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Two Dimensional Gel Electrophoresis Analysis of Mesenchymal Stem Cells

Monique Provansal, Christian Jorgensen, Sylvain Lehmann, and Stéphane Roche

Abstract

Proteomic analysis is a powerful tool to follow physiological modifications and phenotypes of mesenchymal stem cells (MSC). This approach generates informative data on expression and post-translational modifications of proteins which are of interest to assess the true potential of MSC in regenerative medicine. No matter the technologies used, proteomic analysis is always a challenge as the proteome is extremely diverse (in terms of constituents and concentrations), is changing with time, and is highly sensitive to pre-analytical conditions. In the framework of a European project (GENOSTEM <http://www.genostem.org/>), we have set up a multisite two dimensional gel electrophoresis (2DE) proteomic comparison of MSC. The goal is to compare cells from different origins, to follow their differentiation and to ultimately define a specific MSC proteomic signature. One important initial task is the optimization of 2DE protocols such that they are robust enough to be used in a multisite project. In this chapter, we detail these protocols which can be used not only for MSC but also for other cells in culture.

Key words: Mesenchymal stem cell, Proteomics, 2DE electrophoresis, Western blotting, Silver staining

1. Introduction

Stem cells and in particular mesenchymal stem cells (MSC) represent a major hope for regenerative medicine. While their existence in various tissues is undisputed, many questions remain regarding their physiology and their capability to give rise to various functional cell types. To define and exploit their true potential, additional studies on the molecular determinants of MSC isolation, renewal, and differentiation are needed.

Proteomic analysis relies on a set of powerful technologies to study cell physiology (1). This approach has access to expression

levels and post-translational modifications of proteins and adds to protein profiling as changes in the mRNA level do not always correlate with protein expression (2).

Two dimensional gel electrophoresis (2DE) is a core technology for proteomics. It involves the separation of proteins from complex mixtures based on two dimensions. First, proteins are separated by their isoelectric point on a pH gradient gel under denaturing condition. Second, the proteins in the pH gradient are further separated based on their molecular size by SDS-polyacrylamide gel electrophoresis (Fig. 1).

2DE has been used by us and others on MSC cultures to address the important questions mentioned above and to define a MSC-specific proteomic signature (3). However, it has been difficult to compare and combine the different published works which differ in terms of paradigms, protein extraction and 2DE protocols (1). In the present chapter, we detail the methods and

