

Chapter 13

Proteome Analyses of *Jatropha curcas*

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Abstract Plant proteomes are complex and therefore their analyses represent major technical challenges. In fact, proteome analyses depend on several crucial steps, such as the amount of proteins, extraction, separation, visualization, identification, quantification, and the interaction between proteins and other molecules in a plant tissue at a given time.

To date it is recognized, that there is no single method able to describe an entire plant proteome. Even though several alternatives exist, the most widely used methodology for proteome analysis includes two-dimensional gel electrophoresis (2-DE), mass spectrometry, and bioinformatics tools.

The *Jatropha* proteome represents a valuable field of study related to gene functions since many biochemical pathways of fatty acids and secondary metabolites in this species provide an alternative potential source to fossil oil useful for production of biodiesel. Therefore, a detailed proteome analysis of *J. curcas* using gel-based electrophoresis combined with identification by mass spectrometric analyses could help to understand its potentials as a source of biofuel.

Due to the low correlation between protein expression level and mRNA in plant tissues, it is not advisable to predict a final amount of protein from quantities of mRNA. However, it is generally accepted that protein analysis allows to identify so far unknown genes and to assign them a function.

This chapter provides a selection of methods, reproducible with the highest resolution and quality of results from sample preparation to proteins identification of *J. curcas*.

Keywords Sample preparation • Protein extraction • SDS-PAGE • 2-DE • Coomassie staining • Silver staining • Mass spectrometry • Protein sequence databases

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13.1 Introduction

Jatropha curcas has attracted a great deal of attention worldwide, regarding its potential as a new energy plant. The press cake from seeds, rich in protein (60–63 %), could be an ideal protein source with a high content of essential amino acids, even higher than soybean (45 %) (Maghuly and Laimer 2013). However, seeds of toxic *J. curcas* contain a range of toxins and anti-nutritional compounds which render the seedcake and oil unsuitable for use as animal feed and for human consumption (Maghuly and Laimer 2013). Accordingly, in an effort toward better understanding, engineering, and to determine gene function simultaneously, the detail proteomics analysis of *J. curcas* is necessary (Maghuly and Laimer 2013).

It is well known that every plant cell type of *J. curcas* expresses a unique transcriptome and proteome at different stages of differentiation depending on its developmental fate. Knowing when and where a gene product (RNA and/or protein) is expressed can provide important clues to its biological function (Bouchez and Höfte 1998). The facility with which a high-throughput approach can be used for the study of gene expression depends largely on the level of regulation (Bouchez and Höfte 1998). To understand the function of all genes in a given organism, it needs to know not only, which genes are expressed, when and where, but also what the protein end products are and under which conditions they accumulate in certain tissues (Mathesius et al. 2003). At the transcriptome level, however, results must consider that mRNA abundance and protein level are not always correlated (Gygi et al. 1999; Maghuly et al. 2009), that low-copy number mRNAs (potentially very important for regulation) are not detected as readily as abundant mRNAs, and that gene expression studies do not provide reliable information about protein levels, the subcellular localization of gene products or the posttranslational modifications occurring to a protein that may be essential for its function, transport, and activation (Maghuly et al. 2009; Rose et al. 2004). This information will complete the annotation of genes and contribute to the identification of their function (Zivy et al. 2007).

Proteomics, a combination of several methodological approaches used to study the global changes in proteins, provide an essential bridge between the transcriptome and the metabolome, because the levels of various metabolites are the result of direct enzymatic activity and of indirect cellular regulation over transcriptional or biochemical processes (Zivy et al. 2007; Cook et al. 2004; Gray and Heath 2005; Kaplan et al. 2004).

Therefore, comprehensive proteomic studies may provide new information on proteins that play an integral role in functional genomics and on the gene coding sequence and increase the understanding of protein function in specific cellular compartments. Furthermore, proteomics provide major advantages linked to the

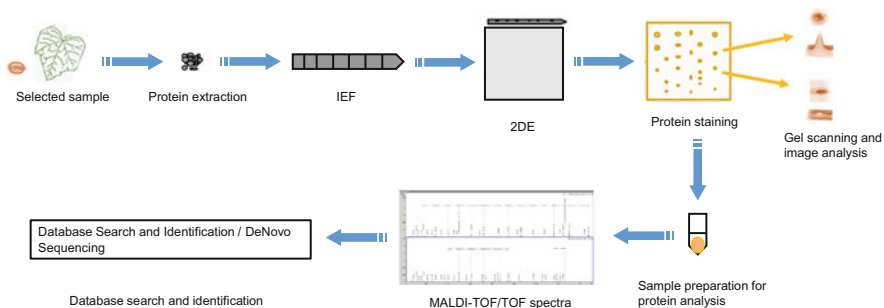


Fig. 13.1 Outline of the experimental approach to proteomic analyses of *Jatropha* tissues

high-throughput capacity due to the ability to perform simultaneously the analysis of hundreds of proteins from the same sample (Maghuly et al. 2011).

Analyses of large portions of the proteome are now possible through techniques such as two-dimensional gel electrophoresis (2-DE) or mass spectrometry (MS). These tools can provide a more direct assessment of biochemical processes by providing information about the main proteins involved in the enzymatic, regulatory, and structural functions encoded by genes and regulated by transcription factors. Although these proteomic techniques are powerful in being able to analyze the complex plant proteome, it constitutes considerable challenges, especially because of low protein concentration and the presence of inhibiting compounds in the cell (Flengsrud 2007). It is crucial to use an effective extraction protocol to avoid interferences and to increase the protein yield because it influences further protein separation and data generation. In addition, the choice of detection methods after protein separation is also important for data visualization.

