Pressure Cycling-Accelerated Digestion with Trypsin: Protein Modifications and Enzyme Specificity

Introduction

The positive effect of Pressure Cycling Technology (PCT) on digestion with trypsin is well established [1-5], and has been shown to result in improved sequence coverage, higher peptide intensities and significantly reduced digestion times. Additionally, the enhancing effect of PCT on the activity of several other enzymes, including Lys-C [6-8], chymotrypsin [9,10], Glu-C [11], thermolysin [12], lysozyme [13], and PNGase F [14,15], has been reported.

Pressure, as well as heat and a variety of chemicals, can be used to denature proteins, but the pressure-perturbed proteins assume conformational forms that are different from those caused by thermal or chemical treatments [16]. Pressure-induced denaturation of substrate proteins leads to better access of enzymes to previously inaccessible, or poorly accessible, target sites. This, in turn, results in improved and accelerated enzyme activity, as long as the level of applied pressure is below the level at which the enzyme itself is denatured and inactivated. In addition, under certain conditions, hydrostatic pressure can have a positive effect on enzyme activity itself, independent of substrate conformation.

Here we report that 90 minute pressure-accelerated trypsin digestion of whole tissue lysate is comparable to, or better than, standard overnight digestion. The goal of this work is to provide the user with the best set of *starting conditions* for pressure-enhanced trypsin digestion of complex samples.

PCT Sample Preparation System (PCT SPS)

Pressure Cycling Technology uses hydrostatic pressure cycles to control biomolecular interactions and to accelerate certain enzymatic reactions. The samples in the pressure chamber are exposed to rapid and repeated pressurization from atmosphere (14.7 psi) to levels as high as 45,000psi (45kpsi), or higher in some applications.

The PCT sample preparation system is comprised of a Barocycler instrument, such as the 2320EXT, and high pressure-resistant sample containers such as PCT MicroTubes with MicroCaps. The specially designed PCT MicroTubes are single-use sample processing containers made of a non-reactive Teflon-like material. These tubes are designed to hold 50-150µl of sample. MicroCaps of different lengths are used to displace excess air in MicroTubes. Other sample containers, that are suitable for larger sample volumes up to 1.4ml, are also available. Up to 16 MicroTubes at a time can be pressure cycled in the 2320EXT, to allow batch-mode processing of multiple samples.

Results and Discussion

Previously we demonstrated the effect of pressure cycling at different pressure levels on digestion efficiency of disulfide-intact proteins [5]. For the current samples, we compared 90 minute digestion with pressure cycling at 45kpsi to standard 18 hour digestion at ambient pressure, using reduced and alkylated kidney tissue lysates.

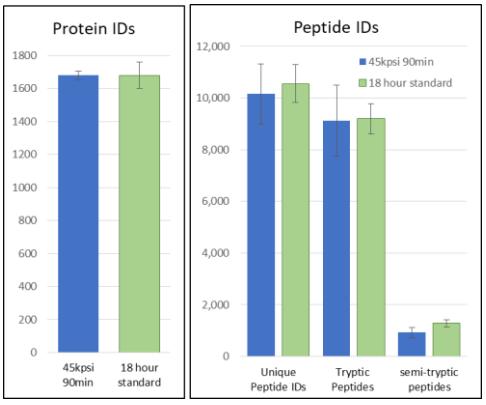

Figure 1. Pressure-accelerated 90 minute digestion with trypsin is as effective as standard overnight digestion. Pressure-treated digests yield the same number of protein IDs (~1680) as standard overnight digestion at atmosphere. The total number of tryptic peptides (trypsin specific peptides with or without miscleavages) is also comparable in the two groups. Standard digestion results in more semi-tryptic peptides, likely due to trypsin over-digestion during the 18 hour incubation.

