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A protocol for protein extraction from recalcitrant tissues of grapevine (*Vitis vinifera* L.) for proteome analysis

Satisha Jogaiah*[Ψ], Anuradha Upadhyay[Ψ], Smita R Maske and Manisha P Shinde
ICAR-National Research Centre for Grapes, Manjri Farm Post, Solapur Road, Pune 412 307, India

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The difficulty in obtaining high quality proteins from recalcitrant plant species like grape (*Vitis vinifera* L.) is mainly due to low concentration of proteins, high activity of proteases and high level of interfering compounds, such as, pigments, polyphenols, tannins, flavonoids *etc*. Utilization of 2-dimentional electrophoresis (2DE) technique in proteome analysis largely depends on more efficient and optimized preparation of samples, which is regarded as one of the most important step for obtaining reliable information on proteins. In the present study, four different previously published protein extraction protocols and commercial protein extraction kits were compared to identify the most suitable protocol for protein extraction from different grape tissues, such as, leaves, buds, clusters and berries. Of the five protocols compared, resolution of protein in 1st dimension and 2nd dimension gel was improved with modified trichloro acetic acid/acetone precipitation with phenol extraction method. This method also yielded higher protein concentration, greater spot resolution with minimal streaking on 2DE gels of grape leaf protein. The same protocol also yielded higher quality protein from other grape tissues, such as, buds, berries and roots. Thus modified tricholoro acetic acid/acetone precipitation with phenol extraction method will be suitable for proteome analysis of different grapevine tissues.

Keywords: Grapes, phenol extraction, protein extraction, proteomics, 2D electrophoresis

Introduction

Grapevine (*Vitis vinifera* L.) is considered as one of the most commercially important fruit crops worldwide. After the completion of its genome sequencing, grape is regarded as a model species for understanding biology of berry development in non-climacteric fruits[1]. Berry development in grape follows double sigmoidal pattern and can be divided into three distinct phases, each characterised by a complex set of physiological and biochemical changes. Post genomic technologies like proteomics and metabolomics are playing a major role in unravelling of these complex processes. Proteomics is defined as the study of all the proteins expressed in the cell, tissues or organism. Proteomes reveal distinct pattern of protein expression in different set of experimental conditions, even in different tissues of the same organism[2]. Majority of the biological processes affecting plant development and responses are controlled by proteins. Analysis of proteins at different stages of life cycle of the plant and in different tissues is vital to understand those biochemical and physiological processes involved in life cycle transitions. The complex dynamic changes occurring during plant and berry development and in response to different conditions have been studied at transcriptome[3] and proteome level in *Muscadine* grapes, woody perennials, wine grape varieties and Tunisian grapevines[4].

Two-dimensional electrophoresis (2DE) is one of the most efficient methods of separating complex protein mixtures based on their isoelectric point and molecular weights. It also reflects changes in protein expression level and post translational modifications as reported by Gorg *et al*[5]. For all proteomic approaches, sample preparation and protein extraction are of prime importance for optimal results. Being a recalcitrant species, obtaining good quality and quantity of protein from grape tissues is problematic due to low protein abundance, high activity of proteases and high levels of interfering substances, such as, pigments, polyphenols, tannins, flavonoids in different vine parts. Several protocols for protein extraction from grapevine leaf, *viz.*, tricholoro acetic acid (TCA)

[Ψ]SJ and AU contributed equally
*Author for correspondence:
Tel: +91-20-26956055; Fax: +91-20-26956000
satilata@gmail.com
Present address: Principal Scientist, Division of Fruit Crops, ICAR-Indian Institute of Horticultural Research, Hesaraghatta Lake Post, Bengaluru 560 089, India

