The Proteomics Protocols Handbook

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Edited by

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Preface

Recent developments in the field of proteomics have revolutionized the way that proteins, and their contribution to cellular functions, are studied. The subsequent increased understanding of the mechanisms of cellular function and misfunction will have particular impact in the area of medical research, where disease processes will be better understood, many new (protein) therapeutic targets identified, and novel therapeutic agents developed. At the basic research level, phenotype will be explained in terms of cellular mechanisms.

The completion of the sequences of an ever-widening range of genomes—not least of all, the human genome—has provided the molecular biologist with a wealth of data that needs to be analyzed and interpreted. For a variety of reasons (including alternative mRNA splicing, varying translational stop/start sites, frameshifting, and the inability to deduce posttranslational modifications), complete sequences of genomes are insufficient to elucidate the protein components of cells. The focus of attention has therefore turned to directly examining these protein components as the means of understanding cell function, as well as the cellular changes involved in disease states. However, the wealth of gene sequencing data now available has produced a glut of information that challenges the protein chemist to develop new tools to utilize this flood of genomic data.

From the beginning, the cornerstone of proteomics has been the use of two-dimensional gel electrophoresis to compare proteomes of different tissues (for example, normal and diseased tissue) with the subsequent identification of protein differences by the use of mass spectrometry and database searching. These still remain valuable techniques and receive appropriate coverage in this book. However, the term proteomics now encompasses a range of newly developed methodologies for determining the structure and function of a protein. I have therefore included in *The Proteomics Protocols Handbook* a number of novel mass spectrometry and LC-MS techniques, protein array technology, new bioinformatics tools, and the range of techniques central to structural and functional proteomics that are needed to deduce the function of newly discovered protein sequences. The use of these techniques, and no doubt further ones that will be developed in the coming years, will lead to achieving the ultimate goal of proteomics, namely to catalog the identity and function of all proteins in living organisms.

The Proteomics Protocols Handbook should prove a valuable resource for molecular biologists, protein chemists, clinical/medical researchers, structural chemists/biochemists, and microbiologists, as well as those involved in bioinformatics and structural/functional genomics.

John M. Walker

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Extraction and Solubilization of Proteins for Proteomic Studies

Richard M. Leimgruber

1. Introduction

For any proteomic study involving various control and experimental specimens, several factors need to be in place. A critical one is the extraction and solubilization of all components, regardless of whether a chromatographic ***(1,2)*** or two-dimensional (2-D) gel electrophoretic fractionation ***(3–6)*** is performed prior to analysis of proteins of interest by mass spectrometry of protein digests. All proteins must not only be extracted, but they must also be completely soluble, free from interacting partners (such as protein–RNA/DNA and protein–protein interactions, metabolites, and so on), and, in the case of 2-D gel electrophoresis, they must remain soluble as they approach their isoelectric points. The solubilization process should extract all classes of proteins reproducibly, such that statistically significant quantitative data can be obtained and correlated with experimental perturbations and the resulting biological responses.

To accomplish this task, various approaches have been presented in the literature ***(7–11)***, and many solubilization cocktails are now available commercially. However, it should be noted that currently, despite several attempts by multiple groups, there is no single solubilization cocktail that works perfectly for all conditions and samples, due to sample source-related interfering compounds and a high degree of heterogeneity among samples. This heterogeneity can lead to differing protein solubilities. Also, the presence of highly abundant proteins complicates the extraction, solubilization, and analysis of the less abundant species. Extracts from certain plant tissues also present their own set of unique issues ***(12)***. In addition to solubilization of all proteins, the solubilization agents used must also be compatible with the subsequent fractionation/analytical method employed. To date, the most efficient solubilization cocktails consist of a mixture of chaotropic agents, a mixture of detergents containing 13–15 carbon long hydrophobic chains, and a reductant ***(13–17)***.

It is important to note that the effectiveness of solubilization is not the only factor that affects the quality of the 2-D protein patterns. Gel strip rehydration, sample application method, sample load, electrophoresis conditions, and so on all have an impact on the quality of the 2-D protein fingerprint or pattern.

Recently, there has been a renewed interest in quantitative protein profiling, a process that is critical for an understanding of biological function ***(18,19)***. Because the proteome is a very complex, dynamic process that represents events at the functional

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level, automated methods *(20)* and approaches to correlate quantitative changes in protein levels (including posttranslational modifications) will be required for system biology studies. Such efforts have been reported for breast carcinoma studies *(21)*, the effects of ultraviolet (UV) irradiation of HeLa cells *(22)*, compound-induced liver toxicity *(23)*, and cell-surface protein characterization *(24)*. Studies of reproducibility of cerebrospinal fluid (CSF) analyses *(25)* indicate that a quantitative 2-D gel electrophoresis approach is a viable one. Sensitive staining methods with greater dynamic ranges *(26–28)*, improvements in peptide detection *(29)*, and image analyses *(30–32)* also support this approach. Chromatographic approaches, such as those pioneered by the isotope-coded affinity tag labeling (ICAT) approach *(1)*, and commercial efforts in development such as that by Protein Forest, Inc., will play critical roles in the development of these rapid, quantitative approaches.

