Proteolysis-PrEP: Pressure-accelerated Activity of Endoproteinase Glu-C

Introduction

The positive effect of Pressure Cycling Technology (PCT) on trypsin digestion is well established [1-6], and has been shown to result in improved sequence coverage, higher recovery and significantly reduced digestion times. Additionally, the enhancing effect of PCT on the activity of several other enzymes, including Lys-C, Proteinase K, PNGase F, chymotrypsin and lysozyme has been reported [7-13].

Pressure, heat, and many different 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 [14]. 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 digestion, as long as the level of pressure that is applied 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, independent of substrate conformation.

Here we report that the activity of Glu-C enzyme is increased by ~40% when the enzyme reaction is carried out under pressure cycling conditions. In addition, we investigate the effect of acetonitrile on enzyme activity under pressure. The goal of this work is to provide the user with the best set of *starting conditions* for pressure-enhanced Glu-C digestion.

PCT Sample Preparation System (PCT SPS)

The Pressure Cycling Technology Sample Preparation System (PCT SPS) uses cycles of hydrostatic pressure between ambient (14.7 psi) and very high levels (up to 45,000 psi, or higher in some applications) to control biomolecular interactions and to accelerate certain enzymatic reactions. The PCT SPS uses semi-automated bench-top Barocycler instruments, in combination with PCT MicroTubes or FT500-ND PULSE Tubes. The specially designed PCT MicroTubes are single-use sample processing containers designed to hold 50-150 µL, while the FT500-ND PULSE Tubes are suitable for larger sample volumes up to 1.4 mL. Samples in MicroTubes can be pressure cycled in sets of up to 12-48 (depending on Barocycler model), to allow batch-mode processing of multiple samples.

Results and Discussion

PCT-Enhanced Glu-C Activity

To assay the effect of pressure on the Glu-C enzyme itself, rather than on substrate proteins, tests were carried out using a small synthetic substrate. Proteolytic cleavage of the substrate leads to an increase in absorbance at 270nm (Abs 270). Increase or decrease in enzyme activity was assayed by comparing the Abs 270 of the pressure-treated samples to controls incubated at ambient pressure (for the same length of time and at the same temperature).

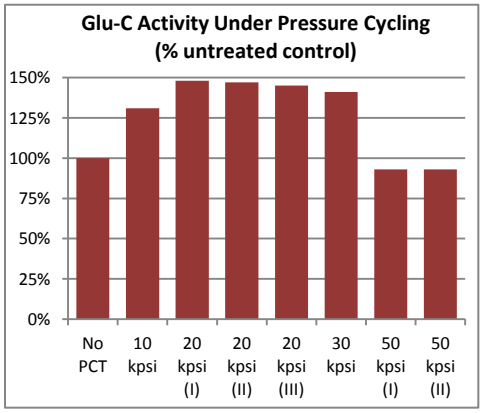

Figure 1. Glu-C activity assay. Reactions were carried out at the indicated pressure at ambient temperature. Abs 270 was measured at the start of the reaction (time zero) and at 10 minute intervals (for a total of 20-30 minute, while the reaction rate was linear). These values were then used to calculate the slope of the Abs 270 increase over time. The slope of the control reaction (carried out at ambient pressure, ie, no PCT) was set at 100% and the treated samples are expressed as the % of control. The graph shows results from multiple experiments (each experiment included its own No PCT control). Enzyme activity at 20 and 50 kpsi was measured several times in independent experiments.

The effect of PCT on Glu-C enzyme activity is shown in Figure 1. As can be seen in all three reactions carried out at 20 kpsi on three separate occasions (I, II, III), the enzyme activity is increased by ~45% when the reaction is carried out under PCT conditions at this pressure. At 10kpsi, the reaction rate is intermediate between the control and the 20 kpsi condition, suggesting that pressures below 20 kpsi are not as effective at accelerating the enzyme. At 30 kpsi, the rate is similar to 20 kpsi, suggesting that there is a range of pressures that can be used for accelerating Glu-C activity. Since Glu-C is a protein, it was predicted that pressure levels above a certain point would induce denaturation of the enzyme and lead to reduction or loss of enzymatic activity. This effect can be seen clearly at 50kpsi, where enzyme activity is close

to, or slightly less than, the control. The fact that Glu-C activity is still quite high even at 50 kpsi suggests that this enzyme is quite pressure-stable and that pressures above 20 kpsi may be useful for Glu-C digestion of very-difficult-to-digest targets, where more extensive denaturation of the target protein may improve enzyme access to cleavage sites.

