Published in final edited form as:

Anal Biochem. 2007 April 15; 363(2): 309–311. doi:10.1016/j.ab.2007.01.033.

Sample preparation for two-dimensional gel electrophoresis using pressure cycling technology

Gary B. Smejkal^{a,b,*}, Frank A. Witzmann^c, Heather Ringham^c, Deena Small^b, Susan F. Chase^b, James Behnke^a, and Edmund Ting^a

Gary B. Smejkal: gsmejkal@pressurebiosciences.com

^aProteomics and Small Molecules Applications Laboratory, Pressure BioSciences, Woburn, MA 01801, USA

^bDepartment of Biochemistry and Molecular Biology, University of New Hampshire, Durham, NH 03824, USA

^cDepartment of Cellular and Integrative Physiology, Indiana University Medical School, Indianapolis, IN 46202, USA

The fast, efficient, and accurate release of proteins from cells and tissues is a critically important initial step in most analytical processes and is essential to reliable proteomic analyses. Two-dimensional gel electrophoresis (2DGE)¹ can be an accurate representation of a proteome only if the entire protein constituency of cells is recovered during the sample preparation process. 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 2DGE and other analytical or preparative methods. Rapid cycling between high and low pressure is more disruptive than high pressure alone, as evidenced by the increased protein yields from *Saccharomyces cerevisiae* correlating to the number of pressure cycles rather than the total elapsed time at high pressure [1]. Similarly, Herrero and coworkers [2] reported a 20% increase in phycobiliproteins yielded from *Spirulina platensis* when multiple iterations of a pressurized liquid extraction method were performed.

Previously, Geiser and coworkers [3] reported the release of 37% more protein from the nematode *Caenorhabditis elegans* by PCT than by sonication. From gram-negative bacteria, PCT reportedly yielded 14.2% more protein from *Escherichia coli* than did bead beating [4,5] and yielded 17.1% more protein from *Rhodospseudomonas palustris* than did enzymatic lysis with lysozyme [6]. For mammalian tissues, PCT also isolated more protein from liver, including several unique proteins that were not isolated by conventional homogenization techniques [7]. From adipose tissue, PCT extracted more protein than did pulverization under liquid nitrogen and detergent extraction of the triturate.

The Barocycler NEP-3229 instrument, disposable polypropylene PULSE Tubes FT-500, and ProteoSOLVE IEF Reagent were obtained from Pressure BioSciences (West Bridgewater, MA, USA). Linear immobilized pH gradients (IPGs, pH 3–10) were obtained from Proteome Systems (Woburn, MA, USA). Nonlinear IPGs and PDQuest version 7.1 image analysis software were obtained from Bio-Rad (Hercules, CA, USA). Ultrafree-CL

© 2007 Elsevier Inc. All rights reserved.

Corresponding author. Fax: +1 781 932 9294.

¹*Abbreviations used:* 2DGE, two-dimensional gel electrophoresis; PCT, pressure cycling technology; IEF, isoelectric focusing; IPG, immobilized pH gradient; MWCO, molecular weight cutoff; TBP, tributylphosphine; C7BzO, 3-(4-heptyl)phenyl 3-hydroxypropyl dimethylammonio propane sulfonate; DTT, dithiothreitol; RCF, relative centrifugal force; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

centrifugal filter devices and Ultrafree-0.5 10kDa molecular weight cutoff (MWCO) centrifugal ultrafiltration devices were obtained from Millipore (Danvers, MA, USA). Tributylphosphine (TBP), protease inhibitor cocktail, 3-(4-heptyl)phenyl 3-hydroxypropyl dimethylammonio propane sulfonate (C7BzO), and Bradford protein assay reagent were obtained from Sigma–Aldrich Chemical (St. Louis, MO, USA). Probe sonication was performed using a Fisher Sonic Dismembrator model 500 (Fisher Scientific, Hampton, NH, USA). *R. palustris* was provided by Oak Ridge National Laboratories (Oak Ridge, TN, USA).