2. Sample Considerations

2.1. General

When one attempts to extract and solubilize proteins, several factors must be addressed. Among these are time, temperature, pH, protein concentration, salts, metal ions, and cofactors. Because each of these will be fairly specific for a given application, they will not be addressed in this brief review.

2.2. Abundant Proteins

The large dynamic range of proteins in biological samples—up to10^{10} in bodily fluids such as serum *(33)*—presents a major problem for whole proteome studies. Because of total protein load limitations by proteomic methods, the most abundant proteins overwhelm the assay and limit or prevent the detection of low-abundance proteins. This issue has been addressed by fractionation procedures *(1,34–42)* and by depletion strategies *(43–47)*. There are several commercial reagents available for depletion of serum albumin, immunoglobin (Ig)G, and so on (Agilent Technologies, Amersham Biosciences, Calbiochem, Pierce, Millipore, Sigma). In addition, approaches using ICAT have been employed to detect proteins quantitatively over a broad dynamic range *(1,48,49)*.

It should be noted, however, that removing and discarding of abundant proteins, such as albumin, may not be advisable in the search for clinical biomarkers *(50)*. A recent publication by Mehta et al. *(51)*, which describes an approach to look at the low-molecular-weight peptides bound to serum carrier proteins, clearly demonstrates that these carrier proteins may be rich repositories of biomarkers. This type of affinity-capture approach is also useful for signal transduction studies *(52)*.

2.3. Chaotropes

A significant advance in increasing the solubility of proteins was the use of urea/thiourea mixtures *(53,54)*. The typical mixtures currently in use consist of 5–8 *M* urea and 2–2.5 *M* thiourea *(13,14,17,41,55)*. It is interesting to note that although the addition of thiourea to this mixture increases both the number and quality of proteins that are detected, these additional proteins are water-soluble proteins, not membrane proteins *(56,57)*.

2.4. Detergents

Although detergents such as sodium dodecyl sulfate (SDS) are extremely efficient at solubilizing hydrophobic proteins, their anionic nature greatly limits their effectiveness for conventional proteomic analyses. As a result, zwitterionic and nonionic detergents have found widespread use in 2-D electrophoresis ***(10,13,17,55,58)***. CHAPS (3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate) is one of the most widely used zwitterionic detergents, and it has been shown to promote solubilization and stabilization by shielding hydrophobic zones from nonspecific aggregation and by stabilizing disordered loops to reduce heterogeneity ***(59)***. Specific instances have been identified in which various sulfobetaine detergents are the better solubilizing agents and CHAPS is not the best detergent of choice ***(58)***. In addition, solubilization of Jurkat membranes with Tween-20, Brij 58, and Lubrol WX yielded significantly more solubilized protein than either Triton or CHAPS ***(60)***. It has been reported that Brij 30 and Brij 96 are very efficient under nondenaturing conditions but are not efficient solubilizing agents when utilized in the presence of urea and thiourea ***(61)***. However, to date there has not been any single zwitterionic or nonionic detergent or detergent mixture identified that will completely solubilize all proteins. In fact, it has been observed that detergent selection for optimal solubilization for a given set of samples will be an empirical, experimentally determined one ***(13)***. It seems logical that the best approach is to combine different types of detergents to obtain a mixture that has the best attributes of each. Approaches to this have been reported ***(13,14,17)***. New polymeric surfactants are being developed for protein solubilization ***(62)***. These hydrophobically modified pullans (HMCMPs) extracted approx 70% of the total protein, without adversely affecting protein structure or function. Therefore, it may be possible to look at active protein complexes using these newer molecules.

2.5. Membrane Proteins

Membrane proteins play critical roles in cellular communication, transportation of nutrients, metabolites and ions, adhesion, signal transduction, and so on ***(15)***. These key proteins are typically not seen or are underrepresented in 2-D protein patterns because the efficient extraction of proteins from membranes is a process that has been difficult to accomplish; but progress is being made ***(8,14,41,53,63–66)***. Short-chain phospholipids have been used to isolate functional membrane protein complexes; however, these molecules interact primarily with the membrane lipids, and there is little if any interaction with the integral membrane proteins ***(67)***. Detergents act through a series of steps, interacting with and destabilizing lipid components of membranes, yielding detergent-lipid-protein complexes, and then effectively replacing the lipids, such that the detergents now interact with and shield the hydrophobic regions of proteins, resulting in better solubilization of these proteins ***(68)***. The ability of detergents to solubilize hydrophobic proteins appears to correlate well with the length of the hydrocarbon chain ***(16)*** and hydrophobic lipid balance (HLB) values ***(61)***. Recently, progress has been made toward obtaining a better representation of these very hydrophobic molecules in proteomic profiles. Zwitterionic detergents have been synthesized and used to solubilize membrane proteins ***(13,55,58)***. Chloroform-methanol extractions of membranes followed by 2-D gel electrophoresis in a detergent/chaotrope mix-

ture resulted in the identification of membrane proteins that had not previously been identified using 2-D gel electrophoresis ***(42)***. Differential extractions of purified chloroplast membranes using different chloroform:methanol ratios and detergents also resulted in the identification of previously unidentified membrane proteins using SDS-polyacrylamide gel electrophoresis (PAGE) and amino acid sequencing ***(64)***. A sequential fractionation procedure ***(65)*** is another possible solution to the study of lypophilic proteins, but this approach complicates the correlation of quantitative changes within the entire proteome with the biological response. Recently, the enrichment of membrane proteins using carbonate extraction coupled with surfactant-free organic solvent-based solubilization ***(69)*** and using a partition phase separation have been reported ***(70)***.

