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Enhanced analysis of human breast cancer proteomes using micro-scale solution isoelectrofocusing combined with high resolution 1-D and 2-D gels

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Abstract

Current methods for quantitatively comparing proteomes (protein profiling) have inadequate resolution and dynamic range for complex proteomes such as those from mammalian cells or tissues. More extensive profiling of complex proteomes would be obtained if the proteomes could be reproducibly divided into a moderate number of well-separated pools. But the utility of any prefractionation is dependent upon the resolution obtained because extensive cross contamination of many proteins among different pools would make quantitative comparisons impractical. The current study used a recently developed microscale solution isoelectrofocusing (μsol-IEF) method to separate human breast cancer cell extracts into seven well-resolved pools. High resolution fractionation could be achieved in a series of small volume tandem chambers separated by thin acrylamide partitions containing covalently bound immobilines that establish discrete pH zones to separate proteins based upon their p*I*s. In contrast to analytical 2-D gels, this prefractionation method was capable of separating very large proteins (up to about 500 kDa) that could be subsequently profiled and quantitated using large-pore 1-D SDS gels. The pH 4.5–6.5 region was divided into four 0.5 pH unit ranges because this region had the greatest number of proteins. By using very narrow pH range fractions, sample amounts applied to narrow pH range 2-D gels could be increased to detect lower abundance proteins. Although 1.0 pH range 2-D gels were used in these experiments, further protein resolution should be feasible by using 2-D gels with pH ranges that are only slightly wider than the pH ranges of the μsol-IEF fractions. By combining μsol-IEF prefractionation with subsequent large pore 1-D SDS–PAGE (>100 kDa) and narrow range 2-D gels (<100 kDa), large proteins can be reliably quantitated, many more proteins can be resolved, and lower abundance proteins can be detected.

Keywords: Proteomics; Sample prefractionation; Solution isoelectrofocusing; Two-dimensional gel electrophoresis

1. Introduction

Proteomics is a relatively new and rapidly expanding research field that includes quantitative comparisons of proteins from two or more closely related experimental samples (protein profiling). Although 2-D polyacrylamide gel electrophoresis (2-D PAGE) is the most commonly used method for detecting changes in protein profiles of cells, tissues or whole organisms, it does not have sufficient dynamic range to detect low abundance proteins [1–3]. Analysis of most eukaryotic proteomes is further complicated by their great complexity. For example, most mammalian cells probably contain more than 20,000

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unique protein components, while 2-D gels typically resolve only about 1500 to 2000 spots. In addition, 2-D gels cannot effectively analyze several major groups of proteins including large polypeptides (>100 kDa), insoluble proteins, and proteins with p*I*s at the pH extremes.

The great complexity of most proteomes together with the limitations of current 2-D gels and alternative current protein profiling approaches indicates that improved proteome analysis methods are needed. Ideally, such methods should be capable of resolving, detecting, and quantitatively comparing the majority of unique protein components present in mammalian cells or tissues, including discrimination of protein isoforms and differences in posttranslational modifications. Previously reported prefractionation methods prior to 2-D PAGE include sequential extractions with increasingly stronger solubilization solutions [4], subcellular fractionation [5], selective removal of the most abundant protein components [6], and fractionation of eukaryotic cell extracts using different chromatographic techniques [7–9]. Although more proteins could be detected when such prefractionation strategies were used, these methods usually resulted in substantial cross-contamination of multiple protein components among two or more fractions. Such incomplete separation of large numbers of proteins seriously complicates quantitative comparisons.

We recently developed a microscale solution isoelectrofocusing (μsol-IEF) method that can reproducibly fractionate samples into well-defined pH pools on a scale compatible with high sensitivity proteome studies [10]. The μsol-IEF device is a simple low volume preparative IEF apparatus that utilizes the basic separation principle originally described by Righetti et al. [11] for the IsoPrime preparative IEF unit (Amersham Biosciences), a complex large scale device designed for purification of individual proteins usually under native conditions. In contrast, μsol-IEF separates proteins under denaturing conditions based on charge in a series of small (typically 500 μl) sealed separation chambers without cross-flow or external mixing but with large pore partition membranes to ensure that large proteins are effectively separated. Subsequently, we showed that μsol-IEF prefractionation enhanced protein profiling of complex proteomes such as human cell extracts and mouse serum [12,13]. One feature of our μsol-IEF method is that the total number of separation chambers, their pH ranges, and their volumes can be easily adjusted to fit requirements of different proteomes and research goals [10].

In the present study, we used μsol-IEF to prefractionate the proteome of human breast cancer cells into seven discrete pools, including four sequential 0.5 pH range fractions in the pH 4.5–6.5 region that contains the majority of cellular proteins. These 0.5 pH pools were separated on high resolution narrow pH range 2-D gels to resolve the large number of proteins that were less than 100 kDa. In addition, large pore 1-D PAGE gels were used to separate and quantitate large proteins (up to about 500 kDa), which are typically not reliably separated or recovered on 2-D gels. The results of this study illustrate a comprehensive strategy for global protein profile analysis of complex proteomes using μsol-IEF sample prefractionation followed by a combination of 1-D and 2-D PAGE.

