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Optimization of Large Gel 2D Electrophoresis for Proteomic Studies of Skeletal Muscle

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Abstract

We describe improved methods for large format, 2-dimensional gel electrophoresis (2-DE) that improve protein solubility and recovery, minimize proteolysis, and reduce the loss of resolution due to contaminants and manipulations of the gels, and thus enhance quantitative analysis of protein spots. Key modifications are: (i) the use of 7M urea + 2 M thiourea, instead of 9M urea, in sample preparation and in the tops of the gel tubes; (ii) standardized deionization of all solutions containing urea with a mixed bed ion exchange resin and removal of urea from the electrode solutions; and (iii) use of a new gel tank and cooling device that eliminate the need to run two separating gels in the SDS dimension. These changes make 2D-GE analysis more reproducible and sensitive, with minimal artifacts. Application of this method to the soluble fraction of muscle tissues reliably resolves ~1800 protein spots in adult human skeletal muscle and over 2800 spots in myotubes.

Keywords

muscle; two-dimensional electrophoresis; proteolysis

INTRODUCTION

Two methods for high resolution, two-dimensional gel electrophoresis (2D-GE), introduced in 1975 by O'Farrell [1] and Klose [2], enable the simultaneous visualization of thousands of proteins in a single analysis. These methods have been applied to monitor changes in proteins that occur under a variety of biological conditions; for example, studies often include comparisons between diseased and normal tissues, which are detectable on the gels as alterations of the position or abundance of individual "spots" of protein. Ensuring quantitative recovery of the proteins through the many steps of the experiment is essential for such comparisons to be reliable. Even if staining and detection methods allowed perfect quantifications, in reality the investigator can analyze only the protein remaining after losses that occur at each stage of the 2D gel procedure, which may differ from sample to sample. Similarly, the integrity of the proteins in the mixture to be analyzed must be preserved by preventing proteolysis, aggregation, or the generation of new species by non-specific cross-linking. In addition, the methods must yield the highest possible resolution of the thousands of different proteins in a tissue, which is most easily achieved by increasing the size of the gels. We describe a simple new gel tank for running large 40 cm × 30 cm slab gels which, when combined with our modifications of earlier methods for sample processing and gel

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preparation, provides highly reproducible and quantitative 2D gel patterns with extracts from human skeletal muscle.

A major cause of variability in the results of 2D-GE studies is protein loss during sample preparation and during the electrophoresis procedure. Many 2D studies utilize IPG strips for isoelectric focusing (IEF) in the first dimension, but the losses of proteins from these strips can be considerable [3,4,5]. In comparison, carrier ampholytes in tube gels yield smaller losses of protein and so may be better suited for quantitative comparisons between biological samples [1,4,5,6,7]. We have therefore developed our methods around the use of carrier ampholytes in tube gels. In addition losses of protein due to precipitation have been documented, with the methods of either Klose (11–16% of total protein; [6,7] or O'Farrell (5–10%; [1]). Further losses may occur during equilibration of the tube gel in SDS solution prior to SDS-PAGE, but this step can be omitted if thin, 0.9 mm gels are used [7], or if the gels are run with 2% SDS in the buffer for 20 min, followed by a buffer change to normal running buffer [1]. Proteins can also be lost as a result of degradation or intermolecular crosslinking of sulphhydryl groups [8]. Even if all these problems could be avoided, the quantitation and identification of particular spots can be compromised by the overlapping of spots [9], especially in a standard 20 cm × 20 cm gel apparatus. The smaller format of these gels requires that the 40 cm long tube gels used for NEPHGE be cut in half and analyzed in two different slab gels.

In this study, we combine recent improvements in 2D-GE with our own modifications, designed to reduce protein losses, aggregation, proteolysis and non-specific crosslinking. We also introduce a new and inexpensive gel tank that allows the full 40 cm tube gel, used in NEPHGE, to be loaded and analyzed on a single, large SDS-PAGE gel. This combination of improvements minimizes many of the problems that we and others have encountered in resolving and quantitating the thousands of spots of protein present in any given tissue. We demonstrate the value of these changes with soluble fractions from myotubes prepared from human skeletal muscle and from biopsy samples of adult human skeletal muscles.

MATERIALS AND METHODS

Adaptations to published procedures to Klose's original technique [6,7,10] are described below. Otherwise, all solutions and steps were as described by Klose and Kobalz [6,7,10].

