Chapter 4
Standardization of protein extraction method from chickpea roots

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4.1. Introduction

The most critical step in any proteomic study is protein extraction and sample preparation. In contrast to the relative ease of mRNA isolation, c-DNA synthesis and analysis, protein extraction presents numerous challenges due to its heterogeneous nature, structural complexity and instability. Such features dramatically complicate their extraction, solubilization, handling, separation, and ultimately identification (Rose et al, 2004). The difficulties involving plant protein extractions especially from roots are quite complicated as compared to other organelles or organisms. Root tissues are highly vacuolated with relatively low protein content. They are often rich in proteases, storage polysaccharides, lipids, phenolics and a broad array of secondary metabolites (Gegenheimer, 1990; Wang et al, 2008). They result in horizontal & vertical streaking, smearing, and reduction in the number of distinctly resolved protein spots in two dimensional gel electrophoresis (2DE) (Saravanan and Rose, 2004). In chickpea, major yield loss is caused by different root invading pathogens like Sclerotium rolfsii (collar rot), Fusarium solani (black root rot), Thielaviopsis basicola (black streak root rot), Phytophthora sp. (Phytophthora root rot), Fusarium sp. (Fusarium root rot), Fusarium oxysporum f.sp.ciceris (Fusarium wilt), and so forth. Hence, root proteins serve to be an excellent target to study early signaling in plant-pathogen interaction. The present chapter deals with protein extraction from chickpea roots. The most common and basic protocols used for protein extraction from plant tissue are TCA-acetone and phenol based extraction methods. But these methods suffer some drawbacks in specific cases and hence, these extraction protocols demand optimization for particular organisms, tissue or cellular compartment. In the current chapter, attempts were made to optimize the phenol SDS method along with sonication for protein extraction from small amount of recalcitrant chickpea roots. Evaluations of other different extraction methods (Figure 4.1) were also done in comparison to the optimized phenol SDS sonication method and its compatibility with high throughput method like mass spectrometry analysed.
4.2. Materials and method

4.2.1. Plant material

Experiments were performed using chickpea seeds (JG62) obtained from International Crops Research Institute for the Semi Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. Seeds sown in sterilized synthetic soil were allowed to grow in natural green house conditions at 22-28°C and 35-40% humidity suited for the crop (Gupta et al, 2010). Roots of 15–20 days old seedlings were thoroughly washed, frozen in liquid nitrogen, and stored at -80°C prior to extraction of protein.

4.2.2. Buffers and solutions:

Compositions of buffers are given at where they have been mentioned and some of them are also mentioned in tables in appendix section.
4.2.3. Extraction methods

(A) TCA-Acetone precipitation method

TCA-acetone precipitation was carried out according to Damerval et al. with some modifications.

- One gram of root tissue was ground in a precooled mortar in the presence of liquid nitrogen. Approximately 100–150 mg of ground tissue powder was precipitated overnight with freshly prepared 2 mL of 10% TCA, 0.07% β-mercaptoethanol in cold acetone.
- Following precipitation the set was centrifuged at 10,000 g for 15–20 min at 4°C and the supernatant was discarded.
- The obtained pellet was rinsed twice in ice-cold acetone with 0.07% β-mercaptoethanol.
- An additional modification was introduced between the rinsing steps by incubating the sample for 60 min at −20°C (Saravanan and Rose, 2004).
- The pellet was air dried, resuspended in 100 μL sample buffer (Biorad) (8 MUrea, 2% CHAPS, 50 mM DTT, 0.2% Biolyte 3/10 Ampholyte, 0.001% Bromophenol Blue), and vortexed for 1 hour at room temperature.
- The supernatant was used for downstream analyses (Figure 4.1).

(B) Phenol extraction methods

Phenol extraction method was used both singly and in combinations of extraction buffer and SDS along with variations of parameters with and without sonication (Figure 4.1).

(B.1) Preparation of Tris saturated phenol

Phenol was distilled and kept at 60°C till it completely melts. Equal volume (50 ml each) of phenol and 25 mM Tris buffer (pH 8.0) was added to it. 50 mg 8-hydroxy quinoline dye was added that gives yellow color to the phenol. The mixture was mixed well which leads to the separation of yellow layer of phenol at the bottom and upper layer of Tris. After three consecutive changes with freshly prepared Tris buffer, Tris saturated phenol was obtained and stored at 4°C.
Phenol extraction of proteins was carried out as described by Hurkman and Tanaka in the presence of SDS buffer designated as phenol-SDS extraction by Wang et al. Root tissues collected from three independent plants (1gm) were pooled to isolate protein and considered as one biological replicate to minimize sample variation. Similarly three biological replicates were also generated.

