Robert J. Isfort[1]
Richard T. Hinkle[1]
Melissa B. Jones[1]
Feng Wang[1]
Kenneth D. Greis[1]
Yiping Sun[2]
Thomas W. Keough[2]
N. Leigh Anderson[3]
Russell J. Sheldon[1]

[1]Research Division, Procter & Gamble Pharmaceuticals, Mason, OH, USA
[2]Corporate Research Division, Procter & Gamble, Ross, OH, USA
[3]Large Scale Biology Corporation, Rockville, MD, USA

Proteomic analysis of the atrophying rat soleus muscle following denervation

A proteomic analysis was performed comparing normal rat soleus muscle to denervated soleus muscle at 0.5, 1, 2, 4, 6, 8 and 10 days post denervation. Muscle mass measurements demonstrated that the times of major mass changes occurred between 2 and 4 days post denervation. Proteomic analysis of the denervated soleus muscle during the atrophy process demonstrated statistically significant (at the $p < 0.01$ level) changes in 73 soleus proteins, including coordinated changes in select groups of proteins. Sequence analysis of ten differentially regulated proteins identified metabolic proteins, chaperone and contractile apparatus proteins. Together these data indicate that coordinated temporally regulated changes in the proteome occur during denervation-induced soleus muslce atrophy, including changes in muscle metabolism and contractile apparatus proteins.

Keywords: Proteomic analysis / Rat soleus muscle / Atrophy EL 3915

1 Introduction

Skeletal muscle atrophy is a process by which skeletal muscle, in response to a variety of stimuli, selectively lose contractile proteins [1–5]. The process of skeletal muscle atrophy can be induced by diverse stimuli such as disuse, denervation, sepsis, and starvation [1–5]. While each mechanism has its own unique initiation signal, it is generally believed that a common mechanism for the reduction of contractile protein loss occurs, independent of the initiating stimuli. The selective loss of contractile proteins, relative to all cellular proteins, results in muscles with smaller myofibers but no loss of myofiber numbers [1–5]. It is generally believed that atrophy is a physiological mechanism that ensures the proper muscle size needed to meet the work demand placed on the muscle and to make available amino acids during times of physiological need.

Skeletal muscle atrophy is achieved by both a decrease in contractile protein synthesis and an increase in contractile protein degradation [6–9]. Severall lines of evidence support this view, including decreases in contractile protein transcription and translation during atrophy [7, 8] and increase in contractile protein degradation, a process which is believed to be mediated *via* increases in several proteolytic systems including the calpain, lysosomal and ubiquitin-mediated proteolytic systems [6, 9]. While contractile proteins have been demonstrated to be selectively lost during skeletal muscle atrophy, the available literature indicates that additional protein alterations occur in noncontractile proteins [1]. Thus the process of skeletal muscle atrophy is probably more complex than what has been previously recognized.

In order to gain a better understanding of the protein changes which occur during the atrophy process, a proteomic analysis was performed on soleus muscle undergoing denervation-induced atrophy. Denervation was chosen as the atrophy-inducing stimuli since the atrophy induced by denervation occurs relatively quickly and is quite profound compared to other atrophy-inducing stimuli [1]. The need for this analysis resulted from the lack of a published systematic proteomic analysis of denervation-induced skeletal muscle atrophy.

2 Materials and methods

2.1 Denervation of rat lower leg muscles

Animal studies were preapproved by the Procter & Gamble Institutional Animal Care and Use Committee and were conducted in compliance with the US Animal Welfare Act and rules and regualtions of the State of Ohio. Female Sprague Dawley rats (Charles River, Raleigh, NC) weighing 260–320 g were used in all studies. Rats were doubled-housed in standard shoebox cages, had free access to water and food, and were subject to standard conditions of humidity, temperature, and 12 h light cycle. For sciatic denervation, rats were anesthetized with a mixture of ketamine/acepromazine/xylazine, the right hindlimb was prepared for surgery, a 1 cm incision was

Correspondence: Dr. Robert J. Isfort, Research Division, Procter & Gamble Pharmaceuticals, Health Care Research Center, 8700 Mason-Montgomery Road, Mason,OH 45040-9317, USA
E-mail: isfortrj@pg.com
Fax: +513-622-1195

