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MudPIT Analysis: Application to Human Heart Tissue

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Abstract

Although two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been used as the standard proteomic approach for separating proteins in a complex mixture, this technique has many drawbacks. These include a limited molecular mass range, poor separation of highly acidic or basic proteins, and exclusion of the majority of membrane proteins from analysis. Considering the important functions of many membrane proteins, such as receptors, ion transporters, signal transducers, and cell adhesion proteins, it is increasingly important that these proteins are not excluded during the global proteomic analysis of cellular systems. Multidimensional Protein Identification Technology (MudPIT) offers a gel-free alternative to 2D-PAGE for the analysis of both membrane and soluble proteins.

The goal of this chapter is to provide detailed methods for using MudPIT to profile both membrane and soluble proteins in complex unfractionated samples. Methods discussed will include tissue homogenization, sample preparation, MudPIT, data analysis, and an application for the analysis of unfractionated total tissue homogenate from human heart.

Keywords

MudPIT; 2D-PAGE; proteomics; membrane proteins; human heart explants

1. Introduction

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) coupled with mass spectrometry has typically been used to identify proteins within complex mixtures. This technique utilizes isoelectric focusing (IEF) to separate proteins in the first dimension according to their charge (pI, isoelectric point) and then in a second dimension using polyacrylamide gel electrophoresis (PAGE), which separates based on the protein's molecular weight (Figure 1) (1).

This method can be used to separate over 1000 proteins; however, membrane proteins are under-represented using this platform due to their poor solubility in the sample buffer and resolution in the gel (2). Membrane proteins are typically insoluble in the non-ionic or zwitterionic detergents utilized in IEF, or they precipitate out of the IEF solution at pH values close to their pI (3). In general, 2D-PAGE techniques can effectively separate membrane proteins that have one or two transmembrane segments, but not those with three or more, which means that approximately 1% of integral membrane proteins are actually resolved on current 2D gels(4,5). Gel based approaches are also generally biased towards detection of highly abundant proteins and housekeeping enzymes and have decreased detection of those proteins with extremes in isoelectric point and molecular weight, a limitation that is exacerbated by the

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fact that up to 7% of proteins have $pI \geq 10$ (6,7,8). An additional limitation of 2D gel approaches is the complexity of the data analysis. There has not been a program developed that is automatic in initial spot recognition and in the subsequent matching of spots between gels when presented with complex 2D gel images that contain thousands of protein spots. Most of the programs conventionally used require a significant amount of user hands-on time to edit images before they can be entered into the database. However, many of these limitations are currently being optimized. For example, immobilized pH gradients that can resolve proteins with extreme acidic or basic pI's are currently being utilized, and these advancements will allow gel based techniques to be more widely used in the future for complex samples (9).

The 2D-GE approach has been used extensively in the study of many complex diseases, including human heart failure. Heart failure is a complex, multi-factorial disease that is characterized by the inability of the heart to maintain cardiac output that is sufficient for the body's needs. In one study of human heart disease using 2D-GE 1600 gel spots were identified, with an unlisted number of proteins identified from these spots (11). However, the study was limited mainly to the examination of soluble proteins and those highly abundant myofilament proteins such as myosin and desmin. In another study using 2D-GE and human heart tissue, 110 proteins were identified from 374 excised gel spots (12). Amounts of membrane proteins in each of these samples were not reported.

As an alternative to 2D gel approaches, several multi-dimensional liquid phase based separation techniques have been investigated. Among these techniques is the Multidimensional Protein Identification Technology (MudPIT) developed by the Yates group (13). This technique uses biphasic capillary columns packed with both strong cation exchange material (SCX) and reverse phase material (RP) to separate whole protein mixtures that have been digested and loaded directly onto the column. The solid phase column packing material allows for the separation of peptides based on both their charge and hydrophobicity. The column is then placed inline with a tandem mass spectrometer to facilitate electrospray ionization, fragmentation, and detection of the ions as they are eluted off the multidimensional separation column (Figure 2).