2. Experimental

2.1. Preparation of cell extracts

A variant of the human breast carcinoma cell line, MCF-7 that has low metastatic potential, MCF-7/AZ [14], was kindly provided by Dr. M.M. Mareel (Ghent University, Belgium). Cells were grown in vitro in a 37 °C incubator with a 5% CO_2–95% air atmosphere in a 1:1 mixture of Dulbecco's Modification of Eagle's Medium/Ham's F-12 (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) as previously described [15]. Prior to protein extraction, 80–90% confluent cells in T75 flasks were washed three times with PBS containing protease/phosphatase inhibitors (0.15 m*M* PMSF, 2 m*M* Na_3VO_4, 50 m*M* NaF, 1 μg/μl leupeptin, 1 μg/μl pepstatin-A) at 0 °C. Cells were immediately lysed in situ on ice with lysis buffer containing 2 m*M* sodium phosphate, 100 m*M* DTT, 5 m*M* EDTA, 4% CHAPS, and protease/phosphatase inhibitors (see above), pH 7.4 for 10 min. The flasks were scraped to remove remaining insoluble material, the extracted samples were transferred to a plastic centrifuge tube, and

sonicated on ice with a probe-tip sonicator using four 2-s bursts with 4-min pauses between sonication cycles to prevent overheating. The supernatant was collected by centrifugation at 40,000 *g* for 30 min at 4 °C. The pellets were resuspended in a small volume of lysis buffer, sonicated and centrifuged as above. This supernatant was combined with the initial supernatant, and represented the soluble component of ~10^7 cells/ml at a protein concentration of about 1.0 mg/ml. The actual protein concentrations of the supernatant and pellet were determined, and samples were stored as aliquots at −80 °C until needed.

2.2. Gel electrophoresis

2.2.1. 2-D PAGE

IPG strips were purchased from Amersham Biosciences (San Francisco, CA, USA) and proteins were isoelectrofocused using the IPGphor™ Isoelectric Focusing System, essentially as described by Görg et al. [16]. Briefly, immediately prior to IEF, samples were diluted into IEF buffer containing 2 *M* thiourea, 7 *M* urea, 2% NP-40, 5 m*M* TBP (tributyl phosphine), 10% sorbitol and 1% IPG-buffer (carrier ampholyte mixture matching the pH range used) to yield the desired protein amount in a volume that could be adsorbed by the IPG strip used. IPG strips were rehydrated with IEF buffer containing the sample followed by automated isoelectrofocusing overnight. Typically, 18-cm IPG strips were rehydrated for 10 h (1 h without current followed by 9 h at 30 V), followed by focusing for 1 h each at 500, 1000, and 2000 V, followed by 8000 V until a total of ~80–90 kVh was reached. Shorter focusing times were used for 7-cm IPG gels (total of ~30–35 kVh).

In most experiments, 10% Tris–Tricine polyacrylamide gels [17] were used for the second dimension. IPG strips were incubated in 10 ml of 50 m*M* Tris, 6 *M* urea, 2% SDS, 30% glycerol, 30 m*M* DTT, pH 6.8, for 10 min, followed by incubation for 10 min in the same solution, except DTT was replaced by 2.5% iodoacetamide. The SDS-equilibrated IPG gel was sealed on top of the second-dimension gel using 0.5% agarose containing 50 m*M* Tris–Cl (pH 6.8), 2% SDS, 30% glycerol and bromophenol blue and the gel was run at ~80 V at room temperature overnight until the tracking dye migrated to within 1-cm of the bottom of the gel.

2.2.2. 1-D PAGE

Two types of 1-D gels were used. The complexity of μsol-IEF fractions and their approximate protein concentrations were estimated using 10% Tris–Tricine gels similar to the above second dimension gels, except a 2.5% stacking gel with 15 or 20 sample wells was used. For analysis of large proteins, 4–9% gradient gels were prepared as described by Hochstrasser et al. [18], where the gel was cast in the absence of SDS, piperazine diacrylyl (PDA) was used as the gel crosslinker, and sodium thiosulfate was included in the gel to reduce silver stain background.

2.2.3. Protein visualization and image analysis

Various protein detection methods were used to visualize proteins resolved on 1-D or 2-D gels, including: Coomassie blue, Colloidal Coomassie, SYPRO Ruby, and silver stain. SYPRO Ruby protein gel stain (Molecular Probes, Eugene, OR, USA) was used to stain gels as described by the manufacturer and gel images were scanned using a FluorImager SI (Molecular Dynamics, Sunnyvale, CA, USA). For silver staining, SilverQuest (Invitrogen, Carlsbad, CA, USA) kits were used as described by the manufacturer. Stained 2-D gels were analyzed using Melanie (version 3.0) 2-D PAGE image analysis software (Genebio, Geneva, Switzerland) and 1-D gels were analyzed using Discovery Series Quantity One (version 4.2.0) software (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. The μsol-IEF method

The μsol-IEF device and method used here was similar to the original method [10] except the partition membrane supports and composition were modified to improve physical integrity and protein transfer [12,13]. Briefly, human breast cancer cell extracts were fractionated into seven pools based on their p*I*s in tandem 500-μl liquid-filled chambers separated by thin porous acrylamide gel membranes containing immobilines at specific pHs (partition membranes). Varying numbers of chambers were assembled into a unit, which included the separation

chambers where proteins were loaded and terminal electrode chambers that were filled with electrode buffers. The assembled unit was placed in an electrophoresis tank and submerged in bulk electrode buffer chambers.

In general, adjacent separation chambers were separated by 3%T acrylamide gels and the terminal separation chambers and electrode chambers were partitioned by 10%T gels, all containing immobilines at specific pHs. The terminal separation chambers were protected from the bulk electrode buffers by electrode chambers that contained 5 kDa cut-off dialysis membranes (MWCO 5K Dialysis Membranes, Harvard Bioscience) on their outer faces.

Porous hydrophilic polyethylene (1.5 mm thickness, medium pores with 45–90 μm DBS) (POREX, Lecester, MA, USA) was used to provide mechanical strength to the partition membrane. Prerinsing in aqueous solutions should be avoided as this can reduce hydrophilicity of the polyethylene and adhesion of the polyacrylamide gel in the pores. Buna O-Rings (12 mm I.D., 2 mm thickness) (Scientific Instrument Services, Ringoes, NJ, USA) were used to form a seal between the chambers and the acrylamide/immobiline membrane discs.

