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Published: 06 Apr, 2018

Journal of Molecular and Cellular Biology Forecast

Evaluation of Different Protein Extraction Protocol for Analysis on Two-Dimensional Electrophoresis from Roots of Eggplant

Gandhi A, Kapadia C, Vasava D and Jajda H*

Department of Plant Molecular Biology and Biotechnology, Aspee College of Horticulture and Forestry, Navsari Agricultural University, Navsari, Gujarat, India

Abstract

We had used three widely used protocols viz., Trichloroacetic acid; TCA/Acetone, Acetone, and Phenol Extraction Followed by Methanolic Ammonium Acetate Precipitation in order to optimize protein extraction and precipitation from eggplant roots. The two-dimensional electrophoresis (2-DE) separated proteins were compared based on protein yield and sum of total spot quantity. The phenol/methanol method yielded more proteins (2.65mg per gram fresh weight) and protein spots (450) in 2-DE gel than TCA and acetone protocols. Moreover TCA/acetone gave higher protein yield but total spot were less compare to phenol/methanol method. This results indicated that phenol based method gave good yield and viable results with better proteins spots for eggplant roots.

Keywords: Electrophoresis; *Solanum melongena L*; Eggplant roots; Proteins

Introduction

Proteome may be defined as the sum of all proteins produced by the cell under any specified condition with respect to tissue or cell types. Proteins are the main workforce of the cell and in eukaryotic systems, although DNA remains constant in all the tissues, the actual number of proteins produced by genes remains mystery, as none of the methods of proteomics could measure the total number of proteins [1]. Proteomic approaches help us in analyzing diverse proteins produced at specific developmental stage or inducible state from the various tissues of the plants. There is more number of genes in plant genome compare to human genome of apparently similar size. In addition to that, gene expression is regulated during transcriptional, Posttranscriptional, splicing, translation and posttranslational level, which will lead to more complex network of proteins [2-5]. Moreover, proteome of plant tissues differ with respect to different types of proteins and it continuously changes during developmental stages, biotic and a biotic stresses. These will lead to the more complexity in the study of the functional part of the cells [2]. With respect to the plant, each tissue has its typical characteristics depending on the role it plays, for instance, leaf tissues shows very little problem during proteomics studies because of less number of interferents or nonprotein components while roots tissue does. These reduce numbers of proteins visualized under two-dimensional gel electrophoresis [3,6]. Therefore, different plant tissues are subjected to different protocols to optimize protein extraction for each tissue. The main drawbacks in any proteomics study are the protein extraction, precipitation and resolubilization of proteins. However, there is no universal protocol available of protein preparation from various kinds of tissue from recalcitrant to simple [7]. Sample prepaparation from plant tissues face several challenges due to the low concentration of cellular proteins, cell wall, vacuoles, proteases, storage polysaccharides, phenolic compounds, lipids and secondary metabolites [8-10]. These interfering compounds create problems in separation and visualization of proteins, which results in the protein smearing as well as horizontal and vertical streaking. In addition to that, interaction of such compounds with proteins gives poor resolution of proteins during 2D-PAGE [9,11,12]. To best our knowledge, no reports on total protein extraction of eggplant have been reported yet. There are several protein extraction and precipitation protocols available in the literature for so many species. Hirakawa *et al.*, [13] published draft genome of eggplant (*Solanum melongena L.*), which gave insight into genetic and genome features but no information available for proteome until date.

The Brinjal, Aubergine or Eggplant (*Solanacerum melongena* L.) is grown by the farmers from tropical and sub tropical region of the world. In 2013, out of 49.4 million tones of total eggplant

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Correspondence:
Chintan Kapadia, Department of Plant Molecular Biology and Biotechnology, Aspee College of Horticulture and Forestry, Navsari Agricultural University, Navsari, Gujarat, India.
Tel: *9725018789*
E-mail: *chintan_bt@yahoo.co.in*
Received Date: *28 Feb 2018*
Accepted Date: *03 Apr 2018*
Published Date: *06 Apr 2018*

