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A Method for Global Analysis of Complex Proteomes Using Sample Prefractionation by Solution Isoelectrofocusing Prior to Two-Dimensional Electrophoresis

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Two-dimensional electrophoresis is a critical technique for proteome research, but currently available methods are not capable of resolving the >10,000 protein components in most eukaryotic proteomes. We have developed and demonstrated the utility of a novel solution isoelectrofocusing device and method that can reproducibly prefractionate cell extracts into well-defined pools prior to 2D PAGE on a scale directly compatible with the high sensitivity of proteome studies. A prototype device was used to separate metabolically radiolabeled *Escherichia coli* extracts in method optimization and proof-of-principle experiments. Samples were loaded into separation chambers divided by thin polyacrylamide gels containing immobilines at specific pH values and isoelectrically focused for several hours, which resulted in well-resolved fractions. Total recoveries in the fractionated samples were greater than 80% and most protein spots in the original sample were recovered after this prefractionation step. Nonideal behavior (precipitation/aggregation), typically encountered when unfractionated samples at high protein loads were applied directly to either narrow- or broad-range IPG gels, was dramatically reduced. Hence this approach allows increases in overall protein loads, resolution, and dynamic detection range compared with either alternative prefractionation methods or direct use of parallel narrow pH range gels without sample prefractionation. The pH ranges and number of fractions can be readily adapted to the requirements of specific types of samples and projects. This method should allow quantitative comparisons of at least 10,000 protein components on a series of narrow pH range gels, and protein detection limits are estimated to be 1000 molecules per cell when mammalian proteomes are fractionated into five or more pools.

Key Words: **proteome analysis; sample prefractionation; solution isoelectrofocusing; immobilized pH gradient; two-dimensional gel electrophoresis; narrow pH range gels.**

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE)[2] is currently the only available method for quantitatively comparing changes in protein profiles of cells, tissues, or whole organisms (1, 2). The basic method utilizes isoelectric focusing under denaturing conditions in gel tubes or strips that contain either soluble ampholytes (3–5) or immobilines (6), followed by a second-dimension separation on a conventional SDS–PAGE slab gel. Existing 2D methods are reasonably adequate for prokaryote proteomes, since most prokaryotes have <3000 genes. In addition, the number of protein spots is typically only moderately larger than the number of genes since the extent of posttranslational modification is much lower in prokaryotes compared with eukaryotes. However, existing methods may not have sufficient dynamic range to detect low-abundant proteins in prokaryotes despite their simpler proteomes. Existing 2D methods are even less adequate for most eukaryote proteome studies. Yeast has over 6000 genes, *Caenorhabditis elegans* has over 19,000 genes, and higher eukaryotes have 100,000 or more genes. Although the number of genes that are

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[2] Abbreviations used: 2D PAGE, two-dimensional polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphate; IEF, isoelectric focusing; IPG, immobilized pH gradient; PMSF, phenylmethylsulfonyl fluoride.

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expressed at any one time in a mammalian cell is not well defined, estimates of at least 5000–10,000 seem reasonable. In addition, the total number of protein spots in higher eukaryotes per gene is further increased by substantial mRNA alternative splicing and posttranslational modifications (7). Current 2D methods lack adequate resolution and sufficient dynamic range for resolving and detecting the majority of the protein components present in eukaryotic proteomes. Hence, effective analyses of eukaryotic proteomes require improved protein separation methods capable of resolving and quantitatively detecting the majority of the >10,000 protein components present in whole-cell extracts.

One strategy for resolving an increased number of protein components in eukaryotic proteomes is prefractionation of samples prior to 2D PAGE. Previously reported prefractionation methods include sequential extractions with increasingly stronger solubilization solutions (8), subcellular fractionation (9), and selective removal of the most abundant protein components (10). Other alternatives include conventional chromatographic techniques, such as gel filtration, ion exchange, or affinity chromatography. However, these methods suffer from incomplete separation of proteins between fractions and poor yields are often encountered. Cross-contamination of specific proteins between fractionated pools can seriously complicate quantitative analyses and comparisons, since many proteins appear in more than one fraction and the degree of cross-contamination is often highly variable.

Prefractionation methods for proteome studies should improve the detection of minor proteins and increase the total number of protein components that can be identified (2, 11). The ideal prefractionation method would resolve complex protein mixtures such as total extracts of eukaryotic cells or tissues into a small number of well-defined fractions. A small number of fractions is essential; otherwise the already labor-intensive 2D separation becomes prohibitively complex. Yet high resolution is essential to minimize cross-contamination of proteins in adjacent fractions. Preparative IEF methods might be useful for prefractionation, but commercially available preparative isoelectric focusing apparatuses typically consume large amounts of sample, result in high protein losses, have much lower resolution than analytical IEF, with cross-contamination of many proteins between adjacent fractions, and yield very dilute fractions that are incompatible with direct subsequent analysis by 2D gels.

Preparative isoelectric focusing as a protein prefractionation procedure was proposed by Bier *et al.* (12) and a commercial version, the Rotofor, was produced by Bio-Rad. It is built as a rotating chamber divided into 20 compartments and uses solution isoelectric focusing to fractionate samples. However, this apparatus has no separation barriers and typically has a low resolution, with relatively large volumes for individual fractions. Righetti *et al.* (13) described a multicompartment electrolyzer where each compartment is separated by a polyacrylamide gel membrane with a specific pH. Immobilines are covalently incorporated into the polyacrylamide membranes analogous to IPG gels. A commercial apparatus, the IsoPrime instrument, incorporating this principle has been marketed (Amersham Pharmacia Biotech). The IsoPrime unit has been developed primarily for large-scale purification of individual proteins starting with partially purified preparations, not for fractionation of crude extracts. The unit has large separation chambers connected to peristaltic pumps and external chambers to further expand the volumes of individual fractions (about 30 ml). While the IsoPrime unit can provide high-quality separations, its large volume and design make it impractical for prefractionation of samples under denaturing conditions for most proteome studies. Overall, current available preparative isoelectric focusing instruments and methods typically suffer from most or all of the following limitations: (1) they require a large sample volume, (2) they produce large-volume, dilute fractions requiring concentration, with attendant losses, (3) they exhibit poor resolution, and (4) they involve expensive, complex instrumentation.

In this study, we evaluated several complex sample prefractionation methods for compatibility with subsequent 2D gel analysis. Particularly, we developed and demonstrated the utility of a novel solution isoelectrofocusing device and method that can reproducibly fractionate cell extracts into well-defined pools on a scale compatible with the high sensitivity of proteome studies. This method was developed using metabolically labeled *Escherichia coli* extracts to monitor and optimize protein yields. On the basis of these results, we predict that this approach can be applied to more complex eukaryotic samples, where reproducible resolution and quantitation of >10,000 protein components should be feasible.

MATERIALS AND METHODS

Preparation of Metabolically Radiolabeled E. coli Extracts

E. coli were cultured as previously described (14), with modifications. Briefly, *E. coli* cells were inoculated in Luria broth (LB medium) and incubated at 37°C with continuous shaking (250–300 rpm) for about 6 h. The LB culture was then inoculated into minimal medium and incubated overnight. When the optical density in the overnight culture reached approximately 1.0 at 550 nm, the culture was diluted 9-fold with methionine- and cysteine-free minimal medium containing 5 μCi/ml of ProMix ^{35}S (Amersham Corp.). The cells were

cultured until the OD_{550} reached 0.9–1.0 to metabolically radiolabel cell proteins to high specific activity.

