

A common Protein Extraction Protocol for Proteomic Analysis: Horse Gram a Case Study

Jyoti Bhardwaj and Sudesh Kumar Yadav

Biotechnology Division, CSIR-IHBT, Palampur-176061 (HP), India

Received 2012-01-25, Revised 2013-10-25; Accepted 2013-10-28

ABSTRACT

Three protocols viz. Trichloroacetic Acid (TCA)-acetone, phenol and multi-detergent were compared for the extraction of proteins from horsegram (*Macrotyloma uniflorum*) which is less studied yet nutritionally and economically very important pulse crop. Almost equal quantity of proteins was extracted by all the three protocols. However, quality of protein from phenol method was superior to that from other protocols. Bands in One-Dimensional (1-D) and Two-Dimensional Electrophoresis (2-DE) of phenol protocol were reproducible and better. We report here a modified and common protocol for the extraction of proteins for proteomic analysis. The developed protocol is applicable not only to other economically important leguminous but also non-leguminous crops.

Keywords: Trichloroacetic Acid (TCA), Pinch of Bromophenol Blue (PBB), Two-Dimensional Electrophoresis (2-DE), One-Dimensional (1-D), Sodium Dodecyl Sulfate (SDS)

1. INTRODUCTION

Legumes form the third largest plant family (Dam *et al.*, 2009). These are also among the largest and most diverse families of flowering plants (Brandon *et al.*, 2007). Grain legumes contribute 33% of daily protein intake of humans (Graham and Vance, 2003). Model legume studies are important for establishing overall legume development (Dam *et al.*, 2009). The most common legume models are peanut, *Lotus*, *Medicago*, soybean, scarletrunner bean, common bean, pea and broadbean (Goldberg *et al.*, 1989; Johnson *et al.*, 1994; Coste *et al.*, 2001; Weterings *et al.*, 2001; Weber *et al.*, 2005). But we chose horsegram (*Macrotyloma uniflorum*) as the study material. Being a pulse legume it is rich in proteins. It is an important yet underexploited legume and is also known as the poor man's pulse. It has been identified as potential food source for the future (Prakash *et al.*, 2008; Kadam and Salunkhe, 1985).

Proteomics is the branch of science dealing with the isolation, identification and characterization of

proteins. On the other hand similar field of genomics is on a high tide. Among legumes, in addition to the soybean genome sequence (Jackson *et al.*, 2006) other legume genome sequences are going to be available soon (Broughton *et al.*, 2003; Gepts *et al.*, 2005; Young *et al.*, 2005). Proteomics still requires huge effort. A large number of groups have published protein reference maps for specific *Medicago* tissues (Lei *et al.*, 2005; Mathesius *et al.*, 2001; Watson *et al.*, 2003; Imin *et al.*, 2004; Gallardo *et al.*, 2003). Still there is no fully characterized proteome equivalent to a fully sequenced genome. Though genomic studies are opening new dimensions every day, but they do not always provide a complete story due to limited correlations in transcript and protein levels (Gygi *et al.*, 1999). Latter could be due to post-translational modifications, proteolysis and protein-protein interactions. The mass spectroscopic analysis, database searching and software for structure prediction allow rapid identification of proteins (Cilia *et al.*, 2009). Thus, proteomics has become a critical complement to genomic data.

Corresponding Author: Sudesh Kumar Yadav, Biotechnology Division, CSIR-IHBT, Palampur-176061 (HP), India
Fax: 91-1894-230425

Proteomics is a fast changing field and new protocols are being established frequently. Still there is a dearth of common protocols for extraction of proteins from variable sources. Since horsegram is an unexplored legume, its proteomic analysis requires special attention. Studies on biochemical parameters, antioxidant enzymes and computational study on miRNAs of horsegram has been already conducted in our lab. In this study, we developed a modified and common protocol using horsegram as a case study plant material. Further, the developed protocol was used to extract proteins from other legume and non-legume crops.

2. MATERIALS AND METHODS

2.1. Sample

Seeds were used for extraction of proteins from legumes. The legumes selected besides horsegram (*Macrotyloma uniflorum*) were rajmah (*Phaseolus vulgaris*), mah (*Vigna mungo*), chickpea (*Cicer arietinum*), masoor (*Lens culinaris*) and mungbean (*Vigna radiata*). Among non-legumes rice (*Oryza sativa*), wheat (*Triticum aestivum*), bajra (*Pennisitum glaucum*) and mustard (*Brassica juncea*) were taken for the study.