PBI
14 Norfolk Avenue South Easton, MA 02375
TEL 508-230-1828 • FAX 508-580-1829
www.pressurebiosciences.com

The data presented in **Figure 1** demonstrate that a 90 minute digest under pressure cycling conditions yields results that are comparable to, or slightly better than, standard overnight trypsin digestion, as indicated by the

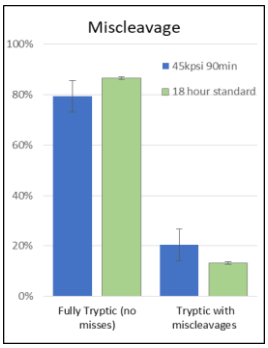

Figure 2. Effect of high pressure on trypsin miscleavage rate. The percentage of tryptic peptides (those with canonical trypsin cleavages at both ends) with and without miscleavages (i.e., "skipped" lysines or arginines), in 90 min pressure-treated and 18 hour control digests is shown. A slight increase in tryptic peptides containing miscleavage sites is observed in samples digested for 90mins at high pressure compared to controls.

total number of proteins identified, and the number of specific tryptic peptides detected. The shorter digestion time reduces trypsin over-digestion, resulting in fewer semi-tryptic peptides, compared to the standard method. The rate of miscleavage (i.e., skipped lysine or arginine sites), has been shown to be slightly increased in samples digested at high pressure [17]. We compared the rate of trypsin miscleavage in samples digested at pressure for 90 minutes to standard method (**Figure 2**), and observed that the rate of miscleavage is slightly higher in the pressure-accelerated samples. For those applications where a slight increase in the rate of miscleavages is unacceptable, additional protocol optimization may be required to bring the rate down to acceptable levels.

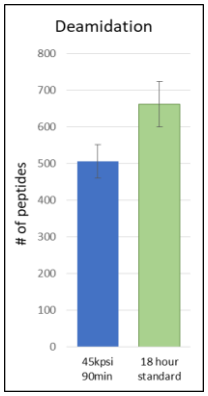

Figure 3. Effect of high pressure digestion on peptide deamidation. The total number of identified peptides that contain (one or more) deamidations, in 90 min pressure-treated and 18 hour control digests. Fewer deamidated peptides are identified in samples digested for 90 mins at high pressure compared to controls.

We also compared the number of peptides containing chemical modifications in pressure-accelerated and standard 18 hour digests. The results in **Figure 3** suggest that deamidation is not increased under pressure, in fact the overall number of deamidated peptides is much higher in standard overnight digests, likely due to the long incubation time. This conclusion is supported by the observation that samples digested for 90 mins without pressure, exhibit deamidation rates similar to those in the 90 minute pressure digests (not shown), suggesting that time is the main factor in increased deamidation.

The rate of methionine oxidation was also examined to determine whether exposure to dissolved air in the PCT MicroTubes results in increased oxidation. During sample pressurization, residual air in the MicroTubes dissolves into the sample solution. The resulting increase in dissolved oxygen concentration can result in an

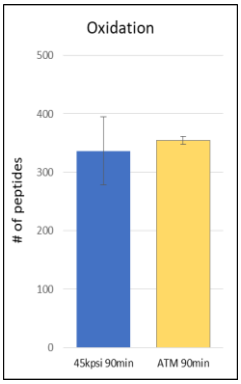

Figure 4. Effect of high pressure digestion on peptide oxidation. The total number of identified peptides that contain (one or more) methionine oxidations, in samples digested for 90 min with pressure cycling or at atmosphere. Comparable numbers of oxidized peptides are identified in samples digested at high pressure compared to controls.

increase in protein oxidation. To look more closely at oxidation in pressurized samples, rather than by total incubation time, we compared samples digested for 90 minutes with and without high pressure cycling. The results presented in **Figure 4** suggest that under the conditions used here, the rate of methionine oxidation is not significantly increased in samples exposed to high pressure for 90 minutes. However, it should be noted that total oxidation is expected to be higher in protocols where samples are exposed to high pressure during multiple steps of the workflow [20].

