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# An effective skeletal muscle prefractionation method to remove abundant structural proteins for optimized two-dimensional gel electrophoresis

Proteomic analysis of biological samples in disease models or therapeutic intervention studies requires the ability to detect and identify biologically relevant proteins present in relatively low concentrations. The detection and analysis of these low-level proteins is hindered by the presence of a few proteins that are expressed in relatively high concentrations. In the case of muscle tissue, highly abundant structural proteins, such as actin, myosin, and tropomyosin, compromise the detection and analysis of more biologically relevant proteins. We have developed a practical protocol which exploits high-pH extraction to reduce or remove abundant structural proteins from skeletal muscle crude membrane preparations in a manner suitable for two dimensional gel electrophoresis. An initial whole-cell muscle lysate is generated by homogenization of powdered tissue in Tris-base. This lysate is subsequently partitioned into a supernatant and pellet containing the majority of structural proteins. Treatment of the pellet with high-pH conditions effectively releases structural proteins from membrane compartments which are then removed through ultracentrifugation. Mass spectrometric identification shows that the majority of protein spots reduced or removed by high-pH treatment were contractile proteins or contractile-related proteins. Removal of these proteins enabled successful detection and identification of minor proteins. Structural protein removal also results in significant improvement of gel quality and the ability to load higher amounts of total protein for the detection of lower abundant protein classes.

**Keywords:** Fractionation / High pH / Skeletal muscle / Structural protein / Two-dimensional gel electrophoresis
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## 1 Introduction

Muscle is a tissue of interest for many biological research areas applying two-dimensional gel electrophoresis (2-DE) as a proteomic tool. Cardiac muscle has been investigated for identification of protein profile changes between normal and diseased heart tissue [1–3]. Skeletal muscle has been studied during muscle atrophy [4–8] and diabetic disease [9, 10]. Initial proteomic studies often used muscle tissue without protein fractionation. However, total protein content in muscle tissue is dominated by contractile proteins, such as actin, myosin, and tropomyosin. The presence of these highly abundant structural proteins impact 2-DE gels in the following ways. First, they mask lower abundant proteins of similar molecular weight and isoelectric point. This is further compounded by the presence of multiple isoforms, fragments, and post-translational modifications of these proteins. Second, they inhibit optimal protein resolution. Actin in particular causes streaking and gel distortion. Third, the protein loading capacity is occupied by abundant structural proteins, preventing sufficient loading for the detection of lower abundant protein classes.

The importance of sample fractionation prior to 2-DE has been recognized by the proteomics research community [11, 12]. One emphasis in this area is to selectively remove the abundant proteins from the targeted tissues. For serum, monoclonal antibodies have been developed to remove albumin, immunoglobulin G (IgG), IgE, and other major abundant proteins [13–15]. This method is highly effective and removes approximately 90% of these abundant proteins. The success of this method enables researchers to use serum for subsequent proteomic studies. For solid tissue, anti-phosphoprotein antibodies have been used for phosphorylated protein enrichment prior to 2-DE analysis [16–18]. Differential centrifugation methods have also been used for subcellular organelle

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isolation [19, 20]. However, there has been no report of a prefractionation method for the removal of abundant muscle structural proteins for 2-DE analysis.

An array of well-developed antibodies would serve as the ideal tool for the depletion of abundant muscle structural proteins, but production and screening of highly specific antibodies takes a considerable amount of time. Concurrent with antibody development, we focused on the development of a simple differential extraction procedure aimed at removing abundant structural proteins from muscle crude membrane preparations. The extraction conditions were designed to segregate the majority of contractile proteins into a crude membrane preparation, allowing for their depletion through high-pH biochemical fractionation. Briefly, when a cell is lysed under biological pH conditions, fragmented membranes can rapidly reseal forming large membrane-bound compartments within the lysate [21, 22]. Highly abundant cytosolic proteins become trapped within these newly formed compartments and readily sediment. Thus, the crude membrane fraction contains abundant structural proteins, as well as other cytosolic and membrane bound components. Under high-pH conditions, membrane vesicles are converted to sheets, thus releasing trapped structural proteins [21, 23]. In addition, high-pH conditions disrupt protein-protein interactions, thus breaking up protein aggregates [21]. The major structural proteins can be effectively removed following their release through ultracentrifugation. The 2-DE profiles from the resulting pellet are free from distortions caused by large and streaky actin, myosin, and tropomyosin spots, resulting in increased sensitivity, gel quality, and reproducibility.

## 2 Materials and methods

### 2.1 Aqueous extraction and centrifugal fractionation

Frozen rat skeletal muscle tissue was powdered with a liquid nitrogen-cooled mortar and pestle. The resulting powder (approximately 100 mg aliquots) was dounce-homogenized in a 1:10 ratio (tissue:buffer) of ice-cold homogenization buffer (10 mM Tris pH 7.6, 30 mM sodium pyrophosphate, phosphatase inhibitor cocktail II (1:100 dilution) (Sigma, St. Louis, MO, USA), complete protease inhibitor (1 tablet/25 mL) (Roche, Mannheim, Germany), and 30 mM DTT). The resulting homogenate was centrifuged at low speed (800 × *g*) to pellet insoluble material. The supernatant (S1) was recovered and the pellet (P1) was subjected to an additional round of homogenization and centrifugation with the supernatant (S1a) being pooled with S1 (Fig. 1, step 1). This supernatant pool was subjected to centrifugation at 37 000 × *g* for 20 min at 4°C resulting in the supernatant (S2) and pellet (P2) fractions (Fig. 1, step 2).

