Sample Preparation Solutions for Cellular Protein Fractionation

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Abstract

2-D gel electrophoresis (2DE) is a widely used, proven method for proteome analysis. The quality and value of the information obtained from a 2DE experiment is highly dependent upon the initial sample preparation. In order to identify the most complete array of cellular proteins it is often necessary to reduce the complexity of the protein sample. A strategy for reduction in sample complexity is especially important when analysis of low-abundance and membrane proteins is the goal. Ideally the method(s) employed should be simple, reproducible, general to a wide variety of cell types, and result in a low conductivity protein sample that is free of substances that interfere with 2DE. With these aims in mind, we will present several solutions for convenient and efficient extraction of cellular proteins into discrete, more easily manageable fractions that are enriched in certain classes of proteins such as cytosolic, nuclear, membrane, and signaling. The majority of these procedures are intended to provide tools that simplify the preparation of membrane proteins that are generally considered difficult to isolate. These proteins are of considerable interest due to their roles in signal transduction and cell-to-cell interactions among other functions. 2DE and MALDI peptide mass fingerprinting data will be presented to illustrate the effective application of these techniques to improved sample prefractionation and protein identification.

Introduction

High-resolution 2DE is the most widely used separation method for global proteome analysis. Continuous improvements in the technique are expanding the capabilities of gel separations and removing limits on the types of proteins that can be resolved. Here, we describe new sample preparation kits that enhance the detection capabilities of 2-D gels.

These protocols address two key issues in applying 2-D gels to proteomics analysis of complex samples. One issue is how to handle both the enormous chemical diversity and the wide dynamic range of proteins in a biological sample so that low-abundance and housekeeping proteins can be monitored and identified. We have developed a number of protocols to quickly prepare highly enriched protein fractions based on protein solubility. One protocol separates a complex sample into enriched fractions of nuclear and cytoplasmic proteins. A second protocol enriches for membrane-associated signaling proteins. Other protocols enrich for membrane proteins. All protocols enhance the capability to monitor and identify proteins of biological interest.

The other issue is how to solubilize these hydrophobic proteins so they can be separated in 2-D gels. A number of new detergents and more stringent chaotropic agents have been incorporated into a complete protein solubilization buffer (PSB), which efficiently solubilizes hydrophobic proteins for immediate application to a 2-D gel. Using both the simplified enrichment and enhanced solubilization protocols, we significantly expanded the capabilities of applying 2-D gels for proteome analysis of complex cellular extracts.

Methods

Sample Preparation

Mouse liver tissue samples were processed with three different sample preparation kits to extract and enrich specific subclasses of proteins. Instructions for each kit were followed exactly. Protease inhibitors were added to the starting buffers immediately prior to use to prevent proteolysis during extraction. Following extraction, if required, protein samples were processed with the ReadyPrep™ 2-D cleanup kit to remove salts and detergents and to create a low-conductivity sample suitable for isoelectric focusing (IEF). This cleanup kit was also employed, if required, to concentrate proteins from dilute samples. Prior to IEF all samples were solubilized in PSB, to which 50 mM DTT, 2 mM TBP, and 0.2% Bio-Lyte® ampholytes (3–10) were added, and applied to 17 cm, pH 3–10 NL, ReadyStrip™ IPG strips. Approximately 455 µg of protein was loaded to each strip. *E. coli* samples were processed with the ReadyPrep membrane II kit protocol. Extracted membrane proteins were applied to 24 cm, pH 4–7 ReadyStrip IPG strips.