Conclusion

The benefit of high pressure for enhanced trypsin digestion in proteomic applications has been well established [1-5, 17-22]. Here we used a model tissue lysate to compare 90 minute trypsin digestion at high pressure, to standard 18 hour digestion at atmospheric pressure. To minimize biological sample-to-sample variability, aliquots of a single tissue lysate (prepared using conventional tissue homogenization) were used for all samples. Therefore, the benefits of high pressure for protein extraction from tissue are not addressed here. The focus of the current work is to examine the effect of hydrostatic pressure on the number of proteins identified, common protein modifications, and enzyme specificity. The data presented in Figures 1-4 demonstrate that overall, the number of proteins and trypsin-specific peptides, identified after 90 minute pressure digestion, is comparable to what is seen after a standard overnight digest. Thus, pressure-accelerated digestion can be used to significantly reduce workflow time in proteomic application, without losing digestion efficiency or reducing protein IDs. Furthermore, we show that the specificity of trypsin does not change under pressure. This agrees with previous data showing that the specificity of trypsin, as well as of many other enzymes tested, is not adversely affected by digestion at high pressure [10,17,19,20].

The best starting conditions for high pressure trypsin digestion are summarized in Table 1. This table is based on results presented here and in earlier Application Notes from PBI [4,5,18,21,22]. These suggested conditions can be used “as-is”, or as a starting point to generate optimized pressure-enhanced protocols for specific applications and sample types.

Table1. Suggested Starting Conditions for Pressure-accelerated Trypsin Digestion

Pressure	20kpsi to 50kpsi, depending on reaction buffer and target proteins.
Cycle profile	50 sec at high pressure/ 10 sec at ambient, per cycle.
Number of cycles	60-90 cycles for most applications
Temperature	37-52^0 C (to avoid carbamylation, 35-37^0 C is recommended when urea is present)
Suggested Buffer	50-100mM ammonium bicarbonate (other buffers can also be used, but may require additional optimization)
Compatible Reagents	Sodium deoxycholate (up to 0.4%), or urea (0.8M is recommended, higher concentrations will require additional optimization), or acetonitrile (up to 10%), or methanol (up to 10%)
Potentially inhibitory reagents	Guanidine HCl (up to 250mM has been shown to be compatible with trypsin, and concentrations <1M may yield acceptable results, but higher concentrations are not recommended)

Materials and Methods

Rat kidney tissue (from Pel-freeze) was manually homogenized in 100mM ammonium bicarbonate (Ambic) with 8M urea, using a glass Dounce homogenizer. The homogenate suspension (~10 mg/ml by Bradford assay) was reduced with 6mM DTT at 28^0 C for 30 mins, and alkylated with 10mM iodoacetamide for 30 mins at room temperature (protected from light). The reduced/alkylated lysate was diluted 1:10 in 50mM Ambic with 10% N-propanol, to bring the protein concentration to ~1mg/ml and the urea concentration to 0.8M in all reactions. Trypsin (Promega sequencing grade, #V5111) was added to a final 1:60 enzyme-to-substrate ratio (w/w).
Digestion was carried out in the Barocycler 2320EXT at 35^0 C, with pressure cycling at 45kpsi (50 seconds at high pressure and 10 sec at Atm, per cycle). Total digestion time in the Barocycler was ~90-100 minutes. Control reactions were digested at ambient pressure overnight (~18-20 hours) at 37^0 C with shaking, or at 35^0 C for 90-100mins. All reactions were carried out in triplicate. Data are presented as average ± standard deviation.

Samples were run by Protifi LLC (Huntington, NY), on Orbitrap-class instruments, and analyzed using Mascot and a suite of home-build informatics tools.

