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TOTAL PROTEIN EXTRACTION FOR PROTEOMIC STUDY IN MEMBERS OF POACEAE FAMILY

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ABSTRACT

The Total Proteins were isolated and purified from leaves of a dwarf hair garden grass *(Elocharis acicularis) and* Jowar *(Sorghum bicolor)*. The total proteins were analysed by 2-Dimension gel electrophoresis (2-DE). Three methods of sample preparation a) Phenol/Acetone, b) TCA/Acetone, without Sonication and c) TCA/Acetone with Sonication were compared based on Proteome maps of different protein preparations for 2DE, it was found that the number of protein spots were significantly reproducible when proteins were extracted by TCA/Acetone precipitation method with four cycle of Sonication in Urea and CHAPS buffer. The spot resolution and numbers were consistently better when protein were extracted in precipitation solution with Urea and CHAPS. The images were compared by using master 2-DE Bio-Rad software.

KEYWORDS
2-dimensional gel electrophoresis (2-DE), Phenol/Acetone, TCA/Acetone and Sonication

INTRODUCTION

Protein extraction of plant samples by 2-dimensional gel electrophoresis (2-DE) is quite challenging due to the high level of contamination. [1] Proteomics has been defined as the systematic analysis of the protein population in a tissue, cell or sub cellular compartment. Proteomics are always associated with two-dimensional electrophoresis because two-dimensional gel is more informative as compared to SDS-PAGE that may be evident from this presentation. The study of plant proteomics is still in its infancy, but is likely to become an active field due to its large impact on plant biology [2].

In the present investigation of three protein extraction methods (a. Phenol/Acetone, b. TCA/Acetone without sonication, c. TCA/Acetone with Sonication.) were evaluated for the study of proteomics in creeping dwarf hair garden grass(*Elocharis acicularis*.) and Jowar (*Sorghum bicolor). S. bicolor* is of main interest in research all over the world after its acceptance as a model plant. It has been ranked fifth in the world and second in United state as an important crop plant. [3] A proper protein extraction method is required to explore the depth of proteomic study and to establish an effective protocol for protein extraction suitable for 2-DE analysis of *S.bicolor*. In the present study of Dwarf hair garden grass (*E. acicularis)* was taken because of its easy availability in all seasons. Protein extraction under denaturing conditions for 2-DE was remarkably improved by the combination of chemically and physically modified processes including Urea and CHAPS, dense buffer and sonication steps [4]

MATERIALS AND METHODS

Extraction of total protein from *E. acicularis* and *S. bicolor*

Three methods for the whole protein extraction were evaluated.

(A.) Phenol/Acetone, Extraction of total protein was performed using a two (+1)-step protein extraction method. Briefly, leaf samples collected from grass plants (approximately 1 gram) were placed in liquid nitrogen and ground thoroughly into a fine powder by using a pre-cooled mortar and pestle. This fine powder (approximately 1

gram) was transferred to a sterile falcon tube containing EDTA 1mM, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propane sulfonic acid (CHAPS 1%), Ascorbic acid 5mM and protease inhibitor (5 µl) extraction buffer and vortex the solution for 10 sec. After vortexing, sonication was done (4 cycle, 10 second on and 2minute off) and 1.5 ml aliquot of suspension was made in 2 ml microfuge tube. Centrifuged at 1200g for 20 minute at 4ºC. The supernatant was collected and proteins were quantified by using established protocol [5]. Benzonase and RNAse were added to the supernatant and incubated at 30ºC for 30 minute. DTT was added (50 mM final concentration) and vortexed. The protein was precipitated by using Phenol/Acetone method as described [6]

(B.) TCA/Acetone method without Sonication, All steps in method B were same as described in method A, only change being Phenol/Acetone replaced by TCA/Acetone for precipitation of proteins [7]

(C.) TCA/Acetone method with Sonication, This is a modified form of total protein extraction method as described [7]**.** Extraction of total protein was performed using a two (+1)-step protein extraction protocol. 1 gram of leaf sample was ground to fine powder in liquid nitrogen by using a mortar and pestle (precooled). This fine powder was then transferred to a sterile falcon tube containing Trichloroacetic acid/Acetone extraction buffer [TCAAEB], acetone containing 10% (w/v) TCA and 0.07% 2-mercaptoethanol (2-BME)] and vortexed for 10 seconds. Aliquot (1.5 ml.) suspension was made, in a 2 ml of centrifuge tube and then incubated at -20ºC for 45 minutes. This suspension was mixed after 5 minutes intervals. The proteins were precipitated, centrifuged at 1200g for 20 min at 4ºC. The supernatant was decanted and the pellets were homogenized by washing twice with wash buffer (acetone containing 0.07% 2-BME), and then centrifuged under same conditions mentioned earlier. After washing, the supernatant was discarded and pellets were air dried. [8]

The pellet were subsequently solubilized in lysis buffer (LB) [8M Urea, 2% CHAPS (w/v)] and for 15 minutes vortexed and cooled alternately which was then followed by Sonication (4

cycle, pulses 10 second on and 2 min off).The solution was then centrifuged at 12000 g at 20ºC for 20 minute to remove the insoluble material. Supernatant was collected and quantified by using established protocol [5] The quantification establishes that the method C is the best method to study total protein extraction in Poaceae family and hence for *S. bicolor* only the Method C was employed for total protein extraction.

