

## Buffer Optimization for *Cynodon dactylon* Proteome

Muhammad Tahir and Samina Noor Shakeel\*

Department of Biochemistry and Molecular Biology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan

### Abstract

Two-dimensional (2D) gel electrophoresis is a powerful technique used for the differential protein expression analysis. High-resolution two-dimensional electrophoresis is still the main applied separation technique in proteomics. A successful 2D gel electrophoresis depends on the proper optimized conditions most importantly an optimized buffer. In this study we checked different factors affecting 2D gel results including rehydration time, reducing agents, chaotropic agents, and detergents. We chose *Cynodon dactylon* as an experimental plant. Proteins were extracted and purified. For the proper solubilization of the protein sample different rehydration buffers of pH 3-10 were applied having different amount of chaotropic agents like urea and thiourea, detergents (NP-40, Triton X-100 and CHAPS) and reducing agents ( $\beta$ -mercaptoethanol and DTT). To further improve the protein profile on 2D gel, solubilized proteins were re-extracted, washed and re-solubilized in a fresh rehydration buffer. Our modified protocol showed a high increase in protein solubility and improvement in protein 2D gel profile for *C. dactylon* proteome. In conclusion, we have successfully optimized the conditions for *C. dactylon* proteome analysis by 2D gel electrophoresis. These optimized conditions will lay the basis for further profound studies in the field of proteomics.

**Keywords:** 2D-PAGE; Buffer optimization; Proteomics; Plant proteome; *Cynodon dactylon*

**Abbreviations:** 2-DE: Two-Dimensional Electrophoresis; 2D-PAGE: Two-Dimensional Polyacrylamide Gel Electrophoresis; APS: Ammonium per Oxidisulfate; BPB: Bromophenol Blue; CBB: Coomassie Brilliant Blue; CHAPS: 3-[(3-Cholamidopropyl) Dimethylammonio]-1-Propanesulphonate; DTT: Dithiothreitol; ESI-MS/MS: Electrospray Ionization Mass Spectrometry/Mass Spectrometry; EtOH: Ethanol; IEF: Isoelectric Focusing; IPG: Immobilized pH Gradients; MALDI-TOF-MS: Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry; MS: Mass Spectrometry; NaEDTA: Sodium Ethylene Diamine Tetra Acetate; NH<sub>4</sub>OAc: Ammonium Acetate; NP-40: Nonidet P-40; RP-HPLC: Reverse Phase High Performance Liquid Chromatography; SDS: Sodium Dodecyl Sulfate; RPM: Revolution per Minute; SDS-PAGE: Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis; SELDI-TOF-MS: Surface Enhanced Laser Desorption/Ionization Time of Flight Mass Spectrometry; SLS: Sodium Lauryl Sulfate; TBP: Tributyl Phosphine; TCA: Tri-Chloro-Acetic Acid; TEMED: N, N, N-Tetramethylethylenediamine; RAPID  $\Delta$ V: Rapid Voltage Ramp

### Introduction

Proteins perform most of activities in the living cells. Under particular circumstances or conditions a comprehensive description of the proteome of an organism not only provides a catalogue of all proteins encoded by the genome but also data on protein expression under defined conditions. Proteomics allows a quantitative description of protein expression and its changes under the influence of biological perturbations, the occurrence of post-translational modifications and the distribution of specific proteins within the cell [1]. Thus, the complexity of a biological system can be approached in its entirety if proteomics allows a multiplicity of proteins to be studied simultaneously [2].

