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Review

Two-dimensional liquid separations–mass mapping of proteins from human cancer cell lysates

David M. Lubman[a,*], Maureen T. Kachman[a], Haixing Wang[a], Siyuan Gong[a], Fang Yan[a], Rick L. Hamler[a], Kimberly A. O'Neil[a], Kan Zhu[a], Nathan S. Buchanan[a], Timothy J. Barder[b]

[a]*Department of Chemistry, The University of Michigan, 930 North University Avenue, Ann Arbor, MI 48109-1055, USA*
[b]*Eprogen, Inc., 8205 S. Cass Avenue, Suite 111, Darien, IL 60561, USA*

Abstract

A review of two-dimensional (2D) liquid separation methods used in our laboratory to map the protein content of human cancer cells is presented herein. The methods discussed include various means of fractionating proteins according to isoelectric point (p*I*) in the first dimension. The proteins in each p*I* fraction are subsequently separated using nonporous (NPS) reversed-phase high-performance liquid chromatography (RP-HPLC). The liquid eluent of the RP-HPLC separation is directed on-line into an electrospray ionization time-of-flight (ESI-TOF) mass spectrometer where an accurate value of the protein intact M_r can be obtained. The result is a 2D map of p*I* versus M_r analogous to 2D gel electrophoresis; however the highly accurate and reproducible M_r serves as the basis for interlysate comparisons. In addition, the use of liquid separations allows for the collection of hundreds of purified proteins in the liquid phase for further analysis via peptide mass mapping using matrix assisted laser desorption ionization TOF MS. A description of the methodology used and its applications to analysis of several types of human cancer cell lines is described. The potential of the method for differential proteomic analysis for the identification of biomarkers of disease is discussed.

Keywords: Reviews; Proteomics; Mass mapping; Proteins

Contents

*Corresponding author. Tel.: +1-734-764-1669; fax: +1-734-936-9463.

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1. Introduction

The ability to profile changes in protein expression has become an important step towards understanding how cells progress through carcinogenesis [1–13]. Although DNA may provide the blueprint for development, it is proteins that perform the functions and dynamic processes that ultimately determine cell phenotype. Cellular mRNA expression levels may not necessarily be correlated directly to protein expression levels; therefore, monitoring the protein content of a cell becomes important. Minor changes in protein expression may be responsible for altering protein cascades that in turn, may eventually lead to transformation and uncontrolled growth of cells. Further mutations may lead to processes which allow the cell to become tumorogenic and ultimately metastatic. Since proteins are the agents that control these processes, it is important to pinpoint changes in global protein expression in order to identify protein biomarkers involved in these cancer-transforming processes. The ability to identify key protein biomarkers in the cancer process may serve as a diagnostic tool for early detection of cancer and may also aid in the development of specific cancer treatments based upon the molecular profile of the tumor.

The present method that is widely used for profiling protein expression in cells is two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (2D-SDS–PAGE) [14–18]. This method provides a 2D separation of proteins based upon isoelectric point (p*I*) in the first dimension and electrophoretic mobility in a polyacrylamide gel in the second dimension. The 2D gel provides an image of the proteins in the cell and also an approximate protein molecular mass (M_r) and p*I*. Standard format 2D gels can separate out over 1000 proteins and can be used to detect proteins of M_r >100 kDa. The sensitivity of the method is such that using silver-stained gels proteins down to the 1–10 ng level or 500–1000 copy numbers can be detected [21]. In addition, various staining methods have been developed to quantify differences in expression between proteins on gels [19–22].

There are also a number of important drawbacks to 2D gel technology for comparisons of protein expression between cell lines or for a cell line at different stages of development. The most important issue is the reproducibility of both protein spot position and intensity which are subject to changes depending on the run conditions. The result is that 2D gel patterns can be difficult to reproduce in interlaboratory comparisons. Such variability in gel conditions can lead to shifts in relative protein spot positions so that quantitative comparisons between spots become difficult. More recent studies have attempted to compensate for this problem using the DIG method [23]; where in comparative studies, the proteins from one cell lysate are labeled with one color dye while those from a second lysate are labeled with a second color dye. When both lysates are run on a gel together, then comparisons can be made using differential color display. However, this method is generally run on two samples simultaneously and it is difficult to compare proteins between large numbers of cell lysates.

There are other disadvantages to the use of 2D gel electrophoresis that make the development of an alternative technology desirable. 2D gels are labor intensive and difficult to automate. Although several companies now sell automated 2D gel platforms, the technology is expensive and available in a limited number of laboratories. Of more concern is that the proteins embedded in gels are not readily interfaced to mass spectrometric analysis. The analysis of proteins in polyacrylamide gels requires excising the spot, destaining, digestion and purification before analysis by either matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) or electrospray ionization (ESI) TOF-MS. In addition, the 2D gels provide a M_r accurate to

only 5–10%. Although methods have been developed to obtain intact M_r directly from gels or by blotting and electroelution techniques followed by MALDI-MS [24–28], these methods are also labor intensive and not readily automatable.

