[13] Proteomic Analysis of the Mammalian Cell Nucleus

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Introduction

Proteomics is the study of the ''proteome'' or the entire protein complement of a genome, a term first coined in 1997.[1,2] It is now known, however, that the proteome is far more complex than previously suggested by the one gene, one protein adage. The proteome consists of all proteins present in a cell or organism at a given time, including not only those translated directly from genetic material, but also the variety of modified proteins arising from events such as alternative splicing of transcripts and extensive post-translational processing.[1] With the advancement and rapid progress in genome sequencing, many new genes have been discovered; however, several of them have no known function or their exact functional roles are poorly understood. In this respect, proteomics can provide valuable information and correlate genome sequence information and the cellular behavior at the molecular level.

In response to chronic moderate hypoxia, cells adjust their normoxia set point such that reoxygenation-dependent relative elevation of pO_2 results in perceived hyperoxia. We have reported that perceived hyperoxia causes growth arrest and differentiation in cardiac fibroblasts. Such a response to oxygen was associated with an increase in the expression of a number of proteins (e.g., p21, cyclin D, cyclin G) localized in the nucleus.[3] To obtain a global view on the response of nuclear proteins in cardiac fibroblasts to perceived hyperoxia, we utilized a subcellular proteomics approach. The methodological approach is described in this article.

Due to the complex nature of the eukaryotic cells, a single proteomics approach cannot be applied to understand the complete proteome. In order to get the entire subset of proteome, it is essential to characterize the proteins of a particular organelle. A subcellular proteomics approach provides a direct correlation of organelle-specific (e.g., nucleus) gene expression and a possible regulatory mechanism of biological process related to a metabolic state of the cell.

[1] D. K. Arrell, I. Neverova, and J. E. Van Eyk, *Circ. Res.* **88,** 763 (2001).
[2] V. C. Wasinger, S. J. Cordwell, A. Cerpa-Poljak, J. X. Yan *et al.*, *Electrophoresis* **16,** 1090 (1995).
[3] S. Roy, S. Khanna, A. A. Bickerstaff, S. V. Subramanian *et al.*, *Circ. Res.* **92,** 264 (2003).

0076-6879/04 $35.00

The nucleus is the organelle, which hosts most of the genetic information and serves as a hub for gene expression. At present about 964 human nuclear proteins are listed in the SWISS-PROT protein database. Such organelle-specific proteomics-based studies can decipher the different subset of proteins compared to the whole cell lysate. This can be helpful in understanding abnormal protein expression under any disease condition.

This article describes approaches to perform proteomics of the mammalian cell nucleus. Major steps involve separation and identification of nuclear proteins using two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS) followed by data analysis and interpretation using advanced bioinformatic techniques (Fig. 1).

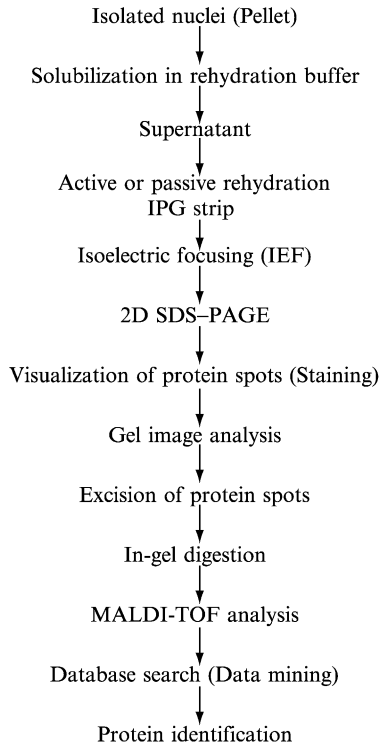

FIG. 1. A schematic flow chart of 2D gel electrophoresis and MALDI-based proteomics assay.

Sample Preparation

The method of sample preparation depends on the specific aim of research. A proper sample preparation method is vital to reduce the complexity of the protein mixture and therefore to the success of the experiment. The protein fraction for 2DE analysis must be devoid of salts, lipids, and nucleic acids and should be prepared in low ionic strength buffer to maintain native charges and the solubility of proteins.

