Organelle Proteomics: Implications for Subcellular Fractionation in Proteomics

Lukas A. Huber, Kristian Pfaller, Ilja Vietor

Abstract—Functional proteome analysis is not restricted to the sequence information but includes the broad spectrum of structural modifications and quantitative changes of proteins to which they are subjected in different tissues and cell organelles and during the development of an organism. Cell biology has provided the means required for the analysis of the composition and properties of purified cellular elements. Subcellular fractionation is an approach universal across all cell types and tissues, including cardiac and vascular system. Subcellular fractionation and proteomics form an ideal partnership when it comes to enrichment and analysis of intracellular organelles and low abundant multiprotein complexes. Subcellular fractionation is a flexible and adjustable approach resulting in reduced sample complexity and is most efficiently combined with high-resolution 2D gel/mass spectrometry analysis as well as with gel-independent techniques. In this study we introduce state of the art subcellular fractionation techniques and discuss their suitability, advantages, and limitations for proteomics research. (Circ Res. 2003;92:962-968.)

Key Words: proteomics ■ subcellular fractionation ■ organelle

The challenge of proteome analysis lies clearly with the task of achieving a combination of high-throughput screening while maintaining high sensitivity for the detection of low copy number proteins. There is no amplification step for proteins, analogous to the polymerase chain reaction method for amplifying DNA or RNA. This means that high abundant proteins without fractionation and enrichment of biological samples muffle low abundant proteins. Most regulatory proteins such as kinases, phosphatases, or GTPases exist in low copy numbers but very specific subcellular localization. In addition, because of the complexity of eukaryotic cells, a single-step characterization of an entire proteome seems at least presently rather unfeasible. Present estimates of the number of genes in the human genome expressed in a particular cell type reach 10 000. However, the number of proteins in the entire human body is expected to be many times higher. Thousands of chemical modifications occur after proteins are created that alter their enzymatic activity, binding ability, how long they remain active, and so on. These modifications and the still-underestimated rate of alternative splicing give rise to a human proteome size that is likely to be significantly larger than the number of estimated genes.1
Subcellular fractionation, allowing the separation of organelles based on their physical properties, was initially applied to separate organelles derived from rat liver. Subcellular fractionation consists of two major steps, disruption of the cellular organization (homogenization) and fractionation of the homogenate to separate the different populations of organelles. Such a homogenate can then be resolved by differential centrifugation into several fractions containing mainly (1) nuclei, heavy mitochondria, cytoskeletal networks, and plasma membrane; (2) light mitochondria, lysosomes, and peroxisomes; (3) Golgi apparatus, endosomes and microsomes, and endoplasmic reticulum (ER); and (4) cytosol. Each population of organelles is characterized by size, density, charge, and other properties on which the separation relies.

However, two major problems have impeded the development of standardized and ready-to-go procedures for subcellular fractionation. First, differential subcellular compartments share similar physical properties and cofractionate at least to some extent in conventional gradients. Second, tissue culture cells are now more commonly used for fractionation, because cells can be manipulated in a manner impossible to achieve in animal-derived tissue. However, after homogenization, tissue culture cells are more difficult to fractionate than most tissues, presumably because of differences in the cytoskeletal organization. It is essential to point out that complete purification is, with few exceptions, hardly possible. However, it is still very powerful, and many laboratories began to apply traditional subcellular fractionation procedures within proteome studies.

The objective of this review was to highlight simple but very useful subcellular fractionation techniques that can be easily combined with proteomics technologies. We will discuss how to assess the quality of such preparations and their limitations and advantages.

How To Get Started

One major limitation in the successful fractionation of tissue culture cells is the production of an ideal homogenate, that is, the release of organelles and other cellular constituents as a free suspension of intact, individual components. Very often cytoplasmic aggregates are observed, which contain cytoskeletal elements as well as various organelles. Consequently, organelles remain associated with the cytoskeletal elements surrounding the nuclei or become entrapped in large aggregates, which readily sediment. Potential sources for aggregate formation are nuclei, which break under harsh homogenization conditions and subsequently release DNA. This in turn results in significant loss of components of the homogenate during the initial centrifugation step for removal of nuclei. Because the cytoplasmic and cytoskeletal organization of different tissue culture cells varies enormously, homogenization conditions must be optimized for each cell line.