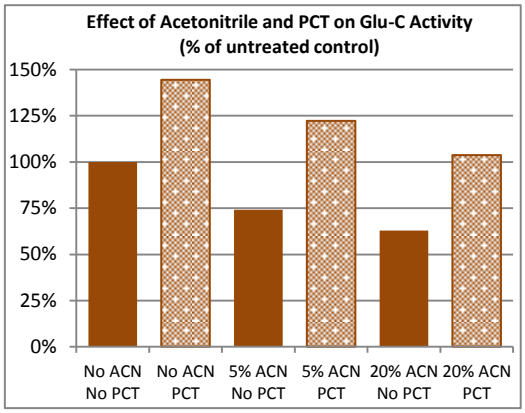

Figure 2. Effect of Acetonitrile and PCT on Glu-C activity. Reactions were carried out at 20 kpsi at ambient temperature in the presence of the indicated concentration of acetonitrile (ACN). Enzyme activity was calculated as described in Figure 1.

Glu-c activity at elevated pressure was also assayed in the presence of acetonitrile, a commonly used denaturant that is often added to improve digestion for proteomic applications at ambient pressure. The synergistic effect of high pressure and chemistry on protein denaturation can result in unexpectedly poor digestion when reactions are carried out at high pressure in the presence of certain denaturants, even though those denaturants may be compatible with digestion at atmospheric pressure (eg., trypsin activity at 20 kpsi in the presence of 2M urea is lower than in 2M urea at ambient pressure) [1]. Figure 2 shows that a reduction in Glu-C activity is associated with increasing concentrations of acetonitrile (0%, 5%, 20%). However, the activity of Glu-C is higher in the pressure treated-samples compared to the ambient-pressure samples, at all ACN concentrations tested. As can be clearly seen from these results, the increase in Glu-C activity under PCT conditions is apparent even in the presence of 20% acetonitrile, supporting the observation that Glu-C enzyme is a very pressure-stable protein.

The results shown in Figures 1 and 2 suggest that Glu-C is resistant to pressure-induced denaturation and subsequent loss of enzymatic activity. However, since Glu-C is a protein, it is expected that exposure to high hydrostatic pressure will lead to protein denaturation over time, and that the extent of this denaturation should be related to the pressure level. Although we have shown that Glu-C activity is increased by 45-50% at 20 kpsi, those results do not tell us whether this increase is due to a very rapid burst of enzyme activity followed by denaturation and inactivation, or whether the enzyme continues to be active for an extended period of time during pressure cycling. This information is important for optimization of PCT-accelerated digestion conditions, since carrying out reactions longer than the enzyme can remain active is both unnecessary and can lead to undesirable non-specific results. To address this question, Glu-C was pre-treated for 30 minutes (30 cycles) at different pressure levels. Substrate was then added to the treated enzyme and the reactions were carried out at ambient pressure to compare the activity of the pressure-treated and control enzyme. The results in Figure 3 show that after 30 minutes of PCT at 10 or 20 kpsi, the enzyme shows only a slight reduction in activity compared to the untreated control. In fact, after 30 minutes at 40 or even 50 kpsi, the enzyme retains ~60% of its original activity, suggesting that the enzyme can remain active for extended periods of time under pressure cycling conditions.

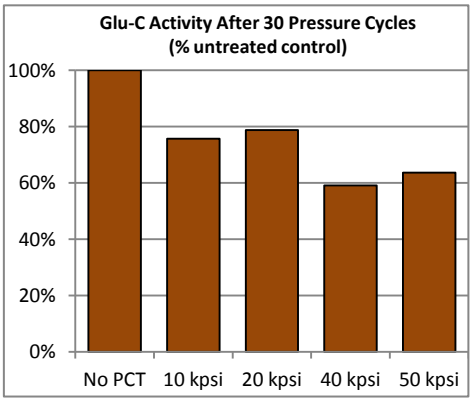

Figure 3. Glu-C Activity Remaining After 30 Minute Pressure Treatment. Glu-C was exposed to pressure cycling in 50mM Tris pH 8.6, at the indicated pressure, for 30 cycles. After pressure treatment, enzyme activity was measured at ambient pressure. Enzyme activity was calculated as described in Figure 1.

Conclusion

Here we examined the effect of hydrostatic pressure and acetonitrile on the activity of Glu-C, in order to determine the best starting conditions for PCT-accelerated digestion. These suggested conditions can be used “as-is” or as a starting point for further optimization by individual users to generate optimized pressure-enhanced protocols for their specific needs and sample types.

Table1. Suggested Starting Conditions for PCT-enhanced Glu-C Digestion Protocol Optimization

Pressure	20-30 kpsi (higher pressures may be used to improve unfolding of tightly folded or hard-to-digest proteins). At higher pressures, additional optimization of PCT conditions may be required.
Cycle profile	50 sec at high pressure/ 10 sec at ambient, per cycle.
Temperature	Ambient temperature (~20-25^0 C). At higher or lower temperatures, additional optimization of PCT conditions may be required.
Acetonitrile	Up to 5% can be used at 20 kpsi without significant loss of enzyme activity (at higher concentrations, additional optimization may be required).