### 2.2 Fractionation of P2 with high-pH sodium carbonate buffer

The pellet from step 2 (P2) was resuspended in 50 mM Tris pH 7.4 (500 μL), diluted to a total volume of 4.0 mL with 100 mM sodium carbonate, pH 11, and incubated on ice for 1 h. Following incubation, the suspension was centrifuged at 100 000 × *g* for 1 h at 4°C (Fig. 1, step 3).

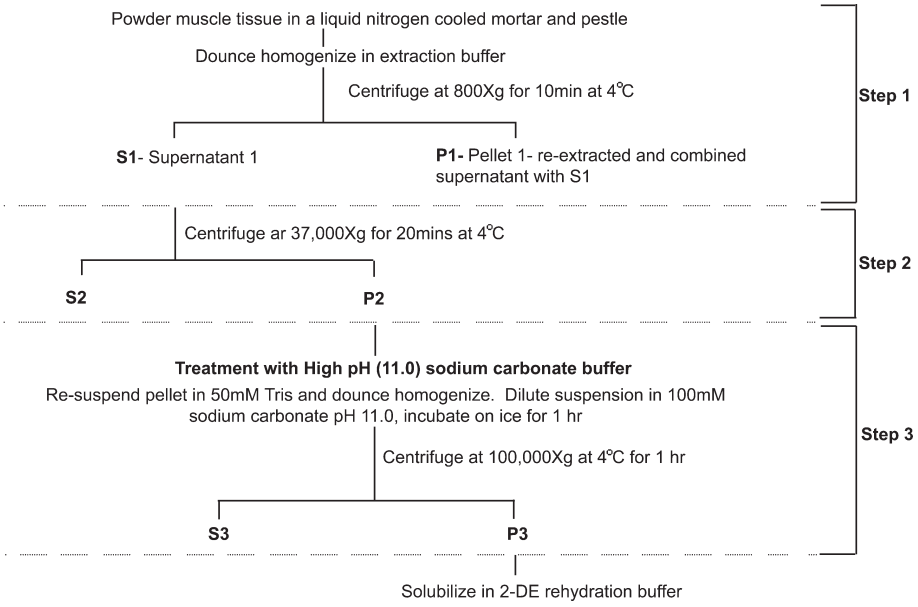


**Figure 1.** Schematic illustration of the differential extraction procedure for the removal of highly abundant muscle structural proteins.

### 2.3 Protein assay

Aliquots of S1, S2, P2, and P3 fractions were solubilized in 5 M urea, 2 M thiourea, 2% CHAPS, 2% 3-(decyldimethylammonio)-propanesufonate inner salt (SB 3–10), and 30 mM DTT and protein content was measured using a Non-Interfering Protein Assay kit (Geno Technology, St. Louis, MO, USA) according to the manufacturer's recommendations.

### 2.4 2-DE

In preparation for isoelectric focusing, 150 μg aliquots of S1, S2, P2, and P3 were diluted to a total volume of 350 μL in 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3–10, and 30 mM DTT. Each sample was incubated at room temperature for 1 h with a 1 min water bath sonication every 15 min. The samples were cleared of insoluble material (if any) by centrifugation for 10 min at 14 000 rpm using a tabletop centrifuge. The resulting soluble fractions were loaded onto 18 cm pH 4–7 immobilized pH gradient (IPG) strips (Amersham Pharmacia Biotech, Uppsala, Sweden) during an overnight in-gel rehydration/sample loading step [24, 25]. Isoelectric focusing was performed on a Multiphor II apparatus at 20°C under the following voltage gradient: 0–300 V over 1 min and held at 300 V for 2 h increased to 3500 V over 2 h and held at 3500 V until a total of 75 000 Vh was reached. The focused strips were immediately equilibrated as follows: reduction through two 15 min incubations in 50 mM Tris, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2% DTT, and a trace of bromophenol blue, followed by alkylation for 15 min in same buffer with 2.5% iodoacetamide replacing the 2% DTT. Once equilibrated, strips were loaded onto Investigator Pre-cast 10% Tricine slab gels (Genomic Solutions, Ann Arbor, MI, USA). Separation was carried out at 15 W per gel. Protein spots were visualized through silver staining using the Investigator silver stain kit (Genomic Solutions), which is a modification of the method described by Rabilloud [26]. Each protein profile shown is a representative image of at least three independent experiments.

### 2.5 Image analysis

Protein profiles of P2 and P3 were compared using the Z4000 Gel Analysis system (Compugen, Jamesburg, NJ, USA). Spots of interest in the P2 fraction were defined as exhibiting reduced expression or missing (removed) when compared to P3.