In this chapter, two-dimensional gel electrophoresis (2-DE), mass spectrometry (MS), and bioinformatics tools suitable for *Jatropha curcas* proteomes are described. The proteins are extracted from the tissues under study, which are also relevant for other plants, and the protein mixtures are separated by isoelectric focusing (IEF) in a conventional Immobiline DryStrip gel (IPG strip). Entire strips are transferred into a set of gels, and proteins are visualized by Coomassie blue (Tu et al. 2005; Sinchaikul et al. 2005) and silver staining for gel image analysis (Sinchaikul et al. 2005) (Fig. 13.1).

The proteins spots are separated, excised from the gel, and digested with trypsin. The tryptic digests are subjected to MS/MS TOF to obtain data on the peptides sequences. MS/MS data are used to identify the proteins through search of protein sequence databases (Giorgianni and Beranova-Giorgianni 2005) for the analysis of *J. curcas* tissue.

13.2 Materials

Sample preparation for protein analysis is prone to various contaminations particularly human keratin from the skin and hair, sheep keratin from clothes, and proteinaceous contaminants from dust. Therefore, try to protect the samples against these influences in every preparation step, especially for protein identification. Important means to achieve this are

1. Wear a clean lab coat.
2. Wear powder-free nitrile gloves; latex gloves contain natural rubber and so significant amounts of keratin and other proteinaceous materials. Pipet tip boxes should not be filled with bare hands, but always in the laminar flow.
3. Work in dust-free/reduced environment: e.g., in a laminar flow.
4. Use clean laboratory equipment: the working area and any tools that will be in contact with the sample should be wiped with an ethanol soaked wipe.

13.2.1 *Sample Preparation and Protein Isolation*

1. 100 mg of *Jatropha curcas* tissue
2. Liquid nitrogen
3. 2 ml Eppendorf tubes
4. Retsch Mill (Retsch GmbH & Co. KG, Haan, Germany)
5. TRIZOL (Invitrogen, San Diego, CA)
6. Cold and room temperature (RT) Centrifuge
7. Chloroform
8. Isopropanol
9. 0.3 M guanidine hydrochloride
10. 95 and 100 % ethanol
11. 8 M urea

13.2.2 *Determination of the Protein Concentration*

1. Bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co Rockford IL)
2. Protein standard: bovine serum albumin (BSA)
3. Incubator 37 °C
4. Late reader: Tecan Infinite M200 reader for 96-Well plates (TECAN, Männedorf, Switzerland)

13.2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Novex[®] 4–20 % Tris–Glycine Mini Gels
2. Running buffer (1×): 25 mM Tris–HCl, 192 mM glycine, 0.1 % (w/v) SDS (store at 4 °C)
3. SDS-PAGE sample loading buffer (2×): 50 mM Tris–HCl, pH 6.8, 0.1 M DTT, 10 % glycerol, 2 % (w/v) SDS, 0.1 % (w/v) bromophenol blue (aliquot and store at –20 °C)
4. Protein marker (PeqLab, Erlangen, Germany)
5. Mini SDS-PAGE electrophoresis, Novex System (Invitrogen, San Diego, CA)
6. Thermos mixer (Eppendorf, Hamburg, Germany)

13.2.4 Separation of Protein by Two-Dimensional Gel Electrophoresis (2-DE)

13.2.4.1 First-Dimension Isoelectric Focusing (IEF)

1. Rehydration solution: 3 M Urea, 2 M Thiourea, 2 % CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 1 % DTT (Dithiothreitol), 2 % Ampholyte, and 0.1 % bromophenol blue
2. 13 cm Immobiline pH 3–10 NL Dry Strip[™] (IPG) (GE Healthcare, Piscataway, NJ, USA)
3. Ettan IPGphor 3 IEF System and Ettan IPGphor 3 strip holders (GE Healthcare, Piscataway, NJ, USA)
4. Paraffin
5. Filter paper blocks (GE Healthcare, Piscataway, NJ, USA)
6. Milli-Q water (deionized water)

13.2.4.2 2-DE

1. Equilibration buffer: 50 mM Tris–HCl (pH 8.8), 6 M urea, 30 % glycerol, 2 % SDS, a trace of bromophenol blue
2. Solution A: Equilibration buffer plus 2 % DDT
3. Solution B: Equilibration buffer plus 2.5 % Iodoacetate
4. 12 % acrylamide gel: 34.4 ml, Milli-Q water, 24 ml 40 % Acrylamide-Mix, 20 ml 1.5 M Tris–HCl (pH 8.8), 0.8 ml 10 % SDS, 0.8 ml 10 % APS (Ammonium persulfate), 0.032 ml TEMED (N,N,N,N-Tetramethylethylenediamine) (add TEMED and APS to the gel solution prior to gel casting)
5. 1 % agarose: 1 g agarose in 100 ml of running buffer (1×) and 100 µl bromophenol blue (Heat them in a microwave oven until the agarose is completely dissolved. Allow the agarose to cool to 40–50 °C before using)

6. Running buffer (1×): 25 mM Tris–HCl, 192 mM glycine, 0.1 % (w/v) SDS (Store at 4 °C)
7. Isobutanol
8. Agarose
9. Bromophenol blue
10. Parafilm
11. Electrophoresis chamber device for 13 × 13 cm vertical gels (PeqLab, Erlangen, Germany)

13.2.4.3 Protein Staining

Silver Staining for Analytical Gels

1. Fixing solution: 40 % (v/v) ethanol, 10 % (v/v) acetic acid, 50 % (v/v) Milli-Q water
2. Glutaraldehyde solution: 1 % Glutaraldehyde and 0.5 M sodium acetate
3. Naphthalene disulfonic acid solution: 0.05 % w/v 2,7-naphthalene-disulfonic acid (250 mg), Milli-Q water (500 ml)
4. Ammoniacal silver nitrate solution: Silver nitrate solution (4 g in 20 ml water). Combine: ammonium hydroxide (5.7 ml, 29 % solution) and sodium hydroxide (1.0 ml, 10 N stock solution) (*see Note 1*)
5. Formaldehyde solution: 0.01 % w/v citric acid (50 mg); 0.1 % formaldehyde (1.35 ml, 37 % stock), water to 500 ml
6. Stop solution: 5 % Tris, 2 % acetic acid