Citation: *Gandhi A, Kapadia C, Vasava D, Jajda H. Evaluation of Different Protein Extraction Protocol for Analysis on Two-Dimensional Electrophoresis from Roots of Eggplant. J Mol Cell Biol Forecast. 2018; 1(1): 1008.*

ISSN 2643-7953

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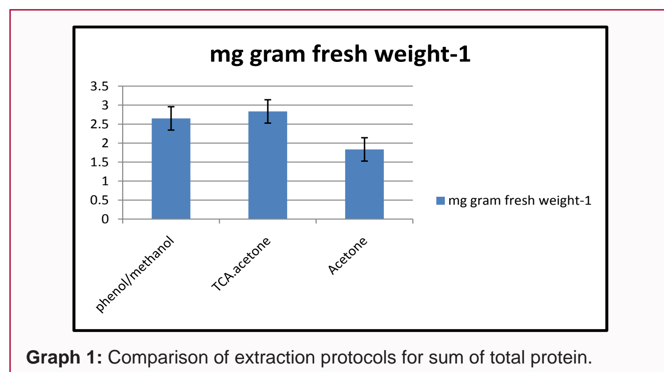
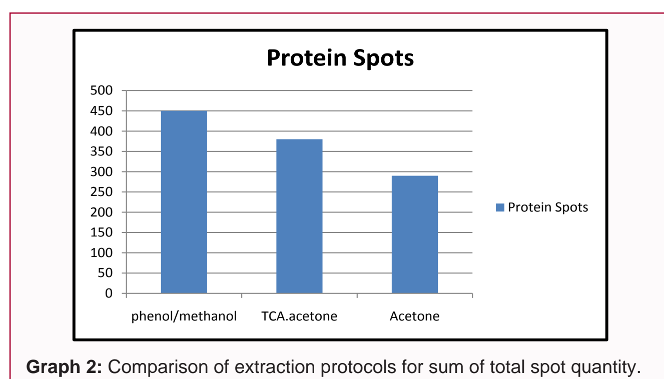
Table 1: Protein yield, concentration and number of spots from banana roots by four different extraction protocols.

Methods	µg/20µl	µg/20µl	µg/20µl	µg/20µl	stddev	µg/100µl	mg/ml	mg gram fresh weight^{-1}	Protein Spots	Sem
Phenol/methanol	24	26.6	28.9	26.5	2.45	132.5	1.32	2.65	450	1.41
TCA/.acetone	30	28	27	28.33	1.52	141.66	1.41	2.83	380	0.88
Acetone	19	16	20	18.33	2.08	91.66	0.91	1.83	290	1.20



Graph 1: Comparison of extraction protocols for sum of total protein.



Graph 2: Comparison of extraction protocols for sum of total spot quantity.

production, 27% of world total comes from India alone. Apart from India, Egypt, China and Iran are also major producers. The commonly used method for proteomics study is 2D-PAGE, which makes use of two independent physicochemical properties (Isoelectric point pI and molecular mass) of proteins for the separation of proteins from the mixture [14,15]. Isoelectric focusing separates molecules based on charges present on them [1]. However, at isoelectric point, the protein molecule does not have any charge, hence no migration. While, in the 2D-PAGE, the protein carry net negative charge only due to SDS (sodium dodecyl sulfate). Therefore, proteins are separated according to their masses.

We have analyzed root proteome using three commonly used protocols (Trichloroacetic acid; TCA/Acetone, Acetone and Tris-Phenol/Methanol extraction) in order to optimized protein extraction and precipitation. These all three protocols are differed with respect to their extraction principles. The basic principle of all these chemicals is to break macro molecular interactions in order to increase the final concentration and purity of proteins and removes polysaccharides and other polyphenolic compounds. We measured yield and purity of protein samples prior to 2D-PAGE using Folin Lowry method. There is no single method/protocol available for protein extraction and precipitation, which could be used for all plant species as well as tissues [9,11]. As a result, there are so many different optimized protocol for protein sample preparation published in the literature for various plant species and their tissues [16,17]. We believe that the outcome of this research help in designing proteomic experiments with eggplant root tissue as an initial material.