 0173-0835/00/1111-2228 $17.50+.50/0

made in the skin along the axis of the femur, and the sciatic nerve isolated. Once isolated, the sciatic nerve was lifted with a forcep and a 3–5 mm segment was resected, thereby denervating the musculature of the lower leg. The incision was closed with 2–3 stainless steel wound clips and the rats were allowed to recover. At a predetermined time after denervation, rats were euthanized by CO_2 asphyxiation followed by cervical dislocation, the soleus muscle was dissected from the hindlimb, cleaned of tendons and connective tissue, weighed and snap-frozen in cyogenic vials by immersion in liquid nitrogen. Muscle were stored at –80°C unti processed.

2.2 Sample preparation

The soleus muscle was prepared for two-dimensional gel electrophoresis (2-DE) as follows. Frozen soleus muscles were crushed to a fine powder in liquid nitrogen using a mortar and pestle; 0.1 g of tissue was solubilized in 0.4 mL of solubilization buffer (9 M urea, 2% CHAPS,Atrophy0.5% dithiothreitol, 2% Pharmalytes; pH 8.0–10.5), (Amersham Pharmacia Biotech, Uppsala, Sweden) homogenized, then shaken for approximately 30 min, centrifuged for 30 min, and the supernatant was removed and aliquoted for analysis.

2.3 2-DE

Sample proteins were resolved by 2-DE using the 20 × 25 cm ISO-DALT 2-D gel system [10–12]. All first-dimensional isoelectric focusing (IEF) gels were prepared using the same single standardized batch of carrier ampholytes (BDH 4–8A). Eight μL of solubilized muscle protein were applied to each gel and the gels were run for 25050 Vh using a progessively increasing voltage protocol implemented by a programmable high voltage power supply. An Angelique computer-controlled gradient casting system (Large Scale Biology, Rockville, MD, USA) was used to prepare second-dimensional SDS gradient slab gels in which the top 5% of the gel was 11% T acrylamide and the lower 95% of the gel varies linearly from 11 to 18%. The first-dimensional IEF tube gels were loaded directly onto the slab gels without equilibration and held a place by agarose. Second-dimensional slab gels were run in groups of 20 in thermal-regulated DALT tanks with buffer circulation. Following SDS electrophoresis, slab gels were stained for protein using a colloidal Coomassie Blue G-250 procedure involving fixation in 50% ethanol/2% phoshoric acid overnight, three 30 min washes in cold deionized water, transfer to 34% methanol/17% ammonium sulfate/2% phosphoric acid for 1 h followed by addition of 1 g/1.5 L of powdered Coomassie Blue G-250 stain and staining for 4 days.

2.4 Quantitative computer analysis

Each stained slab gel was digitized in red light at 133 μm resolution using an Eikonix 1412 scanner (Ektron Applied Imaging, Bedford, MA, USA). Each 2-D gel was processed using the Kepler software system to yield a spotlist giving position, shape and density information for each detected spot. This procedure makes use of digital filtering, mathematical morphology techniques and digital masking to remove background and uses full 2-D least-squares optimization to refine the parameters of a 2-D Gaussian shape for each spot. Each 2-D pattern was matched to an appropriate soleus "master" 2-D pattern. In the matching, a series of about 50 proteins was matched by an experienced operator working with a montage of all the 2-D patterns in the experiment. Subsequently, an automatic program matched additional spots to the master pattern using as a basis the manual landmark data entered by the operator. After the automatic matching, the operator inspected matching for errors. The groups of gels making up an experiment were scaled together by a linear procedure based on a selected set of spots. These spots were selected by a procedure which chooses spots that have been matched in at least 80% of the gels. All gels in the experiment were then scaled together by setting the summed abundance of the selected spots equal to a constant. A Student's *t*-test was used to detect quantitative protein changes between control and treated groups with cutoffs for significance made at the $p < 0.01$ level.

