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Review

Zooming in: Fractionation strategies in proteomics

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The recent development of mass spectrometry, *i.e.*, high sensitivity, automation of protein identification and some post-translational modifications (PTMs) significantly increased the number of large-scale proteomics projects. However, there are still considerable limitations as none of the currently available proteomics techniques allows the analysis of an entire proteome in a single step procedure. On the other hand, there are several successful studies analyzing well defined groups of proteins, *e.g.*, proteins of purified organelles, membrane microdomains or isolated proteins with certain PTMs. Coupling of advanced separation methodologies (different prefractionation strategies, such as subcellular fractionation, affinity purification, fractionation of proteins and peptides according to their physicochemical properties) to highly sensitive mass spectrometers provides powerful means to detect and analyze dynamic changes of low abundant regulatory proteins in eukaryotic cells on the subcellular level. This review summarizes and discusses recent strategies in proteomics approaches where different fractionation strategies were successfully applied.

Keywords: Fractionation / Organelle / Review

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Abbreviations: MudPIT, multidimensional protein identification technology; **PNS**, postnuclear supernatant

1 Introduction

Cells are exceptionally complex and may consist of more than 100 000 protein species with different chemical and physical properties. Because of the limited resolution power of analytical separation techniques presently applied in protein profiling and expression analysis, prefractionation strategies are required to reduce sample complexity [1]. Any complexity reduction strategy greatly increases the number of less abundant proteins that can be subsequently analyzed. Since the magnitude of protein species abundance within a cell may differ by 7–10 orders of magnitude, the relatively low abundant proteins

are usually masked by more abundant ones, *e.g.*, housekeeping and structural ones. When complex protein samples are analyzed by high-resolution 2-DE or by gel-independent techniques, usually only the most abundant proteins are identified by subsequent mass spectrometry. This makes it difficult to relate results of proteome profiling to the biology of the system. Low copy number regulatory proteins such as kinases, phosphatases, or GTPases can be detected only after applying additional fractionation technologies on protein and/or peptide levels, such as subcellular fractionation [2, 3], protein and peptide affinity purification [4], chromatographic protein prefractionation [5], zoom gels of narrow pH ranges for 2-DE and preparative protein isoelectrofocusing [6, 7], or multidimensional peptide separations [8]. Thus, initial fractionation methods coupled with powerful separation methodologies must be employed in functional proteomics to gain a better understanding of the inner workings of a cell.

2 Subcellular fractionation

Subcellular fractionation is the first and essential step among enrichment techniques in proteomics research, which is of special importance for analysis of intracellular organelles and multiprotein complexes. Subcellular fractionation is a flexible and adjustable approach resulting in reduced sample complexity and is most efficiently combined with high-resolution 2-D gel/mass spectrometry analysis as well as with gel-independent techniques. Recent reviews [2, 3, 9–11] describe in detail techniques used for purification of organelles as well as characterization of proteomes of several organelles, such as nucleus, mitochondria, Golgi apparatus, lysosomes, exosomes, peroxisomes and phagosomes. Therefore, they will be discussed here just briefly, on examples of some very recent publications in the field of organelle proteomics.

2.1 Organelle proteomics

Subcellular fractionation, allowing the separation of organelles based on their physical or biological properties, consists of two major steps: (i) disruption of the cellular organization (homogenization), and (ii) fractionation of the homogenate to separate the different populations of organelles. Centrifugation is the most efficient method for organelle isolation [3]. Cells are collected by a low speed centrifugation step and mechanically homogenized. After homogenization, the nuclei are removed by a low speed centrifugation and can be purified for additional analysis from the pellet, which contains cell debris and unbroken cells. The post-nuclear supernatant (PNS) contains the cytosol and the other organelles in free suspension, which can be subsequently separated by gradient centrifugation. Although differences in composition of subcellular components affect relative densities of fractions, the degree of separation obtained also depends on the nature of the gradient medium used. Sucrose is the most commonly used gradient medium, but there are other alternatives, *e.g.*, Ficoll, Percoll, Nycodenz or Metrizamide. Several other techniques, *e.g.*, free flow electrophoresis or immunoisolation have been applied to study organelles [12].

Purity of isolated organelles is essential for comprehensive analysis of total organelle proteomes, but complete purification is almost impossible (see [1 and 3]). On the other hand for functional proteomics studies (*e.g.*, when two or more differentially treated samples are compared) even enrichment of organelles or certain subcellular fractions could be beneficial for detection of low abundant proteins and tracking of their changes after stimulation of cells. An example of the combination of subcellular fractionation, proteomics and a study of cellular signaling is the discovery of p14, a low M_r protein constituent of late endosomes [13]. Purification of cellular endosomes and the subsequent separation of early from late endosomes by subcellular fractionation revealed that p14 is highly enriched in late endosomes. It was shown that p14 functions as an adaptor protein for the targeting of mitogen activated protein (MAPK) kinase signaling to the late endosomal compartment in an alternative epidermal growth factor receptor (EGFR) pathway [14]. An example from our laboratory of a separation of early and late endosomes by sucrose gradient centrifugation is shown in Fig. 1. Cellular endosomes were purified by continuous gradients as described in [15]. Analysis of the enriched endosomal fractions by 2-D differential gel electrophoresis (DIGE) revealed a substantial enrichment of 305 (from 2- to 120-fold) and 292 (from 2- to 25-fold) protein spots in purified late and early endosomal fractions, respectively, in comparison to proteins of the PNS serving as starting fraction (where 1538 spots were detected in total). In addition, the intensity of 286 proteins specifically increased (from two- to ten-fold) in late *vs.* early endosomes. It is important to emphasize here that the major advantage of 2-DE (even if the complexity is reduced by subcellular fractionation) over gel-independent techniques for functional proteomics analysis is still the ability to extract rather easily the proteins of interest from thousands of other species in a biological sample.

