml more of 80 % Acetone (Wash solution III) was added to fill the tube completely after uniformly dispersing the pellet in the wash solution. This was followed by centrifugation at 16000 xg for 3 minutes at a temperature of 4°C in a cooling centrifuge.

The supernatant was discarded and the pellet was drained of the residual 80 % Acetone (Wash solution III) and dried at room temperature or at 50 ° C in a thermo-mixer for at least 10 minutes. Precautions should be taken to avoid over drying of the pellet. An over-dried pellet would be difficult to resuspended in the further steps.

Protein extraction and precipitation

To the dried pellet in the previous step 1 ml of 10 % Water saturated phenol (pH 8.0)/ SDS

PAGE sample buffer (1:1) was added and the pellet was uniformly dispersed by using a micro-pestle. Care should be taken to use the micro-pestle gently while dispersing the pellet. After uniform dispersing of the pellet in 1 ml of 10 % Water saturated phenol (pH 8.0)/ SDS PAGE sample buffer (1:1) mixture more 10 % Water saturated phenol (pH 8.0)/ SDS PAGE sample buffer (1:1) was added to fill the tube completely.

Vortexing was done in an ice bath for not more than 2 minutes followed by centrifugation at 16000 xg for 3 minutes at a temperature of 4°C in a cooling centrifuge. The tubes were removed very carefully from the cooling centrifuge so that the phenol and aqueous phase remain separated and undisturbed.

The upper phenol phase was then taken and collected in a new pre-chilled 2 ml tube and 80 % Methanol plus 0.1 M Ammonium acetate (Wash solution II) was added to the collected phenol phase to fill the tube completely. The solution was then gently mixed by inverting in hand and then was stored in a deep freezer at -20°C for 10 minutes to overnight.

Final washing and air-drying the pellet

Centrifugation of the stored sample from the previous step was done in a cooling centrifuge at 16000 xg for 3 minutes at a temperature of 4 ° C and the supernatant was discarded. The pellet was then washed once with 80 % acetone (Wash solution III) and once with 100 % methanol (Wash solution IV).

During each wash step Vortexing and centrifugation was done as in the previous steps above. The pellet was then dried by inverting the tubes on a blotting paper for not more than 3 to 5 minutes. Care should be taken to avoid over-drying of the pellet.

Resuspension and storage

For resuspension; to the dried pellet in the previous step 250 to 400 µl of Tris-HCl (pH 6.8) was added and the pellet was gently dispersed by using a micro-pestle and micro-pipetting by intermittently keeping in ice. The pellet was tried to dissolve as far as possible as the whole pellet would not dissolve completely due to higher concentration of proteins and/or presence of insoluble proteins. The white turbid solution was then stored in a deep freezer at -80 ° C till further use.

Protein analysis by SDS PAGE

A 12 % SDS PAGE (Sodium dodecyl sulfate Polyacrylamide Gel Electrophoresis) gel was used for visualizing the proteins on the gel. A Biorad Mini-PROTEAN® Tetra Vertical Electrophoresis unit was used to set up the 12 % SDS PAGE gel electrophoresis. For casting the gel first 10 % SDS (Sodium dodecyl sulfate) and 10 % APS (Ammonium per Sulfate) were prepared freshly by dissolving 0.1 g of each of the reagent in a 1 ml volume for each. The glass plates provided in the kit were aligned at the same level in the bottom by keeping it on a flat surface and the pair was fitted into the casting frame. The frame was then fitted to the stand and the set up was checked for gel leak. 5 ml of resolving gel (Table 3) was prepared and introduced into the sealed space in between the two glass plates till it reached the marking of the casting frame by using a 200 µl micropipette with an extended beaked micro-tip provided with the kit to avoid air bubble formation. The excess air bubbles were removed by adding isopropanol to the space above the resolving gel in between the glass plates which was discarded once the resolving gel was solidified. The gel was allowed to solidify for at least 45 minutes. 2.5 ml of stacking gel (Table 4) was then prepared and poured over the solidified resolving gel.