In this review, we describe our work on 2D liquid separations–mass mapping of proteins. Other laboratories have developed liquid-based separations of proteins [29–40,44,63], but this review will focus on work performed in our laboratory. The methods described herein use several different liquid techniques that fractionate proteins based upon p*I* in the first dimension, which are followed by nonporous silica (NPS) reversed-phase high-performance liquid chromatography (RP-HPLC) separation in the second dimension. These methods provide a 2D separation of proteins that generate an image of the cellular protein content analogous to a stained 2D gel. The RP-HPLC separation can ultimately be directly interfaced to ESI-TOF-MS to provide a map of the accurate intact M_r of all the proteins in the cell. Furthermore, the mass accuracy of detected protein M_r values approaches 150 ppm allowing for reproducible interlysate comparisons. In addition, the collection of isolated proteins in the liquid phase can provide an automated method for collection of hundreds of proteins for subsequent enzymatic digestion and identification by MALDI-TOF-MS peptide mass mapping. This method eliminates many of the time-consuming and labor-intensive steps associated with mass spectrometric analysis of proteins in a gel-based format. The result is standardized 2D mass maps of cellular protein expression that can reveal proteins that are either differentially expressed or qualitatively changed as a function of cancer. Herein, we will describe some of the methods and experimental procedures used to accomplish this work.

2. Methods

2.1. pH fractionation

In 2D gel electrophoresis the proteins are initially separated according to p*I* in the first dimension. This is traditionally performed using either carrier ampholyte (CA) tube gels or immobilized pH gradient (IPG) strips [17,41,42]. Carrier ampholyte gels are effective only over a limited pH range of 4–9. They separate proteins effectively in the low pH range but suffer from cathodic drift in the high pH range. This drift often results in smearing of bands over a broad pH range. In addition, the loadability on these CA-based gels is limited where only 200 μg can generally be loaded on the gel before band smearing is observed. Alternatively, IPG-based gels have become widely used in protein analysis. The IPG strips have a broad pH range of 3–12 and a loadability of several mg. IPG strips tend to suffer from precipitation which may result in poor recovery for MS analysis. Reproducibility has often been cited as a problem in IPG-based systems. Precipitation of both large and very hydrophobic membrane proteins in IPG-based 2D gel analysis is often a severe problem.

2.1.1. Liquid isoelectric focusing

As an alternative to gel-based p*I* separations, we have used liquid-based isoelectric focusing (IEF) to fractionate proteins according to p*I*. The following work explores the use of a commercial liquid IEF device (Rotofor cell, Bio-Rad) and an laboratory-developed focusing device that utilizes isoelectric membranes. These methods allow fractionation of proteins in the liquid phase over a broad pH range.

The Rotofor device is a relatively simple instrument that is analogous to separations in carrier ampholyte gels [43]. It consists of a plastic chamber with 20 compartments that are divided by monofilament polyester membranes. The preparative version of the Rotofor has a volume of 55 ml whereas the mini-Rotofor has a volume of 16 ml. The chamber is filled with the sample and buffer which contains a mixture of commercial ampholytes that establish a pH gradient under the influence of an electrical field. A constant power is applied to the chamber and the proteins separate according to their p*I* values. Each of the separated partitions contains a different pH range with the proteins of the corresponding p*I*. The proteins become neutral at their p*I* and so must be properly solubilized so that they do not precipitate out of solution. The device is continually rotated and water-cooled to 4–10 °C to prevent heating in the device and formation of bubbles. The total separation takes between 3 and 5 h. When the separation is

complete, the liquid is suctioned out of each compartment into a vial for further analysis.

The Rotofor has the advantage of providing a liquid-based p*I* separation of proteins that can subsequently be coupled to RP-HPLC analysis. It is also advantageous for preparative and analytical separations where there is a large amount of sample available. In our experiments, typically anywhere from 5 to 100 mg of protein has been separated in this device. The method also allows separation of proteins over a broad p*I* range of 3 to 12. By using commercial Rotolytes (Bio-Rad), the pH range can be adjusted so that a very narrow range pH of even one pH unit can be run over the entire 20 fractions. Furthermore, when using proper solubilization methods, large membrane proteins in excess of 200 kDa can be separated by this device [44] which is a distinct advantage over CA- or IPG-based gels. However, the Rotofor has the disadvantage of incomplete fractionation where proteins do not completely focus and may overlap more then one pH range. This becomes a more serious problem at high pH due to cathodic drift as in CA gels. The Rotofor also has limitations for analysis of samples present in very small amounts due to the large volume of the device.

An alternative method for performing isoelectric focusing in liquids involves the use of multicompartment electrolyzers with isoelectric membranes. This method has been used in our laboratory, but results on this method will not be presented at this time. This method involves the copolymerization of polyacrylamide and immobilines to produce membranes at a defined pH. The method is analogous to that of IPG strip-based p*I* separations. The membranes are used to separate several chambers, and when an electric field is applied to the device, the proteins are fractionated according to the pH ranges defined by the membranes. The method has high loadability of tens to hundreds of mg of protein and is an excellent preparative method. There is a commercial version of this device known as the IsoPrime (Amersham-Pharmacia Biotech). In more recent work, a miniaturized version of the IsoPrime device has been developed by Zuo and Speicher [45] which eliminates many of the problems of the larger device. The method has been used effectively as a prefractionation device prior to 2D gel separations and can be used with HPLC as a second dimension. The isoelectric membrane device still has the disadvantage of not being readily automatable, and substantial precipitation of protein occurs on the membranes.