Different techniques have been used to explore the field of proteomics in different organisms. Some of those commonly used include; 1)- separation techniques as 2-DE (Two-Dimensional Electrophoresis), RP-HPLC (Reverse Phase High Performance Liquid Chromatography), and SELDI-TOF-MS (Surface Enhanced Laser

Desorption/Ionization Time Of Flight Mass Spectrometry) and 2)-protein identification techniques including mass spectrometry such as MALDI-TOF-MS (Matrix Assisted Laser Desorption Ionization Time Of Flight Mass Spectrometry) and ESI-MS/MS (Electrospray Ionization Mass Spectrometry/Mass Spectrometry) [3]. The most commonly used method is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for standard protein separation and display [4], which is very sensitive and accurate. The scope of 2D-PAGE applications extends from drug discovery to diagnostics, therapy, microbiology and biochemistry etc. The technology of high-resolution 2-DE has been considerably improved, making the method more reliable and reproducible. Image analysis software of these complex spot patterns has been developed to such a degree that non-computer experts can also use it and get reliable results. The potential of the method for a systematic approach to create a protein database of human proteins-index has been recognized early [5].

The sample is separated over a large area, increasing the resolution of each component protein and permitting the simultaneous analysis of hundreds or even thousands of gene products [6]. Generally this technique is used for two main purposes, one is to study global protein expression and the second is to analyze the differential protein expression in an organism or tissue. To better understand the physiologic and metabolic responses of the cell to the external environment, comparative proteomic analysis is usually carried out by combined use of two-dimensional gel electrophoresis and mass spectrometry [7]. Although the limitations of the 2-DE approach

\*Corresponding author: Samina Noor Shakeel, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan, E-mail: [snq28@yahoo.com](mailto:snq28@yahoo.com)

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are well-known, i.e., poor solubility of membrane proteins, limited dynamic range, difficulties in displaying and identifying low-abundance proteins. 2-dimensional gel electrophoresis will remain a powerful and versatile tool as most commonly used in proteome analysis [8-10]. Although it is the oldest method to study a given proteome, this technique still needs optimization and expertise for different types of samples under different conditions. The variability in the achievement of optimized conditions depends on the type of sample used.

Most of the proteins can be lost during cell lysis and solubilization process. To avoid this problem complete sample solubilization is required which involves complete disaggregation, denaturation, and reduction of the proteins by disrupting protein-protein and protein non-protein interactions [11,12]. Protein denaturation is usually caused by, chaotrophic agents, urea and thiourea as it has also been found that addition of a thiourea-urea mixture can enhance solubilization of the membrane proteins, nuclear proteins and those prone to isoelectric precipitation [13-16]. Detergents like CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate), NP-40 (Nonidet P-40) and Triton X-100 support the chaotroph action and forms solubilized complexes that are stable and have exposed hydrophobic residues during focusing [17]. For the proper protein solubilization, reduction of the disulphide bonds is also necessary [18].

To deal with protein extraction from plant tissues is little complicated and problematic as compared to the animal or bacterial samples because of higher mechanic resistance of the material itself due to the presence of a cell wall that frequently undergoes secondary thickening and rigidification, a relative low protein content and usually high amount of proteases and interfering compounds e.g. phenolic compounds, carbohydrates, organic acids, pigments, terpenes, and inhibitory ions etc [19,20]. Different methods are available for plant protein extraction like chloroform/acetone method [21], phenol/ammonium acetate method [22], Tris-base/acetone method [14] and TCA/acetone method [23]. The optimal extraction procedure must minimize degradation and eliminate non-protein components that interfere with protein separation during 2-dimension gel electrophoresis.

For optimization of 2-dimension gel electrophoresis *C. dactylon*, also known as Bermuda grass was collected from local area of the Quaid-i-Azam University, Islamabad. As this plant was exclusively found in salt range area of Pakistan, we attempted to optimize buffer conditions for the 2-dimension gel electrophoresis before comparative proteomic studies to find out differentially expressing proteins between control and salt range plants.