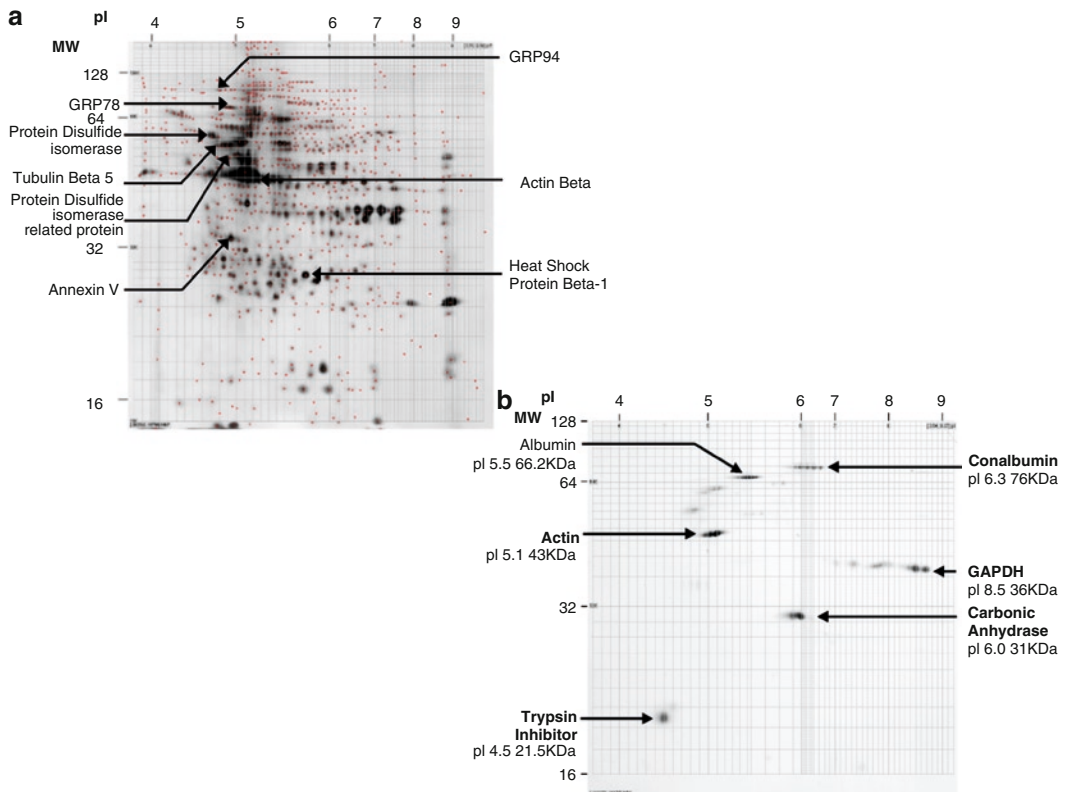


Fig. 1. (a) 2DE silver stained gel with the localization of identified proteins. Lysate of human primary bone marrow mesenchymal stem cells (MSC) was analysed by 2DE electrophoresis using a pH 3–10 IEF strip for the first dimension, a 12% SDS-PAGE for the second dimension. The identification of proteins was performed by peptide mass fingerprints after trypsin digestion and MALDI-TOF. (b) 2DE gel of IEF protein standard. The 2DE protein standard is first analysed alone, then mixed with a sample (not shown). This comigration is important for the calibration in mass and pHi. The analytical software computes automatically corresponding mass and pHi of others spots.

protocols for the 2DE analysis of MSC which was designed through a program that included a multisite comparison of MSC (3–5).

2. Materials

2.1. Cell Lysis

1. Orthovanadate solution: to prepare 100 mL of 10 mM Na_4VO_3 (Sigma) in ddH_2O , boil the solution during 5 min until complete dissolution. The solution will appear yellow to uncoloured. Cool it and adjust the volume to 100 mL with ddH_2O . The solution can be stored in a glass bottle for a few months at room temperature (RT) or at -20°C aliquoted in polypropylene tubes.
2. Wash solution: add 500 μL of orthovanadate solution and 50 μL of protease inhibitor (Sigma) to 50 mL of phosphate buffer saline without Mg/Ca (PBS).
3. Lysis buffer (6, 7): 8 M Urea (GE Healthcare), 1 M Thiourea (GE Healthcare), 4.8% CHAPS (Sigma), 50 mM DTT (Sigma) (see Note 1). When urea is put in solution, the volume increases significantly. Therefore, to prepare 20 mL of lysis buffer, dissolve the components first in 10 mL of water and adjust to 20 mL with ddH_2O afterward (see Note 2). Store lysis buffer at -20 or -80°C (see Note 3). Avoid repeated freeze–thaw cycles.

2.2. Removal of DNA and Protein Quantification

1. Spermine solution: 2.4 M Spermine dihydrate (Sigma). Prepare 5 mL of solution in ddH_2O . Aliquot by 20 μL in eppendorfs, store at -20°C .
2. PlusOne 2-D Quant Kit (GE Healthcare).

2.3. Isoelectric Focusing

1. Rehydration buffer: 9.8 M Urea, 4.8% CHAPS, 50 mM DTT. Store in 250 μL aliquot at -20 or -80°C . To prepare 20 mL of buffer, follow the same recommendations as for the lysis buffer.
2. Low viscosity paraffin oil with a density from 0.827 to 0.89 (Sigma).

2.4. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. SDS-equilibration buffer: 6 M urea, 50 mM Tris–HCl pH8.8, 30% Glycerol, 2% SDS, trace of bromophenol blue. Prepare 200 mL, aliquot by 10 mL and store at -20°C .
2. Gel buffer: 1.5 M Tris–HCl, pH8.8, 0.4% SDS. Prepare 100 mL, store at 4°C .
3. Ammonium persulfate: prepare 10 mL at 10% in water and make 500 μL aliquots stored at -20°C for single use.
4. Running buffer 10 \times : 250 mM Tris, 1.92 M Glycine, 1% SDS. Prepare 3 L, store at 4°C .