Recent progress in proteomics technology has enabled comprehensive profiling strategies of enriched organelle fractions, resulting in identification of hundreds of pro-

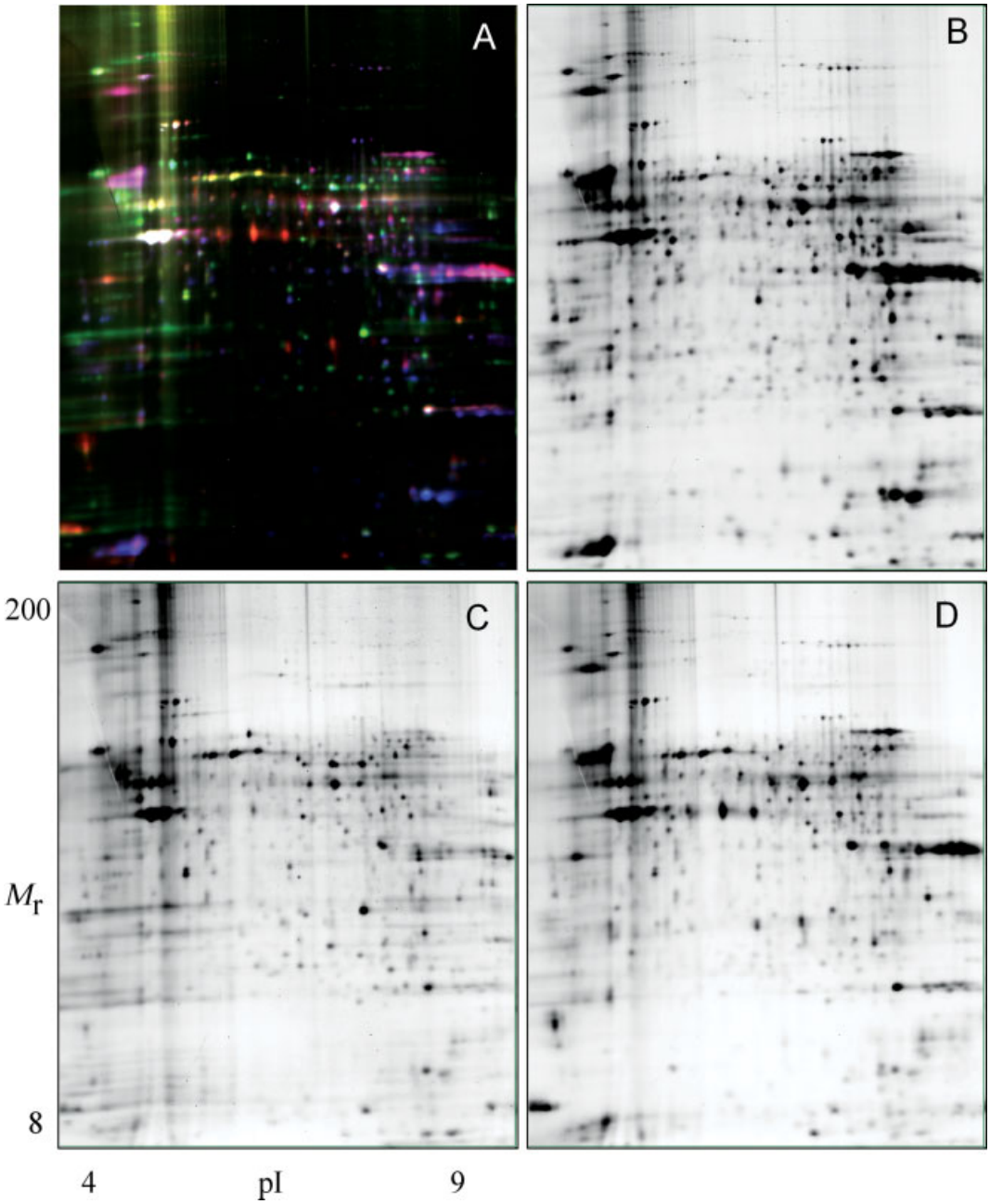

Figure 1. Two-dimensional differential gel electrophoresis (DIGE) of subcellular fractions purified from murine EpH4 cells, merged image (A): PNS (Cy2, blue, B), early (Cy3, green, C) and late (Cy5, red, D) endosomes. Late and early endosomes were purified by sucrose gradient centrifugation [15], 20 μg of protein of each fraction were labeled with CyDye DIGE Fluors according to the manufacturer's recommendations, mixed and separated by 2-DE (3–10 NL IPG strips, 9–16% gradient gel). Labeled proteins were visualized using Typhoon 9410 Imager (Amersham Biosciences, Bucks, UK) and analyzed using DeCyder software (Amersham Biosciences).

teins. Wu *et al.* [16] described an organelle-proteomics analysis in which a stacked Golgi fraction was characterized using multidimensional protein identification technology (MudPIT). The Golgi fraction was enriched from rat liver by classical subcellular fractionation using two different sucrose step gradient centrifugations. Golgi samples were digested to peptides and analyzed by MudPIT using a triphasic chromatography column consisting of

reverse phase, strong cation exchanger and hydrophilic interaction materials. Out of 421 identified proteins many were known Golgi residents (110 proteins), where 64% of these were predicted transmembrane proteins. Proteins localized to other organelles were also identified, strengthening reports of functional interfacing between the Golgi, the endoplasmic reticulum, and cytoskeleton. Two proteins were selected for further analysis, and their Golgi localization was confirmed. One of these, a putative methyltransferase, was shown to be dimethylated arginine, and upon further proteomic analysis, arginine dimethylation was identified on 18 proteins in the Golgi proteome. This organelle profiling study [16] illustrates the utility of proteomics in the discovery of novel organelle functions and resulted in (i) a comprehensive protein profiling of an enriched Golgi fraction; (ii) identification of 41 proteins of unknown function, two with confirmed Golgi localization; (iii) the identification of arginine dimethylated residues in Golgi proteins, generation of a new hypothesis regarding the role of methylation in the Golgi; and (iv) a confirmation of a novel methyltransferase activity within the Golgi fraction.

To isolate peroxisomes from rat liver, Kikuchi *et al.* [17] used classical Nycodenz density gradient centrifugation after homogenization. Organelles were further purified by immunoisolation with anti-PMP70 antibodies (70 kDa peroxisomal membrane protein) bound to magnetic beads. The peroxisomal fraction of high purity was analyzed by SDS-PAGE combined with LC-MS. In addition to several mitochondrial and microsomal proteins that may reside in this fraction 34, known peroxisomal proteins were identified. Furthermore, by treating immunoisolated peroxisomes with Na_2CO_3 at high pH several peroxisomal membrane proteins were identified. With this simple additional fractionation step [17] the authors could identify all 12 known peroxins except for Pex7. One of the two high abundance new peroxisomal proteins of unknown function was a peroxisome-specific isoform of Lon-protease, an ATP-dependent protease with chaperone-like activity. The peroxisomal localization of the protein was confirmed by immunohistochemistry.