2.2. Protocols

The protocols are developed following the earlier used protocols (Cilia *et al.*, 2009). The modifications made in the developed protocol are as follows. The first protocol used for extraction of the proteins was using TCA and acetone. The plant tissue (100 mg) was homogenized in 10% TCA containing 2% β -Mercaptoethanol (β -ME) using liquid nitrogen. It was kept overnight for precipitation at -20°C . Next day, the mixture was centrifuged at 5000 g for 30 min at 4°C . The supernatant was discarded and precipitates were washed thrice with ice cold acetone. The precipitates were agitated vigorously in between each wash. The precipitates were then air dried till they appeared to be damp but not dried or cracked. These precipitates were dissolved in 1 ml of the modified lysis buffer (9 M urea, 2 M thiourea, 1% DTT and 4% CHAPS).

The second protocol involved the extraction of proteins using phenol. Plant tissue (100 mg) was homogenized well in the modified phenol extraction buffer (500 mM Tris (pH 7.5), 2% non-idet P 40 (NP-40), 2% β -ME, 0.7 M sucrose, 0.5 M sodium chloride

and protease inhibitor cocktail). To this, equal volume of cold Tris-saturated phenol (pH 7.5) was added. This mixture was shaken vigorously for 30 min at 4°C and then centrifuged at 5000 g for 30 min at 4°C . The upper phenol phase containing the proteins was collected very carefully. Ammonium acetate (0.1 M) was added five times the volume of the phenol phase. Mixed well and kept for precipitation overnight at -20°C . Next day, the mixture was centrifuged at 5000 g for 30 min at 4°C . The supernatant was discarded and the precipitates were washed twice in methanol and thrice in ice-cold acetone as described for TCA-acetone protocol till their dissolution in the same lysis buffer.

For the third protocol, homogenization of 100 mg of tissue was done in the modified multi-detergent extraction buffer (100 mM dibasic potassium phosphate (pH 7.6), 8 M urea, 1% tritonX-100, 20% glycerol, 0.5 M sodium chloride and protease inhibitor cocktail). This was shaken at room temperature for 10-15 min and then centrifuged at 9500 g for 30 min at 4°C . To the supernatant equal volume of 10% TCA containing 2% β -ME was added and kept overnight for precipitation at -20°C . Next day, the mixture was centrifuged at 5000 g for 30 min at 4°C . The supernatant was discarded and the precipitates were washed thrice in ice-cold acetone as described for TCA-acetone protocol till their dissolution in the same lysis buffer.

2.3. Quantification and 1-DE

The proteins were quantified using Bradford reagent (Bradford, 1976) and samples were analyzed in triplicates. Sodium Dodecyl Sulfate (SDS) PAGE (12%) was used for 1-D electrophoresis analysis. The volume for each sample used was $25\ \mu\text{L}^{-1}$. The gels were run at 30 mA of current per gel in the stacking gel and at 35 mA in the separating gel. The gels were stained in CBB and then destained in a solution containing 10% glacial acetic acid and 30% methanol.

2.4. 2-DE

The samples dissolved in lysis buffer were taken such that their concentration reached to $0.1\text{-}2.5\ \mu\text{g}\ \mu\text{L}^{-1}$ for 2-DE. This concentration of the sample was dissolved in rehydration buffer (8 M urea, 2% CHAPS, 20 mM DTT and 0.2% IPG buffer). This buffer was stored in small aliquots as per requirement at -20°C . The last two ingredients (DTT and IPG buffer) were added fresh to the rehydration buffer just

