On-Membrane Digestion Technology for Muscle Proteomics

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Abstract: High-resolution two-dimensional gel electrophoresis and in-gel digestion are routinely used for large-scale protein separation and peptide generation in mass spectrometry-based proteomics, respectively. However, the combination of isoelectric focusing in the first dimension and polyacrylamide slab gel electrophoresis in the second dimension is not suitable for the proper separation of integral proteins and high-molecular-mass proteins. In addition, in-gel trypsination may not result in a high degree of efficient digestion levels for the production of large numbers of peptides in the case of certain protein species. The application of gradient one-dimensional gel electrophoresis and on-membrane digestion can overcome these technical problems and be extremely helpful for the comprehensive identification of proteins that are underrepresented in routine two-dimensional gel electrophoretic approaches. This review critically examines the general application of on-membrane digestion techniques in proteomics and its recent application for the identification of very large integral membrane proteins from skeletal muscle by mass spectrometry. This includes the discussion of proteomic studies that have focused on the proteomic characterization of the membrane cytoskeletal protein dystrophin from sarcolemma vesicles and the ryanodine receptor calcium release channel of the sarcoplasmic reticulum from skeletal muscle.

Keywords: Dystrophin, on-membrane digestion, mass spectrometry, muscle proteomics, ryanodine receptor.

1. INTRODUCTION

The usage of adsorbent membranes as support material for blotted biomolecules is widely used in analytical biochemistry [1]. The electrophoretic transfer of DNA, RNA and protein is routinely achieved by Southern, Northern and Western blotting techniques, respectively [2-4]. Blotting techniques are highly sensitive and specific for the identification of unique nucleic acid sequences or protein epitopes. Hence, the efficient transfer of DNA, RNA or protein from a gel system onto a membrane support is critical in these frequently used techniques [5, 6]. The electrophoretic transfer of proteins to membrane sheets was originally described by Towbin and co-workers in 1979 [7] and has been extensively applied in basic and applied bioresearch over the last few decades [8-10]. Protein blotting technology is now a key analytical approach in the biochemical identification and characterization of peptides, protein subunits, protein isoforms and protein complexes, as well as post-translational modifications. Western blotting analysis has been continuously modified and improved for novel applications in protein biochemistry [11]. Extensively modified protein blotting methods are represented by Southwestern blotting for investigating DNA-protein interactions [12-14] and Far-Western blotting for the detection of protein-protein interactions [15-17]. Blot overlay methods combine the gel electrophoretic separation of proteins, blotting to a membrane and then incubation with a labeled probe other than an antibody, such as a fluorescently labeled or enzyme-conjugated protein of interest [18-20].

Protein transfer technology has also been integrated into modern mass spectrometry-based proteomics [21], especially exploiting the efficient on-membrane proteolytic digestion of electro-blotted molecules for swift protein identification [22]. Proteomics is a technology-driven and hypothesis-generating approach that combines established biochemical and protein chemical methods for the comprehensive survey of large protein populations [23-25]. Modern proteomics is of crucial importance for the large-scale identification of proteins in diverse fields such as basic cell biology [26], preclinical drug discovery [27] and systems biology [28]. Thousands of proteins from crude tissue extracts can be separated by high-throughput liquid chromatography [29] or gel electrophoresis [30] covering a large portion of the whole tissue proteome. However, if certain classes of proteins from a dynamic cellular system cannot be sufficiently enriched by standard biochemical approaches, organelle proteomics can be employed to reduce sample complexity with the help of sophisticated pre-fractionation steps prior to proteomic analysis [31-33]. In-gel trypsination is the most frequently used method to generate peptides from gel electrophoretically separated protein mixtures [34, 35]. However, this technique does sometimes not result in an efficient proteolysis of distinct protein species for their subsequent identification by mass spectrometry, which necessitates the application of alternative methods such as on-membrane trypsination [21, 22]. Since membrane-bound proteins appear to be more

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prominently exposed to proteases, the controlled digestion of proteins transferred from gel bands or spots to membrane sheets is advantageous when studying certain protein species. This is especially relevant for proteins with unusual properties with respect to charge, hydrophobicity, size and/or density. This review outlines recent applications of on-membrane digestion techniques in muscle proteomics and critically examines its application for the characterization of high-molecular-mass membrane proteins by mass spectrometry.

