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Proteomic profiling of large myofibrillar proteins from dried and long-term stored polyacrylamide gels

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ABSTRACT

A method for the utilization of dried polyacrylamide gels from the pre-proteomic era is described in order to enable the mass spectrometric analysis of long-term stored protein preparations. The in-gel digestion of high-molecular-mass proteins embedded in a 20-year old gel was carried out following gel re-swelling and resulted in the proteomic identification of a large number of proteins, including 3400 kDa titin, 800 kDa nebulin and myosin heavy chains of 220 kDa from rabbit skeletal muscle. These findings demonstrate that dried protein gels from past biochemical analyses can be successfully reused and analyzed by modern and refined mass spectrometric techniques.

One of the most frequently used biochemical methods for the efficient and rapid separation of complex protein samples is one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis, usually referred to as SDS-PAGE [1]. For the long-term preservation of polyacrylamide slab gels or for the usage of gels in autoradiography, gels are routinely dried and can then be conveniently stored at room temperature [2–4]. Prior to the development of large-scale and mass spectrometry-based proteomics [5], separated proteins were usually identified and characterized individually by peptide sequencing or labor-intensive immunochemical methods [6]. Since many bioanalytical research laboratories store large numbers of dried gels from the pre-proteomic era that have not undergone a more refined mass spectrometric analysis, it was of interest to investigate whether re-swelling of old gels followed by standardized in-gel digestion and routine mass spectrometric analysis would result in the identification of sensitive protein species, including very high-molecular-mass proteins.

Building on the findings from a select number of similar experiments that have previously been described for the proteomic analysis of medium size proteins from dried gels [7–11], this study has focused on the retrospective analysis of gel-embedded protein preparations by analyzing a subcellular fraction from a highly complex tissue. The bioanalytical approach was extended to extremely large protein species that are typically found in the contractile apparatus and its associated cytoskeletal network of skeletal muscle fibers. The main emphasis of this report was on sarcomeric proteins including titin, nebulin and myosin heavy chains, which exhibit molecular masses of approximately 3400 kDa, 800 kDa and 220 kDa, respectively [12,13]. In this brief note, we summarize the experimental workflow for the mass spectrometric identification of proteins recovered from dried and long-term stored polyacrylamide gels. Fig. 1 gives an overview of the individual steps involved in this proteomic study. For analytical details on mass spectrometry and bioinformatics, as well as listings of routinely used materials and chemicals, we would like to refer to recent publications from our laboratory on the standardized proteomic profiling of skeletal muscle proteins [14–16]. The approach described here falls under the general category of the GeLC-MS/MS method, which employs a combination of protein extraction, gel electrophoretic separation, in-gel digestion, liquid chromatography and mass spectrometry for the unequivocal identification of individual protein species [17].

The subcellular fraction enriched in proteins from the myofibrils was isolated by a standardized procedure [18], and was originally separated 20 years ago on a 3–12% gradient SDS-PAGE gel [19]. Hind limb muscle tissue from adult New Zealand white rabbits was obtained as freshly dissected post-mortem specimens from the Bioresource Facility of the National University of Ireland. Rabbits were kept under standard conditions according to Irish legislation on the use of animals in experimental research. Transportation of samples to Maynooth University was carried out in accordance with the Department of Agriculture (animal by-product register number 2016/16 to the Department of Biology, National University of Ireland, Maynooth). For rabbit muscle protein separation, a Protean IIxi Cell system from BioRad Laboratories (Hemel Hempstead, Hertfordshire, UK) with 1.5-mm thick and 16-cm long slab gels was used at a constant setting of 200V. Electrophoretic separation was carried out with 60 µg protein per lane until the blue dye front had disappeared from the bottom of the gel, followed by protein staining with Coomassie Brilliant Blue.

