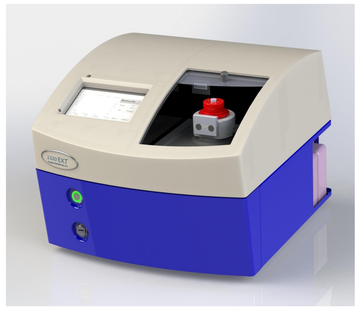
Improved proteolytic digestion under high pressure cycling: rapid digestion with improved sensitivity and sequence coverage.

Vera Gross[1]; John Wilson[2]; Alexander Lazarev[1]

[1]Pressure BioSciences, Inc. Medford, MA; [2]Protifi, LLC, Huntington, NY.

Introduction

Reproducible quantitative proteomics necessitates complete and efficient sample digestion. No amount of improvements in the hardware or software for data acquisition and analysis will compensate for poor sample preparation and incomplete digestion. To improve sample digestion, various approaches such as combinations of multiple proteases, elevated enzyme concentration(s), or addition of detergents or other denaturants, have been used with varying degrees of success. Pressure cycling, in which the reaction is exposed to cycles of high hydrostatic pressure, has been shown to accelerate digestion with proteases. Here we present a systematic comparison between samples digested with and without pressure. We emphasize reduced time, improved quantitation and sequence coverage. In addition we examine the specificity and activity of several enzymes under PCT conditions.



Barocycler 2320EXT

PCT MicroTubes

MicroTube Cartridge

Methods

Fifteen model proteins were reduced/alkylated and combined in equimolar ratio. Rat liver lysates were denatured, reduced/alkylated and buffer exchanged into ammonium bicarbonate containing 0.05% RapiGest detergent (Waters).

Pressure cycling was performed in a Barocycler (Pressure Biosciences) as described below. For parent protein visualization, samples were separated by SDS-PAGE.

All digests were run on Orbitrap-class instruments and analyzed using Mascot and a suite of home-build informatics tools.

Pressure cycling-accelerated digestion was performed as follows:

Trypsin: 20,000psi at 55°C ±10% n-Propanol or 0.05% RapiGest.

Model proteins were digested for 90 minutes. Liver lysates were digested for 60 minutes with Trypsin alone. Double digests were digested with Lys-C at 45,000psi for 45 mins, followed by trypsin for 60 mins at 20,000psi.

Chymotrypsin: 45,000psi at 33°C for 90 mins.

Lys-C: 45,000psi at 55°C for 45 mins.

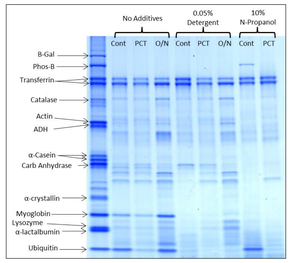
Glu-C: 30,000psi at 30°C for 90 mins.

Tryp-N: 20,000psi at 55°C for 90 mins in reaction buffer supplemented with 0.05% RapiGest.

For all PCT digests, **atmospheric pressure controls** were incubated in the same buffer, at the same time and temperature, as the pressure-treated samples. Additional positive controls were incubated using **standard conditions** (overnight at 37°C on a Thermomixer (Eppendorf) with shaking for all enzymes except Tryp-N. The standard Tryp-N condition used was 3 hours at 55° without shaking). Enzyme to substrate ratios was 1:50-1:60 for all enzymes except chymotrypsin, which was used at 1:100.

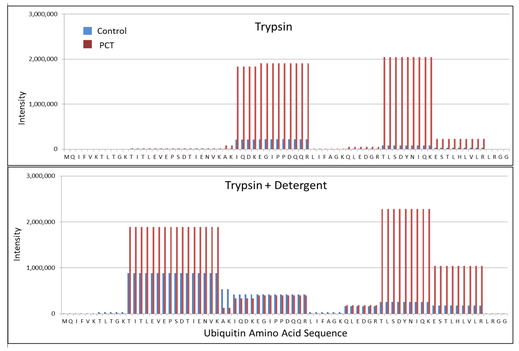
Sequencing grade trypsin, chymotrypsin and Glu-C were purchased from Promega. Lys-C was purchased from Wako Chemicals USA. Tryp-N was from Protifi, LLC. Model proteins were purchased from Sigma. RapiGest from Waters Corporation.