Animals, Tissue Collection and Cells

Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed at the Central Animal Facility, University of Maryland, Baltimore, School of Medicine. Rats were anesthetized by intraperitoneal injection with ketamine (80 mg/kg) and xylazine (7 mg/kg), killed by cervical dislocation and then perfused through the left ventricle with 25 ml of ice cold saline to clear blood from the tissues. Quadriceps muscles were removed and immediately flash frozen in a semi-solid slush of liquid N₂. Procedures involving rats were approved by the Institutional Animal Care and Use Committee. Biopsies of human muscle and cultures of myoblasts and myotubes prepared from those biopsies were generously provided by Drs. K. Wagner, J. Chen and C. Emerson, of the Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center, at the Boston Biomedical Research Institute, Watertown, MA. Methods for their collection and preparation will be provided elsewhere.

Materials

Urea, thiourea, sodium thiosulfate, ethanol, acetic acid, ethylenediamine, glutaraldehyde, silver nitrate, thimerosal, sodium acetate and formaldehyde, tributylphosphine, 2-

vinylpyridine were from Sigma-Aldrich (St. Louis, MO). Acrylamide, bis-acrylamide, SDS, TEMED, Sephadex G-200, and ammonium persulfate were from Bio-Rad Laboratories (Hercules, CA). Duracryl was from Genomic Solutions (Ann Arbor, MI). Tris Ultrapure was from ICN (Aurora, OH). Glycerol was from VWR (West Chester, PA). EDTA was from American Bioanalytical (Natick, MA). Complete protease inhibitor tablets were from Roche (Indianapolis, IN). CHAPS was from Calbiochem (San Diego, CA).

Assessment of proteolysis

Frozen rat quadriceps muscle was crushed to a fine powder under liquid nitrogen and separated into several frozen portions. Four volumes to tissue weight (v/w) of either 1.2X solubilizer 1 (9M urea, 4% CHAPS final concentration) or 1.2X solubilizer 2 (7M urea, 2M thiourea, 4% CHAPS final concentration) were added to the powder in the absence of protease inhibitors. An additional sample was incubated with 4 volumes 4% CHAPS in water. Samples were then quickly mixed on a Vortex mixer and incubated at room temperature for either 1, 2, 3 or 19 hr, with occasional mixing. After centrifugation at $16,000 \times g$ for 30 min at room temperature, supernatants were diluted 10-fold in LDS sample buffer (Invitrogen, Carlsbad, CA). Aliquots containing 20 μ g protein and molecular weight marker proteins (Bio-Rad Laboratories) were separated on precast 3–8% Tris-acetate gels (Invitrogen NuPage[®] LDS-PAGE System), transferred to nitrocellulose membranes (Protran, Schleicher and Schuell, Keene, NH, USA) and blocked for 2 hrs in PBS containing 3% nonfat dry milk and 0.4% Tween-20 (“blocking solution”). Blots were incubated overnight at room temperature with monoclonal antibodies to the fast isoform of the myosin heavy chain (clone MY-32, Sigma), diluted 1:1000 in blocking solution. The membranes were washed extensively and then incubated for 1 hr at room temperature with sheep anti-mouse secondary antibodies conjugated to horseradish peroxidase (Amersham, Piscataway, NJ), diluted 1:100,000 in blocking solution. After additional washes, antibodies bound to myosin were detected with the Lumilight PLUS western blotting substrate (Roche) and Hyperfilm ECL film (Amersham).

Ampholyte mixtures

The broad range ampholyte mixture was prepared by combining 8 ml ampholine pH 3.5–10 (Pharmacia), 8ml Servalyte pH 2–11 (Serva), 24 ml Pharmalyte pH 4–6.5 (Pharmacia), 16 ml Pharmalyte pH 5–8 (Pharmacia), and 8 ml Ampholine pH 7–9 (Pharmacia), mixing well and storing in 8 ml aliquots at -80°C . After thawing, solutions were stored at 4°C . Narrow range Servalyte pH 2–4 (Serva) was used as supplied.

Preparation of solutions, tubes and gels

Two methods essential for optimal resolution (cleaning procedure for the glass tubes used for NEPHGE gels and pre-equilibrating the tops of the NEPHGE gels) are described in Supplemental Materials.

