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Research Article

EVALUATION OF PROTEIN EXTRACTION PROTOCOLS AND EFFICIENT SOLUBILISATION FOR ENHANCED PROTEOMIC ANALYSIS FROM PHENOLICS RICH NON-MODEL RECALCITRANT WILD CRUCIFER *RORIPPA INDICA* (L). HIERN

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Abstract: Prior to 2-dimensional gel electrophoresis (2-DGE), proteins must be denatured, reduced and properly solubilised. Depending on the type of samples, the extraction and solubilisation of protein varies. The purpose of this research was to establish an efficient method of proteome extraction and solubilisation followed by analysis of the proteins from a non-model, recalcitrant, phenolics rich plant *Rorippa indica*. We developed this protocol suitable for 2-DGE and MALDI-TOF based MS analysis of the proteins. The widely used TCA-acetone precipitation, Tris-Cl based extraction and phenol based extraction of proteins have been comparatively evaluated. It was found that phenol based extraction was best suited for the purpose. The extraction was further complemented with efficient protein solubilisation. To increase solubility of precipitated proteins, use of thiourea, supplemented with CHAPS and ASB-14 were found to be very effective. A huge number of high quality unique protein spots in 2-D gel were obtained by this method. The MS analysis resulted in the successful identification of proteins further confirming the compatibility of this protocol for use as a guide method for protein extraction from non-model, recalcitrant tissue.

Keywords: *Rorippa indica;* Non-model plant; Phenol extraction; 2-dimentional gel electrophoresis; MALDI-TOF MS.

Introduction

Two-dimensional gel electrophoresis (2-DGE) is one of the most powerful and efficient techniques used in the field of studying complex pattern of protein expression (Celis *et al.*, 1998; Gygi *et al.*, 2000). Protein extraction, in contrast tof DNA/ RNA isolation and analyses, faces numerous hurdles due to its complexity and instability (Chatterjee *et al.*, 2012). These features make the extraction and solubilisation of proteins tough and results in poor yield, and, since there is no technology currently available that can amplify low abundance proteins, the separation and identification of proteins get complicated (Rose *et al.*, 2004).

Most critical steps in any proteomic study are protein extraction and their solubilisation in desired buffer. Especially, in case of recalcitrant plants the process is affected by interfering agents like proteases, storage polysaccharides, lipids, phenolics and a broad array of secondary metabolites and results in lower yield (Gegenheimer *et al.*, 1990; Tsugita *et al.*, 1999; Wang *et al.*, 2008). Presence of such contaminants also lowers the solubility (Pennington *et al.*, 2004) and lowers the reproducibility and quality of resolution in 2-D gels (Saravanan and Rose, 2004).

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Most common and basic protocols used for protein extraction from plant tissue are TCA-acetone, Tris-Cl based direct extraction and phenol based extraction methods. TCA-acetone precipitation was first developed by Damerval *et al.* (1986). The method increases protein yield with less contaminant interference. However, the protocol appears to be problematic with the tissues those are rich in soluble polysaccharides, and polyphenols. The second method known is direct extraction of protein with Tris-Cl buffer and precipitation of proteins with TCA (Shen *et al.*, 2003; Pavokovic *et al.*, 2012). Third, the phenol based method, involves protein solubilisation in phenol followed by precipitation with ammonium acetate in methanol and subsequent re-solubilisation in IEF rehydration buffer (Saravanan and Rose 2004; Hurkman and Tanaka, 1986; Meyer *et al.*, 1988). The mentioned protocols have three common steps, extraction, precipitation and re-solubilisation of proteome. The methods for proteome isolation, containing all these three steps have several importance. Firstly, this should disrupt macromolecular interactions to yield monomeric polypeptide chains, prevent the modification of the proteins, secondly, it should separate proteins from all interfering compounds those hamper the 2-DGE, thirdly, to maintain the proteins with their original pI value and still they are well dissolved in the buffer solution (Rabilloud and Lelong, 2011).

Proteins from several recalcitrant plants have been extracted using all these three methods (Pavokovic *et al.*, 2012;), but it should be kept in mind that, no single extraction method is ideal to extract the full proteome, thus the protocol must be optimized for each different tissue for particular research objective. Most of the established methods for proteomic analysis are focused on model organisms for basic researches but the 'non-model' organisms (Pavokovic etal., 2012), particularly, plants are neglected in this sense, though they are found to be important for food, pharmaceuticals and agricultural field (Lin *et al.*, 1995). For non-model plants, reproducible, authentic protocols to extract good quality proteins compatible with 2-DGE must be developed, in this scenario.

Here, we compare three classical protein extraction methods on a non-model recalcitrant plant *Rorippa indica* (L) Hiern. *R. indica* is important for its tolerance property against the mustard aphid *Lipaphis erysimi* (L). Kaltenbach. We analysed protein yield by Bradford assay after their extraction and re-solubilisation in three different 2-D rehydration buffers and by performing SDS-PAGE prior to 2-DGE. The 2-D gels were analysed using bioinformatics tools to determine best extraction and re-solubilisation protocol for this non-model recalcitrant plant.