Using a combination of subcellular fractionation and 2-D-LC MS/MS Jiang *et al.* [18] have constructed the proteome database for rat liver (564 rat proteins) and its cytosol (222 rat proteins) and mitochondrial fractions (227 rat proteins). Four fractions from rat liver were isolated: a crude mitochondrial and cytosolic fraction obtained by differential centrifugation, a purified mitochondrial fraction obtained by Nycodenz density gradient centrifugation, and a total liver fraction. Identified rat proteins were annotated according to their physicochemical characteristics and functions [18].

2.2 Purification of protein complexes and microdomains

Proteins rarely function in isolation and are often organized in functional units different in size, number of interacting partners and stability (*e.g.*, ranging from huge and rather stable ribosomes or nuclear pores to small and transient signal transduction complexes). Thus, studying multiprotein complexes and microdomains provides important information about the spatio-temporal organization of signal transduction or metabolic processes within a cell (a major part of this information is lost when the "whole cell lysate" or "total protein digest" is analyzed). On the other hand, isolated protein complexes have dramatically reduced complexity, thereby allowing identification not only of low copy number proteins present in the complex, but also to connect them to particular functions. Multiprotein complexes may be isolated and purified by a variety of techniques, *e.g.*, "affinity"-based methods (*e.g.*, coimmunoprecipitation with specific antibodies, epitope-tagged proteins and tandem affinity purification (TAP)), recombinant protein pull-downs, liquid chromatography, blue native gel electrophoresis and free-flow electrophoresis [1, 3, 12, 19, 20]. Subsequently, proteins associated with complexes can be further separated by standard denaturing electrophoresis followed by MS analysis.

The limiting factor for identifying protein complexes is the method used for their separation. A powerful technique called blue native PAGE (BN-PAGE) was reported for the isolation of intact multiprotein complexes [21]. The resolution of this technique is much higher than that of other methods, such as gel filtration or ultracentrifugation [21]. Electrophoretic mobility of protein complexes in the first dimension of BN-PAGE is determined by the intensity of the negative charge of the bound Coomassie and the size of the complex under native conditions. In 2-D BN-PAGE analysis the second separation step is conventional SDS-PAGE, which allows separation and subsequent identification of proteins in the complex by MS. Dialysis of cell lysates prior to BN-PAGE removes low M_r substances, which interfere with BN-PAGE. This simple additional step for sample preparation allows high-resolution separation of cell lysates. Different multi-protein complexes can be visualized by immunoblotting and identified by MS, showing a wide potential of this method for functional proteomics [21].

Two common methods have been used by Foster *et al.* [22] to isolate membrane microdomains with distinct lipid and protein composition. These microdomains are termed lipid rafts and are biochemically characterized by their resistance to either high pH or nonionic detergents. Foster *et al.* have separated lipid rafts from other

membranes by treatment with either high pH or nonionic detergents and subsequent density gradient centrifugation. Detergent resistance is the much more widely used of the two [23], however, both methods are plagued by contamination from nonraft proteins. This problem was overcome by applying a new method in quantitative proteomics, stable isotope-labeling with amino acids in cell culture (SILAC) [22], to directly determine the subset of cholesterol-dependent proteins in the biochemical preparation. Quantitative high-resolution MS has been used to specifically detect proteins depleted from rafts by cholesterol-disrupting drugs. These results provide large-scale and unbiased evidence for the connection of rafts with cellular signaling. In total, 703 proteins were identified in detergent-resistant fractions and 585 in carbonate-resistant fractions. Of the 703 detergent-resistant proteins, 392 were quantifiable and revealed 241 authentic raft proteins. A large proportion of signaling molecules, highly enriched *versus* total membranes and detergent-resistant fractions has been detected. Interestingly, amongst the identified raft and raft-associated proteins are a significant number of serine/threonine kinases/phosphatases as well as numerous heterotrimeric G protein subunits, suggesting that rafts may be more general signaling coordinators. Very interesting is comparative analysis of this data with previous publications on the proteome of lipid rafts. Less than half of the 19 proteins in a detergent-resistant fraction from Jurkat T cells reported in [23] and about two-thirds of 70 proteins identified in [24] were found to be authentic raft proteins, however, these new data suggest that the remaining ones might be false positives. These data also indicate that the carbonate-resistant preparation is less specific for raft protein isolation and its interpretation is more difficult than that of the detergent-resistant method [25].

Sprenger *et al.* [26] isolated caveolin-enriched membranes by either cationic silica affinity purification or buoyant density methods. They further analyzed more than 100 protein spots in these fractions by comparing a large series of 2-D gel maps and subsequent MALDI-TOF peptide mass fingerprinting. Improved representation and identification of membrane proteins and valuable information on various post-translational modifications were achieved by the optimized procedures for solubilization, destaining and database searching presented above. Whereas the cationic silica purification yielded predominantly known endoplasmic reticulum residents, the cold-detergent method yielded a large number of known caveolae residents, including caveolin-1. Thus, a large part of this subproteome was established, including known membrane, signal transduction and glycosyl phosphatidylinositol (GPI)-anchored proteins.

The molecular complexity of tissues and the inaccessibility of most cells within a tissue limit the discovery of key targets for tissue-specific delivery of therapeutic and imaging agents *in vivo*. Key features of a recent study by Oh *et al.* [27] included tissue subfractionation with subtractive proteomics and bioinformatic analyses that both together reduced tissue complexity by more than five orders of magnitude and unmasked a manageable subset of proteins at the inherently accessible blood-tissue interface. The authors used an affinity-based isolation procedure to enrich and purify parts of blood vessel endothelial cells that contact the blood in organs including rat lung and lung tumors. They accomplished this by infusing colloidal silica particles into the bloodstream of rats, where these particles attached to the endothelial cells. Subsequent centrifugation of tissue homogenates allowed endothelial cell membranes and attached caveolae to be separated from the remainder of the cells. For the final purification step, an antibody that recognizes caveolin, coupled to magnetic beads, was used to isolate caveolae and their associated proteins. Purified caveolae displayed a greater than 20-fold enrichment for specific markers. They were analyzed by 2-DE to produce high-resolution vascular endothelial protein maps of the major rat organs. Thirty-seven proteins identified by this approach were present only in the endothelial membrane; 11 of these possess probably an extracellular portion that could be presented to blood cells. Expression profiling and gamma scintigraphic imaging with antibodies suggested two of these proteins, aminopeptidase-P and annexin A1, as selective *in vivo* targets for antibodies in lungs and solid tumors, respectively. Radioimmunotherapy targeted against annexin A1 selectively decreased tumor size and increased animal survival [27]. This analytical strategy can map tissue- and disease-specific expression of endothelial cell surface proteins to uncover novel accessible targets, useful to design unique molecular tools for organ-specific therapy.