An advantage of the MudPIT approach is that it provides a more unbiased proteomic analysis. It facilitates the identification of both high and low abundance proteins, and hydrophobic proteins that are unresolved by 2D gels with sensitivity in the low femtomole range (14). This technique can be used to overcome many of the current 2D gel limitations. For example, this system has the high resolving power of multidimensional high-pressure liquid chromatography (HPLC), the sensitive peptide detection capabilities of tandem mass spectrometry, the analytical power of highly developed database searching software, and the possibility of creating automated data collection systems (15,16). Most importantly for our discussion of membrane proteins, MudPIT is compatible with sample preparations that optimize for the analysis of membrane proteins that are generally excluded from 2D gel analysis. Washburn and colleagues developed a method in which membranes are solubilized with 90% formic acid, hydrolyzed with CNBr, and digested with endoproteinase-LysC and trypsin, leading to increased analysis of peptides from hydrophobic proteins (17). Similarly, an acid labile detergent, *RapiGest*, developed by the Waters Corporation can be used with the protease trypsin to partially solubilize both cytosolic and membrane proteins for MudPIT analysis (similar detergents are now available through Protein Discovery, Inc.) (18,19). It has been predicted that ~30% of all open reading frames in the human genome encode membrane proteins, and Washburn et. al. showed that using the 90% formic acid solubilization method they were able to identify 19% of the total membrane proteins predicted from the genome (20,21). Even more recently, a high pH method has been used to create intact membrane "sheets" from non-solubilized membrane samples. Proteinase K, a non-specific protease, can then be used to cleave the solvent accessible domains of membrane embedded proteins, which ultimately can

provide information about membrane protein topology (22,23). With the continuing advances in the MudPIT technique it is clear that it will continue to be advantageous for the analysis of membrane proteins, which are generally excluded from traditional 2D gel based analyses.

The MudPIT approach has also been used in the study of heart disease as an alternative to the conventional gel based studies (24). This shotgun proteomics platform for studying heart disease may result in a more comprehensive analysis than to those conducted in gel based systems because it provides a global analysis that is inclusive of membrane proteins and utilizes a dynamic range capable of detecting both high abundance proteins, such as actin and myosin, and low abundance proteins, such as cell surface receptors. Furthermore, MudPIT can be used with unfractionated proteinsamples in conjunction with RNA microarray studies utilizing the same tissue specimen. While microarray studies allow for the unbiased profiling of global changes in mRNA levels, the MudPIT analysis of total cellular proteins allows for the global analysis of myocardial protein expression that originated from the same initial subject. Simultaneous proteome and transcriptome profiling has been shown in plant species using 2D-gel methods; however the sequential extraction of protein and mRNA from human tissue for profiling has yet to be fully explored (25,26). Studies of this type are being performed by our group at the University of Colorado Health Sciences Center using TRIzol protein precipitate from RNA-extracted human ventricular tissue from patients with non-failing hearts and those diagnosed with dilated cardiomyopathy. The advantage of this approach is that by examining un-fractionated protein samples both genomics and proteomics can be applied to create a comprehensive global profile of myocardial gene/protein expression from a single heart sample.

The methods described in this chapter detail 1) tissue homogenization, 2) sample preparation to facilitate concurrent microarray and proteomic analysis, 3) MudPIT, and 4) proteomic data analysis.

2. Materials

2.1 Tissue Homogenization and Protein Precipitation

1. Tissue homogenizer, 4.5mm probe diameter (Biospec Products, Bartlesville, OK)
2. Table top centrifuge, Eppendorf 5417R (Eppendorf, Westbury, NY)
3. 0.4M $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 6.7 or TRIzol (Invitrogen, Carlsbad, CA)
4. Methanol
5. Chloroform

2.2 Protein Digestion I- RapiGest/Trypsin Method

1. RapiGest SF powder (Waters Corporation, Milford, MA)
2. 50mM Ammonium Bicarbonate buffer, pH 7.8
3. 500mM dithiothreitol (DTT)
4. 500mM iodoacetamide (light sensitive)
5. 500mM HCl
6. 250ng/ μl Trypsin, modified, sequencing grade (Promega, Madison, WI)
7. 100mM CaCl_2
8. Thermomixer (Eppendorf, Westbury, NY)

2.3 Protein Digestion II- Formic Acid/CnBr/Trypsin Method

1. 90% Formic acid
2. CNBr
3. 30% Ammonium Hydroxide
4. Saturated Ammonium Bicarbonate in water
5. Solid Urea
6. 500mM DTT
7. 500mM iodoacetamide
8. EndoLys-C protease (Roche, Indianapolis, IN)
9. 100mM Tris-HCl, pH 8.5
10. 100mM CaCl₂
11. 250ng/μl Trypsin, modified, sequencing grade
12. pH paper