## Materials and Methods

### Protein extraction

The *Cynodon dactylon* proteins were extracted using phenol method with some modifications [22] as described below. 1 gram of leaf tissue was ground using a pestle and mortar with liquid nitrogen. 600  $\mu$ l of water saturated phenol (pH-8.0) and 600  $\mu$ l of extraction buffer (0.7 M Sucrose, 0.5 M Tris, 0.05 M NaEDTA, 0.1 M KCl and 2% DTT) was added to grind samples extensively and later on increase to 3 ml by adding extraction buffer. To stop the activity of proteases during protein extraction, 20  $\mu$ l of Halt Protease Inhibitor Cocktails (Cat. # 78415) was used. The slushy was placed in a vortex and gently shaken for about 5 min-1 hr, until the lysate became homogeneous. The samples were centrifuged at 4°C and 1252 $\times$ g for 5 min to separate the phases. The upper phenol phase was transferred to a new tube and

1 volume of extraction buffer with phenol was added to it. Then it was placed on a vortex for 20 min. After centrifugation the phenol phase was again transferred to a new tube and the extraction repeated three more times followed by the addition of 5 volumes of ammonium acetate in methanol (0.1 M  $\text{NH}_4\text{OAc}$ , 1% 2-mercaptoethanol in methanol) and kept overnight at -20°C for protein precipitation. Pellets were washed three times with ammonium acetate in methanol, followed by washing with 10 mM DTT in 80% acetone. Aliquots were made after the last acetone wash. Pellets were dried by placing open at room temperature and kept at -80°C for further protein analysis by 2-DE (Figure 4).

### Sample solubilization for IEF

For plant proteins solubilization, 1 ml of rehydration buffer-I (9.5 M Urea, 1% NP-40, 1% CHAPS, 4% ampholyte pH 3/10, 5% 2-mercaptoethanol and 0.001% BPB) was added to the protein pellet. The pellet was broken down into pieces with the help of a pipette tip and then the tube was vortexed for 30 min at moderate speed. The sample was then sonicated in a water sonicator (Yamato Biosurplus, Cat. # 200-8681), for 20 min followed by centrifugation at 5000 $\times$ g (4°C) for 5 min. The supernatant (containing solubilized proteins) was saved and the pellet discarded. The solubilized proteins in the rehydration buffer II (7 M Urea, 2 M thiourea, 4% CHAPS, 4% ampholyte pH 3/10, 1% ampholyte pH 4/7, 100 mM DTT and 0.001% BPB) were re-extracted by precipitation with ammonium acetate in methanol and further washed three times with ammonium acetate in methanol, two times with 90% EtOH and three times with 80% acetone, both having 10mM DTT. The purified pellets were re-solubilized in the rehydration buffer II and aliquots were made. One aliquot was used for protein quantification and the remaining aliquots were kept at -80°C for further 2-DE analysis.

### Protein quantification

For the quantification of protein Thermo Scientific NanoDrop (1000 Spectrophotometer) was used. After this, proteins were first checked on SDS-PAGE and then calculations were made for loading appropriate concentration of the sample protein on 17cm ReadyStrips IPG Strips, pH 3-10, (BioRad, Cat. # 163-2007).

### Sample preparation for IEF

After protein quantification, 3 mg of the proteins were loaded on the strip as recommended by the manufacturer (Bio-Rad). We first used the in-house rehydration buffer-II (7 M Urea, 2 M thiourea, 1% NP-40, 4% CHAPS, 4% ampholyte pH 3/10, 1% ampholyte pH 4/7, 100 mM DTT, and 0.001% BPB) for loading the protein sample to the strip. The 3 mg of the sample was loaded from +ve to -ve side in focusing tray, avoiding air bubbles. Two paper wicks were placed on the focusing tray terminals. The peeled strip was placed in focusing tray, according to their respective poles, with gel side down. 2 ml of mineral oil was applied on the strip and cover was placed on the tray. IEF programming was set and run.

### First dimension

IEF can be varied according to the sample used, so we optimized the following IEF protocol for plant proteome studies: rehydration (R)-active (50 V), 20°C, 12 hrs which was extended to 14 hrs later on, RAPID  $\Delta$ V; Conditioning Step (S1)-250 V, 20°C, 15 min, RAPID  $\Delta$ V; Final Focusing (S2)-60,000 Vh, 20°C, RAPID  $\Delta$ V; Hold Step-500 V, 20°C, RAPID  $\Delta$ V.