### 2.6 Protein identification

Spots of interest were excised from the gels. In cases where spot intensity was low, several spots were pooled from different gels. Gel plugs were placed in wells of Investigator ProGest microtiter plates. Gel plugs were destained, tryptic-digested, and peptides eluted using an Investigator ProGest Protein Digestion Station (Genomic Solutions). Briefly, gel plugs were destained in 15 mM potassium ferricyanide and 50 mM sodium thiosulfate [27], washed with water, dehydrated with acetonitrile, and subjected to tryptic digestion using 20 ng of sequencing-grade trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate over night at 37°C. Peptides were extracted from gel plugs through two 10 min incubations in a 50% acetonitile and 0.1% trifluoroacetic acid (TFA) solution. Gel extracts from the digestion were concentrated to about 10 μL under reduced pressure in a SpeedVac concentrator (Savant, Holbrook, NY, USA) in preparation for desalting on C18μ ZipTips (Millipore, Bedford, MA, USA). 10 μL of 0.1% TFA was added to each sample prior to binding and elution from the ZipTip according the supplier's instructions. The final peptides eluted from the ZipTip were concentrated to 0.6 μL in the SpeedVac and mixed with 1 μL of matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid, 60% acetonitrile, and 0.3% trifluoroacetic acid) and spotted onto the MALDI-MS target plate. Protein identification was performed using a combination of peptide mass fingerprints (PMFs) and MS/MS fragmentation of the desalted tryptic digests with an Applied Biosystems 4700 Proteomics Analyser. Spectra were collected using 4700 Explorer (Ver. 2.0) software in an automated fashion in positive ion, reflector mode with delayed ion extraction and a mass range of 850–3200 *m/z.* An external calibration was established for the entire plate using a 7-peptide mixture (4700 mix) available from the vendor. Typical peptide mass errors were $<$ 30 ppm. PMFs were searched against an internal database (including the NCBInr entries) using the MASCOT (Matrix Science, http://www.matrixscience.com) search algorithm integrated into the GPSExplorer (Ver. 2.0) software provided by Applied Biosystems. For any sample that produced an ambiguous result from the PMF, fragmentation spectra were collected in MS/MS mode on the 4700 Proteomics Analyser. Data from the resulting spectra were searched either as MS/MS only or in combination with the PMF using the MASCOT algorithms.

## 3 Results

### 3.1 2-DE analyses of differentially fractionated muscle proteins

Figure 2 shows the silver-stained 2-DE protein profile obtained from 150 μg of whole-cell lysate (S1 fraction) following the initial extraction step. It is evident that several groups of proteins dominate and distort the gel

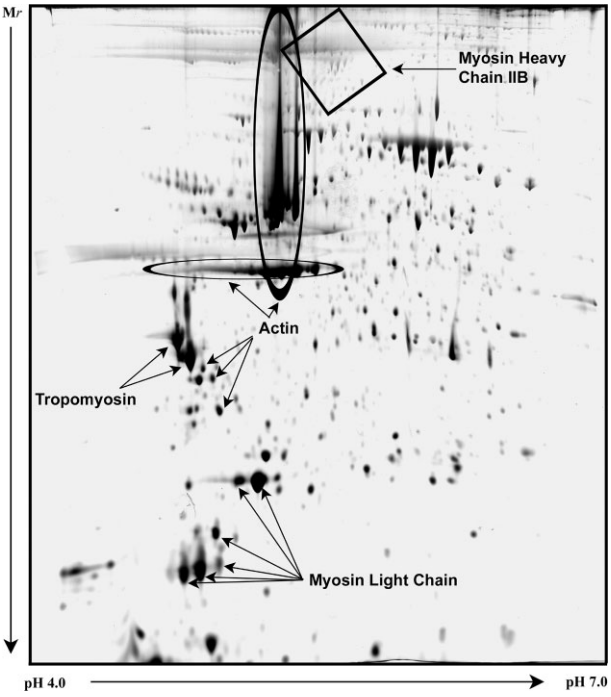


**Figure 2.** Representative 2-DE protein profile generated from S1 fraction. Frozen rat skeletal muscle was powdered under liquid nitrogen, homogenized in 10 mM Tris, pH 7.4 containing protease and phosphatase inhibitors, followed by centrifugation at $800 \times g$ for 10 min, and 150 μg of the resulting supernatant was resolved by 2-DE as described. In this representative gel image the ovals, boxes, and arrows indicate the predominant (less abundant forms exist at other locations) species of various muscle contractile apparatus proteins. These major contractile proteins can be seen as large, poorly resolved, and overloaded spots.

image. We previously identified these as various forms of actin, myosin and tropomyosin, which resolve as large and often saturated spots causing poor resolution and less than desirable reproducibility. After the $37\,000 \times g$ centrifugation of the S1 fraction, equal amounts of protein from both fractions (S2 and P2 pellet) were analyzed by 2-DE.

Comparison of 2-DE gel profiles from S2 and P2 demonstrates that a smaller population of actin, myosin, and tropomyosin is soluble and remains in the supernatant (Fig. 3A), while the majority of these proteins segregate to the pellet fraction (Fig. 3B). Thus, the P2 fraction was subjected to further fractionation. Sodium carbonate buffer at pH 11.0 was used to treat the P2 fraction in order to break the resealed membrane compartments, thus releasing the trapped abundant structural proteins.

Figure 4 demonstrates that high-pH treatment enables the removal of contractile apparatus proteins from the P2 pellet. Comparing protein profiles from P2 and P3 in an overlay (Fig. 5a) demonstrates the improvement in data available from 2-DE gels even in the absence of increased loading of the proteins that remain after removal or reduction of contractile proteins. One can observe that there is noticeable overlap of some protein spots, but, more importantly, the majority of major contractile apparatus proteins have been removed. Many of the abundant proteins that were masked by actin are now visible (Fig. 5b). Moreover, there was a clear enrichment of some proteins that were present in P2 prior to high-pH treatment (Fig. 5c). In addition, spots which were previously undetected in the P2 fraction now become apparent and are available for analysis (Fig. 5d). It is evident that higher protein loads are now possible so that proteins of this and even somewhat lower abundance classes can be detected and analyzed without overwhelming the gel with contractile apparatus proteins. MS identification of 78 newly resolved spots from the P3 fraction indicate that these are not additional structural proteins or their fragments generated by high-pH treatment (full list of data not shown). Nine representative enriched minor proteins are highlighted in Figs. 5b, c, and d, and listed in Table 1.