Fig.1 Step by step method for total plant protein extraction from cotton leaf samples

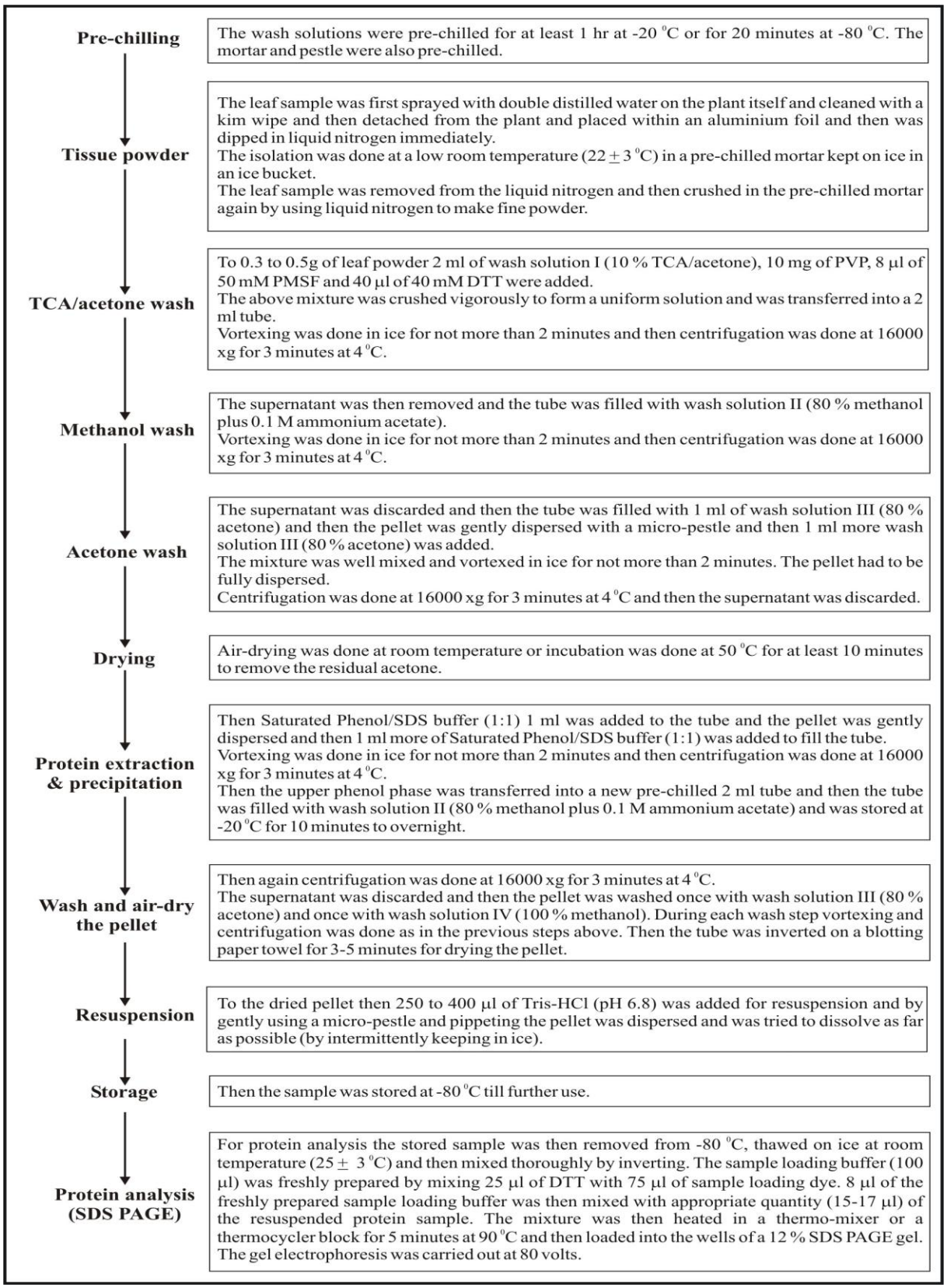
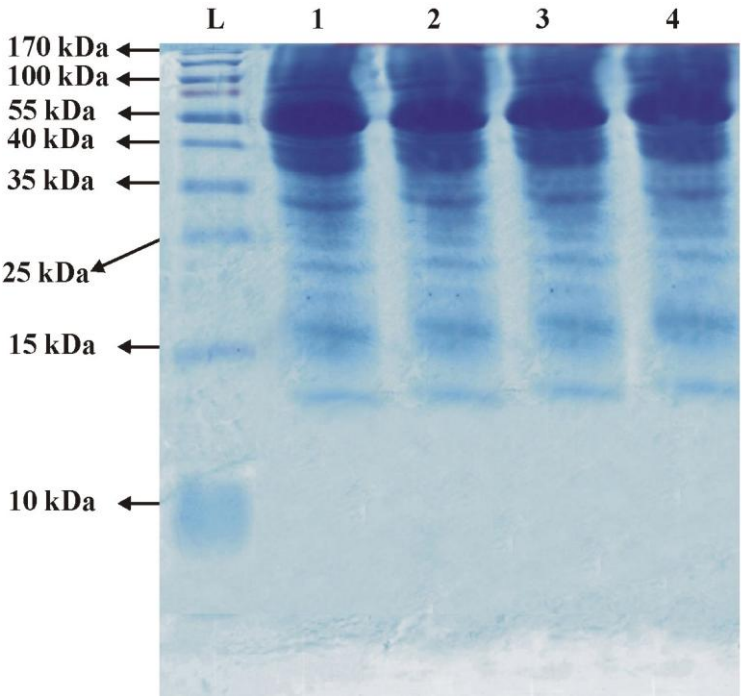