- One gram of root tissue was grounded in a pre cooled mortar in the presence of liquid nitrogen.
- It was then extracted with 3mL of SDS buffer Table 4.1(30% sucrose, 2% SDS, 0.1M Tris-Cl, 5% β- mercaptoethanol, and 1mMphenyl methyl sulfonyl fluoride (PMSF), pH 8.0).
- The extract was sonicated 6 times for 15 seconds at 60 amps.
- Following sonication 3mL of Tris buffered phenol (section B.1) was added to the mixture and vortexed for 10 mins at 4°C.
- The set was centrifuged at 8,000 rpm for 10 min at 4°C, upper phenolic phase was collected and re-extracted with 3mL SDS buffer and shaken for 3–10 min.
- Centrifugation was further repeated using the same settings.
- Upper phenolic phase was collected and precipitated overnight with four volumes of 0.1M ammonium acetate in methanol at −20°C.
Protein pellet obtained by centrifugation at 10,000 rpm for 30 min at 4°C was washed thrice with cold 0.1M ammonium acetate and finally with cold 80% acetone.

The pellet was air dried under laminar air flow and resuspended in 100 μL sample buffer (Biorad) and used for further analyses.

Note: The pellet should not be over dried. Overdried pellets are difficult to solubilize. To enhance solubilization of pellet it can be sonicated 3 times for 5 seconds at 60amp.

(B.3) Phenol-SDS Buffer Extraction without Sonication (PSWOS)

This method was same as mentioned in case of PSWS only with the elimination of the sonication step.

(B.4) Phenol-Extraction Buffer with Sonication (PEWS)

- One gram of frozen root tissue was homogenized in liquid nitrogen
- It was then extracted with 3ml of ice-cold extraction buffer Table 4.2 (500mM Tris-Cl, 50mM EDTA, 700mM sucrose, 100mM KCl, pH 8.0) at 4°C.
- The extract was sonicated 6 times at 60 amps for 15 sec.
- It was further extracted with Tris buffered phenol as described in PSWS.

<table>
<thead>
<tr>
<th>Table 4.2. Composition of extraction buffer without SDS (100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ingredients</strong></td>
</tr>
<tr>
<td>500mM Tris</td>
</tr>
<tr>
<td>50mM EDTA</td>
</tr>
<tr>
<td>700mM sucrose</td>
</tr>
<tr>
<td>100mM KCl</td>
</tr>
<tr>
<td>pH was adjusted to 8.0 with conc.HCL. The buffer solution was then autoclaved and stored at 4°C.</td>
</tr>
</tbody>
</table>
(B.5) Phenol-Extraction Buffer without Sonication (PEWOS)

Protein extraction was carried out in the same way as described in case of PEWS with elimination of the sonication step.

(B.6) Phenol-Extraction Buffer with SDS

This protocol was similar to phenol extraction method.

- The buffer composition was the same as mentioned in PEWS pH 8.0 (Table 4.2) with 2% SDS as an additional component.
- Appearance of a white precipitate following SDS addition to the basal phenol extraction buffer prevented further processing of the sample using this buffer (Figure 4.1).

4.2.4 Protein quantification

Protein concentrations were quantified using the Bradford protein assay method with some modification using BSA as a standard (Bradford, 1976).

- BSA standard stock solution (1µg/µl) was prepared by dissolving 1mg of BSA in 1ml of sample buffer (Biorad).
- Five to seven standard solutions (800µl each) containing 0, 1, 3, 5, 7 and 10 µg/ml BSA were prepared in double distilled water (DD H₂O) from stock solution.
  (Note: 1 µl of sample buffer without protein was also added in blank set up).
- Sample protein solution was prepared (800µl) by adding 1µl of protein sample in DDH₂O. Protein sample solutions were assayed in duplicate or triplicate.
- 200 µl of dye reagent was added to each tube and vortexed.
- Both standard and sample solutions were incubated at room temperature for 5 minutes and absorbance were measured at 595nm with a glass cuvette.