2.5 Protein identification

2.5.1 In-gel tryptic digestion

Protein spots of interest were excised from the Coomassie Blue G-250 stained gels and in-gel tryptic digestion was performed [13, 14]. Briefly, the spots were washed with 100 mM NH_4HCO_3, 50% CH_3CN several times followed by reduction and alkylation with DTT and iodoacetamide, respectively. After washing with 100 mM NH_4HCO_3, 50% CH_3CN and gel dehydration, 0.15–0.2 μg of porcine trypsin in 100 mM NH_4HCO_3 was added to the gel pieces followed by an overnight incubation at 30°C. Protein identification was performed using MALDI-MS as follows. MS of the tryptic fragments was performed by mixing the peptides from in-gel trypsin digestion with an equal volume of saturated α-cyano-4-hydroxycinnamic acid matrix dissolved in 50% CH_3CN/0.3%TFA, then spotted onto a MALDI-TOF target plate. Peptide spectra were collected on a Voyager DE-STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) in the positive-ion reflector mode with delayed ion extraction using the following conditions: nitrogen laser, 337 nm; accelerating voltage, 20 kV; grid voltage, 73%;

ion delay, 100 ns, and mass range, 800–3800 Da. The mono-isotopic masses were calibrated to internal, autodigestion peptides from porcine trypsin at 842.5100 and 2211.1046 such that the unknown peptide masses were accurate to within 25 ppm. Protein identification was facilitated using the MS-FIT module of the ProteinProspector (Version 3.2.1) program, supplied by PerSeptive Biosystems. The accurate tryptic, monoisotopic peptide masses were searched against the entire NCBInr51999 database of 359 734 entries to match with tryptic peptide masses from known proteins and/or predicted protein from DNA sequences. All protein given positive identification matched a minimum of five peptides within 25 ppm.

2.5.2 *N*-terminal Edman sequencing

The sequence of several of the proteins was confirmed by *N*-terminal Edman sequencing as follows. Tryptic digested peptides were extracted with 0.1% TFA in 60% CH_3CN and microblotting was performed. For microblotting, a 173A Microblotter capillary HPLC system (Perkin-Elmer Applied Biosystems, Foster City, CA) with a 0.5 × 150 mm C18 reverse-phase column was used for tryptic peptide separation and collection [15]. The mobile phases used were water and acetontrile, each containing 0.05% TFA. The gradient used was 5% acetonitrile for 20 min, then 5–65% acetonitrile over 120 min with a 5 μL/min flow rate. The microblotter itself is a stage holding a strip of wetted PVDF membrance. The column eluting capillary is held by a solenoid valve above the membrane such that as the solenoid actuates, the capillary is tapping on the surface of the membrane, blotting the column eluate. The solenoid is moved across the membrane at a rate of 1 mm/min. By keeping the UV recorder rate the same as the microblotter rate, alignment of the peaks can be made with the PVDF membrane strip. Edman sequencing of the peptide fractions collected by the microblotter was performed using a 494 HT Micro Protein Sequencer (Perkin-Elmer Applied Biosystems). The PVDF membrane was aligned with the HPLC-UV chromatogram and the spot corresponding to the peak of interest was excised. A small amount of Biobrene (50 mg/mL in 50% methanol) was added to the PVDF, after which the PVDF membrane was air-dried before loading on the sequencer cartridge. A modified sequencing program based on the original NORMAL-BLOT was used.

3 Results

3.1 Soleus muscle mass changes following denervation

Atrophy of the soleus muscle was induced by removal of a section of the right sciatic nerve in 24 rats. The soleus muscle was removed from the right leg of three control rats as well as the right legs of the denervated rats (3 per time point) at 0.5 day, 1 day, 2, 4, 6, 8 and 10 days post denervation, and weighed. The change in soleus muscle mass following denervation are presented in Fig. 1. As can be seen, the soleus muscle begins losing mass between 2 and 4 days post denervation with the loss in mass decreasing at between 8 and 10 days post denervation. By the end of the sampling period, the soleus muscle had lost approximately 40% of its mass.