1.2x urea/thiourea/CHAPS/ampholyte sample diluent

Amberlite MB-150 (5g) was washed with 5 volumes of 18 Mohm deionized water, drained, added to 8.4 M urea (1 g Amberlite/ 20 ml solution) and mixed gently in 50 ml tubes for 20 min at room temperature. The Amberlite was removed by filtration through a disposable chromatography column, pre-washed with water. CHAPS was added to 4.8% w/v, fresh thiourea was added to 2.4M, broad range ampholytes were added to 2.4% and Complete protease inhibitor cocktail was added to 1.2X. The solution was mixed well, divided into 500 μ l aliquots, frozen in liquid nitrogen and stored at -80°C . The 1.2X urea/thiourea/CHAPS/ampholytes/Complete protease inhibitor solution was used to pre-equilibrate the

tops of the tube gels and the Sephadex slurry (see below). The 1.2X solution was also used to prepare the sample alkylating solution.

Sample alkylating solution

The 1.2x urea/thiourea/CHAPS solution was supplemented with 2.4 mM tributylphosphine and 24 mM 2-vinylpyridine. Chemicals were from Sigma Chemical Co. After diluting the protein samples with 4 volumes of this 1.2X concentrated solubilizer, the samples are dissolved in 7M urea, 2M thiourea, 4% CHAPS, 2 mM tributylphosphine, 2% broad range ampholytes and 1X Complete protease inhibitor cocktail.

Electrode solutions

For NEPHGE, the anode solution consisted of 25 ml of phosphoric acid and 475 ml deionized water. The cathode solution consisted of 20 ml of ethylenediamine and 380 ml deionized water. Urea was omitted from both solutions because it introduced variable changes in conductance of the solution during electrophoresis, presumably due to breakdown of urea in the strongly basic ethylenediamine solution. Urea was unnecessary if the sample was protected from the anode solution by overlaying the sample with at least 10 μ l of a “sample protection solution” (4% CHAPS, 2% broad range ampholytes, 9M urea).

Acrylamide gel solution

The 15% acrylamide gel solution for SDS-PAGE was prepared for slab gels by diluting stock solutions of 10% SDS (Bio-Rad), 30% acrylamide/Bis stock (37.5:1, acrylamide/bis, (Bio-Rad), 4x Tris buffer, pH 8.8, and Rhinohide gel strengthener (Invitrogen) as described by the manufacturer, to give final concentrations of 15% acrylamide/bis, 0.1% SDS, 0.375 M Tris-HCl pH 8.8, and 1X Rhinohide. The solution was used to polymerize the 40 cm $\times$ 28 cm slab gels for use in the second dimension, SDS-PAGE. Note that all acrylamide and Rhinohide stock solutions were deionized with Amberlite MB-150 before dilution, and then filtered, to minimize background from subsequent silver stain.

Preparation of samples for 2-DE analysis

Soluble Muscle Protein 2D Sample Preparation—Soluble proteins were extracted from muscle tissue as described [10]. Briefly, tissue was crushed to a fine powder under liquid nitrogen, combined with Tris (20% glycerol, 100 mM KCl, 50 mM Tris-HCl, pH 7.1), sonicated 12 times for 10 sec each, with 50 sec intervals of stirring on ice between sonication steps. The samples were centrifuged at $100,000 \times g$ for 30 minutes at 4°C. The pellet was ground under liquid nitrogen and extracted again with the same buffer after stirring on ice for 30 min, then centrifuged as above. The second supernatant was combined with the first to make the final soluble extract. Protein concentration was determined with the Bio-Rad Protein Assay. Sample alkylating solution was added to the soluble protein solution at a ratio of 4.2:1 and mixed every 10–15 min on a Vortex mixer over 1 hr at room temperature. A solution of 1.4 M dithiothreitol (DTT) was added to a final concentration of 20 mM to eliminate any remaining 2-vinylpyridine. The solution was stirred for 30 min at room temperature. Sample was divided into aliquots of 100 μ g protein, determined using the 2D-Quant kit (GE Healthcare), frozen in liquid nitrogen, and stored at -80°C .

Preparation of unfractionated human myoblast samples

For myoblast samples, 1.2x sample alkylating solution was added to a frozen myotube pellet at proportions of (4.2 ml of 1.2X sample alkylating solution per gram weight of pellet), vortexed until the cells were completely suspended and incubated for 1 hr at room temperature. The samples were vortexed every 15 min and then prepared exactly as described for the soluble muscle extract samples.