4.2.5 Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out following the procedure of Laemmli (1970).
• The separating gel mix was prepared by mixing all the reagents except TEMED according to Table 4.3.
• After thorough mixing TEMED was added, mixed, and the resultant solution was poured between the glass plates separated by spacers, thereafter Isopropanol was added for overlaying to create a barrier against oxygen.
• After allowing sufficient time for the resolving gel to polymerize, the stacking gel mix was prepared by adding all ingredients except TEMED Table 4.3.
• Isopropanol was decanted and TEMED was added to the gel mix and it was poured onto the resolving gel.
• The stacking gel mix was allowed to solidify that was polymerised above the resolving gel with the insertion of a proper slot former.
• All the samples and molecular weight marker (Bangalore, GENEI) were prepared in 4X sample buffer and loaded onto the lanes.
• Approximately 25µl of protein samples obtained from different methods (TCA, PSWS, PSWOS, PEWS, PEWOS) were loaded along with the marker.
• Gel was allowed to run at 75 V using 1X running buffer till the dye front reached the bottom of the gel and optimal resolution was achieved.
• After completion of electrophoresis, the polyacrylamide gel was washed with destaining solution for 15 mins and stained using the staining solution at room temperature for 2 hours.
• After the gel turns blue, it was incubated in the destaining solution till the background was clear and the protein bands were visible.
• The gel was documented using Gel Doc XR imaging system (Bio-Rad, USA).

**Note:** In two dimensional gel electrophoresis stacking gel is often not required.

**4.2.6. Two-Dimensional electrophoresis (2DE)**

• IPG strips (11 cm, 3–10 nonlinear, Readystrip, Biorad) were passively rehydrated overnight with 185µl of rehydration buffer containing 300 µg of isolated protein.
• Isoelectric focusing was carried out on a PROTEAN IEF Cell (Biorad) at field strength of 600 V/cm and 50mA/IPG strip for 5 hours.
The strips were focused initially at 250V for 20 mins, 8000 V for 2 hours 30 mins with linear voltage amplification, and finally to 20,000 volt hour with rapid amplification.

Following IEF, the strips were reduced with 135mM DTT (80mg) in 4mL of equilibration buffer (20% (v/v) glycerol, 0.375M Tris-Cl, 6M urea, 2% (w/v) SDS, pH 8.8, Appendix Table 5) for 15 mins and alkylated with 135mM iodoacetamide (80mg) in 4mL equilibration buffer for 15 mins.

The 2DE was performed using 12% polyacrylamide gels as described in section 4.2.5 (13.8 cm ×13.0 cm × 1mm) in an AE-6200 Slab Electrophoresis Chamber (Atto Biosciences and Technology, China) at constant volt (200V) for 3 hours 30mins in Tris glycine-SDS running buffer.

All 2-D gel separation was performed in triplicates for all the methods. The gels were stained with 0.1% (w/v) coomassie brilliant blue R-250 (Sigma) overnight (Appendix Table 3).

### Table 4.3. preparation of solution for 12% SDS -PAGE analysis

<table>
<thead>
<tr>
<th>12% Separating Gel (30ml)</th>
<th>Stacking Gel (6ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients</td>
<td>Amount (ml)</td>
</tr>
<tr>
<td>Water (DD H₂O)</td>
<td>9.9</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>12</td>
</tr>
<tr>
<td>1.5M Tris pH 8.8</td>
<td>7.5</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.3</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.3</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.030</td>
</tr>
</tbody>
</table>

Note: After focusing the strips can be stored with oil overlaying at -80°C for 2-3 days.
The gels were then destained, and stored in 5% acetic acid at 4 °C for further analysis.

4.2.7. Image analysis of 2D PAGE gels

- Coomassie stained 2-D gels were visualized using Versa Doc (Model 4000) Imaging System (Biorad) and analyzed with PD Quest Advanced 2-D Analysis software (version 8.0.1, Biorad).
- All the replicate gels were used for analyses to generate the master gel.
- Spots were detected automatically by the Spot Detection Parameter Wizard using the Gaussian model with standard parameters.
- Comparison between spot quantities across gels was performed accurately, and normalization was done using local regression model.
- Only spots present in each of the three replicate gels, with high and low intensity, were randomly chosen for subsequent analyses.
- Selected protein spots were excised manually.