First, cells are cooled down on ice and scraped gently with a soft rubber policeman into Ca\(^{2+}\)/Mg\(^{2+}\)-containing PBS to prevent cell breakage (Figure 1, step 1). Cells are then collected by a low-speed centrifugation step and mechanically homogenized (Figure 1, step 2). The quality of the homogenization should be assessed by morphological means; eg, by phase-contrast microscopy, it is possible to assess the extent of cellular disruption, ie, the appearance of unbroken nuclei and the absence of large aggregates or intact cells. Taking these precautions into consideration, one can assume that after homogenization, the nuclei are totally removed by a low-speed centrifugation step (Figure 1, step 3), together with cell debris, unbroken cells, and some larger subcellular components (Figure 2A). For additional analysis, nuclei can be purified from the pellet fraction (Figure 2B). The postnuclear supernatant (PNS) contains the cytosol and the other organelles in free suspension, which can be subsequently separated by gradient centrifugation or other techniques. Detailed experimental protocols can be found as a free download from our Web site (http://www2.ubik.ac.at/ah/).
all soluble proteins (Figure 1, steps 4 and 5). This method is very robust and can also be used with small sample volumes in so-called tabletop ultracentrifuges or even in mini-rotors with a conventional airfuge. This simple protocol allows fractionation of cells into three major constituents, membranes, cytosol, and nuclei. It is suitable for the overall analysis of quantitative changes of proteins as well as for identification of their posttranslational modifications brought about by growth, differentiation, senescence, environmental changes, genetic manipulation, or other events. This analytical step is performed with already less-complex subproteomes, where rare protein species get enriched. In addition, proteins that shuttle between these three major subcellular compartments can be identified rather easily.

Alternatively, the PNS can be additionally fractionated by density gradient centrifugation (Figure 1, steps 6 and 7). The position of membrane particles in density gradients is determined mainly by the ratio of their lipid to protein content; eg, mitochondrial inner membranes are protein-rich and thus have a high density, whereas endosomal membranes are lipid-rich and are of low density. Other parameters that determine density include the contents of vesicles. For example, secretory low-density lipoproteins contained within Golgi vesicles render them more buoyant,

Figure 1. Schematic outline of the stepwise preparation and analysis of subcellular fractions as discussed in the text.

Figure 2. Scanning electron microscopy of a low-speed pellet after cell homogenization (A); see steps 1 through 3 in Figure 1. B, Purified nucleus (for protocol, see http://www2.ubik.ac.at/ahe/histologie-molekularezellbiologie/). Large arrow in A indicates an intact nucleus; arrowhead above cell debris and small arrows, other cellular membranes in this mixed fraction. Arrowheads in B indicate copurifying remnants of the endoplasmic reticulum; small arrows point toward nuclear pores. Magnification ×3000 in A and ×20000 in B, respectively.
whereas the protein contents of secretory granules increases their density (e.g., pituitary secretory vesicles). The presence of attached components (e.g., ribosomes on rough-ER membranes and clathrin on coated vesicles) also affects the density of membranes.5,14

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. Although sucrose is the most commonly used gradient medium, there are many other alternatives, e.g., Ficoll, Percoll, Nycodenz, or Metrizamide.8,15 Discontinuous gradients as well as step gradients have been applied successfully for the separation of early from late endosomes.11 Similar gradients were also applied for the purification of intact Golgi stacks10,16 as well as Golgi-derived transport vesicles.17

For better resolution, equilibrium separations with continuous gradients are the method of choice. After centrifugation to equilibrium, membranes distribute throughout the entire gradient according to their specific densities.14,18 A drawback of continuous gradients can be the low enrichment of organelles, resulting in rather diluted fractions.

