Applications of Pressure Cycling Technology (PCT) to Sample Preparation For Proteomic Analyses by Two-Dimensional Gel Electrophoresis

Gary B. Smejkal[1], Frank Witzmann[2], Heather Ringham[2], Deena Small[3], Edmund Ting[1], Ilyana Romanovsky, and Alexander Lazarev[1]

[1] Pressure BioSciences, Proteomics and Small Molecules Applications Laboratory, Woburn, MA. [2] Indiana University School of Medicine, Department of Cellular & Integrative Physiology, Indianapolis, IN. [3] University of New Hampshire, Department of Biochemistry and Molecular Biology, Durham, NH. [4] Dermtech International, San Diego, CA.

1. Abstract

Pressure Cycling Technology (PCT) uses alternating cycles of high and low hydrostatic pressure to effectively induce the lysis of cells and tissues in preparation for one- and two-dimensional electrophoresis (2DGE) and other analytical or preparative methods. For mammalian tissues, PCT isolated more protein from liver, including several unique proteins which were not isolated in Polytron or glass tissue grinder homogenates. 2DGE of PCT lysates of murine liver detected 2,126 protein spots, compared to 1,853 and 1,739 proteins spots detected in lysates produced by tissue grinding or sonication, respectively. From murine adipose tissue, PCT extracted more protein than pulverization under liquid nitrogen and detergent extraction of the triturate. Non-invasive biopsies of human psoriatic lesions using adhesive films rendered sufficient protein for 2DGE when processed by PCT. In further application to such "microproteomics" approaches, PCT extracted enough protein from submilligram quantities of liver to produce 2D gels in which over 1000 distinct protein spots were arrayed.

2. Introduction

PCT has proven to be more effective in the release of proteins in comparison to other standard methods such as homogenization and sonication. Some of the limitations inherent in the methods of sonication and homogenization include potential for cross contamination, reproducibility and heat production. These specific limitations carry the risk of perturbing structure and function of constituent molecules in a specific sample, resulting in the possibility of bad data. Additional limitations may include loss of protein, depleting the sample of variability which could result in a potential loss of data viable to a study. To this effect, improved instrumentation, such as PCT is not only necessary but critical for facilitation of high yield, high diversity data generation. Fast, efficient and accurate release of proteins from cells and tissues is a critically important initiation step in most analytical processes and the quantity and diversity of the extracted materials will affect all study and analysis further on.

3. Materials and Methods

3.1 Pressure Cycling Technology

Cell suspensions or tissues are placed in disposable PULSE Tubes (**Figure 1**) and are subjected to pressure cycling in the Barocycler NEP-3229 instrument. Maximum and minimum pressures (0-35,000 psi), the time sustained at each pressure

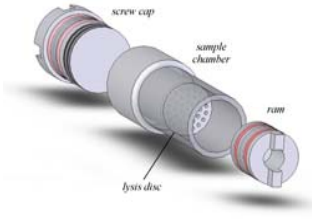

Figure 1. *Exploded view showing the components of the PULSE Tube FT-500. Under high pressure, the ram forces tissue and fluid through the perforated lysis disc. Upon return to ambient pressure, the ram retracts pulling in solvent from the other chamber.*

Level, and the number of cycles is defined using programmable logic controller interface. The Barocycler pressure chamber is thermostated using a peripheral circulating water bath (0-50º C). Recently, two researchers from the U.S. Center for Disease Control were exposed to West Nile Virus aerosols when tubes ruptured during bead mill operation [4]. Safety features in the PCT system design significantly reduce risk of exposure to the researcher to pathogens.