before use. A total of 125 μL of rehydration buffer containing the sample was evenly distributed in the rehydration strip holder. The 7cm IPG strip (pH 3-10 nonlinear, GE Healthcare, Sweden) was placed on it and this assembly was allowed to rehydrate passively overnight. IEF was conducted in Ettan IPGphor II (GE Healthcare, sigma). Current of 50 μA strip as applied. The focusing conditions were: step 1-300 V, step 2-500 V, step 3-1000 V and step 4-5000V. The focused strips were first reduced in equilibration buffer (6 M urea, 50 mM Tris pH (8.8), 30% glycerol, 2% SDS and Pinch of Bromophenol Blue (PBB)) containing 50 mg DTT (added just prior to use) for 15 min on a gel rocker at room temperature. The reduced strips were then alkylated by adding fresh 75 mg Iodoacetamide (IAA) at similar conditions. The reduced and alkylated strips were washed with $1\times$ SDS buffer. These strips were then loaded onto 12% SDS PAGE without any stacking gel. This assembly was sealed using 1% agarose sealing buffer. The gels were run, stained and destained just as for 1-D electrophoresis. The gels were scanned using Imagemaster 2D Platinum (GE Healthcare, Sweden).

3. RESULTS AND DISCUSSION

According to the protein yield results, all the three protocols were almost at par with each other (**Fig. 2**). TCA-acetone and phenol-acetone protocols yielded 15.92 ± 0.02 and 15.35 ± 0.09 mg g^{-1} FW protein while multi-detergent protocol yielded 15.07 ± 0.11 mg g^{-1} FW protein. Our results are in agreement to the earlier reported studies (Cilia *et al.*, 2009; He and Wang, 2008). They have also documented the highest protein yield by TCA-acetone protocol. But based on the quality of 1-D and 2-DE analysis phenol method was chosen for extraction of proteins. Many other groups working with extraction of proteins for 2-DE has chosen phenol method directly or with modifications for their analysis (Wang *et al.*, 2006; Cilia *et al.*, 2009; Dam *et al.*, 2009).

3.1. Phenol-Acetone Protocol was Better Than Other Two Protocols

The pellets following precipitation from the three extraction protocols had unique characteristics. The precipitants from the TCA-acetone protocol were good in amount and easy to handle. These were off white in color, grainy, little viscous but solubilized in lysis buffer moderately giving a turbid solution

initially. Earlier also pellets from TCA-acetone protocol have been found to be light brown in color and grainy in nature (Cilia *et al.*, 2009). Homogenizing the sample in 10% TCA dissolved in acetone was found to almost immediately precipitate proteins. It also allowed interfering substances that dissolved in the acetone to be washed from the precipitated proteins and provided a clean sample for IEF. Although this protocol is effective for some plant tissues, it often results in the co-extraction of polymeric contaminants (Rose *et al.*, 2004). These contaminants precipitate with proteins and cannot be removed by the final washing steps (He and Wang, 2008). The precipitants from the phenol-acetone protocol were whitish in color, flaky, non-viscous, easy to handle and solubilized immediately in the lysis buffer. It has been reported earlier that the protein pellet from phenol extraction is usually white and a yellowish pellet indicates co-precipitation of phenolic compounds. Such pellets have been reported to be easily dissolved in the rehydration buffer. Hence, qualitatively the phenol extraction has been considered to give the cleanest and most soluble pellet (Cilia *et al.*, 2009). Interestingly, another study has reported that non-protein contaminants can be removed using phenol protocol resulting in change in color of the ground powder from brownish to white (He and Wang, 2008). However, the precipitants from the multi-detergent protocol were yellowish in color and very difficult to handle being sticky in nature. These were not easily soluble in lysis buffer. These problems along with lower protein yield makes this protocol unfit according to our studies. It has also been reported earlier that the pellets from multi-detergent protocol are dark-brown in color and viscous in nature (Cilia *et al.*, 2009).

After quantification of the proteins achieved by different methods, SDS-PAGE was run to observe the banding pattern (**Fig. 3**). Samples were run in duplicate to check reproducibility of the three methods. All the methods were reproducible. The TCA-acetone protocol did not give very clear and high intensity bands. The phenol-acetone protocol gave better results. The bands were very sharp and distinct. Similar results have been reported earlier with phenol extraction protocol (Cilia *et al.*, 2009; Wang *et al.*, 2006). Multi-detergent protocol although gave high intensity bands but failed in clarity and sharpness of 1-DE gel-banding pattern. This method has also been reported to be non-reproducible earlier (Cilia *et al.*, 2009).