2.6. Nucleic Acids

The presence of DNA and RNA in samples for proteomic analyses can present problems with respect to both the quality of 2-D gel patterns and the recovery of DNA- and RNA-binding proteins. The presence of these nucleic acids can result in viscous samples that are difficult to pipet (affecting sample loads) and cause streaking in the first dimension because the nucleic acids tend to act somewhat like ion-exchangers, which can slowly release bound protein. Also, if they are precipitated from solution, any associated proteins may also be lost in this discarded fraction unless efforts are made to extract the nucleic acid fraction with a detergent cocktail such as that described by Giavalisco et al. ***(57)***. The DNA and RNA can be digested with DNase and RNase ***(3)*** or sheared mechanically with repeated passes through a tuberculin syringe equipped with a 21-gauge needle. A very convenient alternative method of mechanical shear is to place the tissue extract or cell lysate in a QIAShredder (QIAgen) and centrifuge the sample for 1–2 min in a microcentrifuge ***(17)***. Any buffer with or without detergents can be used, and in addition to the breaking the nucleic acids, cell debris is eliminated in the pellet, without the apparent loss of protein (Leimgruber, R. M., unpublished results). The QIAShredder has been useful for sample preparation for both one- and 2-D electrophoresis.

2.7. Phenolics

The presence of polyphenolic compounds at varying levels in plant samples ***(71–74)*** can adversely affect the 2-D protein patterns, generating streaks. Addition of insoluble polyvinylpyrrilodone (PVP) to the plant extracts effectively removes the phenolics ***(75,76)***.

2.8. Reductants

A consideration for obtaining clearly defined, well resolved protein spots on 2-D gels is the complete reduction of each denatured protein, resulting in very homogeneous proteins, which should be detected as well defined, round spots. Maintaining complete reduction has been complicated by the use of reductants such as dithiothreitol (DTT), a weak acid that migrates out of the very basic end of the first-dimension gel. Efforts to minimize this effect by introducing an excess of DTT in the wick at the cathode appear to help extend the pH range for protein resolution ***(77)***. Another promising approach to extending the pH range beyond an upper pH of around 8.0 to produce

highly resolved proteins is to reduce samples with tributylphosphine (TBP) and to perform the focusing step in the presence of dithiodiethanol (DTDE) ***(78,79)***. Reduction and alkylation of the proteins in this manner also has the advantage of potentially eliminating this step for in-gel tryptic digestions of excised protein spots.

2.9. Plant Tissue

Plant tissues present some of the same challenges for total proteome characterization as those from mammalian sources, including the characterization of membrane proteins ***(80–83)***, the identification of low-abundance proteins, and the presence of high-abundance proteins (e.g., ribulose-1,5-bisphosphate carboxylase/oxygenase [rubisco]). However, for the case of plants, there is also the issue of metabolites from secondary metabolism interfering with the separation process ***(84,85)***. There also is the issue of high protease activity ***(86)***. Because protein levels are low in plant tissues, many of the solubilization procedures involve extraction coupled with trichloroacetic acid (TCA)/acetone precipitation ***(87,88)***. As a result, many proteins may not be readily resolubilized, and some may not be captured by the precipitation step. As a result, recent reports have detailed alternative, much improved extraction procedures. In one procedure reported by Wang et al. ***(40)***, plant tissue is reground into a very fine powder, washed extensively to remove interfering compounds, and subjected to a phenol/dense SDS extraction. In another approach, a sequential extraction procedure yielding three fractions was utilized ***(57)***. The first fraction contains the highly water-soluble proteins in the supernatant resulting from centrifugation of an aqueous extraction of pulverized plant tissue in the presence of protease inhibitors. The resulting pellet sample is extracted with detergents (4% CHAPS and 2% amido sulfobetaine [ASB] 14), followed by the addition of urea and thiourea. The second fraction represents the supernatant after centrifugation of the detergent-solubilized material. Finally, the last pellet is incubated with DNase, followed by the addition of urea and thiourea. Using this approach, the authors detected a threefold increase in the number of total proteins from the stems and leaves of *Arabidopsis*.

*2.10. Labeling With Cyanine Dyes (***see also** *Chapter 24)*

An important consideration for the extraction/lysis cocktail is whether or not proteins will be labeled with fluorescent dyes prior to the first dimension in 2-D electrophoresis. Cyanine dyes (Cy2, Cy3, and Cy5, Amersham Biosciences) containing an *N*-hydroxysuccinomidyl linker are conjugated to solubilized proteins through a lysine residue under carefully controlled conditions ***(26)***. These dyes are designed such that they do not alter the charge on the protein and add only 500 Da. Labeling is performed such that each protein is labeled with only one dye molecule. It is critical that the protein samples do not contain reducing agents, ampholytes, primary amines, or thiols, as they interact with the dye reagent ***(27)***. If any of these agents are required for the extraction of protein from tissue, they must be removed prior to the labeling step. The incorporation of an internal pooled standard for differential in-gel electrophoresis (DIGE) analyses has been reported to improve the accuracy of protein quantitation between gels, facilitating the detection of small changes not readily detected with conventional post-electrophoresis staining ***(89,90)***. A few recent applications of this technology have been in the areas of oncology ***(21)***, inflammation ***(17)***, and compound-

induced liver toxicity ***(91,92)***. Several previously unidentified proteins were identified, including potential biomarkers of liver toxicity ***(92)***. Analyses of the fluorescent 2-D images is performed using either the DeCyder™ software (Amersham Biosciences) or standard image-analysis software ***(93)***. Labeling with reactive thiol dyes has also been reported ***(94)***.

