Preparation of CHO Samples for Evaluating Changes at the Proteomic Level

Jodi M. Zobrist, Jennifer R. Cresswell, Justin Wildsmith,
Richard J. Mehigh, Matthew V. Caple, Rafael Valdes-Camin
Sigma-Aldrich Biotechnology, PO Box 14508, Saint Louis, MO 63103 USA

Abstract

Proteomic sample preparation continues to present a challenge as no universal method of extraction exists for different sample types. Oftentimes, a factorial matrix approach is employed to test detergents in various combinations for obtaining samples optimized for downstream analysis, such as 2-D electrophoresis (2-DE) followed by MALDI-MS.

A detergent matrix was designed to assist in setting up experiments to optimize extraction from various sources. Using Chinese hamster ovary cells (CHO-K1), a variety of detergent extraction reagents were tested to determine which resulted in the greatest number of well-resolved spots in 2-DE. Selected gel spots were identified using MALDI-MS after in-gel tryptic digestion of proteins. The fractionation of proteins with differing solubility was evaluated. By making use of optimized extraction methods for mammalian cell lines, such as CHO-K1, 2-DE coupled with MALDI-MS becomes a more effective tool for studying changes in protein expression.

While there is no universal solution for simplifying the complexity of samples and addressing solubility issues, a systematic approach of optimizing sample preparation methods for 2-DE is extremely powerful.

Introduction

Sample preparation is a critical component when studying different sample types. Separation by 2-DE presents further challenges as the first dimension separation requires a low salt content. Thierry Rabilloud and colleagues have made significant contributions in the area of sample preparation by developing new detergents to help overcome issues of solubility for 2-DE work.[1] Sigma-Aldrich developed a detergent sample kit that contains a variety of nonionic and zwitterionic detergents to aid in determining the optimal detergent for solubilizing specific protein samples.

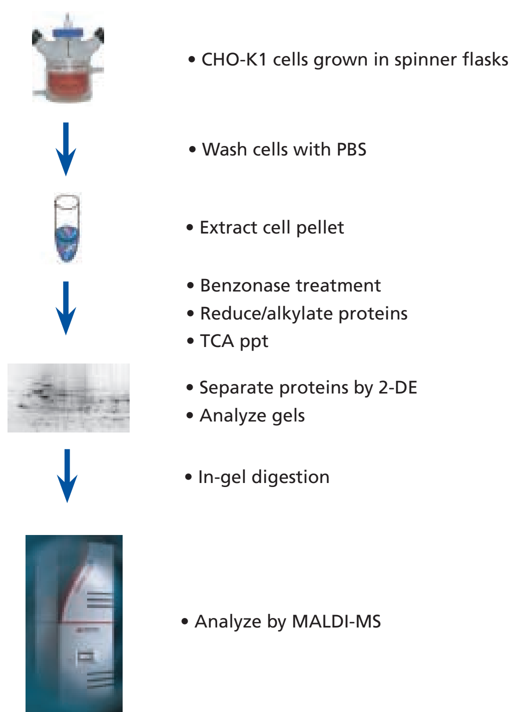
CHO-K1 cells were extracted with a variety of zwitterionic detergent formulations to determine which resulted in the greatest number of well-resolved spots in 2-DE. Zwitterionic detergents were chosen for the total protein extraction based on literature findings for successful solubilization of membrane proteins.[2] While every detergent supplied in the detergent sample kit was not employed, the utility of the kit for setting up a matrix experiment was demonstrated. In addition to detergents, the extraction solutions contained urea, thiourea, and trizma base at concentrations held constant for this investigation. The use of urea–thiourea combination was reported in the literature to prevent solubilization issues of various detergents, while maintaining the chaotropic power.[3] While the optimal extraction method discovered in this work may not be suitable for other cells lines, this approach for studying various extraction methods has demonstrated the utility of the matrix approach.

