Investigation of Pressure-Enhanced Trypsin Proteolysis

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Pressure Cycling Technology and Pressure Effects on Biological Macromolecules

Pressure is a well-understood thermodynamic parameter that affects enzyme activity and protein conformation. High pressure can weaken hydrophobic interactions but can enhance electrostatic ones [1, 2]. The main pressure effects on biological macromolecules are attributed to perturbation of the interactions with solvent, leading to reversible partial protein denaturation, weakening of lipid bilayers and dissociation of multimeric protein complexes [3]. Pressure-perturbed proteins have been shown to assume conformational forms drastically different from those resulting from thermal or chemical treatment alone [4].

Pressure Cycling Technology (PCT) uses alternating high hydrostatic pressure to facilitate thermodynamic perturbation of molecular interactions. PCT has been shown to modulate enzymatic proteolysis and deglycosylation, and improve sequence coverage. In addition, this system offers specific advantages for tissue and cell lysis, and improved recovery of hydrophobic molecules such as integral membrane proteins [5, 6].

While most of the published work to date is based on empirical optimization of high pressure extraction and digestion methods [6-9], we present part of a systematic study investigating the impact of hydrostatic pressure on trypsin specificity. Our goal is to understand the relationship between cleavage site environment and enzyme activity, as a function of high pressure. Our intent 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

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). A mixture of model proteins was denatured and reduced using conventional methods [10], then exchanged into 50mM ammonium bicarbonate with 10% n-propanol using 3kDa MWCO Amicon filters. 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, 2, 4, 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. 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. These results were then grouped by condition/series and were analyzed and plotted using the statistical package R (v.2.14).

