Video Article

Two-dimensional Gel Electrophoresis Coupled with Mass Spectrometry Methods for an Analysis of Human Pituitary Adenoma Tissue Proteome

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URL: https://www.jove.com/video/56739
DOI: doi:10.3791/56739

Keywords: Cancer Research, Issue 134, Two-Dimensional Gel Electrophoresis, Isoelectric Focusing, Acrylamide Gel Electrophoresis, Silver Staining, Coomassie Staining, Image Analysis, Peptide Mass Fingerprint (PMF), Tandem Mass Spectrometry (MS/MS), Pituitary Adenoma, Proteome

Date Published: 4/2/2018

Citation: Zhan, X., Huang, Y., Long, Y. Two-dimensional Gel Electrophoresis Coupled with Mass Spectrometry Methods for an Analysis of Human Pituitary Adenoma Tissue Proteome. *J. Vis. Exp.* (134), e56739, doi:10.3791/56739 (2018).

Abstract

Human pituitary adenoma (PA) is a common tumor that occurs in the human pituitary gland in the hypothalamus-pituitary-targeted organ axis systems, and may be classified as either clinically functional or nonfunctional PA (FPA and NFPA). NFPA is difficult for early stage diagnosis and therapy due to barely elevating hormones in the blood compared to FPA. Our long-term goal is to use proteomics methods to discover reliable biomarkers for clarification of PA molecular mechanisms and recognition of effective diagnostic, prognostic markers and therapeutic targets. Effective two-dimensional gel electrophoresis (2DE) coupled with mass spectrometry (MS) methods were presented here to analyze human PA proteomes, including preparation of samples, 2D gel electrophoresis, protein visualization, image analysis, in-gel trypsin digestion, peptide mass fingerprint (PMF), and tandem mass spectrometry (MS/MS). 2-Dimensional gel electrophoresis matrix-assisted laser desorption/ionization mass spectrometry PMF (2DE-MALDI MS PMF), 2DE-MALDI MS/MS, and 2DE-liquid chromatography (LC) MS/MS procedures have been successfully applied in an analysis of NFPA proteome. With the use of a high-sensitivity mass spectrometer, many proteins were identified with the 2DE-LC-MS/MS method in each 2D gel spot in an analysis of complex PA tissue to maximize the coverage of human PA proteome.

Video Link

The video component of this article can be found at https://www.jove.com/video/56739/

Introduction

PA is a common tumor that occurs in the human pituitary gland in the hypothalamus-pituitary-targeted organ axis systems, which play important roles in the human endocrine system. PA includes clinically functional and nonfunctional PAs (FPA and NFPA)[1,2]. NFPA is difficult in early stage diagnosis and therapy because of only slightly elevated hormone levels (*e.g.*, LH, and FSH) in blood compared to FPA, which has significantly increased levels of corresponding hormones in blood[3,4,5]. The clarification of molecular mechanisms and discovery of effective biomarkers has important clinical significance in the diagnosis, therapy, and prognosis of NFPA. Our long-term goal is to develop and use proteomic methods to study NFPA for the discovery of reliable biomarkers to clarify its molecular mechanisms, and recognize effective therapeutic targets as well as diagnostic and prognostic markers. 2-DE coupled with MS methods have been extensively used in our long-term research program regarding human PA proteome[1,2,6,7], including establishment of proteome reference maps[3,8], analysis of differentially expressed protein profiles[9,10,11,12,13], hormone variants[14,15], post-translational modifications such as phosphorylation[14] and tyrosine nitration[16,17,18], the proteomic variation of invasive relative to noninvasive NFPAs[19], and the proteomic heterogeneity of NFPA subtypes[13], which led to the discovery of multiple important pathway networks (mitochondrial dysfunction, cell cycle dysregulation, oxidative stress, and MAPK signaling system abnormality) that are altered in NFPA[13,19,20].

2DE separates proteins according to their isoelectric point (pI) (isoelectric focusing, IEF) and molecular weight (via sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE)[1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23]. This is a common and classical separation technique in the field of proteomics, since the introduction of the concepts of the proteome and proteomics in 1995[24]. MS is the crucial technique for finding out the identity of 2DE-separated proteins, including PMF and MS/MS strategies. The very rapid development of MS instruments, especially in the aspects of detection sensitivity and resolution, in combination with the improvement of LC system, greatly improves the identity of low or extremely low abundance proteins in a proteome to maximize the coverage of a proteome. It also challenges our traditional concepts that only one or two proteins are present in a 2D gel spot in an analysis of complex human tissue proteome and provides an opportunity to identify multiple proteins in a 2D gel spot in an analysis of complex human tissue proteome and maximize the coverage of NFPA proteome.