precipitation, phenolic extraction with ammonium acetate precipitation or acetone precipitation *etc*, have been reported[6]. However, none of the methods yielded good quality proteins uniformly from other tissues like buds, roots, clusters and mature berries. Each protocol had its own advantages and disadvantages as reported by several workers. The main disadvantage of TCA precipitation is that the proteins are difficult to dissolve as reported by Nandakumar *et al*[7]. In TCA/acetone precipitation method, the extreme pH and negative charges of TCA and the addition of acetone may result in sudden denaturation of proteins along with precipitation, thus leading to instant arrest in the activity of proteolytic and other modified enzymes as suggested by Damervel *et al*[8]. Though Hurkman and Tanaka[9] reported reduced background staining and streaking and better resolution of protein spots on 2D gel in barley tissues, the same method did not yield well resolved spots in recalcitrant tissues like grapes *etc*. Heniz *et al*[10] reported that ammonium acetate precipitation may result in precipitation of nucleic acids along with proteins, which may interfere during protein separation on 2D gels. Hence, the present study was conducted to optimize and standardize the qualitative and quantitative efficiency of the procedures to extract proteins from different grapevine tissues suitable for 2D electrophoresis and other downstream applications.

Materials and Methods

Plant Material

Different tissues, such as, leaves, buds, roots, clusters and berries, from grapevine cv. Thompson Seedless were collected during the fruiting season of 2011-12. Bud samples were collected after 5-6 d of pruning when the buds started swelling. Young leaves of 3 to 4-d-old were collected from fresh growth. Clusters were collected before flowering and ripe berries were collected at the time of harvest. Roots were sampled from the Thompson Seedless vines grown in pots. All the tissues were sampled and snap frozen in liquid nitrogen and stored at −80°C till further use.

Protein Extraction

In the first phase, five different procedures were used to extract proteins from leaves and a protocol to obtain high quality protein was standardized. The optimized protocol was used to extract proteins from other tissues, such as, buds, roots, clusters and berries.

Method A (TCA Precipitation Method)

This method was adopted as per the procedures described by Basha *et al*[11]. In brief, frozen leaves were ground into fine powder in liquid nitrogen and 1 g of powder was suspended into 5 mL solution of 70% acetone containing 20% TCA and homogenized for 2 min on ice. The homogenate was centrifuged at 20,000 g for 15 min at 4°C and the resulting pellet was washed three times with 3 mL ethanol, followed by washing with 80% acetone. The protein pellet was stored in 80% acetone at −80°C till further use.

Method B (Phenol Extraction Followed by Methanolic Ammonium Acetate Precipitation)

Phenol extraction followed by methanolic ammonium acetate precipitation method was adopted from Hurkman and Tanaka[9]. 1 g of frozen tissue was ground to a fine powder in liquid nitrogen in a pestle and mortar. The powder was added to 2.5 mL of Tris buffered phenol (pH 8.0) and 2.5 mL of extraction buffer (0.1 M Tris-HCl pH 8.0, 10 mM EDTA, 0.4% β-mercaptoethanol, and 0.9 M sucrose) in a 15 mL centrifuge tube and homogenized for 1 min. The mixture was agitated for 30 min at 4°C and centrifuged at 5000 g for 10 min. The phenol phase was transferred to a fresh centrifuge tube. Care was taken not to remove the interphase between the phenol and aqueous layer. For second and third extractions, equal volume of Tris-phenol was added to aqueous phase and the procedure was repeated to collect phenol phase. Phenol phases from all the three extractions were pooled and protein was precipitated by adding 5-10 volumes of 0.1 M ammonium acetate in 100% methanol (pre-chilled to −80°C), vortexed and incubated at −80°C for at least 2 h. The precipitate was collected by centrifugation at 4000 g for 30 min at 4°C. The resultant pellet was washed twice with ice-cold 0.1 M ammonium acetate in methanol and two times with ice-cold 80% acetone containing 10 mM DTT. For each wash, pellet was re-suspended completely by vortexing and sonication, and samples were kept at −20°C for at least 20 min in between washes. The purified protein pellet was stored in 80% acetone at −80°C until further use.

Method C (Commercially Available Kits)

Two commercially available ready-to-use kits were used to extract proteins.

Method C1—In this method, P-PER plant protein extraction kit (PIERCE, Rockford, IL, USA) was used. It contained 20 mL of reagent A with

preservative-free HEPES-based buffer, 225 µL of reagent B with protein stabilizer; 20 mL of reagent C with an organic extraction solution and 20 polypropylene mesh bags.