2.1.2. Chromatofocusing

Chromatofocusing (CF) is an alternative liquid separation method for fractionating proteins according to p*I* [46–54]. CF is essentially an ion-exchange technique in which the proteins are bound to an anion exchanger and then eluted by a continuous decrease of the buffer pH so that proteins elute in order of their isoelectric points [46]. This is achieved by using amphoteric buffers titrated to the lower pH to generate a more linear gradient. By changing the buffers used, one can control the pH range used for separation. A pH range of 4–11 can be achieved with this method. Since the pH gradient is generated gradually as the eluting buffer moves down the column, it is possible to add large volumes of proteins to the column or to add a second batch during the run. In the CF separation, identical components with a particular p*I* will focus and elute as a single peak. The CF method has been used as a preparative method for separating proteins. However, more recently, analytical columns have been developed to accommodate proteomic studies where typically 0.5 to 5 mg might be loaded onto a column [54]. The method has the distinct advantage of being readily automatable and can be easily interfaced to other liquid separations. Unlike other ion-exchange techniques, CF also avoids the presence of large amounts of salt which may interfere with downstream mass spectrometric analysis unless removed from the HPLC eluent.

2.2. Nonporous reversed-phase HPLC separations

A key feature of this multidimensional/liquid phase method is the use of NPS as a medium for the second dimension of separation. In any p*I* fraction obtained using IEF or CF methods from a whole cell lysate of a human cell line, there may be from 50 to 150 proteins. In the mid-pH range between 5.5 and 7.0, there are often large numbers of proteins in each fraction; whereas on the basic and acidic ends, there are generally less proteins. In order to separate large numbers of proteins with sufficient resolution, 1.5

μm NPS ODS HPLC columns (Eprogen) have been used [55–64]. NPS RP-HPLC provides rapid and highly reproducible separations of proteins according to their hydrophobicities. The NPS packing material used in these RP separations eliminates problems associated with porous media where proteins adhere within the pores. The result in porous materials is often smearing of protein peaks with loss of resolution and poor recovery of proteins. The use of NPS media improves resolution and reduces separation times by as much as one-third compared to porous media. The separation efficiency remains high due to the use of 1.5 μm particle size in the commercial Eprogen columns. Of greater significance is the reproducibility of the separation which is essential for interlysate comparisons. The reproducibility in retention times for NPS separations has been found to be within 1 s under the conditions used in our work. In addition, the recovery for proteins under 40 kDa may be as high as 90%. These columns have been used for separation of proteins of over 200 kDa M_r. In addition, columns with larger particle sizes have been used to separate proteins of over 400 kDa [55,56]. The NPS RP-HPLC method provides a liquid phase method for separating large intact proteins for further analysis. More specifically, it provides a means of separating proteins for interfacing to mass spectrometric analysis.

2.3. Solubilization of proteins

A key aspect of liquid phase separations is the solubilization of proteins in a manner compatible with all the methods of analysis. SDS is generally used as the detergent in 2D PAGE, but is incompatible with the RP-HPLC separation or the ESI-MS. Other detergents such as Triton X-100 and Tween-20 are not compatible with either the IEF and RP-HPLC dimensions and may provide a polymer background that interferes with the mass spectrometry. We have found that the detergent of choice for this work is the nonionic detergent OG, which can be either *n*-octyl-β-D-galactopyranoside or *n*-octyl-β-glucopyranoside. OG effectively solubilizes large and hydrophobic membrane proteins where large membrane proteins have been recovered from the Rotofor [43] and from RP-HPLC separations. OG is very compatible with RP-HPLC and is compatible in small amounts with the ESI-MS. There are other detergents that are also compatible in small amounts with all stages of analysis such as the sulfobetaine [65] series of detergents; however, these detergents interfere with standard protein quantitation methods. In addition, to the use of detergents for protein solubilization in the liquid phase, the use of denaturing agents and chaotropes also aid in preventing protein precipitation during the course of analysis. In our work, a mixture of urea and thiourea chaotrope coupled with reducing agents dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) have been used. In addition, the use of these denaturing agents prevents further enzymatic degradation of the proteins during analysis.

2.4. Mass spectrometry

The unique feature of the liquid phase experiments compared to 2D gel electrophoresis is the relative ease with which it can be interfaced to mass spectrometric analysis. A key issue here is the ability to obtain an accurate M_r value for the intact protein. The intact M_r serves as an essential piece of information for identification of proteins based upon peptide mapping and database searching. The peptide map alone may provide several matches against a database so that further information is helpful to identify the protein correctly. In the case of 2D gel electrophoresis an approximate M_r can be estimated from the second dimension. For our 2D liquid separations, the eluent of the RP-HPLC is directed into an electrospray source where protein ions in the liquid phase are converted into gas-phase ions for mass spectrometry [62,63]. The ESI source produces an umbrella of multiply-charged ions which are detected according to their m/z in the gas phase. The umbrella is deconvoluted by software; and using modern ESI-TOF-MS systems, an M_r accurate to 150 ppm can be obtained. This accurate M_r also provides an initial means to search for modified proteins and proteins whose modifications change during some process such as the progression of cancer.

The 2D liquid separations method can also be readily interfaced to MALDI-TOF-MS analysis. The proteins from the liquid eluent of the RP-HPLC are collected using a fraction collector as they elute from

the column and are detected by a UV absorption detector. Using the 2D liquid separations, hundreds of proteins can be collected in a day. The proteins are relatively pure after the HPLC separation and the HPLC mobile phase can be readily evaporated since much of it is volatile acetonitrile. The intact protein can then be analyzed by MALDI-TOF-MS to obtain a M_r value or it can be digested and the digest analyzed for protein identification by database searching. In addition, post-source decay and MS–MS can be used for further verification of the protein identification. The use of the protein M_r, p*I* value, peptide and MS–MS data are all invaluable for correct protein identification in human cells because of the large number of proteins where there is substantial sequence homology.