Stocks of six immobilines (p*K*s 3.6, 4.6, 6.2, 7.0, 8.5 and 9.3) from Amersham Biosciences as 0.2 *M* solutions in water or isopropanol were used to make the μsol-IEF partition membranes at specific pHs. The components of the μsol-IEF buffer were the same as in the IEF sample buffer described above. Electrode buffers were 7 m*M* phosphoric acid (anode) and 20 m*M* lysine/20 m*M* arginine (cathode) from Bio-Rad. In some experiments, 10% sorbitol was included in the electrode buffers.

2.4. Preparation of acrylamide/immobiline membranes at desired pHs

Partition membranes with different pHs were prepared using a two-step process as described below.

2.4.1. Step 1. Preparation of immobiline mixtures at desired pHs

Typically, a computer program, ''Dr. pH'', developed by Giaffreda et al. [19] and available from Amersham Biosciences, was used to simulate immobiline recipes to make μsol-IEF partition membranes. Precalculated recipes from pH 3.0 to pH 10.0 using the Dr. pH program have also been published [20]. The final concentration of total immobilines used in the gel solution was ~10–20 m*M*. After preparing immobiline mixtures, their pHs were measured using a pH meter at room temperature. The pH was adjusted, if needed, using small volumes of immobiline stock at the appropriate extreme pH (either p*K* 3.6 or 9.3) to the desired value. This solution was then titrated to pH ~6.5 by addition of 1 *M* solutions of either Tris base or acetic acid to facilitate gel polymerization. These titrants should not affect the final membrane pH since they are not covalently incorporated and are washed out of the gel partition after polymerization.

2.4.2. Step 2. Preparation of partition membranes

To facilitate effective separation of larger proteins and minimize protein precipitation on the partition membranes, large pore acrylamide/immobiline gels, i.e. low total acrylamide (T%) and high crosslinker concentration (C%), are necessary for sample prefractionation. Typically, 3%T/8%C gels were used between separation chambers and 10%T/8%C gels were used between the terminal separation chambers and the electrode chambers. The immobiline mixture prepared in step 1 was mixed with the appropriate amount of an acrylamide/Bis stock (30%T/8%C) as described previously [10], except that 2% NP-40 and 10% sorbitol were also included in the gel solution. Gels were cast between two layers of gel support membranes (Bio-Rad Laboratories) that had been coated with Repel-Silane (Amersham Biosciences). Briefly, the polyethylene discs were placed on a silanized film and surrounded by 2 mm slab gel spacers (Bio-Rad Laboratories) inside a pipet-tip box lid (~10.4×12.6 cm). The gel solution was then loaded onto the polyethylene discs until the solution was completely absorbed inside the polyethylene pores. Additional gel solution was added to cover the remaining areas of film until a 2 mm thickness (height of spacers) was achieved. The gel solution was then covered by another silanized support film. The immobiline/acrylamide gel polymerization conditions were modified compared with our previous method that used glass fiber filter supports and gel polymerization conditions of 1–1.5 h at 50–60 °C

[10]. The use of porous polyethylene supports in the current study necessitated polymerization at lower temperatures; hence, efficient polymerization was obtained within 1 h at room temperature (~23 °C) by increasing the ammonium persulfate and TEMED concentrations by 1.5-fold. After polymerization, the polyethylene acrylamide discs were cut from the surrounding polymerized gel and removed. Membrane discs with continuous and homogeneous gel covering both sides of polyethylene discs were selected and excess gel on the surfaces of the disc was removed using a scalpel or razor blade. Cleaned discs were immediately transferred into individual wells in a 24-well tissue culture plate (Fisher Scientific). The membrane discs were washed three times in 2 ml of 12% glycerol, 10% sorbitol, 2% NP-40 for 30 min with shaking at room temperature to remove polymerization by-products. Washed membranes could be stored in the same solution containing 2 m*M* sodium azide at 4 °C for up to 1 month.

2.5. Loading samples and fractionation of proteins with solution IEF

Protein samples were prepared in a final volume of IEF buffer equal to the total volume of the chambers where sample would be loaded. In the present study, 2.0 mg of human breast cancer cell extract was in a final volume of 3.0 ml buffer and loaded into six separation chambers (each 500 μl). The basic terminal separation chamber was loaded with the sample buffer and the electrode chambers were loaded with the IEF electrode buffers as described above. The assembled unit was placed in the electrophoresis tank and the two compartments of the tank were filled with anode and cathode electrode buffers. A power supply capable of at least 1500 V should be used for μsol-IEF prefractionation. In this study, a model 3000 Xi Electrophoresis Power Supply (Bio-Rad Laboratories) was used at 1 mA constant current. For 2.0 mg of human breast cancer cell proteins, the prefractionation was terminated when ~1200 V (~1 W) was reached (total focusing time was ~4–4.5 h).

After completion of μsol-IEF prefractionation, the resulting fractions (each ~500 μl) were removed. To minimize cross-contamination and loss of fractionated samples during their removal, about half of the sample was withdrawn using a 1-ml insulin syringe with a fixed 28 gauge needle to pierce the terminal dialysis membrane or acrylamide/immobiline partition membrane before the chamber was opened. The pierced membrane was then removed, the remainder of the liquid in the chamber was transferred to a microfuge tube, the walls and membrane surfaces of each chamber were rinsed with 200 μl sample buffer, and the rinse was combined with the fractionated sample. This removal procedure was then repeated for each successive chamber. A few proteins, primarily those with p*I*s equal to the pHs of partition membranes, were retained in the partition membranes after isoelectrofocusing. To recover these proteins, the partition membranes were extracted two times with 350 μl sample buffer for 30 min with shaking at 23 °C and the extracts are pooled. Samples were stored as aliquots at −80 °C until required.