Silver Staining Compatible with Mass Spectrometry

1. Use MALDI-MS compatible silver-staining kit (ProteoSilver™ Plus Silver Stain Kit, SIGMA).
2. Fixing solution: 40 % (v/v) ethanol, 10 % (v/v) acetic acid, 50 % (v/v) Milli-Q water.
3. 30 % ethanol solution (washing solution): 30 ml ethanol 100 and 70 ml water.
4. Sensitizer solution: 1 ml ProteoSilver™ Sensitizer and 99 ml water per 100 ml solution (prepare within 2 h before use).
5. Silver stain solution: 1 ml of ProteoSilver™ Silver Solution and 99 ml water per 100 ml solution (prepare within 2 h before use).
6. Developer solution: 5 ml of ProteoSilver™ Developer 1 and 0.1 ml ProteoSilver™ Developer 2 and 95 ml water per 100 ml solution (prepare immediately before use (<20 min)).
7. Stop solution: 15 ml of the ProteoSilver™ Stop Solution.

Coomassie Staining

1. Coomassie-staining solution: 0.1 % (w/v) Coomassie brilliant blue R250, 40 % (v/v) methanol, 10 % (v/v) acetic acid, 50 % Milli-Q water
2. Coomassie-destaining solution: 40 % (v/v) methanol, 10 % (v/v) acetic acid, 50 % Milli-Q water
3. 5 % acetic acid washing solution

Post-fluorescence Gel Staining (SYPRO[®] Ruby Protein Gel Stain)

1. SYPRO fix solution: 50 % Methanol, 7 % acetic acid (both should be pre-analysed)
2. SYPRO stain solution: SYPRO[®] Ruby protein gel stain (Molecular Probes, Paisley, UK) (1 L of solution provides sufficient material to stain 6–8 gels (13 × 13 cm))
3. SYPRO wash solution: 10 % Methanol, 7 % acetic acid
4. Polypropylene or polycarbonate containers (It should be very clean and rinsed with ethanol before use)
5. Trichloroacetic acid (for IEF gels only)
6. Milli-Q water
7. Orbital shaker

13.2.5 Gel Scanning and Image Analysis

1. Laser scanner Typhoon FLA95000 image scanner (GE Healthcare, Piscataway, NJ, USA)
2. ImageMaster 2D Platinum version 5.0 (GE Healthcare, Amersham Biosciences, Piscataway, NJ, USA)

13.2.6 Protein Identification

1. Spot picker (Serva, Heidelberg, Germany)
2. Ultrasonic bath
3. 100 mM ammonium bicarbonate (ABC 100), pH = 8.5
4. Destaining solution: 50 % Potassium hexacyanoferrate (30 mM), 50 % Sodium thiosulfate (100 mM)
5. Dehydrating solution: Acetonitrile
6. Reducing solution: Dithiothreitol (10 mM in 25 mM ammonium bicarbonate)
7. Alkylating solution: Iodoacetamide (54 mM in 25 mM ammonium bicarbonate)

8. Trypsin solution: 1 µg/µl trypsin in 50 mM acetic acid (Trypsin Gold, Promega)
9. Peptide extraction: 50 % (v/v) acetonitrile, 45 % (v/v) water, 5 % (v/v) trifluoroacetic acid (TFA)
10. Derivatization: 4-sulfophenyl isothiocyanate (SPITC)
11. Matrix for MALDI ionization: α-cyano-4-hydroxycinnamic acid
12. Pre-spotted anchor chip MALDI target plate (PAC target, Bruker Daltonics)
13. Solvent evaporation: Vacuum centrifugation (SpeedVac, Eppendorf)
14. Desalting of peptides: ZipTips C18 (microbed, Millipore)
15. Derivatization of peptides: ZipTip C18 pipette tips (Millipore)
16. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Ultraflex II; Bruker Daltonics)
17. Spectra processing and peak annotation: FlexAnalysis and BioTools (Bruker Daltonics)
18. Mascot Server version 2.3 (Matrix Science, Boston, MA), ProteinScape 2.1 (Bruker Daltonics)

13.3 Methods

13.3.1 *Sample Preparation and Protein Extraction*

Plant tissues should be ground into a very fine powder using liquid nitrogen.

1. Weigh 100 mg plant tissue and cut it in several pieces; place the tissue pieces in a 2 ml Eppendorf tube.
2. Grind the selected tissues with a Retsch Mill into a fine powder using liquid nitrogen because they generally contain low levels of proteins, tightly linked to the cell structures.

13.3.1.1 TRIZOL Protein Isolation Protocol

A number of methods for protein extraction from *J. curcas* tissues for proteomics applications were compared in our lab (PBU), and the TRIZOL reagent was selected for protein isolation.

1. Add 1 ml of TRIZOL to 100 mg pulverized tissue; mix well and keep for 5 min at RT.
2. Add 0.2 ml of chloroform per 1 ml of TRIZOL reagent used, and shake samples by hand vigorously.
3. Centrifuge at $12,000 \times g$ for 5 min at RT. The mixture separates into the three phases; upper (aqueous) phase that contains RNA, interphase (DNA and proteins), and lower phenolic phase.
4. Remove the upper (aqueous) phase.

