Mechanistic Studies of the Pressure-Enhanced Tryptic Digestion

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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.

Materials and Methods

An equimolar mixture of purified proteins was prepared as follows: equine myoglobin, equine cytochrome C and bovine adrenalin (all from Sigma) were dissolved to 2.5 mg/ml in 10 mM urea/50 mM Ammonium Bicarbonate. The protein solutions were mixed with 5mM TCEP, and lyophilized with 15mM iodoacetamide (IAA) and quenched with 5mM TCEP. The protein solutions were exchanged into 5mM Ammonium Bicarbonate using 3KDa MWCO 40-mL Amicon filters. After buffer exchange, the concentration of each protein was adjusted to 0.25 mg/ml and combined in equimolar ratio (~1:100 each).

For Trypsin digestion, the resulting protein mixture was diluted to 0.05 mg/ml (“1xSx” each) with 5xM Ammonium Bicarbonate containing 10% DMSO. The solution was split into 3 aliquots for digestion with trypsin at three different enzyme-to-substrate ratios (1.0, 3.0, 10:1). Trypsin (sequencing grade, Promega) was added and the reactions were quickly aliquoted into PCT MicroTubes (300µl per MicroTube). Pressure cycling was performed in an NEP 3229 Barocycler chamber heated to 50°C. PCT conditions: 50 seconds at 20,000 psi, 10 seconds at atmospheric pressure, per cycle. Control samples were incubated in PCT MicroTubes at 50°C without pressure. Reactions were stopped at 0.5, 1.0, 2.0, 4.0, and “20 hours by the additional 10°C.

All digests diluted 1:4 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 IDA search engine (version 3.5.12; Sage-N Research). The label-free AMT quantitative analysis was performed using Progenesis LC/MS (version 2.5) software (Nonlinear Dynamics, Ltd.), 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 tryptic 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 semistryps 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.

References