5. Agarose: prepare 50 mL of 1% low melting agarose in 1× running buffer, add a few drops of bromophenol blue to colour the solution. The solution needs to be heated at 90°C to dissolve the agarose. Can be stored at 4°C and reheated at 90°C before use.
6. Unstained molecular weight standard (Bio-Rad).
7. MultiTemp III cooling device (GE Healthcare).

2.5. Gel Staining

2.5.1. Silver Staining

1. All solutions are made fresh in ddH₂O; all references are from Sigma. Prepare 250 mL of each solution per gel.
2. Buffer A: 50% ethanol, 10% acetic acid.
3. Buffer B: 5% ethanol.
4. Buffer C: 0.02% sodium thiosulfate anhydrous.
5. Buffer D: 0.2% AgNO₃.
6. Buffer E: 3% sodium carbonate decahydrate, 0.0005% Formaldehyde, 2% buffer C.
7. Buffer F: 1.4% EDTA-Na.

2.5.2. Coomassie Staining

1. Buffer A: 50% ethanol, 10% acetic acid.
2. Buffer B: 5% ethanol.
3. Colloidal Coomassie stain (Fermantas).

2.5.3. Western Blotting

1. The procedure for Western blotting is adapted to the NuPAGE system and gel (Bis/Tris 12% gel); all reagents and associated buffers are from Invitrogen.
2. The MOPS running buffer is diluted to 1× as recommended by the manufacturer.
3. The transfer buffer is prepared as follows: 5% transfer buffer stock solution and 20% ethanol in ddH₂O.
4. Before transfer, the PVDF membrane is incubated in 100% ethanol for 10 s and rinsed in the transfer buffer for 5 min on a rocking platform.
5. PBS-T: Stock solution: 1% Tween-20 in 10× PBS (without Ca and Mg). Dilute at 1/10 in ddH₂O for final solution.
6. Blocking buffer: 5% (w/v) non fat dry milk in PBS-T.
7. Primary and secondary antibody dilutions are performed in the blocking buffer. A multiplex solution of primary antibodies is prepared using anti-14-3-3 (1:1,000, Santa Cruz, Clone K19), anti-Actin (1:500, Santa Cruz), and Sox-9 (1:1,000, kind gift from F. Poulat, IGH, CNRS) antibodies. Secondary antibody multiplex solution is prepared using anti-rabbit and anti-mouse antibodies (Jackson ImmunoResearch) (both at 1:10,000).
8. Enhanced chemiluminescent (ECL) (SuperSignal West Pico) (Pierce Biotechnology). ECL prepared as described by manufacturer by mixing 2 mL of enhancer and 2 mL of

peroxide solution. The ECL camera system is ChemiDoc XRS from Bio-Rad.

2.6. Scan of the 2DE Gels, Detection and Spot Analysis

1. Epson 1680PRO scanner.
2. Greyscale chart (Kodak).
3. Labscan 3 software (GE Healthcare).
4. ImageMaster 2DE Platinum version 6 (GE Healthcare).
5. Progenesis SameSpot version 2 (Non Linear Dynamics).
6. Hierarchical Clustering Explorer v3.5 (8).

2.7. Molecular Weight and Isoelectric Point Calibration

1. 2DE protein standard (Bio-Rad).

2.8. Protein Identification

1. Mass spectrometry qualified tube, 1.5 mL polypropylene tube (TreffLab).
2. 5%, 20% Formic acid, and 100 mM NH_4CO_3 are made fresh.
3. Sequencing grade trypsin (Promega). Reconstitute to a concentration of 0.1 $\mu\text{g}/\mu\text{L}$ using the buffer supplied by the manufacturer. Stored in 10 μL aliquot at -20°C . The working solution is obtained by mixing 2 μL of trypsin to 8 μL of 100 mM NH_4CO_3 .
4. ZipTip C18 column (Millipore).
5. Benchtop speedvac "CentriVac" (Labconco).
6. Ultraflex Mass Spectrometer (Bruker).

