Towards The Understanding of Hydrostatic Pressure Effects on Proteomic Sample Preparation Methods

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Introduction: Pressure Cycling Technology and Pressure Effects on Biological Macromolecules

Pressure is a well-understood thermodynamic parameter orthogonal to temperature, however, its effects on enzyme activity and protein conformation are very complex and present rich opportunities for research. Indeed, high pressure has been shown to weaken hydrophobic interactions between aliphatic amino acid side chains, while electrostatic interactions are known to be enhanced under pressure [1, 2]. Moreover, main pressure effects on biological macromolecules are attributed to pressure perturbation of the interactions of said molecules with the solvent, leading to reversible partial denaturation of proteins, weakening of lipid bilayers and dissociation of multimeric protein complexes [3]. Pressure acts synergistically with chaotropes and detergents leading to protein denaturation, however, pressure-perturbed proteins were shown to assume conformational forms drastically different from those resulting from thermal or chemical treatment [4].

Pressure Cycling Technology (PCT) uses alternating high hydrostatic pressure to facilitate thermodynamic perturbation of molecular interactions. PCT sample preparation systems offer specific advantages for tissue and cell lysis and improved recovery of hydrophobic molecules, including integral membrane proteins [5, 6]. These systems also have been shown to modulate enzymatic proteolysis and deglycosylation and improve protein sequence coverage in quality control of protein pharmaceuticals. However, most of the published work to date is based on empirical optimization of high pressure extraction and digestion methods [6-9].

Our intent in this on-going study is to systematically investigate high pressure effects on proteins within the context of proteomics and sample preparation methods for mass spectrometry analysis.

Materials and Methods

Pressure-Enhanced Digestion of Model Proteins

In-solution digestion was performed at 50°C either at ambient pressure (control) or with pressure cycling at 20,000psi using the Barocycler NEP 3229 (Pressure BioSciences). An equimolar mixture of model proteins in 8M urea/50mM ammonium bicarbonate was reduced using conventional method [10], then exchanged into 50mM ammonium bicarbonate with 10% n-propanol using 3kDa MWCO Amicon filters, diluted to 0.05 mg/ml (~0.3uM each) and split into aliquots. Trypsin (sequencing grade, Promega) was added at three different enzyme-to-substrate ratios (1:10, 1:50, 1:100) and 100ul aliquots of the reaction mixture (in triplicate) were loaded into PCT MicroTubes. PCT conditions: 50 seconds at 20,000 psi, 10 seconds at atmospheric pressure, per cycle. Control samples were incubated in PCT MicroTubes at 50° without pressure. Reactions were stopped at 0.5, 1.0, 2.0, 4.0, and 20 hours by the addition of 5ul of 5% formic acid.

Adipose Tissue Extraction and in-solution Trypsin Digestion

Seven protein extraction methods were compared, (n=6 each, ~100mg of rat adipose tissue each). Six methods (BeadBeater, Sonic Dismembrator, Dounce homogenizer, mortar/pestle, Shredder SG3 and Pressure Cycling) were performed in ProteoSolve-IEF and one in ProteoSolve-SB with pressure cycling. The PS-SB sample proteins were reconstituted in ProteoSolve-IEF prior to further processing. The proteins were reduced, alkylated and buffer exchanged into 25mM Ambic with 10% n-propanol (as described above). Samples were briefly sonicated to re-dissolve any precipitated proteins, adjusted to 100ul and transferred to PCT MicroTubes. Samples were digested with trypsin (1:100 E:S) at 37°C for 2 hours, then more trypsin was added (final E:S ~1:50) and the digests were incubated with shaking overnight and stopped by addition of 5% formic acid.

Adipose Tissue Extraction and in-Gel Trypsin Digestion

Rat adipose tissue was extracted using 3 methods: 1) Shredder and PCT in 2% SDS, 2) Dounce homogenizer in 2% SDS and, 3) Shredder and PCT in ProteoSolve-SB (PS-SB). The PS-SB sample proteins were re-dissolved in 2% SDS buffer. Extracts were reduced, alkylated and separated by SDS-PAGE (30ug per lane x4). Gels were stained and cut into 14 slices per lane. In-gel digestion and peptide extraction was performed according to [10] using 0.6ug trypsin per sample at 37°C with shaking overnight.