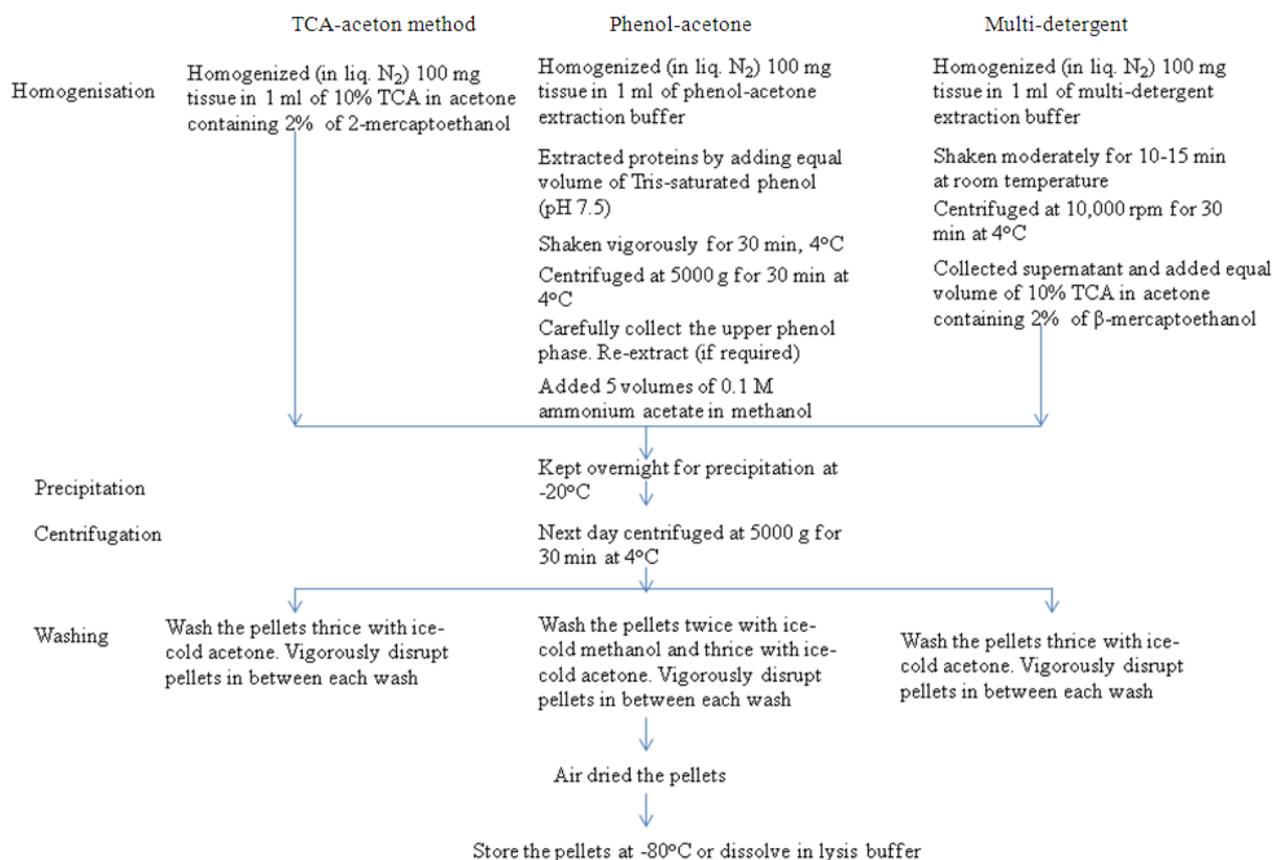


Fig. 1. Flowchart of the three protocols used for the extraction of proteins for 2-DE