2D ELECTROPHORESIS

The protein pellet were finally resuspended in sample rehydration buffer (8M urea, 2% w/v CHAPS, 15 mM DTT and 0.5% v/v IPG buffer pH 3–10). The isoelectric focusing was performed using immobilized pH gradient (IPG) strips (Bio-Rad, USA). IPG strips with a pH range from 3-10 were initially used to determine the distribution of differentially expressed spots. Strips in the pH range of 4-7 were used for all the subsequent experiments. IEF on pre-cast IPG strip gels was carried out. The volume 45 µl of protein (500µg) was mixed with 150 µl rehydration buffer [8M urea,2% CHAPS and 50mM DTT(Dithiothreitol)] followed by pipetting into a 7 cm strip holder tray. IPG strips (pH 3-10; 7 cm) were carefully placed onto the protein samples, overlaid by mineral oil. The IPG strips were placed gel-face down onto the protein samples avoiding air bubbles and were allowed to passively rehydrate themselves with the protein samples overnight.

Next day these strips were linked to a four-step active rehydration and focusing protocol (Step 1.Start voltage at 0 V, end voltage 250 V, time 15 minute, ramp rapid and temperature 20ºC; Step 2.Start voltage at 250 V, end voltage 4,000 V, time 1 hour, ramp slow and temperature 20ºC; Step 3.Start voltage at 4,000 V, end voltage 4,000 VHr, ramp rapid and temperature 20ºC). Gel limit should be, limit/gel (50 µA),the volt hours 10 - 20,000 VHr .Whole procedure was carried out at 20ºC and a total of 10-20,000 VHr ,was used for the 7 cm strip. Following IEF, the IPG strips were removed from the strip holder and immediately used for 2-DE or stored at -20ºC. The strip gels were equilibrate in 5 ml equilibration buffer (6 M urea, 0.375 M Tris-HCL, PH ,8.8, 20% (v/v) glycerol, 2% (w/v) SDS), 2% (w/v) DTT) for 20 minute with gentle agitation

(40 rpm), followed by incubation in (freshly prepared) equilibration buffer(5 ml) supplemented with 2.5% (w/v) Iodoacetamide for 20 minute at room temperature. The IPG strips were rinsed with cathode running buffer [0.025 M Tris, 0.192 M glycine and 0.2% (w/v) SDS] and placed onto Polyacrylamide gels (12-14%) and electrophoresed at 30 mA for second dimensional separation . Standard protein markers (5 µl of prestained 2-DE, Protein Standard; Bio-Rad). 2-DE were repeated thrice to check the reproducibility of the method

Protein visualization and image analysis

To visualize the protein spots, the gels were stained with staining solution (Coomassie brilliant Blue). Protein patterns in the gels were recorded as digitalized images using a digital scanner (resolution 300 dpi, grey scale; Cano Scan 8000F, Canon, Tokyo, Japan). The gels were quantitiesed in profile mode as instructed in the operating manual of the Image Master 2-DE Platinum software (GE Healthcare).

RESULTS

The Method A, that is, Phenol/Acetone resulted in a total of 78 spots resolution of which 6 were unique **(Table and Fig. 1).** The Method B, with TCA/Acetone without Sonication produced 89 spots, among them there were 11 unique spots **(Table and Fig. 2).** On the other hand, TCA/Acetone with Sonication or Method C achieved the best spots resolution as 214 differentially expressed spots were observed; this also showed 63 unique spots **(Table and Fig. 3).** The proteins spots reproducibility was clearly induced or increased after the Sonication treatment and this was analyzed by 2-DE software. Some protein spots were overlapped or may be very close to each other that means not efficiently resolved in previous two methods (A and B). This establishes that the Method C is the best procedure for spot resolution or Total Protein extraction. Hence, it was established that Method C or TCA/ Acetone with Sonication is the best Protocol for Total Protein extraction for Proteomic study in members of Poaceae family. Therefore, the TCA/Acetone with Sonication was the only protocol used for Total Protein extraction in *S. bicolor.*

In *S. bicolor,* the Method C resulted in very high number of differentially expressed spots resolution. The TCA/Acetone extraction method using 2-DE with Sonication, showed higher protein yields as well as resolution of protein separation for leaves of S. *bicolor* with higher spot intensities, in the lower molecular weight or medium isoelectric point (pI) region, which shows the efficacy of Protocol C. It is now expected that the protocol described here can be used to develop high-level proteomic and biochemical studies and can also be applied to other plant tissues .It was observed through the present study that out of the three protocols, the TCA/Acetone with Sonication method is the best for studying Proteomics of Poaceae family.

