Demystifying proteolytic digestion under high pressure cycling: Enzyme specificity, efficiency and synergy of pressure with acid-labile detergents.

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Introduction

Incomplete or inconsistent proteolytic digestion is a significant obstacle for quantitative proteomics. High hydrostatic pressure and, specifically, pressure cycling technology (PCT), have been shown to denature proteins, increase protease activity and accelerate protein digestion. Herein we present the results of an investigation of hydrostatic pressure and higher temperature on the specificity and activity of several proteases including trypsin, chymotrypsin, Lys-C and the new enzyme Tryp-N. Heat and pressure cycling can be used to enhance substrate protein denaturation which minimizes reliance on chemical denaturants and boosts recovery of digestion products, while also speeding up the process. Tryp-N, developed at Cold Spring Harbor laboratory and commercialized by Protifi, LLC., is a thermophilic metalloprotease with N-terminal specificity for arginine and lysine. Since basic centers of peptides (the amino terminus and side chains of K/R) occur in one location, b-ions predominate in MS/MS of Tryp-N peptides. Thermal stability of this enzyme prompted us to investigate its performance at high pressure to assess its resistance to pressure denaturation.

Pressure Cycling Technology (PCT)

Bench-top High Pressure Sample Processing

Barocycler NEP2320

PCT MicroTubes

PCT MicroTube Cartridge

Alternating hydrostatic pressure, or Pressure Cycling technology (PCT) has been shown to reduce digestion time from 18 hours to 1 hour or less, and is currently the method of choice in many labs. At high pressure, enzyme activity is a balance between the benefit effected by pressure-denaturation of the protein substrate and the negative effect of pressure-denaturation on the enzyme itself. Previously, we have shown that optimal trypsin digestion occurs at 20,000psi and that significantly higher pressure levels may be detrimental to trypsin activity. However, many other enzymes relevant to proteomics appear to be more stable and more active at high pressure. The goals of this study were to evaluate the stability, activity and specificity of several enzymes under pressure and at higher temperature, in order to determine their suitability in an optimized sample preparation workflow for high resolution proteomic analysis.

Materials and methods

Pressure cycling was carried out using a Barocycler (Pressure Biosciences). Reduced/alkylated rat liver lysates, containing 0.05% RapiGest, were digested at 55°C. Trypsin and Tryp-N were used at 20,000psi for 1 hour (60 cycles). Double digests were digested with Lys-C at 45,000psi for 45 mins following digestion with trypsin as above.

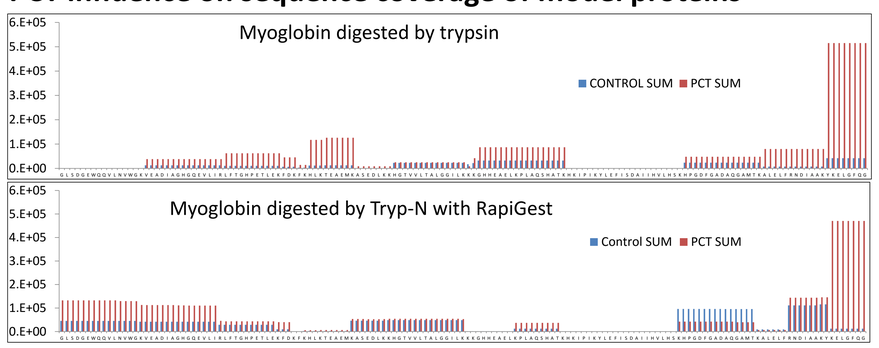
Model proteins were digested with Trypsin (with or without addition of 10% N-propanol) and Tryp-N at 20,000psi. Chymotrypsin and Lys-C were used at 45,000psi and Glu-C at 30,000psi. All model protein digests were incubated for 90 minutes (90 PCT cycles), except Lys-C which required only 45 cycles. Atmospheric pressure controls were incubated at the same time and temperature as the pressure-treated samples. Additional positive controls were incubated using standard conditions (overnight at 35°C on a Thermomixer (Eppendorf) with shaking for all enzymes except Tryp-N. The standard Tryp-N condition used was 3 hours at 55°). Enzyme to substrate ratios was 1:50-1:60 for all enzymes except chymotrypsin, which was used at 1:100. All Tryp-N digestions were supplemented with 0.05% RapiGest.

For parent protein visualization, samples were separated by SDS-PAGE.

Samples were run on Orbitrap-class instruments and analyzed using Mascot and a suite of home-build informatics tools. Signal intensity at each amino acid position was calculated by summing the intensity of all peptides spanning that amino acid, divided by the number of amino acids in each peptide.

