Mechanistic Studies of the Pressure-Enhanced Tryptic Digestion

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Pressure-Enhanced Protein Digestion

Pressure Cycling Technology (PCT) uses alternating high hydrostatic pressure to provide thermodynamic control of protein conformation and molecular interactions. PCT sample preparation systems offer specific advantages for tissue and cell lysis and quantitative recovery of hydrophobic molecules, including integral membrane proteins [4]. These systems also have been shown to modulate enzymatic proteolysis and deglycosylation and improve protein sequence coverage in quality control of protein pharmaceuticals. Nevertheless, most of the published work to date is based on empirical optimization of high pressure digestion methods [3, 7, 8]. While pressure is a well-understood thermodynamic parameter orthogonal to temperature, 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 [6]. 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 [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.

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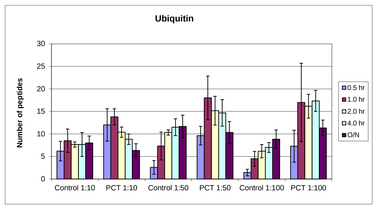
In-solution and in-gel protein digestion can be performed under pressure using Barocycler NEP2320, NEP3229 or HUB440 benchtop high pressure laboratory instruments and a PCT MicroTube Adapter Kit which allows to place up to 48 sample containers into the large pressure chamber. The MicroTube cartridges keep the tubes tightly closed during pressure cycling treatment. The Kit contains convenient Workstation trays, a capping/decapping tool and the MicroTube rack system for compatibility with the microtiter plate liquid handling equipment.

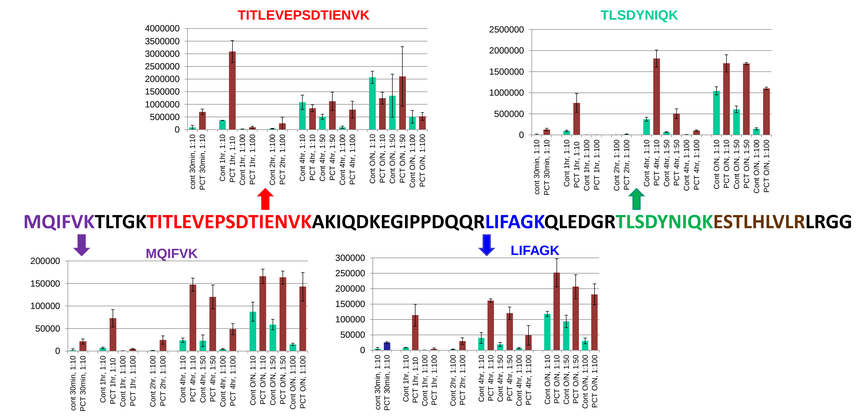
MicroTube Adapter Kit for Barocycler NEP3229

Introduction

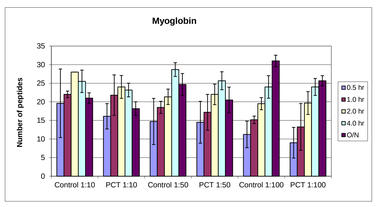
The modern paradigm shift toward quantitative proteomics, especially label-free methods, raises the bar of reproducibility and quantitative recovery to a new level, creating new demands for robust sample preparation methods. Protein extraction and digestion are the two fundamental parts of proteomic sample preparation which have been shown to benefit from high hydrostatic pressure. However, most reports to date have not provided sufficient information on mechanistic aspects of pressure effects on the proteolytic digestion. We present preliminary results from a systematic study aiming to deconvolute pressure effects on protease activity from pressure effects on conformation of substrate proteins. In this study several model proteins were digested in controlled conditions using three enzyme-to-substrate ratios with and without high hydrostatic pressure. Resulting digests were separated on nanoflow HPLC and analyzed by high resolution MS/MS on the LTQ-Orbitrap XL. Pressure effects on tryptic digestion were assessed by relative label-free quantitation of resulting mass spectra.