2. GEL ELECTROPHORETIC PROTEIN SEPARATION

The efficient separation of proteins from complex tissues, the unequivocal identification of individual protein species within heterogeneous protein mixtures, the determination of protein abundance and isoform expression patterns in dynamic cellular systems, and the comprehensive characterization of post-translational modifications are at the core of analytical protein biochemistry. For the cataloguing of entire protein populations, the comparative analysis of differing proteomes or the identification of novel protein biomarkers, the proteomic workflow involves at least five critical steps, i.e. (i) sample preparation from defined body liquids, crude tissue extracts or subcellular fractions, (ii) protein separation *via* liquid chromatography and/or gel electrophoresis, (iii) computer-assisted analysis of proteomic maps, (iv) efficient protein digestion for the generation of meaningful peptide signatures, and (v) the unequivocal identification of individual protein species by highly sensitive mass spectrometry [25]. Since this review focuses on the mass spectrometric identification of very large proteins following gel electrophoretic separation, this section briefly lists the most frequently employed methods that are based on gel matrixes for protein analysis.

Proteins are routinely separated by size and/or charge in one-dimensional or two-dimensional gels depending on the specific method of choice. One-dimensional gel electrophoretic methods used in protein biochemistry include isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis [36], as well as non-denaturing gel electrophoresis, such as Native Blue gel electrophoresis [37]. For large-scale applications, preparative gel systems are available including the Rotofor system and the Prep Cell instrument [38]. These preparative protein separation devices are based on a multi-chamber system using carrier ampholyte focusing or a cylindrical compartment for continuous-elution gel electrophoresis [39, 40]. An interesting alternative to conventional gel electrophoresis is offgel electrophoresis, which is a free-flow protein method based on isoelectric focusing in solution [41-43]. Offgel electrophoretic pre-fractionation of crude tissue homogenates has recently been successfully applied to the subproteomic analysis of basic proteins in aged skeletal muscle [44].

Two-dimensional gel electrophoresis has been extensively used in biochemical research [45] and is one of the most frequently employed separation techniques in large-scale proteomic surveys due to its enormous capacity and high resolution [46-48]. The most commonly used method combines isoelectric focusing in the first dimension and polyacrylamide slab gel electrophoresis in the second dimension [49]. Alternative approaches use native gel electrophoresis in the first dimension [50] or separate protein complexes by diagonal reducing/non-reducing two-dimensional gel electrophoresis [51]. Two-dimensional gel electrophoresis has been continuously advanced for improved separation and characterization of proteins [52], especially with respect to large high-resolution gel systems for the separation of entire proteomes [53]. Hundreds to thousands of individual protein spots can be visualized depending on protein loading conditions and staining methodology [54-56]. Standard staining approaches include Coomassie Brilliant Blue [54] and silver [57, 58], as well as a variety of fluorescent dyes [58-61]. One of the most powerful comparative ways of analyzing proteomes is fluorescence difference in-gel electrophoresis [62], which uses 2-CyDye or 3-CyDye systems to differentially label proteins belonging to dissimilar protein mixtures prior to gel electrophoretic separation [63-65]. Two-dimensional difference in-gel electrophoresis incorporating a pooled internal standard, originally described by Alban *et al*. [66], can routinely analyse over 2,000 proteins using triple fluorescent labeling [67-69]. However, although high-resolution two-dimensional gel electrophoresis is an excellent separation tool for studying urea-soluble proteins, this method usually under-represents certain classes of proteins, such as low copy number proteins, very large proteins, membrane-associated proteins and proteins with extreme isoelectric points [70]. Thus, comprehensive proteomic studies of complex tissues often require additional separation steps to fully cover the membrane and organelle proteome [71-73].

