Isolation of Proteins from Small Subcutaneous Murine Tumor and Adipose Tissues Using a Pressure Cycling Technology

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ABSTRACT

Extremely small and rare tissue samples must be processed with high efficiency to enable reliable proteomic analysis. For small samples of murine tumor and adipose (10-100 mg) or rat liver (0.5-10 mg), Pressure Cycling Technology (PCT) yielded more protein in 30 minutes compared to pulverization of tissue frozen under liquid nitrogen (LNP) followed by buffer extraction of the triturate for 0.5-4.8 hours. For four subcutaneous tumor types, protein extraction efficiency was 4.4 ± 0.3% for PCT compared to 1.5% ± 0.6% for LNP. Further, a coefficient of variation (CV) of 7.8% was observed between the four tumor types for PCT compared to 37.7% for LNP. Also, PCT extracted as much as four times more protein from small adipose tissue samples than LNP. Finally, it is demonstrated that PCT derived sufficient protein less than 1 mg of rat liver (0.7 ± 0.3 mg) which enables highly reproducible analyses through two-dimensional gel electrophoresis (2DGE).

1. INTRODUCTION

The validity of proteomic studies geared towards demonstrating correlations between the expression of specific proteins and disease processes such as diabetes, cancer, heart disease and obesity can only be assured when proteins are isolated reproducibly and in a manner in which they are accurately represented in the downstream analysis. Pressure cycling technology (PCT) uses rapid cycling between high and ambient pressures and has been shown to be more disruptive than sustained high pressure.

Herrera et al. [1] reported increased yields of phycoerythrin from Spirulina platensis when samples were pressurized several times. Similarly, Bart et al. [2] demonstrated increased inactivation of Escherichia coli correlating to the number of pressure cycles, rather than the total elapsed time at high pressure. Geiser et al. [3] reported that PCT released 37% more protein from Caenorhabditis elegans than sonication. PCT yielded 14.2% more protein from Escherichia coli than bead beating [4] and 17.1% more protein from Rhodopseudomonas palustris than by enzymatic lysis [5]. PCT also released proteins from mouse liver, including unique proteins which were not isolated in conventional homogenates [6].

2. MATERIAL AND METHODS

2.1 Tissue samples

Tumors were produced athymic mice by subcutaneous injection of NIH/3T3 cells, NIH3T3 cells, or stable transfectants expressing Jagged1 (SJ1, SJ38-1, SJ38-4) or Delta1 (SD-1, FLDL) proteins. Adipose, brain, and cells, NIH3T3 cells, or stable transfectants expressing Jagged1 (SJ1, SJ38-1, SJ38-4) or Delta1 (SD-1, FLDL) proteins. Adipose, brain, and

2.2 Silanization of glass beads

Polydisperse glass beads (0.5-1.0 mm) were from Nicola Craft Ltd (K. Laurel, NJ, USA). Beads were washed twice in 1M HCl, rinsed copiously in distilled water, and incubated one hour in Rain-X (Blue Coral Limited, Cleveland, OH, USA). The beads were rinsed in 100% methanol, washed thoroughly in distilled water, and dried for two hours at 60°C. All washes were performed on a sintered glass filter on an Erlenmeyer flask under vacuum.

2.3 Pressure Cycling Technology (PCT)

PULSE Tubes and the Barocycler Model NPE-3229 were from Pressure BioSciences (South Easton, MA, USA). Tissues ranging in size from 10-100 mg were weighed into tared PULSE Tubes, and 0.5 mL of radiomunosassay precipitation assay (RIPA) buffer (20 mm Tris, 150 mM NaCl, 1% sucrose, 5% glycerol, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 7.5) supplemented with protease inhibitors was added. Approximately 1.2 grams of silanized glass beads were added to each PULSE Tube in order to displace air volume and to prevent collapse of the tube under high pressure. PCT was performed for 30 cycles – each cycle consisted of 10 seconds at 35 kpsi followed by 5 seconds at ambient pressure. The screw cap was removed and each PULSE Tube was coupled to the inset of an Ultrafree-CL Centrifugal Device (Millipore Corporation, Danvers, MA, USA). These assemblies were centrifuged for one minute at 1,000 relative centrifugal force (RCF) to completely evacuate the contents of the PULSE Tubes. The PULSE Tubes were then removed and centrifuged continued for 10 minutes at 4,000 RCF to separate the glass beads and cellular debris from the clarified homogenate.