4.2.8. Processing of spots for MALDI-TOF MS and MS/MS

Spots were excised from protein gels, and in-gel digestion was performed as described by Shevchenko et al. (2006) with minor modifications.

**Excision of protein spots:**

- The entire slab of two-dimensional gel was rinsed with water for two to three times before it was placed on a white plate in a laminar air flow for spot excision.
- Spots were excised with a clean scalpel and excised gel pieces were again cut into cubes (1mm x 1mm). It should be carefully done as smaller pieces have chances to clog pipette tips.
- The gel pieces were then transferred into microcentrifuge tubes containing water (HPLC grade) and subjected to in-gel digestion for identification by MALDI-TOF MS and MS/MS analyses.

Note: Microcentrifuge tubes should be previously rinsed with 100% acetonitrile. The gel pieces in water could be stored at 4°C.
Destaining of gel pieces

- 100mM stock solution of ammonium bicarbonate was prepared by adding 0.39gms of NH₄HCO₃ to 50ml of water (HPLC grade).
- It was then mixed with acetonitrile in 1:1 ratio to make the final concentration of 50mM (4ml 100mM NH₄HCO₃ + 4ml ACN) and termed as solution 1.
- Initially the excised spots were washed with water for 10 mins. This step was repeated once.
- The water was then thrown out and gel pieces were incubated with 200µl of solution 1 for 15 mins with occasional vortexing.

Note: Ammonium bicarbonate buffer should be made freshly before use. Slight tapping would be preferred over vortexing.

- After 15 minutes the solution was thrown out and the step was repeated.
- 500 µl of neat acetonitrile was then added and mixed properly by pippeting until gel pieces became white and shrink, after which the acetonitrile was removed.
- 250 µl of 25mM of freshly prepared NH₄HCO₃ was then added to the gel pieces followed by equal volume of acetonitrile after 5 mins.
- After 15 mins of incubation the solution was discarded and neat acetonitrile was added as in previous step. (These steps were repeated until the gel pieces are completely destained).
- After the acetonitrile was removed the gel pieces were dried down either in a lyophilizer or in a vacuum centrifuge for 20 mins.

Samples are ready for in-gel digestion. Alternatively, they can be stored at −20 °C for a few weeks.

In gel digestion

Proteins were digested in gel using porcine sequencing grade modified trypsin (Promega). Lyophilized 20µg aliquot of trypsin powder was dissolved in 200µl of trypsin resuspension buffer provided with the enzyme and 10 µl aliquot of enzyme (0.1µg/µl) was prepared and stored at -80 °C.
Trypsin solution was prepared (25ng/µl) Table 4.4 and approximately 20 µl was added to the dried gel pieces. Depending on the volume of gel matrix the amount can be increased. The gel pieces with trypsin solution were then incubated at room temperature for 30 minutes for saturation.

It should be checked that if the solution was entirely absorbed, more trypsin should be added. Gel pieces should be completely covered with trypsin buffer.

After 30 minutes of incubation, excess trypsin solution was thrown out and the tubes with the gel pieces were kept at water bath at 37 °C overnight for digestion.

Note: The gel pieces should not get dehydrated; it can make extraction of peptide difficult

### Table 4.4 Preparation of 100 µl Trypsin solution (25ng/µl)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin stock(0.1µg/µl)</td>
<td>25µl</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>5µl</td>
</tr>
<tr>
<td>100mM NH₄HCO₃</td>
<td>25µl</td>
</tr>
<tr>
<td>Water (HPLC grade)</td>
<td>45µl</td>
</tr>
</tbody>
</table>

The tubes were chilled to the room temperature and spun down. Supernatant from tubes can either be thrown out or can be saved for MS/MS analysis before extraction.

**Extraction of digested peptides and spotting onto an Anchor Chip target plate**

Depending upon the volume of gel matrix approximately 20µl of extraction buffer (25%ACN in 1%TFA) was added to each tube. Gel pieces should be entirely covered with extraction buffer.

100µl of 25%ACN in 1%TFA was prepared by adding 75µl of 1% TFA and 25µl of ACN
• The tubes were then kept at 4°C for 30 minutes. After incubation the tubes were sealed with parafilm and sonicated in water bath for 10 minutes.