Effect of PCT on Trypsin



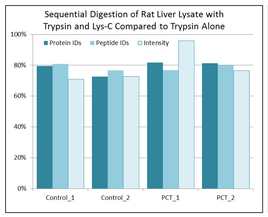
Accelerated Digestion

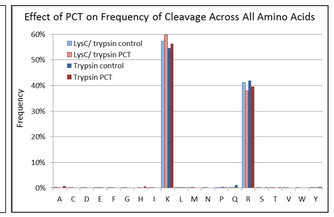
Model protein mix digested with trypsin visualized by SDS-PAGE. The PCT-treated samples show significantly more protein digestion compared to controls. Undigested protein mix is shown on the left for comparison. Addition of detergent or N-propanol further improves the PCT digests (note improved digestion of myoglobin and ubiquitin).



Improved Intensity and Sequence Coverage

Model protein (equine ubiquitin) digested with trypsin for 90 minutes under pressure cycling conditions at 20,000psi (PCT) shows significantly increased signal intensity and improved sequence coverage, compared to control incubated for 90 minutes at ambient pressure. Digests were carried out with or without the addition of mass spec compatible detergent. PCT significantly improves intensity and sequence coverage in both conditions. The addition of detergent serves to further improve digestion and increase coverage. 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.





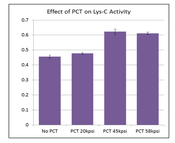
Sequential Digestion with Lys-C and Trypsin

Rat liver lysate was digested with trypsin with or without pre-digestion by Lys-C. For PCT-accelerated samples, digests were carried out with pressure cycling at the appropriate pressures (Lys-C at 45kpsi and trypsin at 20kpsi). The # of unique protein IDs, peptide IDs and total signal intensity were compared. The bar graph (**A**) shows the double digest relative to trypsin alone (ie, the value for the corresponding trypsin digest was set at 100%, and the value for the double digest is shown relative to that). The results of two independent experimental replicates are shown (1 and 2). Amino acid specificity is shown in (**B**). Addition of a Lys-C digest step does not appear to change the ratio of lysine-to-arginine cleavage, compared to trypsin alone.

Trypsin Result: PCT accelerates trypsin digestion compared to controls digested either under similar conditions without pressure, or standard overnight digestion. Improved signal intensity and sequence coverage in PCT-treated digests is evident in samples prepared in 50mM ammonium bicarbonate with or without the addition of detergent or n-Propanol.

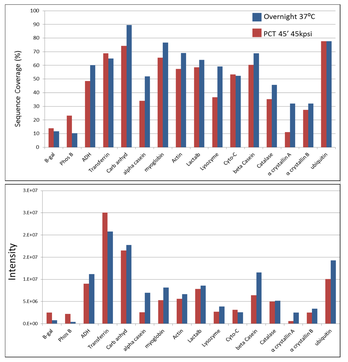
Lys-C / trypsin sequential digests of liver lysate in urea-free buffer produce ~20% fewer protein IDs and ~20% lower signal intensity compared to trypsin alone. The sequential digest does not appear to increase the rate of cleavage at arginines compared to lysines.

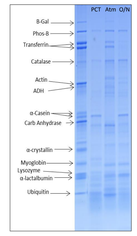
Effect of PCT on Lys-C



Accelerated Activity

Lys-C activity is significantly increased at 45,000psi (45kpsi), compared to control. At 20,000psi, the effect of pressure is almost negligible, suggesting that accelerated lys-C digestion should be carried out at higher pressure. In addition, the enzyme was found to be very pressure-stable, as it showed no significant loss of activity even at 58,000psi. Lys-C activity was measured using a chromogenic synthetic substrate.