3. PROTEIN DIGESTION FOR PROTEOMIC ANALYSIS

Gel electrophoresis and transfer blotting have long been used as preparative tools to isolate and/or immobilize individual proteins for subsequent micro-sequence analysis [74-76]. It is relatively simple to prepare proteins for automated Edman micro-sequencing following gel electrophoresis [77] or blotting [78]. In the case of gel-embedded proteins, their efficient elution from the gel matrix, the removal of excess detergent and renaturing of the target protein are crucial steps prior to biochemical analysis [79]. Micro-sequencing of immobilized proteins following blotting has been used for direct tryptic digestion on nitrocellulose replicas [80] or N-terminal sequencing on polyvinylidine difluoride membranes [81, 82]. For proteomic studies, relatively high yields of tryptic peptides could be extracted from 1 mm thick gel bands at 200 fmol to 10 pmol levels [83], whereby this in-gel digestion method is suitable for automated applications [84]. Both, in-gel trypsination and on-membrane digestion at sub-pmol protein quantities are able to produce a sufficient number and size of released peptides for the successful identification of standard proteins ranging from 17 kDa to 97 kDa by matrix-assisted laser-desorption/ionization mass spectrometry [85].

Although in-gel digestion is a widely used and highly suitable method for the preparation of peptides from the majority of proteins [83-85], the trypsination of certain target proteins may not be efficient enough for routine mass spectrometric analysis and might thus require alternative approaches [86-88]. Importantly, the on-membrane digestion method is faster as compared to conventional in-gel trypsination [89-91], which can considerably reduce technical complications due to trypsin autolysis [92]. In general, the usage of MS grade trypsin, accelerated digestion protocols, suitable solvent systems and specific protease combinations can considerably reduce the appearance of autolytic trypsin fragments in proteomic analyses [93-95]. On-membrane digestion has been reported to result in superior protein sequence coverage as compared to in-gel methods [96], which makes this biochemical technique especially suitable for the proteomic identification of low-abundance proteins, hydrophobic proteins and high-molecular-mass proteins [97, 98]. Interesting applications of the on-membrane method has been the analysis of glycoproteins [99-101], phosphoproteins [102-104] and DNA-binding transcription factors [105-107].

Following protein digestion, highly sensitive mass spectrometric approaches are used for the high-throughput identification of proteins [108]. Several excellent reviews have summarized the importance of mass spectrometry for modern protein analysis [109-111] and its extended usage in large-scale proteomic investigations [112-114]. A key step in proteomics is successful protein digestion and generation of large and representative numbers of peptides. Figure **1** summarizes the main features of in-gel digestion following two-dimensional gel electrophoresis of total extracts versus on-membrane digestion following one-dimensional gel electrophoresis and blotting of subcellular fractions. Ideally, both techniques are used in combination to achieve the near-to-complete coverage of a given tissue proteome. The on-membrane digestion technique has recently been applied to the identification of extremely large proteins from skeletal muscle tissue [97, 98]. Muscle proteomics is concerned with the large-scale biochemical analysis of fibre-associated proteins and attempts to catalogue all components of the muscle proteome and determine protein changes in developing, adapting, transforming, pathological and aging contractile tissues [115-117]. The two investigated high-molecular-mass proteins exist under physiological conditions as supramolecular membrane assemblies in skeletal muscle fibres, i.e. the membrane cytoskeletal protein dystrophin in a glycoprotein-associated complex in the sarcolemma [118] and the ryanodine receptor Ca^{2+}-release channel in a tetrameric structure at the triad junction between transverse tubules and the sarcoplasmic reticulum [119]. On-membrane digestion was instrumental in the proteomic characterization of these two high-molecular-mass skeletal muscle proteins.

4. PROTEOMIC IDENTIFICATION OF DYSTROPHIN BY ON-MEMBRANE DIGESTION

Individual members of the super family of large actin-binding proteins, consisting of spectrins, dystrophins and utrophins, have originated from a common ancestral α-actinin molecule and exist in many different isoforms with a wide tissue distribution throughout the body [120]. The full-length dystrophin isoform Dp427, which absence triggers progressive muscle wasting in Duchenne muscular dystrophy [121], forms tight interactions with integral glycoproteins in the plasmalemma [122]. Dystrophin of 427 kDa constitutes approximately 5% of the actin-associated membrane cytoskeleton in the subsarcolemmal region of skeletal muscle [123]. In healthy muscle tissue, dystrophin binds to β-dystroglycan that in turn interacts with