Materials and Methods

Glu-C enzyme and BOC-L-glutamic acid 1-phenyl ester (synthetic substrate) were purchased from Sigma. Reactions were carried out in PCT MicroTubes in 50mM Tris at pH 8.6, unless indicated otherwise.
Glu-C activity was measured at ambient temperature (~22^0 C) using the synthetic substrate. For each condition, mock reactions were incubated with substrate alone, without enzyme, to measure the background rate of Abs 270 increase, presumably due to spontaneous breakdown of the substrate. These blank Abs 270 values were then subtracted from the Abs 270 of the reactions.
Substrate stocks were made fresh in 1,4 Dioxane for each experiment. All reactions were carried out using 50 µl of reaction per MicroTube. Pressure cycling was performed in PCT MicroTubes using one minute cycling parameters (50 seconds at high pressure and 10 seconds at ambient pressure, per cycle). Absorbance at 270nm was measured at T_0 and after 10, 20 and 30 minutes, using a Nanodrop. The Abs 270 increase over time was plotted to calculate the slope, which was used as a measure of relative enzyme activity. All data are expressed as the percent of control, where the control (100%) is the slope of the Abs 270 over time of the sample incubated at atmospheric pressure in Tris pH 8.6.

References

1. Proteolysis-PrEP: Optimization of Pressure Cycling Conditions for Protein Digestion with Trypsin in Solution. PBI Application Note. http://www.pressurebiosciences.com/downloads/PrEP-files/Trypsin-PrEP-v110912.pdf
2. Rapid mass spectrometric conversion of tissue biopsy samples into permanent quantitative digital proteome maps. Guo T, Kouvonen P, Koh CC, Gillet LC, Wolski WE, Röst HL, Rosenberger G, Collins BC, Blum LC, Gillessen S, Joerger M, Jochum W, Aebersold R. Nat. Med. 2015 Apr 21(4):407-13. doi: 10.1038/nm.3807.
3. Mass spectrometry-based proteomic quest for diabetes biomarkers. Shao S, Guo T, Aebersold R. Biochim. Biophys. Acta. 2015 Jun;1854(6):519-27. doi: 10.1016/j.bbapap.2014.12.012. Epub 2014 Dec 30.
4. Proteomics under Pressure: Development of Essential Sample Preparation Techniques in Proteomics Using Ultrahigh Hydrostatic Pressure. Freeman, E. and Ivanov, A. J. Prot. Res. 2011. 10:5536-5546. http://pubs.acs.org/doi/abs/10.1021/pr200805u
5. Optimization of high pressure-assisted methods for proteomic sample preparation. Lazarev, A., et al., Poster HPBB 2010. http://www.pressurebiosciences.com/downloads/publications/2010-09/HPBB_Final.pdf
6. Label-free mass spectrometry-based relative quantification of proteins separated by one-dimensional gel electrophoresis. Getie-Kebtie, M., Lazarev, A., Eichelberger, M., and Alterman, M. Anal. Biochem. 2011 409:202-212. http://www.sciencedirect.com/science/article/pii/S0003269710006792
7. Pressure-assisted Sample Preparation for Proteomic Analysis. Olszowy, PP., Burns, A., Ciborowski, PS. Anal. Biochem. 2013 Jul 1;438(1):67-72 doi: 10.1016/j.ab.2013.03.023. Epub 2013 Mar 29.
8. Proteolysis (Proteinase K)-PrEP: Efficient and Rapid Extraction of Genomic DNA from Skeletal Muscle using The PCT Shredder and Pressure-enhanced Proteolysis with Proteinase K. PBI Application Note. http://www.pressurebiosciences.com/downloads/2010-03-29/Proteinase-K.II.PrEP.032710.pdf
9. Rapid Release of N-Linked Glycans from Glycoproteins by Pressure-Cycling Technology. Szabo, Z., et al., Anal. Chem., 2010, 82 (6), pp 2588–2593. http://pubs.acs.org/doi/abs/10.1021/ac100098e.
10. A Comparison of Methods for Efficient Digestion of Protein Therapeautics. Maheu, L.L. et al., Poster Amgen, FACSS 2008. http://www.pressurebiosciences.com/downloads/publications/2010-08/Amgen-FACSS-2008-Poster.pdf
11. Proteolysis-PrEP (Lysozyme): Effect of Pressure Cycling on Lysozyme Activity. PBI Application Note. http://www.pressurebiosciences.com/PrEP-files/Lysozyme.PrEP-v2.0.nl_VG.pdf
12. Proteolysis-PrEP : Pressure-accelerated Protein Digestion with Endoproteinase Lys-C.
13. PCT-HD : Homogenization, Extraction and Digestion of Small Tissue Samples for Proteomic Applications.
14. 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
15. In Situ Measurement of pH Under High Pressure. Chaminda P. Samaranayake and Sudhir K. Sastry. J. Phys. Chem. B 2010, 114, 13326–13332.