2.3 Sequential extraction method

A very simple fractionation protocol following the homogenization of cells represents centrifugation of the PNS at $100\,000 \times g$, which separates total membrane fraction from cytosol. Peripheral membrane proteins can then easily be extracted from the membrane pellet in 0.1 M sodium carbonate, pH 11.0 [28]. The remaining integral membrane proteins can be analyzed directly [28]. Alternatively, Triton X-114 phase partitioning can be applied to enrich for the integral membrane protein fraction [29]. Figure 2 demonstrates extraction of peripheral proteins from the total membrane fraction and a comparison of extracted proteins with the cytosolic ones by the 2-D

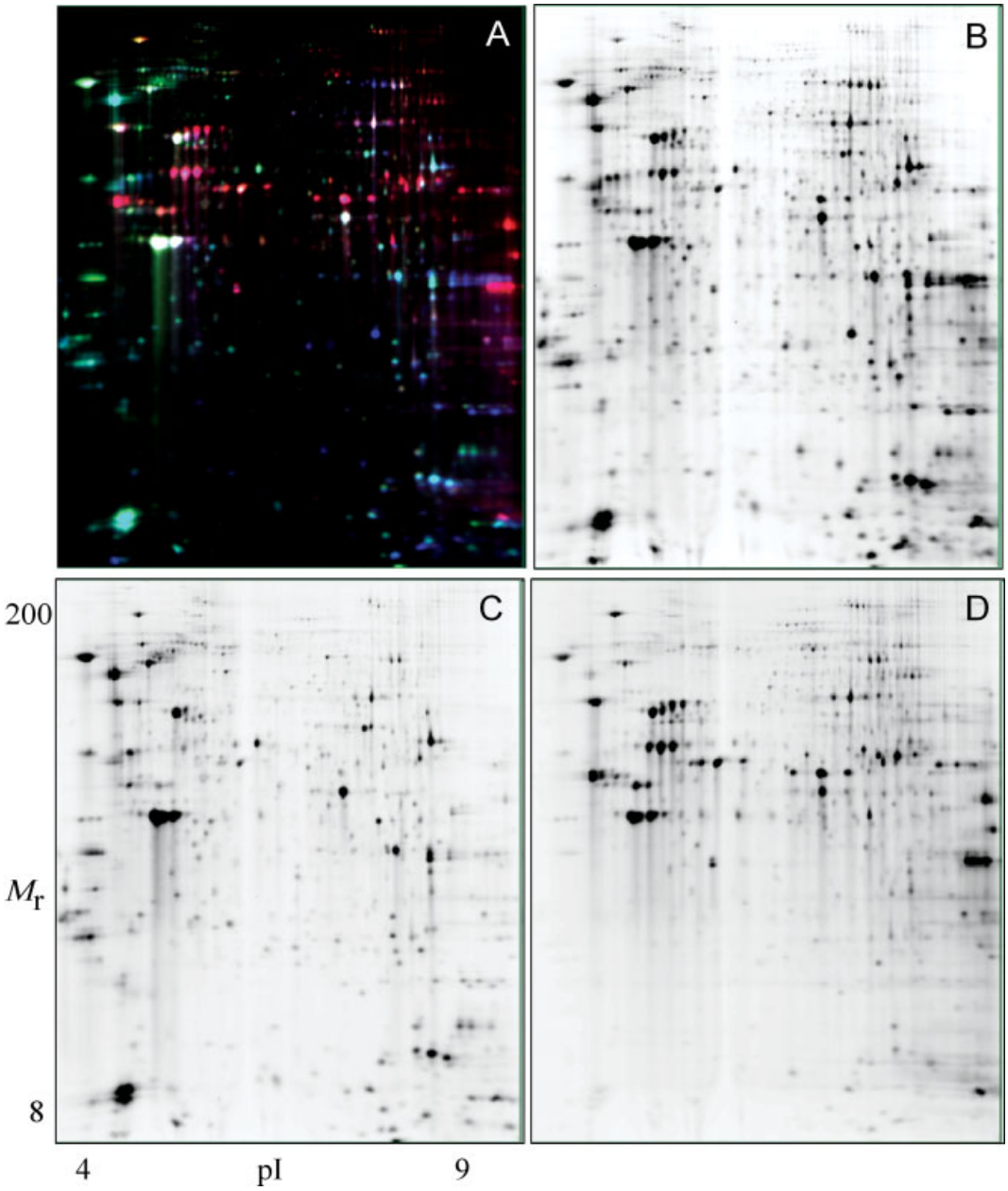

Figure 2. Two-dimensional differential gel electrophoresis (DIGE) of subcellular fractions purified from murine EpH4 cells, merged image (A): PNS (Cy2, blue, B), cytosol (Cy3, green, C), peripheral membrane proteins (Cy5, red, D). Peripheral membrane proteins were isolated by sodium carbonate extraction [28], 50 μg of protein of each fraction were labeled with CyDye DIGE Fluors according to the manufacturer's recommendations, mixed and separated by 2-DE (3–10 NL IPG strips, 9–16% gradient gel). Labeled proteins were visualized using Typhoon 9410 Imager and analyzed using DeCyder software.

DIGE method. In total 2553 protein spots were detected in the mixed sample of PNS, cytosolic and peripheral membrane proteins. However, 441 protein spots were specifically enriched (more than two-fold) by carbonate extraction. Interestingly, many low abundant and cytosolic proteins out of the 252 enriched (from 2- to 15-fold) could be detected by the 2-D DIGE method after purification of cytosol from PNS, indicating that more abundant membrane/organelle proteins masked them before fractionation.