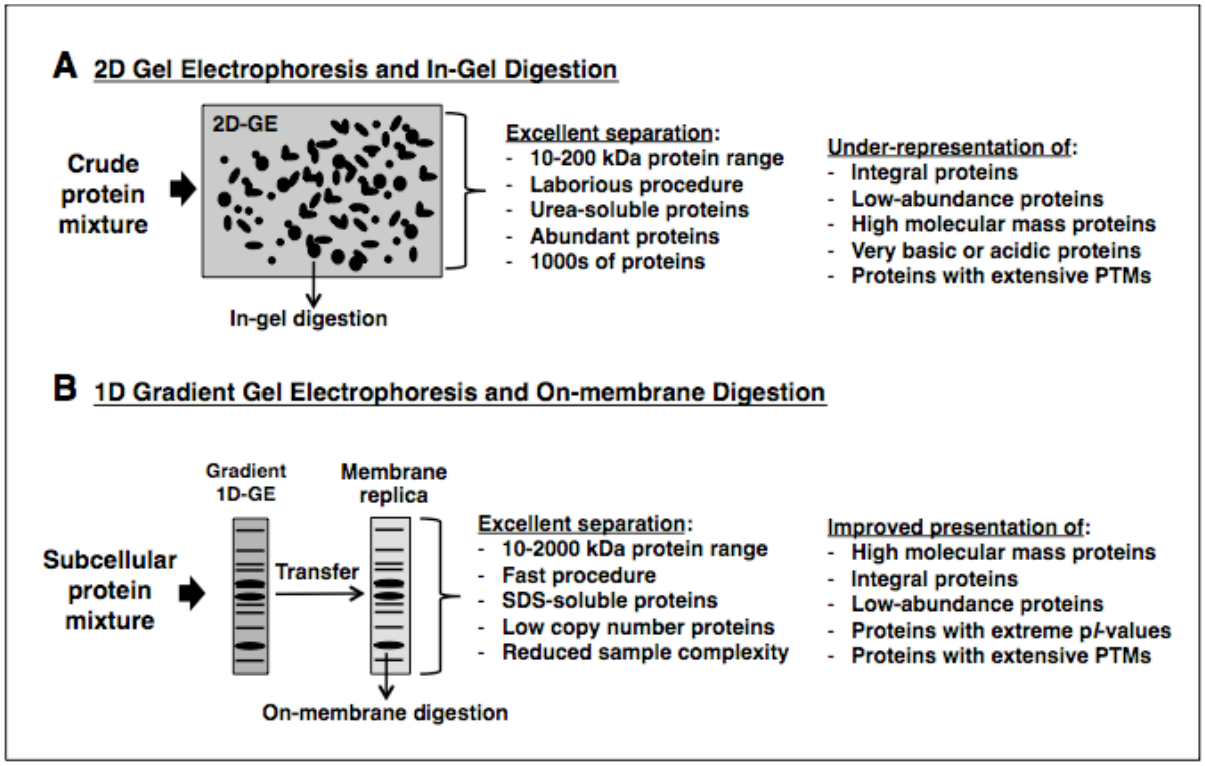

Figure 1: Comparison of major analytical features of two-dimensional gel electrophoresis and in-gel protein digestion versus one-dimensional gradient gel electrophoresis and on-membrane protein digestion. Shown are diagrams of 2D gel-based in-gel digestion (**A**) and 1D gel-based on-membrane digestion (**B**) and lists of advantages and potential disadvantages of these two methods when used as large-scale separation techniques in proteomic profiling studies.

extracellular α-dystroglycan, a receptor for laminin. This membrane assembly provides a trans-sarcolemmal linkage between the intracellular actin cytoskeleton and the extracellular matrix of the basal lamina and thereby stabilizes the muscle periphery during excitation-contraction-relaxation cycles [118]. Loss of dystrophin results in the disintegration of the membrane complex and causes a severe reduction in all dystrophin-associated glycoproteins [124-126].