### 3.2 Selectivity of high-pH treatment

The selectivity of high-pH treatment to remove contractile proteins was further validated through protein identification. Spots of interest in the P2 fraction were defined as exhibiting reduced expression or missing (removed) when compared to P3. We selected 66 of the most abundant spots that met these criteria for identification (Fig. 6); they were excised from the gel, subject to tryptic in-gel digestion, and subsequent MS analysis. To date, 56 of these spots have been identified (Table 2). From these spots 48 were identified as being part of or associated with the contractile apparatus, which included various forms of actin, myosin, tropomyosin, troponin, F-actin capping protein, and myozenin. The other eight spots identified were not members of or associated with contractile apparatus, which included albumin, α-fetoprotein (albumin homolog), sarcoplasmic reticulum 53K glycoprotein, ATPase (mitochondrial), phosphoglucomutase, Pdhb protein, and 3-mercaptopyruvate sulfurtransferase.

### 3.3 Efficiency of differential extraction procedure

The efficiency of extraction was determined by measuring the protein concentration at each step in order to calculate the total protein recovered. The total protein yield at each step and its relative percentage to the initial whole cell lysate (S1) is summarized in Table 3.

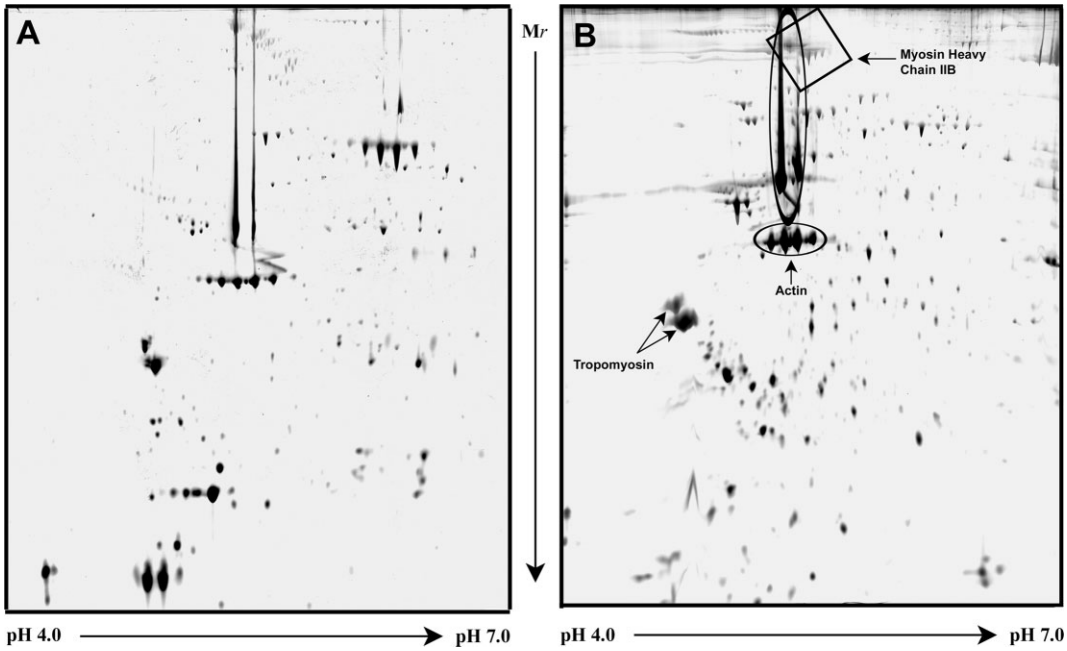


**Figure 3.** Representative 2-DE profiles of initial centrifugal fractionation. Rat skeletal muscle tissue was homogenized and centrifuged at 37 000 × *g* resulting in (A) a soluble supernatant and (B) an insoluble pellet. Both gels were loaded with 150 μg total protein. Note that the majority of the major structural proteins actin (circled region), myosin heavy chain (boxed region) and tropomyosin segregate to pellet.