Materials and Methods

Plant Materials and Tissue Harvesting

Seeds of *Rorippa indica* were collected from Madhyamgram Experimental Farm, Bose Institute, Madhyamgram, India. The surface sterilized seeds of *R. indica* were grown in sterile inorganic soil, Soilrite (KEL, India). Nutrient solution (Half strength Murashige and Skoog liquid medium without sucrose and organic components) (Murashige and Skoog, 1962) was applied twice a week. The plants were grown at 25°C ± 2°C and 62-75% relative humidity at 16/8 hour light/dark photoperiod for 60 days. Mature leaves of 60 days old plants were harvested, washed, weighed and snap-frozen in liquid nitrogen (-196°C) and stored at -80°C before to proceed into next step.

Protein Extraction Protocols

For all protein extraction methods, frozen tissues were ground in pre-cooled mortar-pestle with liquid nitrogen.

(A) TCA-Acetone precipitation

According to Damerval *et al.* (1986), the TCA-acetone precipitation was carried out with some modifications. Briefly, 1 gram of the ground tissue powder was precipitated overnight with freshly prepared 3 ml of 10%TCA in cold acetone, containing 0.07% β-ME. The precipitated material was obtained by centrifugation of the set at 11,200 g at 4°C for 20 minutes. After discarding the supernatant, the pellet was washed thrice with ice-cold acetone with 0.07% β-ME, with an additional incubation of the set at -20°C for an hour (Carpentier*et al.*, 2005).

(B) Direct protein extraction with Tris-Cl buffer followed by TCA precipitation

The direct extraction of protein was carried out following the protocol of Shen *et al.* (2003) with minor modifications. Briefly, 1 gram of fine tissue powder was homogenised in 3 ml of ice cold Tris-Cl extraction buffer (20 mM Tris-Cl pH 8.8, 10 mM EDTA pH 8.0, 250 mM Sucrose, 1 mM DTT, and 1 mM PMSF). This was left in room temperature to thaw. The homogenate was collected and it was centrifuged at 11,200 g for 10 minute at 4°C. The supernatant was collected and the proteins were precipitated by 10% TCA in cold acetone for overnight at -20°C. The precipitated material was obtained by centrifugation as same as stated in case of TCA-Acetone precipitation and pellet was rinsed twice with ice-cold 100% acetone.

(C) Phenol extraction

The phenol extraction procedure was performed according to Wu *et al.* (2014), with some modifications. One gram of frozen plant tissue powder was homogenised in a mortar with 3.5 ml of extraction buffer (500 mM Tris-Cl pH 8.0, 50 mM EDTA pH 8.0, 150 mM NaCl, 100 mM KCl, 2% β-ME and 2 mM PMSF). This was left in room temperature to thaw. The homogenate was collected and it was centrifuged at 25,200 g for 10 minute at 4°C. The supernatant was collected and to it equal volume of Tris-saturated phenol was added. The total mixture was thoroughly mixed by vortexing at room temperature for 3 minutes and further centrifuged as done in previous step. The lower phenolic phase containing proteins was collected. This part was washed by vortexing (2 minute at room temperature) with equal volume of wash buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA pH 8.0, and 700 mM Sucrose) and centrifuged at 11,200 g. The upper layer was collected and subjected to ammonium acetate precipitation. In ammonium acetate precipitation method, five volume of 100mM ammonium acetate in methanol was mixed with one volume of protein sample and was kept at -20°C for overnight. The protein was pelleted by centrifugation at 25,200 g for 10 min at 4°C. The pellet was rinsed twice with ice-cold methanol and finally with 100% acetone.

All dried protein samples were made aliquots and were stored at -80°C for future use.

Protein re-solubilisation, Protein Estimation and SDS-PAGE

It is necessary for 2-DGE (2-Dimensional Gel Electrophoresis), that the protein sample must be well dissolved in the special buffer with an enriched protein amount (in the range of 300 to 500 µg/ml) (according to manufacturer's recommendation, Bio-Rad, for Coomassie Brilliant Blue staining) for the downstream process. There are many protocols available for protein re-solubilisation for 2-DGE (Chinnasamy and Rampitsch, 2006; Nandakumar *et al.*, 2003; Mechin *et al.*, 2003; Zhang *et al.*, 2011). Generally the standard re-solubilisation buffer consists of 8 M Urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10® ampholyte, 0.001% Bromophenol Blue (ReadyPrep 2-D starter kit rehydration/sample buffer, catalog # 163-2106, Bio-Rad). A modified form of this buffer (7 M Urea, 2 M Thiourea, 1% (w/v) ASB-14, 40 mM Tris, 0.001% Bromophenol Blue) (Readyprep 2-D rehydration/sample buffer 1, catalog # 163-2083, Bio-Rad) was also used to dissolve the protein sample, in this experiment after adding 2% CHAPS, 50 mM DTT and 0.2% Bio-Lyte 3/10® ampholyte. The third modification of the re-solubilisation buffer used in this experiment was adding additional 2 M Thiourea to the Readyprep 2-D rehydration/sample buffer 1, catalog # 163-2083, Bio-Rad.