Materials and Methods

Eggplant (Arka nidhi variety collected from Vegetable Research Station, Navsari Agricultural University, Navsari) seeds were grown in the pots under polycarbonate house. The plant growth was maintained at 28°C, under a 16/8h photoperiod. After 40-45 days, Roots tissues were taken, washed several times with sterile deionized water, dried on the filter paper and ground to fine powder under liquid nitrogen using a precooled mortar and pestle. There are several methods are available for extraction of proteins such as Acetone protocol, TCA/Acetone protocol, Phenol/Methanol protocol.

Phenol extraction followed by methanolic ammonium acetate precipitation

Phenol based extraction followed by precipitation with100mM ammonium acetate in 100% methanol was used as described by Jogaiah *et al.* [18] with little modification. 1g of root tissue was ground to a fine powder under liquid nitrogen. The extraction buffer containing 0.5M Tris-HCl pH 8.0, 50mM EDTA, 0.4% β-mercaptoethanol, and 0.9M sucrose was mixed with the grounded tissues. The equal volume of tris buffered phenol (pH 8.0) was added just after incubation of 30min at 4^{0}C. The mixture was kept in shaker incubator for about 10 minutes and centrifuged at 8000 rpm for 10 minutes. The upper phenol phase was carefully transferred in the new tube and again remaining solution was mixed with equal volume of tris buffered phenol (pH 8.0) and same procedure was repeated three times. All the separate phenol phase was pooled to the fresh tube and protein was precipitated by adding five volumes of 100mM Ammonium acetate in 100% methanol (pre-chilled) vortexed and incubated at -20°C for overnight. Proteins were sediment down by centrifugation at 8000 rpm for 15 minutes. They were washed two times with precipitation buffer containing 10mM DTT followed by 80% Acetone. The pellet was dried and stored in -80°C until further use.

TCA/Acetone protocol followed by the phenol precipitation

This protocol was carried out as described previously by Rastegari and Pavoković *et al.,* [11,16] with some modifications. 1gm eggplant root tissues were ground to fine powder using liquid nitrogen and tissue powder was mixed with 5ml of chilled extraction buffer containing 100mM Tris-HCl (pH=8.0), 30% sucrose, 1% SDS and 0.07% β-mercaptoethanol. This was mixed and centrifuge at 8000 rpm for 10 minutes. The supernatant was added to the new tube and proteins were extracted by addition of double the volume of acetone containing 10% (w/v) Trichloroacetic acid, and 0.07% (v/v) β-mercaptoethanol (2-ME). These proteins mixture was incubated at -20°C for overnight, subsequently; it was (13000x*g*, 20min at 4°C) centrifuged to pellet down proteins at 10000rpm for 15 minutes. The supernatant was discarded and the final pellet was washed; first with cold 100% methanol and then with cold 80% acetone respectively. The pellet was dried and stored in -80°C until further use.

Acetone based precipitation protocol

This protocol was performed as described previously by Vaganan

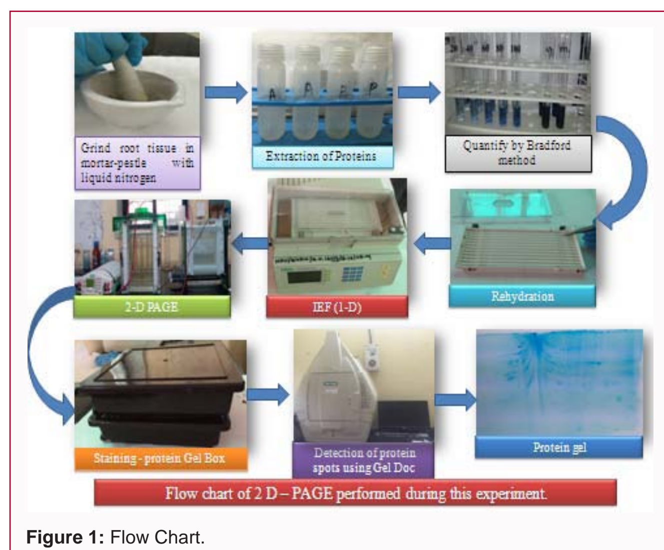


Figure 1: Flow Chart.