3. Results

3.1. A strategy for comprehensive analysis of mammalian proteomes using μsol-IEF prefractionation combined with high resolution 1-D and 2-D gels

Since most mammalian cells or tissues contain more than 20,000 unique protein components, a high resolution prefractionation step combined with multiple types of subsequent analyses are needed to separate and quantitate the majority of the proteome. A strategy using μsol-IEF sample prefractionation to initially separate mammalian proteomes into well-resolved pools is shown in Fig. 1. In this scheme, a μsol-IEF device consisting of seven separation chambers is used to fractionate the sample based upon protein p*I*s. As illustrated, the separation chambers are bounded by polyacrylamide gel membranes with covalently bound immobilines that define discrete pHs of 3.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.5 and 10, respectively. After isoelectrofocusing, each chamber should contain only proteins with p*I*s between the pHs of the boundary membranes of that chamber. A series of four very narrow pH chambers are used between pH 4.5 and 6.5 because the p*I*s of the majority of proteins in eukaryotic proteomes fall within this two pH unit region.

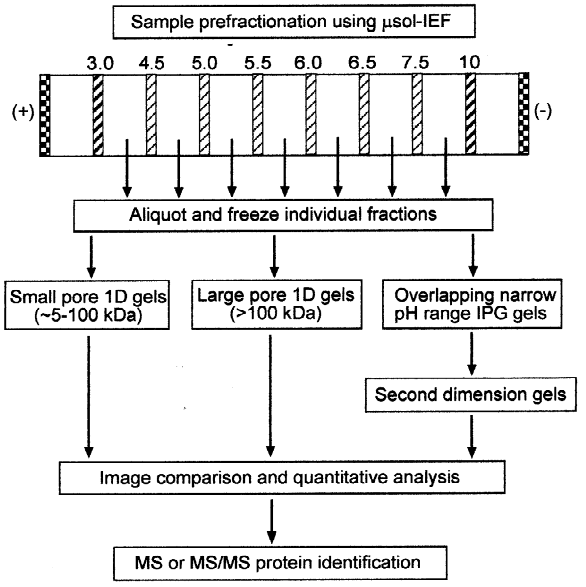

Fig. 1. Schematic illustration of a strategy for comprehensive analysis of mammalian proteomes using μsol-IEF sample prefractionation. This μsol-IEF device contains seven separation chambers divided by acrylamide partition membranes containing immobilines with the pHs indicated above the gel partitions in the diagram. Large pore 1-D gels are used to compare large proteins and 10% 1-D Tris–Tricine gels are used to analyze lower complexity μsol-IEF fractions such as very acidic and very basic fractions. Narrow pH range 2-D gels are used to analyze proteins less than 100 kDa in complex (central pH) fractions.

After μsol-IEF prefractionation, pools are analyzed by a combination of high resolution 1-D and 2-D polyacrylamide gels. Specifically, 18-cm long 10% Tris–Tricine 1-D ge1s can be used to detect and analyze proteins with molecular masses up to about 100 kDa. Complementary data are obtained by parallel analysis of fractions on larger pore 18-cm long 1-D gels for large proteins (greater than about 100 kDa) that cannot be reliably analyzed by 2-D gels. Fractions that contain large numbers of proteins below 100 kDa must also be separated on narrow range 2-D gels. Ideally, these 2-D gels should utilize IPG strips that are only about 0.1 pH units wider than each fraction to maximize separation distances so that a maximum number of proteins can be resolved in each pool. This strategy enables detection and reliable quantitation of a much larger number of proteins, including lower abundance proteins and very large proteins, than alternative methods.

3.2. Analysis of μsol-IEF fractions using high resolution 1-D PAGE

A comparison of fractions from μsol-IEF separation of 2.0 mg of human breast cancer proteins on a high resolution 10% Tris–Tricine 1-D SDS gel is shown in Fig. 2A. This rapid 1-D gel analysis can be used to evaluate differences in total protein content and complexity of different fractions. Interestingly, a substantial number of major bands throughout the gel are sequestered in individual fractions, despite the fact that most fractions have far more proteins than can be resolved on a 1-D gel. Hence, 1-D gels provide an initial rapid assessment of μsol-IEF separation quality and reproducibility. In addition, at least the most acidic fraction appears sufficiently simple that 1-D gel image comparisons should be adequate for quantitative protein profile comparisons of this pool. In addition, much higher protein loads of this fraction can be used to detect very low abundance of very acidic proteins.

In this experiment, SYPRO Ruby was used because it has a much wider linear range compared with silver stain. As a result of the broad detection linear range of this fluorescent stain, total lane densities can be used to estimate protein amounts in individual fractions, and overall recoveries can be estimated by comparisons with the unfractionated sample. As shown in Table 1, about 2/3 of the total protein is recovered in these seven fractions. Additional proteins remain in the membrane partitions because their p*I*s match the membrane pH, while other proteins have p*I*s below 3 or above 10 and therefore migrate to the terminal electrode chambers. Although these proteins were not analyzed in these experiments, they can be recovered if desired to increase the overall protein recovery to about 80–90%.

The μsol-IEF fractions were also separated and analyzed using 18-cm 4–9% gradient gels (Fig. 2B). These large pore 1-D gels effectively separate large polypeptides up to about 500 kDa and therefore complement data from the 10% Tris–Tricine 1-D gels and narrow pH range 2-D gels. We previously showed unfractionated cancer cell extracts could be analyzed on large pore gels and quantitative changes related to metastatic potential could be detected by comparison of band intensities [12]. However, when