The dried protein samples were dissolved into 2-D rehydration buffer solutions (in the standard 2-D rehydration buffer as well as in the modified buffer to test better protein solubility) and were estimated using Bradford method (1976) against BSA as standard. The protein quantity obtained using these three extraction methods, was plotted in graph to compare their relative amount and the SDS-PAGE was run to check their protein profile, following the protocol of Laemmli, 1970.

2-Dimensional Gel Electrophoresisand Image Analysis of 2-D PAGE Gels

IPG strips (17cm, pH 3-10 nonlinear, Readystrip, BioRad) were passively rehydrated overnight with 2-D rehydration buffers containing 300µg of isolated protein mixture. Isoelecrtic focusing (IEF)

was done on PROTEAN IEF cell, BioRad, at field strength of maximum 600V/cm and 50 µA/IPG strip. The strips were focused at 250 V for 20 minutes, 10,000 V for 2 hours 30 minutes with linear voltage amplification and finally to 40,000 Volt hour with rapid voltage amplification.

Following IEF, strips were reduced for 10 minutes with 2% (w/v) DTT, in 2 ml of equilibration buffer (6 M urea, 0.375 M Tris-HCl, 2% (w/v) SDS and 20% Glycerol, pH 8.8) and alkylated with 2.5% (w/v) iodoacetamide, in 2 ml of equilibration buffer for 10 minutes. The 2-DGE was performed using 12% polyacrylamide gels (16 cm X 20 cm X 1 mm) in a PROTEAN II XL cell (BioRad, USA) at constant 250 V for 4 hours 30 minutes in Tris-Glycine-SDS running buffer against PAGERuler Unstained Protein ladder, Thermo Fisher Scientific (catalog # 26614). The gels were stained with 0.05% Coomassie Brilliant blue R-250 (Sigma), for an hour, destained and stored in 5% acetic acid solution at 4°C for downstream analyses.

The Coomassie stained 2-D gels were digitalized using GelDoc™ XR+ Imaging System (Bio-Rad) and analysed with PDQuest Advanced™ 2-D Analysis software (version 8.0.1, Bio-Rad). Spots were detected automatically by the Spot Detection Parameter Wizard using the Gaussian model. Only spots present in all three replicate gels were chosen randomly and were considered for subsequent analysis. Selected protein spots were subjected to in-gel digestion for identification by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)

MALDI-TOF MS Analysis and Database Search

Spots were excised manually from protein gels, and subjected to in-gel digestion as described by Shevchenko *et al.*, 2007, with modifications. Peptides were in-gel digested overnight at 37 °C in digestion buffer [10 ng/µl trypsin gold (Promega) in 100 mM in Ammonium bicarbonate: 100% Acetonitrile (1:1)] followed by stopping the reaction by cold shock. The digested peptides were extracted from gel pieces by ultra-sonication in a bath sonicator for 15 minutes at room temperature. The liquid phase was collected and the total content was dried in rotary vacuum evaporator for 4 hours and stored at -20 °C for future use.

To perform MALDI-TOF MS, the dried peptide mix was dissolved in 1% trifluoroacetic acid (TFA, Merck, Uvasol grade). 1µl of this sample was mixed with equal volume of matrix (á-cyano-4-hydroxy cinnamic acid, HCCA, saturated in acetonitrile: 0.1% TFA, 1:2) (Bruker, Daltonics) and was loaded on an Anchor Chip MALDI Plate (Bruker, Daltonics).

Mass spectra were obtained on an Autoflex II MALDI TOF/TOF (Bruker, Daltonics, Germany) mass spectrometer equipped with a pulsed nitrogen laser (λ_{337nm}, 50 Hz). The spectra were analysed with Flex Analysis Software (version 2.4, Bruker, Daltonics). The processed spectra were then searched using MS Biotools (version 3.0) program, against the taxonomy Viridiplantae (green plants), in the NCBInr 20140323 (38032689 sequences; 13525028931 residues) and Swissprot database using MASCOT search engine (version 2.2, Matrix Science, Boston, MA, USA) for protein identification. The standard parameters used in the search included peptide mass tolerance (±0.5 Da); fragment mass tolerance (±0.8 Da); proteolytic enzyme (trypsin); global modification (carbamidomethyl, Cys); variable modification (Oxidation, Met); peptide charge state (+1) and maximum missed cleavage of 1. The significance threshold was set to a minimum of 79% (and minimum 54%, for Swissprot)($p \leq 0.05$). The criteria used to accept protein identification were based on MOWSE score, the percentage of sequence coverage and minimum ten matched peptides.

Experiments were performed three times for all methods. Data were analysed by one way analysis of variance. Means were separated by Duncan's Multiple range test ($\alpha = 0.05$), using the IBM SPSS statistics 19 software. Graphs were prepared in Graph-Pad Prism 6.02.