Homogenization techniques employed for isolation of organelles usually require relatively large amounts of starting material. Alternative approaches are based on differential detergent extraction methods that enable simple fractionation of the total proteome into distinct subcellular fractions, *e.g.*, cytosolic, cytoskeletal, membrane, and nuclear proteins (reviewed in [30]). This method has the advantage of preserving the integrity of cytoskeletal networks, and is especially useful when the quantity of cells is limited. For adherent cells the extraction can be performed directly on coverslips without the need for cell removal, hence preventing undesirable destruction of cellular structure.

Abdolzade-Bavil *et al.* [31] described recently an optimized sequential extraction method, originally reported in [32]. Fractionation of proteins in their native state according to their subcellular localization yielded four subproteomes enriched in: (i) cytosolic proteins; (ii) membrane and organelle-associated proteins; (iii) soluble and DNA-associated nuclear proteins, and (iV) cytoskeletal proteins, respectively. Four extraction buffers of appropriate ionic and osmotic composition containing defined surfactants enabled stepwise disintegration of cells and selective extraction of certain subcellular compartments. Upon treatment with the first extraction buffer, cells release their cytoplasmic content but remain intact in their overall structure. After the second extraction step, membranes and membrane organelles are solubilized, but nuclei and the cytoskeleton remain intact. The treatment of the residual material with the third extraction buffer solubilizes the nuclear proteins. Finally, the cytoskeleton components are liberated during the fourth extraction. Efficiency and selectivity of this subcellular extraction procedure was demonstrated by fluorescence and phase contrast microscopy, 2-DE, immunohistochemistry and enzymatic analysis. The subcellular extraction method allows the assessment of spatial rearrangements of signaling proteins, which was demonstrated on signal-dependent redistribution of phosphorylated mitogen activated protein kinase (MAPK) and nuclear factor kappa B (NFκB) between cytoplasm and nucleus [31].

Elortza *et al.* [33] have presented recently a general MS-based proteomic "shave-and-conquer" strategy that targets specifically glycosylphosphatidylinositol-anchored proteins (GPI-APs). These proteins have attracted attention because they act as enzymes and receptors in cell adhesion, differentiation and host-pathogen interactions and are potential diagnostic and therapeutic targets. Raft-enriched membranes of human HeLa cells were purified by homogenization of cells and ultracentrifugation in sucrose gradients. After extraction of peripheral membrane proteins by sodium carbonate, lipid rafts were obtained from membrane fraction by two-phase separation in the presence of Triton X-114 [33]. Additionally, microsomal membranes have been purified from *Arabidopsis thaliana* [33]. The isolated membrane fractions were treated with phosphatidylinositol phospholipase C (PI-PLC), which hydrolyzes phosphatidylinositol, releasing the soluble GPI protein from membrane/detergent phase and enabling its recovery in the aqueous phase. Proteins isolated this way were separated by SDS-PAGE and identified by MS. After computational sequence analysis, to eliminate false assignments, six GPI-APs were identified in a *Homo sapiens* lipid raft-enriched fraction and 44 GPI-APs in an *A. thaliana* membrane preparation, representing the largest experimental dataset of GPI-anchored proteins to date [33]. This study demonstrates that membrane fractionation methods in combination with PI-PLC treatment enable significant enrichment of a range of GPI-anchored proteins from human and plant cells.

3 Enrichment strategies

Most techniques currently used in proteomics combine a variety of fractionation and separation steps prior to analysis by MS. Separation steps can be used at the protein level, as well as at the peptide level. Typical experiments include affinity separation methods, 1-D or 2-DE, and 1-D or 2-D chromatographic separation.

3.1 Phosphoprotein analysis

3.1.1 Phosphospecific antibodies

Antibodies specific to phosphorylated amino acids can be used to enrich phosphoproteins by immunoprecipitation from complex cell lysates. In several phosphoproteomics studies effective enrichment of tyrosine-phosphorylated proteins has been used as the first fractionation step prior to immobilized metal affinity chromatography (IMAC) [34, 35], 2-DE [36–38] or 1-D SDS-PAGE [39, 40]. A very limited number of studies were performed using antiphosphoserine/threonine antibodies because of their low specificity [41].

Recently Stannard *et al.* [42] reported a new method for the extraction and fractionation of the phosphoproteome, which has revealed a significant increase in the number of phosphoproteins detected by 2-DE. Three classes of proteins phosphorylated on tyrosine, serine and threonine were individually isolated from human lung fibroblasts stimulated with endothelin-1 using agarose columns with attached anti-phospho-Tyr, phospho-Ser, and phospho-

Thr antibodies. Each of the three classes of extracted phosphoproteins was separated using 2-DE. Extraction of the phosphoproteins led to substantial simplification of the protein patterns and enrichment of low abundant phosphoproteins that were not detectable on 2-D gels of total protein extracts. Overall, about 1500 distinct phosphoprotein spots could be detected on the silver stained gels [42].

A new and very promising alternative to phosphospecific antibodies is a commercially available phosphoprotein purification system, which utilizes a phospho-affinity step to isolate the intact phosphoproteins. Metodiev *et al.* [43] applied this affinity capture in combination with tandem matrix-assisted laser desorption/ionization mass spectrometry to probe signal-induced changes in the phosphoproteome of human U937 cells. Purified phosphoproteins were subsequently characterized by electrophoresis and identified by direct *de novo* sequencing using MS/MS. The capture step ensures minimal interference from nonphosphorylated proteins in all subsequent analyses. Moreover, because phosphoproteins constitute only about 10% of the total cellular proteins, this technique should increase the overall sensitivity by at least one order of magnitude, and thereby enhance the detection of low abundant phosphoproteins. Additionally, a combination of two affinity steps, such as this phosphoprotein purification system and subtractive immunoprecipitation with highly specific anti-phospho-antibodies, could be suitable to separate distinct groups of phosphorylated target proteins.