Quality Control

Many assays can be used to assess the results of subcellular fractionation experiments (Figure 1, step 8). It is important to emphasize that under mild homogenization conditions, sealed vesicles and intact organelles are collected from density gradients and, therefore, luminal proteins and proteins with enzymatic activities will be preserved in their respective subcellular compartments.12,14 For evaluation of the mechanical and functional integrity of organelles, several methods may be used. First, quantitative Western blotting to follow the distribution of specific organelle-marker proteins can be used.5,14 Second, for morphological analysis of all fractions, standard electron microscopy procedures can be applied. Such a quality assessment by scanning electron microscopy is shown in Figure 2. Here, the pellet fraction after low-speed centrifugation (see Figure 1, step 3) is shown (Figure 2A). This fraction contains intact nuclei (large arrow), cell debris (arrowhead), and other relatively heavy cellular membranes (small arrows, Figure 2A). Even when nuclei are additionally purified, they will still carry a considerable amount of contaminating membranes along throughout all steps of the purification procedure (Figure 2B). Arrowheads indicate copurifying membranes, i.e., ER, and for size comparison, small arrows point toward nuclear pores. Electron microscopy provides powerful means for assessing the purity of subcellular fractions. In addition, electron microscopy analysis helps to understand why proteins of the endoplasmic reticulum are the major contaminants in each subcellular fractionation procedure and why they are so prominent on 2D gels of almost every purified organelle fraction. The high-resolution 2D gel in Figure 3 shows an endosomal fraction that we purified according to standard protocols and as described previously.14,18–21 Several proteins were identified...
by mass spectrometry and are indicated accordingly. Actin is indicated as a relatively abundant housekeeping protein. In addition, several proteins of the ER carried along during the purification procedure are indicated: calnexin, endoplasmic binding protein (Grp78; BIP), and calreticulin. Comparison of spot intensities clearly documents the problem of cross-contamination during subcellular fractionation. In our experience, the most common cross-contaminations come from ER, plasma membrane, cytoskeletal elements, and large cytoplasmic protein complexes, such as the proteasome. Therefore, in all fractionation experiments, a balance sheet should be established for the distribution of protein markers and enzymatic activities in all fractions.5,11,14,22 This provides the only appropriate means to judge the efficiency of the homogenization/fractionation steps and to compare the purity of different preparations.

**Protein Fractionation**

The spectrum of techniques applicable for proteomics research can be expanded toward separation of integral and peripheral membrane proteins (Figure 1, step 9).12 Peripheral membrane proteins can easily be extracted either from gradient-purified organelle vesicles (Figure 1, step 7) or from membrane pellets after high-speed centrifugation (Figure 1, step 5). The procedure consists of diluting the organelles or membranes in ice-cold 100 mmol/L Na2CO3 (sodium carbonate), pH 11.0, followed by centrifugation to pellet the membranes.12,23 Closed vesicles are converted to open membrane sheets, and protein content of the vesicles and peripheral membrane proteins are released in soluble form.23 Alternatively, Triton X-114 phase partitioning can be applied to define membrane-protein interactions in organelles.24,25 In general, integral membrane proteins are recovered in the detergent phase and peripheral membrane proteins in the aqueous phase. Both methods are suitable for downstream proteomics analysis; however, the sodium carbonate extraction protocol is easier because it consists only of a simple sedimentation step (for protocol, see http://www2.uibk.ac.at/ahe/histologie-molekulare-zellbiologie/).

**Organelle Proteome Analysis**

Protein patterns of subcellular fractions can be mapped and characterized by high-resolution 2D gel analysis and microsequencing. Proteomics was extremely successful when these technologies were targeted to multiprotein complexes and subcellular organelles.4 Organelle fingerprints based on annotated 2D gel maps have been generated and are available in the public domain. However, subcellular fractions are also applicable for gel-free protein separation and identification techniques,4 such as isotope-coded and biotinylated affinity tags.26 Organelle and subcellular fraction analysis offers many advantages for proteomics research. Complexity is largely reduced and is close to a range where an entire organelle-proteome can be displayed on a single 2D gel or even analyzed by mass spectrometry in gel-free procedures in one shot. In addition, identified proteins can immediately be linked to a functional context, because they were purified together with an organelle or subcellular fraction.