By making use of the optimized extraction method for CHO-K1 cells, 2-DE coupled with MALDI-MS becomes a more effective tool for studying changes in protein expression. To further demonstrate the effectiveness of the optimized extraction method, a heat shock experiment was conducted that demonstrated changes at a proteomic level in cells after exposure to a stressed condition once a specified cell density was reached.

Materials

- ProteoPrep™ Detergent Sample Pack (PROT-DT)
- ProteoPrep™ Reduction and Alkylation Kit (PROT-RA)
- ProteoPrep™ Protein Precipitation Kit (PROT-PR)
- Trypsin Profile IGD Kit (PP0100)
- CHO-K1 cells (ATCC, CCL-61)
- CHO Protein-free Animal Component-free Medium (C5467)
- L-Glutamine solution (G7513)
- Bradford Reagent (B6916)
- IPG strips, pH 3–10 (I3406), pH 4–7 (I3531)
- Equilibration buffer (I7281)
- EZBlue Gel Staining Reagent (G1041)
- 2D gels, 4–20% Tris-HCl-BioRad
- All other reagents were purchased from Sigma-Aldrich

Process Overview



Methods

Cell growth

CHO-K1 cells were grown as a suspension culture in animal component-free CHO medium supplemented with 4 mM L-Gln. Duplicate spinner flasks (Techne Inc., Princeton, NJ) were inoculated at 2 x 10^5 cells/mL in 200 mL media and incubated at 37 °C with humidified air and 5% CO_2 at 80 rpm on a magnetic stirrer platform (Thermolyne, Dubuque, IA). After 4 days, cultures were 96% viable with a cell density of 2.5 x 10^6 viable cells/mL as determined by Vi-Cell™ XR Cell Viability Analyzer (Beckman Coulter, Inc., Fullerton, CA). Cells were harvested by centrifugation at 200 x g for 5 min then washed twice with chilled PBS.

Heat stress

The suspension cultures were transferred to four 50-mL centrifuge tubes. Samples were placed in a hybridization oven (45 °C) and rotated for 0, 10, 60, and 120 min.

Sample preparation

Cells (5 x 10^7) were extracted in 1 mL of extraction solution. The extraction solution contained various detergents, prepared in a solution of 7 M urea, 2 M thiourea, 40 mM trizma. The solutions were sonicated using an ultrasonic probe (4 x 15 sec). Samples were diluted 1:4 in water and incubated on ice with 500 units of benzonase per milliliter of solution for 30 min. Samples were reduced with TBP and alkylated with IAA using the ProteoPrep Reduction and Alkylation Kit. Samples were TCA precipitated using the ProteoPrep Protein Precipitation Kit. Pellets were resuspended in the extraction solution.

Separation by 2-DE

IPG strips (11 cm, pH 3–10 or 4–7) were rehydrated with the samples and were focused at 8,000 V for 85,000 Vhr. The IPG strips were equilibrated for 20 min with IPG equilibration buffer and SDS-PAGE was performed using 4–20% or 8–16% precast gels. The gels were electrophoresed for 10 min at 80 V followed by 70 min at 170 V. All protein bands were visualized in the gels using EZBlue gel stain. The gels were imaged using a Flour-S™ MultiImager (BioRad) and analysis of the gels was performed using Phoretix™ 2D Expression Software (Nonlinear Dynamics).

In-gel digestion

In-gel digestion of protein spots was performed using Trypsin Profile IGD Kit. The tryptic digest was dried and the sample was redissolved in a solution of 10 mg/mL α-cyano-4-hyroxycinnamic acid in 70% acetonitrile, 0.03% trifluoroacetic acid.

MALDI-MS analysis

MALDI-MS data was acquired in positive ion reflectron mode using an Axima-CFR+ mass spectrometer (Shimazdu Biotech).