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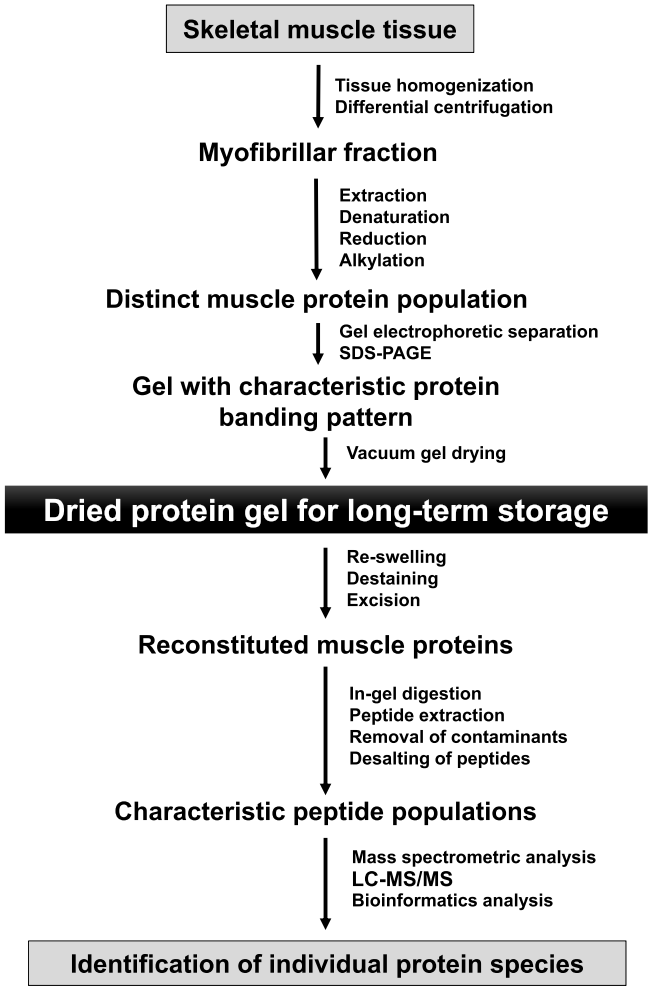

Fig. 1. Flowchart of bioanalytical workflow used for the mass spectrometric identification of muscle proteins recovered from dried and long-term stored polyacrylamide gels.

Standard vacuum drying of the gel was carried out between two layers of acetate film in a solution of 30% methanol and 5% glycerol in order to prevent cracking of the SDS-PAGE gel [4].

Following long-term storage in a lab book at room temperature, SDS-PAGE gels were re-swollen through overnight incubation at room temperature with gentle agitation in 30% methanol, 5% acetic acid and 5% glycerol. The following day gel strips were placed in fresh tubes and incubated at room temperature with shaking for 4 h in 10% glycerol and 1% acetic acid. The gel strips were subsequently incubated with 1% glycerol and 1% acetic acid overnight with shaking at room temperature. The gels were washed with 100% MilliQ water and the acetate sheets were removed. Gel lanes with individual preparations were cut into 6 sections, and destained with 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v) shaking at room temperature for 30 min, followed by incubation with 100% acetonitrile shaking at room temperature for 10 min. In-gel digestion was performed with sequencing grade trypsin from Promega (Madison, WI, USA). The reconstituted protease was added to 50 mM ammonium bicarbonate and each gel mixture treated at a 1:20 ration of trypsin to muscle protein overnight at 37 ˚C with agitation. Extraction buffer (5% formic acid/acetonitrile [1:2, v/v]) was added to gel pieces and incubated at 37 ˚C for 15 min and the supernatant fraction transferred to 1.5 ml micro-centrifuge tubes and dried by vacuum centrifugation. Peptide populations were re-suspended in 0.5% trifluoroacetic acid/5% acetonitrile and centrifuged in 22 μm acetate cellulose spin filter tubes for 20 min to remove any gel particles and then desalted using C18 spin columns (Thermo Fisher Scientific, Dublin, Ireland) and dried by vacuum centrifugation. Peptides were re-suspended in loading buffer consisting of 2% acetonitrile and 0.05% trifluoroacetic acid and analyzed by label-free liquid chromatography mass spectrometry (LC-MS/MS) using an Ultimate 3000 NanoLC system (Dionex Corporation, Sunnyvale, CA, USA) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Dublin, Ireland). The qualitative analysis of myofibrillar proteins was carried out with the Proteome Discoverer 1.4 against Sequest HT (SEQUEST HT algorithm, license Thermo Scientific, registered trademark University of Washington, USA) using the UniProtKB database with 23,072 proteins. Peptides were subsequently filtered using a minimum XCorr score of 1.5 for 1, 2.0 for 2, 2.25 for 3 and 2.5 for 4 charge states, with peptide probability set to high confidence.