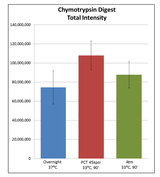
Accelerated Digestion

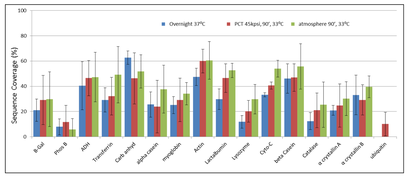
Model protein mix digested with Lys-C visualized by SDS-PAGE. The PCT digest shows significantly more protein digestion compared to controls. Undigested protein mix is shown on the left for comparison.

No Loss of Coverage or Intensity

Sequence coverage and total intensity of all 15 model proteins digested with Lys-C. After just 45 mins of PCT, sequence coverage and intensity are comparable to the overnight digest. For some difficult-to-detect proteins (eg, β-gal and Phos-B), intensity is higher in the short PCT digest than in the overnight control.

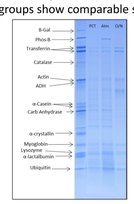
Effect of PCT on Chymotrypsin





Improved Intensity

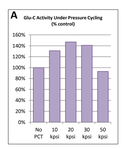
Total intensity and sequence coverage of all 15 model proteins digested with chymotrypsin. Intensity (pooled) is highest in the PCT digest compared to the 2 controls. Sequence coverage is shown individually for each protein. All 3 groups show comparable sequence coverage. Bars represent average of 3 biological replicates (± std dev).

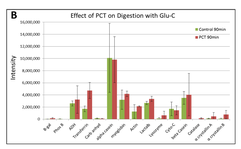


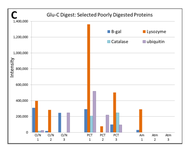
Accelerated Digestion

Model protein mix digested with chymotrypsin and visualized by SDS-PAGE. The PCT digest shows significantly more protein digestion compared to controls. Undigested protein mix is shown on the left for comparison.

Effect of PCT on Glu-C







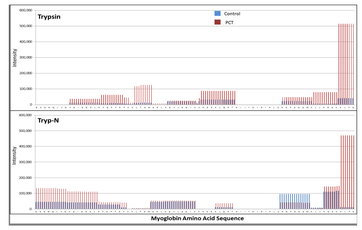
Accelerated Activity and Improved Digestion

A. Glu-C enzyme activity is significantly increased by pressure cycling. Activity is highest at 20,000-30,000psi (kpsi) and declines at higher pressure, likely due to denaturation of enzyme. Glu-C activity was measured using a chromogenic synthetic substrate. **B.** Total intensity of all 15 model proteins digested with Glu-C. Bars represent average of 3 biological replicates (± std dev). **C**. Poorly digested proteins appear to benefit most from PCT digestion (3 replicates shown individually due to high variability between MS runs).

Effect of PCT on Tryp-N

Tryp-N 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.

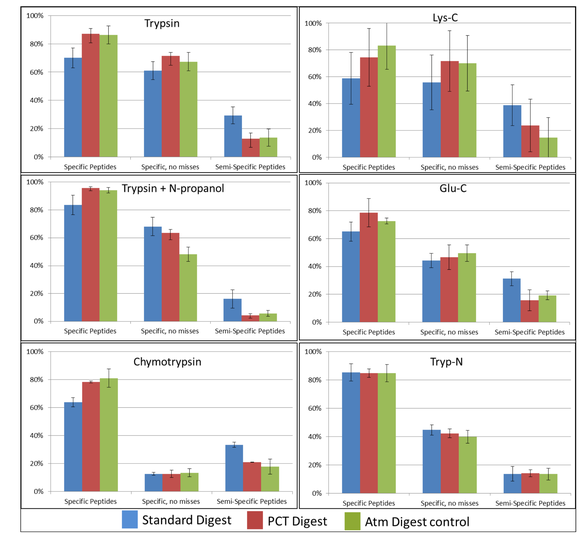
Tryp-N digestion of a model protein shows increased sequence coverage and intensity when the digest is carried out under PCT conditions at 20,000psi. In addition, comparison of Tryp-N and trypsin digested protein shows that regions that are poorly digested by trypsin are better digested by Tryp-N and vice versa. Suggesting that these two enzymes may yield complementary, rather than redundant, data sets.