Results

Comparison of Various Extraction Detergents

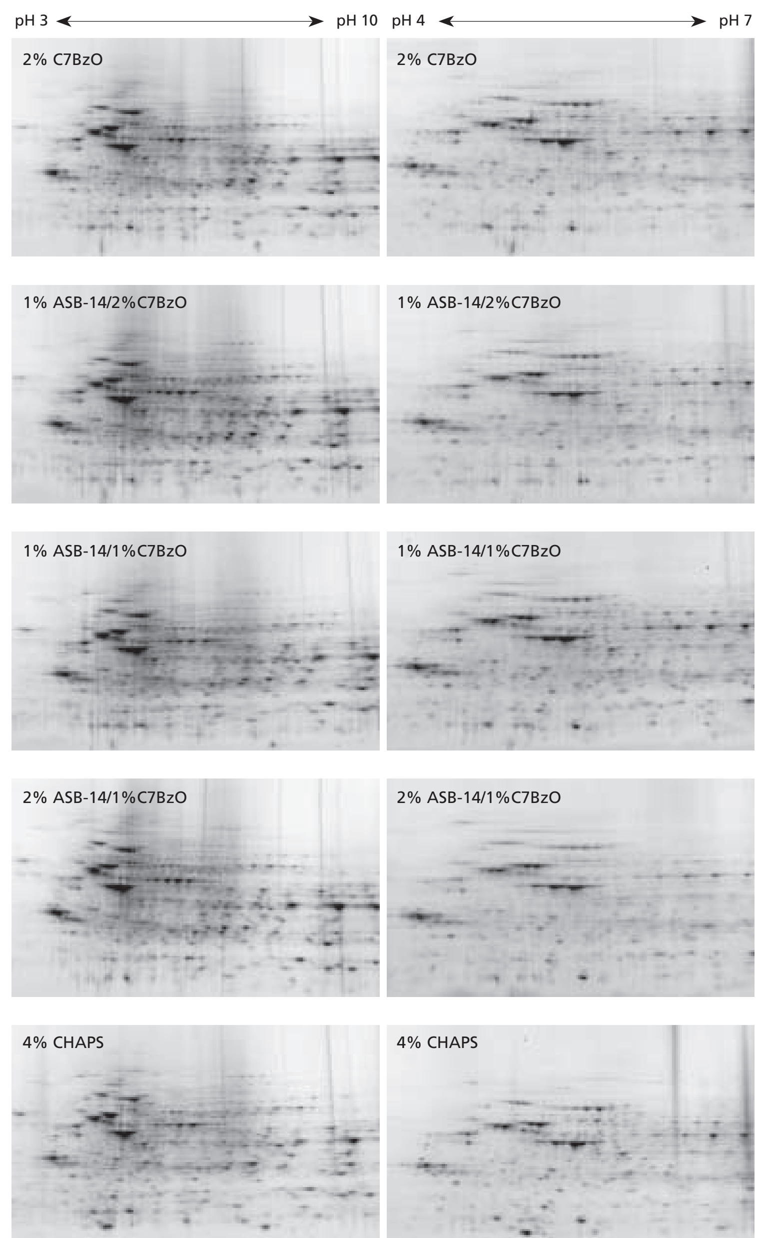


Figure 1. Detergent extraction optimization of CHO cells. Three detergents from the kit were tested in different concentration blends. Gels resulting from five of the detergent combinations tested are illustrated above. In every case, the cell extracts (250 g) were focused on either pH 3–10 or pH 4–7 IPG strips. SDS-PAGE was performed on 4–20% gels.

Extraction with 2% C7BzO demonstrated the greatest number of well-resolved spots of the various conditions as determined by the 2-DE analysis software (Nonlinear Dynamics). Normalizing 2% C7BzO to 100% extraction, 1% ASB-14 and 2% C7BzO, 1% ASB-14 and 1% C7BzO, 2% ASB-14 and 1% C7BzO, and 4% CHAPS extracted approximately 80% of the total proteins.