A standard sample preparation method involves protein solubilization in standard 2D solution *(see solution 1)*. This step can be considered a good starting point for sample preparation.[4,5]

Nuclear Protein Solubilization

Cardiac fibroblast nuclei are prepared as described previously and stored at -80° in aliquots.[6]

1. Thaw the nuclei pellet and resuspend in 200 μl of rehydration buffer *(see solution 2)* by vortexing.
2. Add 5 μg/ml DNase I and 5 μg/ml RNase A[4,7] to the sample and incubate at room temperature for 20 min.
3. Sonicate the sample using a sonic dismembrator (Fisher Scientific, Pittsburgh, PA) at maximum power output for 30 s.
4. Remove the insoluble part by a single centrifugation step at 13,000 rpm on a microfuge (Eppendorf).
5. Collect the supernatant in a fresh tube and use for rehydration.

Caution: Nuclear samples contain high amounts of nucleic acids that can cause problems during sample solubilization procedure and subsequently interfere with the separation of proteins by isoelectric focusing (IEF). The nucleic acids can also be removed by using nucleic acid complex-forming agents, for example, spermine or carrier ampholytes.[4] The composition of sample solubilization solution can be determined empirically.

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis is a powerful proteomics method for separating complex mixtures of proteins into individual components depending on the electric charge and size.[8] This results in an array of

[4] R. J. Simpson. Cold Spring Harbor Laboratory Press, New York, 2003.
[5] B. R. Herbert, M. P. Molloy, A. A. Goooley, B. J. Walsh *et al.*, *Electrophoresis* **19,** 845 (1998).
[6] Y. M. Janssen and C. K. Sen, *Methods Enzymol.* **300,** 363 (1999).
[7] M. Chevallet, V. Santoni, A. Poinas, D. Rouquie *et al.*, *Electrophoresis* **19,** 1901 (1998).
[8] P. H. O'Farrell, *J. Biol. Chem.* **250,** 4007 (1975).

protein spots that are assigned in the *x* and *y* coordinates, as compared to the protein bands in one-dimensional gel electrophoresis. Each spot represents one protein and thousands of proteins can be separated on a single gel. Also, 2DE is a powerful technique for the detection and analysis of various posttranslational modifications in proteins.[9]

Rehydration

1. Load approximately 180 μl of protein sample (supernatant) in a channel in a rehydration tray.
2. Place a immobilized pH gradient (IPG, pH 3–10, 11 cm) strip gel side down in the same channel of the rehydration tray that contains the protein sample.
3. Overlay the entire IPG strip with 2 ml of mineral oil (Bio-Rad, Hercules, CA).
4. Incubate the strip for 12 to 16 h at room temperature for passive rehydration. (*Note*. The incubation step of the IPG strip can also be carried out under low voltage (50 V) to facilitate the entry of high molecular weight proteins.)

During the entire rehydration process, the strip is overlayed with mineral oil to prevent evaporation of sample and the precipitation of urea.

The First Dimension: Isoelectric Focusing of Proteins

In this method, the separation of proteins is based on their net charge (p*I* value). Most of the modern protocols utilize dehydrated, precast IPG strips available from a number of suppliers (e.g., Bio-Rad, Amersham Biosciences). After the strips are rehydrated, they are transferred to focusing trays.

1. First wet two paper wicks with 8 μl of nanopure water.
2. Position the wicks over each electrode inside the focusing tray (Bio-Rad).
3. Now place the rehydrated IPG strip over the wicks.
4. Cover the strip with 2 ml of mineral oil.
5. Place the focusing tray inside the protein IEF cell (Bio-Rad).
6. Program the IEF cell as step 1 on 250 V with a linear voltage ramp, step 2 on 8000 V with linear ramp, and step 3 at 8000 V up to a total of 70,000 volt-hours.

[9] K. Gevaert and J. Vandekerckhove, *Electrophoresis* **21,** 1145 (2000).

7. After completion of the IEF run, remove the IPG strip with forceps and allow the oil to drain completely.

A typical IEF protocol runs through a series of voltages that increase gradually to a set focusing voltage, which can be held for the required time in hours. The total length of focusing time depends on the size of the IPG strip, pH gradient, sample load, composition of sample, and rehydration solution and should be optimized as per requirements of the experiment.

