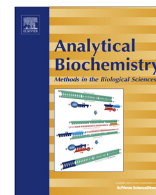
Analytical Biochemistry 446 (2014) 108–115

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio



Comparative proteomic analysis of the contractile-protein-depleted fraction from normal versus dystrophic skeletal muscle

Steven Carberry [a], Margit Zweyer [b], Dieter Swandulla [b], Kay Ohlendieck [a,*]

[a] Department of Biology, National University of Ireland, Maynooth, Kildare, Ireland
[b] Department of Physiology II, University of Bonn, D-53115 Bonn, Germany

ARTICLE INFO

Article history:
Received 7 June 2013
Received in revised form 3 August 2013
Accepted 6 August 2013
Available online 14 August 2013

Keywords:
Dystrophinopathy
Ferritin
Hsp70
Muscle proteomics
Transferrin

ABSTRACT

In basic and applied myology, gel-based proteomics is routinely used for studying global changes in the protein constellation of contractile fibers during myogenesis, physiological adaptations, neuromuscular degeneration, and the natural aging process. Since the main proteins of the actomyosin apparatus and its auxiliary sarcomeric components often negate weak signals from minor muscle proteins during proteomic investigations, we have here evaluated whether a simple prefractionation step can be employed to eliminate certain aspects of this analytical obstacle. To remove a large portion of highly abundant contractile proteins from skeletal muscle homogenates without the usage of major manipulative steps, differential centrifugation was used to decisively reduce the sample complexity of crude muscle tissue extracts. The resulting protein fraction was separated by two-dimensional gel electrophoresis, and 2D-landmark proteins were identified by mass spectrometry. To evaluate the suitability of the contractile-protein-depleted fraction for comparative proteomics, normal versus dystrophic muscle preparations were examined. The mass spectrometric analysis of differentially expressed proteins, as determined by fluorescence difference in-gel electrophoresis, identified 10 protein species in dystrophic *mdx* hindlimb muscles. Interesting new biomarker candidates included Hsp70, transferrin, and ferritin, whereby their altered concentration levels in dystrophin-deficient muscle were confirmed by immunoblotting.

© 2013 Elsevier Inc. All rights reserved.

In high-throughput biochemistry, mass spectrometry is the method of choice for the fast and reliable identification of proteins in large-scale surveys of physiological or pathological processes [1–3]. This makes protein mass spectrometry an integral part of biological network analysis [4] and the discovery of novel disease biomarker signatures [5]. Disorder-specific protein markers play a central diagnostic, prognostic, and therapeutic role in skeletal muscle pathology and the systematic application of proteomics has greatly expanded the range of biomarkers for neuromuscular disorders [6]. Proteome-wide studies combine protein separation methods, such as high-resolution two-dimensional gel electrophoresis [7–9] and liquid chromatography [10], with sophisticated mass spectrometric techniques to determine potential changes in protein concentration, isoform expression patterns, protein–protein interactions and posttranslational modifications [11–13].

However, proteomic findings from comparative studies focusing on total protein extracts from highly complex and dynamic types of tissues, such as skeletal muscle fibers, are often limited to mostly soluble and relatively abundant proteins [14–16], missing especially the classes of very low abundance proteins and hydrophobic proteins. Thus, to cover all of the assessable constituents in a heterogeneous assembly of proteins with a greatly differing abundance and physicochemical properties, as are found in contractile tissues, organelle proteomics should be used to supplement the findings from whole tissue proteomics. The application of prefractionation procedures substantially reduces sample complexity and thus allows more comprehensive cataloging of complex muscle protein mixtures [17–20]. Muscle organelle proteomics has been successfully applied for studying fractions enriched in nuclei, mitochondria, surface membranes, the sarcoplasmic reticulum, the cytosol, and the contractile apparatus in normal, transforming, and pathological muscle [21].

Although the comprehensive analysis of low-copy number proteins in pathological samples is challenging, the proteomic identification of new protein disease markers promises a deeper understanding of pathophysiological mechanisms. To better comprehend the complex changes that occur during X-linked muscular dystrophy [22], we have analyzed here a contractile-protein-depleted fraction from normal versus dystrophic muscle preparations. Since extensive subcellular fractionation protocols may introduce artifacts, we have kept centrifugation steps to a minimum. Hence, the analytical strategy employed in this study is a compromise between using total extracts, which may result in

* Corresponding author. Fax: +353 1 708 3845.
E-mail address: kay.ohlendieck@nuim.ie (K. Ohlendieck).

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.08.004

the underrepresentation of minor muscle proteins, and extensive prefractionation protocols, which often complicate comparative studies by unintended entrapment, desorption, or clustering of certain proteins. Our study complements previous proteomic investigations into global changes in genetic animal models of Duchenne muscular dystrophy [23–27]. The proteomic analysis of the enriched protein fraction from normal versus dystrophic *mdx* muscle has resulted in the identification of 10 novel protein species, including ferritin, transferrin, and various isoforms of the molecular chaperone Hsp70.

Materials and methods

Materials

For the gel electrophoretic separation of muscle proteins, materials and analytical grade chemicals, including CyDye DIGE fluor minimal dyes Cy3 and Cy5, were obtained from Amersham Biosciences/GE Healthcare (Little Chalfont, Buckinghamshire, UK). Digestion was performed with sequencing-grade-modified trypsin from Promega (Madison, WI, USA). Protease inhibitors and chemiluminescence substrate were purchased from Roche Diagnostics (Mannheim, Germany). Primary antibodies were from Abcam (Cambridge, UK; ab92721 to myosin light chain MLC2, ab6588 to collagen, ab11427 to parvalbumin, ab9033-1 to transferrin, and ab69090 to ferritin light chain), Sigma Chemical Co. (Dorset, UK; L-9393 to laminin), Enzo Stressgen (Victoria, BC, Canada; ADI-SPA-811 to heat shock protein Hsp70/72 rabbit), and Santa Cruz Biotechnology (Santa Cruz, CA, USA; sc-3370 to β-dystroglycan). Secondary antibodies were from Chemicon International (Temecula, CA, USA). All other chemicals used were of analytical grade and purchased from Sigma.