Fig.2 12 % SDS PAGE separation of cotton leaf proteins



L: PAGE ruler 10 kDa pre-stained protein ladder, Lane 1-4: Cotton leaf protein samples (Approximately 43-45 µg of protein sample was loaded in each well)

Fig.3 Protein pellet obtained after phenol extraction and precipitation step

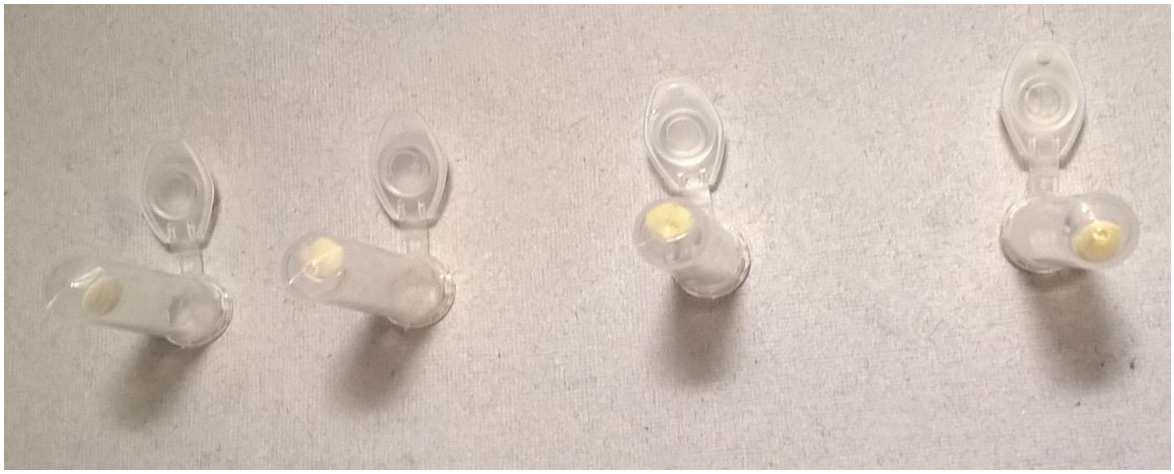


Table.1 SDS PAGE sample buffer (2X)