Here we describe detailed protocols of 2DE-MALDI MS PMF, 2DE-MALDI MS/MS, and 2DE-LC-MS/MS which have been successfully used in the analysis of human NFPA proteome. The protocols include preparation of samples, first dimension (isoelectric focusing, IEF), second dimension (SDS-PAGE), visualization of proteins (silver staining and Coomassie blue staining), image analysis of 2D gel, in-gel trypsin digestion, purification of tryptic peptides, PMF, MS/MS, and database searing[3,8,25,26]. Moreover, this protocol easily translates for the analysis of other human tissue proteomes.

Protocol

The present protocol follows the guidelines of the Xiangya Hospital Medical Ethics Committee of Central South University, China. A head cap and gloves should be worn for the entire experimental procedure to avoid keratin contamination from skin and hair[8].

1. Preparation of Samples

1. Collect PA tissues (0.2 - 0.5 mg) from the neurosurgical department. Immediately freeze in liquid nitrogen, and then transfer to -80 °C for storage.
2. Add 2 mL of 0.9% NaCl in deionized distilled water (ddH_2O). Use this solution to lightly wash the blood from the tissue surface (3x).
   Note: ddH_2O with a conductivity of 18.2 MΩ/cm is used throughout the protocol. Some of the tissuemight be lost when washing off blood from a tissue's surface.
3. Add a volume (10 mL; 4 °C) of the solution containing 2 M acetic acid and 0.1% (v/v) β-mercaptoethanol for every 0.5 - 0.6 g of tissue, homogenize (1 min, 13,000 rpm, 4 °C, 10x) with a tissue homogenizer, sonicate the homogenate for 20 s, lyophilize, and store at −80 °C.
4. Measure the protein content of the lyophilized, homogenized samples using bicinchoninic acid (BCA) assay kit.
   Note: BCA quantification is not an absolute quantification, and the measured result will be altered with a different technician and the experimental agent. A fixed concentration sample standard should be used for different experiments. Therefore, the final loading amount of proteins for each 2D gel will be determined with the silver-stained or Coomassie-stained good-resolution image in the pre-designed experiments.
   1. Add about 300 µg of the lyophilized homogenized sample to one volume (282 µL) of protein-extracting buffer (8 M urea and 4% CHAPS), followed by standing for 2 h, sonicating in a water bath for 5 min, rotating for 1 h, sonicating again in the water bath for 5 min, rotating again for 1 h, and centrifuging at 15,000 x g for 20 min.
   2. Prepare BSA standard solutions with concentrations as mentioned: 0, 25, 125, 250, 500, 750, 1,500, 2,000 µg/mL with the commercial BSA standard (2 mg/mL).
   3. Mix BCA reagent A and B (A:B = 50:1) as BCA working solution prior to use.
   4. Add 0.1 mL of the sample or the standard solution to 2 mL of BCA working solution in a microfuge tube, followed by mixing and then incubating at 37 °C for 30 min. Finally cool at room temperature for 10 min and measure $A_{562\ nm}$ O.D. value.
   5. Calculate the standard linear line ($A_{562\ nm}$ vs. BSA concentration) to obtain a regression equation for calculating the sample protein content with A_{562nm} value.
5. Use 150 µg of the protein equivalent lyophilized sample for silver staining, or 500 µg of protein for Coomassie staining , for an 18-cm immobilized pH gradient (IPG) strip pH 3-10 nonlinear (NL).
   Note: The IPG dry strip is 0.5-mm thick and 3-mm wide, with different lengths including 7, 11, 13, 18, and 24 cm, and different pH ranges including pH 4-5, 4-7, 6-9, and 3-10 in either a linear or nonlinear pH gradient. The IPG buffer used must fit the strip[27,28].
6. 1.6.Add 250 µL of extracting buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 100 mM DTT (add prior to use), 0.5% v/v pharmalyte (add prior to use), and a trace of bromophenol blue).
7. Keep the solution below 30 °C, followed by vortexing for 5 min, sonicating for 5 min, and rotating for 50 min.
8. Add 110 µL of rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 60 mM DTT (add prior to use), 0.5% v/v IPG buffer (add prior to use), and a trace of bromophenol blue). Sonicate for 5 min. Then rotate the sample for 50 min, vortex for 5 min, and centrifuge for 20 min at 15,000 x g.
9. Collect the supernatant (350 µL) as the protein sample solution[8].

2. Two-Dimensional Gel Electrophoresis

1. **IEF (First dimension): Perform IEF on the isoelectric focusing system as described below.**
   1. Add 350 µL of the protein sample solution in the slot of an 18-cm IPG strip holder.
   2. Put an 18-cm IPG strip gel-side-down onto the protein sample solution (avoid bubbles).
   3. Add 3-4 mL of mineral oil to cover the IPG strip.
   4. Assemble the IPG strip holder into the isoelectric focusing systemwith the pointed end on the back (+) plate and square end on the front (−) plate.
   5. Rehydrate overnight (~18 h at room temperature).
   6. Set the IEF parameters: maximum current 30 µA per strip, 20 °C; 250 V, 1 h, 125 Vh, step and hold; 1,000 V, 1 h, 500 Vh, gradient; 8,000 V, 1 h, 4,000 Vh, gradient; 8,000 V, 4 h, 32,000 Vh, step and hold; and 500 V, 0.5 h, 250 Vh, step and hold. Let it run up to a total time of 7.5 h and 36,875 Vh.
   7. After IEF, take out each IPG strip and remove the extra mineral oil with an insoluble paper towel. Now wrap the strip in a sheet of plastic wrap, and store at −80 °C.
      Note: The IPGstrip holder should be cleaned with the IPGstrip holder cleaning solution and distilled water.
2. **SDS-PAGE (Second dimension): Perform SDS-PAGE in a Vertical Cell Electrophoresis System**
   Note: Each SDS-PAGE gel size should be 255 x 190 x 1 mm.
   1. Use a multi-gel caster to cast 12% PAGE resolving gels. For casting 12% PAGE gels, perform the following steps.