2.4 Preparation of Samples for Column Loading

2.4.1 Rapigest/Trypsin Method

1. HCl
2. microcentrifuge

2.4.2 Formic acid/CnBr/Trypsin Method

1. 90% Formic Acid

2.5 Column Packing and Sample Loading

1. P-2000 laser puller (Sutter Instruments; Novato, CA)
2. HPLC packing materials: SCX (Whatman, Florham Park, NJ) and C18 RP (Phenomenex, Torrance, CA)
3. Stainless steel pressure bomb
4. HPLC pump, Agilent 1100 (Agilent, Palo Alto, CA)

2.6 Chromatography

1. Buffer A: 5% acetonitrile, 0.1% formic acid.
2. Buffer B: 95% acetonitrile, 0.1% formic acid.
3. Salt solutions, ammonium acetate: 100 mM, 200 mM, 300 mM, 400 mM, 500 mM, 600 mM, 700 mM, 800 mM, 900 mM, 1 M, 5 M.
4. HPLC pump, Agilent 1100 (Agilent, Palo Alto, CA)
5. Microelectrospray ionization source (built in-house)
6. Temperature controlled autosampler (Agilent, Palo Alto, CA)
7. Ion trap mass spectrometer, Finnigan LTQ linear ion trap (ThermoElectron Corp., Waltham, MA)

8. Xcalibur interface software (ThermoElectron Corp., Waltham, MA)

2.7 Sample Analysis

1. SEQUEST (see <http://www.sequest.org>)
2. DTASelect (see <http://fields.scripps.edu/DTASelect/>)

3. Methods

3.1 Tissue Homogenization and Protein Precipitation

1. Tissue homogenization (1–10 mg) is done in 800 μ L cold phosphate buffer pH 6.7 (or using TRIzol solution to extract intact RNA concurrently, *see Note 1*), using a Tissue Tearor or other tissue homogenizer. For samples from cell culture, a Dounce homogenizer can be used with either cold phosphate buffer or TRIzol.
2. Pellet out nuclei and unbroken cells using a low speed spin (1500 rpm) for 5 min on table top centrifuge.
3. Take post-nuclear supernatant and perform a high speed spin (14000 rpm) for 30 minutes using microcentrifuge to pellet out total cellular membranes. (*see Note 1*)
4. Move total protein pellet or membrane pellet to new microcentrifuge tube and begin to precipitate membrane proteins by adding 4 $\times$ volume of MeOH and 1 $\times$ volume of CHCl_3 .
5. Vortex.
6. Add 3 $\times$ volume of water
7. Vortex and spin down at 14000 rpm for 2 minutes and then remove the bottom CHCl_3 layer.
8. Add fresh CHCl_3 (1 $\times$ volume) and spin down at 14000 rpm for 2 min and again remove the bottom CHCl_3 layer. Also remove the water layer leaving only the interphase layer which contains your protein precipitate.
9. Add fresh CHCl_3 (1 $\times$ volume) and MeOH (3 $\times$ volume) to the protein precipitate, vortex, and spin down at 14000 rpm for 2 min. Remove all the supernatant and leave the protein pellet to air dry for approximately 2 minutes.

3.2 Protein Digestion I- RapiGest/Trypsin Method

1. Make 0.1% RapiGest diluted in 50mM ammonium bicarbonate buffer, pH 7.8 (1mg RapiGest per 1ml 50mM ammonium bicarbonate)
2. Using locking lid microcentrifuge tubes add 50 μ L 0.1% RapiGest solution to membrane protein pellet. Work the pellet back into solution by alternating pipetting and vortexing (*see Note 2*).
3. Once the membrane protein pellet is fully solubilized the sample should be heated to 100°C for 2 minutes and then left to cool until it reaches room temperature.
4. Reduce the solution with DTT (final concentration of 5mM) for 30 minutes at 60°C and then cool the sample to room temperature
5. Alkylate the solution with iodoacetamide (final concentration 15mM) in the dark for 30 minutes at room temperature (*see Note 3*)
6. Add modified trypsin for a final concentration of 1:100, enzyme:protein. Incubate in a thermomixer overnight at 37°C with shaking.