The long-term stored SDS-PAGE gel with the myofibrillar fraction is

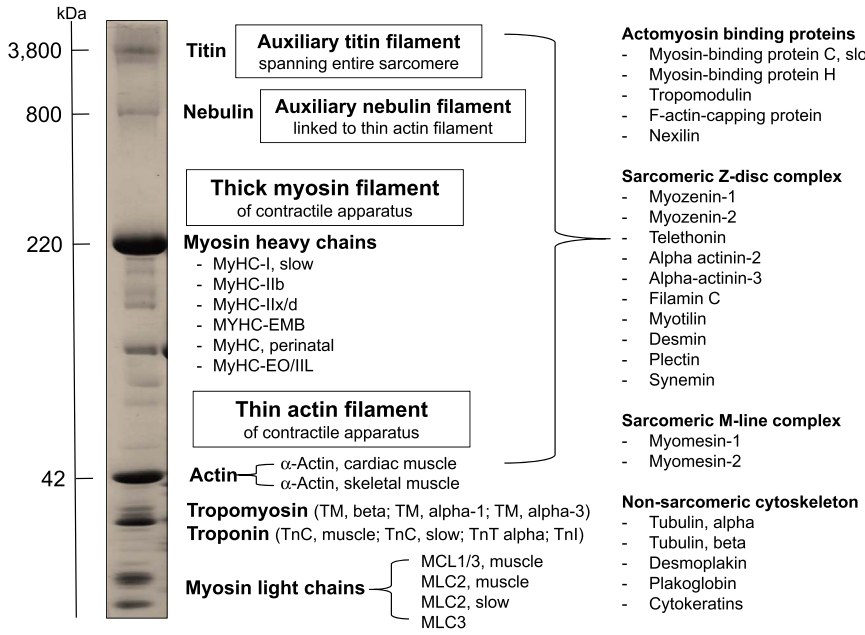

Fig. 2. Image of the long-term stored polyacrylamide gel that was used in this study to identify proteins in the myofibrillar fraction from rabbit skeletal muscle. The apparent molecular mass of the skeletal muscle marker proteins titin, nebulin, myosin heavy chain and actin is marked on the left of the gel. Included in the figure are key results from the proteomic identification of muscle proteins belonging to the contractile apparatus and its associated non-sarcomeric cytoskeleton.

Table 1
List of major proteins of the contractile apparatus that were identified in the myofibrillar fraction from rabbit skeletal muscle by mass spectrometry-based proteomics.

Protein name	Gene	% Coverage	Unique Peptides	Molecular mass (kDa)	p*I*
Titin	TNT	16.38	317	3451.0	6.23
Nebulin	NEB	20.72	115	800.2	9.09
Myosin heavy chain MyHC-IIx/d (Myosin-1)	MYH1	53.50	28	223.4	5.80
Myosin heavy chain MYHC-EMB (Myosin-3)	MYH3	16.75	2	223.3	5.74
Myosin heavy chain MyHC-IIb (Myosin-4)	MYH4	47.06	20	223.1	5.80
Myosin heavy chain MyHC-IIb (Myosin-4)	MYH4	50.83	11	222.9	5.81
Myosin heavy chain MyHC-I/MyHC-β, slow/cardiac (Myosin-7)	MYH7	34.33	42	222.9	5.74
Myosin heavy chain MyHC, perinatal (Myosin-8)	MYH8	39.70	10	222.3	5.78
Myomesin-1	MYOM1	8.67	13	186.5	5.99
Myomesin-2	MYOM2	4.56	6	164.5	6.24
Myosin-binding protein C, slow-type	MYBPC1	8.03	8	119.1	6.05
Alpha-actinin-2	ACTN2	21.48	13	103.8	5.52
Alpha-actinin-3	ACTN3	22.09	13	102.4	5.45
Plectin	PLEC	8.79	6	92.4	8.27
Myotilin	MYOT	6.21	3	55.6	8.98
Myosin-binding protein H	MYBPH	4.35	2	54.3	6.28
Desmin	DES	34.54	16	53.4	5.27
Actin, alpha cardiac muscle	ACTC1	59.68	3	42.0	5.39
Actin, alpha skeletal muscle	ACTA1	59.68	3	42.0	5.39
Tropomodulin 4	TMOD4	7.25	2	39.3	4.70
F-actin-capping protein subunit alpha-2	CAPZA2	5.94	2	32.9	5.85
Tropomyosin beta chain	TPM2	65.49	12	32.8	4.70
Tropomyosin alpha-1 chain	TPM1	67.25	11	32.7	4.74
Myozenin-1	MYOZ1	17.51	4	31.3	8.63
Tropomyosin	TPM	41.30	2	28.9	4.81
Troponin T alpha isoform	TNNT2	22.22	7	27.8	9.54
Myosin light chain 3	MYL	25.36	4	23.1	5.19
Myosin light chain 1/3, muscle isoform	MYL1	40.63	8	20.9	5.03
Troponin I (fragment)	TNNI2	26.26	5	20.9	9.20
Myosin light chain 2	MYL2	40.00	2	19.0	4.92
Troponin C, slow skeletal and cardiac muscles	TNNC1	15.53	2	18.4	4.18
Troponin C, skeletal muscle	TNNC2	33.75	4	18.1	4.20
Myosin regulatory light chain 2, muscle isoform 2	MYLPF	45.33	2	16.7	4.84
Alpha-tropomyosin, smooth muscle (fragment)	TPM1	51.61	2	14.1	4.67