Results and Discussion

Protein Quantification

In this study, we compared three different protein extraction methods followed by the solubilisation of precipitated protein in different modifications of standard 2-D rehydration buffer to determine

which method gives more clearly resolved proteome profile in 2-D gel. We determined the yields of proteins after using equal amount (1 gram) of leave as starting material for the extraction procedure (Figure 1).

Protein yield using the TCA-Acetone precipitation process was quite good when the extracted proteins were dissolved in the standard rehydration buffer (502±15.01 µg/gram), while after adding the additional chaotrope thiourea (2 M), the solubility decreased very much (51±2.04 µg/gram). By increasing the amount of thiourea (4 M) yield of proteins was increased (122±5.19 µg/gram).

The classical direct protein extraction method was found to be least successful. The average protein yield in this method was in the range of 100-220 µg/gram of fresh tissue. The presence of extra chaotropes had no significant effect on protein solubility enhancement.

In case of phenol based method followed by protein solubilisation in standard rehydration buffer as well as in modified rehydration buffer, a quite good amount of protein yield was obtained. Here, it was observed that the increasing amount of thiourea along with zwitterionic detergent ASB-14, results in higher solubility of proteins. The best yield was obtained after using 4 M thiourea with 1% ASB-14 and 2% CHAPS (787±16.23 µg/gram of fresh tissue).

The quality of protein samples were further evaluated by SDS-PAGE (Figure 2). Profiles of proteins obtainedconfirmed the phenol extraction as best method. The phenol based extraction method combined with the re-solubilisation of proteins in modified buffer-2 showed clearest lane with best resolved banding pattern as well as highest number of protein bands, which suggests that, the protein loss was lower than with the other methods.

Secondary metabolites are known to interfere the extraction and solubilisation of proteins (Granier, 1988; Valcu and Schlink, 2006). The plant *R. indica* is rich in carbohydrates, phenolics and other secondary metabolites (Lin *et al.*, 1995), which strongly interfere with the protein extraction and made the protein insoluble in 2-D rehydration buffer. These compounds form Hydrogen bonds (H-bonds) with the proteins, as well as, may form irreversible complexes of proteins by oxidation which leads to charge heterogeneity resulting in streaking of gels (Loomis and Battaile, 1966). Although amount of these compounds are low in young tissues but a better and reproducible proteome extraction method compatible for 2-DGE is required, where we can get a well resolved and highly abundant protein profile with less protein loss. The comparison of protein extraction methods was done on the basis of protein yield, solubility, spot resolution in 2-D gel and number of resolved spots.Quantitative comparison revealed that phenol extraction followed by solubilisation in modified buffer-2 gave highest protein yield than the other methods. The major reason of low protein yield in TCA-Acetone extraction and Tris based extraction, could probably the presence of other interfering compounds which were mostly eliminated from the proteins extracted with phenol. This was further evident that the presence of such compounds made proteins insoluble and thus a poor banding pattern in SDS-PAGE was obtained for these two extraction methods as compared to phenol based method. Though, the TCA and Tris based method are known to be effective for young tissues, but in this case, it was not found to be the best choice (Saravanan and Rose, 2004; Wang *et al.*, 2003).

Data Analysis of 2-D Gels

The best resolved banding pattern as well as highest number of protein bands were obtained from phenol based extraction process (Figure: 2), which suggests that, the protein loss was lower than with the other methods. So we performed the 2-D analysis only with the phenol extracted proteins. 2-D gel profiles of extracted proteins were compared with equal amount (300 µg) of initial protein. After Coomassie brilliant blue staining, proteins dissolved in modified buffer-2 (Readyprep 2-D rehydration/sample buffer 1, catalog # 163-2083, Bio-Rad, supplemented with 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10® ampholyte and additional 2 M thiourea) showed best resolution with less protein loss compared to other two (Figure 3). Here, approximately, 433 protein spots were detected by PDQuest software that was much higher in number compared to

