Mechanistic Investigations of Hydrostatic Pressure Effects on Tryptic Digestion

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Introduction: Pressure Cycling Technology and Pressure Effects on Biological Macromolecules

Pressure is a well-understood thermodynamic parameter orthogonal to temperature. Pressure 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 have been 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 proteases and substrate 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 (E:S) 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.

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.

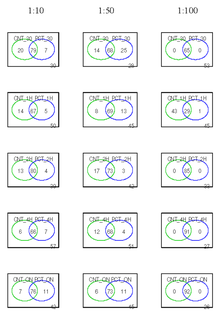
Data Analysis

Pressure effects on tryptic digestion were assessed by extensive data analysis that utilized the NIST_MSQC pipeline (v.1.2.0) and a custom PHP/mySQL data analysis pipeline. Spectra were identified using OMSSA and peptide intensities (MS1) were extracted from the raw files. Subsequently the maximum intensities were calculated using ProMS (NIST, v.May 25,2011). Peptide ion intensities were grouped together to form the basis for detection of reproducible peptide abundances thus accounting for various charge states and/or modifications. Data from these results were then grouped by condition/series and the results were analyzed and plotted using the statistical package R (v.2.14).

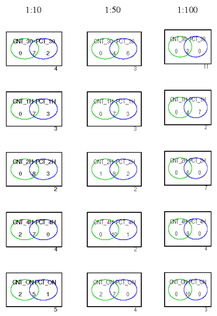
Introduction

Proteolytic digestion is a fundamental bottleneck of proteomic sample preparation. We present the results of a systematic study to deconvolute pressure effects on protease activity from pressure effects on substrate proteins. Model proteins were digested with and without high pressure and analyzed by high resolution MS/MS on the LTQ-Orbitrap XL. The data suggest that pressure effects on digestion are substrate specific, resulting in greater improvements for proteins that are typically resistant to trypsin. Pressure-based digestion appears to be particularly useful for analysis of tough proteins, providing opportunities for time savings and increasing the reproducibility of quantitative analysis.

Pressure effects on peptide identification

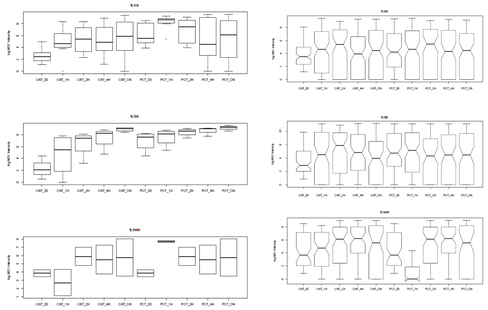


BSA: Effect of pressure on repeatable unique peptide IDs.



Ubiquitin: Effect of pressure on repeatable unique peptide IDs.

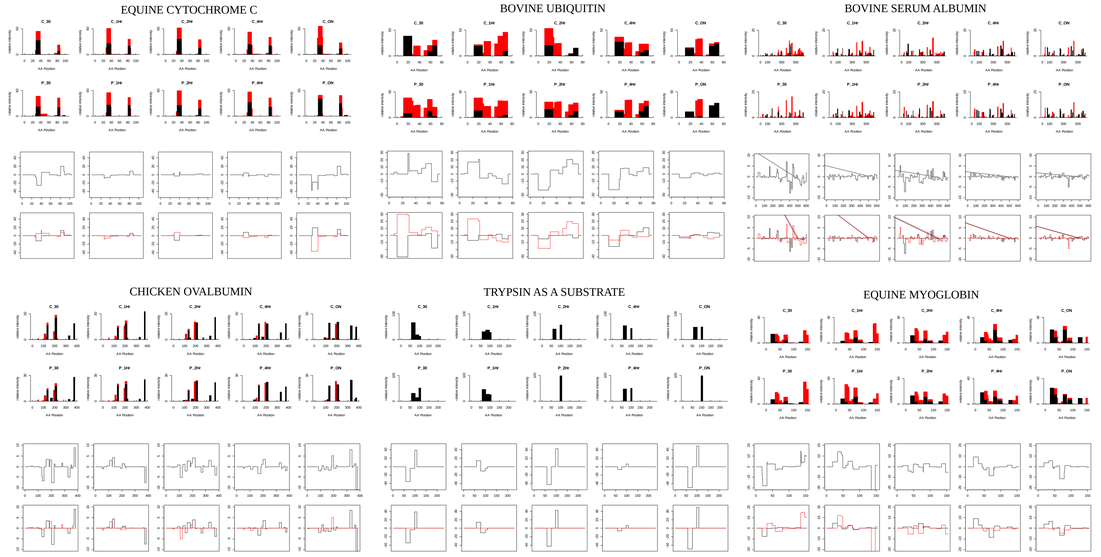
Relative distribution of peptide intensities at different E:S ratios