5. Add 0.3 ml of ethanol to the lower phases, mix by inversion, and keep for 2–3 min at RT (this precipitates the DNA).
6. Centrifuge at $2000 \times g$ for 15 min at 4 °C.
7. Collect the supernatant and add 1.5 ml isopropanol; mix and store the samples at RT for 10 min (this precipitates the proteins).
8. Centrifuge at $12,000 \times g$ for 10 min at 4 °C.
9. Remove the supernatant, and wash the protein pellet three times by adding 2 ml solution of 0.3 M guanidinium hydrochloride in 95 % ethanol for 20 min at RT (keep the samples in washing solution for 20 min at RT before centrifuging).
10. Centrifuge at $7500 \times g$ for 5 min at 4 °C; remove and discard the supernatant.
11. Vortex the protein pellet in 2 ml of 100 % ethanol, store the protein pellet in ethanol for 20 min at RT, and centrifuge at $7500 \times g$ for 5 min at 4 °C.
12. Remove ethanol and dry protein pellet briefly (do not prolong storage of the protein pellet, which can make subsequent solubilization difficult).
13. Dissolve pellet in 8 M urea.
14. Determine protein concentration (*see* Sect. 13.3.2).
15. Aliquot the samples and store at -80 °C until further use.

13.3.2 Determination of the Protein Concentration

1. Determine the protein concentration by the BCA method using a freshly prepared BSA standard curve. Typical protein concentrations range from 2 to 2000 $\mu\text{g/ml}$.
2. Prepare working reagent (WR) by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B).
3. Add 25 μg of each standard or extracted sample into a microplate well, and add 200 μl of WR, mix by careful vortexing.
4. Cover the plate and incubate for 30 min at 37 °C.
5. Cool plate at RT and measure the absorbance at 595 nm on a plate reader.

13.3.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Prepare the Novex[®] 4–20 % Tris–Glycine Mini Gels by removing the comb and rinse the gel wells three times using running buffer (1 \times).
2. Remove the white tape near the bottom of the gel cassettes to give contact between buffer and gel.
3. Place the gels in the Mini SDS-PAGE electrophoresis.
4. Fill the gel wells with 1 \times running buffer.
5. Prepare 10 μl of each sample in 2 \times SDS-PAGE sample loading buffer.

6. Spin down samples and heat them for 10 min at 90 °C to monomerize possibly present complex proteins. Keep samples at 4 °C until ready to use in order to avoid polymerization.
7. Load the appropriate volume of samples and protein marker onto the gel.
8. Run for 2 h at 125 V.
9. At the end of the run, use the gel either immediately for western blotting or for staining with Coomassie brilliant blue (*see* Sect. 13.2.4.3.3).

13.3.4 Separation of Protein by 2-DE

13.3.4.1 First-Dimension Isoelectric Focusing (IEF)

1. Add rehydration solution (prepare it 1 day before isoelectric focusing) to the suitable volume of sample (μg of protein, *see* **Note 2**). Vortex the solution shortly and spin off the vial with a table centrifuge.
2. Open the 13 cm IPG strip (GE Healthcare Immobiline™ DryStrip pH 3–10 NL) package from the acidic end (*see* **Note 3**).
3. Add 250 rehydration solution-containing samples in the middle of the IPG strip in the ceramic strip holder (Ettan IPGphor 3 strip holders).
4. Put the IPG strip onto the strip holder with the gel side down to fit into the holder and to make its code number easily readable. Placing the strip starting from the pointed end helps to avoid bubbles. Move the strip forward and backward along the surface of the solution to ensure the complete and evenly wetting of the strip.
5. Cover the strip by adhesive tape or paraffin oil (to avoid evaporation of solution and crystallization of urea) and incubate at RT for 16 h.
6. Place the strip in the focusing tray (Ettan IPGphor 3 IEF System), with the gel side up. The “+ end” of the IPG strip has to be at “+” pole.
7. Moisturize two filter paper blocks (avoid dust and dirt) with 10 μl of Milli-Q water. Put each wet paper block on both the “+” and “-” ends of the gel on the strip (the pointed end of the IPG strip is at the anode (positive end)). Take care, not to damage the gel.
8. Place electrodes over the paper blocks and fix them in the focusing tray. Cover each strip with 2–3 ml of paraffin oil, close the lid, and switch on the instrument.
9. Start focusing the rehydrated strip with 500 W and higher than 50 μA and continue as described in Table 13.1.
10. Take out the IPG strips and discard the paraffin; transfer the IPG strips into the rehydration tray.
11. Wash the IPG strips very shortly with Milli-Q water to remove the residual paraffin.
12. After isoelectric focusing, the IPG Strip gels should either proceed immediately to the second-dimension separation or to store at $-80\text{ }^{\circ}\text{C}$.

Table 13.1 Program outline for 13 cm IPG strip (3–10 NL) focussing

Step	Voltage	Time (h:min)	kVh
1st step and hold	200	2:30	0.5
2nd step gradient	1000	1:20	0.8
3rd step gradient	8000	2:30	11.25
4th step and hold	8000	0:40	5.2

13.3.4.2 2-DE

Equilibration

1. Incubate the IPG strip for 12 min at RT in 2 ml of equilibration solution A.
2. Discard the solution A (work quickly to avoid unwilling contamination of the samples).
3. Incubate the IPG strip for 12 min at RT in 2 ml of equilibration solution B.
4. The strips can be now transferred to the second-dimension gel.