A large number of gel-based proteomic studies have compared the expression profile of normal versus dystrophic muscle tissue [127], but have disappointingly not identified dystrophin and its associated glycoproteins by standard two-dimensional gel electrophoresis and in-gel digestion approaches [128-130]. This is probably due to the large size of dystrophin and the relative low levels of the constituents of the dystrophin-glycoprotein complex in crude tissue extracts. However, alternative approaches have successfully identified components of this surface complex by mass spectrometry. Figure **2** outlines the various analytical steps involved in the proteomic characterization of the muscle plasma membrane. The experimental conditions and main solutions employed in on-membrane digestion of gel electrophoretically separated proteins are shown in Figure **3**. The flowchart lists the composition of the electrophoretic transfer buffer used in the transblotting from gels to membrane sheets, as well as the trypsin stock solution and extraction buffer that are routinely employed for the proteolytic generation of peptides for the subsequent mass spectrometric identification of proteins of interest.

On-membrane digestion of dystrophin and its associated glycoproteins was carried out with both isolated sarcolemma vesicles and the highly purified dystrophin complex [97]. For studying sarcolemma vesicles, individual fractions from microsomal membranes were separated by density gradient centrifugation and then lectin agglutination was used to affinity purify surface membrane vesicles [122, 123, 131]. Right-side-out sarcolemma vesicles expose a large number of carbohydrate moities that can directly interact with a suitable lectin, such as wheat germ agglutinin [122]. Contaminating material, such as trapped smaller vesicles or adsorbed proteins, can be conveniently removed by mild detergent washing steps [123]. Lectins are finally removed from the agglutinated vesicle fraction by incubation with a competitive sugar, such as N-acetylglucoseamine in the case of wheat germ agglutinin [131]. Mass spectrometry clearly