1. Add 270 mL of ddH_2O, 150 mL of 1.5 M Tris-HCl (pH 8.8), 180 mL of 40% (w/v) acrylamide/bisacrylamide stock solution (29:1, 40% w/v acrylamide and 1.38% w/v N, N'-methylenebisacrylamide), 3 mL of 10% ammonium persulfate, and 150 µL of TEMED to make the gel solution in a beaker. Mix gently and avoid bubbles.
2. Pour the gel solution gently into the multicasting chamber up to the expected gel height (19 cm).
3. Immediately add about 3 mL ddH_2O on each resolving gel to cover the gels. Let the gel polymerize for about 1 h.

2. Prepare 20 L of electrophoresis buffer (25 mM Tris, 192 mM glycerine, and 0.1% SDS). Fill the electrophoresis separation unit buffer tank with this buffer.
3. Take out the focused IPG strips from the freezer, and equilibrate the strips for 10 min by rocking gently in 4 mL of reducing equilibrium buffer (375 mM Tris-HCl (pH 8.8), 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 2% w/v DTT (add prior to use), and a trace of bromophenol blue).
4. Equilibrate for 10 min by gently rocking the reduced IPG strips in 4 mL alkylation equilibrium buffer (375 mM Tris-HCl (pH 8.8), 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 2.5% w/v iodoacetamide (add prior to use), and a trace of bromophenol blue).
5. During gel equilibrium, disassemble the multicasting chamber, and take out the prepared resolving gel cassette. Rinse 3x with ddH_2O. Remove excess ddH_2O with an insoluble paper towel, and put in a gel stander.
6. Rinse the equilibrated IPG strips with electrophoresis buffer, and remove excess liquid on the IPG strip surface with the insoluble paper towel.
7. Put an IPG strip onto the resolving SDS-PAGE gel, and let the IPG strip's plastic side contact the longer glass plate and the pointed end to the left.
8. Pour 3-4 mL of hot 1% agarose in SDS electrophoresis buffer (~80 °C) quickly to seal the IPG strip on the top of each SDS-PAGE gel, and put the top-side of IPG strip down to the top of the shorter glass plate without bubbles, and then polymerize for 10 min.
9. Insert the assembled gel cassette vertically between plastic gaskets in the vertical electrophoresis system. Put the top of the gel with the IPG strip next to the cathode (−).
10. Adjust the level of electrophoresis buffer to immerse the gel cassette.
11. Connect and set the Power Supply/Control Unit in constant voltage mode, and run at 200 V for about 370 min while monitoring the dye.
12. After running, take out the gel cassette from the electrophoresis system, and gently remove the gel from the gel cassette, avoiding tearing the gel, followed by staining of 2DE-separated proteins.

3. **Staining of 2DE-Separated Proteins**
   1. **Silver Staining**
      1. Gently transfer the gel with two hands in a tray containing 250 mL of fixation solution (50% methanol (v/v) and 5% (v/v) acetic acid), and gently shake the gel for 20 min.
      2. Discard the solution and add 250 mL of 50% (v/v) methanol in the tray and gently shake for 10 min.
      3. Discard methanol and add 250 mL of ddH_2O in the tray. Shake gently for 10 min.
      4. Gently shake the gel for 1 min in 250 mL of sensitization buffer containing 0.02% (w/v) sodium thiosulfate, and discard the liquid.
      5. Gently shake the gel in 250 mL of ddH_2O for 1 min (repeat one more time). Discard the liquid after each step.
      6. Gently shake the gel for 20 min in 250 mL silver reaction solution (0.1% (w/v) silver nitrate with 200 µL 37% (v/v) formaldehyde added prior to use).
      7. Gently shake the gel in 250 mL of ddH_2O for 1 min (repeat one more time). Discard the liquid after each step.
      8. Shake the gel gently in 250 mL development solution (3% (w/v) sodium carbonate with 100 µL 37% (v/v) formaldehyde added prior to use) until the desired intensity of staining is obtained (commonly 1-3 min), then discard the liquid.
      9. Gently shake the gel for 10 min in 250 mL stopping solution (5% (v/v) acetic acid) and discard the liquid.
      10. Gently shake for 5 min in 250 mL of ddH_2O and discard the liquid.
      11. Keep the gel in 250 mL storing solution of 8.8% glycerol at 4 °C.