shown in Fig. 2, including key results from the proteomic identification of muscle marker proteins. Select findings from the mass spectrometric analysis of the isolated skeletal muscle fraction are listed in Table 1, including the high-molecular-mass proteins titin, nebulin and various isoforms of myosin heavy chain. In addition, major muscle protein bands of lower molecular mass are marked, including myosin light chains, troponins, tropomyosins and actins. Overall 164 proteins were identified in the myofibril fraction. The entire list of proteomic hits is summarized in Tables S1 in the Supplementary Information in the appendix of this note. Interestingly, a large number of distinct myosin heavy chains, i.e. slow MyHC-I, fast MyHC-IIb, fast MyHC-IIx/d, MYHC-EMB, perinatal MyHC and MyHC-EO/IIL, and myosin light chains MCL1/3, slow MLC2, fast MLC2 and MLC3 were identified [13], demonstrating an excellent degree of sensitivity for protein identification in the long-term stored gel. In addition, both the muscle and cardiac isoform of α-actin, as well as α- and β-tropomyosins and the troponin subunits TnI, TnC and TnT were identified in this study. As summarized in Fig. 2, key proteins belonging to the class of actomyosin binding proteins, sarcomeric Z-disk proteins, M-line complex proteins and cytoskeletal proteins [13] were also covered by the bioanalytical workflow outlined in this report. This shows that all major components of the contractile apparatus and its associated non-sarcomeric cytoskeleton were identified following the above described gel re-swelling, destaining and in-gel digestion procedure.

The decrease in overall yield of protein identification in freshly prepared versus long-term stored dried gels was estimated to be approximately 40%, using the comparative proteomic screening of one-dimensional gel slices containing microsomal membranes isolated from skeletal muscle homogenates (not shown). In relation to the detection of post-translation modifications in proteins following gel drying, as previously studied by Dihazi et al. [8], peptide files were searched for phosphorylation sites. Data analysis via Proteome Discoverer 1.4 using Sequest HT followed by phosphoRS was carried out as recently described by Henry et al. [20]. Of the 2546 identified peptides, 27 contained phosphorylated sites and 24 of these peptides had a site probability score of 75% or greater for S, T, or Y amino acids. Phosphorylation sites in components of the contractile apparatus were identified in titin, plectin, cytokeratin and myosin heavy chain MyHC-IIb.

In conclusion, the large number of identified muscle proteins from a 20-year old SDS-PAGE gel, including components of extremely high molecular mass such as the sarcomeric elements nebulin and titin, demonstrates that the described method can be utilized to re-use dried polyacrylamide gels following very long periods of storage at room temperature. Thus, stored gels from experiments carried out many years or even several decades ago during the pre-proteomic era can be successfully re-analyzed by more recently developed and highly sensitive mass spectrometry [21]. The straightforward combination of SDS-PAGE gel re-swelling, standardized in-gel digestion and routine LC-MS/MS analysis can now be employed to confirm and refine previous findings from gel-based studies, and might also be suitable for the retrospective proteomic profiling of scarce protein samples stored in dried gels. This might result in the identification of previously unknown protein factors and their involvement in biological or pathophysiological processes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2017.11.022.

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