Animal model of muscular dystrophy

Dystrophin-deficient skeletal muscles from the naturally occurring mutant *mdx* mouse model of Duchenne muscular dystrophy are widely used in proteomic screening studies [23]. Since this study employed a subcellular fractionation protocol prior to gel electrophoretic separation of the contractile-protein-depleted fraction and therefore required substantial amounts of starting material, the proteomic analysis was not carried out with a specific skeletal muscle, but combined muscles from the entire hindlimb. Dystrophic muscle from 8-week-old *mdx* mice and normal control muscle from age-matched C57 mice were obtained from the Bioresource Unit of the University of Bonn [26]. Mice were kept under standard conditions and all procedures were performed in accordance with German guidelines on the use of animals for scientific experiments. Animals were sacrificed by cervical dislocation and muscle tissues quickly removed and quick-frozen in liquid nitrogen.

Subcellular fractionation of skeletal muscle homogenates

The subcellular fractionation of skeletal muscle tissue was based on previously optimized protocols for the separation of distinct functional elements from muscle homogenates [28,29]. Each pair of hindlimb muscles of approximately 0.65 g wet wt from *mdx* and control mice was washed in ice-cold phosphate-buffered saline and cut into small pieces with a razor blade. Following suspension in 10 vol of 20 mM sodium pyrophosphate, 20 mM sodium phosphate, 1 mM $MgCl_2$, 0.303 M sucrose, 0.5 mM EDTA, pH 7.0 [28], supplemented with a protease inhibitor cocktail [29], samples were treated with a hand-held homogenizer for 30 s every 10 min for 1 h at 4 °C. Muscle homogenates were centrifuged at 14,000*g* for 15 min and then the resulting supernatant was pelleted at 100,000*g* for 1 h at 4 °C. Microsomal pellets were resuspended in electrophoresis buffer and analyzed by gel-based proteomics. One preparation yielded approximately 0.8 mg of microsomal protein.

Gel electrophoretic analysis of muscle proteins

Fluorescence two-dimensional (2D) gel electrophoretic analysis was carried out in the case of total tissue extracts by postelectrophoretic staining with ruthenium II tris bathophenanthroline disulfonate (RuBPs) [30] and for the comparative analysis of the contractile-protein-depleted fraction from normal versus dystrophic muscle by preelectrophoretic labeling using the difference in-gel electrophoresis (DIGE) method with CyDyes Cy3 and Cy5 [31]. Fluorescent RuBPs dye was prepared by the method of Aude-Garcia et al. [32]. First-dimension isoelectric focusing was performed with nonlinear pH 3–11 strips and second-dimension slab-gel electrophoresis with 12.5% separating gels (gel size 24×16 cm). Our laboratory has optimized fluorescence labeling techniques for the analysis of skeletal muscle proteins. Detailed descriptions of the RuBPs-based analysis and the DIGE method as applied to muscle tissues have recently been published [33,34]. Total protein loading of RuBPs gels and DIGE gels was 600 and 100 μg protein, respectively. Fluorescently labeled proteins were visualized with the help of a Typhoon Trio variable mode imager (Amersham Biosciences/GE Healthcare). Gel image analysis was carried out with Progenesis SameSpots software (Nonlinear Dynamics, Newcastle upon Tyne, UK). The following parameters were employed for the identification of significant differences in the concentration of proteins in the contractile-protein-depleted fraction from normal versus dystrophic muscle: ANOVA $p < 0.05$, $n = 4$, and a power value of >0.8. Proteins in 2D spots with a significant increase or decrease in abundance (differing between the various groups with >1.4-fold change) were subsequently identified by mass spectrometry.

Mass spectrometric identification of muscle proteins

Coomassie brilliant blue (CBB)-stained pick gels were used for the mass spectrometric identification of proteins of interest, as previously described in detail [35]. Total protein loading of CBB pick gels was 600 μg protein. Following the excision, washing, and destaining of 2D spots, characteristic peptide populations were produced by treatment with sequencing-grade trypsin [31]. Peptide samples were dried through vacuum centrifugation and suspended in MS-grade distilled water and 0.1% formic acid, spun down through spin filters, and added to LC–MS vials for identification using a Model 6340 ion trap LC–MS apparatus from Agilent Technologies (Santa Clara, CA, USA). Conditions for the separation of peptide populations generated from individual muscle proteins and elution profiles were previously described in detail [30,31,33–35]. Database searches were carried out with Mascot MS/MS ion search (Matrix Science, London, UK; NCBI database, release 20100212). All searches employed "*Mus musculus*" as taxonomic category and the following parameters: (i) two missed cleavages by trypsin, (ii) mass tolerance of precursor ions ±2 Da and product ions ±1 Da, (iii) carboxymethylated cysteines fixed modification, (iv) oxidation of methionine as variable modification, and (v) at least two matched distinct peptides. MS/MS scores over 40 are listed in Tables 1–3.

Immunoblot analysis of muscle proteins

One-dimensional gel electrophoretic separation and immunoblotting were carried out by standard procedures [36] and used

Download English Version:

https://daneshyari.com/en/article/1176199

Download Persian Version:

https://daneshyari.com/article/1176199

Daneshyari.com