3. Methods

3.1. Cell Lysis

1. Wash twice cultured cells with the wash solution at RT.
2. Remove all residual buffer. The cell layer needs to be near dry (see Note 4).
3. Put the lysis buffer directly on the cells in the culture dish. For a 100 mm Petri dish, use 200 μL of lysis buffer. Do the lysis at RT (see Note 5).
4. Using a clean cell scraper, collect the lysate in the dish, and transfer it to a 1.5 mL eppendorf tube.
5. Do not spin or cool the tube and freeze it as soon as possible at -80°C .

3.2. Removal of DNA and Protein Quantification

1. Thaw samples at RT.
2. Add 1 volume of spermine dihydrate to 100 volumes of sample and incubate at RT during 1 h. DNA that is charged negatively will associate with spermine.

3. Centrifuge at $21,000 \times g$, 1 h, at 20°C to pellet spermine and DNA. Use a refrigerated centrifuge to control the temperature which otherwise increases during centrifugation. Do not set up the temperature below 20°C , otherwise the urea will precipitate.
4. Collect and aliquot supernatant in 1.5 mL eppendorf tubes.
5. Use a fraction of the lysate to measure the protein concentration with the PlusOne 2-D Quant Kit following the supplier recommendations. Briefly, 10 μL of samples (in duplicates) is used and quantify using a standard curve with 2, 5, 10, 20, and 40 μg of proteins.

3.3. Isoelectric Focusing

1. Thaw samples (after spermine treatment) and rehydration buffer to RT.
2. For 13 cm isoelectric focusing (IEF) dry strips, a loading volume of 250 μL is optimal. The concentration of the protein in the samples will indicate if a dilution with the rehydration buffer is needed in order to have 30 μg of protein in 250 μL for silver staining and 300 μg of protein in 250 μL for Coomassie staining.
3. Add 1 μL of IPG ampholine buffer to the 250 μL diluted samples. Mix the samples well and spin briefly to remove bubbles. The IPG buffer is a mix of soluble ampholines which affect sample solubility and focalisation (see Note 6).
4. Deposit each sample along an IPGphor “sarcophagus,” avoiding bubbles. Cover the sample with the IEF dry strip. The acrylamide gel of the strip must be in direct contact with the samples (the acrylamide face is at the bottom). The “plus” or acidic part of the IEF dry strip must be at the head part of the sarcophagus. Cover samples and strips using low viscosity paraffin oil.
5. Place the sarcophagus in the IPGphor apparatus. Set the temperature to 20°C and the current to 60 μA . The program should include passive rehydration, active rehydration and focalisation as follows: 7 h without current, 3 h at 30 V, 2 h at 200 V, 1 h at 1,000 V, 1 h at 3,000 V, then a gradient to reach 8,000 V in 2 h and a final phase of 7 h at 8,000 V.
6. At the end of the focalisation, we expect to have reached 8,000 V with less than 50 μA and a total close to 76,000 $\text{V} \times \text{h}$ (see Note 7).
7. The IPGphor stops automatically at the end of the program. Recover the IEF strips rapidly. Put them in a plastic tube and store them at -20°C . There is no need to rinse the strips.

3.4. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. The SDS-PAGE gels (1.5 mm thick, 12% acrylamide) for the second dimension of the 2DE need to be prepared in advance. The following protocol is adapted to the Hoeffer SE-600 apparatus. One important factor to obtain optimal gels is to