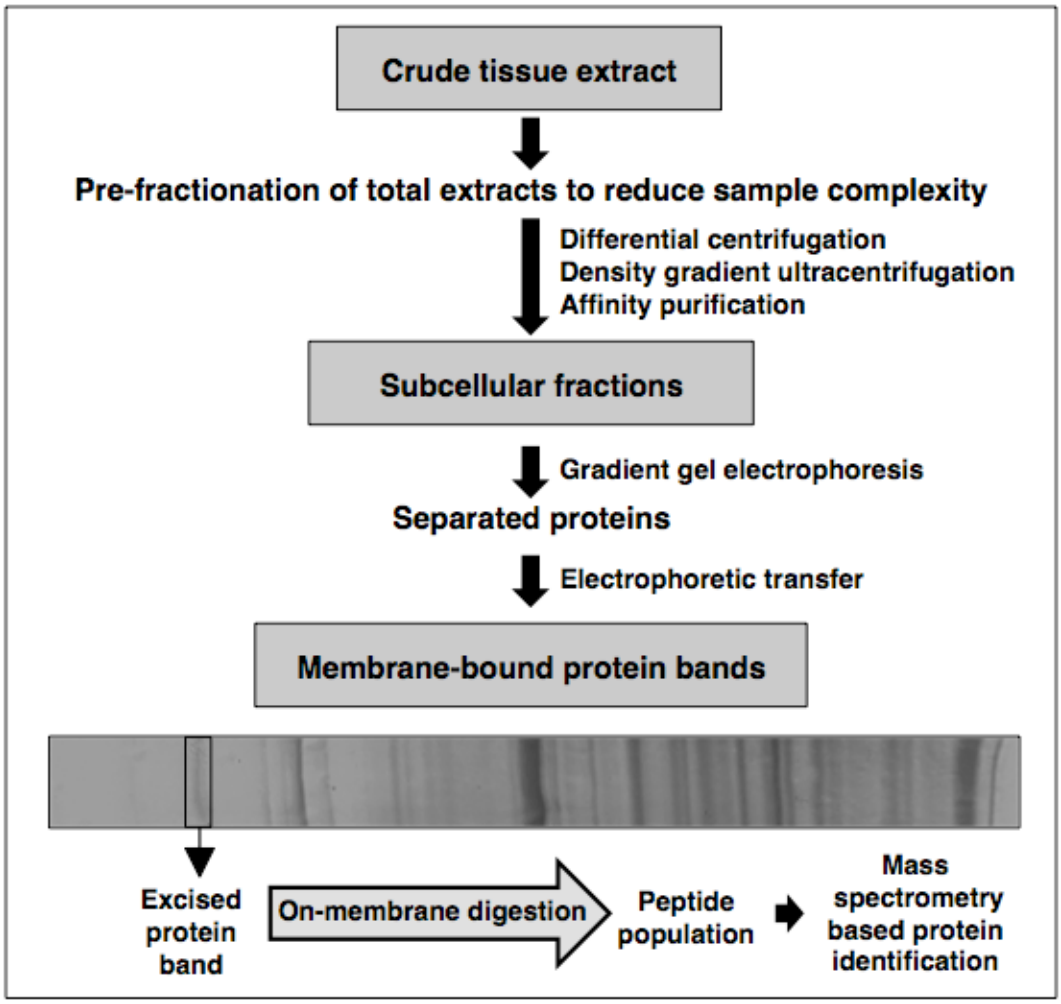

Figure 2: Flowchart of preparative steps employed in the subcellular fractionation of membrane-associated proteins for organelle proteomics. Listed are major biochemical techniques that are routinely used in pre-fractionation procedures to reduce sample complexity in the proteomic analysis of crude tissue extracts, including differential centrifugation, density gradient ultracentrifugation and affinity isolation methods. The protein cohort of a particular subcellular fraction can be further separated by one-dimensional gradient gel electrophoresis, or other suitable methods, and transferred to a membrane sheet for on-membrane digestion. The image in the lower part of the figure represents a nitrocellulose replica of gel electrophoretically separated sarcolemma vesicles from skeletal muscle, labeled with MemCode stain for visualization of individual protein bands. The marked band contains a high-molecular-mass protein that had previously not been identified by standard two-dimensional gel electrophoresis and in-gel digestion, but was clearly recognized as the Dp427 isoform of the membrane cytoskeletal protein dystrophin by on-membrane digestion and mass spectrometry.

identified dystrophin of 427 kDa and its associated glycoprotein α-syntrophin in the sarcolemma fraction following on-membrane digestion [97].

Sophisticated pre-fractionation steps were needed to study the isolated dystrophin complex by mass spectrometry. In one subproteomic approach sarcolemmal β-dystroglycan and tightly associated members of the dystrophin-glycoprotein complex were isolated by immuno precipitation from detergent-solubilized skeletal muscle and then identified by mass spectrometry [132]. In another proteomic study, the dystrophin-glycoprotein complex was purified to homogeneity, separated by gel electrophoresis and then peptides generated by on-membrane digestion for mass spectrometric analysis [97]. Following solubilization with the detergent digitonin, muscle membrane proteins were separated by a combination of ion exchange chromatography, lectin chromatography, sucrose gradient centrifugation and one-dimensional gradient gel electrophoresis. The mass spectrometric analysis of nitrocellulose replicas revealed the presence of dystrophin isoform Dp427 and its associated proteins α-sarcoglycan, γ-sarcoglycan and dystrobrevin [97]. This confirmed the tight linkage between the membrane cytoskeletal protein dystrophin and integral proteins of the muscle plasmalemma by proteomics.