3. Results and discussion

3.1. Rotofor IEF–NPS RP-HPLC: a method for 2D mapping of proteins

Fig. 1 shows an example of a 2D map of a whole cell protein lysate from the MDAH-2774 ovarian cancer cell line obtained using the IEF–NPS RP-HPLC method. The experimental details used to generate this map are described in previous work [61,62]. The sample is an endometrioid sub-type of ovarian tumor [66]. The image is designed to offer the same advantages of pattern recognition and protein profiling provided by a 2D gel, but has the added advantage of being obtained in digitized form. This method thus avoids the problems inherent in

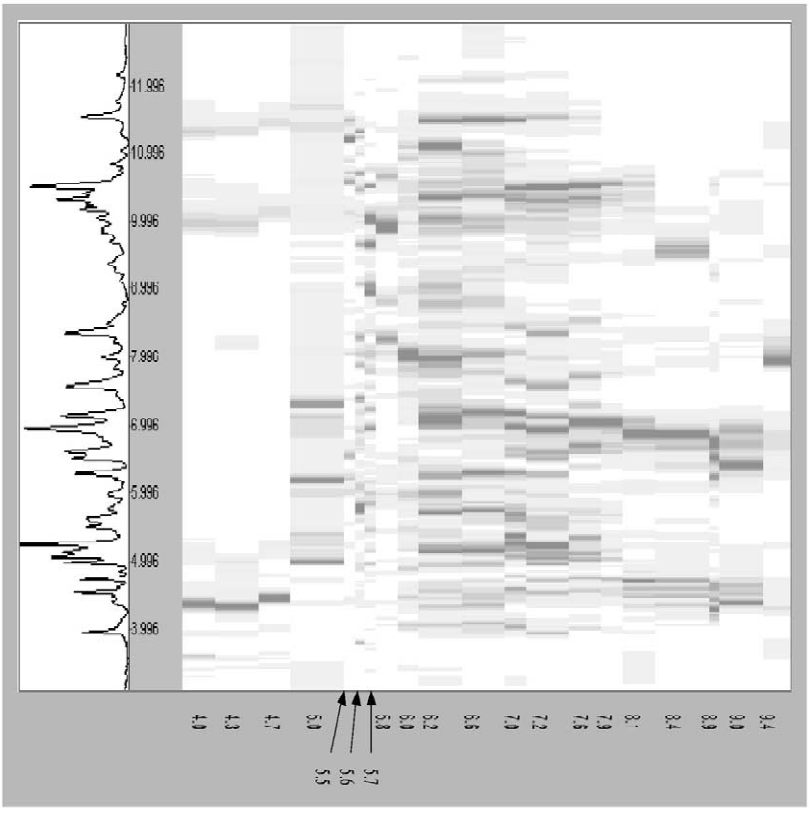

Fig. 1. The 2D image of the soluble proteins from the MDAH-2774 ovarian cancer cell line. The *x*-axis is fraction pH, and the *y*-axis is protein hydrophobicity (LC retention time). The NPS RP-HPLC chromatogram of fraction 13 (pH 7.2) is shown to the left.

staining and developing 2D gels. In this case, the horizontal and vertical dimensions are in terms of isoelectric point and protein hydrophobicity, respectively. The isoelectric focusing step in this run resulted in 20 fractions from pH 3.3 to 9.4, although the method can run to higher pH. These fractions were then each separated by NPS RP-HPLC and detected by UV absorbance (214 nm). The resulting chromatograms for each fraction were converted to ASCII format and then plotted vertically using a 256-step gray scale, such that the peaks are represented as darkened bands against a white background. The software has been designed such that protein profiles may be viewed in greater detail using a built-in zoom feature and/or by selecting a particular Rotofor fraction and observing the HPLC chromatogram. The width of the Rotofor fractions was adjusted to represent their estimated pH range, making the p*I* axis linear.

The molecular mass range of the proteins in this map is estimated to be from 5 to 85 kDa, although experiments have shown proteins of up to 100 kDa in these maps. The total number of peaks observed from all 20 fractions in this map is estimated to be around 700. This map was obtained using a rapid HPLC gradient of 11 min for each fraction. Fig. 2 shows the NPS RP-HPLC separation of Rotofor fraction 7 corresponding to Fig. 1 above. This Fig. presents a typical separation where 35 or more peaks are resolved using a fast gradient. In these separations, 0.05% OG was added to the mobile phase of the RP-HPLC separation to enhance resolution and to ensure that the proteins remained solubilized. If the gradient time is extended to 20 min, improved resolution is obtained and over 1000 protein bands are observed. This number compares favorably to that observed on silver-stained carrier ampholyte 2D gels. The Rotofor required around 5 h to run completely, while the 20 fractions were run by HPLC in around 4 h.