Gel Preparation

1. Clean the glass plates with ethanol or isopropanol using a lint-free cloth to remove dust and fibers.
2. Prepare 12 % acrylamide solution (acrylamide is neurotoxin, suitable precautions should be taken) for each 2D gel of 14 × 16 cm (1 mm thick) before the strip equilibration, and prepare 1 % agarose during the strip equilibration.
3. Pour the acrylamide solution between the glass plates until the solution is about 5 mm below the top of glass plate, carefully avoiding air bubbles.
4. Add 200 µl saturated isobutanol on top of the acrylamide gel to create a flat gel end.
5. Leave the gel to polymerize for at least 1 h at RT.
6. Remove isobutanol by washing the gel with running buffer (Sect. 13.2.4.2, step 7).
7. If the gel is not required immediately, store the gel in the casting apparatus at +4 °C for 72 h covered with running buffer and sealed with Parafilm.
8. Transfer the re-equilibrated IPG strips with forceps to the “+” end, cut the other gel strip end, place the cut end on the large glass plate near the (–) pole, and push the strip down using the flat side of a spatula till the strip touches the surface of the acrylamide gel (without damaging the gel).
9. Fill the strip slot with hot agarose to the edge of the glass plate avoiding air bubbles. Let the agarose polymerize completely before transferring the gel cassette to the electrophoresis chamber device.
10. Fill the buffer chamber with 1 × running buffer. Run the gels for 5 h at 160 V, 35 mA, and 5 W. Cool the chamber at 15 °C during the run.

11. Finish the run, when the bromophenol blue front reaches nearly the end of the gel (about 0.5 cm to the bottom).
12. Stop the run, remove the running buffer, disassemble the electrophoresis chamber device, and open the glass plates.

13.3.4.3 Protein Staining

Silver Staining for Analytic Gels (*see Note 4*)

1. Fix the gels before staining in fixing solution (*see Sect. 13.2.4.3.1, step 1*) for 30 min at RT or up to 72 h at 4 °C.
2. Soak the gels in glutaraldehyde solution for 30 min.
3. Wash the gel (2×) in Milli-Q water for 10 min.
4. Soak the gel in naphthalene disulfonic acid solution for 15 min, in order to obtain homogeneous dark brown staining of proteins.
5. Wash the gel (4×) in Milli-Q water for 15 min.
6. Stain the gel with freshly prepared ammoniacal silver nitrate solution for 30 min.
7. Wash the gel (4×) in Milli-Q water for 4 min.
8. Develop the gel with formaldehyde solution from 5 to 10 min or even shorter, depending on the protein load and background levels until the desired spot intensity is achieved.
9. Stop the process with stop solution and wash the gels (2×) in water for 10 min.

Silver Staining Compatible with Mass Spectrometry

Silver staining compared to Coomassie staining increases the sensitivity from 10 to 100-fold; however, glutaraldehyde from normal silver staining is not compatible with mass spectrometry, which causes protein–protein cross-linkage.

1. Remove the fixing solution (*see Sect. 13.2.4.3.2, step 2*) and wash the gel in 30 % ethanol for 10 min.
2. Remove the 30 % ethanol solution and wash the gel in Milli-Q water for 10 min or for 72 h in Milli-Q water.
3. Remove the water and incubate the gel in the sensitizer solution for 10 min.
4. Remove the sensitizer solution and wash the gel (2×) in Milli-Q water for 10 min.
5. Remove the water and equilibrate the gel in silver stain solution for 10 min.
6. Remove the silver stain solution and wash the gel in Milli-Q water for 1 min (washing longer than 1.5 min decrease sensitivity).
7. Remove the water and develop the gel in developer solution (*see Sect. 13.2.4.3.2, step 6*) from 3 to 7 min (*see Note 5*).

8. Add 15 ml of the ProteoSilver™ Stop Solution (see Sect. 13.2.4.3.2, step 7) to stop the developing reaction and incubate for 5 min. Bubbles of CO₂ gas will form in the mixture.
9. Remove the developer/stop solution and wash the gel in Milli-Q water for 15 min.
10. Store gel in Milli-Q water at 4 °C.

Coomassie Staining

1. Directly after electrophoresis, rinse gel in Milli-Q water for 1 min.
2. Incubate the gel in Coomassie staining solution at RT for 3–4 h with gentle agitation on an orbital shaker.
3. Incubate gel in destaining solution until spots are visualized.
4. Incubate the gels in 5 % acetic acid with gentle agitation and change the solution until the background is transparent.
5. Store the gel in Milli-Q water at 4 °C.

Post-fluorescence Gel Staining (SYPRO® Ruby Protein Gel Stain) (see **Note 6**)

SYPRO® Ruby protein gel stain is an irritant; therefore, it should be handled with care and using powder-free gloves.

1. Perform all fixation, staining, and washing steps with continuous, gentle agitation (e.g., on an orbital shaker at 50 rpm).
2. Place the gel into a clean polypropylene container (one gel per container, see **Note 7**) with 100 ml of SYPRO fixing solution for at least 15 min (2×) on orbital shaker.
3. Wash the gel in Milli-Q water for 10 min (3×). Incubate gels in SYPRO® Ruby gel stain for at least 3 h or overnight on an orbital shaker (use a sufficient staining volume so that staining solution flows over the surface of the gel).
4. Cover the gel container during staining and subsequent wash steps to exclude light, as with any other fluorescent stain.
5. Transfer the gel to a clean container with 100 ml of SYPRO wash solution and wash for 30 min.
6. Rinse the gel with Milli-Q water for 5 min (2×).
7. Visualize the gels stained with the SYPRO® Ruby protein gel stain are using imaging systems Typhoon at 450, 473, 488, or 532 nm and evaluate with 2-D gel evaluation software (e.g., ImageMaster 2D Platinum).
8. Store the gel wet, seal the stained gel in a small volume of fresh SYPRO® Ruby stain (approximately 5–10 ml, enough to keep the gel wet) containing a preservative, press out any air, and store at 4 °C.