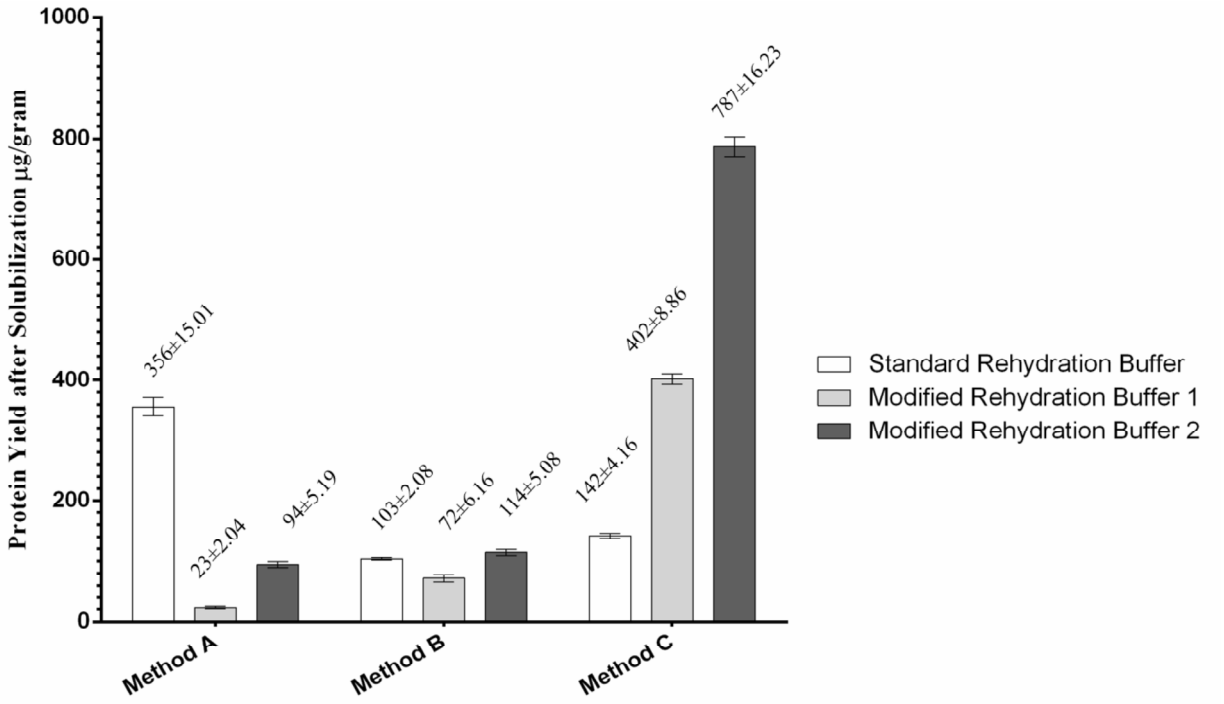

Figure 1: **A comparative graphical representation showing the average protein yield (µg/gram of fresh tissue) after extraction and re-solubilisation in standard rehydration buffer, modified buffer-1 and modified buffer-2. Method A: TCA-Acetone precipitation; Method B: Direct protein extraction with Tris-Cl buffer followed by TCA precipitation and Method C: Phenol extraction**

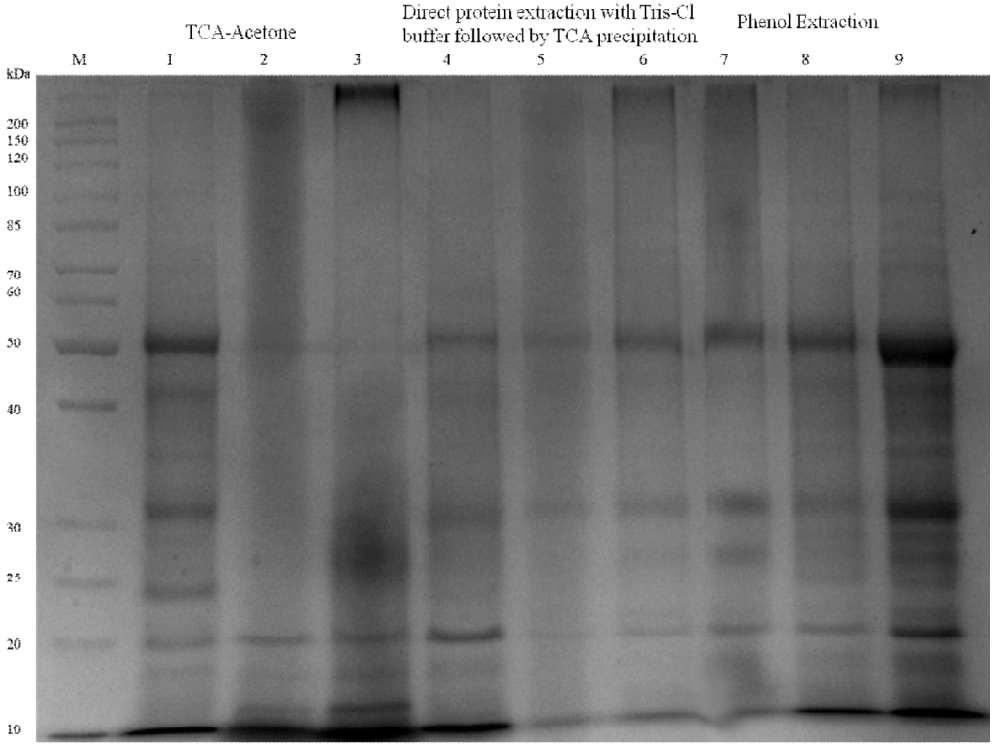

Figure 2: **SDS-PAGE of protein samples extracted using different methods and run on 12% gels. An equal amount of protein (10 ìg) was loaded in each lane. Proteins were visualized with Coomassie Brilliant Blue staining. M: Molecular weight markers in kDa; 1,4,7: Proteins dissolved in standard 2-D rehydration buffer; 2,5,8: Proteins dissolved in modified buffer-1; 3,6,9: Proteins dissolved in modified buffer-2.**