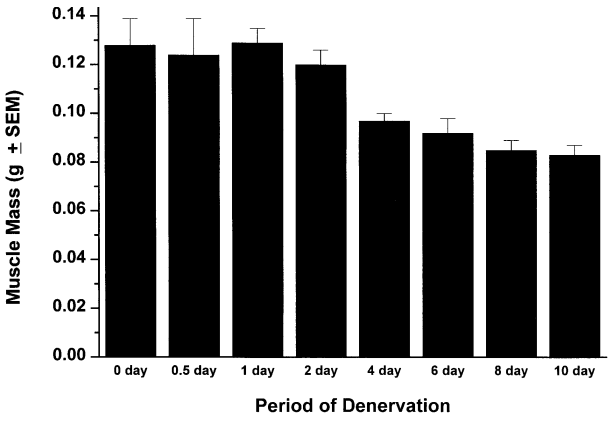

Figure 1. Analysis of rat soleus muscle mass changes following sciatic denervation. The right sciatic nerve was severed and the soleus muscle was removed at either 0, 0.5, 1, 2, 4, 6, 8, or 10 days post denervation and weighed ($n = 3$ for each time point).

3.2 Changes in soleus muscle protein levels following denervation

2-DE of normal and denervated soleus muscle proteins was performed using samples isolated at 0, 0.5, 1, 2, 4, 6, 8, and 10 days post denervation. The normal soleus protein pattern was used to create a soleus muscle proteome master map which was subsequently used for landmarking and identifying proteins whose levels change during denervation-induced atrophy. As can be seen in Fig. 2A, 73 proteins were identified whose levels changed at the $p < 0.01$ level of significance in any group (these proteins are denoted by their coordinate numbers and dark shading relative to all proteins resolved – represented by light shading). As can be seen, these proteins cover a broad range of molecular weights, isoelectric points, and abundance levels (the size of the spot indicates relative abundance). Figure 2B demonstrates the relative level of change of 73 proteins at varying times following denervation. Each tic separates a group of three protein spots (the same protein), each from a different soleus muscle from three different animals. As is visible, a multitude of different patterns of change are observed, including a

striking increase in relative abundance between day 4 and 6 post denervation (a total of 25 spots: 157, 169, 176, 177, 178, 182, 215, 239, 253, 283, 357, 388, 393, 395, 515, 531, 540, 621, 661, 693, 737, 821, 1021, 1219, 1997); a decrease in expression between days 4 and 6 post denervation (a total of 16 spots: 38, 49, 75, 137, 172, 211, 244, 254, 259, 366, 373, 435, 551, 1987, 1989, 1991); a transient increase followed by a decrease (a total of 11 spots: 38, 66, 255, 261, 263, 280, 289, 303, 333, 373, 417); a gradual increase following denervation (a total of 6 spots: 201, 229, 455, 459, 541, 598); a gradual decrease following denervation (a total of 1 spot: 66); a sharp decline or increase in protein between and 0 and 0.5 days post denervation (a total of 9 spots: 111, 114, 159, 222, 261, 364, 433, 827, 1990); and others (a total of 5 spots). The decreases or increases observed are compared as the average change observed, and are typically observed in two or more samples/group.

3.3 Protein identification

Several proteins whose levels changed during denervation atrophy were identified either by Edman sequencing or MALDI-TOF. Table 1 provides the protein identification for ten soleus proteins altered during denervation atrophy. As can be seen, four of the proteins identified were metabolic enzymes (β-enolase, S263, ATP synthase β, S280, carbonic anhydrase III, S515; fatty acid binding protein, S293), one was a charperone protein (p20, S333) and five were contractile apparatus proteins (skeletal muscle myosin light chain, S229, troponin T, S114, myosin alkali light chain 3, S1987, cardiac myosin light chain 2, S222; actin, S1991).

4 Discussion

This paper describes a proteomic analysis of the rat soleus muscle during the denervation-induced atrophy process. In this analysis we observed quantitative differences in the level of 73 proteins, at the $p < 0.01$ level of significance, during atrophy. Importantly, classes of protein changes were observed indicating coordinated regulation of protein levels. Also of significance was the observation that proteins with similar functionality were altered, indicating a coordinated regulation.