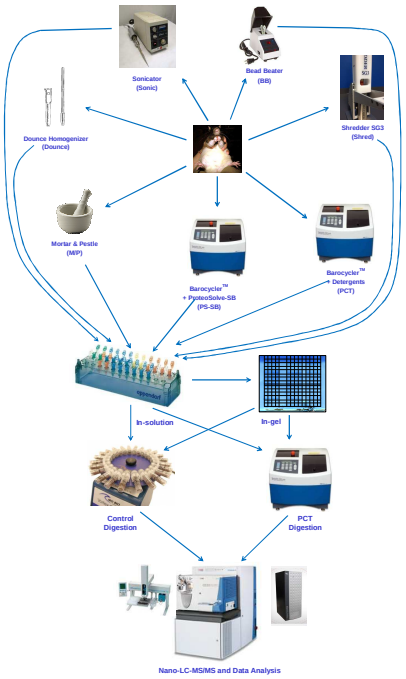
High Resolution Nano-LC-MS/MS

All digests diluted 1:6 with mobile phase were separated by nanoflow liquid chromatography; the eluent was introduced into the LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) via nanospray from the tip of the nano-LC column, and the peptide ion species were fragmented using the collision-induced dissociation mode. The database search was performed using the SEQUEST-Sorcerer algorithm and the Sorcerer IDA2 search engine (Sage-N Research). The label-free AMT quantitative analysis was performed using ProGenesis LC_MS software (Nonlinear Dynamics, U.K.), to obtain peptide abundance measurements from the .raw files.

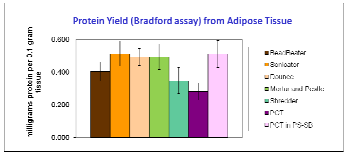
Abstract

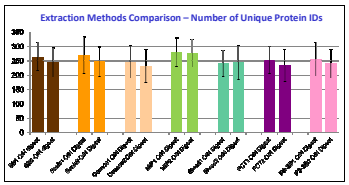
The growing emphasis on quantitative proteomics and label-free methods creates higher demands for robust sample preparation methods. Protein extraction and digestion are the fundamental parts of proteomic sample preparation that have previously been reported to benefit from high hydrostatic pressure. However, earlier reports relied on empirical optimization and did not provide sufficient information about the molecular mechanisms of pressure effects on proteins. We present our initial results obtained in a systematic study aiming to deconvolute pressure effects on protease activity from pressure effects on the conformation of substrate proteins. In addition, we used extraction of the complex adipose tissue proteome as a model to explore the synergistic action of pressure with detergents, organic solvents and chaotropic reagents in comparison with several conventional extraction methods.

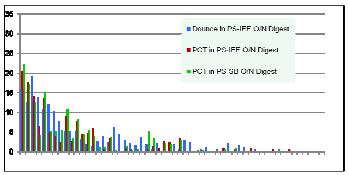
Comparison of Protein Extraction Methods

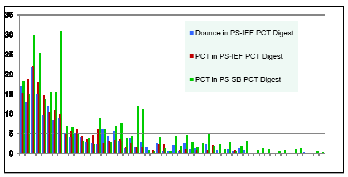


Adipose Extraction Followed by In-solution Digestion





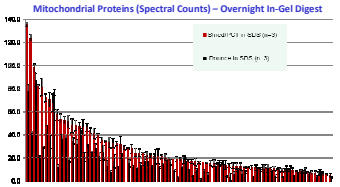


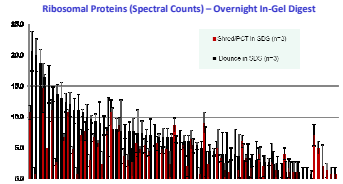


Adipose Extraction Followed by SDS-PAGE Fractionation and In-gel Digestion

Extraction Methods Comparison – Number of Unique Protein IDs

	Shred/PCT in PS-SB	Shred/PCT in SDS	Dounce in SDS
In-Gel Digest (O/N) Protein IDs	847.3± 36.5	837.0± 24.3	961.3± 41.0