Reagents	Volume (ml)
0.5 M Tris-HCl (pH 6.8)	3.75
50 % Glycerol	15.0
10 % SDS	6.0
ddH_2O	Volume adjusted to 30

Table.2 SDS PAGE sample loading dye

Reagents	Volume
100 mM Tris-HCl (pH 6.8)	200 µl
200 mM DTT	100 µl
20 % Glycerol	400 µl
Bromo Phenol Blue	4 mg
4 % SDS	800 µl
ddH_2O	Volume adjusted to 2 ml

Table.3 Composition of 12% SDS PAGE resolving gel (5 ml)

Reagents	Volume
ddH_2O	1.65 ml
30% Acrylamide mix	2 ml
1.5 M Tris-HCl (pH 8.8)	1.25 ml
10% SDS	50 µl
10% APS	50 µl
TEMED	3 µl

Table.4 Composition of 12% SDS PAGE stacking gel (2.5 ml)

Reagents	Volume
ddH_2O	1.7 ml
30% Acrylamide mix	425 µl
1.5 M Tris-HCl (pH 8.8)	312.50 µl
10% SDS	25 µl
10% APS	25 µl
TEMED	2.5 µl

The comb was inserted carefully to avoid introduction of air bubbles just after the stacking gel was poured and the gel was allowed to solidify for at least 30 minutes.

The set up was then removed from the stand and attached with the electrode unit and then immersed into the tank buffer for electrophoresis.

The concentration of the protein samples before loading was checked by Lowry method (methodology not mentioned). For analysis each individual protein sample was removed from -80^{o}C and thawed on ice at room temperature followed by mixing thoroughly by inverting and micro-pipetting. 100 µl of fresh sample loading buffer (Table 1) was then prepared by mixing 25 µl of DTT (Dithiothritol) with 75 µl of sample loading dye (Table 2). 8 µl of sample loading buffer was then mixed with 15 to 17 µl of individual resuspended protein sample and the mixture was heated for denaturation to monomers on a thermomixer or a thermocycler block for 5 minutes at 90^{o}C and then each of them was loaded into the wells of a 12% SDS PAGE gel alongside a PAGE RULER 10 kDa prestained ladder and electrophoresis was done at 80 volts.

Results and Discussion

This protocol facilitated the extraction of high-quality protein samples from cotton leaf suitable

for SDS PAGE electrophoretic analysis. The overall quality of the protein was good with less vertical streaking and smearing in the SDS PAGE gel (Figure 2). The protein estimation was done by Lowry method. The average protein yield obtained was approximately 510 µg/g fresh weight of cotton leaf.

The efficient extraction of proteins from the cotton leaf tissue depends on the time interval in between plucking the leaf and snap freezing (i.e. dipping in liquid nitrogen), the quality of sample disruption, the room temperature at which the extraction is carried out and the time gap in between two consecutive steps. The key components of this protocol were water soluble PVP and DTT (those prevent oxidation of phenolic compounds); and PMSF which acted as a protease inhibitor.

The 10 % TCA/Acetone wash turned the tissue pellet white or light colour indicating the removal of majority of the secondary metabolites such as pigments and phenolics (Wu *et al.*, 2014). The key step in this protocol was the phenol extraction step in which the proteins were first extracted as a pellet after washing and then dispersed in the 10% water saturated phenol (pH 8.0)/ SDS PAGE sample buffer (Wang *et al.*, 2006) causing proteins to denature and dissolve in the phenol phase, whereas other water-soluble substances (e.g., salts, nucleic acids, and carbohydrates) remained in the aqueous phase (Wu *et al.*, 2014).

Proteins in the phenol phase were then purified and concentrated simultaneously by subsequent methanol precipitation (Figure 3). The protocol required very low starting material i.e. maximum 1 g of leaf powder for extracting proteins enough to conduct SDS PAGE for at least four times and was found to be very simple and effective for extracting high-quality total plant proteins from cotton leaf.

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