**Some Successful Examples**

Phagosomes are the key organelles within macrophages that provide these cells with the innate ability to participate in tissue remodeling, to clear apoptotic cells, and to restrict the spread of intracellular pathogens. The establishment of a comprehensive 2D gel database enabled Garin et al27 to analyze how phagosome composition is modulated during phagolysosome biogenesis. In a follow-up study, this proteome characterization provided also new insights into phagosomes as endoplasmic reticulum-mediated entry site for pathogens regardless of their final trafficking in the host.28

A comprehensive proteomics analysis of human nucleoli was recently performed.29 The authors of this study identified 271 proteins in the nucleoli and showed that nucleoli have a surprisingly large protein complexity. The fact that many novel factors and separate classes of proteins were identified supports the view that the nucleolus might perform additional functions beyond its known role in ribosome subunit biogenesis. This extensive proteomics analysis also demonstrated for the first time that the protein composition of nucleoli is not static and can alter significantly in response to the metabolic state of the cell.

Starting from a classical subcellular fractionation approach combined with high-resolution 2D gel electrophoresis and mass spectrometry analysis, the proteome of endosomes was investigated.14 In continuation of this work, the authors have identified a novel adapter molecule, named p14, for a mitogen-activated protein kinase scaffolding complex on the cytoplasmic face of late endosomes.19 Finally, reconstitution of this multiprotein signaling complex and depletion of endogenous levels of the involved proteins by RNAi (RNA interference) revealed that the late endosomal localization of the p14–mitogen-activated protein kinase scaffold complex is essential for signal transduction.30,31

**Subcellular Fractionation and Protein Identification in Cardiac Research**

Simple protocols like subcellular fractionation of homogenized myocardium in nonaqueous media were applied already many years back to compare cytosolic adenylates and adenosine release between whole tissue and cytosolic nonaqueous fractions.32 Applying similar subcellular fractionation techniques, the localization, macromolecular associations, and function of the small heat shock–related protein HSP20 in rat heart was investigated. Subcellular fractionation demonstrated that HSP20, αB-crystallin, and myotonic dystrophy kinase binding protein (MKBP) were predominantly in cytosolic fractions. Chromatography with molecular sieving columns revealed that HSP20 and αB-crystallin were associated in an aggregate of ≈200 kDa, and the phosphorylation of HSP20 was determined by 2D gel electrophoresis and immunoblotting.33

Signal transduction protein complexes and their activation, eg, protein kinase C activation, were analyzed as described above by subcellular fractionation, detergent extraction, and Western blotting in cultures of neonatal rat ventricular myocytes after electric stimulation.34 Caveolae are plasma membrane invaginations that are enriched in cholesterol, sphingolipids, and the marker protein...
caveolin. Muscle cell caveolae may function as specialized membrane microdomains in which the dystrophin-glycoprotein complex and cellular signaling molecules reside. These fractions were prepared directly from hearts from wild-type and caveolin-3 knockout animals with very similar protocols as used, for example, for cultured epithelial cells. Proteomic analysis of rabbit ventricular myocytes after detergent extraction revealed a novel posttranslational modification to myosin light chain. Specialized detergent extraction protocols in combination with subcellular fractionation allowed solubilization and mapping of cardiac sarcoplasmic reticulum proteins. Recently, mitochondria were purified from bovine heart by a combination of detergent extraction and sucrose gradient centrifugation as described above and finally separated on high-resolution 2D gels. In another study, sucrose in-gel processing and subsequent detection by mass spectrometry and rigorous bioinformatic analysis yielded a total of 615 distinct protein identifications.

Concluding Remarks

Subcellular fractionation and purification of organelles had always been a challenge for cell biologists. Most fractionation protocols take advantage of physical properties of intracellular membranes, eg, their density or charge. These are simple methods, applicable for proteomics; however, they do have limitations, too. The use of high-resolution 2D gel electrophoresis uncovered the problem that is common to all of those techniques, considerable cross-contamination with other subcellular organelles (membranes). Nevertheless, subcellular fractionation and proteomics are an ideal combination. 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, eg, between the cytoplasm and nucleus. Importantly, subcellular fractionation is a flexible and adjustable approach that may be efficiently combined not only with 2D gel electrophoresis but also with gel-independent techniques.

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