## Equilibration and second dimension SDS-PAGE

When IEF was over, 10 ml of fresh equilibration buffer (6 M Urea, 2% Sodium Lauryl Sulfate (SLS)/Sodium Dodecyl Sulfate (SDS), 0.375 M Tris-HCl, pH 8.8, 20% glycerol and 130 mM DTT) was used to equilibrate the strip by placing in the disposable tray with gel side up for 15 min, with slow shaking.

Duplicate gels were prepared to run the samples. The IPG strips were then placed on top of 12% SDS-PAGE with the gel side towards the large glass plate. And separation was performed at 80 V constant current with external cooling.

## Staining of proteins

After electrophoresis, the gel was removed from electrophoresis apparatus and transferred into fixation solution (10% v/v acetic acid, 10% v/v methanol, and 40% v/v ethanol), and fixed for 1 h. After fixing, the gel was placed into the sensitization solution (1% v/v acetic acid, 10% v/v ammonium sulfate) and stirred for an additional 2 h. The staining solution consists of 5% v/v acetic acid, 45% v/v ethanol and 0.125% (w/v) CBB R-250. The gel was then placed into staining solution and leaved for overnight. The gel was transferred to the destaining solution-I (5% v/v acetic acid, 40% v/v ethanol) and stirred for 1 h, then into the destaining solution-II (3% v/v acetic acid, 30% v/v ethanol) until the background was clear [24]. The 2-D gels were scanned and gels were imaged at 1.38 MPixels (1×1 bin) resolution by using SynGene gel documentation system.

## Results and Discussion

For optimization of buffer to study a plant proteome, we selected *Cynodon dactylon* plant. We chose this plant for 2DE optimization because this was the most predominating plant found to be salt tolerant in the Kheora salt range, and we are interested in the comparative protein expression with control plants growing in other parts of Islamabad, Pakistan. The extracted proteins were solubilized in the rehydration buffer-I. The solubilized proteins were loaded on the IPG strip pH 3-10 in accordance with the manufacturer instructions (Bio-Rad). The resulted 2D gel (Figure 1a) shows very few spots of proteins. This was may be due to insufficient rehydration of the IPG strip prior to IEF. The experiment was repeated with same conditions and rehydration buffer-I; only the duration of rehydration step was increased from 12 hrs to 14 hrs at 20°C as the increased rehydration time gives the proteins and buffer to enter the strip properly and also the strip gains its original thickness. Rehydration at higher temperature (>37°C) holds the risk of protein carbamylation, whereas lower temperatures (<10°C) should be avoided to prevent urea from crystallization on the IPG strip. The resulted 2D gel again has few protein spots (Figure 1b).

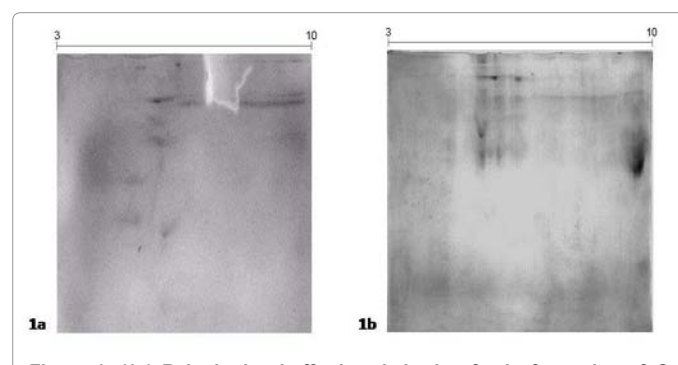
Proteins were re-extracted from leaf tissues and solubilized in the rehydration buffer, called rehydration buffer II, with some modifications in rehydration buffer-I; addition of thiourea in combination with urea [25,26], ampholyte pH 4/7 and replacement of 2-mercaptoethanol with DTT. 3 mg of proteins were loaded for IEF and figure 2a shows improvement in the number of protein spots. This result unveils the fact that the addition of thiourea in combination with urea increases the protein solubility, as reported in the literature [27]. Ampholytes are added for improved protein solubility, but also as cyanate scavenger.