The Bio-Rad Bradford-based protein assay was used to determine the quantity of protein in the whole cell lysate that was loaded into the Rotofor for isoelectric focusing. The assay was also used to quantify each Rotofor fraction after the first dimension separation. The recovery of proteins summed over all the Rotofor fractions compared with the amount of protein in the whole cell lysates loaded was 64%. This is a relatively high level of protein recovery and together with the high recovery of the

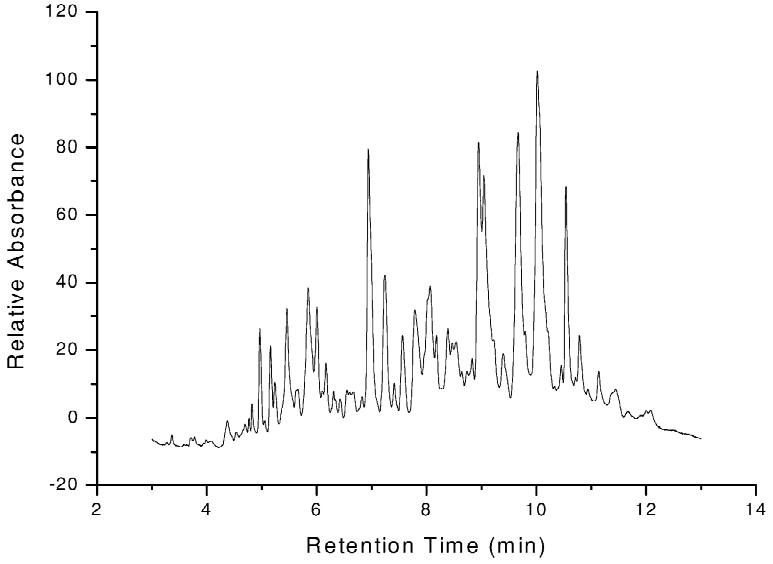

Fig. 2. NPS RP-HPLC of Rotofor fraction 7 from Fig. 1 for the MDAH-2774 cell line.

NPS separations, the result is a substantial recovery of protein for analysis by mass spectrometry. The protein quantification pattern shows that the fractions near the mid-pH range contain the greatest amount of focused proteins as expected.

3.2. 2D liquid separations–mass mapping—differential display of proteins

An important aspect of the liquid separation of proteins described above is that the liquid eluent can be analyzed on-line using an ESI-TOF-MS system. The result is that a 2D plot of the protein content of the cell can be produced with the M_r values plotted for each p*I* range. This map provides a direct analog to that of 2D gel electrophoresis; however, using ESI-TOF-MS, the M_r for each protein can be determined within 150 ppm. This highly accurate M_r has proven important for identifying proteins along with peptide mapping and for identifying the presence of posttranlational modifications (PTMs). Moreover, the relatively high reproducibility of the M_r value as determined by ESI-TOF-MS allows for accurate interlysate comparisons between cell lines. This is an important issue which is difficult to accomplish with 2D gels. In addition, there is also a hydrophobicity value from the RP-HPLC for each protein, which can be used together with the p*I*, M_r, and peptide map to tag each protein in future interlysate comparisons.

Fig. 3 shows one p*I* fraction of a 2D liquid separation–mass map as described above. The p*I* of this fraction is 6.4, and the M_r values are plotted in band format. This figure shows the same p*I* fraction from two different cell lines for comparison, where the map on the left is from a pool of immortalized surface epithelial cells and the map on the right is from the ES2 clear cell carcinoma cell line. The band format is only a representation which does not display the true resolution of the M_r bands which is

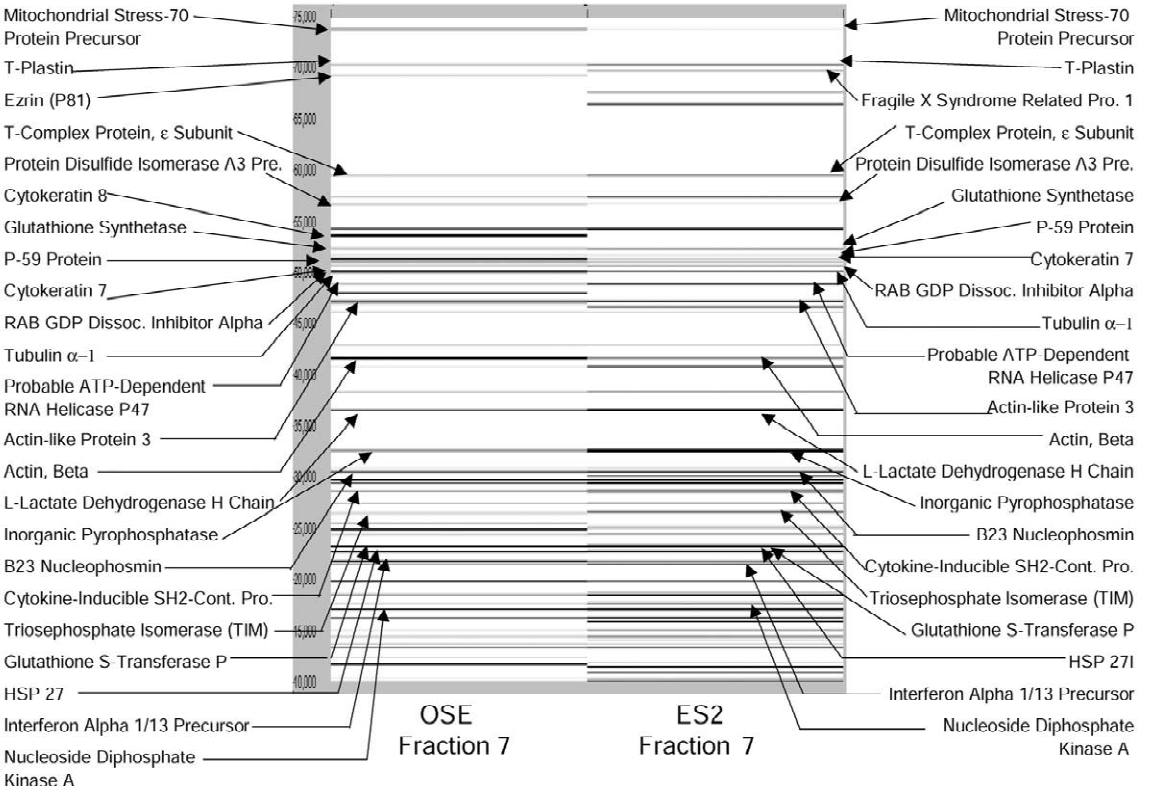

Fig. 3. Two-dimensional mass map of M_r versus p*I* comparing the ES2 cell line to the OSE cell line for Rotofor fraction 7.