2.11. Laser Capture Microscopy (see also *Chapter 8*)

Recent advances in sample generation include laser capture microscopy (LCM), which can be utilized to generate large populations of homologous cells from tissue sections, from which the proteins can be solubilized. This approach also has the potential to aid in the characterization of heterogeneous samples, such as some tumor types, and to identify key biomarkers that may be missed when analyzing the entire tumor ***(96–100)***. This method can in some cases be utilized as an alternative to histological staining of brain tissue ***(101)***. A recent report has utilized LCM for the study of plant cells ***(102)***, in which LCM and microarrays were used to analyze global gene expression.

2.12. Protein Determination

Because many of the additives employed to solubilize and extract proteins from biological samples interfere with many protein assays, it is often difficult to accurately determine the total protein present in a given sample. These additives at levels typically used tend to interfere with many of the commonly used assays, such as the Bradford and modified Bradford assays. One of the best is that marketed by Cytoskeleton (Advance Protein Assay 01), because it can tolerate relatively high levels of chaotropes, detergents, and reductants. This assay is very rapid, requires little sample, and has a fairly good dynamic range.

3. Current Basic Solubilization Protocol

3.1. Sample Generation

3.1.1. Lysis/Extraction/Rehydration Solution

3.1.1.1. General Solubilization Cocktail for Mammalian Tissues and Cells

The typical lysis solution ***(17)*** consists of the following:

- 5 *M* Urea.
- 2 *M* Thiourea.
- 0.25% (v/v) CHAPS (Sigma).
- 0.25% (v/v) Tween-20 (Bio-Rad).
- 0.25% (v/v) sulfobetaine (SB) 3-10 (Sigma).
- 0.25% (w/v) carrier ampholytes (1:1:1:1 mixture of Bio-Lyte 3-10 [Bio-Rad], Servalyte 3-10 [Serva], Ampholine 3.5-9.5 [Amersham Biosciences], and Resolyte 4-8 [BDH]).
- 2 m*M* Tributylphosphine (TBP).
- 10% Isopropanol.
- 12.5% (v/v) water-saturated isobutanol.
- 5% (v/v) glycerol (Bio-Rad).
- 1 m*M* Sodium vanadate (phosphatase inhibitor, Sigma).
- 1X complete protease inhibitor cocktail (Boehringer-Mannheim).

This lysis solution can be stored tightly sealed at –80°C for several weeks. Care must be taken with respect to the potential evaporation of the alcohol over prolonged time periods. It may be necessary to change the detergent mixture and level for a given application. In some cases, 0.25–0.5% Triton X-100 yields better results than Tween-20, or increased levels of CHAPS solubilize more proteins. If TBP cannot be used, 100 m*M* dithiothreitol or 5% 2-mercaptoethanol can be used, but the results are not as good. The ampholyte level may also need to be increased if high amounts of sample are applied to the gel strip. In general, it is best to keep the detergent (0.75–2%) and ampholyte (0.25–1.5%) levels as low as possible for optimal resolution.

3.1.1.2. General Solubilization Cocktail for Plant Seeds

The typical plant extraction/solubilization solution consists of the following:

- 6 *M* Urea.
- 2 *M* Thiourea.
- 0.5% (v/v) CHAPS (Sigma).
- 0.25% (v/v) Triton X-100 (Bio-Rad).
- 0.25% (v/v) SB 3-10 (Sigma).
- 0.35% (w/v) carrier ampholytes (1:1:1:1 mixture of Bio-Lyte 3-10 [Bio-Rad], Servalyte 3-10 [Serva], Ampholine 3.5-9.5 [Amersham Biosciences] and Resolyte 4-8 [BDH]).
- 2 m*M* Tributylphosphine (TBP).
- 16% Isopropanol.
- 5% (v/v) glycerol (Bio-Rad).
- 1X complete protease inhibitor cocktail (Boehringer-Mannheim).

This lysis solution can be stored tightly sealed at –80°C for several weeks. As noted above, care must be taken with respect to the potential evaporation of the alcohol over prolonged time periods. As noted above, this cocktail may need to be modified empirically for specific plant tissue types (Leimgruber, N. L., et al., and Ruebelt, M. C., et al., unpublished data).

3.1.2. Cell Lysates

Cells such as those of the U937 human monocytic cell line are typically solubilized directly in lysis/rehydration solution at a level of approx 20,000 cells/µL or approx 1.5 mg protein/mL. Much smaller cells (e.g., monocytes and splenocytes) are solubilized at approx 80,000 cells/µL, which represents approx 1.28 mg protein/mL). Following extraction for 30 min at room temperature on a Nutator mixer, the samples are clarified by centrifugation in a microcentrifuge at 15,300*g* for 5 min. The presence of alcohol appears to precipitate the nucleic acid out of solution, while the chaotropes-detergents mixture solubilizes and releases the RNA- and DNA-binding proteins.