Heat Shock Experiment

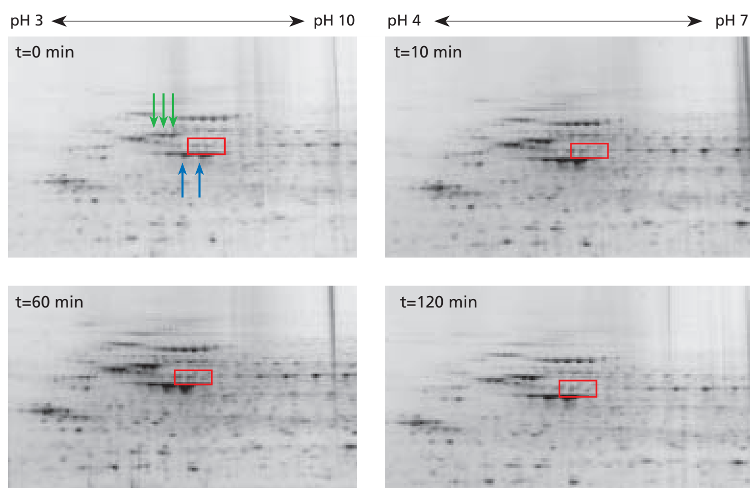


Figure 2. Identification of heat shock induced proteins in CHO cells. A heat shock experiment was performed and proteins were extracted using optimized conditions developed in **Figure 1**. Cells at a density of 5 x 10^7 were extracted with 2% C7BzO prepared in a solution of 7 M urea, 2 M thiourea, and 40 mM trizma. The cell extracts (250 µg) were focused on pH 4–7 IPG strips. SDS-PAGE was performed on 4–20% gels. Comparing the gel images and using 2-DE analysis software (Nonlinear), two proteins were identified (indicated by red boxes) as having increased expression levels as a function of heat shock duration. These protein bands, along with additional protein markers (indicated by arrows) were excised and digested, and analyzed by MALDI-MS.

After database searching the resulting peptide mass fingerprints, one set of the marker proteins were identified as β-actin isoforms. β-actin has a molecular weight of 40 kDa and a pI of approximately 5.4. The second set of marker proteins were identified as vimentin isoforms. Vimentin has a molecular weight of approximately 57 kDa and a pI of 5.4.

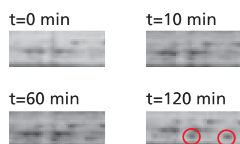


Figure 3. Enlarged sections of gel images from Figure 2. The proteins of interest (indicated by red circles) were identified as CAA26846 using the NCBI rodent database with a molecular weight of approximately 40 kDa, and an estimated pI of 5.6. The two distinct spots resulted in the same protein identification demonstrating that the spots are isoforms of one another. The molecular weight in the database report correlates with gel data in **Figure 2**.

Conclusion

- An optimal method for total protein extraction from CHO cells was determined based on total protein spots and clarity of the gel using the detergent sample pack.
- After in-gel digestion and analysis by MALDI-MS, database searchable spectra were obtained and resulted in positive identification of proteins from CHO cells.
- The optimized extraction method was successfully applied to a heat shock experiment which demonstrated its utility for the study of proteomic changes in CHO cells.
- A protein (CAA26846) was identified and shown to have an increased expression level upon exposure to heat shock.

References

1. Rabilloud, T.; et al. Structure-efficiency Relationships of Zwitterionic Detergents as Protein Solubilizers in Two-dimensional Electrophoresis. *Proteomics* **2003,** 3, 111–121.
2. Rabilloud, T.; et al. Evaluation of Nonionic and Zwitterionic Detergents as Membrane Protein Solubilizers in Two-dimensional Electrophoresis. *Proteomics* **2003,** 3, 249–253.
3. Lunardi, J.; et al. Improvement of the Solubilization of Proteins in Two-dimensional Electrophoresis with Immobilized pH Gradients. *Electrophoresis* **1997,** 18, 307–316.

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