4.1 Metabolic enzymes

Previously, analysis of atrophying muscle by 2-DE has been performed including an analysis of denervated chicken skeletal muscle which focused on C-protein [16], an analysis of sepsis-induced atrophy in rat gastrocnemius muscle [17], and an analysis of rat hindlimb muscles (including the soleus) following spinal cord injury [18]. Unfortunately, none of these studies were performed under conditions which allowed for either a rigorous quantitative analysis or a systemic temporal analysis of the atrophying muscles, thereby limiting the amount of data which could be utilized for a cross study comparison. In the study reported in this publication, we have performed a rigorous quantitative and temporally comprehensive analysis of soleus muscle undergoing denervation-induced atrophy. In this study, we have observed changes in the levels of over 70 proteins. Interestingly, of the ten identified proteins, several have been previously reported to be altered during atrophy when investigated by a variety of means. These proteins fall into several classes including metabolic enzymes, chaperones and contractile apparatus proteins. Four proteins identified as metabolic enzyme were altered in the soleus muscle following denervation including:

(i) β-Enolase: In the current study, the level of this protein was increased following denervation. In previous reports β-enolase has been observed to decrease following denervation, with two weeks post denervation being the earliest time point investigated [19, 20]. This result differs from the changes in β-enolase protein levels observed in this report, although it should be noted that the differences observed may be due to the difference in sampling time point utilized.
(ii) ATP synthase β: This protein was observed to increase transiently at 2 and 4 days post denervation. A previous publication reported that ATP synthase β mRNA levels increase approximately twofold following electrical stimulation-induced altrophy in rabbit tibialis anterior muscle [21].
(iii) Carbonic anhydrase III: This protein decreased as early as 12 h post denervation only to increase above nondenervated levels at 6 days post denervation. One previous publication reported a decrease in carbonic anhydrase III protein and mRNA levels in the soleus muscle by two days post denervation with the levels returning to baseline levels later in the denervation time course. In contrast, the base levels of carbonic anhydrase III were lower in the fast twitch extensor digitorum longus and anterior tibialis muscle and increased dramatically by 8 days post denervation [22]. A second publication reported a decrease in soleus carbonic anhydrase III mRNA levels at 2 and 5 days post immobilization [23] while a third publication demonstrated a large increase in medial gastocnemius skeletal muscle carbonic anhydrase III protein levels in sepsis-induced atrophy five days following the induction of atrophy [17].
(iv) Fatty acid binding protein: The level of this protein decreased between 4 and 6 days post denervation. One previous publication reported that fatty acid binding protein is reduced in atrophied rat adductor longus muscle following 14 days of space flight [24].