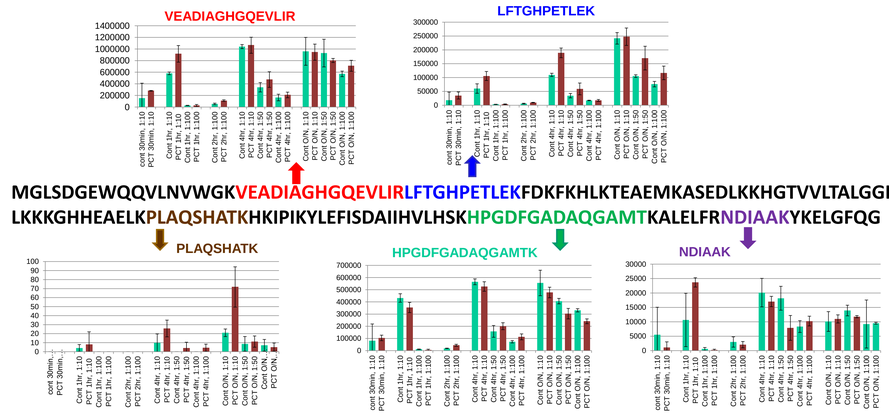
Ubiquitin



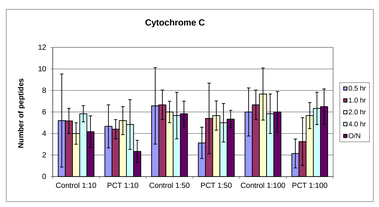


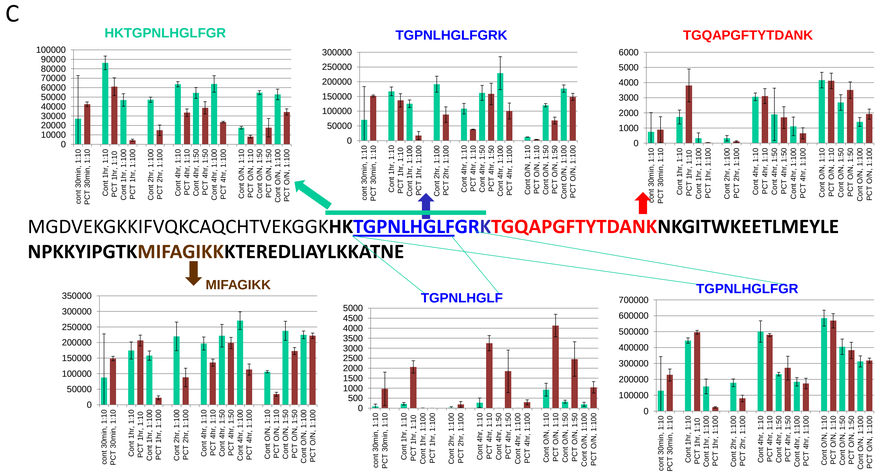
Myoglobin





Cytochrome C





Materials and Methods

An equimolar mixture of purified proteins was prepared as follows: equine myoglobin, equine cytochrome C and bovine ubiquitin (all from Sigma) were dissolved to 2.5 mg/ml in 8M urea/50mM Ammonium Bicarbonate. The protein solutions were reduced with 5mM TCEP, alkylated with 10mM iodoacetamide (IAA) and quenched with 5mM TCEP. The protein solutions were exchanged into 50mM Ammonium Bicarbonate using 3kDa MWCO 4ml Amicon filters. After buffer exchange, the concentration of each protein was adjusted to 0.25mg/ml and combined in equimolar ratio (~1.6uM each).

For Trypsin digestion, the resulting protein mixture was diluted to 0.05 mg/ml (~0.3uM each) with 50mM Ammonium Bicarbonate containing 10% n-propanol. The solution was split into 3 aliquots for digestion with trypsin at three different enzyme-to-substrate ratios (1:10, 1:50, 1:100). Trypsin (sequencing grade, Promega) was added and the reactions were quickly aliquoted into PCT MicroTubes (100ul per MicroTube). Pressure cycling was performed in an NEP 3229 Barocycler chamber heated to 50˚. 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.

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 (version 3.5 RC2; Sage-N Research). The label-free AMT quantitative analysis was performed using ProGenesis LC_MS (version 2.5) software (Nonlinear Dynamics, U.K.), to obtain peptide abundance measurements from the .raw files.

Conclusions

The data obtained to date suggest that pressure effects on digestion efficiency are substrate protein-specific, resulting in significant improvements in quantitative recovery of peptides for those proteins that are otherwise resistant to trypsin digestion. The benefits of pressure digestion of “easy” protein substrates may not be as obvious; however, there is no significant decrease in peptide recovery or increase in number of semitryptic or miscleaved peptides for such proteins digested under pressure, as long as digestion conditions do not severely affect trypsin conformation. Therefore, pressure-enhanced protein digestion could shed additional light on the “*The Dark Side of the Proteome*” by improving quantitation of difficult protein species in complex samples. 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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