3.1.2 Phosphopeptide enrichment by immobilized metal ion affinity chromatography (IMAC)

Isolation/enrichment of phosphorylated peptides from the protein digest is a crucial step for successful peptide sequencing and identification of phosphorylation sites by MS. The methodology for profiling tyrosine phosphorylation, considered herein as the assignment of multiple protein tyrosine phosphorylation sites in a single analysis, was reported recently [34]. The authors described a sensitive approach based on multidimensional LC-MS that enables the rapid identification of numerous sites of tyrosine phosphorylation on a number of different proteins from human whole cell lysates. The technology platform included the use of immunoprecipitation, IMAC, LC, and MS/MS. This methodology was used to follow changes in tyrosine phosphorylation patterns occurring either during the activation of human T cells or inhibition of the oncogenic BCR-ABL fusion product in chronic myelogenous leukemia cells in response to the treatment with STI571 (Gleevec, Novartis, Basel, Switzerland). Together, these experiments rapidly identified 64 unique sites of tyrosine phosphorylation on 32 different proteins. Half of these sites have been documented in the literature, validating the merits of the approach [34], whereas motif analysis suggests that a number of the previously undocumented sites are also potentially involved in biological pathways.

Another report from the same group [35] showed that when using complex mixtures of peptides from human cells, methylation improved the selectivity of IMAC for phosphopeptides and eliminated the acidic bias that occurred with nonmethylated peptides. The IMAC procedure was significantly improved by desalting methylated peptides, followed by gradient elution of the peptides to a larger IMAC column. These improvements resulted in assignment of approximately three-fold more tyrosine phosphorylation sites from human cell lysates, than were uncovered by the previous methodology. Nearly 70 tyrosine-phosphorylated peptides from proteins in human T cells were assigned in single analyses [35]. These proteins had unknown functions or were associated with a plethora of fundamental cellular processes. This robust technology platform should be broadly applicable to profiling the dynamics of tyrosine phosphorylation.

In order to identify serine- and threonine-phosphorylated proteins on a proteome-wide basis, Shu *et al.* [44] treated WEHI-231 cells with calyculin A, a serine/threonine phosphatase inhibitor, to induce high levels of protein phosphorylation. Phosphorylated peptides were enriched from a tryptic digest using IMAC and identified by LC-MS/MS. A total of 107 proteins and 193 phosphorylation sites were identified using these methods. Forty-two of these proteins have been reported to be phosphorylated, but only some of them have been detected in B cells. Fifty-four of the identified proteins were not previously known to be phosphorylated. The remaining 11 phosphoproteins have previously only been characterized as novel cDNA or genomic sequences. Many of the identified proteins were phosphorylated at multiple sites. The proteins identified in this study significantly expand the repertoire of proteins known to be phosphorylated in B cells [44].

Since the first introduction of IMAC [45] several different materials based on agarose, sepharose, polystyrene, silica or cellulose have been used and many of them are commercially available from several suppliers. Iminodiacetic and nitrilotriacetic are two functional groups commonly used to chelate metal (Fe^{3+} or Ga^{3+}) ions. The retention of phosphopeptides to immobilized metal ions is based on electronic interactions, therefore, other acidic peptides can also bind to these surfaces. Selectivity of IMAC can be effectively improved by methyl esterification of carboxyl groups on aspartic and glutamic amino acid residues [46]. IMAC is widely used for phosphopeptide enrichment with different degrees of success, which depends mainly on the complexity of protein samples as

well as sample preparation and the quality of the IMAC material. Recent developments on IMAC-optimized protocols for methyl esterification [35], synthesis of new IMAC materials based on cellulose [47] and glycidyl methacrylate/divinylbenzene (Aprilita *et al.*, submitted for publication) and new open tubular (OT-IMAC) methods [48], where the functional groups are attached directly to the inner surface of a glass tube, significantly improve the selectivity and the reliability of the IMAC method. These technical advances, especially in combination with other fractionation strategies, *e.g.*, strong anion exchange chromatography [49], are expected to make possible the specific isolation of phosphopeptides from complex mixtures for large-scale phosphoproteome analysis.

3.1.3 Isolation of chemically modified peptides

Chemical derivatization of the modifying group potentially allows attachment of a tag for affinity purification. It should be noted, however, that only very simple and extremely efficient chemical derivatization steps are compatible with proteomics. If any heterogeneity is introduced by the chemical reaction, the peptide samples become even more complex and then it is possible to analyze only modifications of the most abundant proteins.

Several methods have been reported that use chemical modification of the phosphate moiety as a strategy to enrich phosphopeptides from complex mixtures. Oda *et al.* [50] designed a strategy in which the phosphate group on serine and threonine was replaced with ethanedithiol by a beta-elimination and Michael addition reaction followed by introduction of a biotin-containing tag. Biotinylated peptides could be selectively captured using immobilized streptavidin. Phosphorylated serine residues undergo this reaction quite easily whereas it is not as reliable for threonine residues. This method [50] does not distinguish between *O*-glycosylated and phosphorylated serine/threonine residues, therefore, requiring additional experiments to confirm phosphorylation.

Recently, an improved and more sensitive method for beta-elimination based phosphopeptide enrichment has been demonstrated [51], where the incorporated thiol group is used as the ligand for affinity purification. A nonspecific side reaction of the beta-elimination chemistry was described, in which non-phosphorylated serine residues were modified by the affinity tag at the level up to 2%. Despite the presence of the side reaction, the strategy was shown to be effective at enriching phosphopeptides from complex peptide mixtures and *in vitro* phosphorylated proteins, resulting in the identification of new phosphorylation sites.

An elegant approach for mapping sites of protein phosphorylation has been proposed by Knight *et al.* [52], which is also based on beta-elimination transformation of phosphoserine and phosphothreonine residues into lysine analogs (aminoethylcysteine and beta-methylaminoethylcysteine, respectively), which can be cleaved then by Lys-C or trypsin. This reaction has been adapted to the solid phase providing phosphopeptide enrichment and modification in one step. Using a mixture of synthetic peptide capture and modification of phosphoserine peptides has been shown to be highly selective. This interesting approach needs to be optimized for more complex protein samples.

The method, developed by Zhou *et al.* [53], is applicable to phosphotyrosine-containing peptides in addition to those containing phosphoserine and phosphothreonine residues. A more complex reaction scheme is used to capture phosphorylated peptides on a solid support containing immobilized iodoacetyl groups. This approach requires several chemical reactions and purification steps before MS analysis, which could lead to substantial losses of analyzed material. In general chemical modification-based approaches require rather large amounts of sample, therefore, only abundant proteins are easily identified. However, an improved protocol [51] has increased the sensitivity to the subpicomolar level. Chemical approaches coupled to other fractionation steps could improve recovery of low abundance proteins.