Method C2—In this method, plant total protein extraction kit (Sigma, MO, USA) was used. It contained 23 mL of protein extraction reagent Type 4, protease inhibitor cocktail for plant cell and tissue extracts packed in amber vials.

The protocols for protein extraction with the kits were as per the manufacturer's instructions.

Method D (TCA-Acetone Precipitation with Phenol Extraction)

The TCA-acetone precipitation with phenol extraction protocol was used as described by Wang *et al*[12]. The sample was ground to a fine powder in liquid nitrogen. About 0.2 g of powder was transferred into 2 mL centrifuge tube containing chilled 10% TCA prepared in acetone, vortexed and incubated at 4°C for 15 min. The tubes were centrifuged at 13,000 rpm for 15 min at 4°C and supernatant was discarded. The pellet was washed with 1 mL of chilled 80% methanol containing 0.1 M ammonium acetate, vortexed and centrifuged for 15 min at 4°C and supernatant was discarded. The pellet was air dried at room temperature to remove residual acetone. The air dried pellet was suspended in phenol-SDS buffer pH 8.0 (0.1 M Tris-HCl, 30% sucrose, 1% SDS and 5% β-mercaptoethanol) and incubated at room temperature (25°C) for 1 h. The tubes were centrifuged at 16,000 g for 15 min at 4°C. The upper phenol phase was transferred to a fresh microcentrifuge tube and mixed with 2 mL of chilled methanol containing 0.1 M ammonium acetate and incubated at –20°C overnight. The tubes were centrifuged at 16,000 g for 15 min at 4°C and supernatant was discarded. The resultant white pellet was washed; first with 100% methanol and then with 80% acetone. The pellet was stored in 80% acetone at –80°C until further use.

Method E (Modified TCA-Acetone Precipitation with Phenol Extraction)

The TCA-acetone precipitation with phenol extraction protocol of Wang *et al*[12] was modified to obtain consistently good quality and quantity of proteins from varied tissue types. Precipitation buffer was modified by including 20 mM DTT, while phenol-SDS buffer was modified by including 1 mM EDTA and 1% PVPP. Each washing step was repeated 3 times instead of once. The modified protocol was as follows. The sample was ground to a fine powder in liquid nitrogen. About 0.2 g of powder was transferred into 2 mL centrifuge tube and 1 mL of chilled 10% TCA prepared in acetone with 20 mM DTT was added to it, vortexed and incubated at 4°C for 15 min. The tubes were centrifuged at 16,000 g for 15 min at 4°C and supernatant was discarded. The pellet was washed with 1 mL of chilled 80% methanol containing 0.1 M ammonium acetate, vortexed and incubated for 15 min at 4°C and supernatant was discarded. The washing procedure was repeated with cold acetone as explained above. The pellet was air dried at room temperature to remove residual acetone. The air dried pellet was suspended in phenol-SDS buffer pH 8.0 (0.1 M Tris-HCl, 30% sucrose, 1% SDS and 5% β-mercaptoethanol, 1 mM EDTA, 1% PVPP) and incubated at room temperature (25°C) for 1 h. The tubes were centrifuged at 16,000 g for 15 min at 4°C. The upper phenol phase was transferred to a fresh microcentrifuge tube and mixed with 2 mL of chilled methanol containing 0.1 M ammonium acetate and incubated at –20°C overnight. The tubes were then centrifuged at 16,000 g for 15 min at 4°C and supernatant was discarded. The resultant white pellet was washed, first with 100% methanol and then with 80% acetone, each three times. The pellet was stored in 80% acetone at –80°C until further use.

Protein Quantification

The protein pellets stored in 80% acetone at –80°C were centrifuged and air dried. The protein pellets were then dissolved in rehydration buffer (250 µL Destreak reagent supplied by GE Healthcare). And protein concentration was determined by Bradford method[13] using bovine serum albumin as standard.

SDS-PAGE and Gel Staining

Solubilized protein 50-75 µg per sample was used for the separation of proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gel as per the method described by Laemmli[14]. Electrophoresis was carried at 30 mA for 6 h using SE600Ruby™ vertical slab gel electrophoresis unit (Amersham Biosciences). For staining, gel was fixed for 1 h with 30% (v/v) ethanol in 10% (v/v) acetic acid and was stained with 0.025% (w/v) Coomassie G250

in 10% (v/v) acetic acid for 45 min, followed by destaining with 10% (v/v) acetic acid. Stained gels were stored in 10% glycerol (v/v).