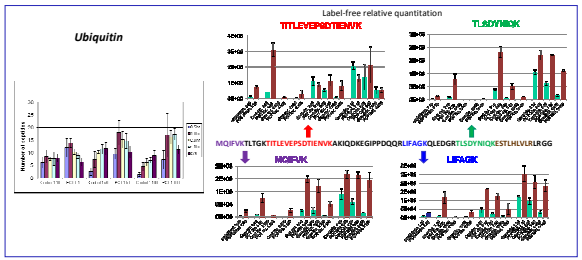


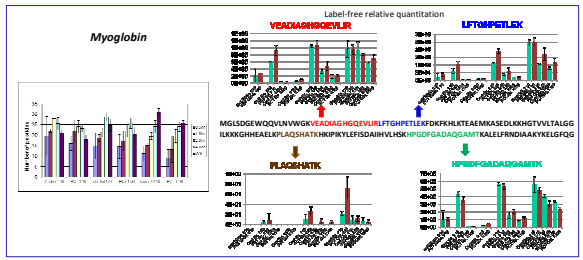


Examples of Proteins Enriched in PS-SB-Extracted Samples

	Shred/PCT in PS-SB	Shred/PCT in SDS	Dounce in SDS
P38650 Cytoplasmic dynein 1 heavy chain 1	51.0± 6.24	28.3± 1.15	22.7± 14.43
P27139 Carbonic anhydrase 2	37.3± 5.03	20.0± 2.00	23.7± 4.04
Q6P6Q2 RAT Keratin, type II cytoskeletal 5	8.3± 3.06	0.0± 0.0	0.0± 0.0
B2RYD3 Myh11 protein (Fragment)	6.3± 1.15	0.3± 0.58	2.7± 2.31
P82471 Guanine nucleotide-binding protein G(q) subunit alpha	6.0± 1.00	1.7± 1.53	1.3± 2.31
Q9QY44 ATP-binding cassette sub-family D member 2	5.3± 0.58	0.7± 1.15	1.3± 1.53
B5DFK1 Copa protein	5.3± 1.53	0.0± 0.0	2.0± 1.00
P61459 Pterin-4-alpha-carbinolamine dehydratase	4.0± 2.00	2.3± 2.52	0.0± 0.0
D3ZF54 Putative uncharacterized protein Ano10	4.0± 1.00	0.0± 0.0	1.3± 1.15
Q6IFC5 Tumor protein D54	3.0± 1.00	0.0± 0.0	0.7± 1.15
P18652 Phosphoglycomutase-1	3.0± 0.0	0.3± 0.58	1.3± 2.31
P63086 Mitogen-activated protein kinase 1	3.0± 1.00	0.0± 0.0	0.0± 0.0

Pressure-Enhanced Protein Digestion

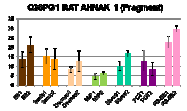




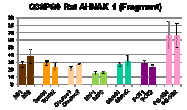
Comparison of Protein Extraction Methods: Synergy of Pressure and Chemistry and Influence of Downstream Separation Methods

Extraction in IEF/SB; In-Solution Digestion; NanoLC-MS/MS*

Extraction in SDS Buffer; SDS-PAGE In-Gel Digestion NanoLC-MS/MS*



	Shred/PCT in PS-SB	Shred/PCT in SDS	Dounce in SDS
Q3SPG1 RAT AHNAK 1	38.0± 5.0	31.0± 3.6	46.0± 10.4



	Shred/PCT in PS-SB	Shred/PCT in SDS	Dounce in SDS
Q3SPG0 RAT AHNAK 1	55.0± 1.0	55.7± 4.0	70.0± 10.2

*Relative quantitation: Spectral counts

AHNAK 1 is a giant protein of approximately 700 kDa originally identified in human neuroblastomas and skin epithelial cells and implicated in murine diet-induced obesity [11] and several other disorders. The protein contains 39 conserved repeat elements consisting of alternating hydrophilic and hydrophobic aminoacid residues. The model predicts a polyionic rod with hydrophobic residues facing inward and charged residues facing outward, resulting in clear separation of the hydrophobic and hydrophilic residues [12]. Such extraordinary structure could potentially explain unusual extraction/digestion behavior of this protein and its susceptibility to high pressure thermodynamics. On-going data analysis of the results obtained in this study may shed more light on mechanism of pressure impact on protein solubility and proteolytic digestion.

Conclusions

PCT-based extraction of complex proteomic samples results in selective enrichment of membrane-associated proteins. Pressure acts synergistically with chaotropic agents and organic solvents on many proteins, particularly useful in less stringent extraction buffers that are more compatible with downstream chromatography and mass spectrometry analysis.

The data also suggest that pressure effects on digestion efficiency are substrate protein-specific. Pressure does not appear to negatively affect digestion of "easy" protein substrates (no significant decrease in peptide recovery or increase in number of semitryptic or miscleaved peptides). Moreover, pressure digestion sheds additional light on the "*The Dark Side of the Proteome*" by providing significant improvements in quantitative recovery of peptides for those proteins that are otherwise resistant to trypsin digestion. Further investigation of this hypothesis is currently being conducted using model integral membrane proteins and complex proteomic samples such as protein extracts from adipose tissue.

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