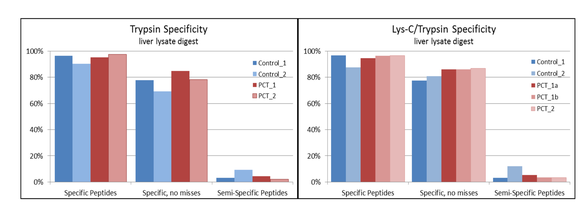


Improved Sequence Coverage and Intensity

Example of a model protein (myoglobin) digested by trypsin (top) or Tryp-N (bottom) at atmospheric pressure (control) or pressure cycling at 20,000psi (PCT). Total signal intensity at each amino acid position is shown. Signal intensity at each amino acid was calculated by summing the intensity of all peptides spanning that amino acid, divided by the number of amino acids in each peptide. All digests performed at 55°C for 90 minutes.

Effect of PCT on Enzyme Specificity





Consistent Enzyme Specificity

A. Model proteins (15 protein mix) were digested with or without PCT with the indicated enzymes. Results shown are averages of three biological replicates (±std dev). **B.** Rat liver lysates were digested with or without PCT, either with trypsin alone or by sequential digestion with Lys-C and trypsin. Results of two independent replicates (1 and 2) are shown.

All unique peptides were grouped into 3 categories based on cleavage specificity (Specific, with or without missed cleavage sites. Specific with no missed cleavage sites. Semi-specific, with or without missed cleavage sites). Results are expressed as percent of all unique peptides detected. No loss or change of enzyme specificity was observed in any of the PCT-treated digests compared to controls, indicating that at the pressures and temperatures used here, the enzymes do not show significant increase in cleavage at non-specific sites.

Conclusions: Our data strongly suggest that use of pressure cycling technology (PCT) increases the recovery of observed peptides and improves sequence coverage, while also reducing sample preparation time. The effects of pressure cycling on trypsin proteolysis are, in part, substrate protein-specific, and can result in significant improvements in digestion and quantitative recovery of peptides from difficult-to-digest proteins that may be underrepresented in samples prepared using traditional workflows. Digestion of model proteins with trypsin for 90 minutes under pressure cycling conditions at 20,000psi shows significantly increased signal intensity and improved sequence coverage, compared to controls incubated for 90 minutes at ambient pressure. The improved digestion in PCT-treated samples is evident in samples prepared in 50mM ammonium bicarbonate with or without the addition of acid-labile detergent (0.05% RapiGest). Addition of 10% n-Propanol serves to further improve the PCT digest without compromising trypsin specificity. Pressure-accelerated digestion with trypsin alone was comparable to, or better than, sequential digestion with lys-C and trypsin, suggesting that under the conditions used here (PCT, urea-free buffer, model proteins), trypsin alone is sufficient for optimal digestion. However, it is likely that for samples in urea-containing lysis buffers, the benefit of including a lys-C step may be significant, as this enzyme is far more active in urea than is trypsin. In addition, we show that PCT can be used to accelerate the specific activity of other enzymes, including chymotrypsin, Lys-C, Glu-C, and Tryp-N without compromising enzyme specificity. Pressure Cycling has previously been shown not to cause an increase in non-specific chemical modifications of proteins such as methionine oxidation and asparagine or glutamine deamidation, which, combined with the specificity results presented here, strongly suggests that there are few, if any, drawbacks to incorporating PCT into current Proteomics workflows.

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