   2. **Coomassie brilliant blue staining**
      1. Prepare Coomassie staining solution by dissolving Coomassie brilliant blue G250 0.3 g, ammonium sulfate 25 g, and phosphoric acid 25 mL in ddH_2O and up to a total volume of 200 mL. Prior to use, add methanol 50 mL.
      2. Add 250 mL of Coomassie staining solution to the gel and gently shake at room temperature until clear protein spots appear (~2-4 h). Discard the liquid.
      3. Add 250 mL of ddH_2O and shake slowly for 5 min, then twice more. Discard the liquid.
      4. Add 250 mL of destaining solution (20% (v/v) ethanol) and shake slowly until the background color become almost colorless, and discard the liquid.
      5. Replace with the fresh destaining solution when the old one is getting dark, and discard the liquid.
      6. Add 250 mL of ddH_2O and shake slowly for 5 min. Discard the liquid.
      7. Add 250 mL of storing solution (8.8% glycerol) and keep at 4 °C.

4. **Two-dimensional gel electrophoresis image analysis**
   1. Digitize the stained gels with a flatbed scanner.
   2. Input the image into the 2D gel image analysis system.
   3. Detect and quantify each spot using a 2D gel image analysis software according to the manufacturer's protocol.
   4. Match the spots between different gels.

3. Identification of 2DE-Separated Proteins with MS

1. **Preparation of tryptic peptide mixture**
   Note: Siliconized or low-retention tubes and pipette tips should be used to avoid any loss of proteins or peptides.
   1. **Silver-gel destaining**

1. Excise the gel spots into a 1.5 mL siliconized tube, and wash in 500 µL ddH_2O 6 times.
2. Put the washed gel into a new 1.5 mL siliconized tube, and mince it into many small pieces (0.5-1 mm^3).
3. Destain the gel pieces in 20 µL of the destaining solution that mixes a part of 30 mM potassium ferricyanide and a part of 100 mM sodium thiosulfate (1:1) prior to use to let the brownish color disappear (commonly 1-2 min). Discard the liquid.
4. Wash the gel pieces with 20 µL of ddH_2O 5-6 times to let the yellow color disappear. Discard the liquid after each step.
5. Add 20 µL of 200 mM ammonium bicarbonate to the gel pieces, and stay for 20 min. Wash the gel pieces with ddH_2O (20 µL, 1 time). Discard the liquid.
6. Dehydrate the gel pieces with 30 µL acetonitrile at least twice to let the gel pieces turn an opaque white, and vacuum-centrifuge dry for 30 min.

2. **Coomassie brilliant blue gel destaining**
   1. Cut gel spots into a 1.5 mL siliconized tube and wash with 500 µL ddH_2O at least 3 times.
   2. Put the washed gel into a new 1.5-mL siliconized tube, and mince it into several pieces (0.5-1 mm^3) with a pipette tip.
   3. Destain the gel in 50-100 µL destaining solution (1:1 v/v mix 200 mM NH_4HCO_3 with acetonitrile) depending on the gel volume, incubate at 37 °C in a water bath. Repeat and add fresh destaining solution until the color disappears (~1 h). Discard the liquid after each step.
   4. Wash the gel pieces once with 20 µL of ddH_2O.
   5. Dehydrate the gel pieces in 100 µL acetonitrile (at least twice) to let the gel pieces turn an opaque white.
   6. Vacuum dry for 30 min.

3. **In-gel trypsin digestion of the proteins in the dried gel pieces**
   1. Dissolve 20 µg (833 pmol) of lyophilized trypsin powder in 100 µL of 50 mM acetic acid.
   2. Dilute 20 µL of trypsin solution (200 ng/µL) to 16 ng/µL with 230 µL of 50 mM ammonium bicarbonate.
   3. Add 20-30 µL (0.32-0.48 µg) of the diluted trypsin solution to each tube containing dried gel pieces. Incubate for 18-20 h in a 37 °C in water bath. Let the solution stand at 4 °C for 30 min.
   4. Centrifuge the solution gently for 10 s. Sonicate in a water bath for 5-6 min at 30 °C and then centrifuge at 12,000 x g for 2 min.
   5. Collect the supernatant containing tryptic peptide mixture into a 0.5-mL siliconized tube.

4. **Purification of tryptic peptide mixture with C18 microcolumn.**
   1. Wash each C18 column with acetonitrile (10 µL, 5 times), and then with 50% acetonitrile (10 µL, 5 times).
   2. Equilibrate with 0.1% trifluoro acetic acid (TFA) (10 µL, 5 times).
   3. Pipette the sample up and down through the prepared C18 column for 15 times.
   4. Wash the sample 2x with 10 µL 0.1% TFA.
      NOTE: The purified tryptic peptides are retained in the C18 column.
   5. Add a volume (6 µL) of solution (85% v/v acetonitrile/0.1% v/v formic acid) in a clean 0.5-mL siliconized tube, pipette this solution gently and slowly up and down through peptide-C18 beads for 10x to wash the bond peptides into the solution, air-dry, and store (−20 °C) for MS analysis.