First Dimension NEPHGE

The first dimension gels were prepared as described [7]. Polymerization proceeded for 3 d at room temperature. Samples, prepared as described above and containing 50–100 µg protein, were injected into the gel tubes without disturbing the Sephadex layer. Each sample was overlaid with 10 µl of sample protection solution (9M urea, 4% CHAPS, 2% broad range ampholytes). NEPHGE was performed as described [7]. The first dimension gels were run using a standardized protocol of at 100V for 1hr, 300V for 1 hr, 1000V for 21 hr, 1500V for 30 min, and finally 2000V for 10 min. Gels were extruded from the glass tubes into equilibration solution using water pressure delivered through a 1ml syringe and pipette tip that fit the inner bore of the tubing. After extrusion and equilibration for 10 min, the gels were sucked into 1.5 mm inner diameter TEFLON tubing (Thermo Scientific, part 8050–0125) and frozen on dry ice for storage at –80C.

Apparatus for SDS-PAGE

The materials, dimensions and method of assembly of the gel tanks designed for the second, SDS-PAGE dimension, are described in detail in Fig. 1, Supplementary Fig.3, Table S1, and the text of Supplementary Materials. Images of the apparatus and the plate assembly are presented in Figure 1 and Supplementary Figure 3.

Introduction of tube gels

Equilibration of tube gels with the detergent solution preparatory to SDS-PAGE was performed as described [7] with minor modifications (see Supplementary Materials). A 45.7 cm × 0.95 cm × 2.54 cm glass bar was clamped even with the notched gel plate surface to create a temporary transparent shelf for placing the tube gels while loading them onto the second dimension slab gel. The tube gels were thawed at room temperature and the Teflon tubing holding the gels was tilted back and forth until they moved freely. The gels were allowed to fall out of the plastic tube, end to end, onto the glass shelf. Stretching was minimized by allowing the gel to fall vertically onto the shelf, not at any angle. The tube gels were then shifted using insulin syringe needles from the shelves to the top surfaces of the notched gel plates. This step brought the tube gel as close as possible to the SDS-PAGE gel surface to avoid stretching, and to drain most of the remaining equilibration solution from the tube gel before placing it onto the slab gel. The slab gel tank was propped up on one end to allow any excess fluid to flow away from the incoming tube gel. Finally, tube gels were transferred, end to end, to the SDS-PAGE gel surface using syringe needles. Seating of the tube gel onto the slab gel surface without any fluid displacing the contact between the gel surfaces was critically important to good spot resolution. With the tank still tilted, excess fluid was removed from the slab gel surface and to prevent spot streaking.

After the tank was re-leveled, the glass shelf was removed, the slab gel was clamped to the top buffer tank, and a 0.5% solution of low melting temperature agarose (Invitrogen, Carlsbad, CA), dissolved in running buffer, was gently overlaid to seal the tube gel onto the slab gel. Agarose (0.8 %, dissolved in water) was also applied around the gasket where the slab gel meets the top tank to prevent leaks.

While the agarose solution solidified, cold tap water was added to the water cooling jackets. After the agarose solution solidified, 2L of running buffer was added to both the top and bottom gel tanks (see Fig. 1 and Supplementary Figure 3). Electrophoresis was started immediately after addition of the buffer and run at 25 mA per slab for 17 hr. Electrophoresis was terminated when the bromophenol blue dye front reached the bottom of the gel or when Coomassie blue, placed as a small droplet in 90% glycerol on one edge of the slab gel immediately before electrophoresis, approached within 2.5 cm of the bottom of the slab gel.

Silver Staining

Gels were fixed and stained with silver as described [7], with minor modifications. After fixation and incubation with glutaraldehyde, the gels were washed once with 4 L water, then washed again in water supplemented with 10 µl/L of fresh 10% w/v solution of sodium thiosulfate (1 ppm (w:v), final concentration). This minimized the formation of a yellow-brown background during the 30 min incubation in the silver nitrate solution. In addition, 5 µl/L of the sodium thiosulfate solution was added to the sodium carbonate wash solution to reduce background. Thimerosal was omitted from the stop solution because no increase in spot intensity was observed with its use.