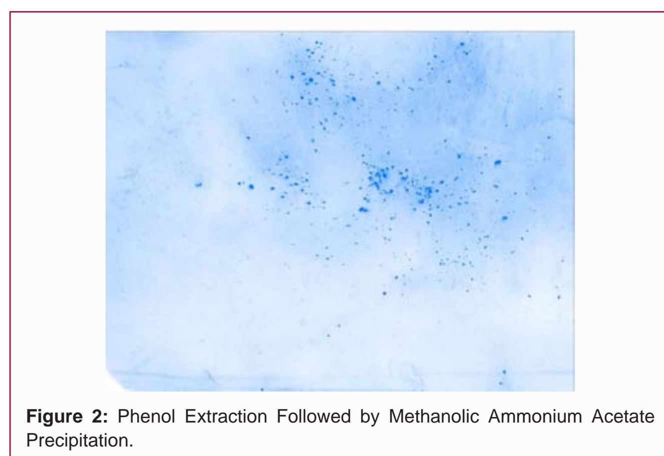


Figure 2: Phenol Extraction Followed by Methanolic Ammonium Acetate Precipitation.

et al., [19] with little modifications. 1gm of eggplant root tissue was ground to fine powder using liquid nitrogen followed with addition of 5ml of ice cold extraction buffer containing 50mM Tris (pH=8), 5M urea, 2M thiourea, 1.5% (w/v) PVPP and 2% (w/v) DTT and 1 mM PMSF. These mixtures were vortex for a short period of time and centrifuged for 20 minutes at 10000rpm at 40°C. These proteins were precipitated by adding four volume of ice cold acetone containing 0.07% DTT and left overnight at -80°C, These precipitated proteins were pellet down by centrifugation at 10000rpm for15 minutes and washed twice with acetone. All the left over acetone was removed by air drying.

The final pellet was resuspended in the pellet in 750µL of IEF extraction solution (8M urea, 2M thiourea, 4% CHAPS, 2% Triton X-100, 100 mM DTT and 1% pH 3-10 ampholytes) by pipetting and vortexing at 25°C. Incubate sample for 1h at room temperature with agitation. Total proteins in all the above extracts were estimated according to Bradford [20]. The samples were immediately subjected to 2-DE or stored at -80°C for further analysis.

Protein separation through IPG Strips in first dimension followed by SDS-PAGE for second dimension using electrophoresis

The isoelectric focusing was carried out in 17 cm IPG bluestrips (pH 3–10, linear gradient, Biorad) by using a Biorad protein IEF cell. IPG strips were passively rehydrated with 350 µl rehydration buffer (8M urea, 2M thiourea, 2% CHAPS, 1% ampholytes and 0.002% bromophenol blue) containing 350µg of root proteins for 12h. The voltage settings for IEF were: 250V for 20min. linear, 8000V for 2.50h linear and 20,000V/hr rapid at working temperature of 20°C. The proteins in the strips were denatured by keeping the strip gel - side up into the 5ml of Equilibration buffer 1 (2% SDS, 0.375M Tris pH 8.8, 2% DTT, 20% glycerol, 6M Urea) and Equilibration buffer 2 (0.4% SDS, 0.375M Tris pH8.8,20% glycerol, 6M Urea, 2.5mg iodoacetamide per ml) for 10-10 mins. The second dimension separation of proteins was carried out with 12% (v/v) SDS-Polyacrylamide gels with 5% stacking gels in PROTEAN II XL (Bio-Rad) vertical gel electrophoresis apparatus at 22°C. Electrophoresis was carried out at a constant current of 15mA for 30min, followed by at 50mA for 6h at 20°C. The gels were stained using Coomassie brilliant blue G250 followed by destained in 7% acetic acid until a clear background. The gel was analyzed using PDQUEST 8.0 software (Bio-Rad). Protein spots were detected on scanned gels using the default spot detection setting.