>5000. The actual resolution and mass accuracy can be recovered from the original data. In addition, each protein band has been collected and digested by trypsin and analyzed by MALDI-TOF-MS peptide mapping. The band identity is displayed for each protein for which there was a strong positive identification based upon database searching. In addition, changes in quantitative expression of proteins in the two cell lines are shown based upon the relative intensities of the bands.

In Fig. 3, the protein bands shown are the more highly expressed proteins that have been deconvoluted, where many of the proteins that are detected at low expression levels have not been fully analyzed or are lost in the noise of the Max Ent processing. Nevertheless, over the full p*I* range, we observe a total of around 900 protein bands of which 750 are unique proteins over a range of 5–75 kDa with typically 50–60 proteins detected in each p*I* fraction. Over the full p*I* range of this cell line we could identify around 30% of the proteins with confidence based upon our criteria. The bands identified include many of the common proteins found in normal or cancer cells such as actin, the tubulin isoforms, heat shock protein (hsp) 71, hsp 27, vimentin, and NDP kinase, and also other bands of proteins expressed at lower levels. Using this method we can detect <1 ng of a 40 kDa protein, which is comparable to silver staining methods. In addition, we have obtained each of these p*I* band spectra using an injection of ~50 μg of protein which corresponds to $\sim 10^5$ cells. We thus estimate that we are analyzing close to 1000 copy numbers per cell which is comparable to that of a silver stained gel. It should be noted that the 2D gel method can still detect more proteins over a larger M_r range. The mass mapping method is highly efficient below 40 kDa, but loses sensitivity at increasing M_r due to the increased number of multiple charges for larger proteins. Improvements in detection of proteins will be accompanied by enhancements in the detection and deconvolution process for the ESI-multiply charged umbrellas.

An important advantage of the 2D liquid separation–mass mapping method over 2D gels is the use of an accurate M_r as the standard for interlysate comparison. A major problem with 2D gels in interlysate comparisons is the lack of run-to-run reproducibility. This becomes a problem especially in interlaboratory comparisons where one may want to compare a protein profile image to that of a database. The use of the M_r of the intact protein becomes the standard for comparison. This is shown in Fig. 3 where proteins have been mapped according to M_r and these proteins can be compared between normal and cancer cell lines. In addition, there is also the hydrophobicity, which can be compared in terms of % acetonitrile elution. Thus, there are at least three parameters in which to initially compare the proteins in each fraction. The peptide map for each protein further serves to tag each protein in these interlysate comparisons. For example, Table 1 lists the proteins that have been identified in both the OSE and ES2 cell lysates from Rotofor fraction 7. The M_r of a protein in each fraction is often detected within 100–200 ppm from that of the other fraction. There are proteins such as protein disulfide isomerase A3 precursor, keratin type II cytoskeletal 8, glutathione synthetase and β-actin in which the M_r values in the two cell lines are detected within 1 Da or less. Other proteins such as T-plastin and P-59 protein show a measured difference of ~150 ppm between the M_r values of the OSE and ES2 cell lines. This difference may be due to an as yet unidentified PTM or just an error in measurement. However, the protein maps and the hydrophobicities of these proteins do correlate. The key issue is that in interlysate comparisons proteins can be matched based upon the p*I* and intact M_r of the protein between cell lines. The protein matches being compared can then be verified by peptide mapping patterns to confirm the identity of the protein.

The ultimate application of these maps remains the ability to study differences in expression between cancer cell lines. This means studying differences in quantitative expression of proteins in interlysate comparisons. Although the ESI efficiency is different for each protein, in these cancer differential expression studies, the same proteins are being compared between different samples rather than proteins within the same sample. In order to normalize the quantitation between different runs an internal standard, insulin, has been used. In addition, several of the major housekeeping proteins can be compared since these generally do not change significantly in expression. This method is often used for quantitation

Table 1
Proteins identified in ES2 and OSE fraction 7 with quantification and hydrophobicity comparison