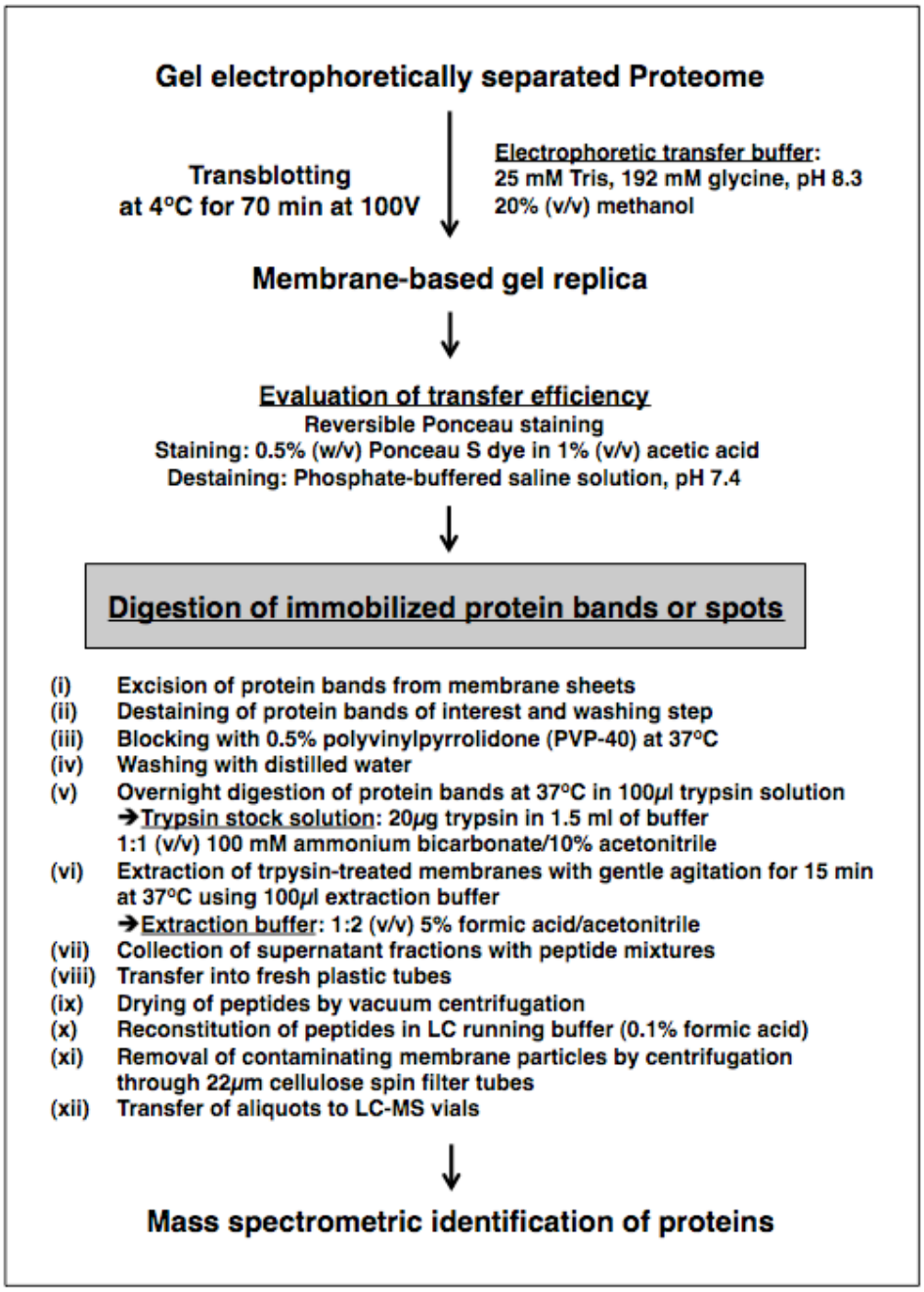

Figure 3: Flowchart of the main preparative steps used in the on-membrane digestion of gel electrophoretically separated proteins. Listed are the experimental conditions and main buffers employed in transblotting from gels to membrane sheets, as well as the proteolytic generation of peptide populations for the mass spectrometric identification of individual protein species.

5. RYANODINE RECEPTOR IDENTIFICATION BY ON-MEMBRANE DIGESTION

In skeletal muscle fibres, excitation-contraction coupling is mediated by complex spatiotemporal interactions between several Ca^{2+}-handling proteins [133]. The sarcoplasmic reticulum contains very high levels of Ca^{2+}-ions and cycling of the second messenger molecule through the lumen of this organelle determines the contractile status of muscle fibres [134]. Following excitation of the sarcolemma above a critical physiological threshold level and subsequent propagation of an action potential along the transverse tubular membrane system, the α_{1S}-subunit of the dihydropyridine receptor acts as a voltage sensor and upon activation this protein complex interacts directly with ryanodine receptor Ca^{2+}-release channels at the triad junction [135]. Ca^{2+}-ions are buffered by the luminal ion-binding proteins calsequestrin and sarcalumenin, and the energy-dependent re-uptake of Ca^{2+}-ions is achieved by the

