Novel efficient alternatives for essential sample preparation techniques in functional proteomics

Emily Freeman and Alexander R. Ivanov

Harvard School of Public Health, Department of Genetics and Complex Diseases

Overview

Good control, efficiency, and reproducibility of protein extraction from cells and tissues are essential aspects for diverse biological and medical research applications. Effective and specific enzymatic digestion of proteins prior to mass spectrometry (MS) analysis is one of the fundamental techniques most often used in the proteomics laboratory. It is problematic that neither routine procedures for cell and tissue lysis nor for in-solution protein digestion have been significantly altered in common practice for over a decade. Here we have tested and optimized several alternative techniques for preparing lysates of mammalian cells, as well as in-solution enzymatic digestion compatible with downstream qualitative and quantitative MS-based proteomics applications.

Introduction

There are obvious benefits to both minimize the total time required for enzymatic protein digestion (which often exceeds 12 hours), and also maximize digestion efficiency without sacrificing results or enzyme specificity. For this purpose we have analyzed the role of hydrostatic pressure as an alternative thermodynamic parameter to control enzyme kinetics. We have compared the use of conventional protocols and those utilizing pressure-assistance via Pressure Cycling Technology, or PCT. This provides pressures of up to 35 kpsi, and has been shown to be efficient for proteolytic digestion, disruption of cells, micelles and membrane fragments, as well as for efficient protein recovery from cultured cells and tissues [Gross V., et al., J Biomol Tech. 2008, 19(3); and López-Ferrer D, et al., J Proteome Res. 2008;7(8)]. Using high performance LC-MS analysis, we have tested the role of pressurization, organic solvents, chaotropic agents, reducing reagents, enzyme/substrate ratios, temperature, and incubation time on efficiency, selectivity, and throughput of proteolytic digestion. The tests were performed using a mixture of protein standards, and the most effective conditions were applied to HepG2 cells lysates that were prepared using conventional lysis methods, as well as those using PCT and organic solvent buffers. Application of PCT resulted in significant improvement of throughput and reproducibility of sample preparation. Superior extraction rates for cytosolic, nuclear, mitochondrial, ribosomal, and membrane-associated proteins, as well as for proteins related to vital GO processes (RNA splicing, chromatin assembly, organelle organization among others) were observed in pressure-assisted and organic solvent-assisted sample preparation. There was also an enrichment in proteins which contained putative transmembrane domains (TMDs) with the use of pressure- and organic-assistance.

Methods

1. Optimization of basic sample preparation methods using Protein Standards

- A 1 pmol/μL mixture of standard proteins (“Protein Mix”, Table 1) was used for examination of various digestion protocols.
- Sample digestion was done either in an incubated shaker or in a barocycler NEP2320 (Pressure BioSciences, MA), and used 3 replicates per method
- Each replicate sample was analyzed twice using NanoLC-2D HPLC system (Eksigent, CA) and LTQ Orbitrap (ThermoElectron, CA). MS data was analyzed with the SEQUEST-Sorcerer algorithm on the Sorcerer IDA2 (SageN Research, CA).

2. Development of an efficient cell lysis procedure for proteomic studies:

- HepG2 cells were grown with 10% FBS in (3) separate 10-cm dishes to >80% confluence, and were scraped into separate flasks
- A urea-based lysis buffer was applied to the three cell suspensions, and hexafluoroisopropanol (HFIP), an organic solvent, was added to one of the three cell suspensions to the concentration of 30%.
- The first lysis method used only sonication to solubilize cellular proteins (“conventional”)
- The second method involved sonication and membrane disruption / protein solubilization using HFIP (“HFIP-assisted”)
- The third method used PCT to break up the cells (“PCT-assisted”).
- PCT was used to simultaneously homogenize the sample, to facilitate the dissolution of cells, micelles and membrane fragments, and to increase the efficiency of hydrophobic protein recovery.
- After digestion and analysis by LC/MS, the number and properties of proteins identified in each lysate were determined and compared.

Results and Discussion

1.1. Optimization of Enzymatic Protein Digestion

Protein Description	SwissProt accession #	MW (Da)	PI	AAs
Ubiquitin human	P62988	9382	7.30	82
Myoglobin equine	P68082	16941	7.36	154
Cytochrome C bovine	P62894	11565	9.52	104
β –Casein bovine	P02666	23568	5.13	209
Bovine serum albumin	P02769	66390	5.60	583
α1- Casein	P02662	22960	4.91	199
α2- Casein	P02663	24333	8.34	207
κ -Casein	P02668	18963	5.93	169