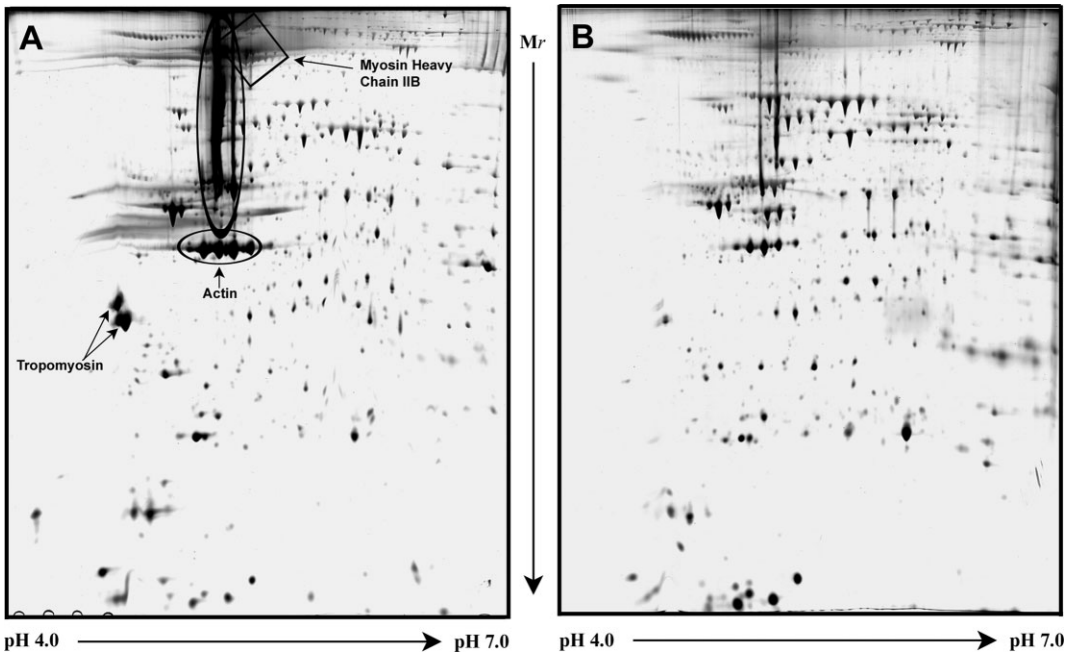


**Figure 4.** Removal and reduction of muscle major structural proteins with high-pH sodium carbonate treatment followed by high-speed centrifugation. Representative 150 μg loaded 2-DE profiles of (A) P2 prior to high-pH treatment and (B) P3 insoluble pellet following high-pH treatment and 100 000 × *g* centrifugation. The removal of highly abundant structural proteins resulted in improved gel quality, the detection of protein spots that were previously masked, and new protein spots. The improved quality allows for the loading of larger amounts of total protein, enabling the detection of lower abundant proteins.

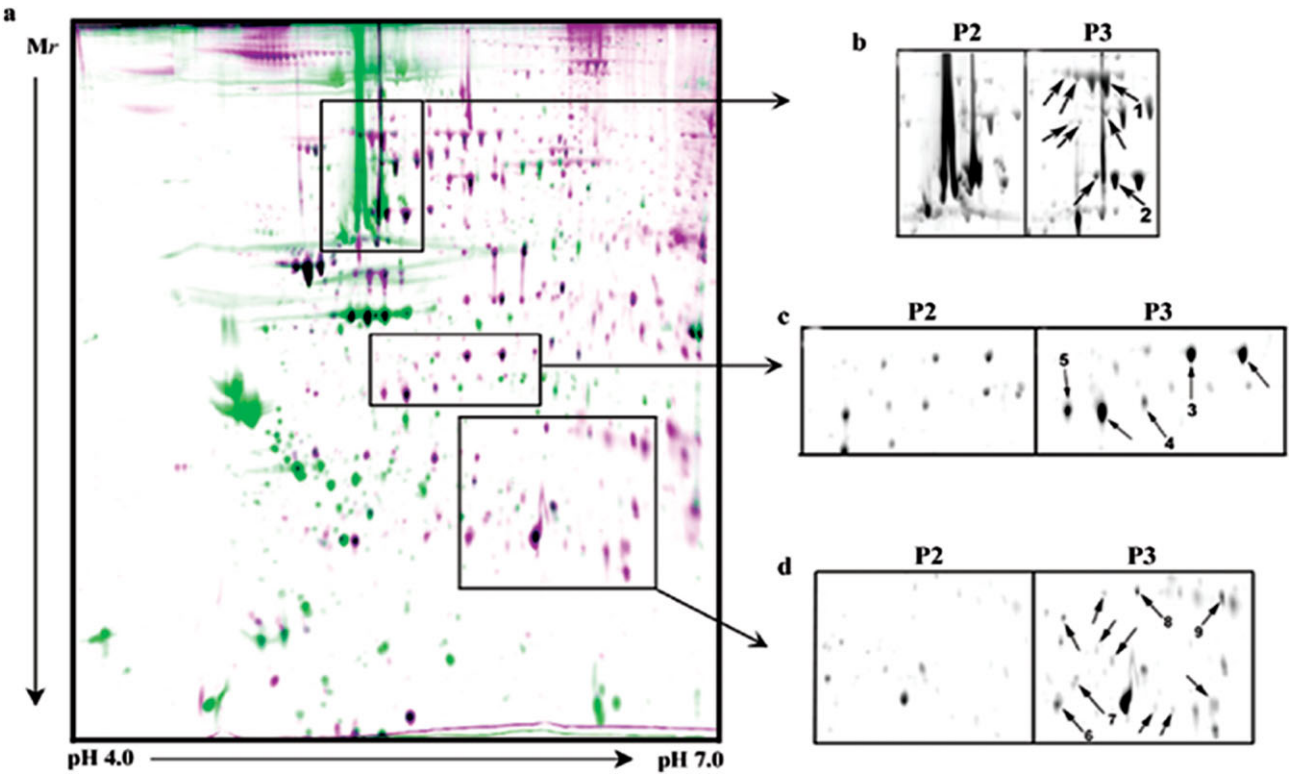


**Figure 5.** Comparison of gel images from P2 prior to and P3 following high-pH treatment. (a) Overlay image of P2 (green) and P3 (purple), both gels were loaded with 300 μg of total protein. The overlay allows visualization of global similarities and differences between the two gel images. Spots present only in the P2 appear green. While spots only present in the P3 appear purple. Spots that match between the two images appear in shades of black. (b)–(d) Comparison of zoomed regions of protein profiles from P2 and P3. The arrows in (b) indicate protein spots that could not be detected prior to high-pH treatment because they were masked by highly abundant structural proteins. The arrows in (c) indicate protein spots that have been enriched following high-pH treatment. The arrows in (d) indicate protein spots that were undetectable prior to high-pH treatment. Numbered arrows indicate spots which were identified. Protein identification results accompanied by their MS data are listed in Table 2.