3.2 Sample Preparation

Unless otherwise specified, samples were processed in ProteoSOLVE CE Lysis Reagent (Pressure BioSciences, West Bridgewater, MA, USA) supplemented with 100 mM DTT and protease inhibitor cocktail P-2714 (Sigma-Aldrich Chemicals, St. Louis, MO, USA). For PCT, samples were typically subjected to 20 pressure cycles; each cycle consisting of 20 seconds at 35,000 psi followed by 20 seconds at ambient pressure. Following PCT, the samples were centrifuged for 10 minutes at 25,000 RCF and 500 uL of the sample was transferred to an Ultrafree Centrifugal Filtration Device (Millipore, Danvers, MA, USA) and centrifuged until a retentate volume of 100 uL was obtained. Four hundred microliters of ion-exchanged ProteoSOLVE IEF Reagent was added (reducing the DTT concentration to 20 mM) and the sample was alkylated for two hours following the addition of 40 mM acrylamide and 40 mM Tris. The alkylation reaction was terminated by resuming centrifugation in the ultrafiltration device [5].

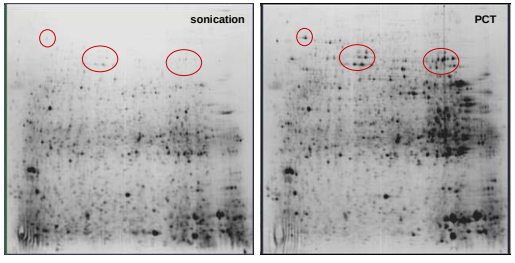

Figure 2. *2DGE of mouse liver proteins isolated by sonication (left) or PCT (right). PCT isolated more high molecular weight species (red ellipses). IPGs were pH 4.5-6.5.*

4. Results

4.1 PCT vs. Sonication

PCT was compared to sonication for the extraction of proteins from mouse livers macerated in a ground glass douncer. 2DGE of PCT lysates of mouse liver (250 mg) detected 2,126 protein spots, compared to 1,739 proteins spots detected in the sonication lysates. As shown in **Figure 2**, high molecular weight proteins were diminished in lysates produced by probe sonication. This was commensurate with an increase in low molecular weight proteins in the sonication lysates.

4.2 Extraction of proteins from psoriatic cells

Human skin was sampled using adhesive films from Dermatech (San Diego, CA, USA) from a psoriasis patient. Psoriatic lesions were cleansed thoroughly with 70% isopropanol and dried thoroughly before exfoliation with adhesive films. Two films were placed in each PULSE Tube and PCT was performed for 20 cycles. The adhesive films were removed from the PULSE Tube and replaced with two unprocessed films and PCT was repeated. The lysates were concentrated six times by ultrafiltration for 2DGE, shown in **Figure 3.**

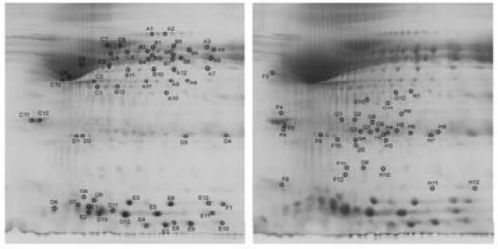

Figure 3. *Proteins released from psoriatic exfoliates by PCT of four (left) or eight (right) adhesive films. Spots excised for protein mass fingerprinting are indicated. The two predominent proteins were putatively identified as keratin (C3-C10) and IgG (A5-A6, B5-B9). Numerous other proteins of lesser abundance are currently being investigated as potential biomarkers of the psoriatic condition.*

4.3 PCT vs. Pulverization

PCT was compared to pulverization under liquid nitrogen followed by buffer extraction of the triturate for the isolation of proteins from mouse adipose tissue. Tissue samples (20-80 mg) were placed in a porcelain crucible and pulverized under liquid nitrogen. The triturates were transferred to polypropylene tubes containing 500 uL of NPB buffer (20 mm Tris, 150 mM NaCl, 60 mM KCl, 9% sucrose, 5% glycerol, 1% Triton X-100, 2 mM EDTA, pH 7.5) and protease inhibitors and incubated 30 minutes to 48 hours at 4º C. PCT extracted more protein from adipose in 30 minutes than buffer extraction from pulverized triturates. "Longer is not better" as significant protein losses were observed commensurate with extended incubation times of PCT and pulverized lysates. 2D gels shown in **Figure 4** showed no accumulation of small molecular weight proteins suggesting proteolysis was not the culprit. It is hypothesized that this loss of protein was due to aggregation of hydrophobic proteins from adipose.