13.3.5 Gel Scanning and Image Analyses

1. Transfer the gel to a suitable scanner, and set the scanner for generation of 16 Bit positive images with a resolution of 400 dpi in TIFF format.
2. Import the gel images into ImageMaster 2D Platinum software for spot matching and evaluation. Use viewing tools like contrast, overview, and profile, 3-D to assess image quality (poor quality images can cause insufficient spot detection and/or matching).
3. Place one or several landmarks to support automatic matching. Keep landmarks at a minimum. Place the landmark on well-defined smaller spots clearly identifiable on all gels in the dataset.
4. Use the ratio between means and gaps to find the most interesting potential marker proteins or use statistical analyses such as ANOVA, Wilcoxon, or Kolmogorov. They are automatically calculated within classes (experimental groups) and presented in the class analysis table (*see Note 8*).

13.3.6 Protein Identification and Data Interpretation

13.3.6.1 In-Gel Digestion of Proteins

1. Excise selected spots from the gel using a spot picker to minimize the amount of unstained gel area around the spot in order to minimize the amount of contaminants from the gel.
2. Transfer the excised gel plug into a clean, dust-free, correctly labeled 500 μ l Eppendorf tube, and add enough Milli-Q water to cover the gel piece. The spots can be stored at 4 °C at this point.
3. Remove the Milli-Q water, and wash each spot with 100 μ l ABC 100 using an ultrasonic bath (US) for 5 min. Remove the supernatant.
4. Destain the silver-stained spots by adding 30 μ l of destaining solution (Sect. 13.2.6 protein identification). After about 2 min, supernatant can be discarded (Gharahdaghi et al. 1999).
5. Wash the destained gel pieces with 100 μ l Milli-Q water followed by 100 μ l ABC 100 (US, 5 min each).
6. Prior to the reduction step, dehydrate the gel pieces using 100 μ l acetonitrile (US, 5 min), remove the supernatant, and evaporate the remaining acetonitrile in a SpeedVac (RT, 5 min)
7. Reduce disulphite bonds adding 50 μ l dithiothreitol (10 mM in 25 mM ammonium bicarbonate) to the sample and incubate at 56 °C for 60 min on a thermo mixer (*see Note 9*).
8. For the alkylation (*see Note 10*), add 50 μ l of 54 mM iodoacetamide solution after removing the dithiothreitol and incubate for 45 min at RT in the dark by covering the thermo mixer with a dark lid (Jiménez et al. 2001).
9. After alkylation, wash the spots with 100 μ l ABC 100 as before (US, 5 min).

10. Dehydrate the gel pieces again using 100 μl acetonitrile (US, 5 min) and evaporate the remaining drops in the SpeedVac.
11. Reconstitute the lyophilized trypsin (*see Note 11*) powder (Trypsin Gold, Promega, Germany) in 50 mM acetic acid to a concentration of 1 $\mu\text{g}/\mu\text{l}$ (trypsin stock). Before use, dilute 5 μl of trypsin stock solution with 120 μl water, 120 μl ABC 100, and 10 μl calcium chloride (120 mM) to a final concentration of 20 ng trypsin per μl . Perform the in-gel digest using 5 μl of this 20 ng/ μl solution (Schevchenko et al. 1999).
12. Allow the gel piece to swell in the trypsin solution at 4 $^{\circ}\text{C}$ for 20 min and remove the remaining solution from the gel spot.
13. Cover the rehydrated pieces with 30 μl of a 50 mM ABC for the digest and put them on the thermoMixer.
14. For the digestion procedure, program the thermoMixer at 37 $^{\circ}\text{C}$ for 8 h and then cool down to 4 $^{\circ}\text{C}$.
15. Extract the peptides from the gel three times using 30 μl of acetonitrile: water: TFA (50:45:5 v/v) supported by ultra-sonication for 15 min each time. Pool extracted peptides in a fresh 500 μl Eppendorf tube, and dry down the solution in a SpeedVac at 45 $^{\circ}\text{C}$ for 150 min.

13.3.6.2 ZipTip Purification

To eliminate salts from the sample and to concentrate the extracted peptides, carry out a ZipTip purification as follow (*see Note 12*):

1. Resuspend the dried samples in 10 μl 0.1 % TFA.
2. Freshly prepare a solution by mixing 350 μl ACN with 150 μl 5 % TFA for eluting peptides.
3. The pipette plunger is depressed to a dead stop. Using the maximum volume setting of 10 μl , elution solution is aspirated into the tip and dispensed to waste.
4. Equilibrate the ZipTip by aspirating and dispensing 3.3 μl of 0.1 % TFA and discard to waste container (*see Note 13*). Repeat this step three times (total 10 μl).
5. Bind the peptides to the ZipTip pipette tip by aspirate and dispense the sample ten times for maximum binding of peptides to the reversed phase material.
6. In a further step, wash the tip 3 \times with 3.3 μl 0.1 % TFA (total 10 μl).
7. Elute the peptides from the tip by carefully aspirating and dispensing 2 μl elution solution through the ZipTip pipette tip 2 \times without introducing air into the sample. Repeat once and add the second 2 μl eluent to the first vial (total 4 μl eluted peptides).
8. Spot desalted peptides (0.5 μl) onto a disposable PAC target (MALDI target plate pre-spotted with α -cyano-4-hydroxycinnamic acid) (*see Note 14*).