Scanning

Gels were scanned with an Epson Perfection 4990 scanner (20 cm × 20 cm gels in Supplementary Figure 1) or an Epson Expression 10000XL photo scanner (40 cm × 35 cm gels), in transparency mode. The scanner was calibrated with an IT8 calibration target using Silverfast Ai Studio (LaserSoft Imaging, Sarasota, FL) calibration and scanning software. All gels were scanned at 150 or 300 dpi and 16 bit depth resolution, and data were stored as TIFF files in grayscale mode using the full dynamic range of the scanner. Protein spots were evaluated qualitatively by visual inspection and quantitatively with Melanie 7.1 gel analysis software (GeneBio, Geneva, Switzerland).

Data Analysis

Protein spots were quantified with Melanie 7.1 software (GeneBio, Geneva, Switzerland). Quantities were calculated as percent of total volume of matched spots on each gel using the default settings in Melanie software. Quantitative values for spot pairs were imported into Kaleidagraph graphing software (Synergy Software, Reading, PA). Data pairs were plotted on scatter plots using logarithmic coordinates due to the broad dynamic range of spot intensities and to the fact that variations in the spot quantities are much more readily spotted in the graphical output. As the correlation of the data points was always above $R = 0.98$ using a linear curve fitting line, we used power correlation lines to fit the data to the logarithmic output.

RESULTS

To obtain highly reproducible results from large format, two-dimensional gels, we standardized every step in our procedure. To minimize sample-to-sample variation, we prepared most solutions in large batches and stored them in frozen aliquots at -80°C . The following steps were crucial.

Deionization

As the electrode solutions normally contain large amounts of urea, even minor impurities can affect the final resolution. Without deionization, proteins at the basic end of the pH gradient created streaks throughout the entire 40 cm length of the gel, spots were poorly resolved and the total number of clearly resolved spots was reduced (see Fig. S1), compared to gels for which all urea solutions were treated with mixed bed ion exchange resin (Amberlite MB-150). Our solution to this was to exclude urea from the electrode solutions.

Use of thiourea

Protein degradation is common in 2D gel results [12], despite the fact that samples are routinely prepared with a mixture of protease inhibitors, probably because the charged inhibitors migrate away from the proteins as electrophoretic separation starts. This occurs just as the proteins (and any proteases that may remain active) reach their highest

concentrations at the top of the tube gel. Following an earlier suggestion [13, 16], we tested the ability of 7M urea + 2M thiourea to prevent proteolysis in extracts of skeletal muscle (Figure S2), compared to urea alone. Western blots showed that fast myosin heavy chain was degraded in the solution containing 9M urea, as well as with no urea at all (Fig. S2). By contrast, in solutions containing 7M urea + 2M thiourea (Fig. S2), most of the myosin remained intact. Thus, 2M thiourea + 7M urea effectively minimizes proteolytic artifacts in extracts prepared from mammalian skeletal muscle.

In addition to its effectiveness as an inhibitor of proteolysis, thiourea enhances the solubility of some proteins in IEF gels where they concentrate at their isoelectric points or at the top of the gels, where proteolysis and precipitation may occur [14,15,16,17,18]. For these reasons, we routinely incubate the tops of our tube gels and the Sephadex mixture applied to them with a solution containing 7M urea, 2M thiourea, 4% CHAPS and 2% broad range ampholytes before applying our protein samples for NEPHGE. Before first dimension electrophoresis, most of the urea/thiourea solution is removed leaving only a minimal volume covering the Sephadex layer. After the layer of protein sample is applied, the sample is finally covered with sample protection solution (9M urea, 4% CHAPS, and 2% broad range ampholyte mixture).

Large gel tank

We constructed a tank and cooling apparatus for the second, SDS-PAGE dimension of 2D-GE that accommodates a large gel, 40 cm wide $\times$ 28 cm long, appropriate for separating all the proteins resolved in a 40 cm tube gel by NEPHGE in a single slab (Fig. 1). Details of the design of the tank and the materials used are presented in Supplementary Materials (Figures 1, S3, and Table S1).

The apparatus has several key features. First, the glass plates that house the slab gel are glued directly to additional plates that form a cooling jacket. This improves cooling and eliminates the need to bathe the gel plates in large volumes of cooled buffer to control temperature during electrophoresis. The design has the added advantage of preventing leakage of electrical current around the gel spacers.