2. **MS Analysis**
   1. **MALDI-MS PMF analysis**
      1. Add 4 µL of 85% v/v acetonitrile/0.1% v/v TFA to dissolve the purified tryptic peptide mixture. Load 2 µL dissolved tryptic peptide mixture onto a MALDI plate and immediately add 2 µL of a-cyano-4-hydroxycinnamic acid (CHCA) solution that contains 10 g/L in 500 mL/L acetonitrile 1 mL/L TFA, then air-dry.
      2. Load the MALDI plate with tryptic peptide mixture into a MALDI-MS mass spectrometer.
         Note: The instrument parameters are set as reflector mode with 20 kV accelerating voltage, positive polarity, and 160 ns delay extraction time. Each MS spectrum is acquired with 200 laser shots with a range of m/z 1,000 to 4,000 after external calibration with the standard peptide mass.
      3. Use a MS spectrum-processing software to process each MS spectrum, including baseline correction, noise removal (5%), and peak deisotoping.
      4. Correct the mass list from each MS spectrum with the removal of contaminating masses from trypsin, matrix, keratins, and other unknown contaminants in the parallel of blank-gel experiments.
      5. Input the corrected mass list into an PMF-based protein searching software to identify proteins in the Swiss-Prot protein database with the following search parameters, including PMF search type, human, trypsin with 1 maximum missed cleavage, fixed carbamidomethyl cysteine, variable methionine oxidation, monoisotopic, peptide mass tolerance 50 ppm, peptide charge state (1+), and signal to noise (S/N) 5.0.
      6. Identify proteins with statistically significant protein scores, and where protein score = -10 × log(p), where p is the probability that the observed match is a random event.

   2. **MALDI-MS/MS analysis**
      1. Add 4 µL of 50% v/v acetonitrile/0.1% v/v TFA to dissolve the purified tryptic peptide mixture. Each purified tryptic peptide mixture (0.5 µL) was spotted on a 384-well MALDI-plate, immediately spotted 0.5 µL of saturated CHCA matrix in 50% acetonitrile/0.1% TFA on the top of the peptide sample, and then dried in the air.
      2. Let the MALDI-TOF-TOF MS/MS to be managed automatically, using 100 laser shots to acquire one MS1 spectrum, and accumulating 6 repeated MS1 spectra into one synthetic MS1 spectrum.
      3. Set 4 most-intense precursor ions from each synthetic MS1 spectrum for MS/MS analysis. Use 100 laser shots for each precursor ion to acquire one MS/MS spectrum, and accumulate 4 repeated MS/MS spectra into one synthetic MS/MS spectrum.
      4. Use the synthetic MS/MS data to search for proteins through an MS/MS-based protein searching software with parameters including MS/MS Ion Search, Swiss-Prot human database, trypsin with a maximum of 1 missed cleavage, fixed carbamidomethyl (C), variable Oxidation (M), monoisotopic mass value, peptide mass tolerance ± 100 ppm, and fragment mass tolerance ± 0.7 Da.

5. Identify a protein with a statistically significant probability based on Mowse score, and ions score = -10 x Log(P), where p is the probability that the observed match is a random event.

3. **LC-ESI-MS/MS analysis**
   1. Add 6 µL of 5% v/v acetonitrile/0.1% v/v formic acid to dissolve the purified tryptic peptide mixture.
   2. Use a high-performance liquid chromatography (HPLC) system coupled online with an electrospray ionization (ESI)-MS/MS mass spectrometer with an MS/MS data-acquired management software to manage the entire operation.
   3. Use the C18 trap column (300 µm i.d. × 5 mm length), and reverse-phase C18 column (75 µm i.d. x 15 cm length) with a separation gradient at 98% solvent A (0.1% formic acid) and 2% solvent B (0.1% formic acid in 100% acetonitrile) for 2 min, 2% to 40% solvent B for 45 min, 40% to 95% solvent B for 5 min, and 95% solvent B for 10 min (a total of 65 min at 300 nL/min).
   4. Set positive-ion mode and data-dependent automatic survey mode to acquire MS and MS/MS spectra, and set collision-induced dissociation (CID) for ion fragmentation.
   5. Set each MS scan to a mass range of m/z 350-1,800 with a resolution of 100,000 at *m/z* 400. Perform the scan for the 7 most-intense ions in the MS scan.
   6. Use MS/MS-based protein searching software I to search the database for identification of proteins.
      1. Select the human protein database.
      2. Select trypsin with one missed cleavage site allowed.
      3. Set peptide tolerance (±10 ppm), fragment ion tolerance (±0.8 Da), peptide charge (2+, 3+, and 4+), fixed carbamidomethylation at cysteine, and variable oxidation at methionine.
      4. Set false discovery rates (FDR) <1%, and only peptides with rank 1 are accepted.
      5. Identify protein with PSM≥1 (PSMs: The total number of identified peptide sequences (PSMs) for the protein, including those redundantly identified) and at least two unique peptides per protein.