Fraction 7 protein name	Accession No.	MALDI % coverage	MS-Fit M_r	OSE LCT M_r	% B	ES2 LCT M_r	% B	% Change in expression ES2 versus OSE
Mitochondrial stress-70 protein precursor	P38646	17	73 780.3	73 812	50.33	73 780	50.45	−351
T-Plastin	913797	31	70 436	70 388	49.35	70 377	49.58	121
Fragile X mental retardation syndrome	P51114	33	69 692.3			69 778	43.09	†
Ezrin (P81) (cytovillin) (villin 2)	P15311	18	69 399.4	69 308	42.51			†
T-Complex protein 1, ϵ subunit	P48643	29	59 621	59 627	49.56	59 605	49.60	195
Protein disulfide isomerase A3 precursor	P30101	21	56 782.9	56 782	42.16	56 782	42.43	−89
Keratin, type II cytoskeletal 8	P05787	31	53 674	53 643	45.88	53 643	45.51	
Glutathione synthetase	P48637	20	52 385.3	52 311	50.01	52 312	50.10	88
P59 protein	Q02790	28	51 805	51 732	39.56	51 739	39.73	−12
Keratin, type II cytoskeletal 7	P08729	26	51 335	51 326	44.41	51 320	44.43	
RAB GDP dissociation inhibitor α	P31150	23	50 583.2	50 721	46.83	50 735	47.10	82
Tubulin α-1 chain, brain-specific	P04687	29	50 158	50 168	46.83	50 179	47.10	
Probable ATP-dependent RNA helicase P47	Q13838	30	48 991	48 913	49.01	48 921	49.11	272
Actin-like protein 3	P32391	22	47 371	47 315	48.65	47 300	48.76	111
Actin, cytoplasmic 2 (γ-actin)	P02571	27	41 793	41 677	48.65	41 674	48.76	
Actin, cytoplasmic 1 (β-actin)	P02570	30	41 737	41 735	48.65	41 736	48.76	
L-Lactate dehydrogenase H chain (LDH-B)	P07195	12	36 638.8	36 561	50.33	36 566	50.35	296
Inorganic pyrophosphatase	Q15181	31	32 660	32 714	39.56	32 712	39.79	578
B23 Nucleophosmin	X16934	18	30 938.4	30 887	39.56	30 906	39.79	144
Cytokine-inducible SH2-containing protein	Q9NSE2	31	28 663.2	28 645	49.01	28 644	49.16	−59
(TPI1..)Triosephosphate isomerase (EC 5.3.1.1) (TIM)	P00938	17	26 538.5	26 584	45.26	26 584	45.43	82
Glutathione S-transferase P	P09211	25	23 356	23 232	44.51	23 221	44.81	81
Heat shock 27 kDa protein (hsp 27)	P04792	25	22 782	22 785	38.62	22 782	38.87	−261
Interferon α-1/13 precursor	P01562	36	21 725.3	21 810	45.21	21 812	45.43	−71
Nucleoside diphosphate kinase A (NDK A) (NDP)	P15531	37	17 148.9	17 073	43.64	17 067	43.49	127

† % Change greater than 10 000 (reproduced with permission from Ref. [63], copyright 2002 American Chemical Society).

methods in 2D gel patterns. The result is that we can compare protein bands in terms of intensity and M_r so that changes in expression and structure can be determined. The relative changes in quantitation for Fig. 3 are shown in Table 1 in terms of a % change between the proteins in the normal versus ES2 cell line. A 100% change means a factor of 2× in expression. Many of the proteins in fraction 7 show only relatively small changes between 1 and 3× in upregulation compared to the normal cells. However, in many other cases there are strong differences in expression between proteins in the normal versus cancer cells. For example, there are two bands around 69 kDa identified as ezrin (P81) and fragile X mental retardation syndrome related protein 1. Ezrin is detected in the normal cells but in a different fraction compared to ES2 cells, while the fragile X mental retardation syndrome related protein 1 is detected in the ES2 cells but not in normal cells. These types of differences in protein expression may serve as potential markers for tumor detection for this specific type of ovarian cancer. Other proteins are differentially expressed in varying degrees as shown in Table 1. Many of these have not yet been identified such as the two proteins in fraction 7 around 68 kDa which are strongly expressed in the ES2 cell line, but not in the normal fraction. Using our mass mapping method, may also provide the ability to search for subtle differences in expression; although, it remains as yet to be determined whether such subtleties are biologically significant. The validation of such markers will require extensive studies on large numbers of such samples. However, the 2D liquid separation–mass mapping method can provide a reproducible method of comparing the expression of intact proteins from cellular samples based upon

M_r. Furthermore, using internal standards any number of related cell lines can be compared against a standard mass map.

3.3. Chromatofocusing–NPS RP-HPLC

An alternative method for fractionating proteins by p*I* used in this work is CF. CF is a column-based method which can be used to isolate proteins in a specific pH zone of interest. As the p*I* fractions elute from the column they can be collected in intervals of 0.1 to 0.2 p*I* units. The proteins in these fractions can then be separated by NPS RP-HPLC to produce a 2D UV map or can be directed into an ESI-TOF-MS to produce a M_r map. A p*I* fraction from such a 2D mass map is shown in Fig. 4. A major advantage of this method is the level of highly reproducible p*I* fractionation over narrow pH intervals. In Fig. 4 is shown the mass map for a fraction of 0.2 p*I* units at p*I* 6.68–6.48. This fractionation was performed over the entire range from p*I* 4 to 7 so that 30 p*I* lanes were run by RP-HPLC to produce an entire map (not shown). In Fig. 4, we compare the same p*I* fraction from two different samples where the one on the right is from a colon cancer sample and the one on the left is from a drug treated colon cancer sample. The two samples have been normalized using an internal standard so that quantitative comparisons in