Dithiothreitol (DTT), Tributylphosphine (TBP), and 2-mercaptoethanol are the reducing agents that break disulfide bonds

increasing protein solubility and also streaking reduction. Usually DTT is preferable in comparison to TBP [26].

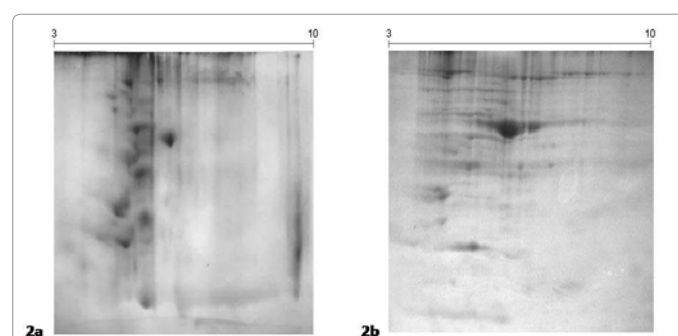
It is cleared from the figure 2a, that the proteins are not properly focused; this may be due to the incomplete protein solubilization. We confirmed this by increasing CHAPS concentration up to 4%, affecting the protein resolution, resulted in high spot numbers and comparatively minimal streakings [26]. The protein focusing was also better than before as shown in the figure 2b, which proves that proteins solubility increases by increasing CHAPS concentration in rehydration buffer as addition of the detergents to rehydration buffer helps in solubilizing the hydrophobic residues that have been exposed by denaturation [26].

In figure 2b, there are less protein spots and more horizontal and vertical streaking. These types of streakings often results due to presence of nonprotein interfering substances in the sample. This can interfere with IEF causing horizontal streaking, particularly toward the acidic side of the gel. There is possibility of presence of some impurities



**Figure 1: (1a) Rehydration buffer-I optimization for leaf proteins of *C. dactylon* with rehydration buffer-I:** Proteins were extracted with phenolic/ammonium acetate method from *C. dactylon* leaves and isolated. The sample was solubilized in the rehydration buffer-I and run for IEF according to the conditions as given in the materials and method section. 3 mg of proteins were loaded for IEF separation using immobilized pH gradient strips (3-10). SDS-PAGE gel was stained with coomassie brilliant blue.

**(1b) Optimizing rehydration time:** The 2-DE was performed according to the same conditions as for figure 1a with the exception of 14 hrs rehydration time rather 12 hrs.



**Figure 2: (2a) Buffer optimization for leaf proteins of *C. dactylon* by thiourea, DTT, and ampholyte pH 4/7 addition:** *C. dactylon* leaf proteins were solubilized in the rehydration buffer with the addition 7 M urea in combination with 2 M thiourea, 1% ampholytes (pH 4/7) and replacing 2-mercaptoethanol with DTT while IEF conditions were kept the same.

**(2b) Buffer optimization for leaf proteins of *C. dactylon* by increasing CHAPS Conc. 1-4%:** *C. dactylon* proteins were solubilized in the rehydration buffer-II with the increase in CHAPS concentration 1-4%. IEF conditions were not changed.

in the chemicals, especially urea, which we used for the rehydration buffer preparation, usually needed in high concentration or impurities from the plant tissues.

Similarly we added ethanol and acetone washings step with 10 mM DTT to improve the pure protein extraction, [28]. 2-DE was done with the same rehydration buffer-II and IEF conditions. We found comparatively more protein spots (Figure 3a) as compared to previous result (Figure 2b) having same streaking problem. The addition of 10 mM DTT at this step may help in making protein more soluble for the rehydration buffer II. Extensive vertical and horizontal streakings, and incomplete focusing, in both low and high molecular weight regions of the gel can be due to the NP-40 (non-ionic detergents) [26]. Excluding NP-40 from rehydration buffer-II conformed these streaking problems.