- use clean and unscratched glass plates. Avoid using detergents to clean the plates, prefer ddH₂O and ethanol.
2. For one gel, prepare an acrylamide solution as follows: mix 15 mL of acrylamide/bisacrylamide solution (37.5:1, T40%), 12.5 mL of gel buffer and 22.25 mL of ddH₂O. Add 25 µL of TEMED and 250 µL of 10% ammonium persulfate. Pour the solution between the glass plates, leaving 5 mm on the top empty for the IEF strip. No stacking gel is needed. Pour isopropanol on the top of the gel to flatten the surface. To reduce the amount of unpolymerized acrylamide monomers, let the gel polymerize at least 2 h. The gels can be prepared 24 h in advance and kept at 4°C in a wet atmosphere.
 3. Thaw the IEF strips and incubate them for 30 min on a rocking platform in 5 mL of SDS-equilibration buffer supplemented with 0.25% DTT. Remove the buffer and replace it with 5 mL of SDS-equilibration buffer supplemented with 4.5% iodoacetamide for another 30 min on the rocking platform.
 4. Before using the SDS gel, verify the absence of bubbles. Pour off isopropanol and rinse the top of the gel with ddH₂O.
 5. Using small tweezers, place the IEF strip on the top of the gel. Usually, the “plus” end of the strip is positioned on the left of the gel. On the right part, insert a small 1.5 mm thick piece of plastic to create a molecular weight (MW) well. To secure the strip to the gel, pour warm agarose on the top paying attention not to leave any bubbles between them. Place the gel at 4°C for 5 min to solidify the agarose.
 6. Remove the above piece of plastic to create a small well in the agarose. Add to this well 10 µL of unstained molecular weight standard and seal the well with a drop of warm agarose.
 7. Mount the gel on the apparatus and connect it to the power supply. Run the gel for 17 h at 70 V. To obtain an optimal migration, the electrophoresis apparatus needs to be associated to a MultiTemp III cooling device, maintaining the system at 10°C.

3.5. Gel Staining

3.5.1. Silver Staining

1. At the end of the SDS-PAGE, carefully disassemble the plates and put the gel in the buffer A in order to fix proteins. Incubate for a minimum of 2 h on a rocking platform (Fig. 1a).
2. Rehydrate the gel in buffer B for 15 min on the rocking platform and rinse 3 times for 5 min with ddH₂O.
3. Incubate the gel in buffer C for 2 min on the rocking platform and rinse 3 times for 30 s with ddH₂O (9).
4. Incubate the gel in buffer D for 25 min on the rocking platform and rinse twice for 1 min with ddH₂O (9).
5. Stain proteins using buffer E (9). The staining takes place from 2 to 10 min. It needs to be stopped at the optimal time

in order to obtain the best signal/background ratio (see Note 8).

6. To stop the staining, replace the buffer E with the buffer F for 10 min in a rocking platform.
7. Dry gels or conserve them in 10% glycerol in a sealed plastic pouch.

3.5.2. Coomassie Staining

1. At the end of the SDS-PAGE, carefully disassemble the plates and put the gel in buffer A in order to fix proteins. Incubate for a minimum of 2 h on a rocking platform.
2. Rehydrate the gel in buffer B for 15 min on the rocking platform and rinse 3 times for 5 min with ddH₂O.
3. Incubate the gel in colloidal Coomassie stain for at least 1 h and remove excess stain by rinsing 2–3 times in ddH₂O.
4. The gels can be dried or conserved in 10% glycerol in a sealed plastic pouch.

3.5.3. Western Blotting

1. For Western blotting, the first dimension of the 2DE is performed using a 7 cm IEF strip. The only modification in the protocol (when compared to the 13 cm IEF strip) is the lower amount of samples (125 µL) and IPG buffer (0.5 µL) used (Fig. 2).
2. The second dimension is performed with the NuPAGE system with one well Bis/Tris 12% gels.
3. After the final incubation in the SDS-equilibration buffer, carefully position the IEF strip on the top of the gel. Usually, the “plus” end of the strip is positioned on the left of the gel. To secure the strip to the gel, pour warm agarose prepared in 1× MOPS buffer on the top, paying attention not to leave any bubbles between them. Place the gel at 4°C for 5 min to allow the agarose to solidify. The NuPAGE migration at 70 V will take 2 h. No cooling system is necessary for this migration.