References

1. High-Throughput Proteomic Analysis of Fresh-Frozen Biopsy Tissue Samples Using Pressure Cycling Technology Coupled with SWATH Mass Spectrometry. Zhu, Y. and Guo, T. (2017) Methods in Molec. Biol. https://link.springer.com/protocol/10.1007%2F7651_2017_87
2. Minimal sample requirement for highly multiplexed protein quantification in cell lines and tissues by PCT-SWATH mass spectrometry. Shao, S. et al (2015). Proteomics. 2015 Nov;15(21):3711-21. https://www.ncbi.nlm.nih.gov/pubmed/2628712
3. Rapid and efficient protein digestion using trypsin coated magnetic nanoparticles under pressure cycles. Lee, B., et al. (2011). Proteomics 11(2):309-318.
4. Proteolysis-PrEP: Optimization of Pressure Cycling Conditions for Protein Digestion with Trypsin in Solution. http://www.pressurebiosciences.com/downloads/PrEP-files/Trypsin-PrEP-v110912.pdf
5. Pressure Cycling-Accelerated Digestion with Trypsin: Digestion of Native Proteins in Whole Tissue Lysate
6. Fast and efficient non-reduced Lys-C digest using pressure cycling technology for antibody disulfide mapping by LC-MS. Cheng Y., Chen Y., and Yu C. (2016) J. Pharm. Biomed. Anal. 129:203-209.
7. High-resolution physicochemical characterization of different intravenous immunoglobulin products. Washburn N., et al., (2017) PLoS ONE 12(7) :e0181251.
8. Proteolysis-PrEP: Pressure-accelerated Protein Digestion with Endoproteinase Lys-C.
9. Pressure-assisted Sample Preparation for Proteomic Analysis. Olszowy, PP., Burns, A., Ciborowski, PS. Anal. Biochem. 2013 Jul 1;438(1):67-72.
10. Proteolysis-PrEP: Activity and Specificity of Chymotrypsin Under Pressure Cycling Conditions
11. Proteolysis-PrEP Pressure-accelerated Activity of Endoproteinase Glu-C.
12. Proteolysis-PrEP: Pressure-accelerated Protein Digestion by Thermolysin Enzyme
13. Proteolysis-PrEP (Lysozyme): Effect of Pressure Cycling on Lysozyme Activity.
14. PrEP Pressure-accelerated Native Protein Deglycosylation by PNGase F Enzyme
15. Rapid Release of N-linked Glycans from Glycoproteins by Pressure Cycling Technology. (2010). Anal. Chem. 82(6):2588-2593
16. High-pressure EPR reveals conformational equilibria and volumetric properties of spin-labeled proteins. McCoy and Hubbell PNAS 2011. http://www.pressurebiosciences.com/downloads/publications/2011-02/McCoy-pressure.pdf
17. Streamlining PCT-HD for tissue biopsy samples: Better digestion in a shorter time. ASMS 2017 poster.
18. Proteolysis-PrEP: Optimization of Pressure Cycling Conditions for efficient Digestion of Native IgG. https://www.pressurebiosciences.com/view/db/444/5524/pdf/Proteolysis-PreP.pdf
19. Improved proteolytic digestion under high pressure cycling: Rapid digestion with improved sensitivity and sequence coverage. ASMS 2016 poster. https://www.pressurebiosciences.com/publications/detail/5748/improved-proteolytic-digestion-under-high-pressure
20. PCT-HD for tissue biopsy samples: Comparison to a standard method. US HUPO 2017 poster
21. Proteolysis-PrEP: High Pressure-Accelerated Trypsin Digestion in the HUB880 Explorer: Rapid Digestion of Unreduced IgG (document in preparation)
22. Addition Trypsin Digestion Application Notes from PBI
    A. Pressure Cycling-Accelerated Digestion with Trypsin: Effect of Urea on Digestion at Pressure
    B. PCT-HD: Homogenization, extraction and digestion of small tissue samples for proteomic applications.
    C Compatibility of deoxycholate detergent with pressure-enhanced digestion by trypsin and Lys-C: Alternative PCT-HD workflow