The *E. coli* were lysed to extract ^{35}S-radiolabeled proteins essentially as previously described (14). Briefly, the cell culture was harvested by centrifugation at 4000*g* at 4°C for 20 min and the cell pellet was resuspended in fresh minimal medium and washed once by centrifugation. After the supernatant was discarded, the pellet was resuspended in 5 ml of lysis buffer containing 50 mM NaCl, 50 mM Tris, 5 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.15 mM PMSF, 1 mM DFP, pH 8.0, and was sonicated on ice using a probe-tip sonicator at the lowest power setting for 20 cycles of 15 s each with a 1-min pause between sonication cycles to prevent overheating. The cell lysate was centrifuged at 48,000*g* at 4°C for 20 min and the supernatant was retained. SDS was added to the *E. coli* extract supernatant to a final concentration of 0.05%. The sample was dialyzed with two buffer changes for about 15 h at 4°C against the lysis buffer containing 0.05% SDS using 12-kDa cutoff dialysis membranes to remove unincorporated radiolabel. After the protein amounts in the dialyzed sample were determined using the BCA protein assay (Pierce Chemical Co., Rockford, IL), samples were stored in aliquots at −80°C until required. Immediately before use, samples were thawed, treated with DNase/RNase as described by Harper *et al.* (15), lyophilized, and dissolved in appropriate IPG sample buffers described in individual experiments.

Two-Dimensional Electrophoresis

Isoelectric focusing equipment, IPG gels, and relevant reagents were purchased from Amersham Pharmacia Biotech (San Francisco, CA) unless otherwise indicated. Proteins were isoelectrofocused using different pH range IPG strips (pH 3–10NL, 4–7L, and 4.8–6.2L, 18-cm length) on the IPGphor isoelectric focusing system. Narrow pH range IPG gels (pH 4.8–6.2L) were cast in the laboratory using commercial immobilines as detailed in the IPG application manual (16). Immediately prior to IEF, dried IPG strips were rehydrated for 8 h with sample in IPG sample buffer (350 μl) in the ceramic strip holders (1 h without current followed by 7 h at 30 V) as described by Görg *et al.* (17). The IPG sample buffer contained 2 M thiourea, 7 M urea, 0.1 M DTT, 4% Chaps, and 2% IPG buffer (carrier ampholyte mixture matching the pH range used). After the 8-h rehydration, samples were focused for 1 h each at 500, 1000, and 2000 V, respectively, followed by 8000 V, for a total of 60 kV h.

Immediately prior to loading focused IPG strips on second-dimension gels, the IPG strips were incubated in 10 ml of 50 mM Tris, 6 M urea, 2% SDS, 30% glycerol, 30 mM DTT, pH 6.8, for 10 min, followed by incubation for 10 min in the same solution, except that the DTT was replaced by 2.5% iodoacetamide. The second-dimension SDS–PAGE was performed in 10% acrylamide separating gels prepared as described by Laemmli (18) using the Iso-Dalt gel format (25 × 20 cm, 1.5-mm thickness) (Amersham Pharmacia Biotech). The SDS-equilibrated IPG gel was sealed on top of the second-dimension gel using 0.5% agarose containing 50 mM Tris–Cl (pH 6.8), 2% SDS, 30% glycerol, and bromophenol blue. SDS gels were run overnight (at 10°C) until the tracking dye was within 1 cm of the gel bottom. The 2D gels were typically stained using Coomassie blue R-250. In some experiments, autoradiography was also used to visualize radiolabeled proteins. Briefly, gels were fixed in a solution containing 10% acetic acid and 30% methanol for 1 h, incubated with EN^{3}HANCE autoradiography enhancer (NEN Life Science Products Inc., Boston, MA) for 1 h, and dried under vacuum with heat (60–80°C). The dried gel was exposed to a preflashed BioMax MS film using a Transcreen-LE intensifying screen (Eastman Kodak Co., Rochester, NY) at −80°C for 1.5–7 h. The 2D gels were analyzed using Melanie II 2D PAGE analysis software (Bio-Rad).

Prefractionation of Samples by Solution Isoelectrofocusing

Varying numbers of Teflon dialysis chambers with 500-μl volumes in each chamber (Amika Corp., Columbia, MD) were connected in tandem and used in initial proof-of-principle experiments. In general, adjacent separation chambers were separated by 3% acrylamide gel membranes containing immobilines at desired pH values, terminal separation chambers used 10% acrylamide gels with appropriate immobilines, and terminal separation chambers were protected from the electrode buffers by electrode chambers containing 5-kDa dialysis membranes. Immobiline gels were cast in different concentrations and thicknesses and with several alternative supports for strength. Typically, Whatman GF/D glass fiber filters were imbedded in the gels for mechanical strength using a Bio-Rad minigel plate (7 cm × 10 cm) with 1-mm spacers to cast gels. Two slab gels could be cast with 25 ml of gel solution as prepared in Tables 1 and 2. After the gel was polymerized at 60°C for about 1.5 h, 12-mm-diameter gel membrane discs were excised from the slab gel using a stainless steel core borer. These gel membrane discs were washed three times with 1 ml of Milli-Q water for 1 h each and soaked in the IPG sample buffer for at least 30 min before use. Unused membranes could be stored in the buffer at 4°C for up to 3 weeks without affecting the effectiveness of sample prefractionation.

TABLE 1

Preparation of Immobiline Mixtures

Immobilines	pH 3.5	pH 5.0	pH 6.0	pH 9.5
p*K* 3.6	299 μl	158 μl		410 μl
p*K* 4.6	223 μl	863 μl	863 μl	
p*K* 6.2	157 μl	863 μl	803 μl	
p*K* 9.3			338 μl	694 μl
Water	6.82 ml	5.62 ml	5.50 ml	6.40 ml

Note. The mixture should be within 0.05 pH unit of the desired pH. If not, the pH should be adjusted using immobilines.

Determination of Protein Recoveries

Protein recoveries and losses throughout alternative prefractionation methods were determined using liquid scintillation counting. Any surfaces that came into contact with samples were extracted with 1% SDS to remove adsorbed or precipitated proteins. Adsorbed proteins were shown to be effectively extracted from surfaces by this method since all fractions and surface losses were analyzed in most experiments, and total recovered counts were equal to the starting sample amount within experimental error, which was typically $<\pm 5\%$ (data not shown). Typically, a small volume of these SDS extracts or sample solutions (5 μl) was mixed with 4.5 ml of Bio-Safe II scintillation cocktail (Research Products International Corp., Mt. Prospect, IL) and radioactivity was counted using a Model 1500 TRI-CARB liquid scintillation analyzer (Packard Instrument Co., Downers Grove, IL). The radioactivity left in the gels after elution was counted after solubilization using 1 N NaOH at 60°C for 3 h, followed by neutralization with concentrated HCl and addition of the scintillation cocktail.

RESULTS

Evaluation of Maximum Protein Loading Capacity without Sample Prefractionation

Prior to analysis of prefractionation methods, the effects of different protein loads on resolution and spot detection were evaluated using metabolically radiolabeled *E. coli* extracts. The maximum protein loading capacity of any given 2D gel method is an important parameter since higher protein loading capacities should permit detection of lower abundant proteins for any given staining and protein identification method.