   7. Also, use MS/MS-based protein searching software II to search the database for identification of proteins.
      1. Convert the raw MS file to a .mgf file.
      2. Selected the human protein database (uniprothuman_20161031.fasta which contains 70940 sequences and 23897047 residues).
      3. Select trypsin digestion with a maximum of 2 missed cleavages.
      4. Set fixed carbamidomethyl (C), variable deamidated (NQ) and oxidation (M), precursor ion mass tolerance 10 ppm, daughter ion mass tolerance 0.8 Da, and monoisotopic peak.
      5. Identify proteins with a significance threshold of 0.05 and at least 2 unique peptides.

Representative Results

1. 2DE-MALDI MS PMF: With the experimental procedure described above, a total of 150 µg proteins were extracted from FSH-expressed NFPA tissues (female; 50 years old, ACTH (-), GH (-), PRL (-), LH (-), FSH (+), and TSH (-)) and arrayed with an 18 cm IPG strip (pH 3-10 NL) and a large-format SDS-PAGE gel, then visualized with silver staining. We obtained a reproducible and good 2DE gel pattern of NFPA tissue proteome (**Figure 1**), with an average positional deviation of 1.98 ± 0.75 mm in the IEF direction and 1.62 ± 0.68 mm in the SDS-PAGE direction, and with an approximately 1,200 protein spots detected in the 2DE gel map that were mainly distributed within the area of pH 4-8 and mass 15-150 kDa[3]. In total, 337 gel spots were excised and subjected to gel destaining, trypsin digestion, purification of tryptic peptides, and MALDI-TOF-MS PMF analysis. PMF data was used for protein identity with MASCOT search against the human protein database. A total of 192 redundant proteins (representing 107 nonredundant proteins) were identified from 141 gel spots out of 337 analyzed gel spots (141/337 = 42%), and no protein was identified in 196 spots (196/337 = 58%) (**Table 1**). For those 141 spots, only one protein per spot was identified in 121 spots, 2 proteins per spot were identified in 12 spots (Spots 31, 32, 33, 41, 42, 43, 44, 68, 75, 137, 148, and 224), 5 proteins per spot were identified in 3 spots (Spots 19, 22, and 23), 6 proteins per spot were identified in 3 spots (Spots 24, 91, and 93), and 7 proteins per spot were identified in 2 spots (Spots 72, and 92).

2. 2DE-MALDI MS/MS and 2DE-LC-MS/MS: Using the experimental procedure described above, a total of 500 µg of protein extracted from an invasive NFPA tissue (male, 22 years old, ACTH (-), hGH (-), PRL (++), LH (-), and FSH (-)) was arrayed with an 18 cm IPG strip (pH 3-10 NL) and a large-format SDS-PAGE gel, then visualized with Coomassie Blue G250 staining. This staining yielded a reproducible and high quality 2DE gel pattern of invasive NFPA tissue proteome (**Figure 2**), with an average positional deviation of 1.95 ± 0.85 mm in the IEF direction and 1.85 ± 0.79 mm in the SDS-PAGE direction, and with approximately 1,100 protein spotsdetected in the 2DE gel map that were mainly distributed within the range of pH 4-8 and mass 15-150 kDa[29]. The 10 spots randomly selected and labeled in **Figure 2** were excised and subjected to gel destaining, trypsin digestion, purification of tryptic peptides, followed by MALDI-MS/MS and LC-ESI-MS/MS analyses. Each MS/MS dataset was used for protein identity with MASCOT search against the human protein database. For all spots, an average of 1 protein per spot was identified with MALDI-TOF-TOF MS/MS analysis, whereas many proteins (an average of 63 proteins in a single gel spot, an average of 71 proteins in a pool of 2 matched spots, and an average of 118 proteins in a pool of 3 matched gel spots) in each corresponding spot were identified with LC-ESI-MS/MS analysis (**Table 2**). Here, one must mention that the LC-ESI-MS/MS system has a much higher sensitivity and resolution relative to the MALDI-MS/MS analysis system. This data clearly demonstrates that each 2D gel spot contains many proteins, not just one or two proteins in the complex human cancer tissue proteome.