**Table 1.** MS identification of minor protein spots which were unmasked or enriched through high-pH treatment

| Spot No. | Protein name | Database accession No. | Theoretical $M_r$ | Theoretical p*I* | # Peptides matched |
|---|---|---|---|---|---|
| 1 | NADH dehydrogenase (ubiquinone) Fe-S protein 1 | gi\|21704020 | 79698 | 5.51 | 15 |
| 2 | Mitochondrial precursor /Hsp60) | gi\|3219998 | 60917.4 | 5.9 | 15 |
| 3 | Isocitrate dehydrogenase 3 (NAD+) α | gi\|16758446 | 39588.0 | 6.5 | 8 |
| 4 | Pyruvate dehydrogenase (lipoamide) β | gi\|18152793 | 38912.0 | 6.4 | 7 |
| 5 | Pdhb protein | gi\|12805431 | 34813.8 | 5.6 | 6 |
| 6 | Subunit d of mitochondnal H-ATP synthase | gi\|220904 | 18769.6 | 5.8 | 3 |
| 7 | Ras-related protein Rab-11A | gi\|4758984 | 24378.4 | 6.1 | 4 |
| 8 | 3-Mercaptopyruvate sulfurtransferase | gi\|20304123 | 32919.4 | 5.9 | 5 |
| 9 | Chain A, dienoyl-CoA isomerase | gi\|4699607 | 30359.4 | 7.1 | 6 |

## 4 Discussion

A major challenge of conducting 2-DE experiments of any tissue is evident when one considers the large variation in the level of expression of various proteins in a tissue (which can range from 3 to 10 orders of magnitude). Inevitably, the more highly expressed proteins, which are usually structural or homeostatic in nature, mask those with lower expression (more biologically relevant proteins, including those involved in regulatory or signaling path-

**Table 2.** MS identification of protein spots reduced or removed through high-pH treatment

| Spot No. | Protein name | Database accession No. | Theoretical $M_r$ | Theoretical p*I* | # Peptides matched |
|---|---|---|---|---|---|
| 1 | Actin α, cardiac-mouse (fragment) | gi\|627834 | 41758 | 5.23 | 13 |
| 2 | Actin α, cardiac-mouse (fragment) | gi\|627834 | 41758 | 5.23 | 13 |
| 3 | Actin α, cardiac-mouse (fragment) | gi\|627834 | 41758 | 5.23 | 13 |
| 4 | Actin α, cardiac-mouse (fragment) | gi\|627834 | 41758 | 5.23 | 12 |
| 5 | Tropomyosin β 2-mouse | gi\|346655 | 32781 | 4.64 | 17 |
| 6 | Tropomyosin 1 α chain (α-tropomyosin) | gi\|20178271 | 32661 | 4.69 | 21 |
| 7 | Actin, fetal skeletal/adult cardiac muscle-mouse (fragment) | gi\|90263 | 39226 | 5.83 | 8 |
| 8 | Actin, fetal skeletal/adult cardiac muscle-mouse (fragment) | gi\|90263 | 39226 | 5.83 | 8 |
| 9 | Actin, fetal skeletal/adult cardiac muscle-mouse (fragment) | gi\|90263 | 39226 | 5.83 | 8 |
| 10 | Actin, fetal skeletal/adult cardiac muscle-mouse (fragment) | gi\|90263 | 39226 | 5.83 | 6 |
| 11 | Myosin light chain 1, skeletal muscle isoform (MLC1F) (A1 catalytic) | gi\|127131 | 20666 | 4.99 | 10 |
| 12 | Actin, fetal skeletal/adult cardiac muscle-mouse (fragment) | gi\|90263 | 39226 | 5.83 | 4 |
| 13 | ATP synthase, H+ transporting, mitochondrial F1 complex, δ subunit | gi\|20806153 | 17584 | 5.16 | 3 |
| 14 | Actin α, cardiac-mouse (fragment) | gi\|627834 | 41758 | 5.23 | 5 |
| 15 | Actin α, cardiac-mouse (fragment) | gi\|627834 | 41758 | 5.23 | 7 |
| 16 | Actin α, cardiac-mouse (fragment) | gi\|627834 | 41758 | 5.23 | 5 |
| 17 | Actin α, cardiac-mouse (fragment) | gi\|627834 | 41758 | 5.23 | 9 |
| 18 | Actin α, cardiac-mouse (fragment) | gi\|627834 | 41758 | 5.23 | 9 |
| 19 | Actin α, cardiac-mouse (fragment) | gi\|627834 | 41758 | 5.23 | 12 |
| 20 | α-Fetoprotein | gi\|191765 | 47195 | 5.47 | 5 |
| 21 | Albumin (*Rattus norvegicus*) | gi\|19705431 | 68674 | 6.09 | 13 |
| 22 | x | | | | |
| 23 | x | | | | |
| 24 | Sarcoplasmic reticulum 53K glycoprotein precursor-rabbit | gi\|109376 | 54416 | 6.21 | 8 |
| 25 | Sarcoplasmic reticulum 53K glycoprotein precursor-rabbit | gi\|109376 | 54416 | 6.21 | 8 |
| 26 | Chain A, phosphoglucomutase Mol_id: 1; | gi\|1310948 | 61389 | 6.62 | 2 |
| 27 | Actin prepeptide | gi\|178067 | 36783 | 5.19 | 5 |
| 28 | Pdhb protein (*Mus musculus*) | gi\|12805431 | 34813.8 | 5.63 | 3 |
| 29 | Actin γ 2, smooth muscle, enteric (*Mus musculus*) | gi\|6752952 | 42852 | 5.36 | 4 |
| 30 | F-actin capping protein α 2 subunit (CapZ α 2) | gi\|18206239 | 13574 | 4.73 | 1 |
| 31 | Slow skeletal muscle troponin T, low $M_r$ isoform (*Mus musculus*) | gi\|3449358 | 29992 | 6.34 | 8 |
| 32 | Troponin T fast skeletal muscle isoform (*Mus musculus*) | gi\|2340062 | 31827 | 5.31 | 1 |
| 33 | Troponin T fast skeletal muscle isoform (*Mus musculus*) | gi\|2340062 | 31827 | 5.31 | 3 |
| 34 | Troponin T class IIIb alpha (*Rattus norvegicus*) | gi\|1256739 | 28300 | 9.35 | 6 |
| 35 | 3-Mercaptopyruvate sulfurtransferase (*Rattus norvegicus*) | gi\|20304123 | 32919 | 5.86 | 6 |
| 36 | x | | | | |
| 37 | x | | | | |
| 38 | Troponin T class IIIb α (*Rattus norvegicus*) | gi\|1256739 | 28299.9 | 9.35 | 6 |
| 39 | Troponin T fast skeletal muscle isoform (*Mus musculus*) | gi\|2340066 | 31020 | 5.59 | 11 |
| 40 | Actin prepeptide | gi\|178067 | 36783 | 5.19 | 5 |
| 41 | Actin prepeptide | gi\|178067 | 36783 | 5.19 | 5 |
| 42 | Actin, fetal skeletal/adult cardiac muscle-mouse (fragment) | gi\|90263 | 39226 | 5.83 | 2 |
| 43 | Actin, γ 2, smooth muscle, enteric (*Mus musculus*) | gi\|6752952 | 42852 | 5.36 | 6 |
| 44 | Actin, α, cardiac-mouse (fragment) | gi\|627834 | 41757.8 | 5.23 | 8 |
| 45 | Actin Prepeptide | gi\|178067 | 36783 | 5.19 | 3 |
| 46 | Actin, aortic smooth muscle-bovine | gi\|71616 | 41748 | 5.24 | 6 |
| 47 | Actin α, cardiac-mouse (fragment) | gi\|627834 | 41757.8 | 5.23 | 8 |
| 48 | x | | | | |
| 49 | Striated muscle α tropomyosin (aa 81-284) (*Rattus nornegicus*) | gi\|57406 | 23532 | 4.61 | 2 |
| 50 | Myosin, light polypeptide 3; myosin light chain 3, alkali, cardiac ventricles | gi\|6981240 | 22142.1 | 5.03 | 4 |
| 51 | Myosin, light polypeptide 3; myosin light chain 3, alkali, cardiac ventricles | gi\|6981240 | 22142.1 | 5.03 | 7 |
| 52 | x | | | | |
| 53 | Myozenin 1; skeletal muscle-specific protein; calcineurin-2; calsarcain-2 | gi\|10946924 | 31437.6 | 8.57 | 4 |
| 54 | Troponin T, fast skeletal muscle splice form α-rabbit (fragment) | gi\|346621 | 27793 | 9.53 | 4 |
| 55 | α-Smooth muscle actin-rabbit (fragment) | gi\|2136927 | 26054 | 8.46 | 1 |