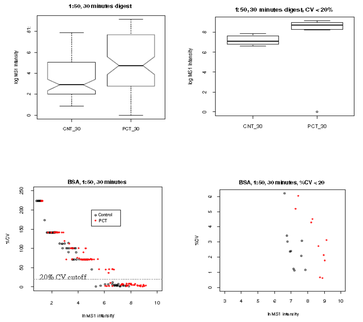
Ubiquitin **BSA**

Relative intensity by amino acid position for all proteins digested at 1:50 enzyme:substrate ratio, pressure treatment vs. control

Legend: ***BLACK*** – fully tryptic peptides, ***RED*** – peptides with 1 or more miscleavage; Panels below: Relative change by AA position:. *GRAY* trace – Pressure treatment minus Control, ***BLACK*** – fully tryptic peptides, ***RED*** – peptides with 1 or more miscleavage.



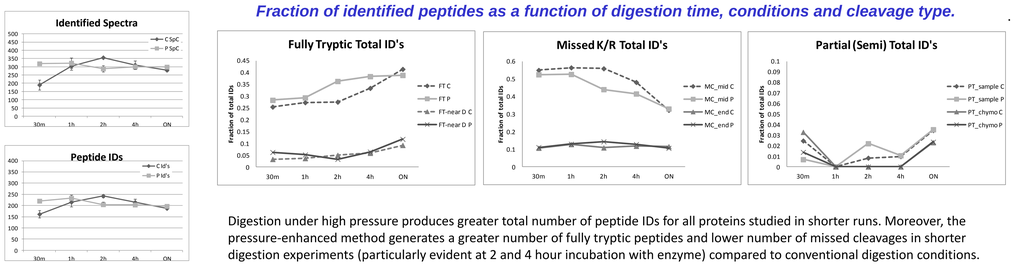
BSA: repeatable peptide intensities



Bovine serum albumin peptides identified with CV≤20% (independent of digestion time) at 1:50 E/S. Pressure-enhanced digestion results in significantly greater intensities for most identified peptides in 30 minute digestion method, thus offering greater throughput for targeted proteomics workflows.

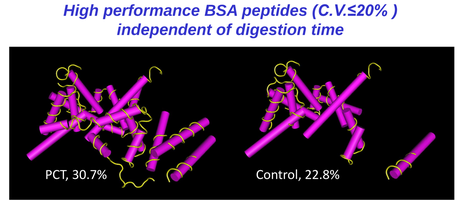
Greater number of peptides suitable for reliable quantitative analysis (C.V.≤20%) was obtained in pressure-based digestion workflow compared to control (figure below). These peptides covered 30.7% and 22.8% of BSA sequence, respectively.

Fraction of identified peptides as a function of digestion time, conditions and cleavage type.



Digestion under high pressure produces greater total number of peptide IDs for all proteins studied in shorter runs. Moreover, the pressure-enhanced method generates a greater number of fully tryptic peptides and lower number of missed cleavages in shorter digestion experiments (particularly evident at 2 and 4 hour incubation with enzyme) compared to conventional digestion conditions.

High performance BSA peptides (C.V.≤20%) independent of digestion time

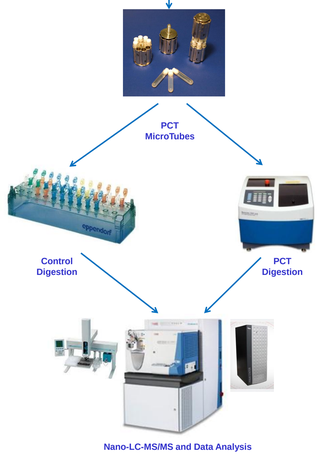


Peptides with low CV: green= in the CONTROL, blue= in the PCT, teal= in both. Peptides quantified with high accuracy are shown with respect to their location on the native protein structure.

Pressure-Enhanced Protein Digestion Workflow

Bovine Serum Albumin, Chicken Ovalbumin, Equine Heart Myoglobin, Bovine Cytochrome C, Bovine Ubiquitin

+ Promega Sequencing Grade Trypsin



Conclusions

The data suggest that pressure effects on digestion efficiency are substrate protein-specific, affecting to the greater extent proteins (e.g. ubiquitin) that are more resistant to conventional tryptic digestion. Pressure digestion does not appear to negatively affect digestion of "easy" protein substrates such as BSA (no significant decrease in peptide recovery or increase in number of semitryptic or miscleaved peptides). Moreover, pressure-enhanced digestion in contrast to conventional method produces greater number of proteotypic peptides that are reproducibly quantified even when short digestion protocols are employed. Thus, pressure can be used to produce more reproducible digests with higher throughput.

Specialized data analysis approach using custom spectral libraries in conjunction with NIST MSQC Pipeline and MS1 peptide intensities obtained directly from raw files by NIST ProMS algorithm offered in-depth analysis of peptide intensities relative to their position in a protein sequence.

References

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