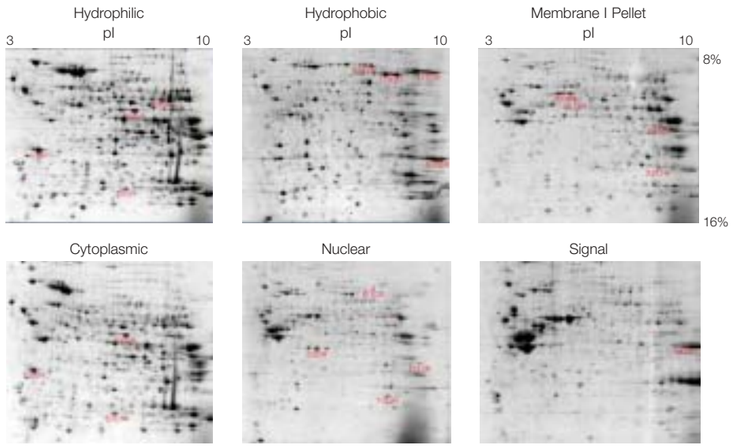


Fig. 1. Mouse liver samples fractionated using three sample preparation protocols.
Enriched samples from fractions in each protocol were run on 2-D gels. Each gel is identified by the fractionation procedure used to generate the sample. The gels labeled "Hydrophilic", "Hydrophobic", and "Membrane I Pellet" were of samples generated using the ReadyPrep protein extraction kit (membrane I), which employs a temperature-dependent phase partitioning in the presence of Triton X-114 detergent to partition the sample into hydrophobic and hydrophilic protein fractions (Bordier 1981, Santoni et al. 2000). The gels labeled "Cytoplasmic" and "Nuclear" were of samples generated using the ReadyPrep protein extraction kit (cytoplasmic/nuclear) (Dignam et al. 1983, Zerivitz & Akusjarvi 1989). Proteins from both phases were recovered for further analysis. The gel labeled "Signal" was of a sample generated from the ReadyPrep protein extraction kit (signal). This kit takes advantage of the limited solubility of plasma membrane microdomain structures, i.e., lipid rafts and caveolae, in nonionic detergents at 4°C to yield a protein pellet that is enriched in membrane-associated signaling proteins (Simons & Ikonen 1997, Brown & Rose 1992, Parton & Simons 1995, Anderson et al. 1992). PDQuest identification numbers and a red triangle on the gel images indicate spots that were cut for MS analysis. The protein identifications for these spots are listed in Table 1, and an example of the ProteinLynx Global *SERVER* 2.0 results screen for identification of lamin A protein from the mouse liver nuclear fraction is shown to the right of Table 1.

Table 1. Protein identifications from enriched fractions of mouse liver samples.

Gel Name	SSP #	Protein Name	Gene Name	Subcellular Location
Cytoplasmic	1202R	Senescence marker protein 30	SMP30	Cytoplasm
Cytoplasmic	5102R	Antioxidant protein 2	AOP2	Cytoplasm
Cytoplasmic	5404R	-Enolase	ENO1	Cytoplasm
Nuclear	3203R	Retinoic acid receptor alpha	RARA	Nuclear receptor
Nuclear	6702R	Lamin A	LMNA	Nuclear structural protein
Nuclear	7102R	Glutathione S-transferase, theta 1	GSTT1	Nucleus
Nuclear	8212R	Heterogeneous nuclear ribonucleoprotein A2/B1	HNRPA2B1	Ribonucleosomes
Signal	8403R	Argininosuccinate synthase	ASS1	Plasma membrane caveolae
Hydrophilic	1303R	Senescence marker protein 30	SMP30	Cytoplasm
Hydrophilic	5103R	Antioxidant protein 2	AOP2	Cytoplasm
Hydrophilic	6501R	-Enolase	ENO1	Cytoplasm
Hydrophilic	7603R	Glutamate dehydrogenase precursor	GLUD	Mitochondrial matrix
Hydrophobic	5811R	Succinate dehydrogenase flavoprotein subunit	SDHA	Inner mitochondrial membrane
Hydrophobic	7703R	Electron transfer flavoprotein oxidoreductase	ETFDH	Inner mitochondrial membrane
Hydrophobic	9106R	Voltage-dependent anion channel protein 1	VDAC1	Outer mitochondrial membrane
Hydrophobic	9702R	Carnitine palmitoyltransferase II	CPT2	Inner mitochondrial membrane
Membrane I pellet	4613R	Hydroxymethylglutaryl-CoA synthase	HMGCS2	Mitochondria
Membrane I pellet	8211R	Guanine nucleotide-binding protein	GNB2L1	Intracellular membrane
Membrane I pellet	8415R	Acyl-CoA dehydrogenase, medium chain	ACADM	Mitochondrial matrix
Membrane I pellet	3614R	Formiminotransferase-cyclodeaminase	FTCD	Golgi