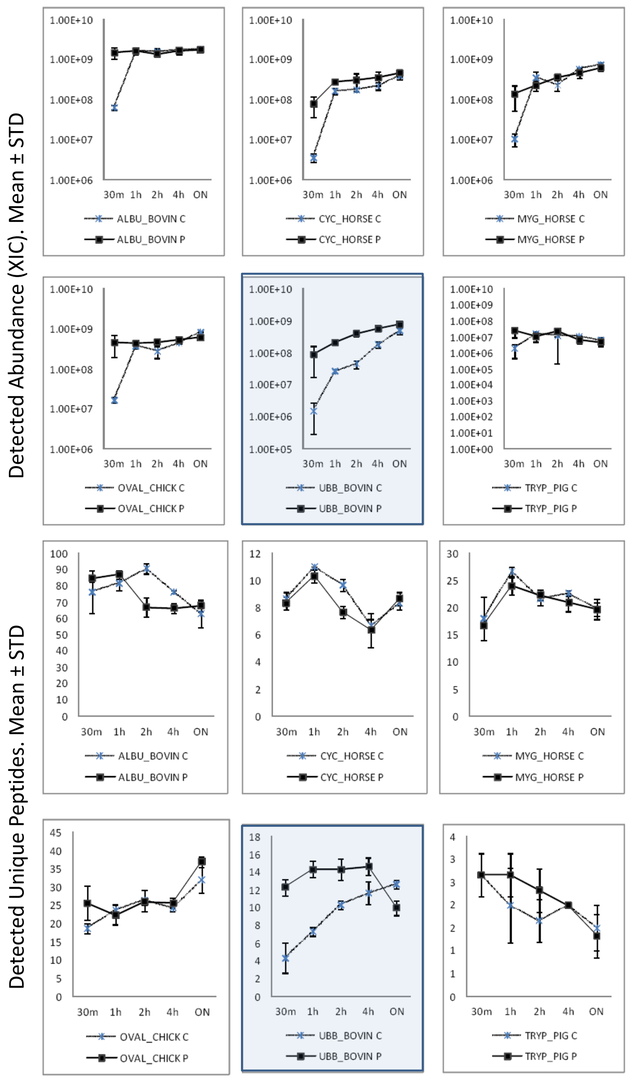
Conclusions

The specialized data analysis approach described above offers in-depth analysis of peptide intensities relative to their position in the protein sequence. The data thus generated suggest that pressure effects on digestion efficiency are substrate-specific, affecting to a greater extent proteins, such as ubiquitin, that are more resistant to conventional tryptic digestion. Pressure digestion does not appear to negatively affect digestion of “easy” substrates such as BSA, as indicated by the absence of any significant decrease in peptide recovery or increase in number of semitryptic or miscleaved peptides. Moreover, pressure-enhanced digestion produces a greater number of proteotypic peptides that are reproducibly quantified even when short digestion protocols are employed. Thus, pressure can be used to generate more reproducible digests using shorter digestion times, leading to higher throughput.

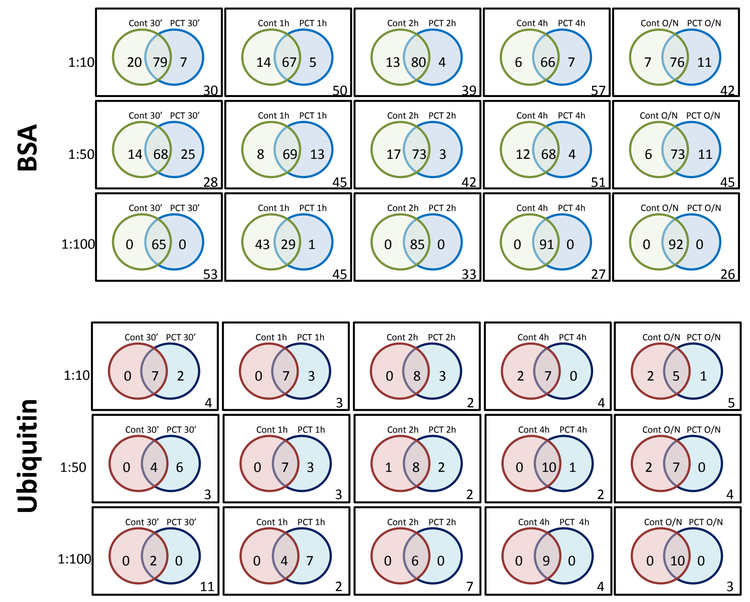
Pressure-Enhanced Protein Digestion Workflow

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

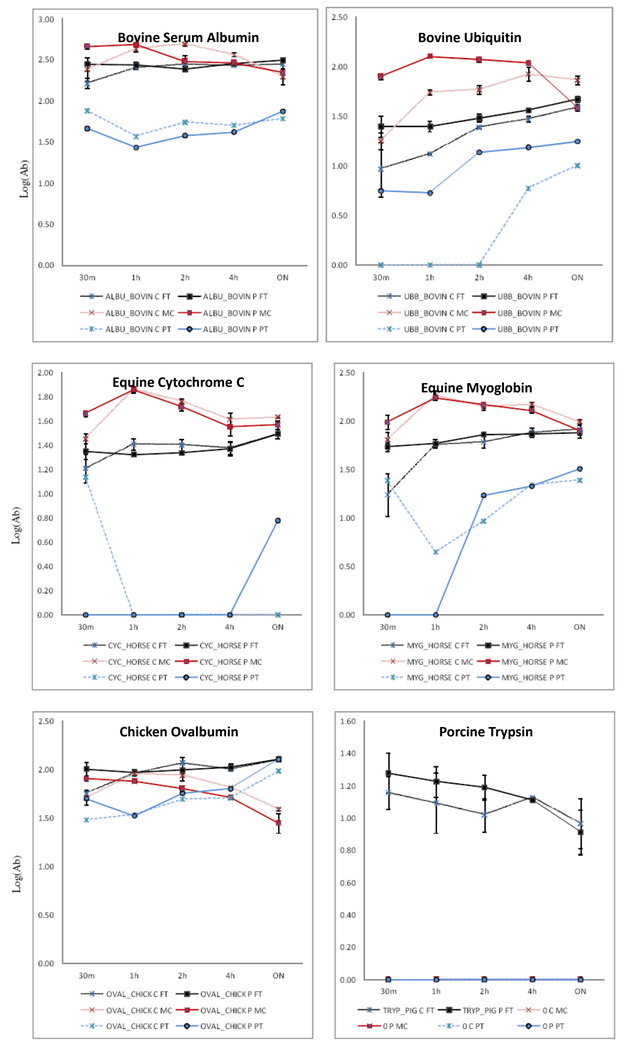
+ Promega Sequencing Grade Trypsin



Pressure improves digestion of some proteins, such as ubiquitin (highlighted), but has little effect (positive or negative) on other proteins, such as BSA and ovalbumin.