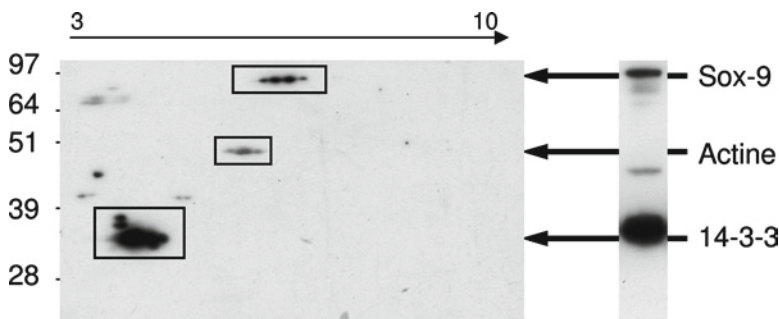


Fig. 2. 2DE Western blotting of BM-MSC. Proteins were separated by 2DE electrophoresis using a 7 cm dry strip pH 3–10 for the first dimension and a Bis/Tris 12% NuPAGE for the second dimension. After transfer on PVDF membrane, targeted proteins are detected by multiplex Western blotting using antibodies against 14-3-3, actin, and Sox-9 proteins. Note the different spots of 14-3-3 and Sox-9 indicating the presence of post-translational modified isoforms.

4. At the end of the migration, open the gel cassette, discard the IEF strip and the agarose, then incubate the gel a few minutes in the transfer buffer. The transfer, using pads and Whatman paper sheets on a PVDF membrane, is performed as per the manufacturer instruction. The transfer is performed at 30 V for 1 h at RT.
5. After transfer, rinse the membrane for 30 s in ddH₂O and incubate it for 30 min in the blocking buffer on a rocking platform.
6. Discard the blocking buffer and incubate the membrane in the primary antibodies for 1 h at RT.
7. Wash 4 times for 5 min with PBS-T and incubate the membrane with the secondary antibodies for 1 h at RT.
8. Wash 4 times for 5 min with PBS-T, incubate with the ECL mix for a few minutes and detect the signal using the CCD camera. Save the digital file. Include in the file name the gel number, date, nature of sample and nature of antibodies.

3.6. 2DE Gel Scan, Detection and Protein Spot Analysis

1. Scan the stained gels at 300 dots per inch using the Labscan 3 software after a calibration procedure using the greyscale chart.
2. For spot detection and quantitation of the silver stained gels, use the ImageMaster 2DE Platinum or the Progenesis SameSpot software. A value for each spot is obtained by an integration of the grey level of each spot and normalisation with the total spot volume. In this method, the volume of each spot is divided by the total volume of all of the spots in the gel.
3. Run three to five replicates with independent samples to ensure reproducibility of the results. The software includes many functions for selected spots and can perform statistical comparison between experimental conditions. Select a gel with the maximum number of spots to serve as a reference.
4. As a threshold for significant differential spot expression, use a fold increase/decrease ratio of 1.7 and a *p*-value of 0.05 as calculated by Progenesis SameSpot.
5. A global analysis of the data can be performed using the Hierarchical Clustering Explorer 3.0 software (8). This software relies on an average linkage hierarchical clustering algorithm based on the centred Manhattan Distance coefficient as the similarity metric.

3.7. Molecular Weight and Isoelectric Point Calibration

1. Run a 2DE gel with a mixture of 10 µg of whole cell extract and 5 µL of 2DE standard (Fig. 1b).
2. These standard spots are associated with their respective MW and pI. After a comparison step with this gel, the software calibrates all homologous gels with pHi and MW.