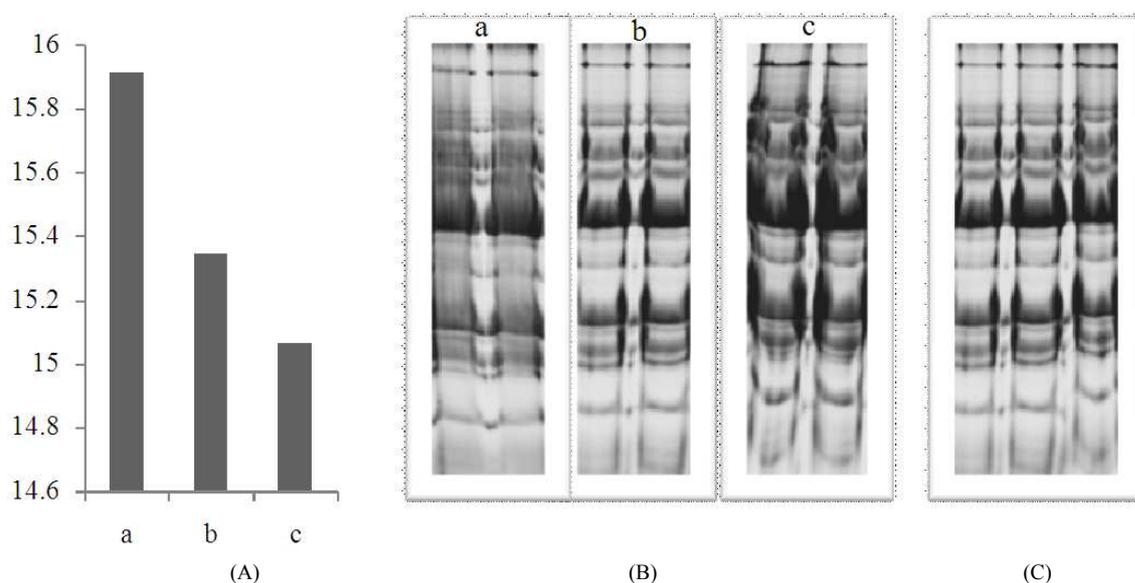


Fig. 2. Comparison of the extracted protein from horsegram seeds by quantification and 1-DE analysis. (A) Protein yield from three different protocols; (a) TCA-acetone (b) phenol-acetone and (c) multi-detergent. (B) SDS-PAGE analysis of the three protocols. (C) Triplicates of the phenol-acetone protocol