Result

There is prerequisite of successful optimization of extraction and purification protocol from any plant tissue to study changes in proteome. The extraction buffer and protocol is depends on the type of plant tissue and their chemical composition, which often need ample time and money. Our aim is to optimize the method of protein extraction from eggplant roots for 2D-PAGE analysis (Figure 1). We had evaluated three most common protein extraction methods for higher protein concentration and well resolved spots with less streaking and fewer interfering substances, which may be more desirable in IPG stripes. The methods used were Acetone, TCA/Acetone and Phenol/Methanol. Out of three protocols, TCA/acetone and phenol/methanol based extraction gave considerably superior protein yield of 2.83 ± 0.88 and 2.65 ± 1.41 mg g^{-1} root tissue respectively, compared to acetone protocol (Table 1). On the other hand, the protein from phenol/methanol extraction method had much lower protein concentration than other methods with relatively little impurities but spot were very sharp and distinguishable. The almost equal quantities of the proteins were analyzed on the IPG strips under same conditions (17cm IPG strips (Non linear pH gradient of 3-10), passive rehydration, 12% acryl amide gels and revealed distinctively different spots patterns. There was noticeable dissimilarity observed in the protein resolving pattern between the methods evaluated and more number of protein spots detected in the gels (Graph 1 & 2). Proteins extracted from roots using phenol/methanol protocols (Figure 2) exhibited clear protein profiles with less horizontal and vertical streaking, since the proteins in the 2-DE gels of TCA/acetone and acetone protocols (Figure 3 & 4) did not show clear separation with prominent streaking and distortion of spots, thus reducing the distinctly resolved spots. The average number of protein spots (Table 1) observed in 2-DE using phenol/methanol extraction method (450) and TCA/acetone method (380) was higher than that with acetone method (290). Higher yield of proteins by the TCA/acetone and phenol/methanol protocols was reflected in the more number of protein spots detected in the gels, compared to acetone methods. Consequent on detection of highest number of protein spots, the sum of protein spots quantity was highest by the phenol/methanol protocol, followed by the TCA protocol and the acetone protocols had very low sum of proteins intensity. Also, phenol/methanol protocol showed highest average protein spot intensity, followed by TCA protocol (Figure 3). There was unsatisfactory protein separation observed since the distance between two spots was very small as

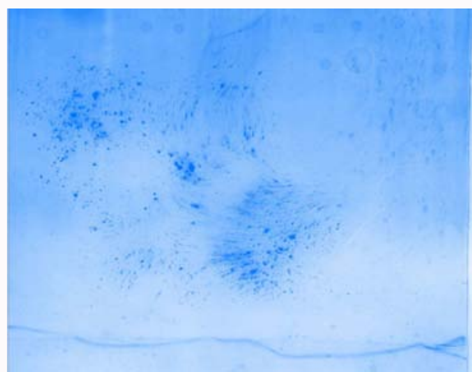


Figure 3: TCA/Acetone protocol followed by the phenol precipitation.

Figure 4: Acetone based precipitation protocol for 2DPAGE.

well as streaking and smearing made them undistinguishable. It was common belief that most of the soluble proteins come under the pI (isoelectric point) range of 4-8, and acidic and basic regions of the gel showed fewer spots.