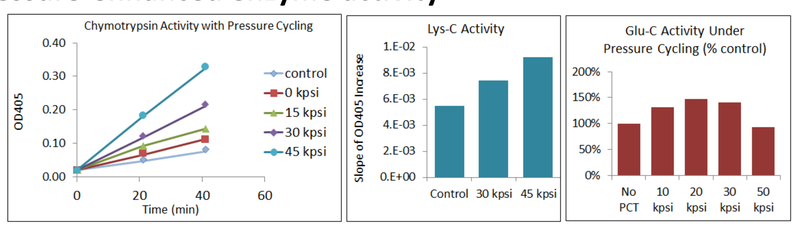
Sequencing grade trypsin, chymotrypsin and Glu-C were purchased from Promega. Lys-C was purchased from Wako Chemicals USA. Tryp-N was from Protifi, LLC. Model proteins were purchased from Sigma. Rapigest was obtained from Waters Corp.

PCT influence on sequence coverage of model proteins



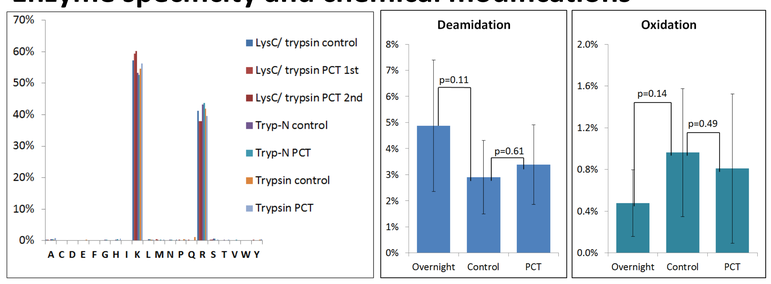
Signal intensity was calculated for each amino acid in the protein sequence. The improved signal intensity and coverage in the pressure-treated digest can be easily seen here.

Pressure-enhanced enzyme activity



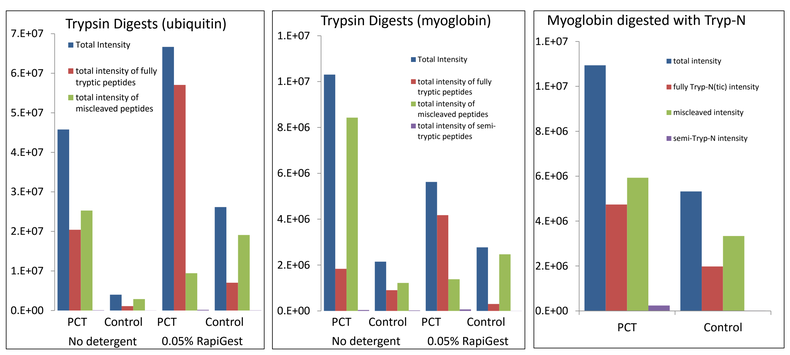
Pressure effects on enzymatic activity of chymotrypsin, Lys-C, as Glu-C were measured spectrophotometrically using appropriate chromogenic substrates to eliminate any influence of pressure on protein substrate conformation.

Enzyme specificity and chemical modifications



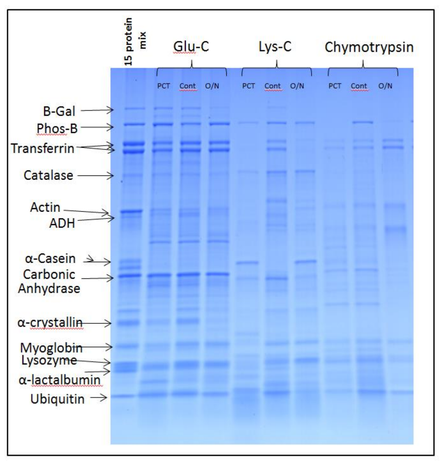
Pressure Cycling was shown not to have any significant influence on enzyme specificity or non-specific chemical modifications of proteins such as methionine oxidation and asparagine or glutamine deamidation. Reduced and alkylated rat liver lysate was subjected to digestion by several enzymes and their combinations with or without pressure cycling. The occurrence of Lysine or Arginine –terminated peptides (at the appropriate terminus) predominate in digests by trypsin, Lys-C/Trypsin and Tryp-N in all conditions tested, indicating than the rate of non-specific cleavage is not increased by PCT. Rates of deamidation and oxidation were derived from digests of a mixture of 15 model proteins, and also show no significant increase under pressure cycling.