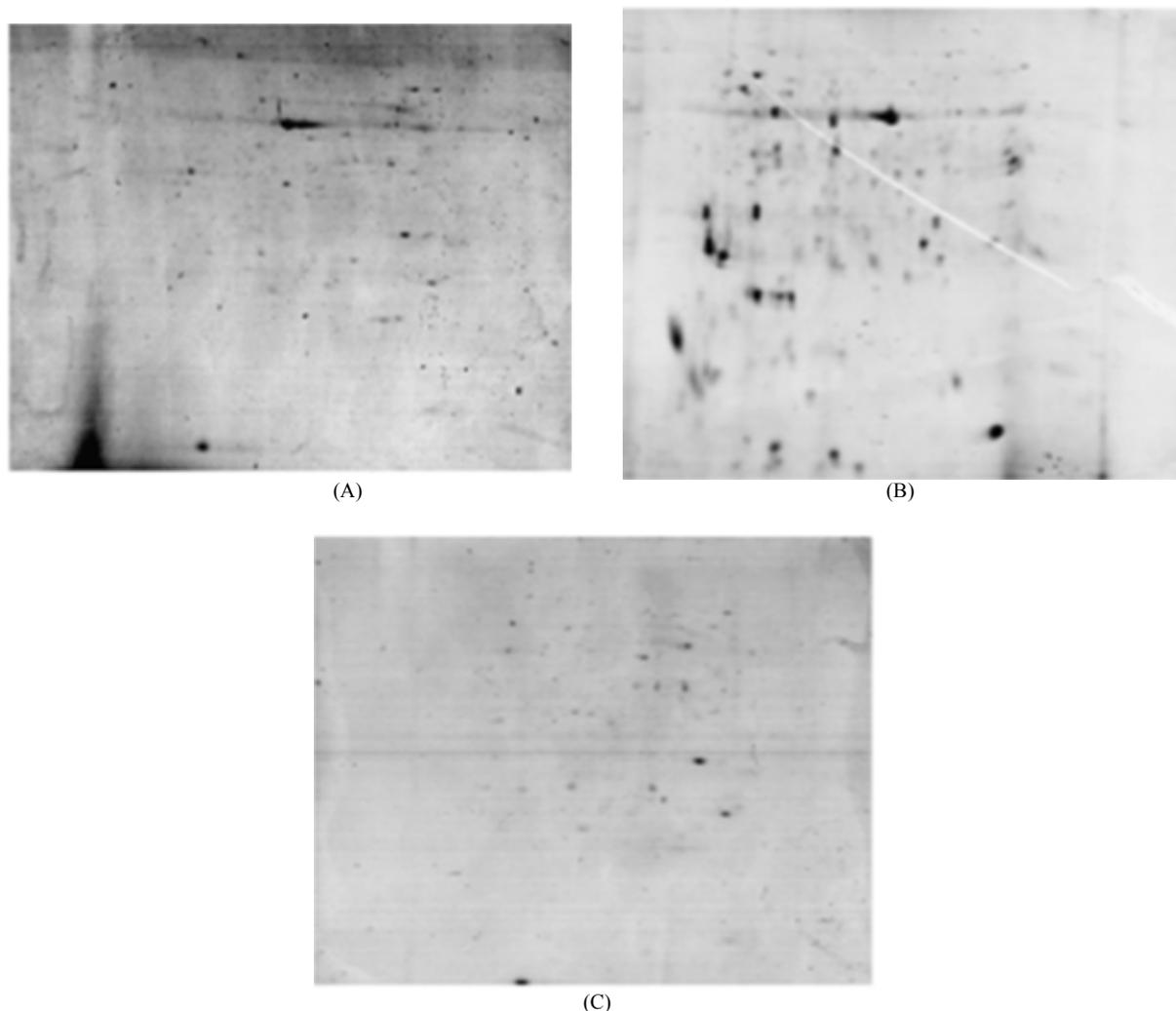


Fig. 3. Two-dimensional electrophoresis analysis of the three protocols compared for extraction of proteins from horsegram seedlings. (A) TCA-acetone (B) Phenol-acetone and (C) Multi-detergent

After the initial screening at the 1-D electrophoresis level, the quality of proteins were checked on 2-DE (**Fig. 3**). We observed that the phenol-acetone method gave good and clear spots on the 2-DE gel. The other two protocols gave lesser number of protein spots being difficult to observe. Most of the spots present in the phenol-acetone protocol were either absent or less visible in the 2-DE pattern of other two protocols. The enhanced performance of the phenol-acetone extraction protocol could be due to the fact that contaminants were eliminated more efficiently with the aqueous phase. It has been reported earlier that phenol protocol resulted in better 2-DE quality than TCA-acetone although latter gave higher protein yield (He and Wang, 2008). Taken together, the quantitative, qualitative, 1-D and 2-DE analysis of the protocols tested, we found the

phenol-acetone protocol better for extraction of protein from horsegram for 2-DE.

3.2. Phenol-Acetone Protocol Can be Applied to Both Legumes and Non-Legumes

Proteins from different legumes and non-legumes were extracted using phenol-acetone protocol and then quantified (**Fig. 4**). Among legumes the highest protein concentration was shared by rajmah and chickpea ($14.25 \pm 0.005 \text{ mg g}^{-1} \text{ FW}$), then followed by lentil ($14.02 \pm 0.005 \text{ mg g}^{-1} \text{ FW}$), mungbean ($13.87 \pm 0.015 \text{ mg g}^{-1} \text{ FW}$) and mah ($13.83 \pm 0.02 \text{ mg g}^{-1} \text{ FW}$). Among non-legumes the order was mustard showing the highest concentration ($14.09 \pm 0.045 \text{ mg g}^{-1} \text{ FW}$) followed by rice ($13.05 \pm 0.01 \text{ mg g}^{-1} \text{ FW}$), bajra ($12.92 \pm 0.005 \text{ mg g}^{-1} \text{ FW}$) and wheat ($12.28 \pm 0.07 \text{ mg g}^{-1} \text{ FW}$).