Protein Separation by IPG Strips in First Dimensional Electrophoresis Followed by Second Dimensional SDS-PAGE

Protein separation in the first dimension, *i.e.*, iso electric focusing (IEF), was achieved on 13 cm, 3-10 pH gradient immobiline dry-strip (GE Healthcare Life Sciences). The IPG (immobiline pH gradient) strips were rehydrated overnight at 20°C with 250 µL of DeStreak rehydration buffer (GE Healthcare Life Sciences, Product Code. 71-5028-42AD) in 2% IPG buffer (pH 3-10) (GE Healthcare Life Sciences, Product code 17-6004-40) containing 300 µg proteins. IEF was performed on Multiphor II (GE Healthcare Life Sciences) at 300 V (1 min), from 300 to 3,500 V (1 h & 30 min), 3500 V (4 h & 30 min) at 20°C.

After IEF, the strips were equilibrated, first with 0.1% DTT in 10 mL of equilibration buffer (6 M urea, 30% glycerol, 2% SDS, and 50 mM Tris-HCL, pH-8.8) for 15 min, followed by 2.5% iodoacetamide in 10 mL of equilibration buffer for 15 min. The second dimensional electrophoresis (SDS-PAGE) was performed on 10% acrylamide separation gel using SE600 Ruby™ vertical gel electrophoresis unit (Amersham Biosciences). Electrophoresis was carried out at a constant current of 15 mA for 30 min, followed by at 30 mA for 8 h at 20°C.

Gel Staining, Imaging and Data Analysis

The gels were visualized by Coomassie G250 staining procedure as described above. The stained gels were scanned at a resolution of 300 dpi using an Image Scanner (GE Healthcare Life Sciences), and image analysis was performed with Image Master™ 2D Platinum Software Version 7.0 (GE Healthcare Life Sciences) as per the instructions given in user's manual.

Results

Successful 2DE separation of proteins is a crucial step in understanding changes in proteome and their further characterization. But, for an accurate analysis of proteins on 2DE, efficient protein extraction and purification are the prerequisites. Generally, no single extraction protocol is found adequate for proteome analysis in all the tissues and the protocol must be optimized for each tissue in a given species, which is time consuming and costly. In the present study, we first optimized the method of protein extraction from grape leaves and then assessed the suitability of the optimized method for protein extraction from other grape tissues.

Protein Yield

The protein yield (0.79 to 2.07 mg/g) from grape leaves was comparable in all the methods used except for method C2 (10.80 mg/g) (Table 1). Although some differences were observed in protein yield, the differences were statistically non-significant. Method C2 yielded significantly higher quantity of protein.

Protein yield from different tissues, such as, buds, clusters, berries and roots were compared using TCA-acetone precipitation with phenol extraction (method D) and modified TCA -acetone precipitation with phenol extraction method (method E). For both the methods, the difference in protein yields from grape leaves and berries was statistically insignificant, while values from buds, berries and clusters differed significantly (Table 2). The significantly higher protein yield of 4.83 mg/g in buds, 1.75 mg/g in cluster and 1.02 mg/g in roots was obtained from method E.

Table 1—Protein yield from leaf of grapevine using different extraction protocols (n=4)

Extraction method	Protein yield* (mg/g FW)
Method A	1.16 ± 0.66^{A}
Method B	0.79 ± 0.29^{A}
Method C1	2.07 ± 0.52^{A}
Method C2	10.80 ± 2.43^{B}
Method D	1.59 ± 0.50^{A}
Method E	1.98 ± 0.13^{A}

*Values with same alphabet are not significantly different

Table 2—Comparison of protein yield from different grapevine tissue using phenol extraction and modified methods

Extraction method	Protein yield (mg/g FW)				
	Leaf	Bud	Cluster	Berries	Roots
TCA-acetone/phenol extraction method (Method D)	1.59	1.13	0.32	1.10	0.42
Modified TCA-Acetone/ Phenol extraction method (Method E)	1.98	4.83	1.75	1.14	1.02
Significance level	*	NS	*	NS	*