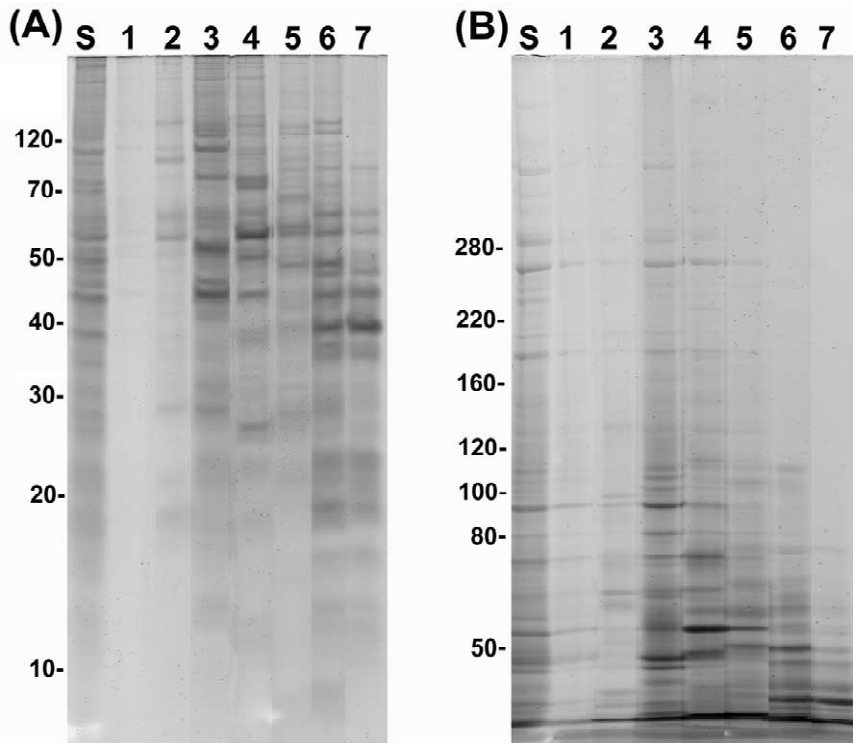

Fig. 2. Characterization of human breast cancer cell proteins after μsol-IEF prefractionation using SYPRO Ruby stained 1-D gels. (A) 10% Tris–Tricine gel; (B) 4–9% gradient gel. S, sample prior to fractionation (30 μg); 1–7, μsol-IEF fractions 1 to 7 from prefractionation of 2.0 mg of a human breast cancer cell extract using the scheme shown in Fig. 1; amounts of the original unfractionated sample that each fraction was derived from were: 1, 300 μg; 2, 240 μg; 3, 120 μg; 4, 120 μg; 5, 120 μg; 6, 240 μg; 7, 300 μg.

unfractionated cell extract supernatants were analyzed, many high molecular mass bands contained several proteins and major proteins could mask quantitative changes in underlying minor proteins. In the current study, the large proteins visible in the unfractionated sample could be readily detected in one or more μsol-IEF fraction indicating most large proteins were recovered after μsol-IEF. Although some bands appeared to be distributed between multiple fractions, this is unlikely to indicate incomplete separation since many other bands were specifically sequestered in individual fractions. Instead, as mentioned above, the previous study showed that multiple unrelated proteins with identical migration rates were present in unfractionated samples [12] (and data not shown). In addition, some proteins are expected to have heterogeneous p*I*s due to variable posttranslation modifications, especially variable glycosylation. Hence, these initial comparisons of large proteins strongly suggest that μsol-IEF coupled with quantitative image analyses of large pore 1-D gels is an effective high throughput method for detecting changes of large proteins in protein profile

Table 1
Protein recoveries after μsol-IEF prefractionation

Fractionated samples	Protein recoveries (%)
Fraction 1 (pH 3.0–4.5)	2.5
Fraction 2 (pH 4.5–5.0)	4.2
Fraction 3 (pH 5.0–5.5)	18.0
Fraction 4 (pH 5.5–6.0)	16.3
Fraction 5 (pH 6.0–6.5)	10.6
Fraction 6 (pH 6.5–7.5)	8.2
Fraction 7 (pH 7.5–10.0)	6.1
Total recovery	65.9

Protein recoveries of μsol-IEF fractions were calculated from total lane densities on 10% Tris–Tricine gels and compared to total lane densities of unfractionated control lanes (see Fig. 1A) using Discovery Series Quantity One (version 4.2.0) software.

studies. The utility of this method is being further tested by LC–MS–MS analyses of matching bands from different fractions to evaluate protein band complexity.

3.3. Detection of μsol-IEF fractionated proteins using 2-D PAGE

The effectiveness of μsol-IEF sample prefractionation based upon protein p*I*s could be rapidly evaluated by analyzing small aliquots of each pool on appropriate 3 pH unit mini-gels. For example, results from four central fractions (each with 0.5 pH units) after prefractionation of 2.0 mg of human breast cancer proteins on pH 4–7 mini 2-D gels are shown in Fig. 3. These data illustrate the sharp separations based on p*I* between fractions. Only a few proteins outside the expected pH zones were detected and typically the degree of cross contamination of these proteins was very minor. Comparison of the protein pattern of the unfractionated sample with the individual fractions shows good recovery of most major spots on these relatively low resolution mini-gels. While these mini 2-D gels with Coomassie blue staining are not optimal for either resolution or detection sensitivity, they represent a rapid economical screen for checking effectiveness of μsol-IEF prefractionation prior to systematic analysis of these fractions on large narrow pH range 2-D gels using more sensitive stains.

Subsequent analysis of the four 0.5 pH unit fractions on 18×18-cm narrow pH range 2-D gels with silver staining is shown in Fig. 4. Consistent with the above mini 2-D gels, most proteins in