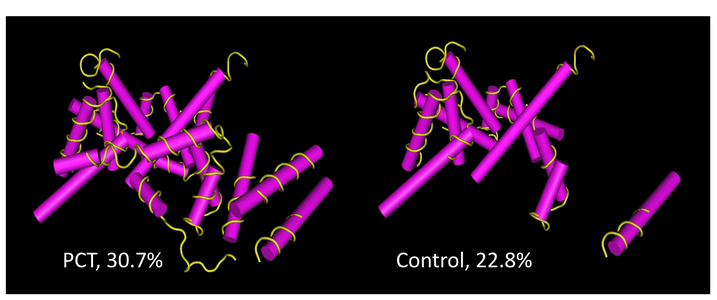
Repeatable unique peptide IDs in control and PCT-enhanced digests shown over time (BSA and Ubiquitin). Enzyme-to-substrate ratios are indicated at the left (1:10, 1:50 or 1:100). Peptides not identified in either PCT or control sample are shown at the bottom right of each panel.



Peptide total abundance in control (C) and PCT-enhanced (P) digests. Data are shown for each individual protein and each cleavage classification.

FT= Fully Tryptic peptides **MC= Miscleaved Peptides** **PT = Partly (Semi) Tryptic Peptides**

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

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  1 MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYGDMADCCEKQEP
121 ERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETMREKVLASSARQRLRCASIQKFGERALKAWSVA
241 RLSQKFPKAEFVEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRR
361 RPEYAVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRTTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYLSLIL
481 NRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEACFAVEGPKLVV
601 STQTALA
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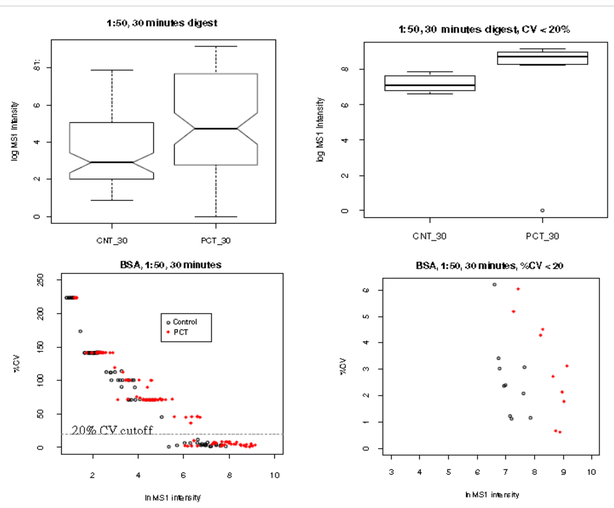
PCT, 30.7%

Control, 22.8%

Peptides with Low CV:
Green= in the Control Digest,
Blue= in the PCT-enhanced Digest,
Teal= in Both.
Peptides quantified with high accuracy are shown with respect to their location on the native protein structure.
Greater numbers of peptides suitable for reliable quantitative analysis (C.V.≤20%) were obtained in the pressure-enhanced workflow compared to control. These peptides covered 30.7% and 22.8% of BSA sequence, respectively .



BSA Peptides: Total peptides vs Low CV (≤20%) peptides.
30 minute digestion with 1:50 E/S, **PCT** vs **Control**.
Pressure-enhanced digestion results in significantly greater intensities for most identified peptides in as little as 30 minutes, thus offering greater throughput for targeted proteomics workflows.

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