3.1.3. Serum

Rat serum (typically around 55 mg protein/mL, but ranges from approx 40–100 mg protein/mL) is either analyzed by dilution directly into lysis/rehydration solution or is first depleted with an affinity column to remove albumin, IgGs, and so on. The depleted serum sample is then concentrated back to the starting protein level and diluted into lysis/rehydration solution.

3.1.4. Tissue

Tissue is frozen in liquid nitrogen and pulverized in liquid nitrogen with a BioPulverizer (sizes to accommodate from 10 mg to 10 g of tissue, Biospec Products, Inc.). The pulverized tissue is extracted at a level of approx 2–3 mg of tissue/mL of lysis/rehydration solution for 30 min at room temperature. The extract is clarified by centrifugation, and the supernatant is either analyzed immediately or stored at –80°C. Fresh bone samples can be pulverized and processed in a similar manner using the MicroCryoPulverizer (Biospec Products, Inc.).

3.1.5. Urine

Urine (typically less than 1.5 mg protein/mL in a 24-h collection) is concentrated using membrane filtration devices (Centricon 3, Amicon) or lyophilized prior to dilution into the lysis/rehydration solution at a final concentration of approx 1–2 mg protein/mL.

3.2. Focusing Parameters

Because the solubility of proteins is also dependent upon the amount of salts (and the resulting loss of water as joule heating occurs), the isoelectric focusing steps are slowly ramped up at the start of each electrophoretic run. The rehydrated immobilized pH gradient (IPG) strips are typically focused in Bio-Rad Protean IEF units using the following protocols. In general, the total number of volt-hours should be 50,000–75,000. Narrower range pH gradients require longer focusing times than broad pH ranges.

1. *pH 3.0–10, Linear or Nonlinear, 18-cm-Long IPG Gel Strips, "Normal Samples."* 150-V rapid ramp for 1 h; 250-V rapid ramp for 1 h; 400-V rapid ramp for 4 hs; 10,000-V linear ramp for 14 h; 10,000-V rapid ramp as a hold step for additional volt-hours. The typical total volt-hours for focusing are 60,000–75,000.
2. *pH 3.0–10, Linear or Nonlinear, 18-cm-Long IPG Gel Strips, Samples Containing Salts up to 150 m*M. 50-V rapid ramp for 4 h; 150-V rapid ramp for 1 h; 250-V rapid ramp for 1 h; 400-V rapid ramp for 4 h; 10,000-V linear ramp for 12 h; 10,000-V rapid ramp as a hold step for additional volt-hours. The typical total volt-hours for focusing are 60,000–75,000.
3. *pH 3.0–10, Linear or Nonlinear, 11-cm-Long IPG Gel Strips "Normal Samples."* 150-V rapid ramp for 1 h; 250-V rapid ramp for 1 h; 400-V rapid ramp for 4 hs; 8,000-V linear ramp for 14 h; 8,000-V rapid ramp as a hold step for additional volt-hours. The typical total volt-hours for focusing are 55,000–60,000.
4. *pH 3.0–10, Linear or Nonlinear, 11-cm-Long IPG Gel Strips, Samples Containing Salts up to 150 m*M. 50-V rapid ramp for 4 h; 150-V rapid ramp for 1 h; 250-V rapid ramp for 1 h; 400-V rapid ramp for 4 h; 5,000-V linear ramp for 14 h; 5,000-V rapid ramp as a hold step for additional volt-hours. The typical total volt-hours for focusing are 55,000–60,000.

3.3. In Strip Equilibration of Focused Proteins for the Second Dimension

Once the proteins are focused in the first dimension, the gel strips can be frozen and stored sealed at –80°C or equilibrated immediately for electrophoresis in the second dimension (SDS-PAGE). If each gel strip is to be analyzed directly, it is equilibrated directly in 2 mL of a solution containing 62.5 m*M* Tris-HCl, pH 6.8, 2.3 % SDS, 5% 2-mercaptoethanol (or 100 m*M* DTT), trace of bromphenol blue for 3 min at room

temperature. If reduction alkylation is to be performed, the strips are incubated for 6 min in 2 mL of a solution containing 5 *M* urea, 50 m*M* Tris-HCl (pH 6.8), 2.3% SDS, 20-50 m*M* DTT, 5% glycerol, trace of bromphenol blue. The first equilibration solution is removed and replaced with the same solution lacking DTT and containing 40–100 m*M* iodoacetamide. For either case, the IPG strips are incubated in the second solution for 12 min protected from light. For either case, the gel strips are embedded on top of the second-dimension gel with 1% agarose in 5 *M* urea, 50 m*M* Tris-HCl (pH 6.8), 2.3% SDS, 20–50 m*M* DTT, 5% glycerol, trace of bromphenol blue.