*Differences significant at $p<0.01$

Protein Quality

SDS-PAGE

Though all the protocols except method C2 resulted in comparable protein yield, the protein quality as determined on SDS-PAGE was not comparable (Fig. 1). Distinct banding pattern was indicative of good quality proteins. TCA protocol (method A) is technically simpler but on SDS-PAGE only smears were detected on gel without any distinct bands (Fig. 1, lane 2). Similarly, although method B (Fig. 1, lane 3) and the kits (method C) yielded fairly good quantity of proteins (Fig. 1, lanes 4 & 5), no distinct bands were obtained on SDS-PAGE. The protein extracted by method D, *i.e.*, acetone precipitation with phenol extraction, was comparable to that of method E in terms of yield. Distinct protein bands were obtained for both the methods D and E, but background smearing was observed for method D (Fig. 1, lane 6). Method E yielded good quality protein from leaf, bud and cluster with distinct bands on SDS gel (Fig. 1, lanes 7-9).

2DE

Due to poor quality of protein from the method A, we could not perform 2DE for the proteins extracted from this method. 2DE of protein samples from method B resulted in streaks and no spots were observed (Fig. 2). Any attempts to resolve protein spots from the proteins extracted from kit methods failed due to overheating of IPG strips during IEF. Though method D yielded fairly good quality and quantity proteins, it resulted in few horizontal streaking on 2DE gel (Fig. 3). 2DE of proteins extracted from method E resulted in large number of distinct spots (Figs 4-6) without any smearing and streaking.

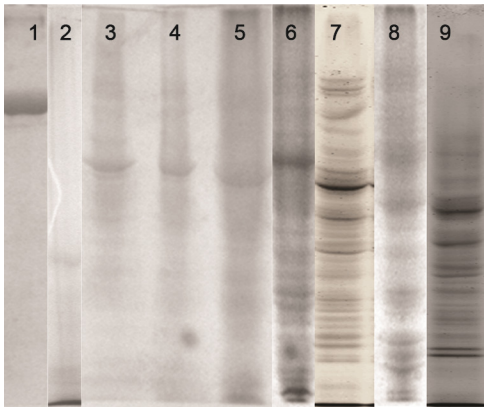

Fig. 1—SDS-PAGE of protein extracted using different methods. [Lane 1, BSA marker; Lane 2, Method A (leaves); Lane 3, Method B (leaves); Lane 4, Method C1 (leaves); Lane 5, Method C2 (leaves); Lane 6, Method D (leaves); Lanes 7-9, Method E (leaf, bud and berry, respectively).]

2DE of Protein from Different Tissues

On 2DE among different grape tissue types, maximum number of protein spots was recorded in leaves (581), followed by those in clusters (520) and least spots were recorded in buds (320) (Fig. 7). In leaves, the abundance of protein spots was in the mol wt range of 30-50 kDa distributed in the pH range of 4-8. Very few spots were detected at pH <4 and >9. In case of cluster, protein spots were abundant in the pH range of 5 to 8 and very few spots

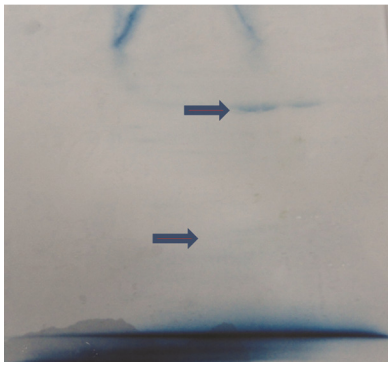

Fig. 2—2DE gel showing no spots except streaking in grape leaf proteins extracted by method B.

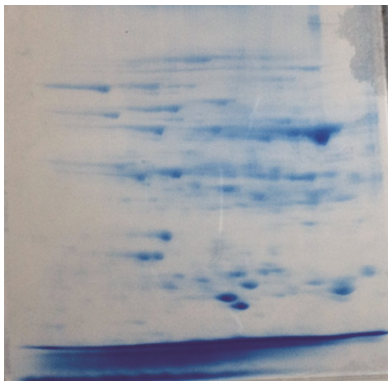

Fig. 3—2DE gel showing protein spots from grape leaves (Method D).