• After sonication the tubes were spun down and the supernatant was collected in a microfuge tube and dried down in a lyophilizer.

Note: Dried extracts can be safely stored at −20 °C for a few months.

• Tryptic peptides were redissolved by adding 3-5µl of 0.1% TFA in a PCR tube and then it was vortexed or tapped properly for complete dissolution.

• 1µl of peptide sample was then spotted onto Anchor Chip target plate (Bruker, Daltonics) and allowed to dry in a laminar air flow.

• After the sample was dried, 1µl of CHCA matrix (α-cyano-4-hydroxy cinnamic acid, Bruker, Daltonics) Table 4.5 was spotted and dried.

<table>
<thead>
<tr>
<th>Table 4.5 Preparation of 1ml matrix solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCA (Bruker)</td>
</tr>
<tr>
<td>ACN</td>
</tr>
<tr>
<td>TFA (0.1%)</td>
</tr>
<tr>
<td>NH₄H₂PO₄ (100mM stock)</td>
</tr>
<tr>
<td>Water (HPLC grade)</td>
</tr>
</tbody>
</table>

4.2.9. MALDI-TOF MS and MS/MS analysis and database search.

• Mass spectra were obtained on an Autoflex II MALDI TOF/TOF (Bruker, Daltonics, Germany) mass spectrometer equipped with a pulsed nitrogen laser (λ-337 nm, 50 Hz).

• Then the spectra were analysed with Flex Analysis Software (version 2.4, Bruker, Daltonics).

• The processed spectra were then searched using MS Biotoools (version 3.0) program, against the taxonomy of Viridiplantae (green plants) in the MSDB database using MASCOT search engine (version 2.2).

• The peptide mass fingerprinting parameters included peptide mass tolerance (≤100 ppm); proteolytic enzyme (trypsin); global modification
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(carbamidomethyl, Cys); variable modification (oxidation, Met); peptide charge state (1+) and maximum missed cleavage 1.

- The significance threshold was set to a minimum of 95% (P ≤ 0.05).
- The criteria used to accept protein identification were based on molecular weight search (MOWSE) score, the percentage of the sequence coverage, and match with minimum five peptides.
- MS/MS was performed to confirm the identification with matched peptides, selected on the basis of suitability for fragmentation (signal strength and relative isolation).

4.3. Results

4.3.1. Protein quantification

**TCA-Acetone precipitation method.** Protein yield using the classical TCA-acetone precipitation method was extremely low. However, a modification of incubating the sample at −20°C for 60 mins in between the rinsing step yielded a measurable amount of protein. Approximately seventy three micrograms of protein were obtained from one gram of root tissue using this method Table 4.6. However, when the obtained protein was subjected to electrophoresis in SDS-PAGE (polyacrylamide gel electrophoresis) gel, no banding profile was visualized Figure 4.2. Hence, this protocol was eliminated from further downstream analysis.

**Phenol-based methods**

In case of phenol-based methods, protein yields obtained from PSWS, PSWOS, PEWS, and PEWOS were 603μg, 406 μg, 302 μg and 408 μg, respectively, Table 4.6. One gram of fresh chickpea roots yielded maximum amount of protein with PSWS method as compared to protein obtained by methods PSWOS, PEWS, and PEWS.

4.3.2. Data analysis of 2DE gels.

The 2DE patterns of extracted protein when compared with equal amount of initial protein load revealed that protein extracted by PSWS method displayed a comparatively good resolution with lesser contamination, whereas proteins extracted with methods PSWOS, PEWS and PEWOS resolved fewer protein spots (Figure 4.3). Approximately 446 detectable spots (as estimated by PD Quest
standardization of protein extraction method

software were obtained by PSWS method while 287 spots by PSWOS method, 338 by PEWS, and 348 by PEWOS method were detected (Table 4.7). The number of spots described in Table 4.7 is the average number of spots across the triplicates. In addition we also found that many spots were diffused or absent in these methods (PSWOS, PEWS, PEWOS) as indicated in the marked areas (Figures 4.4 a, b, c, and d). Intensities of all the spots randomly selected for downstream MS and MS/MS were more in PSWS method as compared to other methods (Figures 4.5 and 4.6).