SERCA-type Ca^{2+}-ATPases during fibre relaxation [133]. The ryanodine receptor Ca^{2+}-release channel of the sarcoplasmic reticulum exists at the contact sites between the terminal cisternae and the transverse tubules as an integral tetramer with a molecular mass of over 2,000 kDa [119]. The gigantic size of the native ryanodine receptor complex makes it difficult to study this type of hydrophobic protein by conventional biochemical methodology. Routine two-dimensional gel electrophoresis can separate proteins from crude skeletal muscle extracts with a molecular mass not much greater than 200 kDa [67-69]. In stark contrast, one-dimensional gels using 3-12% gradients can properly separate very large membrane proteins with molecular masses up to 2,000 kDa [51, 136].

This separating capacity of one-dimensional gradient gel electrophoresis for proteins of very high molecular mass was exploited in a recent on-membrane digestion-based characterization of the isolated sarcoplasmic reticulum [98]. Mass spectrometric analysis identified proteins in 31 distinct bands. This included the main Ca^{2+}-regulatory proteins involved in excitation-contraction coupling, muscle relaxation and ion homeostasis, such as the RyR1 isoform of the junctional ryanodine receptor Ca^{2+}-release channel of 565 kDa, the SERCA1 isoform of the Ca^{2+}-ATPase of 110 kDa, the luminal Ca^{2+}-shuttle protein sarcalumenin of 160 kDa, and the high-capacity Ca^{2+}-buffering protein calsequestrin of 63 kDa [98]. The position of these Ca^{2+}-handling proteins in one-dimensional gradient gels was confirmed by immunoblotting. Previous structural studies have indicated that glycolytic enzymes are present on the sarcoplasmic reticulum [137]. This was confirmed by on-membrane digestion of the purified sarcoplasmic reticulum, which demonstrated the presence of aldolase and phosphofructokinase in the purified membrane fraction [98]. These proteomic findings agree with the concept of close physical coupling between the energy-dependent sarcoplasmic reticulum and the ATP-producing glycolytic pathway [138].

6. CONCLUSIONS

Mass spectrometry-based proteomics is a powerful approach to catalogue entire protein populations present in body liquids, cells or tissues and is widely used to determine protein changes in biological systems in health and disease. Since current biochemical separation methods may under-estimate the presence of certain classes of proteins in whole tissue proteomics, organelle and membrane proteomics has been developed to fully cover the entire spectrum of proteins in a given tissue. This makes subproteomics an important part of modern protein biochemistry. In the case of large-scale protein separation approaches using two-dimensional gel electrophoresis, in-gel digestion is widely employed to produce peptides for subsequent mass spectrometric analysis. However, in some cases trypsination is inefficient for comprehensive in-gel digestion and alternative techniques with membrane replicas of gels have been shown to result in superior results. On-membrane digestion of gel electrophoretically separated proteins is especially useful for the identification of proteins with a low abundance, high molecular mass, a considerable degree of hydrophobicity and/or extreme isoelectric points. The recent application of on-membrane digestion in skeletal muscle proteomics has resulted in the mass spectrometric identification of extremely large membrane-associated proteins, i.e. the Dp427 isoform of dystrophin and the RyR1 isoform of the ryanodine receptor Ca^{2+}-release channel. These muscle proteins were previously not detected by routine proteomic surveys of various skeletal muscle tissues. This shows that mass spectrometry-based subproteomics can be successfully utilized for the identification and biochemical characterization of membrane-associated muscle proteins with a low density in muscle fibres. Large muscle proteins adsorbed onto nitrocellulose sheets seem to be more accessible to tryptic digestion, which increases the number of generated peptides for mass spectrometry. Hence, the usage of subcellular fractionation, in combination with gel electrophoresis and on-membrane digestion, can be extremely helpful for the future identification of protein species that are currently not fully recognized in proteomic studies of total cellular extracts.

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