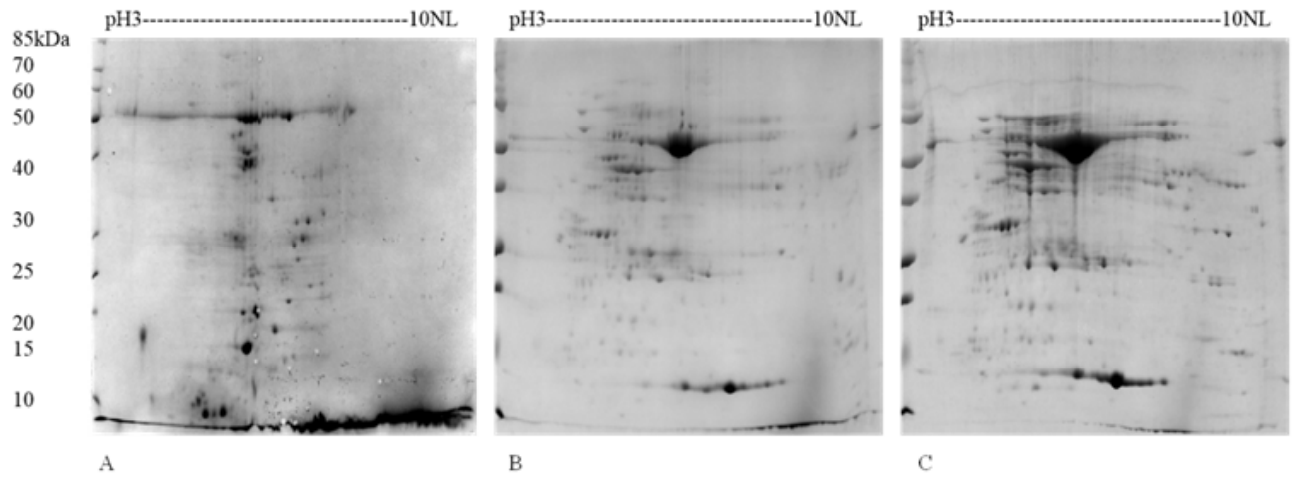

Figure 3: **2-DE gels of protein extracts from *R. indica* by phenol based extraction method. A: Proteins dissolved in standard 2-D rehydration buffer; B: Proteins dissolved in modified buffer-1; C: Proteins dissolved in modified buffer-2.**

other two re-solubilisation buffer used (in case of standard rehydration buffer 277 spots and by modified buffer-1, 312 spots were detected; Table 2). In addition, we found that there was a zonal aggregation of protein spots in case of samples dissolved in standard 2-D rehydration buffer and some spots were diffused or absent after dissolved in modified buffer-1 (Readyprep 2-D rehydration/sample buffer 1, catalog # 163-2083, Bio-Rad, supplemented with 2% CHAPS, 50 mM DTT and 0.2% Bio-Lyte 3/10® ampholyte).

In the present paper, we managed to visualize about 440 detectable protein spots using the phenol based extraction method followed by protein solubilisation in modified buffer-2 (Readyprep 2-D rehydration/sample buffer 1, catalog # 163-2083, Bio-Rad, supplemented with 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10® ampholyte and additional 2 M thiourea) after Coomassie brilliant blue staining. These spots only represent the most abundant proteins in the tissue.

In case of phenol extraction, the protein yield is quite good as the phenol may have some role to eliminate most of the phenolics, carbohydrates and other secondary metabolites. Also, the high pH of extraction buffer (pH 8.0) inhibited the common protease activity (Hochstrasser *et al.*, 1988) and caused the ionization of phenolic compounds, preventing them from forming H-bonding with proteins (Cherif *et al.*, 2007). β-ME and PMSF are known to function as reducing agent irreversibly inhibit the serine protease activity respectively, were used in the extraction buffer to prevent the protein oxidation. NaCl and KCl used facilitated the extraction by their salting in effect, while EDTA used, inhibits the metalloprotease and polyphenoloxidase by chelation of metal ions (Carpentier *et al.*, 2005).

The solubilisation of proteins was found to be increased by the addition of thiourea as an additional chaotropic agent. The best solubility and thus the protein yield obtained after using 4 M thiourea along with 7 M urea and 1% (w/v) ASB-14. The ASB-14 is known as zwitterionic detergent that helps proteins to maintain their pI value in solution and thus a better resolution of peptides may occur during the iso-electric focusing (IEF) which ultimately helps to get well resolved, reproducible and unique 2-D gel proteome profile. Streaking was absent in all gels. Besides, the protein spots we got in 2-D gel after dissolved in modified buffer-2, showed higher intensity and presence of a number of unique spots indicate a lower protein loss as compared to other two rehydration buffers.