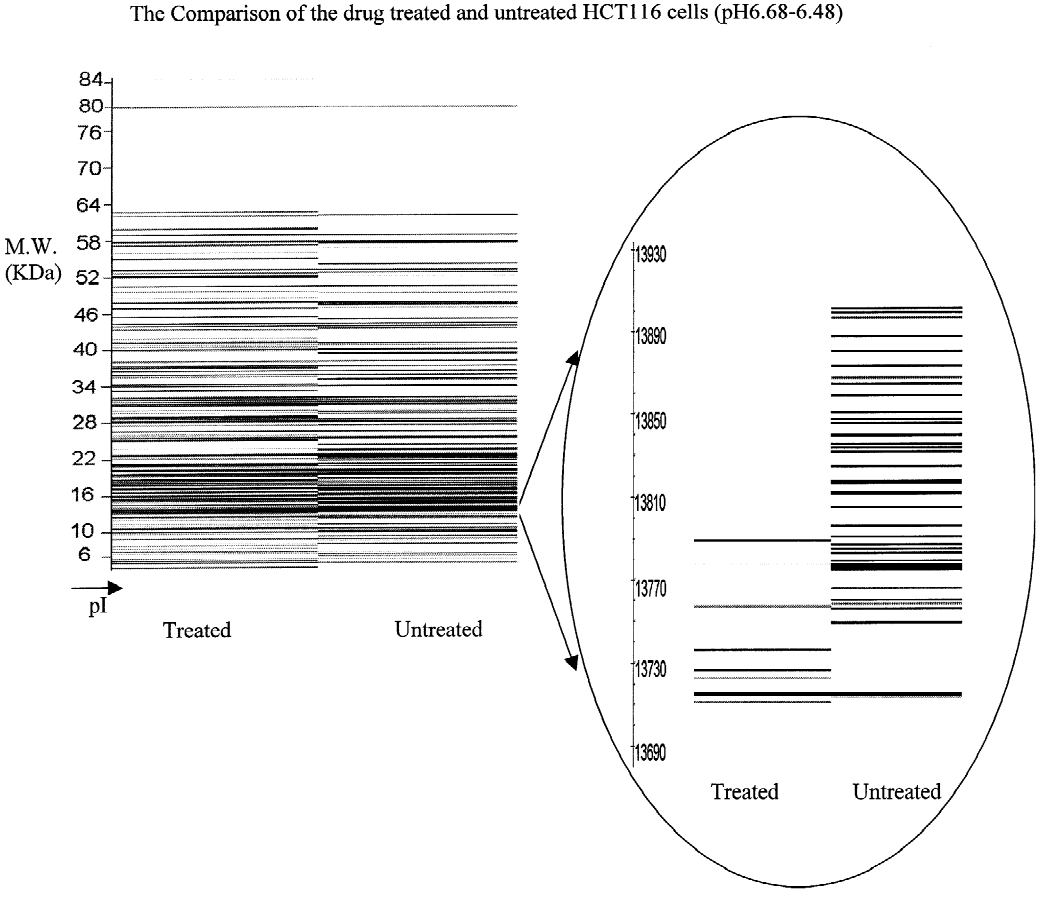

Fig. 4. Two-dimensional mass maps of M_r versus p*I* obtained using chromatofocusing as the first dimension. The maps illustrate a comparison of drug treated versus untreated HCT116 colon cancer cell lines for a narrow p*I* lane (pH 6.68–6.48).

expression can be made. In this p*I* lane there are nearly 80 unique proteins, and over the entire map it is presently estimated that over 2000 unique proteins can be detected. The degree of fractionation is excellent where few proteins overlap p*I* fractions. Also, the narrow range of fractionation allows the detection of many more unique proteins than in previous work. It also offers the same type of comparison between mass maps as in previous work, where major changes in protein expression are observed between the colon cancer sample and the drug-induced sample. This is observed in Fig. 4 where there is a highly overexpressed protein at around 14 kDa in the untreated colon cancer sample, while the same band is barely observed in the drug treated cancer sample. Upon expansion of this band (see inset) it is shown that this band actually consists of many protein bands that are not resolved on the original figure. It is seen that there are actually many of these low M_r proteins in the untreated colon cancer sample that are no longer observed in the drug-treated sample. The identity of these bands is presently unknown. The arduous task of identifying proteins in this sample is presently underway, and it is hoped that markers related to drug treatment in cancer samples may be found. This two-column based method also has the potential of being automated so that the possibility for large-scale comparisons of different cancer samples or drug treated cancer samples could be realized.

4. Conclusion

The use of 2D liquid separations has the potential to become an important alternative to the use of 2D gels for profiling the protein content of the cell. The methodology used in our work uses a p*I* fractionation of proteins followed by RP-HPLC separation and mass spectrometric detection. The result is a 2D map that provides the same type of image as 2D gels which can be used for searching for changes in protein expression and structure in interlysate comparisons. The 2D liquid method has the potential advantage of speed and automation compared to 2D gels. The use of liquid separation methods also provides a convenient means of interfacing the technique to ESI-TOF-MS where accurate intact M_r values can be obtained. These M_r values together with the p*I* and hydrophobicity serve as a highly reproducible method for tagging proteins in interlysate comparisons. In addition, hundreds of proteins can be collected from the liquid eluent of the HPLC separation, subsequently digested, and identified based upon peptide mapping and the M_r value. The method has the advantage of being performed in preparative mode where large amounts of protein can be recovered for further analysis and one may be able to eventually access low level proteins. The method can also be performed in analytical mode where smaller amounts of sample may be available. It can also be performed on the cytosolic and membrane fraction under 100 kDa using the proper solubilization methods. In ongoing work, NPS columns are being utilized to study the microsomal membrane fraction of various cancer cell lines [67]. These 2D liquid separation methods are still in early stages of development; however, they have the potential to play an important role in the search for protein markers important in cancer progression.

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