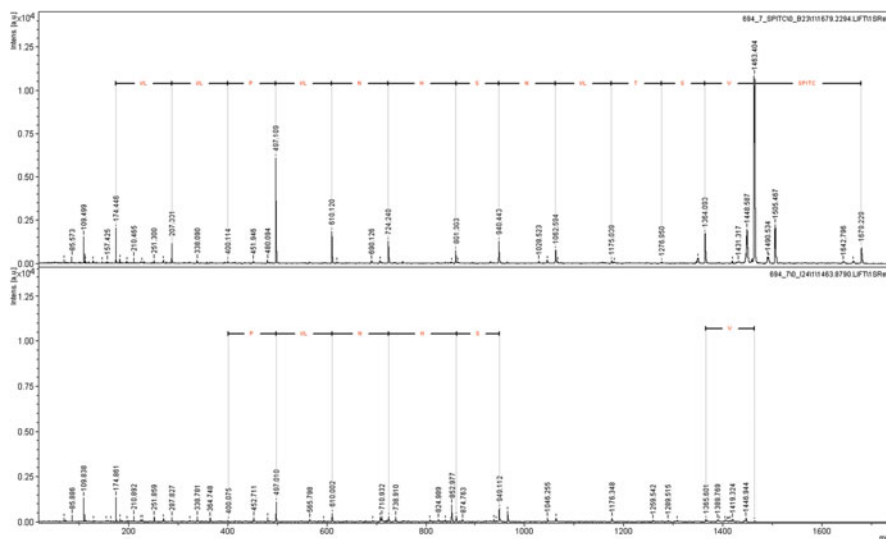


Fig. 13.2 Derivatization of internal peptides with SPITC

13.3.6.3 Derivatization of Peptides for De Novo Sequencing

In case of organisms or species with insufficient or poor database entries and spectral libraries, the only way to identify the proteins is to perform de novo sequencing. In order to improve the identification rate and obtain complete peptide sequences, the proteolytic peptides are derivatized by 4-sulphophenyl isothiocyanate (SPITC). This N-terminal chemical derivatization increases the structural information and the efficiency of post-source decay (PSD) fragmentation (Fig. 13.2). The reaction delivers a negative sulfonic acid group to the N-terminus of the proteolytic peptides leading to suppression of b-ions (*see Note 15*). Consequently, only positively charged ions (y-ions) are observed in MALDI-TOF/TOF spectra (Fig. 13.3) (Joss et al. 2006; Gevaert et al. 2001).

1. After the first spotting, dry the remaining 3.5 μl of the eluted peptides in the SpeedVac and dissolve in 10 μl 0.1 % TFA.
2. To avoid a too low pH for derivatization, repeat the ZipTip procedure without TFA in the elution solution.
3. Dry the eluted peptides again in the SpeedVac.
4. Prepare a 10 mg/ml solution of SPITC (*see Note 40*) in 20 mM NaHCO_3 buffer (pH 9.5) (Wang et al. 2004).
5. Add 4 μl the SPITC solution to the dried peptides and incubated for 30 min at 56 $^\circ\text{C}$.
6. Stop the reaction by acidification of the solution with 1 μl 5 % TFA.

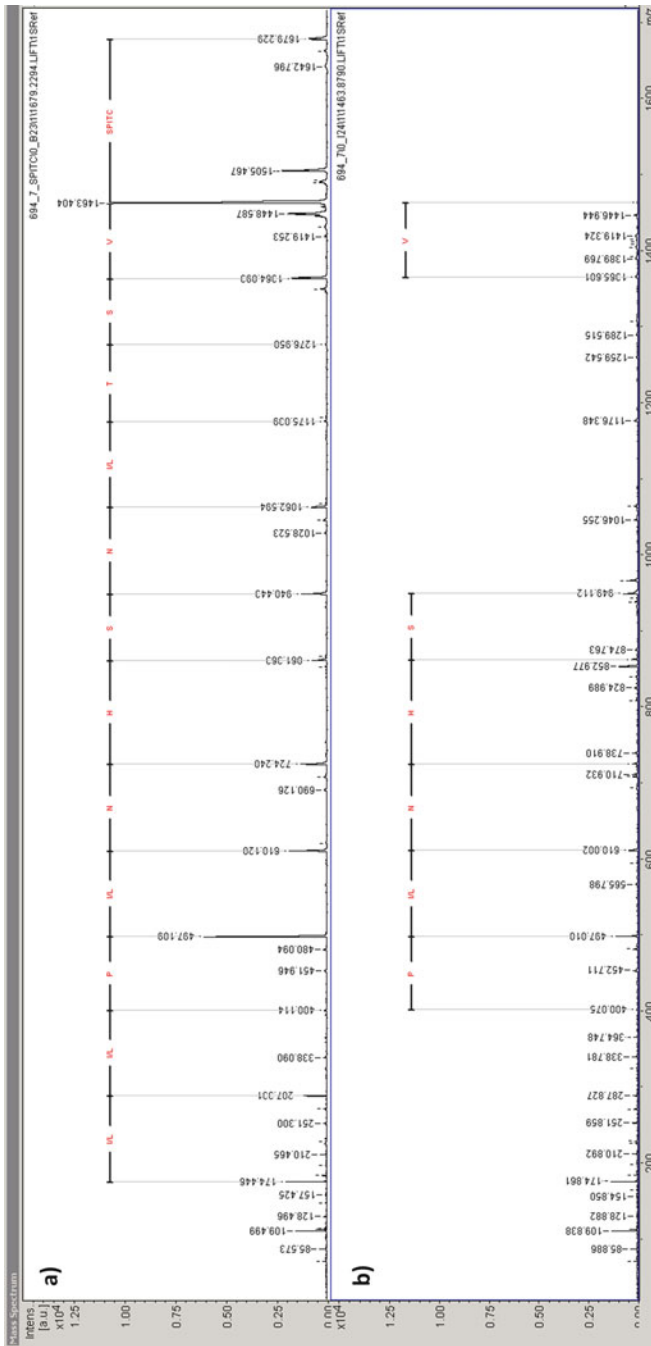


Fig. 13.3 MALDI-TOF/TOF spectra, (a) with and (b) without SPITC derivatization

7. Desalt the derivatized peptides using μ ZipTips C18 (see Sect. 13.3.6.2).
8. Spot the eluted, derivatized, and purified peptides on a PAC target as described above.