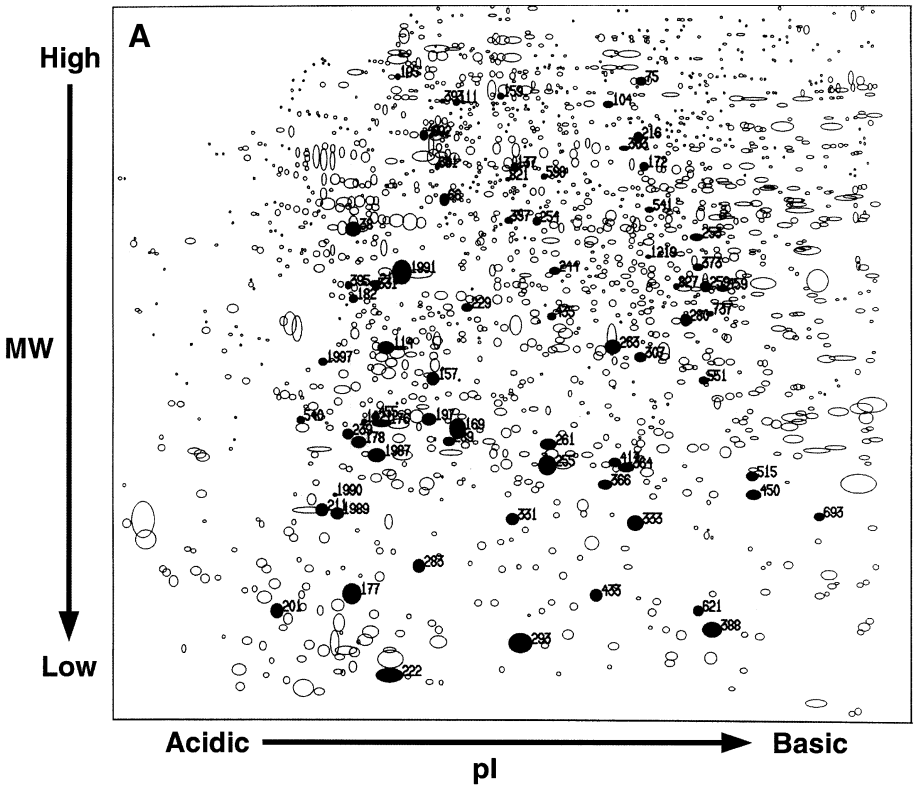

Table 1. Protein identification of ten differentially regulated soleus proteins

Spot No.	Protein identification
S253	β-Enolase
S280	ATP synthase β
S515	Carbonic anhydrase III
S293	Fatty acid binding protein
S333	p20
S229	Skeletal muscle myosin light chain
S114	Troponin T
S1987	Myosin alkali light chain
S222	Cardiac myosin light chain
S1991	Skeletal actin

4.2 Chaperone protein

One protein identified as the P20 chaperone protein was changed during atrophy. P20 is a member of the small heat shock protein family with sequence similarity to two other members of the small heat shock protein family, HSP27 and alpha B-crystallin. P20 has been reported to be expressed in soleus muscle [25]. In the analysis published in this paper, P20 levels increased as early as 12 h post denervation, returning to predenervatioin levels at 4 days post denervation, followed by a decrease in expression beginning at 6 days post denervation. Previous publications have demonstrated that P20 mRNA levels decreased 3 days post denervation in the soleus [25]. In addition, it has been reported that HSP27 protein levels increase at 3 days post denervation followed by a decrease at 6 days post denervation in the rat soleus muscle [26].

4.3 Contactile apparatus proteins

Several proteins identified as contactile apparatus proteins were altered in the soleus muscle following denervation including:

(i) Skeletal muscle myosin light chain: This protein increased following denervation. A previously published report demonstrated that skeletal muscle myosin fast and slow light chain protein levels either increase or decrease, depending on the specific isoform, after denervation-induced atrophy of the chicken pectoralis major muscle [27].
(ii) Troponin T: Levels of troponin T protein decreased by 12 h post denervation and remained lower throughout the period of denervation. A previous publication reported that troponin T protein levels decrease in vitamin E deficiency-induced atrophying muscle [28].
(iii) Myosin alkali light chain: The levels of myosin alkali light chain decreased at 6 days following denervation.