PCT boosts recovery of model protein peptides

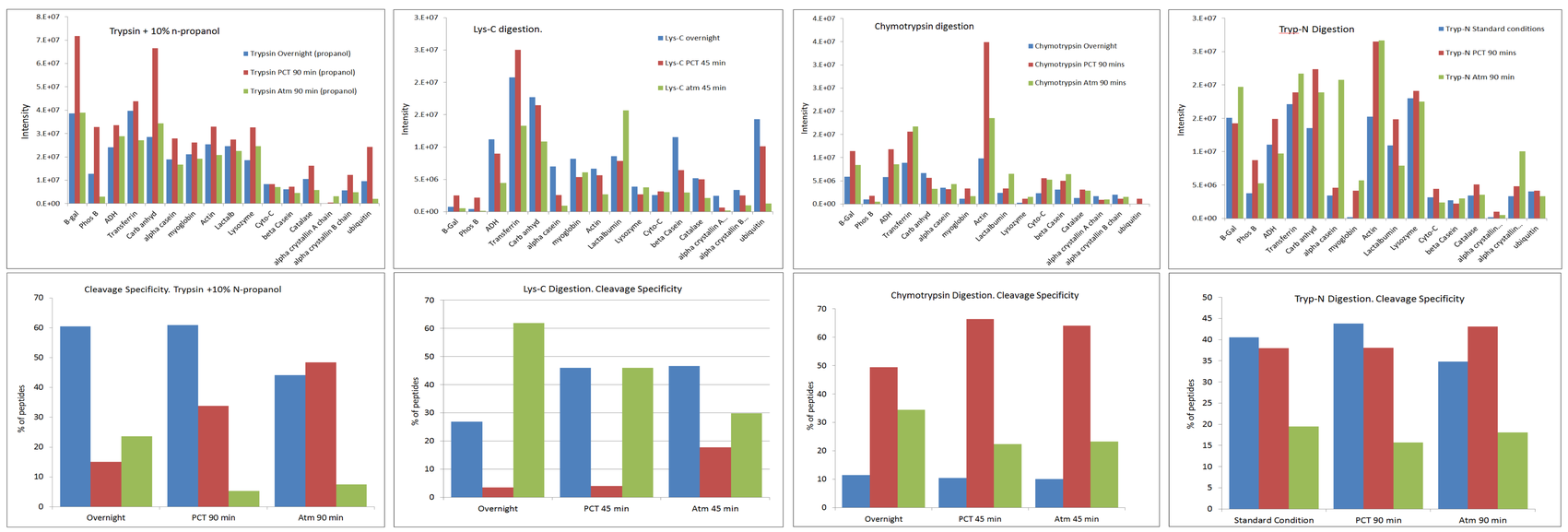


Digestion under pressure resulted in higher proteolysis and subsequent intensity of detection for many proteins that are not efficiently digested in conventional methods. Horse heart myoglobin and bovine ubiquitin were used as model proteins to illustrate digestion efficiency with and without influence of high pressure. As measured by total intensity (counts) of identified peptides, PCT produced higher recovery of peptides in each digest. Addition of Rapigest at half of manufacturer-recommended concentration led to further enhancements in peptide recovery, and highlights the synergy of pressure and chemistry. Similar effects of other chemical additives such as n-propanol are observed in experiments with 15 model protein mixture shown below.

Analysis of a mixture of 15 model proteins



A mixture of 15 model proteins was used in this study. These proteins were chosen based on their availability in purified form, properties, abundance, solubility, and susceptibility to proteolysis and analysis by mass spectrometry.



Conclusions

Pressure Cycling Technology is a unique and rapid option for proteomic sample preparation. In general, pressure cycling results in the same or better results with all proteases tested in significantly reduced time. Although mechanistic aspects of pressure effects on protein substrate conformation, enzyme kinetics and specificity are still not entirely understood, substantial empirical evidence suggests that pressure acts in a manner that is analogous to, or synergistic with, chemical denaturants and temperature to enhance proteolytic digestion by several enzymes relevant to proteomic analysis. Moreover, pressure offers thermodynamic conditions orthogonal to thermal effects that are not attainable with any other popular sample preparation methods. However, pressure is synergistic with temperature and chemical agents in its ability to efficiently predispose protein substrates to enzymatic digestion, as long as enzyme activity is preserved under those conditions. Increased signal intensity and improved sequence coverage with Trypsin, Lys-C, Chymotrypsin and Tryp-N enzymes has been demonstrated. No significant change in enzyme specificity, deamidation or methionine oxidation rate was observed in any conditions tested.

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