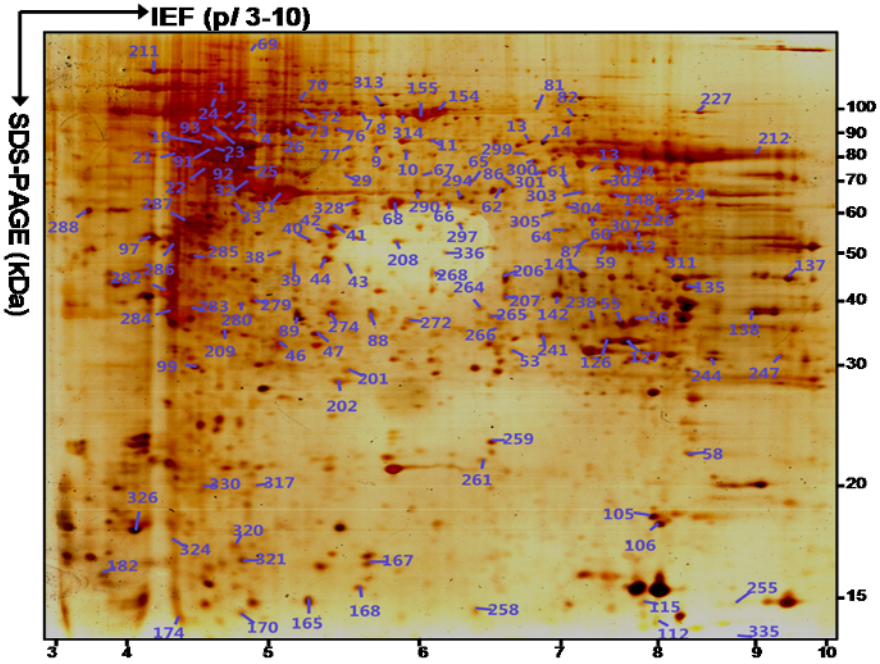

Figure 1: Silver stained 2-DE pattern of an FSH-expressed NFPA proteome analyzed with IPGstrip pH 3-10 NL and 12% gel concentration of SDS-PAGE. The red-brown spots are the silver-stained proteins, the orange is the background of silver-stained gel. The labeled spots were analyzed with MALDI-TOF-MS PMF. Reproduced from Wang X, *et al.* (2015)[3], with permission from Wiley-VCH. Please click here to view a larger version of this figure.

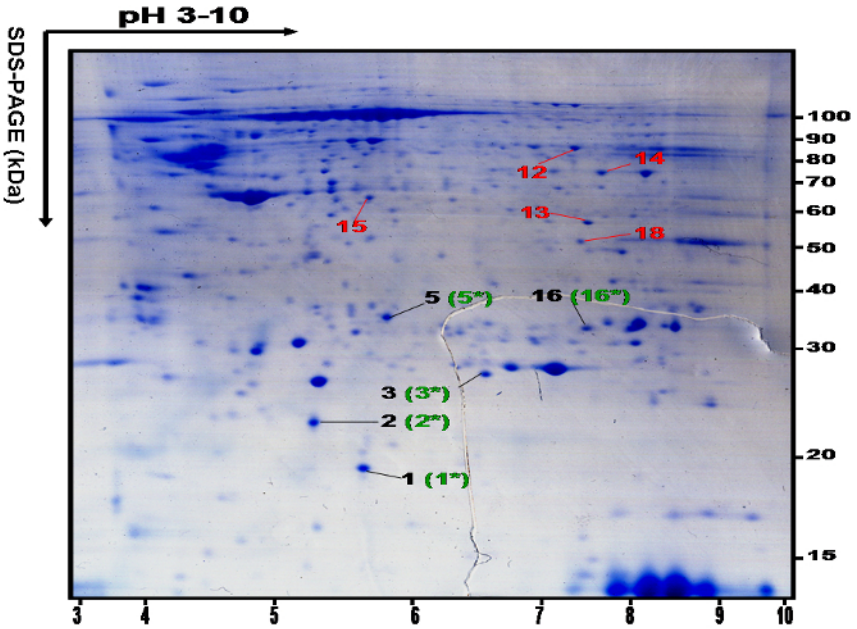

Figure 2: Coomassie blue stained 2-DE pattern of an NFPA proteome analyzed with IPGstrip pH 3-10 NL and 12% gel concentration of SDS-PAGE. The labeled spots were analyzed with MALDI-TOF-TOF MS/MS and **LC-ESI-MS/MS**. Spots 1*, 2*, 3*, 5*, and 16* were matched to the corresponding spots 1, 2, 3, 5, and 16, and pooled from 2 matched gels. Modified from Zhan X, *et al.* (2018)[29], with permission from Wiley-VCH. Please click here to view a larger version of this figure.

The number of proteins	The number of spots (n = 337)	Marks
7	2	Spots 72, 92
6	3	Spots 24, 91, 93
5	3	Spots 19, 22, 23
2	12	Spots 31, 32, 33, 41, 42, 43, 44, 68, 75, 137, 148, 224
1	121	The rest spots marked in Figure 1
0	196	No protein was identified.

Table 1: The number of proteins that were identified in each 2DE gel spot with MALDI-TOF-MS PMF analysis.