4.3.3. MALDI-TOF MS and MS/MS analysis for protein identification

All the 9 spots selected for MALDI analysis (Figures 4.5 and 4.6), consisting of both less abundant (sp 36, 80, 212) and more abundant (sp 19, 55, 109, 165, 248, 267) proteins, were successfully identified and listed in Table 4.8 (Figure 4.7). Data listed in the table include assigned spot number, spot identity, protein identity (MSDB database), number of peptide matches, sequence coverage (%), MOWSE score, accession number, experimental and theoretical molecular weight and pl.
### Table 4.6. Protein yield /fresh weight of material (μg/gm) using Bradford method

<table>
<thead>
<tr>
<th>Methods</th>
<th>Protein yield (μg/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSWS</td>
<td>603 ± 6.08</td>
</tr>
<tr>
<td>PSWOS</td>
<td>406 ± 5.77</td>
</tr>
<tr>
<td>PEWS</td>
<td>302 ± 5.51</td>
</tr>
<tr>
<td>PEWOS</td>
<td>408 ± 7.64</td>
</tr>
<tr>
<td>TCA</td>
<td>73 ± 2</td>
</tr>
</tbody>
</table>

### Table 4.7. Total number of spots using different methods

<table>
<thead>
<tr>
<th>Methods</th>
<th>Average number of spots</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSWS</td>
<td>446 ± 9.07</td>
</tr>
<tr>
<td>PSWOS</td>
<td>287 ± 6.43</td>
</tr>
<tr>
<td>PEWS</td>
<td>338 ± 6.11</td>
</tr>
<tr>
<td>PEWOS</td>
<td>348 ± 1.53</td>
</tr>
</tbody>
</table>
Figure 4.2. SDS PAGE of proteins obtained from different extraction methods. Lane M represents protein molecular weight marker. Lane 1 to 5 represents 25µl of total protein obtained by TCA, PEWS, PEWOS, PSWS, PSWOS respectively.

Figure 4.3. A comparative graphical representation showing the average number of protein spots detected in 2DE gels using PSWS, PSWOS, PEWS, and PEWOS protein extraction protocols.
Figure 4.4. 2-DE profiles of chickpea root proteins of JG 62. Profile of proteins isolated using PSWS (A), PSWOS (B), PEWS (C), and PEWOS (D) extraction protocols. Inset a, b, c, d represents a close-up view of an area showing spot resolution: in PSWS (A), PSWOS (B), PEWS (C), and PEWOS (D), respectively.
Figure 4.5. 2-DE profiles with marked spots selected for MALDI-TOF MS and MS/MS. (A) 2-DE profile using PSWS, (B) 2-DE profile using PSWOS, (C) 2-DE profile using PEWS, and (D) 2-DE profile using PEWOS.
Figure 4.6. 2-DE gel profiles showing individual spots and their relative intensities in graphical form using PSWS, PSWOS, PEWS, PEWOS protein extraction protocols. a, b, c, d represent the spot obtained by PSWS, PSWOS, PEWS, PEWOS, respectively.
Table 4.8. Proteins identified by MALDI-TOF MS and MS/MS analyses