After silver staining, the gels dimensions are approximately 40 $\times$ 35 cm, which can be scanned in a single image (see Methods) and then analyzed. Fig. 2 shows silver stained gels of proteins in the soluble fraction from human skeletal muscle biopsies, and from unfractionated myotubes (Fig.3), analyzed with our methods and apparatus. We typically resolve 3000–4000 protein spots from the unfractionated myotube samples and 1500–1800 spots from the soluble protein fraction from muscle biopsies. The reduced resolution in the biopsy samples is due to the presence of highly abundant serum proteins present in the tissue that partially obscure the overall protein pattern (see Fig. 2). The use of a single slab is superior to a previous method, in which the 40 cm tube gels were cut in half and then analyzed in separate 20 $\times$ 20 cm, acrylamide slab gels. Using that method, we failed to resolve many of the protein spots along the site of the cut.

Quantitative Reproducibility

We assessed the quantitative reproducibility of our methods by comparing replicate gels of soluble proteins extracted from the muscle biopsies and the myotubes (Figs. 2, 3). We prepared scatterplots of the results, representing the quantity of protein present in each spot in one silver stained *vs.* the same spot in the replicate gel. The linear correlation coefficients for muscle and myotube extracts across the entire gels were $R = 0.9893$ and $R = 0.9904$, respectively (not shown). As the wide range of spot intensities is better represented logarithmically, we replotted the data in logarithmic coordinates and recalculated the

correlation coefficients with the power fitting method in Kaleidagraph graphing software. As the quantitative reproducibility may vary depending on the region of the gel analyzed, we prepared separate scatterplots of the data from spots present in each quadrant of the gel. Figures 2 and 3 show that each of the 4 quadrants give R values of 0.989 (power fitting) in the gels containing muscle extract and 0.986 in the myotubes extracts. Thus, our methods yield highly reproducible, quantitative results in all areas of the gels.

DISCUSSION

We describe methods to optimize the performance of large-gel 2-D electrophoresis, specifically for proteomic studies of skeletal muscle. We based our approach on Klose's methods [6,7,10], adding steps to reduce streaking and improve resolution, to minimize proteolysis and to enhance the solubility of proteins as they enter the NEPHGE gel. In addition, although we alkylated the samples before electrophoretic steps, as recommended by Righetti [12] to prevent proteolysis and the formation of mixed sulfhydryl crosslinking of protein species, we did not test the efficiency of the alkylation steps in this study. We also developed a new gel tank that allowed proteins over the entire range of isoelectric points resolvable in NEPHGE to be analyzed in a single, 40 cm SDS-PAGE gel. Our method quantitatively and reproducibly resolves 1800 and 2800 protein spots in the soluble fractions of adult skeletal muscle and myotubes, respectively. Although developed specifically for skeletal muscle, our modifications should be compatible with large gel 2D electrophoretic studies of the proteome in many mammalian tissues.

Only two basic high resolution approaches for separating proteins in the first dimension gels are available, one based on carrier ampholytes in tube gels and the other on immobilized pH gradient (IPG) gels. Tube gels using carrier ampholytes cover a broad pH range and can resolve proteins with isoelectric points between 4 and 10 in a single 40 cm long gel. Single commercial IPG strips do not offer the same 40cm spatial resolution and thus several strips with overlapping ranges are needed to analyze proteins with isoelectric points from 4 to 10 over such long separation to give comparable resolution. Alternatively, commercial IPG strips that span the entire range can be prepared. Tube gels are preferable, however, as protein losses from them are lower than those from IPG strips [3,4,5]. This is especially important when comparing precious samples, such as those from biopsies of human tissue with rare diseases, or those obtained by microdissection. We have therefore used tube gels routinely; we also took additional precautions to minimize protein loss and to improve resolution.

A key modification that we made to the earlier methods of Klose [6,7,10] is the replacement of 9 M urea in all sample solutions with 7M urea, 2 M thiourea and 4% CHAPS. This mixture was more effective for preparing samples for NEPHGE because it increased the solubility of proteins and so reduced losses due to precipitation [5, 15], and because it minimized proteolysis (ref [13] and Fig. S2). Application of this solution to the tops of the tube gels and in the Sephadex resin, used to filter insoluble materials there, is especially helpful. Early in IEF, the sample compresses at the top of the gel and proteins (and proteases) reach their highest concentrations simultaneously, while some of the small, charged protease inhibitors (e.g. aprotinin, EDTA, e-aminocaproic acid, benzamidinium-HCl, pepstatin) migrate away from the proteins. Inhibiting proteolysis with 7M urea + 2M thiourea at this critical stage is therefore a significant improvement.