2-D Electrophoresis

The IPG strips were focused using a PROTEAN® IEF cell for ~60,000 V-hr at a final focusing voltage of 10,000 V. The cell was set for rapid voltage ramping. Focused IPG strips were loaded onto 8–16% SDS-PAGE gels following equilibration for 10 min with DTT containing buffer followed by 10 min with iodoacetamide containing buffer. Following electrophoresis, gels were fixed for 30 min, stained with Bio-Safe™ Coomassie stain for 1 hr, and then destained for at least 2 hr before scanning with a GS-800™ densitometer.

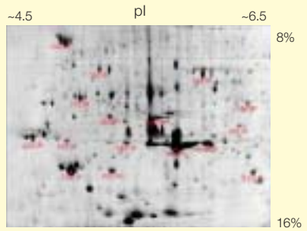
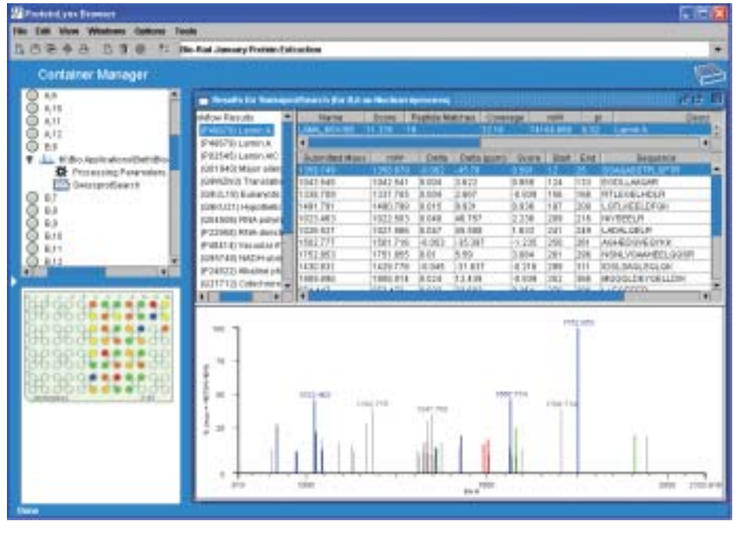


Fig. 2. *E. coli* membrane proteins.
An *E. coli* sample was treated using the ReadyPrep protein extraction kit (membrane II) protocol, which employs ice-cold sodium carbonate (pH 11) and ultracentrifugation to generate a pellet enriched with membrane proteins (Molloy et al. 2000, Nouwens et al. 2000). Membrane enriched proteins were resolved on a 2-D gel. Extracted membrane proteins were cut from the gel and identified. PDQuest identification numbers and a red triangle on the gel image indicate spots that were cut for MS analysis. The protein identifications for these spots are listed in Table 2.

Table 2. *E. coli* protein identification.
Proteins were cut from the gel of membrane-enriched proteins (Figure 2). ProteinLynx Global *SERVER* 2.0 software was used to identify proteins based on their peptide mass fingerprints.