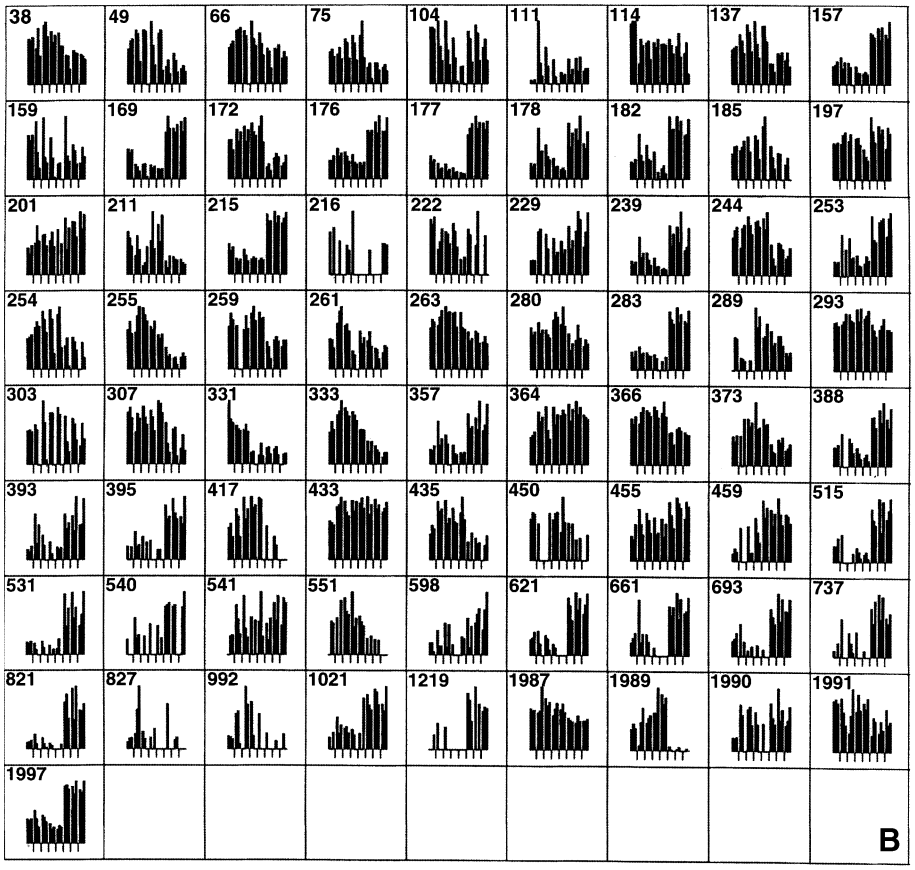

Figure 2. Alterations in the levels of soleus muscle proteins following denervation. Soleus muscle proteins were prepared and separated by 2-DE as described in Sections 2.1–2.3. (A) Master map of soleus muscle proteins (clear spots) showing proteins whose levels changed following denervation (black spots). (B) Graphical representation of the 73 soleus proteins whose levels changed following denervation. Each tic separates a group of three protein spots (the same protein), each from a different soleus muscle from three different animals. Time points following denervation are identified by each tic mark (from left to right: 0, 0.5, 1, 2, 4, 6, 8, 10 days post denervation). Each panel, which represents the integrated Coomassie Blue G-250 absorbance measurement for one protein, is the relative measure of the normalized abundance of that protein from the different experiment groups (*y*-axis) and experimental group (*x*-axis).

(iv) Cardiac myosin light chain: Levels of cardiac myosin light chain decreased as early as 12 h post denervation and remained lower throughout the period of denervation. A previous publication demonstrated that cardiac myosin light chain is a relatively abundant light chain in soleus and cardiac muscle whose levels increase during cardiac hypertrophy [29].

(v) Skeletal muscle actin: The level of skeletal muscle actin decreased between 4 and 6 days post denervation. Previous reports have demonstrated decreases in skeletal muscle actin protein and mRNA levels following denervation [30, 31].

4.4 Denervation-induced atrophy

What do these changes in the level of metabolic, chaperone and contractile apparatus proteins tell us about the process of denervation-induced atrophy? It is well known that a slow twitch muscle like the soleus muscle, upon denervation, transitions to a fast twitch muscle phenotype with a coordinated change in expression of metalbolic, chaperone and contractile apparatus proteins [32, 33]. The change in metabolic, chaperone, and contractile apparatus proteins from slow twitch isozymes to fast twitch isozymes occurs *via* changes in transcription in a coordinate manner [32, 33]. Thus the observed changes in these three classes of proteins is consistent with fiber type switching resulting from a decrease in tonic activity of the soleus muscle. This study demonstrates the utility of proteomics to delineate mechanistic pathway information about the system under study *via* changes in proteome. Future protein identifications should provide us with increased understanding of additional processes which are modulated during skeletal muscle atrophy.

Received October 12, 1999

5 References

[1] Herbison, G. J., Jaweed, M. M., Ditunno, J. F., *Arch. Phys. Med. Rehabil.* 1979, *60*, 401–404.

[2] Goldspink, D.F., *Biochem. J.* 1976, *156*, 71–80.

[3] Goldberg, A. L., *Biol. Chem.* 1969, 244, 3223–3229.

[4] Zeman, R. J., Lundemann, R., Etlinger, J. D., *Am. J. Physiol.* 1987, *254*, E152–E155.

[5] Booth, F. W., Gollnick, P. D., *Med. Sci. Sports Exercise* 1983, *15*, 415–420.

[6] Furuno, K., Goodman, M. N., Goldberg, A. L., *J. Biol. Chem.* 1990, *265*, 8550–8557.

[7] Boissonneault, G., Tremblay, R. R., *FEBS Lett.* 1989, *257*, 329–332.