<table>
<thead>
<tr>
<th>S no.</th>
<th>Spot ID.</th>
<th>Protein identity</th>
<th>Peptides matched</th>
<th>Sequence coverage (%)</th>
<th>MOWSE score</th>
<th>Accession number (NCBI)</th>
<th>Mr(kDa)/pI experimental</th>
<th>Mr(kDa)/pI theoretical</th>
<th>Plant species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>sp165</td>
<td>NADP specific isocitrate dehydrogenase</td>
<td>10</td>
<td>17%</td>
<td>70</td>
<td>Q9XGU7_ORYSA</td>
<td>46.4/6.29</td>
<td>(46.0/6.0)</td>
<td>Oryza sativa</td>
</tr>
<tr>
<td>2</td>
<td>sp212</td>
<td>Glyceraldehyde 3 phosphate dehydrogenase</td>
<td>9</td>
<td>24%</td>
<td>86</td>
<td>Q6K5G8_ORYSA</td>
<td>36.7/7.68</td>
<td>(37/6.5)</td>
<td>Oryza sativa</td>
</tr>
<tr>
<td>3</td>
<td>sp109</td>
<td>Triose phosphate isomerase</td>
<td>6</td>
<td>20%</td>
<td>71</td>
<td>Q38IW8_SOYBN</td>
<td>27.4/5.87</td>
<td>(25/5.5)</td>
<td>Glycine max</td>
</tr>
<tr>
<td>4</td>
<td>sp55</td>
<td>Fructokinase like protein</td>
<td>9</td>
<td>40%</td>
<td>94</td>
<td>Q8LPE5_CICAR</td>
<td>26.2/5.03</td>
<td>(35.5, 4.5)</td>
<td>Cicer arietinum</td>
</tr>
<tr>
<td>5</td>
<td>sp36</td>
<td>ATP synthase (sub unit D chain)</td>
<td>13</td>
<td>36%</td>
<td>88</td>
<td>ATPQ_ARATH</td>
<td>19.4/5.09</td>
<td>(20/5.0)</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>6</td>
<td>sp267</td>
<td>Porin of Pea, Channel protein</td>
<td>2</td>
<td>11%</td>
<td>134</td>
<td>T12558</td>
<td>29.7/8.56</td>
<td>(30/9.5)</td>
<td>Phaseolus caceinus</td>
</tr>
<tr>
<td>7</td>
<td>sp19</td>
<td>Plasma membrane intrinsic polypeptide</td>
<td>10</td>
<td>38%</td>
<td>74</td>
<td>Q9SMK5_CICAR</td>
<td>23.3/4.95</td>
<td>(24.5/5.0)</td>
<td>Cicer arietinum</td>
</tr>
<tr>
<td>8</td>
<td>sp248</td>
<td>Unidentified protein</td>
<td>11</td>
<td>35%</td>
<td>80</td>
<td>CAA0649_1</td>
<td>22.1/9.91</td>
<td>(44.0,9.0)</td>
<td>Cicer arietinum</td>
</tr>
<tr>
<td>9</td>
<td>sp80</td>
<td>Putative pyruvate dehydrogenase E1 beta subunit isoform 1 protein</td>
<td>2</td>
<td>6%</td>
<td>55</td>
<td>Q6Z1G7_ORYSA</td>
<td>40.2/5.25</td>
<td>(38.5/5.3)</td>
<td>Oryza sativa</td>
</tr>
</tbody>
</table>
Figure 4.7. Spectral profiles obtained by MALDI MS and MS/MS. 
(A) MALDI spectra of sp55 and a, b, shows MS/MS spectra of the same. 
(B) MALDI spectra of sp 212 and c shows MS/MS spectra of the same.
4.4. Discussion

Secondary metabolites are known to play important role in structural composition and defense of plants. These metabolites accumulate in various soluble forms in vacuoles and cause severe interference in protein extraction as well as separation in 2DE gels (Granier, 1988; Vâlcu and Schlink, 2006). Chickpea roots are rich in phenolic compounds like tannic acid, gallic acid, o-coumaric acid, chlorogenic acid, cinnamic acid; flavanoids, isoflavanoids like daidzein, genistein, as well as tannins, lignins, and carbohydrates (Maurya et al, 2005; Chérif et al, 2007). These compounds form hydrogen bonds with proteins. Besides they also form irreversible complexes with proteins by oxidation and covalent condensation which leads to charge heterogeneity resulting in streaking of gels (Loomis and Bataille, 1966). Carbohydrates block gel pores causing precipitation and prolonged focusing time, which also results in loss of protein spots and streaks in the gels (Carpentier et al 2005). Although the amount of these secondary metabolites is comparatively low in etiolated tissues like roots, but low protein content and limiting tissue amounts demand for a competent protein extraction method. In our study TCA-acetone method and phenol-based method using two different extraction buffers (SDS buffer and extraction buffer without SDS) with and without sonication were evaluated. Comparison was done on the basis of protein yield, spot focusing, resolution, number of resolved spots, and also intensities of the spot and their downstream analysis using high throughput technology (MALDI/MS) of the optimized method.