Discussion

Protein isolation and that to be in pure form, is one of the most critical steps in any proteomics studies. The results of the any successful proteomics approach are based on the well resolve spots with minimum inhibitory substances. We analysed three different protein extraction protocols to optimise the extraction method, which could yield high quantity and quality of proteins. The cell is made up of the complex charged and uncharged bio molecules like nucleic acid, proteins, polysaccharide, phenolic compounds and insoluble organics, which strongly hinder in extraction, purification followed by the solubilization of proteins [21]. Negatively charged Polysaccharides bind with proteins through electrostatic interactions can often choke the pores and subsequently lead to precipitation and increase in focusing time during IEF. Nucleic acids can also clog gel pores and enhance viscosity of the reaction mixture. Phenolic compounds modify proteins through enzyme catalyzed oxidation reaction [22]. Salts bind with proteins affect its movements under electrophoresis, which lead to heating of IEF strips, high strip conductivity and protein streaking [23]. There are various reports indicated the TCA/Acetone precipitation as superior protocol than Phenol precipitation for protein purification for various plants such as *Brassica* sp, Rice and Date palm [24-26]. On the other hand, the Hurkman and Tanaka [27] exploited phenol into protein extraction process for improvement in protein purification. On the other hand, Phenol method gives reliable and interference free proteins from diverse plant species and tissues such as potato [28] and rapeseed seedlings [29]; roots of Jerusalem artichoke, potato, apple and banana leaves [30,31]; olive leaf; and tomato, avocado and banana fruits [12]. The phenol method efficiently separate polysaccharides and other impurities into aqueous phase and thus gives a higher protein yields with less interference and good resolve spots. TCA/Acetone based extraction method yielded considerable quantity of proteins; but there was less number of distinct protein spots observed and also with horizontal streaking [19]. Acetone and TCA/Acetone precipitation does not sufficiently remove nucleic acids, lipids carbohydrates and polyphenols, which cause precipitation, worst focusing and protein streaking [16,18,19,30,32,33]. The TCA or Acetone alone could not precipitate all proteins [31] though TCA denatures proteins under low pH may cause protein degradation or modifications [16,34,35]. Oxidase are the main culprits in the isolation of proteins, which convert phenol into toxic quinine, henceforth, it is quite necessity to inactivate by any means the activities of various oxidases. The TCA and Acetone at low pH does the same things and ultimately prevent complex formation of proteins with debris. Machin *et al.* [36] explained the possible role of acetone during purification of proteins. The organic molecules does solubilize in the organic chemicals, therefore due to its chemical nature acetone tends to solubilize the lipids, pigments and other secondary metabolites. An alternative method is based on the solubilization of proteins in phenol, followed by their precipitation with ammonium acetate in methanol. Phenol extraction was initially served to remove proteins from carbohydrates based sample as well as nucleic acids. The basic methanol wash was found to be useful in removing phenolic compounds [34]. Phenol is an effective protein solvent and that can considerably reduce molecular interaction between proteins and other compounds that inhibit electrophoresis [34]. Phenol dissolves proteins and lipids while leaving water-soluble substances such as polysaccharides, nucleic acids, etc in the aqueous phase [7]. Subsequently the proteins can be separated from lipids by adding precipitation with 100% methanol containing 0.1M ammonium acetate [37]. The slightly alkaline pH of the buffered phenol helps to reduce protein degradation [38]. This helped to minimize the impact of phenolics compounds. Furthermore, a methanol wash was also added as an aid to extract residual phenolic compound [34]. Here, we have described the methods to visualize more or less 300–500 protein spots per gel using the phenol extraction method. This could be the most solubilize and abundant protein of the cell as at one time and in one buffer all the protein could not be dissolve. Protein spots resolution and their numbers differentiate various methods for its use for 2D analysis. Differences were observed in both the number and resolution of protein spots. The acetone precipitation produced the lowest number of protein spots. The TCA/acetone method gave intermediate results while highest numbers of protein spots were detected in the gels after phenol extraction. The high intensity and number of spots for phenol based extraction suggests that this method more efficiently preserved proteins, inhibiting their hydrolysis, while TCA/Acetone was less effective. The phenol extraction method gave higher protein concentration than the other two methods under study. Similar results were found for protein extracts of tomato pollen and grapevine leaves [30,37,38]. But our results did not coincide with Shen's results. Many previous evaluation studies resulted in the similar findings and supported the present results. Phenol based method gave better protein yield and solubilization; moreover protein spots were also well resolved. This is the first report on the evaluation of various protein extraction methods for 2-DE of eggplant roots and these

could be useful for proteomics research in eggplant.

Conclusion

The present study provides practical proof for the root proteomics research as phenol/methanol method gave good yield and viable results with better proteins spots for eggplant roots. These could be helpful for future downstream processing application.

Acknowledgement

The authors gratefully acknowledge the financial assistance from NAU, Navsari, Gujarat for carrying out this work.

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