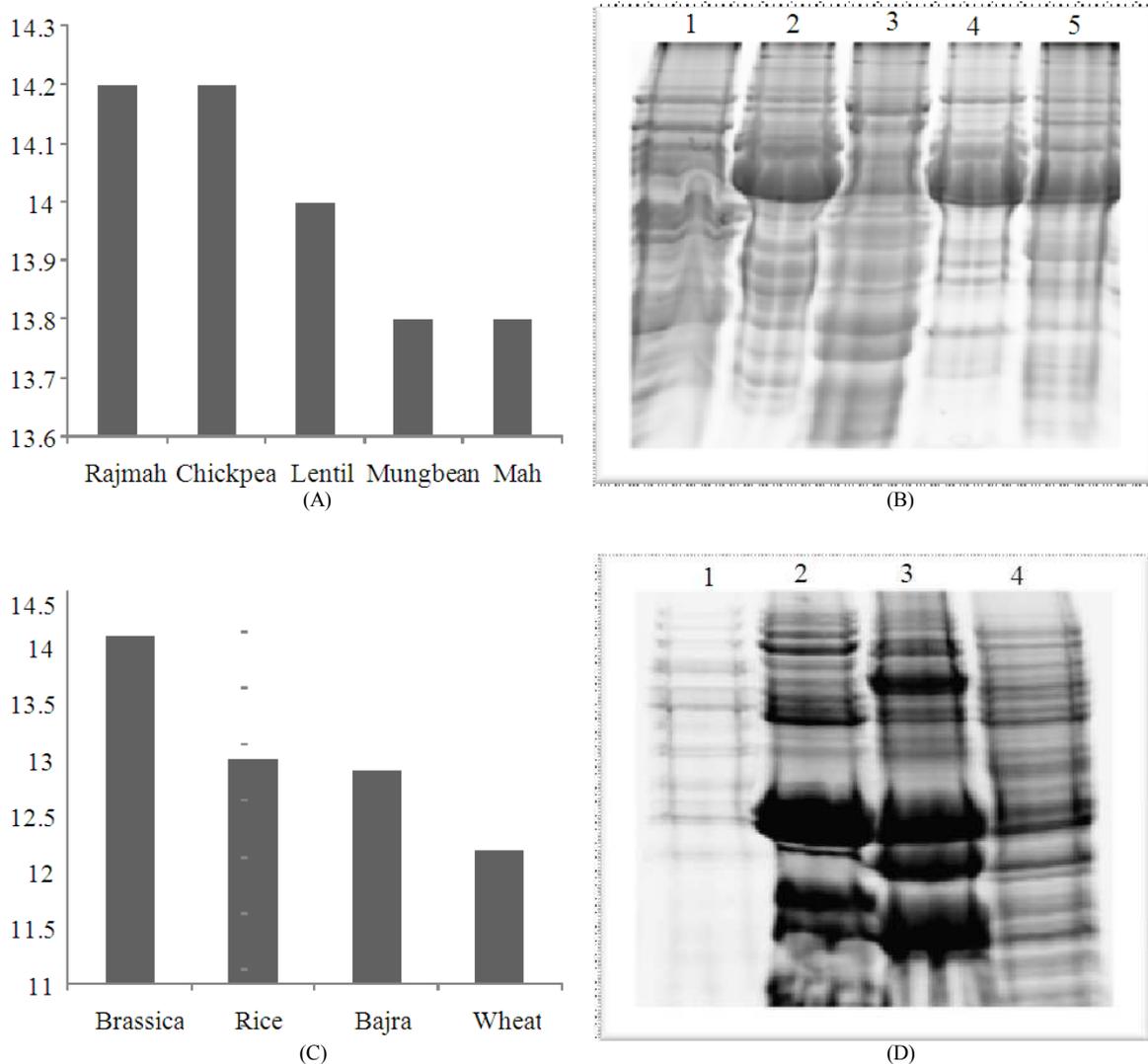


Fig. 4. Extraction of proteins from different legumes and non-legumes using phenol-acetone protocol. Protein yields in legumes (A) and non-legumes (C). SDS-PAGE of proteins from (B) legumes (Lane 1- rajmah, Lane 2- mah, Lane 3- chickpea, Lane 4- mungbean and Lane 5- lentil) and (D) non-legumes (Lane 1-wheat, Lane 2-rice, Lane 3-mustard and Lane 4-bajra)

Good banding patterns were observed in 1-D electrophoresis for different legumes and non-legumes (**Fig. 4**). Among non-legumes, the intensity of bands was less for wheat while rice and mustard showed good bands. Best protein bands among non-legumes were observed for bajra. This protocol extracts good and fairly equal amount of proteins from leguminous as well as non-leguminous samples as is shown by quantification of proteins. Earlier, a protocol based on phenol extraction has been reported to be highly effective for the production of high-quality protein samples from only recalcitrant plant tissues (Wang *et al.*, 2006). Phenol protocol has also been applied for extraction of proteins from different plants. However,

there was no quantification data (Cilia *et al.*, 2009; He and Wang, 2008).