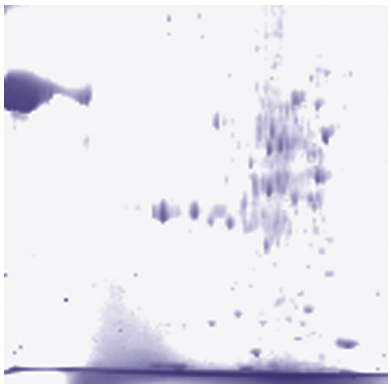

Fig. 4—2DE gel showing protein spots from grape buds (Method E).

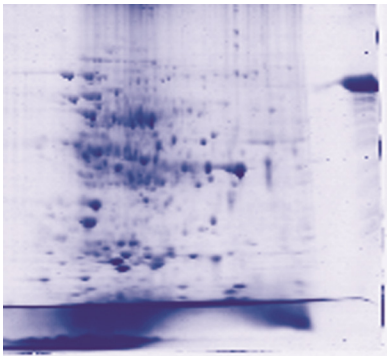

Fig. 5—2DE gel showing protein spots from grape leaves (Method E).

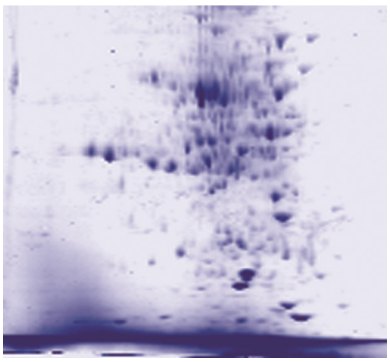

Fig. 6—2DE gel showing protein spots from grape clusters (Method E).

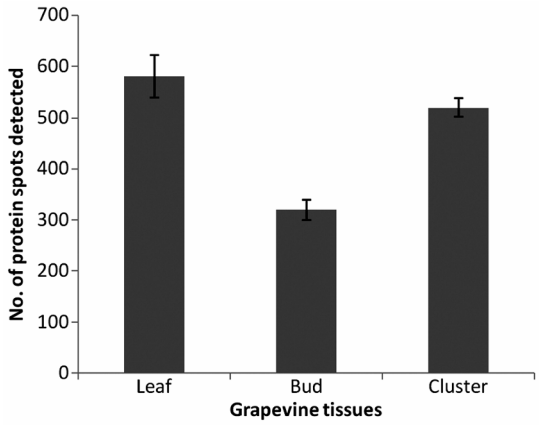

Fig. 7—No. of protein spots in different grape vine tissues detected on 2 D gel from the proteins extracted using method E. [Vertical bars represents ±SD, n=3]

were seen at lower pH. The mol wt of protein spots from cluster was mainly in the range of 4.5-100 kDa and a few spots were observed in high mol wt range. The protein spots detected in buds were in higher pH range of 6 to 9 and mol wt range of 35-90 kDa.

Discussion

Sample preparation is one of the most crucial yet problematic steps for high-quality resolution of proteins in 2DE. We used five different protein extraction protocols to identify the extraction method that could yield high quantity and quality of proteins from different grape tissues. Of the five protocols, TCA/acetone precipitation combined with phenol extraction method with modifications resulted in best resolution of protein on 1D and 2D gel.

An optimal extraction protocol should yield good quality proteins suitable for obtaining well resolved protein spots on 2-DE gels with reproducibility. Method A (TCA/acetone method) though yielded comparable quantity of proteins, no distinct protein spots were observed on 2DE with horizontal streaking. These results were in contrast with results of Gomez-Vidal *et al*[15] who reported this method to be suitable for rice and date palm proteome analysis, respectively. Damervel *et al*[8] also reported that TCA-acetone precipitation method improved the protein yield and helped in removing contaminants, although some polymeric contaminants are often co-precipiated. Chaturvedi and Kumar[16] reported that lipids and polysaccharides, which bound tightly to proteins, were not easily removed by TCA/acetone method. Horizontal streaking and smearing at basic end of the strip during 2DE is caused by factors like the presence of nucleic acids, sugars, lipids in the crude protein extract as explained by Gorg *et al*[17]. Grapevine tissues are rich in polyphenols and polysaccharides and our results suggest that these interfering compounds probably not been removed by TCA-acetone precipitation method, resulting in charges heterogeneity and streaking on SDS-PAGE[18].