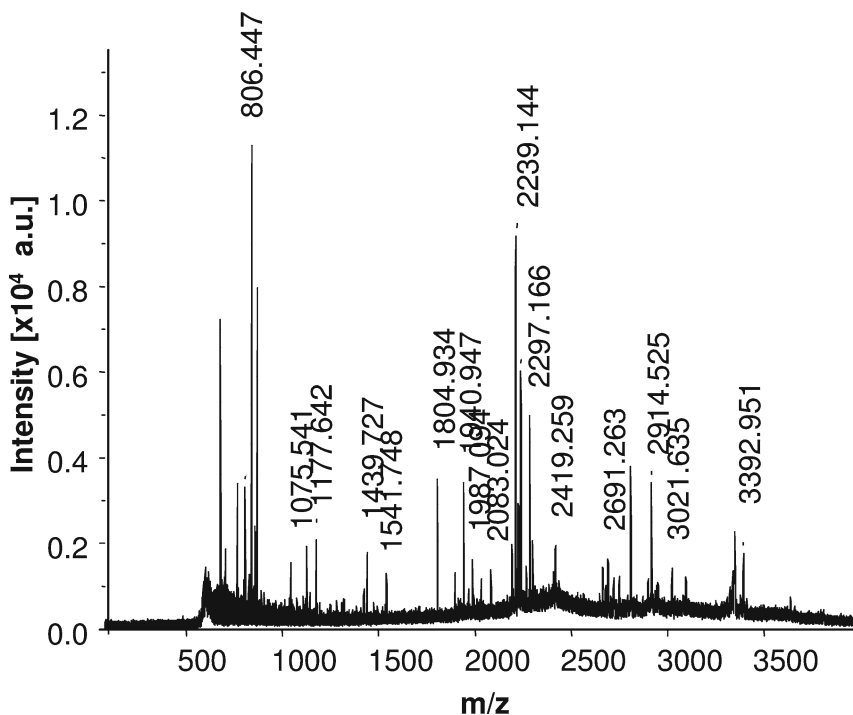


Fig. 3. Mass spectrum of trypsin in gel digestion of candidate protein. A selected spot is excised from the gel, dehydrated, and rehydrated with a sequencing grade trypsin. After digestion, the peptides are extracted, purified, and submitted to MALDI-TOF mass spectrometry. After database query, the protein can be identified with a good probability.

3.8. Protein Identification

1. Excise spots from fresh Coomassie stained 2DE gels run with 300 μg of proteins. Put them separately in 1.5 mL specific tubes (Fig. 3).
2. Incubate pieces of gels with 15 μL of 100 mM NH_4HCO_3 for 10 min. Add 15 μL of acetonitrile for 10 min. Remove and discard supernatant, repeat the procedure.
3. Use a speedvac to evaporate the remaining buffer. Rehydrate in 10 μL of trypsin working solution and digest overnight at 25°C.
4. Extract the digested peptides as follows: add 10 μL of 100 mM NH_4HCO_3 for 10 min then 10 μL of acetonitrile for 10 min. Collect the supernatant, repeat the procedure twice, and combine the supernatants. Add 10 μL of 5% formic acid for 10 min then 10 μL of acetonitrile for 10 additional min. Collect the supernatant, repeat the procedure twice, and combine all the supernatants.
5. Dry the supernatants using the speedvac and put back in solution with 10 μL of 20% formic acid. Desalt using a ZipTip C18 column as recommended by the manufacturer.

6. Determine peptide masses in the positive-ion reflector mode using the Ultraflex mass spectrometer. Compare peptide mass fingerprints to mammalian databases (SWISS PROT and Trembl) using MASCOT (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF) and Aldente (<http://www.expasy.org/tools/aldente/>) algorithms with one miss trypsin cleavage site and a mass deviation smaller than 20 ppm. The probabilistic score of MASCOT/Aldente is required for the identification of an unnamed protein.

4. Notes

1. 2DE results are affected by the composition of the lysis buffer used. This is in relation with differences in protein solubility vs. extraction and with the negative effect of salt on protein focalisation in 2DE. Other types of lysis exist for example design to solubilised membrane proteins (7).
2. Do not heat the lysis buffer in order to speed up dissolution as it will alter urea lysis properties.
3. To optimize the use of the lysis buffer it is recommended to store it in 250 μ L aliquots.
4. Do one dish at a time to reduce the risk of complete drying of the cells. Dry cells disrupt and fix the proteins and limit extraction efficacy.
5. Low temperatures induce urea crystallisation and reduce extraction efficacy.
6. It is recommended to use the same pH range of ampholines in the IPG buffer and in the IEF strip. Increasing the volume of ampholines may alter focalisation performance.
7. If the power supply do not reach 8,000 V and/or 76,000 V \times h, the focalisation might be poor. The decrease of final voltage or V \times h value is due to the interferences of salts and amino acids originate from culture medium and PBS.
8. Above 10 min, no additional spots will generally appear. The staining is the most critical step of 2DE electrophoresis.

Acknowledgments

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