IPG Equilibration

Immediately after the IEF is over, the IPG strips are equilibrated to solubilize the focused proteins and allow SDS binding as done for one-dimensional SDS–PAGE.

1. Place the IPG strip (gel side up) in channel of rehydration tray.
2. Incubate the strip at room temperature in 4 ml of buffer I *(see solution 3)* for 10 min with gentle agitation.
3. Decant the DTT buffer and place the strip into another channel.
4. Again incubate the strip in buffer II *(see solution 4)* for 10 min.

The equilibration step should also be optimized for each sample.

Second-Dimension SDS–PAGE

1. After equilibration, position the strip on top of the precast 4–20% acrylamide Criterion gels (Bio-Rad) with the plastic backing against the plate.
2. Slide the strip between gel plates and place it directly on top of the second-dimension gel.
3. Overlay the strip with 0.5% molten agarose prepared in SDS–PAGE running buffer containing a small amount of bromphenol blue.
4. Place the gel into a gel running chamber with electrophoresis buffer.
5. Load 8 μl of precision-plus protein molecular weight standards (Bio-Rad) into the single well.
6. Run the gel on 200 V for 60 min at room temperature.

Visualization of Protein Spots by Staining 2D Gels

A large number of protocols are available to detect proteins on 2D gels, and the method of choice depends on the sensitivity of detection, compatibility of mass spectrometry, and objectives of the experiment. This section describes three of the most widely used protocols to stain 2D gels.

Coomassie Brilliant Blue R-250 Staining

a. After the second-dimension SDS–PAGE is over, place the gel in staining solution *(see solution 5)*.
b. Stain with gentle agitation for 3 h to overnight.
c. Destain the gel with an ample volume of Coomassie Blue R-250 (Bio-Rad) destaining solution *(see solution 6)*.
d. Store the destained gels in pure Milli Q water for further analysis.

Sypro Ruby Protein Gel Stain (Bio-Rad)

With the recent development of the ruthenium-based fluorescent dye Sypro Ruby, the sensitivity to detect protein spots on the gels has been enhanced up to the level of silver staining.

a. Fix the gel for 30 min in fixing solution *(see solution 7)*.
b. Remove the fixing solution and incubate the gel in 100 ml of Sypro Ruby stain (Bio-Rad) for 3 h to overnight.
c. Wash the gel for 1 h in an aqueous mixture of 10% methanol and 7% acetic acid.
d. Store the gel in pure Mili Q water for further analysis.

Mass Spectrometry Compatible Silver Staining

A number of kits are available from a variety of the sources such as Bio-Rad, Pierce, and Invitrogen that can be used for this type of staining. Each kit comes with a detailed procedure optimized by the manufacturer.

Image Analysis of Two-Dimensional Gels

Once the 2D gels are ready, the next step of proteomics is gel image acquisition and analysis. Images of the gels, stained with Coomassie, Sypro Ruby, or silver stain, are acquired on the Versadoc 3000 gel imaging system (Bio-Rad) interfaced with computers. Using commercially available 2D analysis software systems, such as PDQuest (Bio-Rad), it is easy to (1) detect and quantify even faint protein spots, (2) make comparisons between 2D gel images, and (3) detect the expression patterns with up- or downregulation of target proteins. A representative gel image of a cardiac fibroblast nuclear protein is shown in Fig. 2.

Once the images are taken, they need to be edited for proper orientation and size by rotation or crop functions. The contrast level and other parameters for the gel images are optimized based on faint spots. The