Method B, phenol extraction followed by methanol ammonium acetate precipitation described, by Hurkman and Tanaka[9] is reported to be inefficient in removing nucleic acids, which interfere during protein extraction and result in poor resolution and high background in 2DE. The present study confirmed this observation as horizontal streaking with high background was observed on 2D gels of grape protein extracted using Method B. The incomplete re-solubilisation of the precipitated proteins, which we encountered frequently may also be the reason for absence of distinct protein spots.

The TCA-acetone precipitation with phenol extraction method is mainly reported for recalcitrant

plant tissues or organs, such as, apple and banana leaves, wood, potato and rapeseed seedlings, tomato, avocado and banana fruits, and olive leaves[18,19]. In our study also, this method resulted in relatively good quality protein from grape leaf, a recalcitrant tissue. These results are in accordance with reports of Carpentiere *et al*[18] who compared TCA/acetone and phenol extraction methods and observed that though both the methods minimize protein degradation, often encountered during sample preparation owing to endogenous proteolytic activity, the phenol extraction procedure had a high clean up capacity.

Modification of phenol extraction protocols has been reported by several workers. Chaterjee *et al*[20] complemented the phenol extraction method with sodium dodecyl sulphate (SDS) and pulsatory treatments, which was proved to be the most suitable method represented by greatest spot number, good resolution and spot intensities in chick pea roots. In our studies, modification of phenol extraction method considerably improved protein yield from recalcitrant tissues like buds and clusters and subsequent resolution on 2DE. Grape buds and clusters are known to contain higher concentration of phenolic compounds, such as, gallic acids, cinnamic acids, flavonoids and tannins, besides high levels of sugars. Binding of polyphenols to proteins causes precipitation and prolonged focusing time leading to inefficient resolution and streaks in gels[21]. Phenolic compounds bind to the proteins through a variety of mechanisms in aqueous media, including hydrogen bonding[22], covalent bonding[23], hydrophobic interactions[24], and ionic bonding[25]. The separation of proteins from phenolic compounds is complicated because of these complex-forming mechanisms. Besides, phenolic compounds form irreversible complexes with proteins by oxidation and covalent condensation, which leads to charge heterogeneity resulting in streaking of gels. PVPP is a strong H-acceptor making it effective in adsorbing polyphenols[26]. Xu and Diosady[27] reported that PVPP treatment reduces the phenolic acid content in the acidic soluble protein extracts by more than 50% without affecting final protein concentration. EDTA, a chelating agent, minimizes the activities of proteolytic enzymes and thus improves protein yield. Addition of 1% PVPP and EDTA in extraction buffer of the proposed method might be the reason for improved protein yield and quality. Washing steps also play a major role in improving the spot resolution on 2D gel. In our method, the pellets were washed three times each with 0.1 M ammonium acetate in methanol, in cold methanol and in cold acetone. We observed that increased washing steps also improved solubility of the final pellet in rehydration buffer, besides improved spot resolution. These results are in accordance with earlier reports where more number of washes could reduce horizontal streaking on 2D gels[28]. In addition to all these factors, use of rehydration buffer and IPG buffer, while loading samples, also helps in two ways. IPG buffer offers improved resolution, sensitivity and increased spot intensity as well as additional protein spots in high mol wt and basic pH areas, while rehydration buffer during IEF is known to transform the protein thiol groups into stable disulfides and protects the disulfide groups from unspecific oxidation, thus reducing streak between spots in pH range of 7-9.

To summarize, the modified protocol of TCA-acetone precipitation with phenol extraction not only improved protein yield but also resulted in well-resolved and reproducible spot pattern on 2-DE gels in proteins extracted from different grapevine tissues. Our sample preparation procedure helped in minimizing protein losses and facilitated reproducibility. The modified method presented in this study proved to be efficient, simple, economical and appropriate for proteomics studies with different grape tissues.

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