[8] Jakubiec-Puka, A., Kordowska, J., Catani, C., Carraro, U., *Eur. J. Biochem.* 1990, *193*, 623–628.

[9] Taillander, D., Aurousseau, E., Meynial-Denis, D., Bechet, D., Ferrara, M., Cottin, P., Ducastaing, A., Bigard, X, Guezennec, C.-Y., Schmid, H.-P., Attaix, D., *Biochem. J.* 1996, *316*, 65–72.

[10] Giometti, C. S., Anderson, N. L., *Clin. Chem.* 1979, *25*, 1877–1884.

[11] Giometti, C. S., Barnay, M., Danon, M. J., Anderson, N. G., *Clin. Chem.* 1980, *26*, 1152–1155.

[12] Giometti, C. S., Anderson, N. G., *Clin. Chem.* 1981, *27*, 1918–1921.

[13] Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., Mann, M., *Nature* 1996, *379*, 466–469.

[14] Hellmann, U., Wernstedt, C., Gonez, J., Heldin, C.-H., *Anal. Biochem.* 1995, *224*, 451–455.

[15] Bauer, M., Sun, Y., Wang, F., *J. Prot. Chem.* 1999, *18*, 337–347.

[16] Kojima, T., Sano, K., Hirabayashi, T., Obinata, T., *J. Biochem.* 1990, *107*, 470–475.

[17] Owens, E. L., Lynch, C. J., McCall, K. M., Carter, N. D., Vary, T. C., *Shock* 1994, *2*, 171–178.

[18] Lee, J. C., Braun, A. M., *J. Neurochem.* 1990, *54*, 96–101.

[19] Matsushita, H., Yamada, S., Satoh, T., Kato, K., Adachi, M., *Exp. Neurol.* 1986, *93*, 84–91.

[20] Kato, K., Shimizu, A., Semba, R., Satoh, T., *Biochim. Biophys. Acta* 1985, *841*, 50–58.

[21] Williams, R. S., Garci-Moll, M., Mellor, J. Salmons, S., Harlan, W., *J. Biol. Chem.* 1987, *262*, 2764–2767.

[22] Carter, N. D., Wistrand, P. J., Isenberg, H., Askmark, H., Jeffery, S., Hopkinson, D., Edwards, Y., *Biochem. J.* 1988, *256*, 147–152.

[23] Brownson, C., Loughna, P. T., *Pfugers Arch.-Eur. J. Physiol.* 1996, *431*, 990–992.

[24] Riley, D. A., Ellis, S., Giometti, C. S., Hoh, J. F. Y. Ilyina-Kakueva, E. I., Oganov, V. S., Slocum, G. R., Bain, J. L. W., Sedlak, F. R., *J. Appl. Physiol.* 1992, *73*, 33S–43S.

[25] Inaguma, Y., Hasegawa, K., Kato, K., Nishida, Y., *Gene* 1996, *178*, 145–150.

[26] Inaguma, Y., Goto, S., Shinohara, H., Hasegawa, K., Ohshima, K., Kato, K., *J. Biochem.* 1993, *114*, 378–384.

[27] Matsuda, R., Spector, D., Strohman, R. C., *Proc. Natl. Acad. Sci. USA* 1984, *81*, 1122–1125.

[28] Dayton, W. R., Schollmeyer, J. V., Chan, A. C., Allen, C. E., *Biochim., Biophys. Acta* 1979, *584*, 216–230.

[29] Zhu, H., Garcia, A. V., Ross, R. S., Evans, S. M., Chien, K. R., *Mol. Cell. Biol.* 1991, *11*, 2273–2281.

[30] Jakubiec, P. A., *Comp. Biochem. Physiol. Comp. Physiol.* 1992, *102*, 93–98.

[31] Shimizu, N., Kamel-Reid, S., Zak, R., *Develop. Biol.* 1988, *128*, 435–440.

[32] Pette, D., Vrbova, G., *Muscle & Nerve* 1999, *22*, 666–677.

[33] Brown, J. M. C., Henriksson, J., Salmons, S., *Proc. R. Soc. London* 1989, *235*, 321–346.