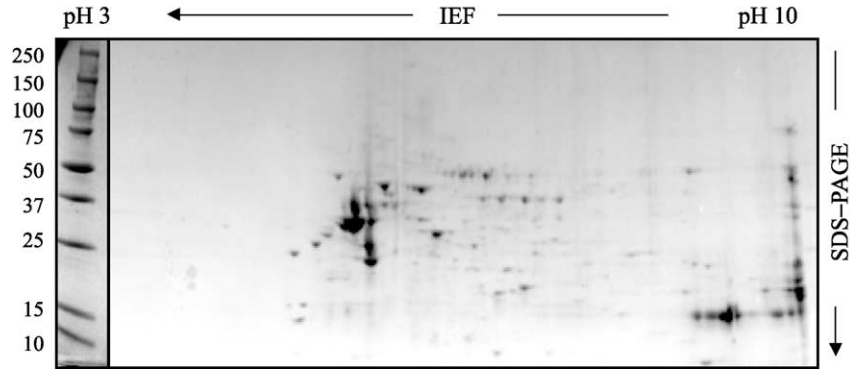

FIG. 2. Separation of cardiac fibroblast nuclear proteins by 2D gel electrophoresis. The first dimension was run on an 11-cm immobilized pH gradient (IPG; pH 3–10 nonlinear) strip. For the second dimension, a 4–20% SDS–PAGE was used. Following electrophoresis, the gel was stained with Coomasie Blue R-250. IEF, isoelectric focusing.

PDQuest program allows all these manipulation for transformations of the gel images.

Spot Detection

The software uses an automated detection system based on unique algorithms. The different detection parameters, such as sensitivity, spot size, background subtraction, and smoothness, are optimized manually to locate the proteins of interests.

Spot Matching

Once the spots are identified on all the gels in the data set, spot matching can be performed. A well-resolved gel is used as a reference gel for this purpose to determine the marker proteins. After the protein spots are selected and matched, data can be normalized, and spot characteristics and statistical data are produced with the help of a software program. Once information about the protein spots on the gel has been generated, it is added to the gel images using an annotation system available with the software.

Spot Cutting

After the gel image analysis, gels are placed on the spot-cutting platform of the Proteome Works spot cutter (Bio-Rad) interfaced with a camera and a computer system. The gels are reimaged, and protein spots

are selected for the spot cutter. After assigning each spot, the automated robotically controlled spot cutter cuts the spots and deposits them to an assigned well of a 96-well plate.

In-Gel Digestion

The MassPREP station robotic protein handling system (Perkin-Elmer, Boston, MA) is used for fully automated destaining, reduction, alkylation, and in-gel digestion of proteins. The whole process is controlled and programmed through the MassPREP software system.

a. First destain the excised gel pieces (containing proteins) by incubation in 50 m*M* ammonium bicarbonate and 15 m*M* potassium ferricyanide and 50 m*M* sodium thiosulfate for 15 min at room temperature.

b. Remove the destaining solution and then incubate the gel fragments for 30 min at 56° in 25 μl of 10 m*M* dithiothratol (DTT) in 100 m*M* ammonium bicarbonate.

c. Replace the DTT solution by 25 μl of 55 m*M* iodoacetamide in 100 m*M* ammonium bicarbonate, and incubate the gel fragments for 20 min at room temperature.

d. Wash the gel pieces for 10 min with 100 μl of 100 m*M* ammonium bicarbonate and for 10 min with 100 μl of 50 m*M* ammonium bicarbonate and 30% acetonitrile.

e. Gel pieces obtained from 2DE are only treated with 50 m*M* ammonium bicarbonate and 30% acetonitrile for 20 min at room temperature because disulfide bond-containing proteins are already reduced and alkylated after IEF.

f. Once the gel pieces are dried, initiate the digestion by the addition of 25 μl of a solution of 50 m*M* ammonium bicarbonate containing sequencing grade trypsin (Promega, Madison, WI) at 6 ng/μl.

g. Extract all the peptides with 30 μl of 1% formic acid and 2% acetonitrile at room temperature.

Control extractions (blanks) should be performed using pieces of gels devoid of proteins. Digestion of 96 samples takes from 8 to 10 h according to the specificity of the protocol.[10]

Peptide Mass Fingerprinting by Matrix-Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry

MALDI-MS is used to determine the accurate mass of a group of peptides derived from a protein by digestion with a sequence-specific protease, for example, trypsin, and thus generating a peptide mass map or

peptide mass fingerprint. Depending on a specific trypsin cleavage site at the amino acids arginine and lysine, the masses of tryptic peptides can be predicted theoretically for all the proteins in the database. The experimentally acquired peptide masses are compared with those obtained theoretically, and the protein can be identified correctly if there are enough peptide matches for a protein in the database. Proteins separated on a 2D gel system provide the required information about protein molecular weights and isoelectric points, which in turn help in the MALDI analysis and final identification.

Before peptide mass fingerprinting, 1 μl of peptide-containing solutions from each sample is mixed with an equal volume of matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% AcN, 0.1% TFA) and then deposited on a 96-well MALDI target plate and dried at room temperature.