Unless otherwise specified, 250 mg of each sample was loaded between the ram and lysis disc of the PULSE Tube, and 1.25 ml of 7 M urea, 2 M thiourea, and 25 mM C7BzO supplemented with 100 mM dithiothreitol (DTT) and protease inhibitors was added. PULSE Tubes were subjected to 20 pressure cycles, with each cycle consisting of 20 s at 35,000 psi followed by 20 s at atmospheric pressure. Following PCT, each PULSE Tube was coupled to the insert of an Ultrafree-CL device, and the contents of the tubes were fully evacuated by centrifugation at 1000 relative centrifugal force (RCF) for 1 min. The PULSE Tubes were removed from these assemblies, and centrifugation was continued at 5000 RCF for 5 min. Then 500 μ l of each filtrate was transferred to an Ultrafree 0.5 ml ultrafiltration device and centrifuged until a retentate volume of 100 μ l was obtained. Then 400 μ l of ionexchanged ProteoSOLVE IEF Reagent (<10 μ S/cm) was added and ultrafiltration was repeated. Sample volumes were adjusted such that the final DTT concentration was approximately 10 mM. The sample was alkylated for 2 h following the addition of 40 mM acrylamide and 40 mM Tris. The alkylation reaction was terminated by resuming centrifugation in the ultrafiltration device [7]. Protein concentrations were determined by Bradford assay.

For murine adipose tissue, 20 to 80 mg of tissue was placed in a PULSE Tube with 500 μ l of RIPA buffer (20 mM Tris, 150 mM NaCl, 60 mM KCl, 9% sucrose, 5% glycerol, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 2 mM ethylenediaminetetraacetic acid [EDTA], pH 7.5) with protease inhibitors and was processed for 30 pressure cycles. Each cycle consisted of 10 s at 35,000 psi followed by 5 s at atmospheric pressure. Alternatively, samples were pulverized under liquid nitrogen in a porcelain crucible. The triturates were transferred to polypropylene tubes containing 500 μ l of RIPA buffer and were incubated for 0.5 to 48 h at 4 °C.

IEF and 2DGE were performed as described previously [5,7]. For IEF of adipose tissue extracts, proteins were first precipitated in 90% acetone at 4 °C. The flocculants were pelleted by centrifugation at 20,000 RCF for 20 min and were resolubilized in ProteoSOLVE IEF Reagent. The proteins were reduced and alkylated for 2 h following these addition of 5 mM TBP, 10 mM acrylamide, and 40 mM Tris. The alkylation reaction was terminated after 2 h by ultrafiltrative exchange into ProteoSOLVE IEF Reagent.

In this study, PCT was compared with sonication for the extraction of proteins from murine livers macerated in a ground glass tissue homogenizer. 2DGE of PCT lysates produced from intact 250 mg pieces of murine liver detected 2126 protein spots as compared with 1739 protein spots detected in the sonicated lysates. Spots were quantified using the spot detection utility of the PDQuest image analysis software. As shown in Fig. 1, high-molecular weight proteins were less abundant in lysates produced by sonication of macerated tissue. This was commensurate with an increase in low-molecular weight proteins observed in the sonicated lysates. It is hypothesized that the gradual deterioration of sonicator probes and the release of metal ions into the sample could facilitate the reactivation of proteolytic enzymes.

Moreover, sonication rapidly generates heat that accelerates the hydrolysis of urea and risks the carbamylation of proteins. When 1 ml samples of 7 M urea, 2 M thiourea, and 25 mM

C7BzO were sonicated 6×10 s and the sample temperature was measured between each interval, the temperature of the samples increased to 75 °C. Cooling the samples on ice for 30 s between each 10 s of sonication resulted in thermal cycling between 12 and 50 °C. This potentially compromises protein solubility because lowering the temperature below 18 °C results in the precipitation of chaotropes and detergents, as suggested by increased turbidity (Fig. 2). In comparison, the temperature in the Barocycler pressure chamber is controlled by a peripheral thermostated circulating water bath. In these experiments, temperature was maintained at 22 ± 1 °C throughout 50 pressure cycles at 35,000 psi.

PCT extracted more protein from murine adipose tissue in 30 min than did RIPA buffer extraction of pulverized tritirates for 48 h. Significant protein losses were observed commensurate with extended incubation times of both PCT and pulverized lysates. 2DGE, shown in Fig. 3, showed no accumulation of low-molecular weight proteins at the Kolrausch boundary, suggesting that this loss of proteins was not due to proteolysis. It is hypothesized that this general loss of protein was due to aggregation of hydrophobic proteins from adipose tissue.

Previously, Smejkal and coworkers [6] reported that PCT yielded 17.1% more protein from *R. palustris* than did enzymatic lysis with recombinant lysozyme. Two-dimensional gels in Fig. 4 show that PCT isolated multiple proteins from *R. palustris* that were not isolated by enzymatic lysis.