MALDI-TOF MS Analysis

Protein spots were chosen randomly for MALDI-TOF MS analysis from the 2-D gel of protein dissolved in modified buffer-2. All 7 protein spots selected for MALDI-TOF MS analysis were successfully identified and listed in Table 2 (Figure 4). Data listed in the table include spot

Table 1
Comparison of the number of phenol extracted protein spots detected on 2-D gels after re-solubilisation in different 2-D rehydration buffers

	Average number of spots		
Re-solubilisation → ↓ Methods used	Standard 2-D rehydration buffer	Modified buffer-1	Modified buffer-2
Phenol extraction	277±4.81	313±4.33	433±5.61

Table 2
Proteins identified by MALDI-TOF MS analysis [Protein scores >79, (NCBInr) and > 54, (Swissprot) are significant (p<0.05)]

Spot no.	*Spot ID*	*Protein Identity*	*Peptides matched*	*Sequence coverage (%)*	*MOWSE score*	*Accession no. (NCBInr/ Swissprot)*	*Mr (kDa)/pI Experimental (Theoretical)*	*Plant species*
1	2903	ATPase alpha subunit (Chloroplast)	25	35%	118	NCBInr YP_001123271	55.227/5.19 (55/5.1)	*Barbarea verna*
2	3804	Ribulose bisphosphate carboxylase large chain	26	46%	157	SwissProt O03042	53.435/5.88 (54/6.2)	*Arabidopsis thaliana*
3	3904	Ribulose bisphosphate carboxylase/ oxygenase activase, chloroplastic	14	25%	57	Swissprot P10896	52.347/5.87 (53/6)	*Arabidopsis thaliana*
4	4501	PREDICTED: oxygen-evolving enhancer protein 1-1, chloroplastic-like	14	47%	130	NCBInr XP_013686285	35.287/5.56 (34/5.9)	*Brassica napus*
5	6103	Chloroplast ribulose 1,5-bisphosphate carboxylase/ oxygenase small subunit, partial	9	34%	85	NCBInr AIF75291	18.828/8.55 (14/7.5)	*Arabidopsis arenosa*
6	6601	Probable fructose-bisphosphate aldolase 2, chloroplastic	11	28%	69	Swissprot Q944G9	43.132/6.78 (43/7.1)	*Arabidopsis thaliana*
7	6605	Ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit, partial (chloroplast)	30	42%	96	NCBInr BAO57034	49.272/6.23 (48/7)	*Arabidopsis thaliana*

number, spot identity, protein identity, number of peptides match, sequence coverage (%), MOWSE score, NCBInr/Swissprot Accession number and experimental and theoretical molecular weight (kDa) and pI value.

Spots with higher intensity (like spot number 4501) as well as less intense spots (like spot number 6601) both generated high quality spectra with low background noise (Figure 5). This result evidenced the compatibility of phenol

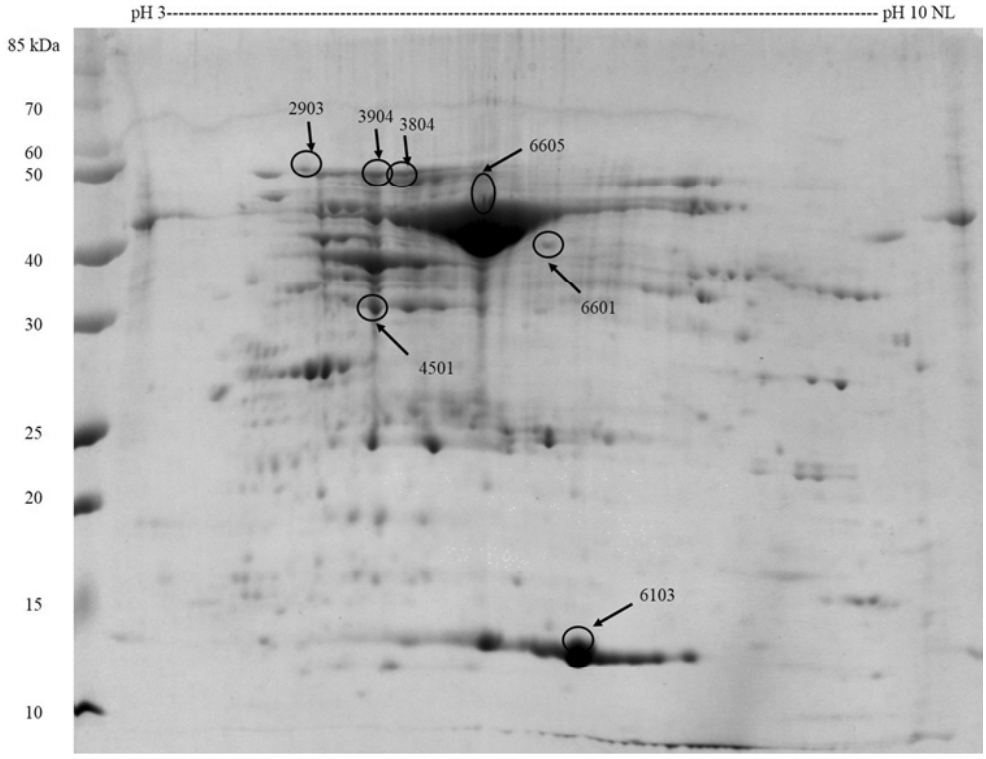

Figure 4: **2-DGE profile of phenol extracted Proteins dissolved in modified buffer-2, with marked spots selected for MALDI-TOF based MS analysis.**