MALDI-MS/MS			LC-ESI-MS/MS		
1 spot	2 pooled spots	3 pooled spots	1 spot	2 pooled spots	3 pooled spots
2 (1)	1 (1*)	2 (12)	76 (1)	50 (1*)	102 (12)
1 (2)	1 (2*)	0 (13)	32 (2)	48 (2*)	91 (13)
0 (3)	2 (3*)	1 (14)	60 (3)	93 (3*)	141 (14)
1 (5)	1 (5*)	2 (15)	86 (5)	78 (5*)	156 (15)
1 (16)	1 (16*)	1 (18)	61 (16)	84 (16*)	98 (18)
Averaged 1 /spot	**Averaged 1 /spot**	**Averaged 1 /spot**	**Averaged 63 /spot**	**Averaged 71 /spot**	**Averaged 118 /spot**

Note: The number in the bracket is the spot number labeled in Figure 2. Spots 1*, 2*, 3*, 5*, and 16* matched to the corresponding spots 1, 2, 3, 5, and 16.

Table 2: The number of proteins that were identified in each 2DE gel spot with MS/MS analysis.

Discussion

2DE, coupled with MS methods including 2DE-MS PMF and 2DE-MS/MS, has been successfully used in our long-term program - the use of proteomics to study human NFPA proteomic variations and molecular network variations for the elucidation of molecular mechanisms and discovery of effective biomarkers for NFPAs. 2DE-based comparative proteomics with good reproducibility plays an important role in the identification of NFPA proteomic variations[9,10,11,12,13,19], and 2DE coupled with the antibody method demonstrates its advantages in the detection of a given post-translational modification and variants of a given protein, such as detection of tyrosine nitration[16,17,18] and GH variants[14,15].

However, 2DE coupled with a MS method is considered a labor-intensive technique with difficulties in analyses of low abundance proteins, hydrophobic proteins, extremely basic or acidic proteins, and extremely low or high mass proteins[2]. For instance,extremely low-mass (<10 kDa) or high-mass (>150 kDa) proteins, and extremely basic (pI>7.5 or 8) or acidic (pI<3.5 or 4) proteins are not easily detected by 2DE with an 18-cm IPG strip of pH 3-10 NL[3]. With the rapid development of gel-free separation methods, including two-dimensional liquid chromatography (2D-LC) coupled with stable isotope labeling such as iTRAQ and TMT in the last ten years[23], it seems that 2-DE has gradually withdrawn from its central status in proteomics research in the analysis of the complex tissue proteome because 2D-LC coupled with stable isotope labeling and MS/MS methods effectively overcome the drawbacks of 2DE-based methods.

Recent studies found that, with the use of high-sensitivity mass spectrometry, such as an LC-ESI-MS/MS (its sensitivity is in 1-10 amol range) in the identification of 2DE-separated proteins, the new characteristics of the 2-DE method were discovered which show that a 2D gel spot contains many proteins (**Table 2**). This ran counter to the traditional concept of only one or two proteins per spot, as is evidenced by the World 2DPAGE database (World-2DPAGE portal: http://world-2dpage.expasy.org/portal). This clearly demonstrates that the throughput of 2-DE in the identity of proteins in a complex human proteome is much higher than one previously assumed, namely that there were only one to two proteins in a 2D gel spot. This techinique offers the promise of use for 2DE in the field of proteomics. One 2DE can separate over a thousand to ten thousand spots[3,8,22] in the analysis of the complex human proteome, thus 2DE is a more detailed separation technique to maximally simplify the complexity of proteome samples before LC-MS/MS analysis relative to the 2D-LC system that the first-dimensional LC commonly generates 18 to 30 fractions before LC-MS/MS analysis. Therefore, 2DE in combination with high-sensitivity mass spectrometry can be used to establish the tremendous information reference map for the complex human proteome, especially for those low or extremely low abundance proteins. In addition, 2-DE in combination with stable isotope labeling (such as iTRAQ, TMT, and SILAC) and high-sensitivity mass spectrometry can be used to quantify large-scale proteomic variations among different conditions. Moreover, 2DE coupled with stable isotope labeling and high-sensitivity mass spectrometry has absolute advantages in large-scale detection and quantification of protein PTMs, protein variants, protein speciation, and protein species. These advantages will have 2DE revitalized in the field of proteomics.

Furthermore, the present protocols for the analysis of human PA tissue proteome described here are easily translated to study other human disease proteomes.

Disclosures

The authors declare that there is no conflict of interests.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 81572278 and 81272798 to XZ), the grants from China "863" Plan Project (Grant No. 2014AA020610-1 to XZ), the Xiangya Hospital Funds for Talent Introduction (to XZ), and the Hunan Provincial Natural Science Foundation of China (Grant No. 14JJ7008 to XZ). The authors also acknowledge the scientific contribution of Dr. Dominic M. Desiderio in the University of Tennessee Health Science Center. X.Z. continuously developed and used 2DE-MS methods to analyze pituitary adenoma proteome starting from 2001, conceived the concept for the present manuscript, wrote and revised the manuscript, coordinated the pertinent work, and was responsible for the financial support and corresponding work. Y.L. participated in revision of the manuscript. Y.H participated in collection of references, and revision of the manuscript. All authors approved the final manuscript.

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