3.2 Glycoprotein analysis

Lectins are carbohydrate-binding proteins that recognize specific carbohydrate structures, and they can be used to enrich for glycoproteins and glycopeptides. Lectins such as Concanavalin A (Con A) and wheat germ agglutinin (WGA) have been widely used in glycoprotein research [54, 55]. In addition to the advantages of reducing the complexity of samples, the specificity of different lectins for different sugar moieties may indicate the important features of carbohydrate chains on glycoproteins.

Bunkenborg *et al.* [56] demonstrated recently a procedure for mapping *N*-glycosylation sites in complex mixtures by reducing sample complexity and enriching glycoproteins. Glycosylated proteins were selected by an initial lectin chromatography step and digested with endoproteinase Lys-C. Glycosylated peptides were then selected from the digest mixture by a second lectin chromatography step. The glycan components were removed with *N*-glycosidase F and the peptides digested with trypsin before analysis by on-line reversed-phase LC-MS. Using Con A and wheat germ agglutinin, 86 *N*-glycosylation sites in 77 proteins were identified in human serum [56].

Lectin-based affinity enrichment of glycopeptides in combination with glycosidase-catalyzed ^{18}O stable isotope labeling and MS/MS allowed isolation, detection and sequencing of *N*-glycosylated peptides in another study. This method revealed 400 *N*-glycosylation sites in 250 glycoproteins in a Caenorhabditis elegans protein extract [57].

3.3 Affinity purification of ubiquitinated proteins

Ubiquitination of membrane-associated proteins can direct their proteasome-mediated degradation or activation at the endoplasmic reticulum (ER), as well as their endocytosis and intracellular sorting. Hitchcock *et al.* [58] combined proteomics analysis with yeast genetics to identify 211 ubiquitinated membrane-associated proteins in *Saccharomyces cerevisiae* and mapped precisely more than 30 ubiquitination sites. Major classes of identified ubiquitinated proteins include ER-resident membrane proteins, plasma membrane-localized permeases, receptors, enzymes and components of the actin cytoskeleton. Hence, 83 of these identified ubiquitinated membrane proteins were identified as potential endogenous substrates of the ER-associated degradation (ERAD) pathway. These substrates are highly enriched for proteins that localize to or transit through the ER. Interestingly, several novel membrane-bound transcription factors were identified that may be subject to ubiquitin/proteasome-mediated cleavage and activation at the ER membrane.

The methodology described by Peng *et al.* [59] provides a general tool for large-scale analysis and characterization of protein ubiquitination. Ubiquitin conjugates from a *S. cerevisiae* strain expressing 6xHis-tagged ubiquitin were isolated, proteolyzed with trypsin and analyzed for amino acid sequence determination by multidimensional liquid chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS). 1075 proteins in total have been identified and 110 precise ubiquitination sites were found in 72 ubiquitin-protein conjugates. Finally, ubiquitin itself was modified at seven lysine residues providing evidence for unexpected diversity in polyubiquitin chain topology *in vivo*.

4 Fractionation of proteins and peptides according to their physicochemical properties

4.1 Chromatographic protein prefractionation

Different classical chromatographic approaches have been successfully used to prefractionate crude protein extracts for proteomics studies. Ion exchange chromatography, size-exclusion chromatography, hydrophobic interaction chromatography and affinity chromatography can serve as powerful tools for protein separation from total cell lysates or subcellular fractions into distinct groups with different physicochemical properties, *e.g.*, surface charge (ion exchange chromatography), molecular mass of proteins and protein complexes (gel filtration), hydrophobicity (hydrophobic interaction chromatography), and according to differences in affinity to particular compounds (affinity chromatography), reviewed recently in detail [5, 7].

4.2 Preparative IEF

A number of techniques are available now for fractionation of proteins according to their isoelectric characteristics, *e.g.*, several devices for electrophoretic prefractionation on IEF steps and their applications were reviewed recently [7] and will, therefore, not be discussed here. Recently, one of these instruments has been used for prefractionation IEF to examine alkaline proteins [60]. The genome of *Helicobacter pylori* is dominated by genes encoding basic proteins, and is therefore a useful model for examining methodology suitable for separating such proteins. Proteins were separated into two fractions using Gradiflow technology (Gradipore, Frenchs Forest, Australia), and the extremely basic fraction subjected to both SDS-PAGE and LC-MS/MS post-tryptic digest. This experimental approach allowed the identification of 17 proteins with p*I* > 9.0 [60].

Görg *et al.* [6] developed recently a simple prefractionation procedure based on IEF in granulated Sephadex gels. Complex protein mixtures were prefractionated in Sephadex gels, containing urea, thiourea, zwitterionic detergent (CHAPS), DTT and carrier ampholytes of pH range 3–10, *i.e.*, very similar to the standard for 2-DE sample buffer. After IEF, up to ten gel fractions alongside the pH gradient were separated and directly applied onto the corresponding narrow range IPG strips as first dimension of 2-DE. This technology has been successfully applied for prefractionation of mouse liver proteins. The major advantages of it are highly efficient transfer of the prefractionated protein into the IPG strips and its compatibility with subsequent 2-DE analysis. This prefractionation dramatically reduces sample complexity, allowing loading of higher protein amounts for systematic analysis of fractions with narrow p*I* range, thereby facilitating the detection of low abundant proteins.

4.3 1-D SDS-PAGE – LC-MS/MS

The powerful alternative to size-exclusion chromatography for fractionation of proteins according to molecular mass is a combination of protein separation by 1-D SDS-PAGE and peptide fractionation with identification by LC-

MS/MS. Proteins from complex mixtures, subcellular fractions *e.g.*, purified organelles or affinity enriched protein fractions, can be separated by SDS-PAGE with subsequent gel slicing, digestion of gel slices with trypsin or other enzymes and analysis of the resulting peptides using LC-MS/MS. A big advantage of this approach is that 1-D SDS-PAGE is a well established and highly reproducible method for protein separation under denaturing conditions in a broad molecular mass range (*e.g.*, from 7–250 kDa in gradient gels). Alternatively, using linear gels one can focus on proteins of a certain molecular weight range. Gels can be sliced corresponding to molecular mass markers into several well-defined fractions. Another interesting advantage of this approach is the possibility to stain protein bands, separate gel slices containing more abundant protein bands from those less abundant and analyze them separately. This approach can increase the possibility of identification of low copy number proteins.