Table 1. Composition of Protein Mix

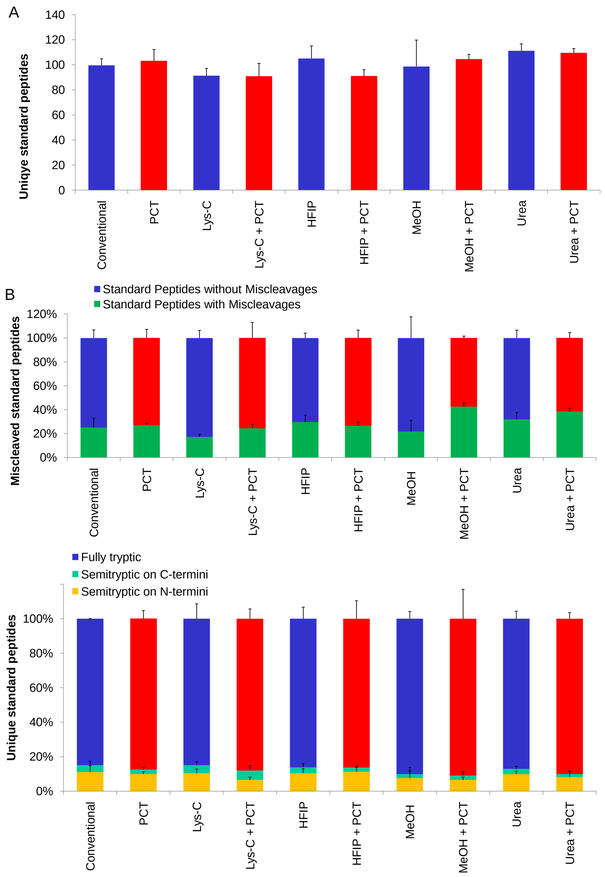


Figure 1. Protocol optimization using Protein Mix in MicroTubes.
BLUE = atmospheric pressure, **RED** = PCT.
(A) Digestion efficiency assessed using the number of identified unique peptides in Protein Mix using MicroTubes (n=6).
(B) Rate of miscleavages in identification results for unique standard peptides resulted from digestion of Protein Mix using MicroTubes (n=6).
(C) Percentage of semi-tryptic standard peptides identified using MicroTubes (n=6). Peptides semi-tryptic on the C-terminus are shown in **aqua**, and those semi-tryptic on the N-terminus are in **orange**.

Run-to-run reproducibility (R^2)	LC-MS Runs 1 and 2, R^2	LC-MS Runs 2 and 3, R^2	LC-MS Runs 1 and 3, R^2	Mean R^2	CV
Conventional Digestion	0.945	0.815	0.892	0.884	7.4%
PCT Digestion	0.908	0.938	0.950	0.932	2.3%
Sample-to-sample reproducibility,(R^2)	Samples 1 and 2, R^2	Samples 2 and 3, R^2	Samples 1 and 3, R^2	Mean R^2	CV
Conventional Digestion	0.862	0.918	0.883	0.888	3.2%
PCT Digestion	0.966	0.944	0.939	0.950	1.5%

Table 2: Run-to-run and sample-to-sample reproducibility for quantitative peptide peak attributes of the Protein Mix in MicroTubes.

1.2. Optimization of Enzymatic Protein Digestion. Reproducibility.

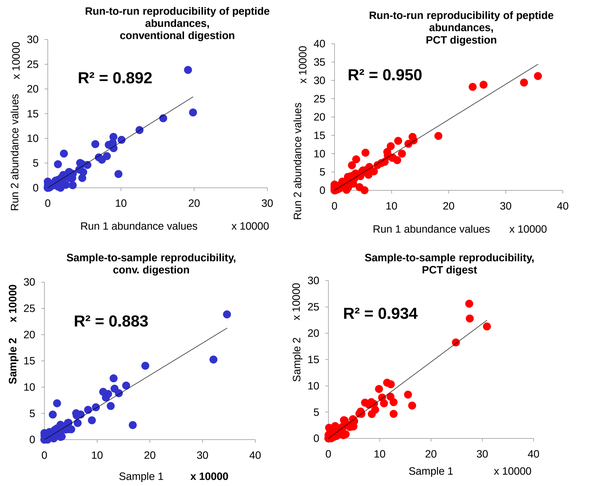
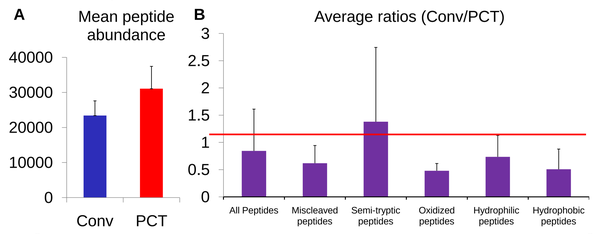


Figure 2: Run-to-run and sample-to-sample reproducibility for peptide peak volumes in LC-MS analyses of tryptic digests resulted from conventional and PCT-assisted protocols.



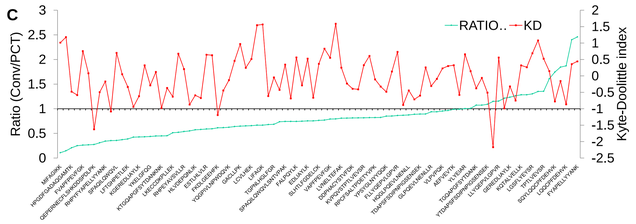


Figure 3: Differential analysis of peptide abundances by label-free AMT LC-MS.