About 530 protein spots could be detected on Coomassie blue R-250 stained gels when 0.5-mg aliquots of the *E. coli* extracts were separated using 18-cm pH 4–7L IPG strips and 18-cm-long second-dimension gels (Fig. 1). The greatest population of protein components fell within the pH 5–6 range, where approximately 240 spots were detected. Although most spots were well separated at the 0.5-mg load, some horizontal streaking of higher molecular weight and basic proteins was observed. Horizontal streaking, which indicates protein precipitation and/or aggregation, decreases the ability to reliably quantitate the proteins involved, can obscure other proteins underlying the streaking, and may induce coprecipitation of other proteins. Use of higher protein loads did not substantially increase the number of spots detected, but at the 2.0-mg level, lower resolution occurred due to both increased horizontal streaking and merging of major protein spots with their neighbors. These experiments show that about 1.0 mg was the maximum feasible load of unfractionated *E. coli* extracts for this 2D gel system.

The maximum protein loading capacity of narrow pH range IPG strips was also evaluated, since one recommended method for improving 2D gel capacities is to separate replicate aliquots of a total cell extract on parallel narrow pH range gels (1, 19). The results of separating different amounts of unfractionated *E. coli* extract on pH 4.8–6.2 IPG gels are shown in Fig. 2. At a 0.25-mg loading, approximately 320 spots were detected in the pH 5–6 range of the gel and most spots were well resolved, with only moderate horizontal streaking of some higher molecular weight proteins (>60 kDa). Increasing sample loads (0.5 and 1 mg) did not substantially increase the total number of protein spots detected, although more severe horizontal streaking was observed, even at lower molecular weights. As expected, the narrower pH range gels (pH 4.8–6.2) increased the total number of spots detected by increasing the resolution within this pH range. For example, the total number of spots between pH 5 and 6 was about 320 in the pH 4.8–6.2 gel when 0.5 mg was separated (Fig. 2), but only about 240 spots were detected when the same sample load was separated in a pH 4–7 gel (Fig. 1). However, the narrower pH gels did not increase the maximum sample load capacity when cell extracts were analyzed without prefractionation.

Chromatographic Prefractionation of Cell Extracts Prior to 2D PAGE

Several HPLC methods were initially evaluated as potential prefractionation methods prior to 2D PAGE.

TABLE 2

Preparation of Gel Membranes

	3% T/8% C gel	10% T/8% C gel
Immobiline mixture	7.5 ml	7.5 ml
Acrylamide/Bis (30% T/8% C)	2.5 ml	8.33 ml
Glycerin (87%)	3.45 ml	3.45 ml
Ammonium persulfate (40%)	25 μl	25 μl
TEMED	15 μl	15 μl
Water	11.51 ml	5.68 ml

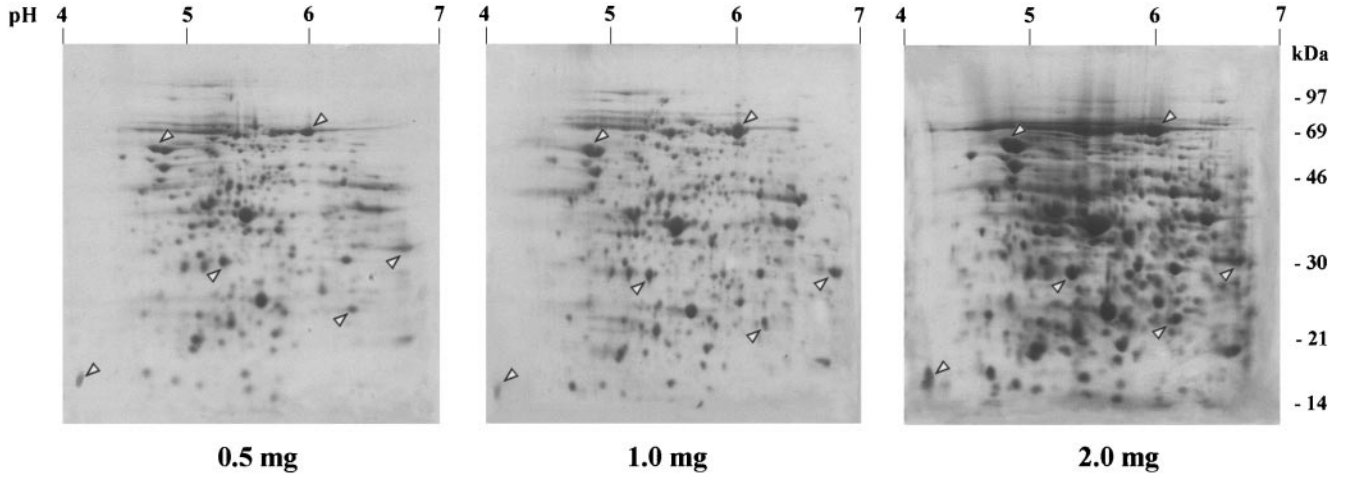

FIG. 1. Effects of increasing protein amounts on 2D PAGE separations of unfractionated *E. coli* extracts. Different amounts of sample were loaded to pH 4–7L IPG strips by rehydration and samples were focused for 60 kV h followed by separation in 10% SDS gels. Proteins were visualized using Coomassie blue staining. The pH range and location of molecular weight markers are indicated. Arrowheads highlight corresponding landmark spots in each 2D gel to aid comparisons.

HPLC gel filtration in the absence of denaturants resulted in dilute samples and elution of specific proteins in multiple fractions due in part to the moderate resolution of this chromatographic method and in part to heterogeneous migration of multiple oligomer states (data not shown). Gel filtration in the presence of denaturants such as urea or SDS primarily separates samples by size, similar to the separation that occurs in the SDS gel dimension. This method does not improve the overall capacity of the separation method since the SDS dimension typically does not limit loading capacity or resolution in 2D PAGE.

FPLC ion exchange separation in the presence of 7 M urea with step pH elutions was attempted in an effort to approximate the separation achieved in the isoelectric focusing dimension. The rationale was that prefractionating sample prior to direct loading onto parallel narrow range IPG gels might minimize precipitation and horizontal streaking. Unfortunately, the volumes of eluate at a given pH were too large to directly load onto IPG gels. In addition, losses were high, the method was cumbersome, and resolution at the ion exchange step was inadequate, with extensive occurrence of particular proteins in several pools, hence severely complicating quantitative comparisons (data not shown).