**Table 2.** Continued

| Spot No. | Protein name | Database accession No. | Theoretical $M_r$ | Theoreretical p*I* | # Peptides matched |
|---|---|---|---|---|---|
| 56 | α-Smooth muscle actin-rabbit (fragment) | gi\|2136927 | 26054 | 8.46 | 6 |
| 58 | Actin, skeletal muscle-rabbit | gi\|71611 | 41790 | 5.23 | 6 |
| 59 | x | | | | |
| 60 | Troponin T class IV/d β-4 (*Rattus norvegicus*) | gi\|207407 | 27980.8 | 9.53 | 5 |
| 61 | x | | | | |
| 62 | α-Smooth muscle actin-rabbit (fragment) | gi\|2136927 | 26054 | 8.46 | 1 |
| 63 | x | | | | |
| 64 | x | | | | |
| 65 | α-Smooth muscle actin-rabbit (fragment) | gi\|2136927 | 26054.3 | 8.46 | 7 |
| 66 | Actin, skeletal muscle-rabbit | gi\|71611 | 41790 | 5.23 | 2 |

x, insufficient signal for identification

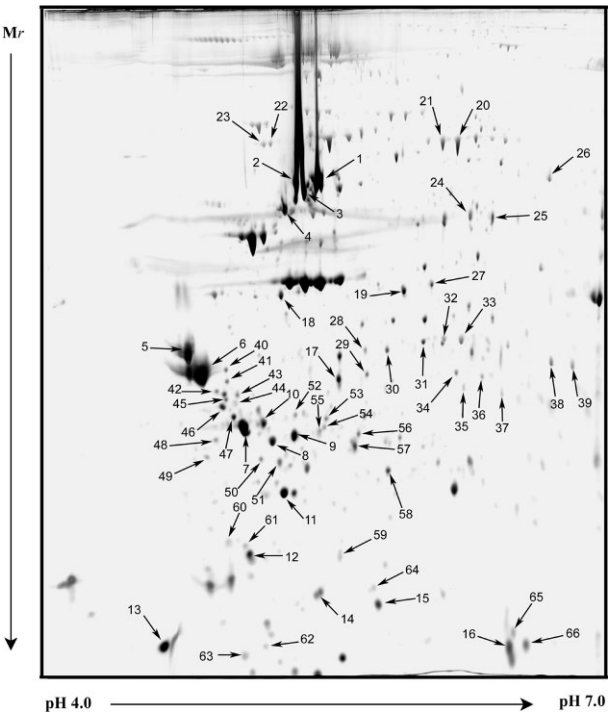


**Figure 6.** Representative 2-DE protein profile generated from 300 μg of the P2 fraction. The numbered arrows indicate spots that were either removed or reduced when compared to the P3 fraction gel image. These spots were excised from the gel, digested with trypsin, and analyzed by MS. Protein identification results accompanied by their MS data are listed in Table 2.