4. 2-D Gel Electrophoresis Images

Representative 2-D protein patterns resulting from solubilization of proteins using the chaotropes-detergents cocktail described in **Subheading 3.** are shown in **Figs. 1–5**. Analyses of conditioned media from two sister human stromal cell lines are presented in **Fig. 1**. These proteins are highly water soluble proteins secreted by the cells into the medium. In **Fig. 2**, a profile of a typical cell lysate is shown, using U937 cells following experimental treatment. In panel A, the proteins were labeled with Cy5 prior to separation by 2-D gel electrophoresis, and the Cy5 signal is detected following the 2-D fractionation. The same gel was subsequently fixed and stained with the fluorescent dye SYPRO Orange ***(103)***, and the resulting image is seen in panel B. Detection of U937 cellular proteins by staining with the fluorescent dye SYPRO Orange after electrophoresis is shown in panel C. There is a good representation of proteins covering the entire pH and molecular-weight ranges, although very high molecular weight proteins are not present. The absence of the high-molecular-weight proteins is likely due to the lack of their diffusion into the gel and/or inefficient extraction. We have found that by using slightly higher than typical rehydration volumes, it is possible to increase the representation of higher-molecular-weight proteins. However, these proteins remain under-represented. Analysis of sera from two different rats resulted in a well-resolved 2-D protein pattern using the solubilization cocktail described in **Subheading 3.1.1.1.** (**Fig. 3A,B**). Similarly, use of this cocktail efficiently solubilized proteins from rat pancreas (**Fig. 3C**) and rat heart (Leimgruber, R. M., unpublished data).

The two-dimensional pattern of proteins extracted from soybean, *Arabidopsis*, and wheat seeds are shown in **Fig. 4**. Well-resolved protein patterns are obtained in high yield. It is usually much more difficult to obtain well-resolved two-dimensional protein patterns from leaf tissue, due to the presence of interfering substances. The results of two different extraction/solubilization cocktails are shown for *Arabidopsis* leaf proteins in **Fig. 5A,B**. Leaf proteins were precipitated with TCA/acetone as described previously ***(104)***. Much more protein is extracted under the conditions employed for panel **A**. Although it has been previously reported with mammalian samples that the presence of alcohol does not appear to have adverse affects on extraction efficiency ***(17)***, in this case for *Arabidopsis*, the presence of alcohol (panel **B**) appears to decrease the solubilization efficiency, but has no adverse effect on the quality of the 2-D pattern. However, the overall representation of *Arabidopsis* proteins is very similar for leaves extracted in the presence and absence of isopropanol. Note also the presence of the very abundant protein, rubisco, and some lower-molecular-weight forms of rubisco near the middle of the pH range.

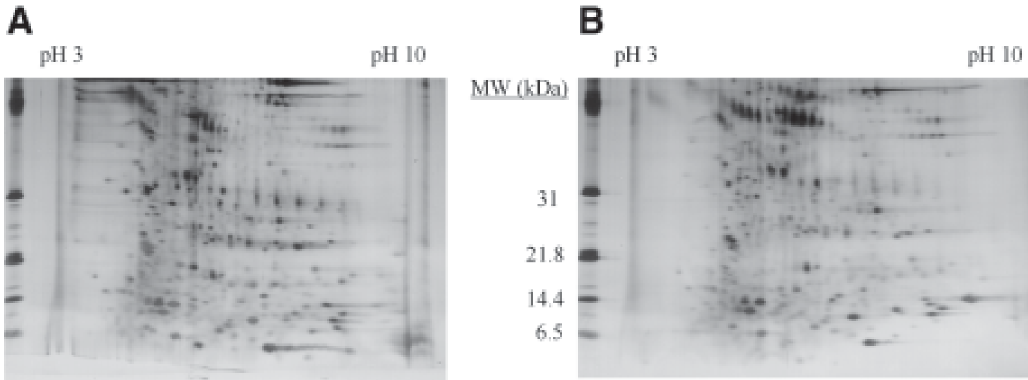

Fig. 1. Solubilization of proteins in conditioned media from human stromal cell lines. Focusing was performed using a nonlinear pH 3.0–10.0 gradient (Amersham Biosciences immobilized pH gradient [IPG] gel strips) for 65,000 V-hours. Second dimension analyses were performed using 10–20% polyacrylamide DALT (25 × 20 cm × 1.5 mm) gels. Protein (approx 800 µg) was detected with SYPRO® orange. **(A)** Cell line 1. **(B)** Cell line 2.