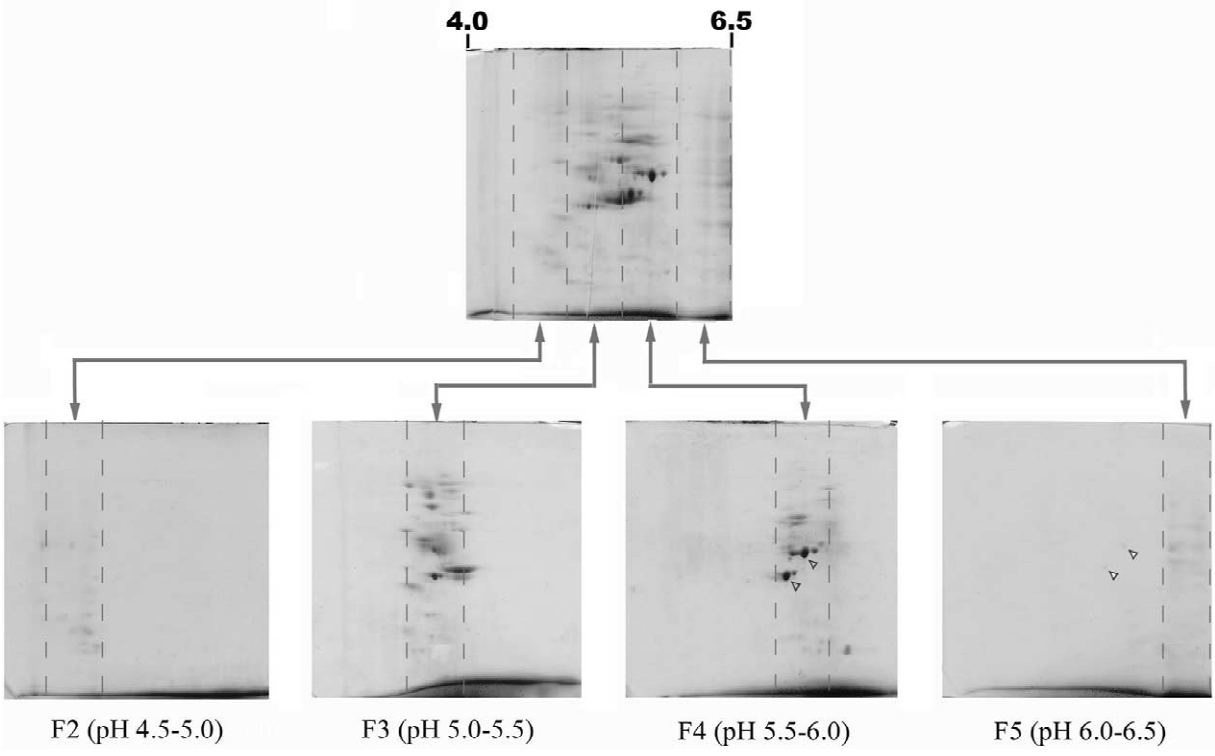

Fig. 3. Rapid screening of μsol-IEF fractions on Coomassie blue stained 2-D minigels. Unfractionated (200 μg) and fractionated samples from 200 μg of extract were focused on 7-cm pH 4–7L IPG gels, followed by 10% Tris–Tricine SDS minigels. Upper panel, the unfractionated sample. Lower panels, μsol-IEF fractions 2 to 4 (pH boundaries of fractions are indicated). Note that the actual pH range of the commercial ''pH 4–7L'' IPG strips is indicated as 4.0–6.5 as described on the manufacturer's website [28]. To illustrate the extent of overlap between μsol-IEF fractions, two minor spots outside the expected pH range in fraction 5 and matching major spots in fraction 4 are highlighted by triangles. About 7–10% of these two proteins were recovered in the incorrect fraction (fraction 5).

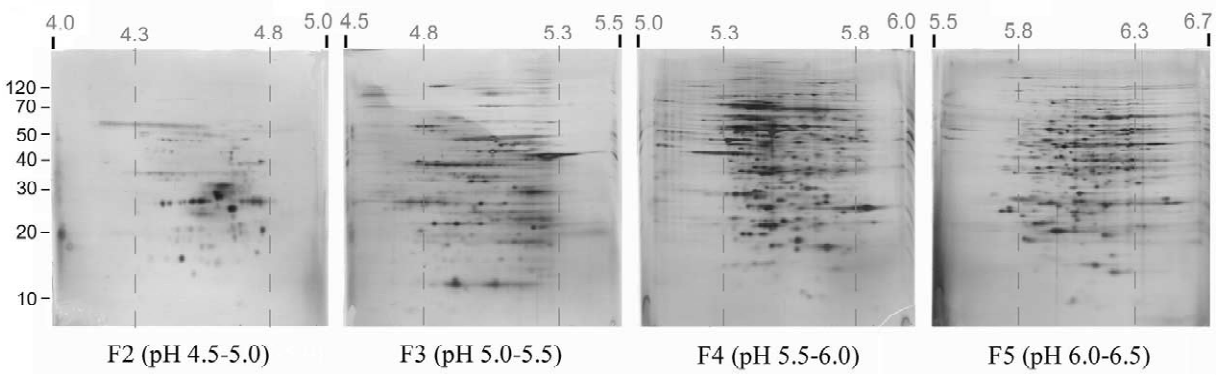

Fig. 4. Separation of human breast cancer cell μsol-IEF fractions on full-sized narrow pH range silver stained 2-D gels. Aliquots of each pool equivalent to 400 μg of unfractionated sample were analyzed on 18×18 cm gels. As indicated by the pH boundaries in the images, all actual pH ranges on the 2-D gels were shifted ~0.2 pH units below the original pHs of the gel solutions used for the partition membranes (values in parentheses).

individual fractions were found within sharp pH boundaries with minimal overlapping spots or proteins outside the pH zone of the pool. In these experiments, relatively high protein loads (equivalent to ~400 μg of unfractionated sample) were combined with silver staining to maximize detection of lower abundance proteins. Despite the resulting over staining of high abundance proteins, most proteins were well separated with only moderate horizontal streaking of some major proteins. The 2-D separations of prefractionated samples showed greatly improved overall protein resolution, recovery and consistency compared with proportional loads of unfractionated samples, which resulted in extensive streaking and loss of proteins due to coprecipitation (Fig. 5 and data not shown). These differences in protein load capacity between unfractionated and fractionated samples were similar to the results observed with serum samples and human melanoma cell extracts [12,13].