**Table 3.** Quantitation of protein recovery[a)]

| | Total protein recovered | | Recovery relative to S1 | |
|---|---|---|---|---|
| | Mg | SD | % | Standard |
| S1 | 9.5 | +/− 1.7 | − | − |
| S2 | 9.3 | +/− 1.0 | 98.5 | +/− 11.0 |
| P2 | 1.3 | +/− 0.1 | 13.8 | +/− 1.1 |
| P3 | 0.340 | +/− 0.052 | 3.6 | +/− 0.5 |

a) 100 mg of rat skeletal muscle was differentially extracted as described in Sections 2.1–2.3. The resulting fractions (S1–3 samples, S2–9 samples, P2–3 samples, and P3–3 samples) were solubilized in 2-DE rehydration buffer (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3–10, and 30 mM DTT) and quantitated using Genotech's Non-Interfering Protein assay according to supplied recommendations

ways). In muscle tissue this problem is compounded by the presence of many highly abundant contractile apparatus proteins including actin, myosin, tropomyosin, and troponin. The protocol established here exploits high-pH extraction to reduce or remove contractile apparatus proteins from muscle crude membrane preparations in a manner which is suitable for protein analysis through 2-DE. There are several key points to this procedure. First, once the majority of structural proteins are removed, we demonstrate that additional low abundant proteins can be detected and identified. We have identified 78 of the newly unmasked protein spots, enriched protein spots, or protein spots which were previously undetected. Second, it is now possible to load higher amounts of protein, which may allow for the detection of lower abundant, more biologically relevant proteins. Third, by removing abundant structural proteins that distort the gel image, we gain significant improvement in overall 2-DE gel quality, reducing run-to-run variability. This is important for studies in which multiple samples are analyzed (*e.g.*, time course studies). By simplifying the protein pattern, spot detection and pattern matching becomes more manageable and relia-

ble. Fourth, this procedure requires relatively small quantities of sample. In these experiments we started with 100 mg of powdered tissue, but much less could be used. Moreover, the procedure is practical, reproducible, and minimizes protein loss through the application of only three steps.

We, along with others, have observed that the majority of muscle contractile apparatus proteins, particularly actin, are insoluble in low-ionic-strength detergent free media and segregate into crude membrane preparations when sedimented [28]. These observations suggest that the majority of contractile proteins are involved in some type of protein-protein interaction (aggregates) or interaction with membranes and or membrane-associated proteins. When a cell is lysed in an aqueous, detergent-free environment, the cell membrane along with other membrane structures will fragment. However, under biological pH conditions, these fragmented membranes can rapidly reseal forming large membrane-bound compartments within the lysate [21, 22]. Highly abundant cytosolic proteins become trapped within these newly formed compartments and segregate to the membrane-containing fraction when the lysate is sedimented [29]. The results presented here suggest the high pH conditions could serve two functions; (i) disrupting resealed membrane compartments, thus releasing the trapped structural proteins, and (ii) acting as a stripping agent, perturbing any noncovalent protein-protein interactions [21, 29]. In either case, the now soluble contractile proteins can be easily removed through ultracentrifugation, resulting in a much improved 2-DE separation and detection of the remaining pelleted proteins.

While this differential extraction procedure has enabled the removal of structural proteins from a membrane-containing fraction, the soluble protein (S2) fraction generated still contains an appreciable amount of contractile proteins, although much reduced when compared to the whole-cell lysate (S1). This fraction, although not ideal, may be utilized for the analysis of soluble proteins without any further fractionation. In fact, we have conducted 2-DE analysis of this soluble fraction as it pertains to models of muscle atrophy (data not shown) with only minimal interference from the remaining structural proteins. In those experiments we were able to detect and analyze protein spots which were not resolved from whole-cell lysates because they were masked by highly abundant structural proteins. To truly optimize 2-DE analysis of this fraction, a specific and efficient method of abundant protein depletion would be required. For example, several groups have implemented antibody affinity columns to remove albumin and other abundant proteins from serum [16–18]. The utility of this approach to remove abundant proteins from muscle tissue would depend on the development of highly specific antibodies that would efficiently remove large quantities of contractile proteins along with their post-translationally modified forms and fragments.

Disease of muscle tissue can have profound physiological effects and much research is focused on understanding the underlying molecular causes of various muscular dystrophies, myopathies, metabolic disorders, and neural muscular diseases. However, the masking effect of a few highly abundant contractile proteins poses major obstacles for the 2-DE resolution and detection of lower abundant, more biologically relevant proteins. The practical differential extraction procedure we have developed, effectively removes these structural proteins from crude membrane preparations facilitating access to lower abundant proteins of interest. In addition, removal of these proteins results in significant improvement in gel quality and the ability to load higher amounts of total protein for the detection of even lower abundant protein classes.

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