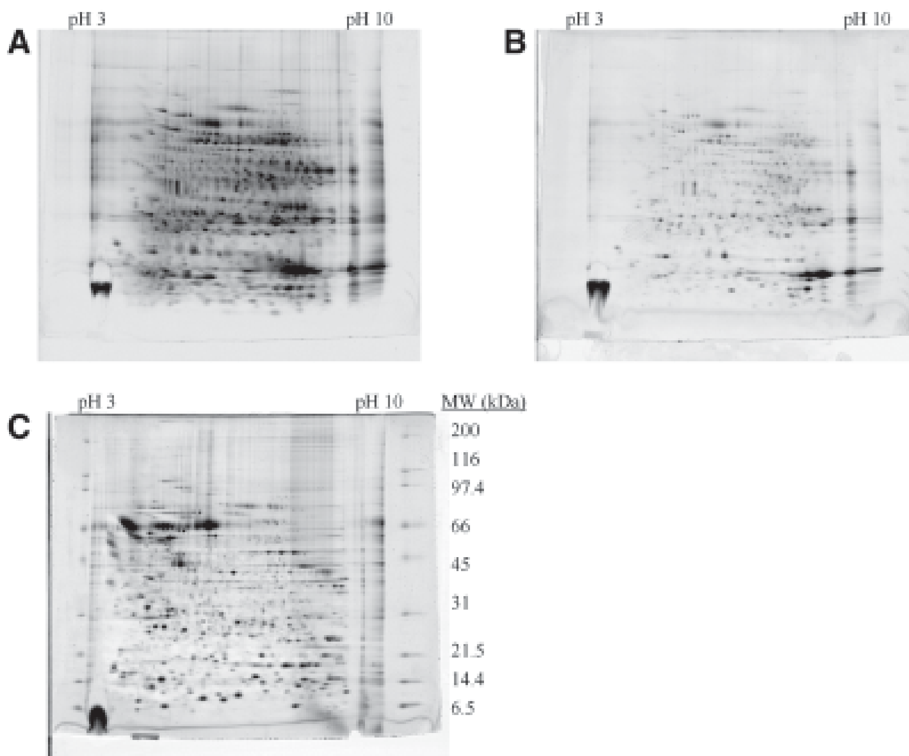

Fig. 2. Lysis and solubilization of U937 cells. Focusing was performed using a nonlinear pH 3.0–10.0 gradient (Amersham Biosciences immobilized pH gradient [IPG] gel strips) for 65,000 V-hours. Second dimension analyses were performed using 10–20% polyacrylamide DALT (25 × 20 cm × 1.5 mm) gels (Leimgruber, R. M., and Malone, J. P.). **(A)** protein (approx 450 µg) was detected by staining with Cy5. **(B)** Protein was detected by staining gel in panel **A** with SYPRO® Orange. **(C)** Protein (approx 900 µg) was detected by staining with SYPRO Orange.

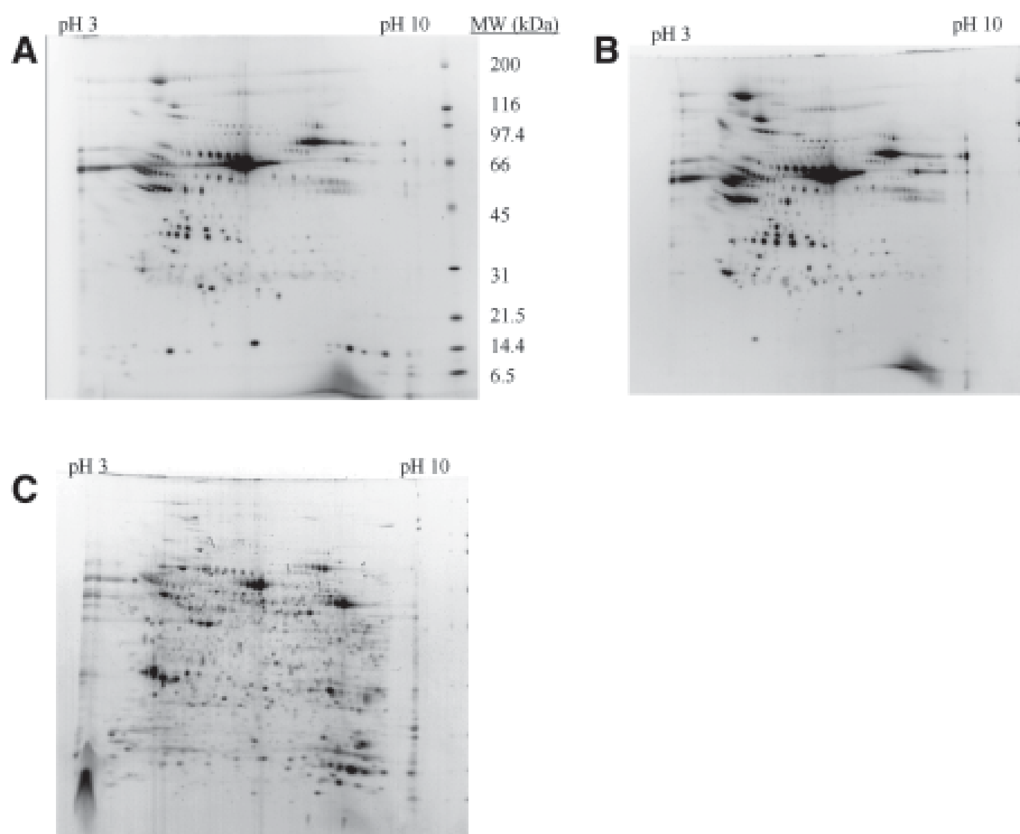

Fig. 3. Solubilization of proteins in rat serum and rat pancreas. Focusing was performed using a nonlinear pH 3.0–10.0 gradient (Amersham Biosciences immobilized pH gradient [IPG] gel strips) for 60,000 V-hours. Second dimension analyses were performed using 10–23% polyacrylamide DALT (25×20 cm $\times$ 1.5 mm) gels. Protein was detected by staining with SYPRO® Ruby **(A,B)** and SYPRO Orange **(B)**. (Gels are courtesy of Cabonce, M. C., Pfizer, Inc., Pfizer Global Research and Development.)

5. Conclusion

Unfortunately, no magic method or solution has been identified that solubilizes all proteins completely and reproducibly, free of interfering substances from all sample sources. Progress is being made, however, toward the identification of new detergents that are compatible with downstream analyses, and fractionation procedures are being developed to facilitate protein solubilization, as well as to address the issues associated with the large dynamic range seen for protein levels in a given sample.