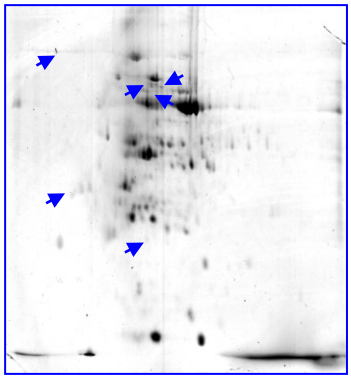
DISCUSSION

The protocol is rapid, and compatible with silver staining, it could be used for routine protein extraction from plants other than *E.acicularis* and *Sorghum bicolor* for proteomic analysis. In TCA/acetone method Sonication step play pivotal role. Using sonication from the present study we observed in our protocol that Sonication is required for optimization of this step. When sonication, apply for short pulses(4 cycle,10 second on and 10 second off with two minute gap), and let the sample were cooled in between sonications, the protein yield increased approximately 5 folds (20µg/10µl to 100µg/10µl). Sonication buffers include Urea, CHAPS and protease inhibitors improve sonication efficiency also [9]. The influence of continuous ultrasonic extraction to the yield of dispersed protein and increase of sonication in the recovery of dispersed protein progressively [10]

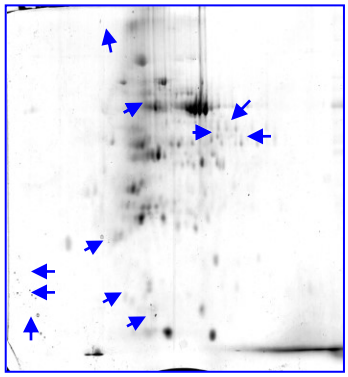
When the sample was sonicated, the polysaccharides were completely solubilized whereas without sonication the polysaccharides still remained in the supernatant hindering the following steps of protein extraction. This difference was also visible that more protein spots were observed in the sonicated sample than in the sample without sonication [11].

In conclusion, the present result (2-DE profiles) demonstrates the suitability of the TCA/Acetone extraction buffer with a modified form for improved total protein extraction and solubilization in the *E.acicularis* and *S. bicolor* (leaf).

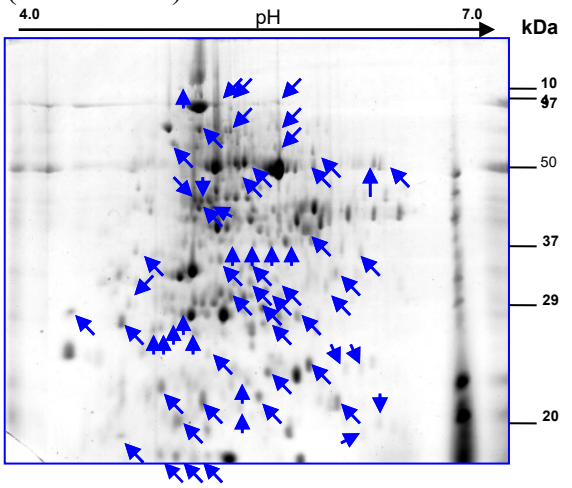
This (method establishment) will provide a new land mark in the study of proteomics for the plants of poaceae family.

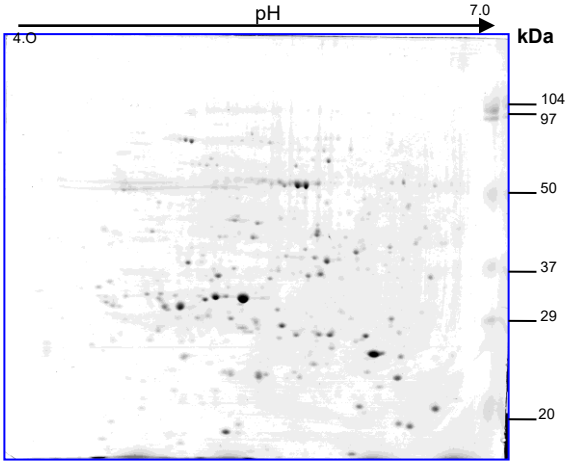


Fig;1. Phenol /Acetone (*E.acicularis*) (*E.acicularis*)



Fig; 2. Tca /Acetone, Without sonication





Fig; 3. Tca /Acetone, with sonication (*E.acicularis*) Fig**; 4.**Tca /Acetone, with sonication (*Sorghum bicolor*)

Table. 1 Differential number of protein spots among three methods (method A, B and C)

Name of methods	**No. of total spots**	**No.of unique spots(Showing by blue arrows in Fig)**
Method **A.** (*E.acicularis*)	78	6
Method **B.** (*E.acicularis*)	89	11
Method **C.** (*E.acicularis*)	214	63

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