SSP #	Accession	Protein	Molecular Mass	pI
0503R	YFGL_Ecoli	Hypothetical 41.9 kD protein in XSEA-H	42 kD	4.7
1501R	NLPB_Ecoli	Lipoprotein-34 precursor	37 kD	5.4
1902R	OSTA_Ecoli	Organic solvent tolerance protein precursor	90 kD	5.0
2301R	YAEC_Ecoli	Putative lipoprotein YAEC precursor	29 kD	5.2
2703R	ATPB_Ecoli	ATP synthase beta chain	50 kD	4.9
3801R	BTUB_Ecoli	Vitamin B12 receptor precursor	68 kD	5.3
4301R	NLPA_Ecoli	Lipoprotein-28 precursor	29 kD	6.1
4601R	RECA_Ecoli	RECA protein	38 kD	5.1
5401R	OMPA_Ecoli	Outer membrane protein A precursor	37 kD	6.3
6602R	EFTU_Ecoli	Elongation factor TU	43 kD	5.4
8402R	OMPA_Ecoli	Outer membrane protein A precursor	37 kD	6.3
8405R	OMPA_Ecoli	Outer membrane protein A precursor	37 kD	6.3
8802R	DHSA_Ecoli	Succinate dehydrogenase	64 kD	6.2
9301R	GLNQ_Ecoli	Glutamine transport ATP binding protein	27 kD	6.5
9502R	TOLB_Ecoli	TOLB protein precursor	46 kD	7.7
9601R	ARGA_Ecoli	Amino acid acetyltransferase	49 kD	6.5



Protein Identification

2-D gel images were processed for analysis with PDQuest™ 2-D analysis software and spots of interest were cut from the gels using the ProteomeWorks™ spot cutter. Peptide mass fingerprint data was obtained from excised 2-D gel spots using the Mass*PREP*™ station robotics system for protein digestion and MALDI target spotting. Gel spots were destained, reduced, alkylated, dried, digested with modified trypsin (Promega) while heated at 37°C for 5 hr, and extracted with an acidic solution. Peptides were then automatically spotted onto MALDI target plates mixed with a matrix solution of 2 mg/ml -cyano-4-hydroxycinnamic acid. MALDI mass spectra were acquired on a M@LDI-LR™ instrument in reflector mode. A nitrogen laser (= 337 nm, Laser Science) was pulsed at 20 Hz. Ions were accelerated to 15 kV after a time-lag focusing pulse of 2,750 V at 500 ns. Data were collected from the sample well and a near point lock mass (ACTH clip 18–39, Sigma). The MALDI mass spectra were processed to identify monoisotopic peaks using the Micromass algorithm MaxEntLite. The search engine used to identify peptide mass fingerprint data was ProteinLynx™ Global *SERVER*™ 2.0 software.

Results

Three separate sample preparation protocols were used to fractionate and enrich mouse liver protein samples prior to 2DE. Although the proteins for each gel were prepared from the same starting material, the final gel results following fractionation provide completely different views of the mouse liver proteome (Figure 1). Proteins that were unique to the fractionated sample were selected for identification. Table 1 shows selected proteins from these gels that are associated with the cellular structures enriched for in the sample separation protocols. Many of these identified proteins are membrane associated.

E. coli samples were treated using a carbonate extraction procedure to extract and enrich for membrane proteins (Figure 2). Table 2 shows that many of the proteins that were extracted using this procedure are membrane-associated *E. coli* proteins.

Conclusions

Applying specialized sample preparation solutions for the fractionation of cellular proteins from complex protein mixtures provides a simple and rapid method for enrichment of both low-abundance and membrane-associated proteins prior to sample analysis by 2-D electrophoresis. Understanding the cell biology of membrane proteins is especially important in the search for new drug targets, as ~50% of the known drug targets are membrane proteins. We have shown that application of four new kits for protein fractionation can provide a simple way to differentiate the proteome of a complex sample into a number of manageable fractions. Key features of these kits include:

- Rapid separations — Extraction from a crude sample to an enriched, gel-ready sample takes <3 hr
- Reproducible results — Each kit is based on proven chemistries and provides detailed instructions for application
- Increased protein solubilization — The PSB solution has been optimized to enhance solubilization of membrane-associated proteins for 2-D separation
- Improved 2-D results — The ReadyPrep 2-D cleanup kit provides quantitative recovery of samples with elimination of detergent and salt contaminants

Applying these sample prep kits to enrich proteins from complex biological samples should enhance the quality of the data that is obtained with any 2-D proteomics program.

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