4.4 PCT in "microproteomics"

PCT was used for extraction of microgram quantities of tissue emulating needle biopsy samples. PCT extracted sufficient amounts of protein from 0.7 ± 0.3 mg liver samples to produce the 2D gels in **Figure 5.**

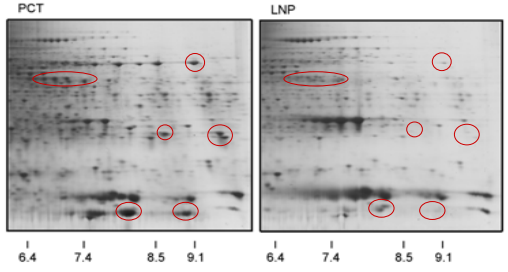

Figure 4. *Enlarged basic regions of 2D gels showing adipose tissue extracted by PCT (left) or pulverization under liquid nitrogen (LNP) and incubation of the triturate in RIPA buffer for 48 hours (right). Several proteins were isolated by PCT that were isolated in much lower abundance by RIPA buffer extraction (red ellipses).*

5. Discussion

Pressure Cycling Technology is novel approach to sample preparation which helps to achieve higher recovery of nucleic acids, proteins, and small molecules from cells and tissues compared to traditional methods. Rapid cycling between high and low pressure is more disruptive than high pressure alone. Recently, Barl *et. al* [1] reported increased inactivation of *Escherichia coli* 0157:H7 correlating to the number of pressure cycles, rather than the total elapsed time at high pressure. Similarly, extraction of proteins of increasing molecular mass from the chitinous exoskeletons of the arthropod *Daphnia pulex* correlates with the number of pressure cycles [2]. Previously, Geiser *et al.* [3] reported that PCT released 37% more protein from the nematode *Caenorhabditis elegans* than sonication. PCT yielded 14.2% more protein than bead mill from *E. coli* [4] and 17.1% more protein from *Rhodopseudomonas palustris* than by enzymatic lysis with lysozyme [5]. PCT extracted more protein from several components of the plant *Strelitzia reginae* inflorescence than a centrifugal homogenizer [2]. PCT also released more protein from mouse liver, including unique proteins, which were not isolated in Polytron or Douncer homogenates [6].

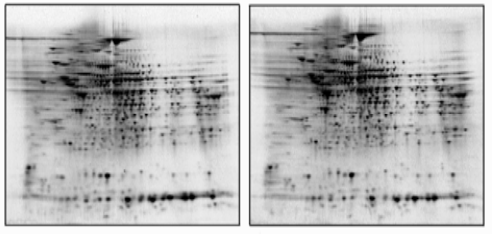

Figure 5. Submilligram quantities of rat liver tissue processed in standard PULSE Tubes using silanized glass beads for volume displacement. Duplicate tissue samples (0.7 ± 0.3 mg) were processed to illustrate the reproducibility of protein extraction from extremely small tissue samples. Following protein assay, gel loads were normalized to 100 µg total protein per gel.

6. References

[1] Barl L, Mori M, Hoque M, Kawamoto S, Yamamoto K. *Fourth International Conference on High Pressure Biosciences and Biotechnology.* Tsukuba, Japan, September 26-29, 2006.

[2] Smejkal G, Lazarev A, Tao F, Lawrence N, Schumacher RT. *CHI Sixth Annual Proteomics Sample Preparation Summit*. Boston, MA, USA, April 24-25, 2006.

[3] Geiser H, Hanneman A, Reinhold V. *Glycobiology* 2002, 12, 650.

[4] Smejkal GB, Robinson MH, Lawrence NP, Tao F, Saravis CA, Schumacher RT. *J. Biomolecular Techniques* 2006, 17, 159-161.

[5] Smejkal GB, Li C, Robinson MH, Lazarev A, Lawrence N, Chernokalskaya E. *J. Proteomic Res.* 2006, 5, 983- 987.

[6] Ringham H, Pedrick N, Smejkal GB, Behnke J, Witzmann FA. *Electrophoresis* 2006, in review.

For more information on Pressure Cycling Technology, visit www.pressurebiosciences.com