In preparing samples for electrophoresis, PCT frequently yields more total protein than do conventional sample preparation methods. In some instances, PCT yielded proteins that were either deficient or totally absent in samples prepared by other methods. Furthermore, PCT enables precise control over the sample processing conditions and ensures more reproducible protein yields.

Detergents disrupt biological membranes by mimicking the lipid bilayer environment. At sufficiently high concentrations, detergents effectively disrupt cellular membranes and membrane lipids are sequestered into mixed detergent–lipid micelles. Hydrophobic membrane proteins remain soluble as detergent–protein, lipid–detergent–protein, or lipid–protein complexes; otherwise, they may form insoluble protein aggregates. It is hypothesized that cycling between high and low pressure enhances the solubility of such hydrophobic proteins. At high pressure, protein aggregates are dissociated. On returning to atmospheric pressure, and when the detergent concentration is sufficiently high, the formation of primarily detergent–protein complexes is thermodynamically favored.

Acknowledgments

Frank Witzmann and Heather Ringham were funded by Air Force Office of Scientific Research (AFOSR) grants F49620-03-1-0089 and FA9550-05-1-0216. Deena Small was funded by National Institutes of Health (NIH) grant R15DK070599-01.

References

1. Smejkal, G.; Robinson, M.; Lazarev, A.; Li, C.; Behnke, J.; Tao, F.; Schumacher, R.; Lawrence, N. Application of pressure cycling technology (PCT) in proteomics: Increased cell lysis and protein yields from model organism. Paper presented at Second Annual U.S. HUPO Conference; Boston, MA. March 12-15, 2006;
2. Herrero M, Simó C, Ibáñez E, Cifuentes A. Capillary electrophoresis–mass spectrometry of *Spirulina platensis* proteins obtained by pressurized liquid extraction. Electrophoresis. 2005; 26:4215–4224. [PubMed: 16200528]

3. Geiser H, Hanneman A, Reinhold V. HTP proteome–glycome analysis in *Caenorhabditis elegans*. *Glycobiology*. 2002; 12:650.
4. Smejkal GB, Robinson MH, Lawrence NP, Tao F, Saravis CA, Schumacher RT. Increased protein yields from *Escherichia coli* using pressure cycling technology. *J Biomol Tech*. 2006; 17:159–161.
5. Smejkal GB, Lazarev A. Solution phase isoelectric fractionation in the multi-compartment electrolyser: A divide and conquer strategy for the analysis of complex proteomes. *Brief Funct Genom Proteom*. 2005; 4:76–81.
6. Smejkal GB, Li C, Robinson MH, Lazarev A, Lawrence N, Chernokalskaya E. Simultaneous reduction and alkylation of protein disulfides in a centrifugal ultrafiltration device prior to two-dimensional gel electrophoresis. *J Proteom Res*. 2006; 5:983–987.
7. Ringham H, Pedrick N, Smejkal GB, Behnke J, Witzmann FA. Application of pressure cycling technology to tissue sample preparation for 2-DE. *Electrophoresis*. 28 in press.

Fig. 1. Two-dimensional gels showing mouse liver proteins isolated by PCT (top) or sonication (bottom). PCT isolated more high-molecular weight species than did sonication. Molecular mass (kDa) is indicated at right. Estimated *pI* is indicated at bottom.

Fig. 2. Decreased solubility of 7 M urea, 2 M thiourea, and 25 mM C7BzO as a function of temperature. Cooling samples on ice between sonication cycles results in thermal cycling and risks precipitation of chaotropes and detergent required to maintain protein solubility during isoelectric focusing.

Fig. 3. Enlarged basic regions (pH 6–10) of two-dimensional gels showing proteins extracted from adipose tissues by PCT (top) or pulverization under liquid nitrogen (LNP) and buffer extraction (bottom). Samples were normalized to total protein load for 2DGE. Molecular mass (kDa) is indicated at right. Estimated *pI* is indicated at bottom.

Fig. 4. Enlarged regions (pH 4–8) of two-dimensional gels showing proteins extracted from *R. palustris* by PCT (top) or enzymatic lysis with recombinant lysozyme (bottom). Several proteins were isolated by PCT that were not isolated by enzymatic lysis (circles). Estimated *pI* is indicated at bottom.