Quantitative comparison of protein extracts revealed that phenol-based methods gave higher protein yield as compared to TCA-acetone method. The major reason for low protein yield in TCA-acetone method which constrained it for further downstream processing could probably be attributed to the insolubility of protein pellet in IEF buffer as compared to phenol-based methods (Chen and Harmon 2006). Moreover TCA-acetone protocol is known to be effective with tissues from young plants and was found not to be the best choice for more complex tissues (Saravanan and Rose, 2004; Wang et al, 2008; Carpentier et al 2005). In case of phenol extraction, the proteins were first homogenized in two different extraction buffers; both the buffers contained sucrose which was added to create phase inversion. These buffers formed the aqueous lower phase containing carbohydrates, nucleic acid, insoluble cell
debris, while the upper phenol phase contained cytosolic and membrane proteins, lipids, and pigment (Carpentier et al. 2005). SDS buffer contained about 30% sucrose which helped in better phase separation as compared to extraction buffer (24%). The high pH buffers inhibit common activity of the proteases (Hochstrasser et al, 1988) and cause ionization of phenolic compounds, thus preventing them from forming hydrogen bonding with the protein (Loomis et al, 1966). It also neutralizes the acids that are released by disrupted vacuoles. PMSF and β-mercaptoethanol which were used in both buffers in the present study were reported to irreversibly inhibit serine protease action and act as a reducing agent which prevents protein oxidation, respectively. KCl and EDTA were used in case of extraction buffer without SDS (PEWS and PEWOS). KCl facilitates the extraction of proteins by its salting in effect and EDTA inhibits metalloprotease and polyphenoloxidase by chelating metal ions (Carpentier et al 2005). Although the salting in effect or chelation of metal ions could not improve the protein yield as compared to SDS buffer with sonication, SDS is known to act as an excellent solubilizing agent, which allows the recovery of membrane bound proteins (Wang et al, 2008). The solubilization of protein was found to increase with sonication as evident from the increase in protein yield and spot resolution after sonication in PSWS compared to PSWOS. Sonication results in better disruption of cell membrane and release of intracellular proteins and thus provides explanation for SDS to have efficiently solubilized the protein in PSWS method. In contrary, in case of extraction buffer, sonication could not improve protein yield or resolution, presumably due to the interference with constituents of buffer (KCl or EDTA) or due to lack of better solubilizing agent like SDS and/or both. The phenol used in this method was buffered to pH 8.0 to ensure that nucleic acids are partitioned to the buffer phase and not to phenol-rich phase (Pusztai, 1966), and thus proteins in phenol phase were purified and concentrated simultaneously by subsequent methanol ammonium acetate precipitation. Phenol acts as one of the strongest dissociaters known to decrease molecular interaction between proteins and other materials (Carpentier et al 2005). It can minimize protein degradation resulting from endogenous proteolytic activity (Schuster and Davies, 1983). Phenol extraction method though with high clean-up capacity has a little tendency to dissolve polysaccharides and nucleic acids. We found that in PSWS method the spots obtained were well resolved and showed high intensity (Figures 4.4 and 4.6) as compared to PSWOS, PEWS, and PEWOS. About 25%
unique spots were obtained in PSWS and the rest 75% spots though existed in PSWOS, PEWS, and PEWOS, however, resolved with variable clarity. Streaking was absent in all the gels. We could see that the difference in number of spots between PSWS and PSWOS was more as compared to PEWS and PEWOS, which confirmed that the effectivity of SDS increased in presence of sonication. However in the latter case (PEWS, PEWOS) sonication did not have much influence. Improvisation of the extraction buffer was also made by adding 2% SDS, which resulted in precipitation. Interference between constituents of the extraction buffer and SDS was assumed to be the cause of such precipitation. However further experimentation needs to be performed for confirmation of such predictions. All protein spots selected for MALDI-TOF/MS and MS/MS from PSWS resulted in successful identification. High intense spot like sp 55, (fructokinase-like protein) and less intense spot like sp 212, (glyceraldehyde 3-phosphate dehydrogenase) both resulted in high quality spectra with low background noise (Figure 4.7). These results further indicated the compatibility of PSWS method with MS and MS/MS and its reliability for downstream processing.

4.5. Summary

- The present study emphasizes Phenol-SDS Buffer Extraction with Sonication as the optimized phenol-based method for chickpea root protein extraction.
- This method successfully isolated high quality protein suitable for two dimensional gel electrophoresis and mass spectrometric analyses.
- Hence, the data obtained projects this protocol as an effective and efficient one that could be applied for other recalcitrant leguminous root tissues as well.

Nevertheless, it should be kept in mind that one generalized protein extraction protocol though may be applicable in global protein profiling but requires improvisation with variable tissues.