Taylor *et al.* [61] described an approach to elucidate the mitochondrial proteome by a combination of several fractionation methods: subcellular fractionation to purify human heart mitochondria by differential and gradient centrifugation: (i) sucrose density gradient fractionation to separate intact protein complexes; (ii) followed by a separation of obtained 12 fractions by 1-D SDS-PAGE; and (iii) protein identification by peptide mass fingerprinting (PMF) by MALDI-TOF mass spectrometer [62] linked to LC-MC/MS [61]. Total in-gel processing (gradient gel, 65 gel slices for each fraction) of partially resolved protein complexes and subsequent detection by MS and bioinformatic analysis yielded a database of 615 mitochondrial and mitochondrial-associated proteins [61].

The most recent demonstration of an application of this technology is large-scale characterization of HeLa cell nuclear phosphoproteome [63]. These authors used a strategy which combined different protein as well as peptide fractionation methods, such as subcellular fractionation, preparative SDS-PAGE, strong cation-exchange (SCX) chromatography with subsequent reverse-phase chromatography – MS/MS. HeLa cell nuclear proteins (8 mg) were separated in a gradient SDS-PAGE. The entire gel was then cut into ten regions and subjected to in-gel digestion with trypsin followed by phosphopeptide enrichment by off-line SCX chromatography. Such a strategy exploits the difference between the charge of tryptic phosphorylated and nonphosphorylated peptides. Because tryptic peptides contain Lys or Arg at the *C*-terminus most of them have at pH 2.7 a charge of 2^+ in SCX solvents. At acidic pH the phosphate group maintains a negative charge. Therefore, after single phosphorylation the charge state of the posphopeptide is 1^+. SCX chromatography separates peptides primarily based on their charge, and therefore phosphopeptides containing a single basic group elute first and are highly enriched. When early eluting fractions, containing mainly monophosphorylated peptides (charge state of 1^+), were subjected to reversed-phase LC with on-line sequence analysis by MS/MS, 2002 phosphorylation sites from a totall of 967 proteins were determined [63]. Interestingly, all detected sites were exclusively phosphorylated Ser and Thr. This study represents the largest data set of PTMs reported so far.

4.4 Peptides: 2-D LC-MS/MS

An alternative or even complementary step to protein fractionation is pre-fractionation at the peptide level. Proteins are digested in solution and resulting peptides are separated using 2-D chromatography: in the first dimension according to their charge (typically SCX chromatography) and in the second dimension according to hydrophobicity by reversed-phase chromatography. The latter column is directly coupled through ESI with the tandem mass spectrometer [64]. This approach is known also as multidimensional protein identification technology (MudPIT) [65], multidimensional chromatography coupled to tandem mass spectrometry (LC/LC-MS/MS) [66] or shotgun proteomics.

Recently, several large-scale proteome studies have been published using this approach. Washburn *et al.* [65] optimized the DALPC system (direct analysis of large protein complexes) developed by Link *et al.* [64] and carried out an analysis of yeast proteome by the MudPIT method. The excessive capacity of the matrix of a single SCX-RP biphasic column and fully automated 15-step multidimensional chromatography analysis enabled the identification of low abundant transcription factors and kinases. All together 1484 yeast proteins (5540 peptides) were detected and identified [65]. A principal advantage of this on-line approach is automation and high-throughput.

A more recent publication on yeast proteomics utilized a similar 2-D off-line approach prior to MS/MS [66]. An off-line approach has increased loading capacity and better peptide separation (80 fractions in this study) and is more flexible. A total of 1504 proteins (7537 peptides) were unambiguously identified in this single analysis. The total number of identified proteins in both of these publications seems to be very close to the resolution limit of such an approach, suggesting a requirement of additional protein or peptide separation methods to reduce the complexity of an entire proteome. To overcome the limitations of the MudPIT method due to the presence of high abundance proteins and limited chromatographic resolution, protein prefractionation with fast performance liquid chromatography (FPLC) has been successfully used [67].

Durr *et al.* [68] presented recently a comprehensive proteomic *in vivo* investigation of luminal endothelial cell plasma membranes isolated from rat lungs. Using the MudPIT method 450 proteins were identified, 29% of them were signaling proteins and 26% were proteins with unknown function. Comparative proteomics analysis revealed that 41% of the proteins expressed *in vivo* were not detected in cultured rat lung microvascular endothelial cells *in vitro*, suggesting that distinct protein expression is apparently regulated by the tissue microenvironment, and is therefore different in cell culture [68]. In addition, a very useful estimation of the reproducibility and relative comprehensiveness of MudPIT is presented in this study [68]. Statistical analysis revealed that 7–10 MudPIT measurements are necessary to achieve $\geq 95\%$ confidence of analytical completeness (number of measurements required to achieve a statistically defined level of completeness) with the equipment, database and analytical approach used in this study [68].

Gaucher *et al.* [69] presented results of a study of the mitochondrial proteome by three different methods. The sucrose density gradient protein fractionation combined with 1-D SDS-PAGE separation and LC-MS/MS (see [61], described in Section 4.3) provided the greatest proteome coverage in terms of the total number of identified proteins as well as the dynamic range and functional classification when compared to two different MudPIT methods. These results emphasize that different proteomics methods are complementary to each other and in combination provide information about different subproteomes of complex protein samples.

5 Concluding remarks

The main task of many proteomics studies in recent years has been to develop strategies for the separation of low abundant regulatory proteins and corresponding peptides in quantities sufficient for identification by mass spectrometry. Subcellular fractionation allows access to intracellular organelles and multiprotein complexes; low abundant proteins and signaling complexes can be enriched, and at the same time complexity of the sample can be reduced. Analyzing subcellular fractions and organelles allows also tracking proteins that shuttle between different compartments. Protein and peptide fractionation technologies have the advantage of simplifying the complexity of crude cell or tissue extracts. Rational combination of different fractionation strategies such as subcellular fractionation, affinity enrichment of certain classes of proteins and protein complexes, chromatographic protein fractionation into distinct groups with different physicochemical characteristics could provide a realistic approach to deeper and more sensitive proteome analysis. Furthermore, subsequent chromatographic fractionation of peptides obtained by protein digestion using similar strategies, but on a peptide level (2-D or multidimensional chromatography) can significantly reduce sample complexity and increase separation efficiency thereby maximizing the probability of identification of low abundance proteins in the mass spectrometer.

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