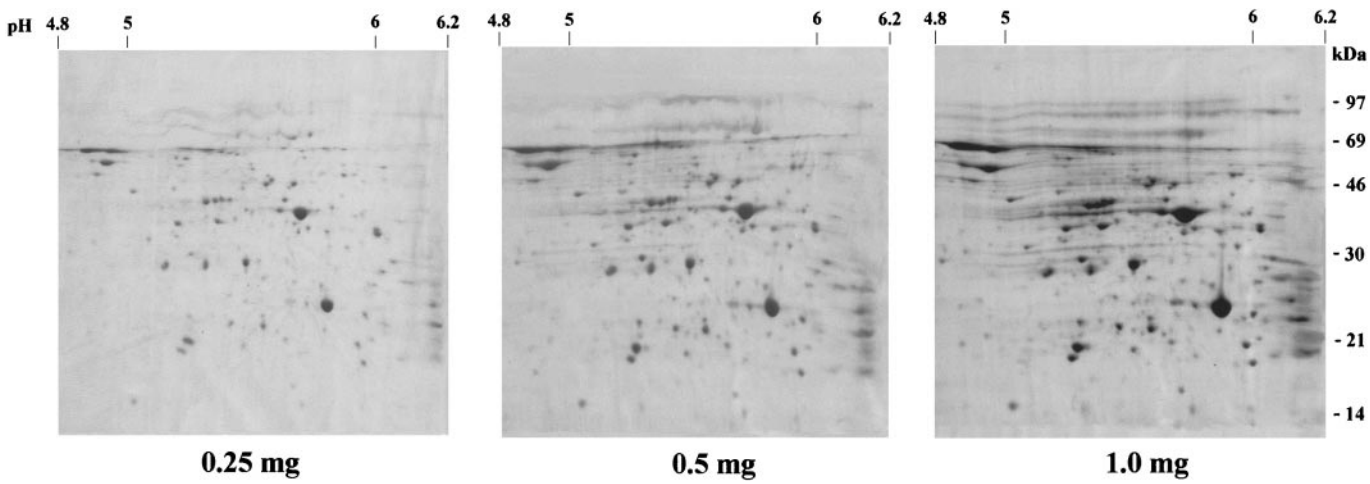

FIG. 2. Separation of unfractionated *E. coli* extracts on narrow pH range IPG-based 2D PAGE. Different amounts of sample were loaded to pH 4.8–6.2L IPG strips by rehydration and proteins were focused for 60 kV h followed by separation in 10% SDS gels. Proteins were visualized using Coomassie blue staining.

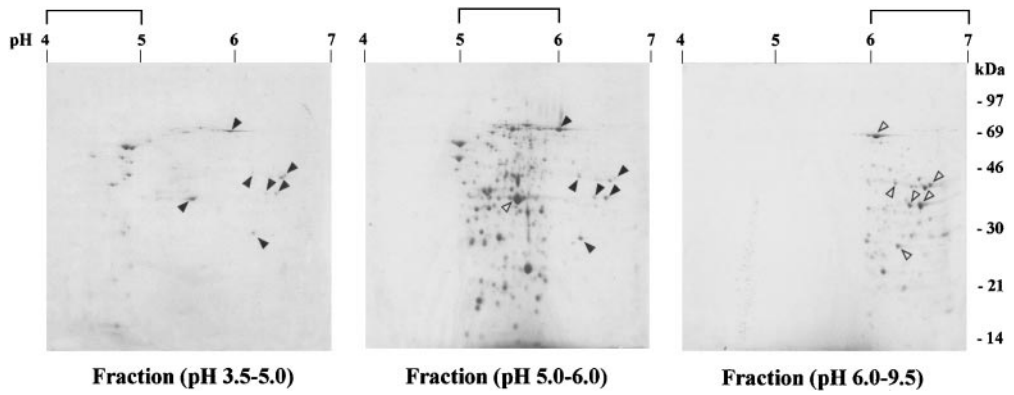

FIG. 3. Evaluation of samples prefractionated using an IPG gel. A 1.0-mg aliquot of *E. coli* extract was initially fractionated on a pH 4–7L IPG gel, the focused gel was cut into three sections, and proteins were eluted. To check the effectiveness of the initial separation, eluted pools were rerun on pH 4–7L IPG gels for 60 kV h followed by separation in 10% SDS–PAGE gels. Proteins were visualized using Coomassie blue staining. The pH range of the section from the initial IPG gel that was eluted is indicated above each 2D gel by brackets on the pH scale. Solid arrowheads highlight several incorrectly focused spots; the matching spots in the correct pH range fractions are indicated with open arrowheads.

Prefractionation of Cell Extracts Using Gel-Based Isoelectric Focusing

The incomplete success of the ion exchange chromatography method suggested that the only separation method with adequate resolution to prevent extensive cross-contamination of many proteins between multiple pools would be a high-resolution isoelectric focusing method closely analogous to the actual analytical IPG gel method itself. While a same-mode prefractionation approach would not improve overall separation by providing a true third mode of separation, or 3D method, it could offer the potential of improving capacity and resolution when multiple slightly overlapping narrow pH range gels would be used in parallel.

To evaluate the feasibility of this approach, 1-mg loads of the *E. coli* extracts were initially focused in pH 4–7L IPG gels. After both ends of the gel that were beyond the electrode locations were removed, the remainder of the gel was cut into three parts having pH ranges of 4–5, 5–6, and 6–7, respectively. The gel sections were separated from the supporting plastic film and each section was extracted with three 500-μl volumes of the IPG sample buffer for 1 h per extraction. The three extractions were pooled (~1.5 ml) and concentrated to about 50 μl using a 10-kDa cutoff Centri-Con. The volumes of concentrated samples were adjusted to 350 μl with the IPG sample buffer, loaded onto new IPG strips (pH 4–7L), and analyzed by 2D gel electrophoresis to evaluate the feasibility of an IPG gel-based prefractionation approach (Fig. 3). Typically, all the proteins in the pH 6–7 pool were cleanly separated from other fractions. However, some protein spots recovered in the pH 4–5 and pH 5–6 pools were focused at other pH values when the fractionated samples were refocused on the second IPG strips (Fig. 3). This demonstrated that some proteins failed to focus correctly when the 1.0-mg cell extract sample was initially isoelectric focused in the first 4–7L IPG gel. This incorrect focusing is consistent with the observed moderate degree of horizontal streaking of some proteins on the 2D gel at 1-mg loads described above (Fig. 1). Most spots that migrated outside the expected pH range appear to be due to incorrect isoelectric focusing rather than proteolysis or artifactual modifications since these spots usually precisely overlap spots in the correct pH range fractions (e.g., see arrowheads in Fig. 3).

Losses of sample proteins during prefractionation using IPG gels were impractically high. Typically, about 16% of the total sample protein was recovered in the strip holder after 1.0 mg of *E. coli* extract was initially isoelectrofocused using a pH 4–7L IPG gel with the IPGphor system. The loss of sample proteins at this stage was primarily proteins with p*I* values outside the pH 4–7 range that run out of the gel and remained in the strip holder. Another 5.5% of total sample protein was lost in the ends of the IPG gel beyond the electrodes, which contained unfocused proteins and was trimmed off after isoelectric focusing, and 3.6% of the total sample was recovered on the IPG gel plastic supporting film. However, the greatest loss (approximate 23%) resulted from the proteins that were not eluted out of the focused IPG gel fractions after the sequential elutions. The total protein recovery in the three eluted fractions was only about 47% of the original sample applied to the IPG gel. This low recovery and incomplete separation of fractions indicated that a scale-up of gel-based isoelectric focusing would not be a practical routine method for prefractionating complex samples prior to 2D PAGE.

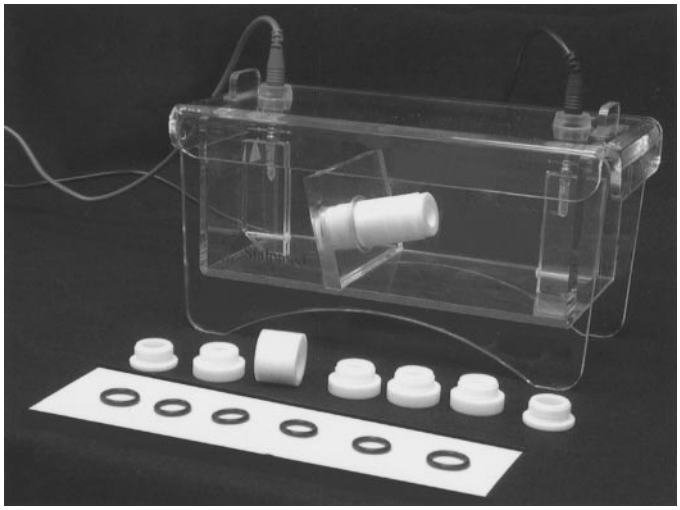

FIG. 4. A photograph of the solution isoelectrofocusing device used for sample prefractionation in this study. The electrophoresis tank (Amika Corp.) is designed for electroelution of proteins or nucleic acids from gels. Inserted in the tank is a complete five-chamber prefractionation device consisting of three separation chambers and terminal electrode chambers (see Fig. 5 for schematic). Components for another five-chamber device are shown in front of the tank and include five Teflon chambers plus terminal Teflon caps with holes (multichamber Teflon dialyzer system, Amika Corp.) and 12-mm-i.d. O-rings (Scientific Instrument Services, Inc., Ringoes, NJ). To assemble chambers, 12-mm-diameter immobiline gel membranes with imbedded glass fiber membranes are inserted into the O-rings and the membrane/O-ring assembly is then placed between two adjacent 500-μl Teflon chambers.