4. CONCLUSION

Sample preparation is one of the most crucial and problematic step when high-quality resolution of proteins is desired in proteomic analysis (Wang *et al.*, 2006). To deal with these challenges, we tested and compared three protein extraction protocols with modifications: TCA-acetone, phenol and multi-detergent (**Fig. 1**). Earlier also these basic protocols have been compared for samples ranging from insects to recalcitrant plants (Cilia *et al.*, 2009; Wang *et al.*,

2006; He and Wang, 2008; Barraclough *et al.*, 2004). However, we have standardized a common protocol for protein extraction from legumes and non-legumes. We tried different combinations of chemicals and detergents to modify the extraction and lysis buffers. Finally, we decided not to make any changes in the first protocol. But the extraction buffers for second and third protocol were modified for better extraction as is evident by protein yield. The reason behind this could be that TCA-acetone protocol is simpler than phenol protocol. The number of steps in the phenol or multi-detergent protocol might have caused some loss of proteins quantitatively. The lysis buffer common to all three protocols was also modified for better solubilization. In phenol protocol the extraction buffer was modified to suit extraction of proteins from plant. Instead of potassium chloride we used sodium chloride as latter is more commonly used for extraction of proteins from plant sources. Polyvinylpyrrolidone (PVPP), PMSF and EDTA were not used. We used NP-40 and protease inhibitor cocktail in the phenol extraction buffer. One washing step with acetone was increased for the phenol protocol. Therefore, we call our method phenol-acetone protocol. In multi-detergent extraction buffer, 8 M urea was used instead of 7 M urea. Amidosulfobetaine-14 and 1% dodecyl maltoside were omitted. We used sodium chloride and tritonX-100. CHAPS and thiourea were omitted from the extraction buffer. In the lysis buffer, 9 M urea was used instead of 7 M and 1% DTT was added to enhance solubility besides the usual components.

Earlier also a combination of TCA/acetone and phenol protocol has been shown to provide enhanced 2-DE based proteomic analysis of most plant tissues (Cilia *et al.*, 2009; Wang *et al.*, 2006). Protein extraction from plant tissues is challenging due to relatively low protein concentration and high levels of polysaccharides, polyphenols, pigments and lipids. These all can contribute to horizontal or vertical streaking, proteolytic degradation and artificial migrations on 2-DE gels (Carpentier *et al.*, 2005). Protein extraction methods to obtain well-resolved 2-DE maps from different samples have been reported previously (Wang *et al.*, 2006; Charmont *et al.*, 2005; Mijnsbrugge *et al.*, 2000). Methanol is often used to extract polyphenolic compounds. However, in combination with ammonium acetate it facilitates the extraction of proteins by phenol at an increased pH (Wang *et al.*, 2006). It is known that exposure to low pH may lead to protein degradation or modifications,

therefore buffers were kept within pH 7-8. Earlier also in a study on *Aloe vera*, a lysis buffer with pH around or above 8 for all three methods has been used resulting in better performance at 2-DE (Wang *et al.*, 2006; He and Wang, 2008). The strips chosen (pH 3-10) have allowed a good resolution of the legume proteins that mostly range in pI from 4-7. Despite the fact that phenol-acetone protocol involves more number of steps, its reproducibility, good protein yield and high intensity bands/spots makes it a better protocol.

4.1. Funding Information

This study was supported by Department of Science and Technology, GOI in the form of project (SR/WOS-A/LS-243/2009) granted to JB under WSS (WOS-A) scheme.

5. ACKNOWLEDGEMENT

Researchers are full of gratitude towards the Director, CSIR-IHBT for his support and facility. Council of Scientific and Industrial Research, GOI is duly acknowledged for financial support in the laboratory.

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