Acknowledgments

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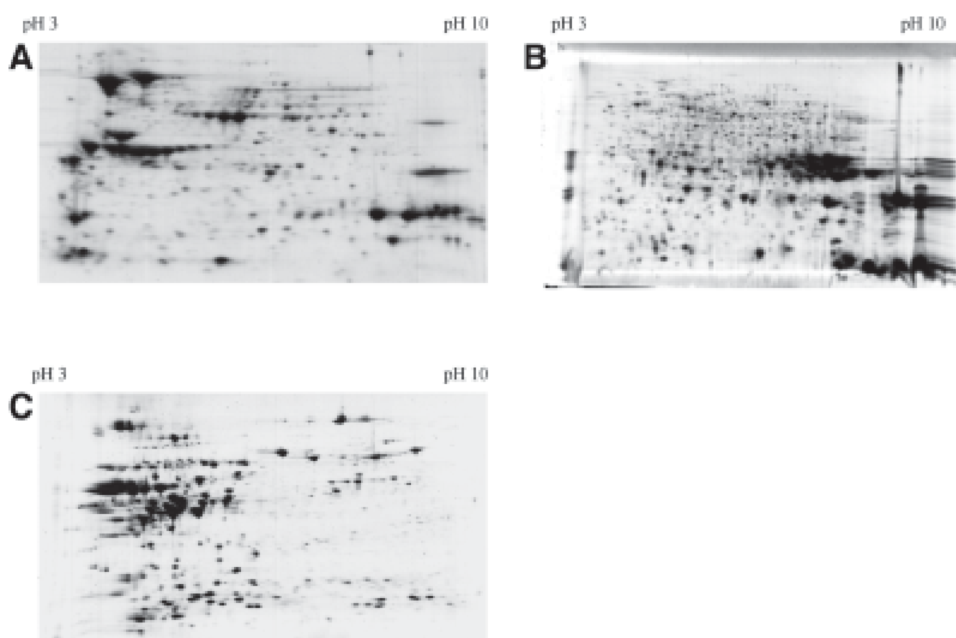

Fig. 4. Solubilization of proteins from plant seeds. Focusing of 150 μg of protein/gel was performed using a nonlinear pH 3.0–10.0 gradient (Bio-Rad immobilized pH gradient [IPG] gel strips) for 50,000 V-hours. Second dimension analyses were performed using 10–20% (soybean and wheat) and 8–16% (arabidopsis) Criterion Tris-HCl polyacrylamide gels (Bio-Rad). Proteins were detected by staining with colloidal Coomassie brilliant blue. **(A)** Glycine max L. Merr (soybean). **(B)** *Arabidopsis thaliana* [ecotype Columbia] (gel is courtesy of Ruebelt, M. C., Monsanto Company, Regulatory Sciences). **(C)** *Triticum aestivum* (bread wheat).

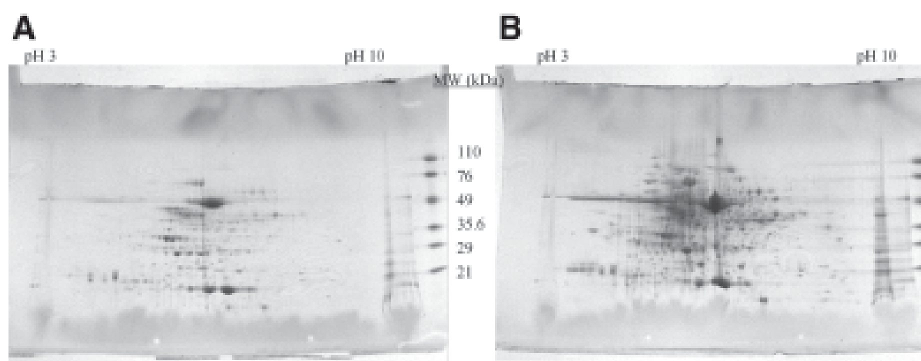

Fig. 5. Solubilization of proteins from *Aridopsis thaliana* [ecotype Columbia] plant leaves. Sample loading per gel was from the extraction of 2.7 mg of plant leaf tissue. Focusing was performed using a linear pH 3.0–10.0 gradient (Bio-Rad immobilized pH gradient [IPG] gel strips) for 50,000 V-hours. Second dimension analyses were performed using 10–20% Criterion Tris-HCl polyacrylamide gels (Bio-Rad). Proteins were detected by staining with colloidal Coomassie brilliant blue. (Gels are courtesy of Ruebelt, M. C., Monsanto Company). A, leaves, trichloroacetic acid (TCA)/acetone precipitation, re-solubilized and analyzed in 7 *M* urea, 2 *M* thiourea, 2.0% CHAPS, 2.0% Triton X-100, 0.4% carrier ampholyte mixture, 20 m*M* dithiothreitol (DTT). **(B)** leaves, TCA/acetone precipitation, resolubilized and analyzed in 7 *M* urea, 2 *M* thiourea, 1.0% CHAPS, 1.0% Triton X-100, 1% SB 3–10, 0.5% carrier ampholyte mixture, 2 m*M* TBP, 20% isopropanol.

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