Development of a Solution Isoelectrofocusing Method for Sample Prefractionation

A solution isoelectrofocusing method was developed and used to separate a protein extract in tandem small-volume, liquid-filled chambers separated by thin porous acrylamide gel membranes containing immobilines at specific pH values. In initial proof-of-principle experiments, a series of tandem Teflon dialysis chambers with 500-μl internal volumes and an electroelutor electrophoresis tank from Amika Corp. (Columbia, MD) were used to construct a simple sample prefractionation device (Fig. 4). This unit consists of five chambers and four gel membranes having pH values 3.5, 5.0, 6.0, and 9.5, respectively, as illustrated in the schematic drawing in Fig. 5. Adjacent separation chambers were divided by 1-mm-thick 3% acrylamide gel membranes containing immobilines at the desired pH values. Terminal separation chambers used immobiline gel membranes with 10% acrylamide and these chambers were protected from electrode solutions by electrode chambers containing dialysis membranes (5-kDa cutoff, Amika Corp.). O-Rings were used between chambers to assist sealing of gel membranes and chamber compartments by placing an appropriate gel membrane inside an O-ring before the tandem chambers were assembled.

An *E. coli* extract (3 mg) was solubilized in 1.5 ml of IPG sample buffer and divided among the three separation chambers. The terminal electrode chambers were filled with Bio-Rad premade IEF electrode buffers, 7 mM phosphoric acid (anode), and 20 mM lysine/20 mM arginine (cathode). The assembled chambers were placed into the electrophoresis tank and the two compartments of the tank were filled with anode and cathode electrode buffers, respectively. A PS500X power supply (Amersham Pharmacia Biotech) was used for focusing the sample. Typically, 100 V was used for 1 h (initial ~2–3 mA, final ~1 mA), followed by 200 V for 1 h (initial ~2–3 mA, final ~1 mA), and then 500 V (initial ~3–4 mA) until the current fell to 0 mA (about 1.5 h). After fractionated samples (each ~500 μl) were removed, the surfaces of the gel membranes and the inside walls of the separation chambers were rinsed with 500 μl of the sample buffer and these rinses were combined with the fractionated samples. The gel membranes were removed and extracted twice with 500 μl of sample buffer for 1 h each to elute proteins from the gel matrix. To evaluate the effectiveness of this prefractionation method, one-third of each fractionated sample, which was proportional to 1.0 mg of the original sample, was separated on a pH 4–7L IPG-based 2D PAGE (Fig. 6). These results showed that the cell extracts were well separated into three discrete pools and only a few overlapping spots were found in the pH 3.5–5.0 and 6.0–9.5 fractions. Only a few proteins were eluted from the separation membranes and most of them had p*I* values close to the membrane pH (±0.1) (Fig. 6).

A composite image (Fig. 7A) of the five individual gels shown in Fig. 5 can be compared to a 2D gel separation of a 1.0-mg *E. coli* extract without prefrac-

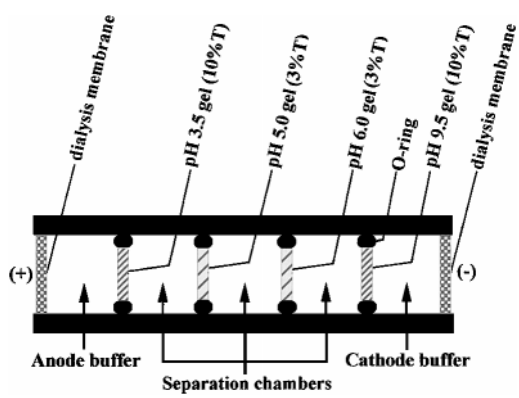

FIG. 5. Schematic illustration of the prototype solution isoelectrofocusing device. It consists of five chambers separated by four gel membranes containing immobilines at the desired pH. Proteins with p*I* values within the pH range 3.5–9.5 were fractionated into three pools in the separation chambers.

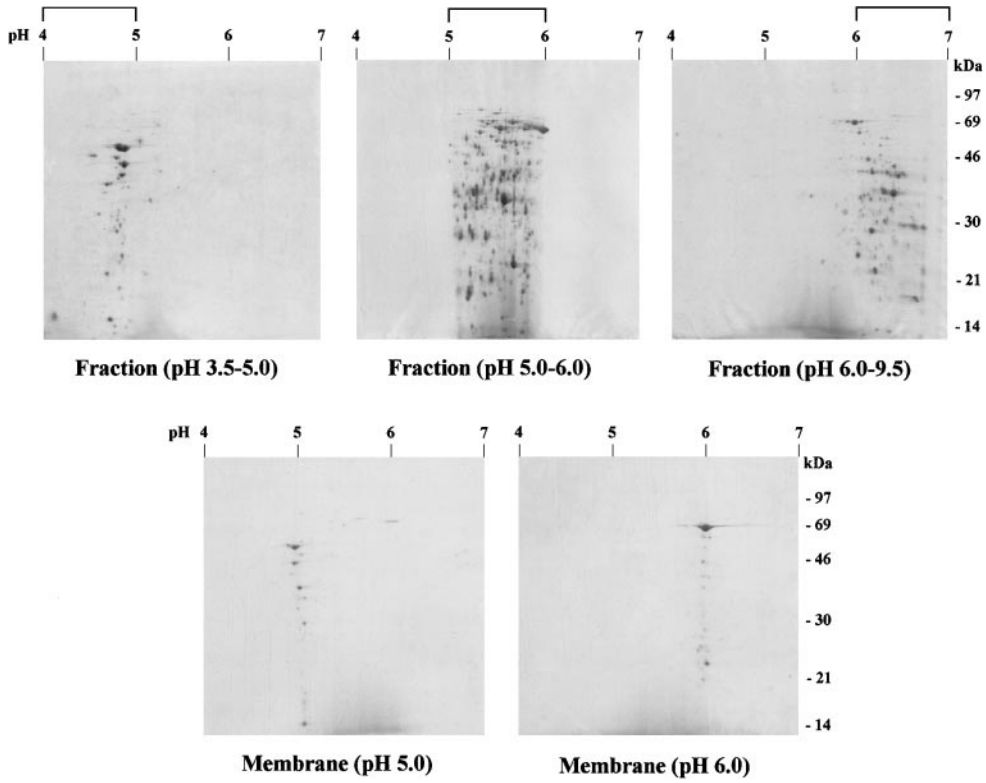

FIG. 6. Evaluation of sample fractionation using solution isoelectrofocusing in a representative experiment. After prefractionation of 3 mg of *E. coli* extract using solution isoelectrofocusing, the samples from the three separation chambers and the proteins extracted from the separation membranes were evaluated by 2D PAGE. One-third of each recovered sample (proportional to 1 mg of original sample) was separated using pH 4–7L IPG gels followed by separation in 10% SDS gels. Proteins were visualized using Coomassie blue staining.

tionation (Fig. 7B). The composite image showed that most protein spots in an unfractionated sample were recovered with good yield in prefractionated samples and resolution was improved (Fig. 7A). Specifically, the total number of spots in the 2D gel without prefractionation was 545 (Fig. 7B), compared with 610 spots (Fig. 7A) in the prefractionated composite image. More importantly, no horizontal streaking of proteins was observed on the composite image with the prefractionated samples (Fig. 7A), while substantial streaking occurred on the gel with the unfractionated sample (Fig. 7B).