3.3 Protein Digestion II- Formic Acid/CNBr/Trypsin Method

1. Begin with a membrane pellet that contains approximately 200µg protein and add 50µl CNBr solution (500mg/ml CNBr in 90% formic acid) (*see Notes 4 and 5*).
2. Resuspend the pellet in the CNBr/formic acid solution by alternating vortexing and pipetting. If you cannot achieve a homogenous solution, then add another 10µl CNBr/formic acid solution. However, do not exceed 100µl in a single microcentrifuge tube because you will run out of room in subsequent neutralization steps.
3. Incubate the homogenous solution in the dark overnight at room temperature.
4. The next day, neutralize your highly acidic sample by SLOWLY adding 2 volumes of 30% ammonium hydroxide (1 volume = volume of CNBR/formic acid solution initially added).
5. Neutralize the sample further by SLOWLY adding 1–2 volumes of saturated ammonium bicarbonate solution. Check the pH by spotting a drop of the sample onto pH paper and continue neutralization until the sample has reached a pH of 8.0–8.5 (*see Note 6*).
6. Add solid urea to the sample to a final concentration of 8.0M and vortex until completely solubilized.
7. To reduce the proteins add DTT to a final concentration of 25mM and incubate at 60°C for 30 minutes.
8. Follow by alkylating the proteins using iodoacetamide (final concentration 100mM) and incubate at room temperature in the dark for 30 minutes.
9. The EndoLys-C protease should be added to your sample (1:100, enzyme:protein). Incubate in a thermomixer overnight at 37°C with gentle shaking.
10. The next day, dilute the initial solution volume by a factor of two with 100mM Tris, pH 8.5, and add CaCl₂ (final concentration 1mM).
11. Add modified trypsin (1:100, enzyme:protein) and incubate at 37°C in a thermomixer overnight with shaking.

3.4 Preparation of Samples for Column Loading

3.4.1 RapiGest/Trypsin Method

1. Add HCl (final concentration of 100mM) to the trypsin digested sample and incubate at 37°C for 45 minutes.
2. Spin sample at 14000 rpm (4°C) for 10 minutes. A cloudy pellet should appear.
3. Separate the supernatant from the pellet and place the supernatant into a fresh eppendorf tube (spin again if needed to make sure all cloudy material has been removed).

3.4.2 CNBr/Formic acid/Trypsin Method

1. Microfuge sample (14000 rpm) for 10 minutes to pellet any insoluble debris. Move supernatant to a new tube and adjust it to 5% formic acid for loading onto the chromatography column.

3.5 Column Packing and Sample Loading

1. Laser puller is used to pull a 5µm tip from a fused silica tube (~17cm long) of 100µm inner diameter (i.d.) and 365µm outer diameter (o.d.).

2. The column is then packed with 8cm 5 μ m C18 RP material followed by 4cm of 5 μ m SCX material using a pressure bomb that uses helium (27) (*see* **Notes 7 and 8**).
3. Equilibrate column on HPLC with Buffer A for 30 minutes.
4. Load sample onto column using pressure bomb and follow by washing the column for 10 minutes with Buffer A. (*see* **Notes 9 and 10**)

3.6 Chromatography Conditions

1. Chromatography set-up is controlled through the Xcalibur interface on the tandem mass spectrometer (*see* **Notes 11 and 12**)
2. Following the 10 minute wash with Buffer A done in step 4 of 3.5 (see above), step 1 of the MudPIT run is initiated by applying a short pulse of water onto the column (50 μ l).
3. The initial water pulse is followed by a long shallow increase in Buffer B until 80% acetonitrile is reached.
4. Buffer A (100%) is used to re-equilibrate the column for 10–15 minutes.
5. Step 2 of the 12 step MudPIT is initiated by addition of 100mM ammonium acetate salt (50 μ l) directly onto the column by means of the autosampler. This is followed by the shallow gradient of Buffer B to 80% acetonitrile and re-equilibration with 100% Buffer A for 10 minutes.
6. Steps 3–11 in the 12 step MudPIT are done similar to step 2 described above with increasing concentrations of ammonium acetate salt loaded on to the column (50 μ l) followed by the shallow gradient of Buffer B to 80% acetonitrile and re-equilibration with 100% Buffer A for 10 minutes. The salt concentrations for steps 3–11 are 200mM, 300mM, 400mM, 500mM, 600mM, 700mM, 800mM, 900mM, and 1M respectively.
7. Step 12 is done using 5M ammonium acetate salt solution (100 μ L) loaded onto the column, followed by the shallow gradient of Buffer B to 80% acetonitrile and re-equilibration with 100% Buffer A for 10 minutes.