We also modified established procedures to use 4-vinylpyridine [19], a reagent specific for the free sulfhydryl groups of cysteine residues [20], to prevent the formation of mixed disulfides or other protein modifications, which Herbert et. al. demonstrated could create artifacts in two-dimensional electrophoresis [8, 12].

Another key modification we have introduced is the use of a large SDS-PAGE gel tank capable of resolving proteins from the entire 40 cm length of the first dimension tube gel. This eliminates the loss and distortion of spots that can occur on smaller slab gels, which require cutting the tube gel in half and analyzing the spots that are transferred with each piece. The cooling jacket we incorporated in the apparatus serves both to cool the gels, allowing nearly isothermal, overnight runs at currents of 20–25 mA, while reducing current leak around the gel spacers. The tanks have the additional advantage of being inexpensive and easy to assemble. Combined with our additional modifications of published methods, our procedures and new tank design yield gels that resolve several thousand spots clearly, reproducibly and quantitatively. Positional variation was minimal for repeated runs of a single sample but increased between different samples. The greatest reproducibility between different samples was with samples from first degree relatives. The relative positions of the spots were reproducible but the absolute position of the spots was influenced by the presence of salts in the sample which, as a result, is more pronounced with increasing protein load. For a detailed discussion, see O'Farrell [11]. Our methods allow two people using 6 gel tanks to run 12 gels per week, routinely, or more, if the tube gels were first generated in larger numbers and stored frozen. Comparisons of our data to those reported by Klose suggest that, although his results are outstanding, our methods are more effective in reducing variation and in improving reproducibility in all quadrants of the gels [21]. We are now applying these improvements to analyze the proteomic differences between biopsies of healthy and dystrophic skeletal muscles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Gel tank for the SDS-PAGE step in 2D gel electrophoresis: (A) The gel tank is shown assembled. The top gel tank is reversibly clamped to its adjustable stand with Erwin clamps (vertical clamps in back). The individual glass plates and gel spacer assembly are connected with stainless steel office binder clips. The gel assembly is connected to the top gel tank with the Erwin clamps (horizontal clamps). (B) A skeletonized drawing of the electrophoresis chamber is shown, without the gel. Additional dimensional drawings and gel tank pictures are provided in the Supplementary Materials.

Quantitative Reproducibility of Muscle Extracts

Figure 2.

Quantitative reproducibility of soluble muscle proteins.

Upper panels: Sixty micrograms of soluble muscle protein extract were prepared and replicate gels of the same sample were run at the same time in the same first dimension apparatus. The orientations of the gels are acidic proteins on left, basic proteins on right, and all proteins arrayed from high to low molecular weights from top to bottom. Gels were stained with silver. Each replicate was run in separate tube and SDS-PAGE gels under identical conditions. Lower panels: Left panels: Spots matched in the each quadrant of the resulting gels were plotted as indicated on a logarithmic scale and the correlation coefficient was determined with the power fitting algorithm in Kaleidagraph software. There was a very

high correlation between spot intensities in all regions of the gels. Right upper panel: The entire gels showed excellent reproducibility with a correlation coefficient of $R = 0.988$. Right lower panel: A 99 point running average of the coefficient of variation (CV) is plotted against the spot intensities. On average, the lower abundance spots showed the highest CVs ($\approx 20\%$), with CV decreasing as the intensity increased. Overall, the gels showed very high reproducibility.

Quantitative Reproducibility of Myotube Extracts

Figure 3.

Quantitative reproducibility of myotube protein extracts with new gel apparatus.

Upper panels: Fifty micrograms of an unfractionated myotube protein extracts were processed and analyzed as in Fig. 2. Lower panels: All gel regions showed correlation coefficients of $R \approx 0.99$ when entire gels were compared: The 99 point running average of the coefficient of variation against the spot intensities revealed that the average spots showed CVs of < 20%. The cultured cell samples showed slightly more quantitative reproducibility than the tissue extracts.