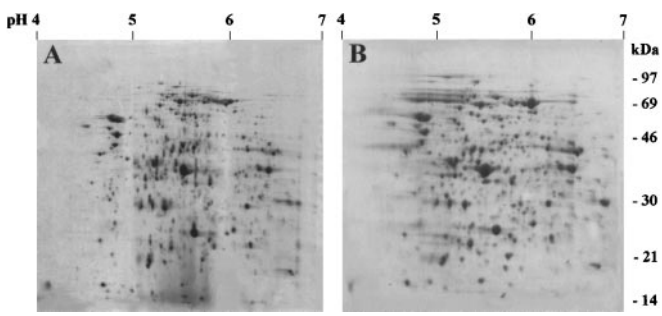

FIG. 7. Comparison of a composite 2D image from prefractionated samples with the 2D image of an unfractionated *E. coli* extract. (A) Composite 2D protein image produced by cutting and pasting the protein-containing sections from the five gels shown in Fig. 5. (B) A pH 4–7L 2D gel containing 1.0 mg of unfractionated *E. coli* extract. Proteins were visualized using Coomassie blue staining.

Total protein recovery of the three solution focused fractions was 65% in these pilot experiments. Another 20% of the total sample proteins was associated with the four gel membranes. About three-quarters of the proteins retained in the membranes could be readily extracted and combined with an adjacent fraction to increase overall yield to about 80%. Finally, about 5% of the total sample was found in the two electrode chambers since a small proportion of proteins in *E. coli* have p*I* values outside the pH 3.5–9.5 range of the separation chambers used in these pilot experiments. Most of these proteins could presumably be recovered by modifying the experimental design to cover a wider overall pH range. Hence, these initial experiments demonstrated that this solution isoelectrofocusing technique can rapidly separate complex protein mixtures into a small number of discrete well-defined pools in very high yield (>80%) for subsequent separation on parallel, slightly overlapping, narrow pH gradient gels.

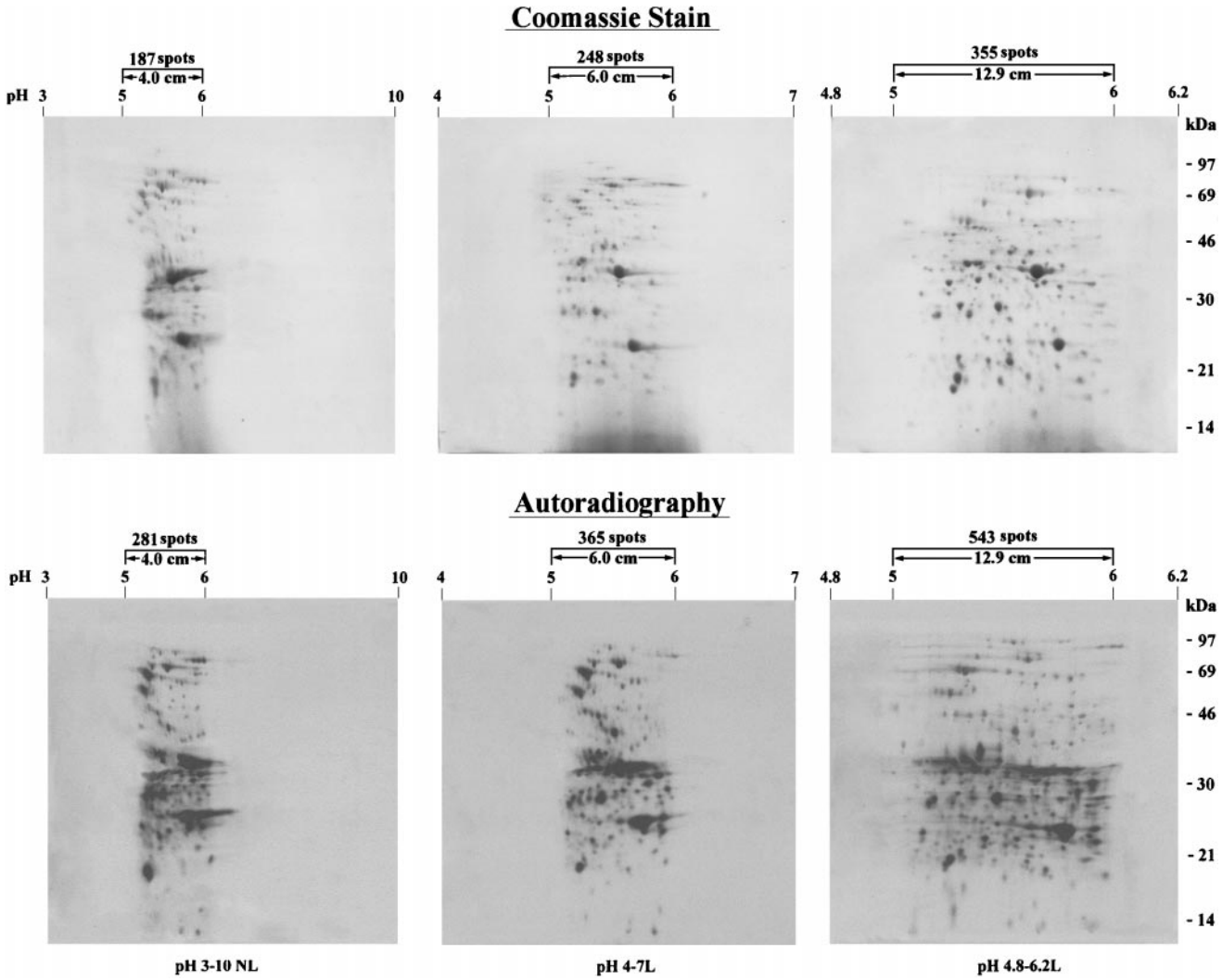

FIG. 8. Effects of IPG strip pH ranges on protein resolution. Replicate pH 5–6 range samples (proportional to 1 mg of unfractionated *E. coli* extract), which were prefractionated using solution isoelectrofocusing, were focused using pH 3–10NL, 4–7L, and 4.8–6.2L IPG strips, respectively, followed by separation in 10% SDS gels. Proteins were visualized using Coomassie blue staining (upper panels) and autoradiography (lower panels). Spots were detected and counted using Melanie II software. These values and the effective separation distances for proteins with p*I* between 5 and 6 are shown above each 2D gel.