Representative base peak chromatogram from one 12-step MudPIT run containing total cellular protein from a non-failing human heart sample is shown in Figure 1.

3.7 Sample Analysis

1. Acquired tandem mass spectra can be analyzed using correlation software such as SEQUEST, which correlates the experimental tandem mass spectra with theoretical mass spectra from a protein sequence database in order to identify the peptide sequences detected.
2. The identified peptide sequences can then be assembled back into proteins using software such as DTASelect (28) (*see* **Note 13**).
3. The Contrast program is used for the comparison and filtering of data sets generated by multiple MudPIT runs to determine the protein content of multiple related samples.

4. Notes

1. To look at total protein content (soluble and membrane) perform the methanol/chloroform protein precipitation on the post-nuclear supernatant following the low speed spin in step 2. In addition, if microarray and proteomic analyses are to be done concurrently on the same tissue sample, a TRIzol extraction of the tissue is done to

yield RNA, DNA, and total protein fractions (29). A methanol/chloroform protein precipitation can then be done on the total protein fraction. For the experiments described here a TRIzol extraction was done on human heart tissue followed by protein precipitation to yield total cellular protein components.

2. The membrane protein pellet can be difficult to get back into solution. To facilitate its solubilization an insulin syringe can be used to pull the solution in and out in order to break up the pellet.
3. To reduce the amount of chymotryptic activity the trypsin enzyme is capable of add CaCl_2 (final concentration 1mM) to the solution following reduction with iodoacetamide.
4. CNBr is a toxic reagent and all solutions containing CNBr should be handled in a hooded chemical area and disposed of properly.
5. If you have no idea how much protein is in your pellet, a rough approximation of 200 μg protein would be a pellet the size of an "o" at 12 point font.
6. Be sure to add the basic solutions drop by drop, otherwise the highly acidic sample will bubble over and some of your sample may be lost. In addition, it is encouraged that following neutralization you let the sample sit for 15 minutes and then re-check the pH before adding the solid urea to be sure the pH was adjusted correctly.
7. C18 RP and SCX material should be placed in HPLC grade methanol forming a slurry to be utilized for column packing.
8. There is a large variety of C18 RP material commercially available. Aqua C18 (Phenomenex, Torrance, CA), Luna C18 (Phenomenex, Torrance, CA), Prodigy C18 (Phenomenex, Torrance, CA), Zorbax C18 (Agilent, Palo Alto, CA), and Resolve C18 (Waters Corporation, Milford, MA) are examples. Each RP material will yield slightly different retention times depending on the sample composition (30). For the experiment shown here, Aqua C18 was used.
9. Samples need to be free of particles/debris before loading onto the column since the small inner diameter of the column makes it prone to clogging. If problems with clogging occur, consider using a 3cm C18 RP packed in a 250 μm i.d. column and connect to the biphasic column using an in-line filter assembly (Upchurch, Oak Harbor, WA). This facilitates the loading and desalting of viscous samples and/or large volumes.
10. Loading of Formic acid/CnBr/Trypsin samples can take up to 5 times longer than *RapiGest*/Trypsin samples.
11. Using a column heater at $\sim 40^\circ\text{C}$ during all chromatography steps can improve peak resolution.
12. The chromatography conditions listed here are for a typical 12 step (24 hour) MudPIT run. The longer acetonitrile gradients (80–110 min) may allow for more peptides to be eluted from the column onto the mass spectrometer and detected and analyzed. The number of salt pulses and the length of acetonitrile gradients can be modified depending on the complexity of the sample to achieve the best resolution and number of peptides identified.
13. Other peptide identification software is commercially available. SEQUEST is available through ThermoElectron Corporation and Mascot is available through Matrix Science Inc (31).

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Figure 1.

Two-dimensional polyacrylamide gel electrophoresis

Chromatography profile for a human heart sample prepared using the *RapiGest*/Trypsin digestion protocol. In a typical 12-step MudPIT analysis >1500 unique proteins are identified with an FDR < 5%, approximately 13.5% of which are predicted by the TMHMM prediction algorithm to be membrane proteins.

Figure 2.
Overview of Multidimensional Protein Identification Technology