13.3.6.4 MALDI-TOF/TOF Analysis of Peptides

1. Acquire the MS and MS/MS spectra on an Ultraflex II MALDI-TOF/TOF instrument (Bruker Daltonics, Germany) in positive reflector mode with a nitrogen laser operating at 337 nm.
2. Prior to each measurement, calibrate the mass spectrometer using the pre-spotted calibration spots on the PAC target with an accuracy of 10 ppm (see Note 16–17).
3. Measure MS spectra in a mass range from 500–4400 Da.
4. Acquire MS/MS spectra of the peaks with the highest signal-to-noise ratio using the LIFT mode using the instrument default calibration.
5. Analyze spectra processing and peak annotation using FlexAnalysis 3.0 (Bruker Daltonics, Bremen, Germany).
6. For standard database searches, processed spectra are analyzed using a Mascot Server version 2.3 (Matrix Science, Boston, MA) enabling the search in customized databases (in this case genome sequences of *J. curcas*) and the software ProteinScape 2.1 (Bruker Daltonics, Germany) in a generic database built on genomic data of the organism using the following search parameters: (a) taxonomy all entries, (b) global modifications carbamidomethylation on cysteine, (c) variable modifications oxidation on methionine, (d) deamidation of asparagine and glutamine, (e) N-terminal pyro-glutamic acid and a N-terminal SPITC derivatization, (f) enzyme specificity trypsin, (g) charge state $z = 1$, (h) MS tolerance 100 ppm; MS/MS tolerance 1 Da, and (i) one missed cleavage allowed; significance threshold $p < 0.05$.
7. Carry out manually peptide de novo sequencing using FlexAnalysis. Use these sequences for a homology search by MS-homology search (<http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mshomology>) (Zakharova et al. 2014).

13.4 Notes

1. Add the ammonium/sodium hydroxide solution to the silver nitrate solution; a transient brown precipitate will form. After it cleared, bring to 500 ml with water. Drop the silver nitrate solution carefully into the solution.
2. Calculate the volume of sample solution that will contain the required amount of protein. Recommended sample loads for 13 cm Immobiline™ DryStrip

pH 3–10 NL for silver (analytic, 10–20 µg of protein) and Coomassie (preparative, 50–240 µg of protein) staining.

3. This end of the gel is usually more stable and less prone to damage. In all steps, move strip with the forceps. Strictly avoid touching the strip on the surface, not to damage the gel.
4. Silver nitrate is extremely sensitive and dirty. Be very careful in weighing and pouring solutions. After staining, wash both the inside and the outside of trays very carefully with deionized water to avoid tracking silver.
5. Three to seven min is sufficient to produce the desired staining intensity for most gels. Development times as long as 10–12 min may be required to detect band or spots with very low protein concentrations (0.1 ng/m²).
6. Post-fluorescence staining using SYPRO[®] Ruby or Orange stains can be used subsequent to staining with other fluorescence protein stains. However, SYPRO[®] Ruby stain should always be used the last, because its bright fluorescent signal tends to dominate the signal from other stains. SYPRO[®] Ruby stain does not work well for colorimetric stains such as Coomassie[®] and silver stains. It provides challenging characteristics like linear quantitation range from 0.25 to 1 ng and is capable of visualization of different protein types including glycoproteins, phosphoproteins, lipoproteins, calcium-binding proteins, fibrillary proteins, and proteins that are difficult to stain. SYPRO[®] Ruby stain can be multiplexed with other gel stains to obtain more information from one gel (Pro-Q[®] Diamond phosphoprotein gel stain, Pro-Q[®] Emerald 300 glycoprotein gel stain, Pro-Q[®] Sapphire and InVision[™] oligohistidine-tag gel stains, and Pro-Q[®] Amber transmembrane protein gel stain). Further, it is compatible with subsequent analysis of proteins by Edman-based sequencing or mass spectrometry.
7. Polypropylene or polycarbonate containers adsorb minimal amounts of the dye.
8. There are different tables providing the result of the analysis. To find the appropriate information, check the appropriate table that summarizes the data needed.

Spot table: Gives summarized information about enabled spots.

Gel analysis table: Gel analysis table and histograms provide valuable tools for checking spot detection, verifying matches, and analyzing expression changes within a class.

Class analysis table: Provides information about expression changes between classes (experimental groups).

9. Disulfide bonds stabilize the 3D structure of proteins and are very important for their biological functionality. An important step in peptide chemistry is the reduction of disulfide bonds by using reducing agents like dithiothreitol.
10. The reduced disulfide bonds can spontaneously be oxidized. Iodoacetamide is used in order to stabilize the thiol groups and prevent the building of disulfide bonds.
11. Trypsin as proteolytic enzyme hydrolyses peptide bonds selectively at the carboxyl side of lysine and arginine amino acids. Its optimum pH is between

- 7 and 8 (pH = 7.6). However, in order to prevent auto-proteolysis, 50 mM acetic acid should be used as storage solution. This prevents generating fragments, which would interfere with peptide analysis during ionization in mass spectrometry.
12. ZipTip is a sample preparation strategy based on reversed phased chromatography. The pipette tips contain C18-modified silica material. This step is used for desalting and concentrating the peptides (volume: 1–10 μ l).
 13. The equilibration of reversed phase material helps to reach a better adsorption of peptides to the C18 side chains.
 14. Matrix in MALDI ionization plays a key role and needs to be of highest purity.
 15. When using soft ionization techniques like MALDI or ESI, structural information by fragmentation of molecular ions of peptides is necessary. The fragmentation of peptides according to (Nishimura and Tojo 2014) is a post-source decay delivering predominantly b- and y-ions.
 16. Since the reaction is pH-dependent, it is important to maintain a pH value of 9.5.
 17. Calibration of mass spectrometer is a crucial point determining the accuracy of mass measurement and therefore the confident protein identification. Therefore, always calibrate the instrument prior to analyses.

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