Effects of Narrow pH Range IPG Gels on Protein Detection and Resolution

The feasibility of using narrow pH gradient gels with prefractionated samples described above was evaluated. Replicate fractionated samples (pH 5–6) prepared using solution isoelectrofocusing were separated on different pH range IPG strips (pH 3–10NL, 4–7L, and 4.8–6.2L) followed by SDS–PAGE (Fig. 8). The protein spots in the 2D gels were found only within the pH 5–6 range regardless of the IPG gel pH ranges or detection methods used. These results verify that the components in the pH 5–6 fractionated sample were well resolved from other pH fractions, with no detectable cross-contamination of proteins after the prefractionation step even when the more sensitive autoradiography detection method was used (Fig. 8). The advantage of using narrow pH gradient gels with sample prefractionation was clearly demonstrated by the improved resolution of a fractionated pool on narrow pH range gels. Specifically, about twice as many protein spots were detected on an 18-cm pH 4.8–6.2L gel compared with an 18-cm pH 3–10NL gel with either Coomassie blue detection (355 spots vs 187 spots) or the more sensitive autoradiographic detection (543 spots vs 281 spots).

The advantages of sample prefractionation are further illustrated by comparing 2D gels using pH 4.8–6.2L IPG strips (Fig. 8) to the 2D PAGE analysis of unfractionated samples using the same narrow pH range (Fig. 2). No horizontal streaking of proteins was

observed on the 2D gel with prefractionation (Fig. 8), but substantial horizontal streaking occurred on 2D gels loaded with unfractionated samples (Fig. 2). Apparently, the prefractionation using solution isoelectrofocusing eliminates the components with p*I* values beyond the pH range of a given narrow IPG gel which otherwise may precipitate or aggregate in the gel. Hence, the prefractionation step results in overall load capacity increases over alternative 2D PAGE methods such as direct use of parallel narrow pH range gels without prefractionation. Increased sample loads using the prefractionation method should improve reliability of quantitative comparisons, increase the number of spots that can be resolved, and allow detection of lower abundance spots, because precipitation and aggregation are minimized. Finally, prefractionation more effectively utilizes samples that are available in limited amounts compared with replicate application of unfractionated samples to multiple different narrow pH range gels.

DISCUSSION

In this study, several alternative methods for improving the capacities of 2D gels were evaluated. While the ultimate goal is to improve analysis for complex eukaryote proteomes, metabolically radiolabeled *E. coli* extracts were used in these studies to systematically evaluate protein recoveries. *E. coli* was selected since this relatively simple organism could be readily metabolically radiolabeled to high specific activity to provide sensitive and reliable detection of protein losses. In contrast to chemical labeling methods such as iodination of a portion of the sample, metabolic radiolabeling of the entire sample ensured that the labeling method would not alter the properties of the proteins and that each protein was a homogeneous population of molecules.

A major disadvantage of existing 2D gel methods when applied to proteome analyses of higher eukaryotes is that the maximum sample loading capacity of whole-cell or tissue extracts is fairly low, which results in detection of only the most abundant proteins when currently available visualization methods are used (1, 2, 11). Increasing the amount of sample above the optimal level, e.g., 1–2 mg of the *E. coli* extracts in the present study, results in horizontal streaking of many proteins as shown in Figs. 1 and 2. Although current IPG-based 2D gels have much higher resolution than alternative separation methods, not all proteins in whole-cell extracts can be resolved by a single IPG gel. This incomplete resolution contributes to errors in subsequent quantitation and identification of proteins; that is, a single spot on the gel is frequently not a single protein.

The complexity of eukaryotic proteomes coupled with the limited resolution and inability to detect less abundant proteins using current 2D gels suggests that a prefractionation step could be a conceptually attractive approach to reduce complexity and facilitate detection of less abundant components (11). Unfortunately, most proposed prefractionation methods have substantial limitations. Conventional protein separation methods have much lower resolution than either of the two modes of separations used in 2D PAGE, which invariably results in substantial cross-contamination of multiple protein components among two or more fractions (see above and Ref. 2). Also, as noted above, currently available preparative isoelectric focusing instruments and methods typically require large sample volumes, result in large dilute fractions that need to be concentrated, with attendant substantial losses, and involve relatively complex, expensive instrumentation. Often, these methods result in low- or moderate-resolution separations and/or do not produce optimal fractions where the entire sample can be directly applied to analytical 2D gels at high protein loading levels.

In contrast, the prefractionation device and method we developed allow reproducible fractionation of cell extracts into a small number of well-defined pools on an analytical scale such that the entire sample can be applied to duplicate or triplicate narrow pH range IPG strips for comprehensive, or "global," 2D PAGE analysis of most proteomes. After evaluating alternative chromatographic prefractionation methods, we tested both gel-based and solution isoelectric focusing approaches. We found that using a solution isoelectrofocusing method is much better than eluting proteins from sections of focused IPG gels for prefractionation of samples. Although eluting proteins from a focused IPG gel can result in fractions that are better resolved than alternative methods, more than 50% of the sample was lost. More importantly, some protein spots in the original sample were selectively lost by this prefractionation method (compare Fig. 3 with Fig. 1) and other proteins were partially recovered in the wrong pH range fractions due to precipitation and aggregation in the initial IPG strip (see Fig. 3). In contrast, prefractionation using the solution isoelectrofocusing method developed in this study results in a higher yield (~80%) and more importantly, most protein spots in the original sample can be recovered (Fig. 7). Initial isoelectric focusing in solution minimizes nonideal behavior of proteins (precipitation/aggregation) encountered if samples are applied to narrow pH IPG gels without prefractionation (Fig. 2). The fractionated proteins exhibit good solubility when applied to narrow pH range IPG gels, which results in better resolution and more spots detected compared to direct 2D PAGE without prefractionation (Figs. 6–8). The major advantage of this prefractionation method is to maximize the

amount of total extract that can be analyzed on the final parallel narrow pH range 2D gels while simultaneously decreasing protein aggregation and precipitation in the IPG strips. Hence less abundant proteins can be detected (see below) and quantitative comparisons are more reliable since incorrect isoelectric focusing of some proteins at high protein loads is minimized. Prefractionation is less important for low protein loads (<250 μg) since direct application of unfractionated samples to parallel narrow pH range gels results in less extensive protein streaking. Nevertheless, even at low protein loads, prefractionation can reduce this moderate protein streaking and will conserve samples that are only available in limited amounts. For example, a single 50-μg sample can be fractionated into five fractions and analyzed on five parallel narrow pH range gels compared with use of a 250-μg sample if replicate unfractionated 50-μg aliquots of sample are loaded onto five narrow pH range gels. Prefractionation using the method described in this study is relatively fast (less than 4 h), requires only a simple, easy-to-use device, and yields well-separated fractions that can be applied directly to subsequent narrow pH IPG strips.