3.4. Effects of μsol-IEF prefractionation on protein spot detection

To further evaluate the utility of sample prefractionation on analysis of complex proteomes, different amounts of unfractionated samples (20 μg and 200 μg of human breast cancer cell proteins) and an aliquot of the pH 5.5–6.0 fraction equivalent to 200 μg of extract supernatant were analyzed on full size pH 5.0–6.0 2-D gels (Fig. 5). When 20 μg of unfractionated proteins were separated, reasonably good resolution was obtained but few spots were detected due to the low protein load. More total spots were detected with a higher load (200 μg) of unfractionated sample, but resolution was poor and many proteins appeared as smears. Protein streaking near the electrodes was substantial at the 20 μg load and severe at the 200 μg load because many proteins in the unfractionated sample with p*I*s outside the pH of the IPG gel migrated toward the electrodes and precipitated. In contrast, resolution was much better on the 2-D gel of the fractionated sample and this gel yielded the largest number of spots in the pertinent pH range. Differences between the unfractionated and fractionated samples are particularly evident in the enlarged areas of the gels shown in the lower panels of Fig. 5. That is, the 200 μg load of the unfractionated sample resulted in loss of ~38% of the total spots that were detected in the fractionated sample because these proteins coprecipitated with proteins outside the pH range of the IPG strip near the electrodes. In addition, many of the proteins that were present on both the unfractionated and fractionated samples were under recovered on the unfractionated gel for the same reason. These experiments show that μsol-IEF prefractionation enables use of greatly increased protein loads on narrow pH range 2-D gels while maintaining good resolution and spot recovery. As a result, lower abundant proteins can be detected and quantitative analyses of protein profiles

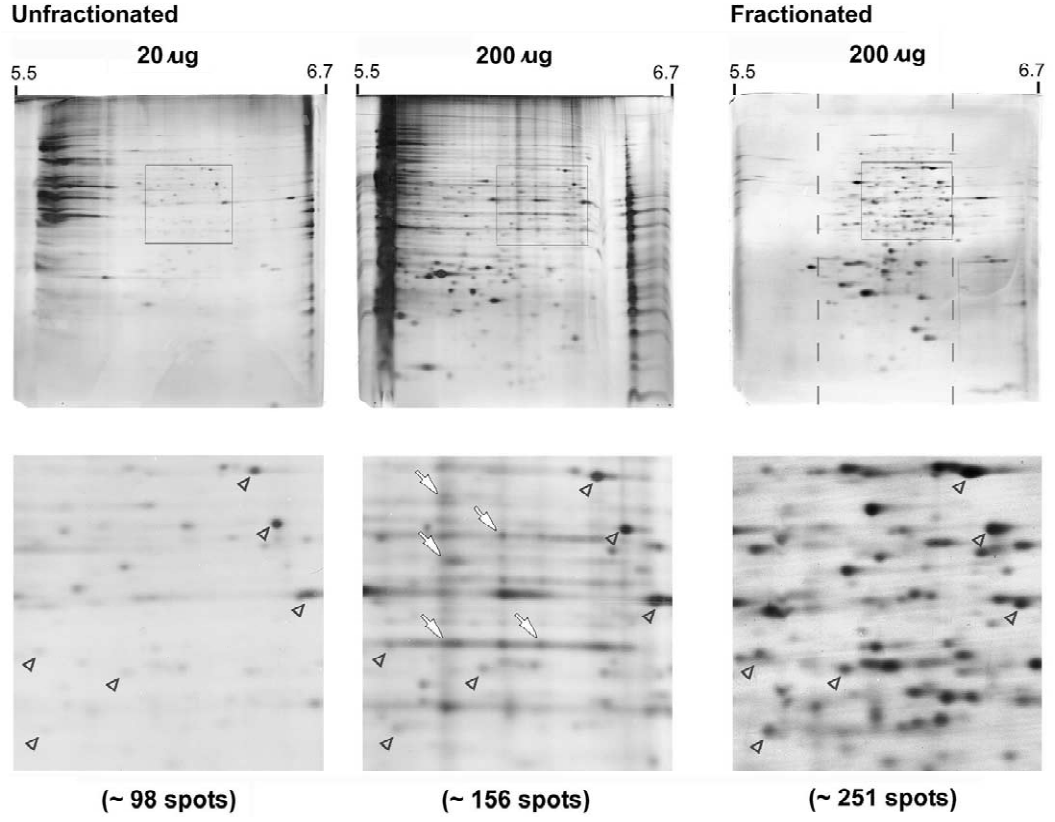

Fig. 5. Comparison of unfractionated and fractionated human breast cancer cell proteins on full-sized silver stained narrow pH range 2-D gels. Samples and electrophoresis conditions were as described in Fig. 4, except that 20 μg and 200 μg of unfractionated extract, and fraction 5 (pH 6.0–6.5) from 200 μg of extract were analyzed. Upper panels, complete 2-D gel images with a highlighted 0.3 pH wide region. Lower panels, enlargements of the highlighted regions. Triangles indicate landmark proteins to facilitate visual comparison and arrows highlight some poorly focused proteins in the higher load of unfractionated sample that co-migrated with either vertical or horizontal streaks and were detected at incorrect positions.

are more reliable due to minimization of protein streaking and co-precipitation artifacts.

4. Discussion

Over the past several years, it has become apparent that more powerful and reliable methods are needed for prefractionation of complex proteomes prior to 2-D gels or alternative LC–MS analysis methods, which would facilitate detection of low abundant proteins and increase the total number of proteins that can be separated, detected, and quantitated [3,9,12,21]. However, most prefractionation methods have substantial drawbacks when applied to proteome analysis. Commercially available preparative IEF devices, such as the Rotofor (Bio-Rad) and the IsoPrime (Amersham Biosciences), require large sample volumes, result in large dilute fractions that need to be concentrated, and often involve complex instrumentation. Recently, Herbert and Righetti described a related device that used immobiline/acrylamide partitions, which they called a multicompartment electrolyzer (MCE) and used to prefractionate *E. coli* and human serum samples prior to 2-D gel analysis [21]. However, the initial MCE contained large chambers (~100 ml) that required large sample amounts and resulted in large fraction volumes that

are not readily compatible with subsequent analysis on analytical 2-D gels.

