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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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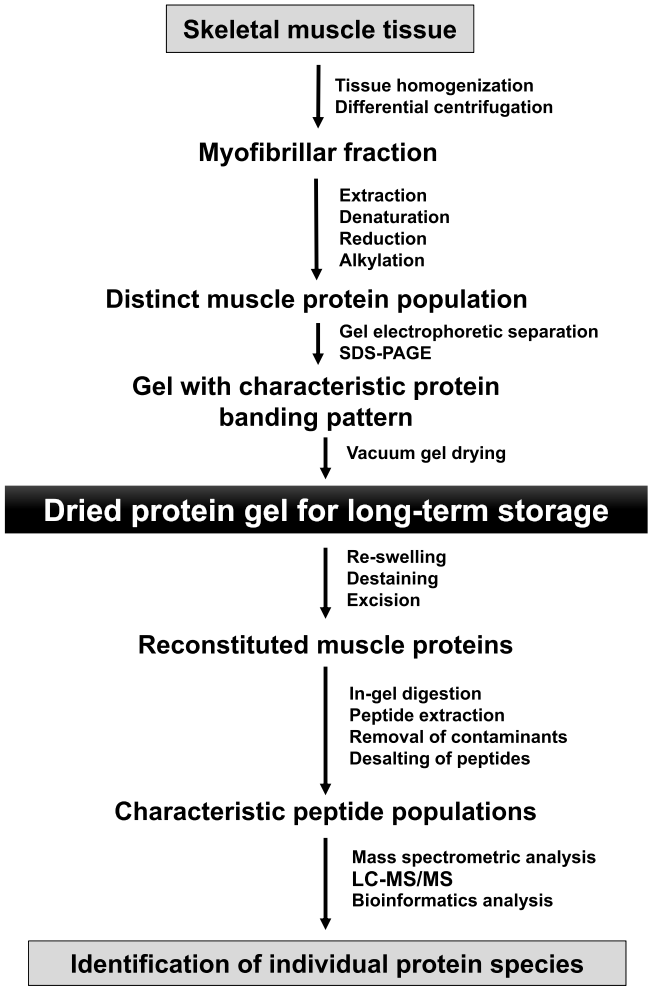

Fig. 1. Flowchart of bioanalytical workflow used for the mass spectrometric identification of muscle proteins recovered from dried and long-term stored polyacrylamide gels.

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

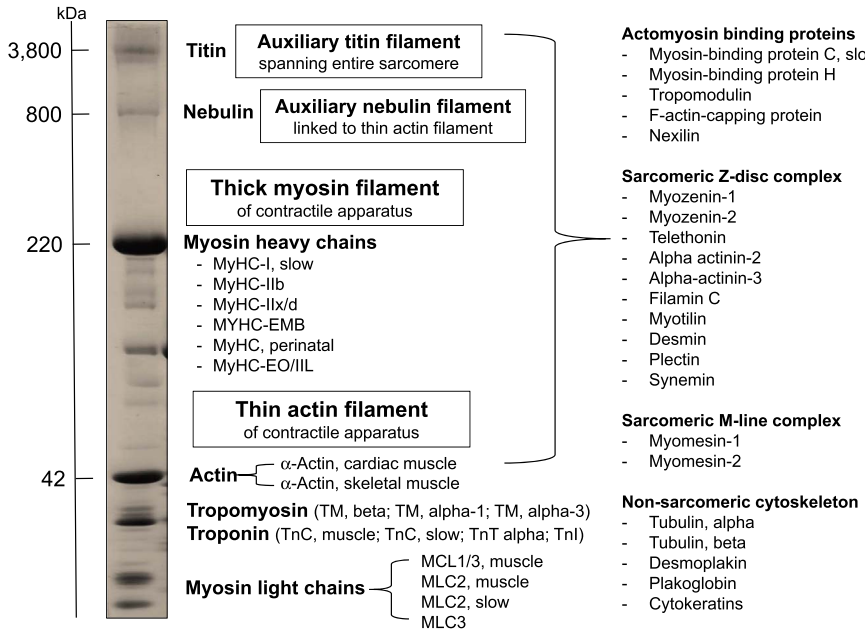

Fig. 2. Image of the long-term stored polyacrylamide gel that was used in this study to identify proteins in the myofibrillar fraction from rabbit skeletal muscle. The apparent molecular mass of the skeletal muscle marker proteins titin, nebulin, myosin heavy chain and actin is marked on the left of 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.