In initial tests of solution isoelectrofocusing, 5% gels were used for the separation membranes and 10% gels for the electrode membranes, as suggested for most applications of the IsoPrime method (13, 20–22). However, when 1-mm 5% gels were used for separation membranes, many higher molecular weight proteins with p*I* values not equal to the membrane pH precipitated on the gel matrix, thus resulting in a low overall yield (only ~40%) of fractionated samples and poor separation (data not shown). When the 5% gels were replaced with 3% gels as separation membranes, the yield and separation of fractions were improved, and typically only proteins with p*I* values equal to the membrane pH were retained in the 3% gel matrix (see Fig. 6). These results suggest that even lower gel densities and thinner gel dimensions may be beneficial if mechanical strength can be maintained to prevent membrane rupture during isoelectric focusing. Also, in the present study, we found that gel membranes could be stored at 4°C for up to 3 weeks. Longer storage should be feasible if the membranes are dried and frozen similar to commercial IPG strips. The presence of 2% ampholytes in the solution IEF is advantageous; otherwise current during the electrophoresis is too low to effectively focus the sample (data not shown). The 2 M thiourea/7 M urea present in the sample buffer is superior to 9 M urea alone for solubilizing sample proteins (23). Dialysis membranes (5-kDa cutoff) are found to be advantageous for protection of the terminal separation chambers and prevent the proteins with p*I* values beyond the pH range of the separation chambers from migrating out into the electrophoresis tank,

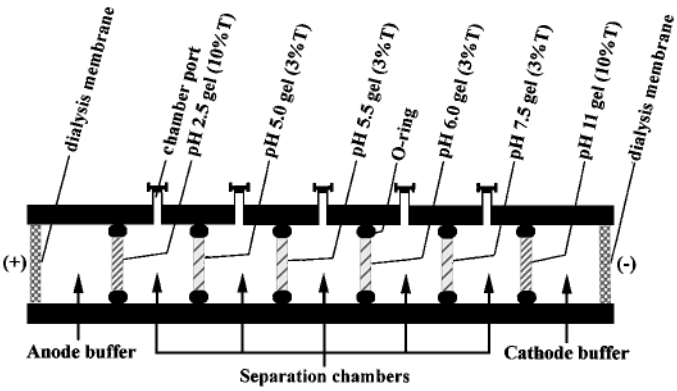

FIG. 9. A proposed second-generation solution isoelectrofocusing device. This modified device contains more separation chambers to cover the full pH range of most proteomes and to increase the total number of spots that should be detected. Each chamber has an access port for improved sample loading and removal.

which permits reliable measurement of overall protein losses during prefractionation. The sample loading capacity to the device used in these studies (up to 3 mg of cell extract) and chamber volumes (500 μl) were selected to permit downstream processing of fractionated samples; i.e., each fraction could be divided into two or three parts and analyzed on duplicate or triplicate 2D gels as desired.

The simple prototype solution isoelectrofocusing device (Figs. 4 and 5) used in this proof-of-principle study had three separation chambers and covered the pH 3.5–9.5 range, where most protein spots occurred in the test sample. A second-generation device is schematically illustrated in Fig. 9. The larger number of chambers and wider pH range should be appropriate for comprehensive or global analyses of complex eukaryotic proteomes. Each chamber has a fill port for more convenient loading and removal of samples. The volumes of separation chambers can be adjusted to fit experimental design such that fractionated sample volumes match the IPG gel rehydration volumes for the number of replicate 2D gels desired. Similarly, the total number of separation chambers and the pH values of divider gel membranes can be altered to fit requirements of specific proteome studies.

A strategy using the second-generation isoelectric focusing device for global analysis of eukaryotic proteomes is illustrated in Fig. 10. Prior to 2D gel electrophoresis, a complex sample is fractionated into approximately five pools by solution isoelectrofocusing. The pH ranges of each pool should be selected so that similar numbers of spots are obtained on each subsequent 2D gel. For example, in the illustrated scheme the pH 5–6 range has been divided into 0.5 pH unit increments since the largest proportion of proteins in eukaryotic proteomes fall in this pH range. As described under Results, proteins with p*I* values equal to the

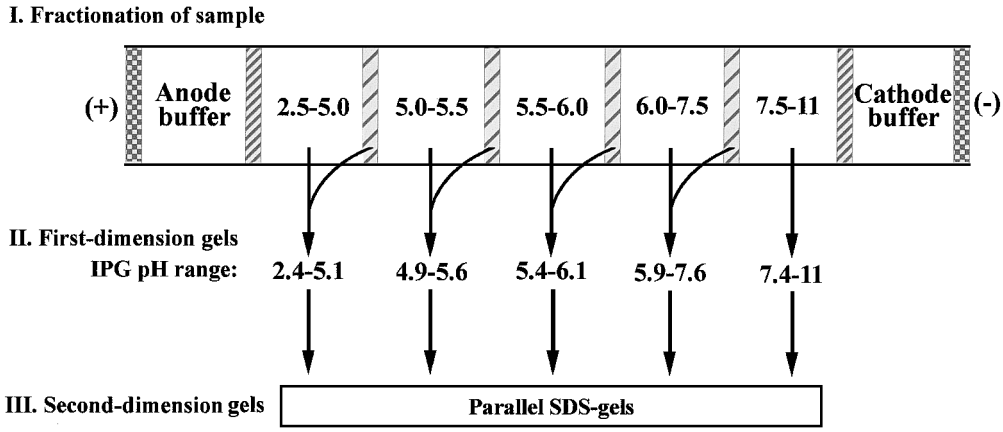

FIG. 10. Schematic illustrating a method for global analysis of complex proteomes. The specific number of separation chambers and the pH values of the separation membranes can be adjusted to fit different conditions and sample properties. It should be feasible to optimize the pH ranges so that the full resolving capacity of each gel can be utilized. Since each full-sized (18 cm × 18 cm) gel should be readily capable of resolving 2000–3000 spots when a high-sensitivity detection method is used, the illustrated scheme should be capable of resolving on the order of 10,000–15,000 protein spots when complex eukaryotic proteomes are analyzed.

separation gel membranes are retained in the gel matrix and can be recovered in reasonable yields by extraction with a small volume of sample buffer. The proteins eluted from the gel membranes can then be combined with the adjacent solution fraction to minimize sample losses at these boundaries. Fractionated samples are then loaded onto narrow pH range gels that are 0.1 pH unit wider than the flanking gel membranes. It should be noted that we used gels that were ±0.2 pH unit wider than the solution pools in this study, but the accuracy of the p*I* values from these pools was sufficiently precise that ±0.1 pH unit wider gels should be adequate in future experiments. Parallel SDS gels are used for second-dimension separation electrophoresis.

The ability to increase the protein load per narrow pH range gel using prefractionated samples compared with unfractionated samples will improve the dynamic range of the global proteome analysis by permitting detection of less abundant spots for any given detection method and by increasing separation distances between minor components and major components. In addition, the prefractionation step more efficiently utilizes samples available in limited amounts compared with analysis of unfractionated samples on narrow pH range gels. Since each narrow pH range gel has the capacity to resolve approximately 2000–3000 protein components, the scheme shown here with separation of a sample into five fractions to be loaded onto five overlapping narrow pH range gels results in a robust method for reliably detecting at least 10,000–15,000 protein components.

The scheme shown in Fig. 10 should permit the routine detection of most proteins in eukaryotic proteomes, with an estimated detection limit of about 1000 molecules per cell. For example, 2×10^7 cells equal approximately 4 mg of protein, which can be efficiently fractionated in a five-chamber separation unit followed by analysis of each fraction on duplicate narrow pH range gels. When a high-sensitivity silver stain is used, the detection limit is less than 0.5 ng for well-focused spots. A 0.5-ng spot of a 50-kDa protein is 10 fmol, or 6×10^9 molecules. Each duplicate gel represents a well-resolved fraction recovered in high yield from 10^7 cells; hence this 0.5-ng spot represents 6×10^9 molecules/10^7 cells, or 600 molecules per cell, and if an overall recovery of the protein is about 60%, then any protein present at the 1000 copy per cell level or higher should be readily detected by this technique. It is likely that even the lowest abundance proteins (i.e., between 100 and 1000 copies per cell) could be detected by using a more sensitive detection method such as metabolic labeling and phosphoimage analysis of the gels and/or by fractionating the samples into approximately 8–10 pools followed by 8–10 narrow pH range 2D gels.

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