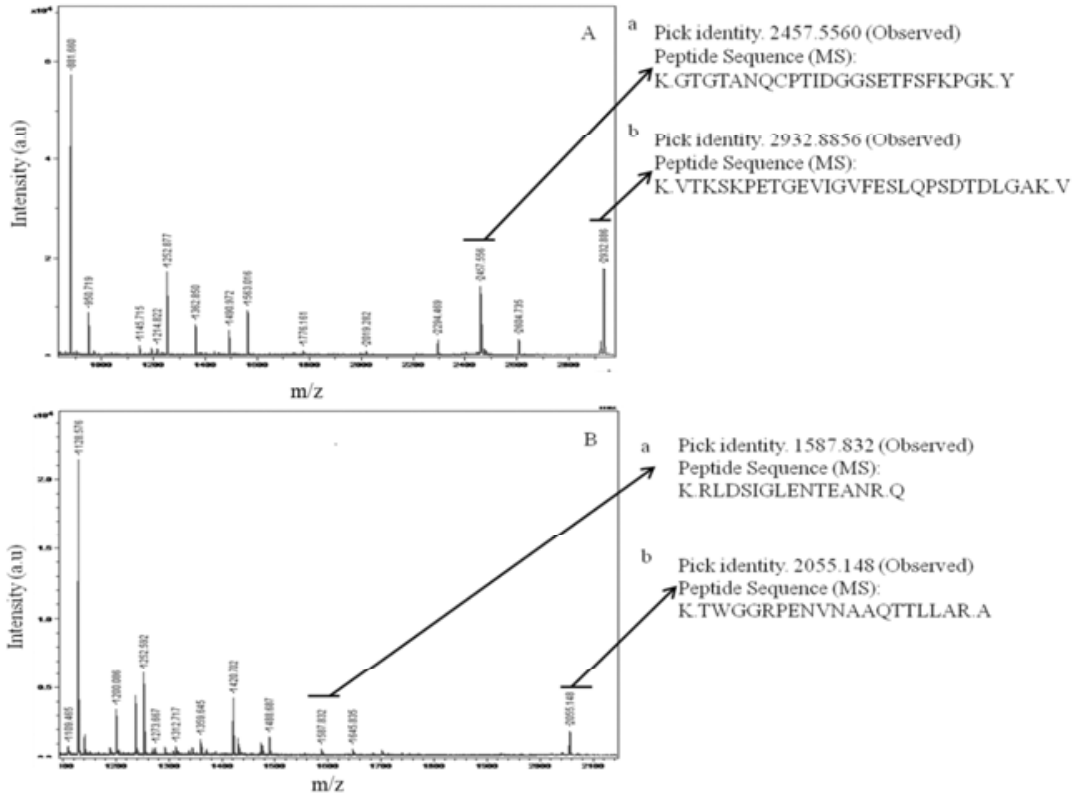

Figure 5: **Spectral profiles obtained by MALDI-TOF MS. A: MALDI MS spectra of spot 4501; a and b: Peptide sequence of selected pick. B: MALDI MS spectra of spot 6601; a and b: Peptide sequence of selected pick.**

based method combined with proper solubilisation of proteins in proper 2-D rehydration buffer (modified buffer-2) with MS analysis and its reliability for downstream processing.

Conclusion

This study, to our knowledge, is the first attempt to establish a proteome map of *R. indica*, which has not yet been studied in detail at protein level. The present study highlights a modified phenol based protein extraction method and solubilisation of extracted proteins from non-model, phenolics rich, recalcitrant tissue of *R. indica*, in a modified form of standard 2-D rehydration buffer (ReadyPrep 2-D starter kit rehydration/sample buffer, catalog # 163-2106, Bio-Rad). This method successfully isolated high quality proteins suitable for downstream 2-DGE and MALDI-TOF MS analysis. Thus, the data obtained indicates this protocol as an effective and efficient one that could be applied for other non-model, phenolics rich, recalcitrant tissue as well. But it should be kept in mind that no protocol for protein extraction and solubilisation is applicable as global. Each and every tissue must have their own standardized protocol depending on the nature of experiment.

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Conflict of Interest

The authors do not have any conflict of interest of this manuscript.

Abbreviations

2-DGE: 2-dimensional gel electrophoresis; BSA: Bovine serum albumin; â-ME: Beta marcaptoethanol; CHAPS: 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate; DTT: Di-thiotheritol; EDTA: Ethylene diamine tetra acetic acid; IEF: Iso-electric focusing; IPG: Immobilized pH gradient; MALDI-TOF: Matrix Assisted Laser Desorption Ionization-Time of Flight; mM: mili Molar; MS: Mass Spectrometry; PMSF: Phenylmethylsulfonyl fluoride; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA: Tri-chloro acetic acid.

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