2.4 Liquid Nitrogen Pulverization

Tissues were pulverized under liquid nitrogen and the triturates were transferred to polypropylene tubes with 500 mL of RIPA buffer. The samples were incubated for 48 hours at 4ºC with 360º circumrotation at 8 rotations per minute. Control PULSE Tubes were not immediately evacuated, but instead were incubated 48 hours at 4ºC alongside the tubes containing LNP triturates. Following this incubation, the samples were transferred to Ultrafree-CL Centrifugal Devices and clarified by centrifugation for 10 minutes at 4,000 RCF.

2.5 Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE)

Samples were analyzed by SDS PAGE in which volumes were normalized to 2 mg initial tissue mass, except for adipose extracts in which volumes were normalized to 8 mg initial tissue mass. Samples were diluted in NuPAGE LDS sample buffer supplemented with 60 mM dithiothreitol. Electrophoresis was performed on NuPAGE Novex 4-12% Bis-Tris gels using MOPS SDS running buffer (Invitrogen, Carlsbad, CA, USA). Gels were stained with ProteomIQ Blue colloidal stain (Proteome Systems, Woburn, MA, USA).

2.6 Two-dimensional gel electrophoresis (2DGE)

Mouse liver tissues ranging in size from 0.5 to 10 mg were processed in PULSE Tubes with 0.5 mL of lysate reagent containing 9M urea, 75 mM CH3COOH and 5 mM tributylhydroxylamine with silanized glass beads added to displace volume to 1.5 mL. Following PCT, the PULSE Tubes were evacuated as described above and the clarified supernatants were alkylated for two hours following the addition of 10 mM acrylamide and 40 mM Tris. The proteins were precipitated with 50% acetone and resolubilized in 0.25-Ml of ProteoSilver REF Reagent (Pressure BioSciences, South Easton, MA, USA). Protein concentrations were determined using the Bradford Reagent (BioRad, Hercules, CA, USA). Samples were diluted to 0.4 mg/mL in the ProteoSilver REF Reagent and were used to hydrate immobilized pH gradients (IPGs) with pH range 3-10 (Amerham Biosciences, Piscataway, NJ, USA). Isoelectric focusing (IEF) was performed for 100-120 kV hours. IPG strips were equilibrated for 10 minutes in 375 mL Tris-HCl pH 8.8 containing 3M urea and 4% SDS, followed by 10 minutes in the same buffer containing only 2% SDS, and transferred to second dimension 8-16% polyacrylamide gels (BioRad, Hercules, CA, USA). Gels were stained with SYPRO Ruby fluorescent stain (Invitrogen, Carlsbad, CA, USA) or silver stained using the ProteoSilver Plus Silver Stain Kit (Sigma-Aldrich, St. Louis, MO, USA).

3. RESULTS

For adipose tissues, prolonged incubation of the triturates in RIPA buffer resulted in the loss of proteins, as shown in Figure 1, presumably due to the aggregation and precipitation of hydrophobic proteins. As an alternative to passive buffer extraction of triturates, PCT enabled rapid extraction of proteins while minimizing protein losses. In some instances, PCT extracted as much as four times more protein from small adipose tissue samples than LNP. Figure 4 shows additional proteins that were detected in 2D gels of adipose tissues processed by PCT.

Protein extraction efficiency from four subcutaneous tumor types was 4.4 ± 0.3% for PCT compared to 1.5 ± 0.6% for LNP. CV of 7.8% was observed between four tumor types for PCT compared to 37.7% for LNP. Figure 3 demonstrates that PCT derived sufficient protein from microgram quantities of rat liver to enable highly reproducible 2DGE.

4. DISCUSSION

Exacerbated in small tissue samples, the synthesis of tissue fluids during pulverization under liquid nitrogen frequently results in the inability to quantitatively recover the entire sample constituency from the crude and contributes to the poor reproducibility of the method. In contrast, PCT is a highly reproducible method for processing small samples. For tumor and adipose tissues extracted in RIPA, the samples could be analyzed directly by ELISA, SDS PAGE, and protein assay and were compatible with other downstream applications such as immunoprecipitation or affinity chromatography.

Recent recent development, specifically the ProteoSilver LRS Kit, has led to improved yields of hydrophobic proteins in adipose tissue and enabled more comprehensive analysis of the proteomes of lipid rich samples. See U.S. HUPO Poster No. 194 Unified Sample Preparation Approach Using Hydrostatic Pressure Cycling: Simultaneous Isolation of Proteins, Nucleic Acids and Lipids from a Single Sample by Groos et al.

5. REFERENCES


PDF available at www.pressurebiosciences.com