MS measurements are conducted with a matrix-assisted laser desorption ionization/time of flight (TOF) mass spectrometer MS (MicroMass, Altrincham, UK) equipped with a 337-nm nitrogen laser. Analyses are performed in the reflectron mode with an accelerating voltage of 20 kV, a delayed extraction parameter of 100–140 ns, and a low mass gate of 850 Da. The laser power is set slightly above threshold (10–15% higher than the threshold) for molecular ion production. Automated spectral data acquisition is programmed with MssLynx software (Micromass). Spectra are obtained by the summation of several consecutive laser shots. Masses of the peaks are extracted from the spectra and used for protein identification using the SmartIdent peptide mass fingerprint tool. The data search is conducted against SWISS-PROT and TrEMBL databases.[10]

Reagents

Urea, thiourea, DTT, TBP, CHAPS, DTT, leupeptin, pepstatin, aprotinin, DNase I, RNase A, α-cyano-4-hydroxycinnamic acid, acetonitrile, ammonium bicarbonate, potassium ferricyanide, sodium thiosulfate (Sigma, St. Louis, MO)

Trypsin (Promega, Madison, WI)

Tris base, glycerol, methanol, acetic acid (Fisher Scientific, Pittsburgh, PA)

Carrier ampholytes, bromphenol blue, IPG strips, criterion gels, mineral oil, prestained protein standards, iodoacetamide, Coomassie Blue R-250 (Bio-Rad, Hercules, CA)

MS-compatible silver-staining kit (Invitrogen, Carlsbad, CA)

[10] J. R. Chapman, "Mass Spectrometry of Proteins and Peptides: Methods in Molecular Biology." Humana Press, New Jersey, 2000.

Solutions

Solution 1: 8 *M* urea, 50 m*M* DTT, or 2 m*M* TBP (tributylphosphine), 4% CHAPS, 0.2% carrier ampholytes, 0.0002% bromphenol blue
Solution 2: 5 *M* urea, 2 *M* thiourea, 4% CHAPS, 40 m*M* Tris, 2 m*M* TBP, or 100 m*M* DTT, 0.2% carrier ampholytes, 0.0002% bromphenol blue, 1 μg/ml leupeptin/pepstatin/aprotinin
Solution 3: 6 *M* urea, 2% SDS, 0.05 *M* Tris–HCl, pH 8.80, 20% glycerol, and 2% DTT
Solution 4: 6 *M* urea, 2% SDS, 0.05 *M* Tris–HCl, pH 8.80, 20% glycerol, and 2.5% iodoacetamide in place of DTT
Solution 5: aqueous solution of 0.1% Coomassie Blue R-250 (w/v) in 40% methanol (v/v) and 10% acetic acid
Solution 6: aqueous solution of 40% methanol and 10% acetic acid
Solution 7: aqueous solution of 10% methanol and 7% acetic acid.

[14] Oxygen-Dependent Regulation of Erythropoiesis

By JAROSLAV JELINEK and JOSEF T. PRCHAL

Introduction

Hypoxia inducible factor 1, subunit α (HIF-1α) is a central molecule in oxygen sensing and the response to hypoxia. HIF-1α is unstable in normoxic conditions: specific proline hydroxylases convert its proline residues 402 and 564 to hydroxyprolines, the hydroxylated HIF-1α is captured by the von Hippel Lindau protein (pVHL), ubiqutinated, and degraded in proteasomes. Under hypoxic conditions, HIF-1α is not recognized by pVHL; it forms a heterodimer with the HIF-1β subunit and acts as a transcription factor stimulating the expression of genes involved in glucose metabolism, angiogenesis, erythropoiesis, blood coagulation, and other essential processes.[1]

This article describes methods for the quantitative detection of mRNA for hypoxia-regulated genes in blood cells and for assessing the response of red blood cell progenitors to erythropoietin—a hormone playing an essential role in the adaptation to hypoxia. Real data of studies of the congenital defect of the germ-line mutation of *VHL* gene leading to the constitutive

[1] G. L. Semenza, *Curr. Opin. Cell Biol.* **13,** 167 (2001).

0076-6879/04 $35.00