Proteome analyses of samples available in limited quantities require small scale reproducible fractionation of complex proteomes into well-resolved pools. The prefractionation method recently developed in our laboratory, μsol-IEF, is capable of slicing complex proteomes into variable numbers of well-resolved fractions on a small volume scale compatible with direct subsequent analysis by 1-D gels, narrow pH range 2-D gels, or direct LC–MS–MS. Complex proteomes, such as human cell extracts and mouse serum, can be fractionated into well-resolved pools according to their p*I*s. Total recoveries are at least 65% and can be increased to greater than 80% if the partition membranes are extracted and the terminal chambers (very acidic and very basic proteins) are included in the analysis.

In the present study, we optimized the μsol-IEF prefractionation method for human breast cancer cell extracts and demonstrated the utility of a larger number of pH range fractions that include four sequential very narrow pH range chambers. The thin acrylamide partition membranes can be cast with the same pH precision as is inherent in IPG technology [22], which allows reproducible fractionation of a complex proteome into a relatively large number of very narrow pH range pools. Such narrow p*I* range pools can be analyzed on IPG strips encompassing the same pH range as the fractionated pools to maximize protein separation and detection. In practice it is advantageous to have slightly wider pH IPG strips than the pH range of the fraction in order to avoid ambiguities that always occur near IPG gel electrodes as well as minor variations in membrane pHs.

The μsol-IEF prefractionation approach allows the use of high proportional protein loads on narrow pH range 2-D gels that increase the number of protein spots which can be resolved and the dynamic detection range compared with direct use of parallel narrow pH range gels without sample prefractionation. The combination of high protein loads with sensitive stains such as SYPRO Ruby or silver stains enables the detection of lower abundant proteins compared with direct analysis of unfractionated samples. The maximum sample amounts that can be effectively prefractionated using μsol-IEF depend upon sample complexity, the total separation chamber volumes, and the number of separation chambers initially loaded with sample. Typically, when bacterial cell extracts or serum samples were prefractionated, about 3 mg of total protein could be fractionated using three to five 500-μl separation chambers [10,12,13]. In contrast, sample loading capacities were slightly lower, about 1.5–2 mg, for fractionation of more complex mammalian cell extracts using seven separation chambers as described in this study.

The buffering pHs of μsol-IEF partition membranes are critical since these pHs define the p*I* range of each fraction. Typically, several different immobilines are blended to obtain the desired pHs at final immobiline concentrations of ~10–20 m*M*, because higher immobiline concentrations could cause excessive gel swelling [19]. For example, Wenger et al. clearly showed erratic behavior of membranes containing >50 m*M* immobilines in the membrane partitions of the IsoPrime device [23]. In our experiments, fairly consistent 0.2 pH variations were observed between the calculated and measured pH of partition membrane solutions and the apparent pH ranges of resulting μsol-IEF pools (Fig. 4). There are several potential reasons for this moderate pH deviation. Firstly, the μsol-IEF partition membrane pH was determined in the absence of urea, while protein p*I*s on the IPG strips are in the presence of urea/thiourea. Secondly, different immobilines may be incorporated into the gel matrix with varying efficiencies, which would skew the actual pH. Unfortunately, the pH values on polymerized immobiline gels cannot be measured directly even with an advanced surface electrode [20]. Therefore, when deviations are observed between different immobiline gels such as μsol-IEF membranes and IPG gels, it is not straightforward to independently verify the pH accuracy of immobiline buffered acrylamide partitions. Regardless, these minor pH differences are reproducible and do not interfere with matching IPG strips with μsol-IEF fractions.

Partition membrane additives and μsol-IEF solution composition are other important experimental variables. In the present study, 2% NP-40 and 10% sorbitol were included in the partition membrane gel solutions prior to polymerization. These additives did not appear to adversely affect polymerization or

subsequent separations, and we observed that addition of 2% NP-40 to gel solutions prior to polymerization of partition membranes decreased potential protein precipitation on the membrane partitions. Addition of sorbitol to the sample buffer and the electrode buffer as well as to the gel membranes prior to polymerization appears to reduce electroosmosis during μsol-IEF similar to its previously reported role in gel-based preparative IEF [24]. Very large pores in partition membranes are essential for fractionation of large proteins as shown in Fig. 2B. This study showed that the 3% gels used in these experiments allow effective migration of very large proteins (up to 500 kDa). Presumably, even lower concentrations and thinner gel membranes may be advantageous but thinner, more porous gels are likely to be too fragile using the current porous polyethylene supports.

A conceptually attractive alternative to sample prefractionation is direct analysis of samples using multiple narrow pH range IPG gels in parallel for enhancing proteome analysis [25–27]. However, using this approach without sample prefractionation results in only moderate increases in proteins detected compared to use of a single broad pH range gel, because only low protein loads can be applied to narrow pH range gels (see Fig. 5). When high protein loads of unfractionated complex samples are applied to narrow range IPG gels, some proteins with p*I*s outside the pH range of the gels cause massive precipitation and aggregation [12,13]. In contrast, initial μsol-IEF separation minimizes non-ideal behavior (precipitation/aggregation) by segregating complex mixtures into separate pools.

In summary, this study demonstrates the value of μsol-IEF prefractionation of mammalian cell extracts followed by a combined 1-D and 2-D gel analysis approach for comprehensive quantitative protein profile comparisons. The 1-D PAGE analysis allows efficient high throughput quantitative comparisons of large soluble proteins as well as smaller proteins from simple fractions such as very acidic and very basic fractions. These three groups of proteins are difficult or impossible to analyze by 2-D gels. This integrated protein profiling strategy allows more comprehensive comparisons of proteomes in mammalian cells, tissues, and physiological fluids.

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