1.3. In-Solution Digestion. Quantitative Analysis.

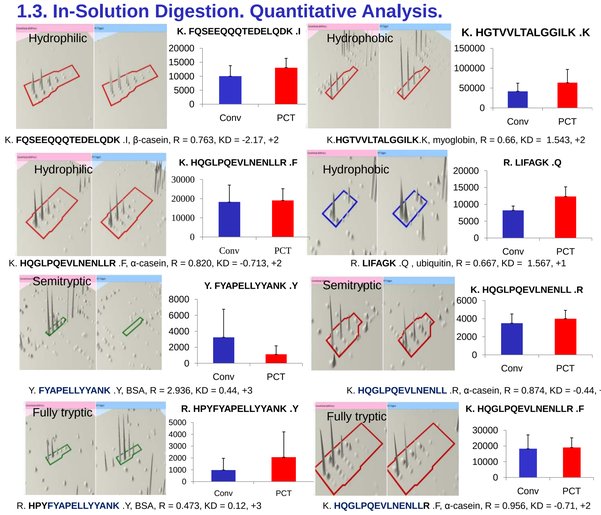


Figure 4: Peptide abundance in protein digests resulted from PCT-assisted and conventional protocols. Representative examples of hydrophilic and hydrophobic peptides (R- ratio Conv/PCT for mean peptide abundance; KD – Kyte-Doolittle hydrophobicity index; n=6).

2. Optimization of Cell Lysis Conditions. Pressure Assistance.

Lysis: Sonication	+		+	+	+	
Lysis: PCT-assisted		+				
Lysis: Solvent assisted						+
Digestion: atmosp.press	+	+	+		+	
Digestion: PCT-assisted				+		+

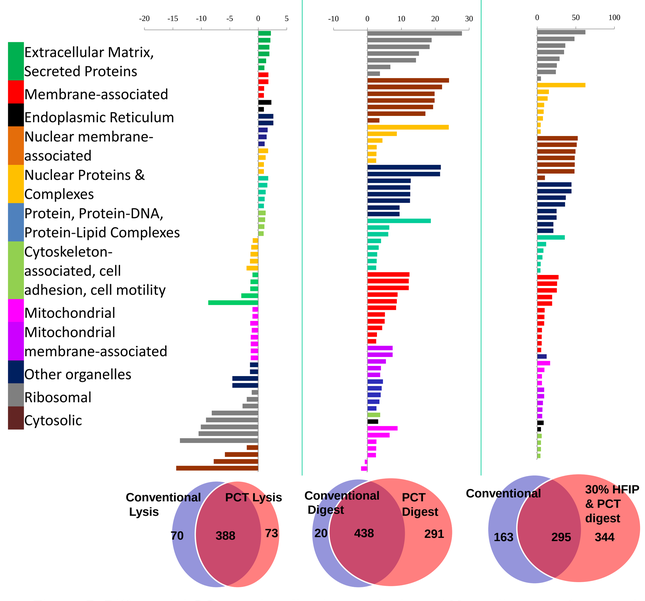
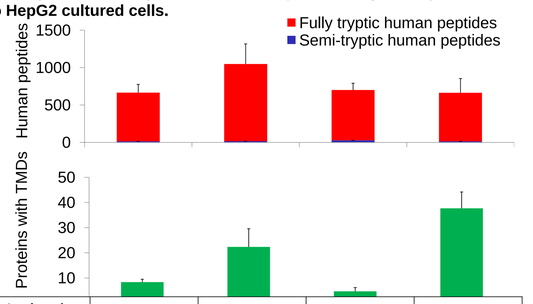


Figure 5: Differential GO term localization analysis and Venn diagrams for protein LC-MS profiling data resulting from alternative lysis and digestion protocols applied to HepG2 cultured cells.



Lysis: Solvent Assisted				+
Lysis: Sonication	+	+		+
Lysis: PCT-assisted			+	
Digestion: atmosp.press	+		+	
Digestion: PCT-assisted		+		+

Figure 6: Human proteins identified containing predictive transmembrane domains (TMDs) resulting from alternative lysis and digestion protocols applied to HepG2 cultured cells.

Conclusions:

Application of pressure assistance resulted in significant improvement of cell lysis and digestion procedures prior to proteomic profiling analyses. Specifically, pressure cycling assistance enabled
(1.) higher throughput;
(2.) higher efficiency;
(3.) superior reproducibility of enzymatic digestion;
(4.) more efficient cell lysis;
(5.) superior recovery of membrane, organelle, and complex forming proteins in comparison to the conventional protocols, as well as increased identification of proteins containing TMDs.

Acknowledgements: We would like to thank Drs. A. Lazarev and V. Gross for their kind guidance in experimental aspects of PCT and the members of sPRG for invaluable discussions. This work has been funded by the NIEHS Center NIH grant GM59780.