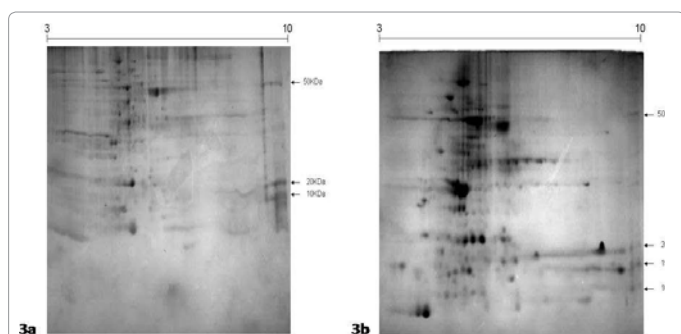
The same procedure of protein extraction and solubilization was repeated and re-extracted only the solubilized proteins from

the rehydration buffer-II for further washing steps with ethanol and acetone in combination with DTT (Figure 3a). After washings, the purified proteins were re-solubilized in the rehydration buffer, which did not include NP-40. The protein focusing was amazingly improved (Figure 3b) although little vertical streakings were still there.

Conclusively, we tried to optimize the buffer (Rehydration Buffer-II) and IEF conditions for 2D gel electrophoresis for *Cynodon dactylon*. These conditions can be helpful to analyze the comparative proteome of salt tolerant and control *C. dactylon* from salt ranges of Pakistan.

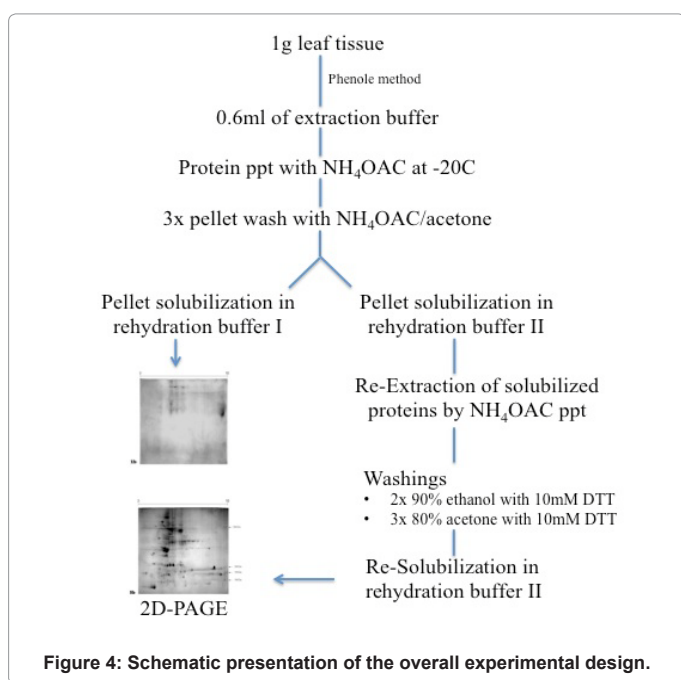
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**Figure 3: (3a) Buffer optimization for leaf proteins of *C. dactylon* with rehydration buffer-II and 10mM DTT:** Protein extraction was carried out from *C. dactylon* with 10 mM DTT in acetone and followed by ethanol washings. The rehydration buffer-II composition and IEF conditions were kept constant.

**(3b) Re-extracting solubilized proteins:** The proteins were re-extracted from the rehydration buffer II by precipitating with 0.1M NH<sub>4</sub>OAC, washed with 90% ethanol and 80% acetone having 10mM DTT and re-solubilized in the rehydration buffer II.



**Figure 4: Schematic presentation of the overall experimental design.**

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