Proteolysis-PrEP: Optimization of Pressure Cycling Conditions for Protein Digestion with Trypsin in Solution

Introduction

Pressure Cycling Technology (PCT) has been proven to accelerate enzymatic protein digestion. The positive effect of PCT on trypsin digestion has been demonstrated by several laboratories [1, 2, 3] who have reported improved sequence coverage, higher recovery and significantly reduced digestion times when the trypsin reactions were carried out under pressure. Not only has PCT been shown to accelerate and improve digestion in solution, but it can also accelerate in-gel trypsin digestion [4, 5]. Additionally, the enhancing effect of PCT on the activity of several other enzymes, including Proteinase K, PNGase F, Lys-C and lysozyme has been reported [6, 7, 8, 9].

Pressure induces protein denaturation, but the pressure-perturbed proteins assume conformational forms that are different from those caused by thermal or chemical treatment [10]. The combination of pressure and certain chemistries was previously examined using a chromogenic trypsin substrate to measure trypsin activity during pressure cycling [11]. Pressure-induced denaturation, when combined with chaotropes and/or detergents that are often used in standard (atmospheric pressure) digestion reactions, can lead to denaturation and inactivation of the trypsin itself. Thus, it is important to determine which reagents, and at what concentrations, are compatible with pressure cycling-enhanced trypsin digestion protocols.

In addition to optimizing the chemistry for pressure-enhanced trypsin digestion, here we also report the effect of pressure cycling profile and other parameters on digest efficiency of protein substrates. The goal of this work is to provide the user with the best set of *starting conditions* for pressure-enhanced trypsin digestion of hard-to-digest proteins.

PCT Sample Preparation System (PCT SPS)

The Pressure Cycling Technology Sample Preparation System (PCT SPS) uses rapid cycles of hydrostatic pressure between ambient and very high levels to control biomolecular interactions and to accelerate enzymatic reactions. The PCT SPS uses a small, semi-automated bench-top instrument (Barocycler NEP2320 or NEP2320-Enhanced) 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.

Materials and Methods

Trypsin (sequencing grade) was purchased from Promega. Bovine ubiquitin and human gamma globulin (IgG) were purchased from Sigma. Protease inhibitor cocktail was purchased from Sigma and used at 1X final concentration.

Ubiquitin was prepared using standard methods. The protein was dissolved at 2.5 mg/ml in denaturing buffer (8M urea in 50mM ammonium bicarbonate), reduced with 5mM TCEP, alkylated with 10mM iodoacetamide and quenched with additional 5mM TCEP (note: although ubiquitin does not contain disulfide bonds, it was treated in the manner that is commonly used to prepare protein solutions for digestion by trypsin). To remove urea, the ubiquitin was buffer-exchanged to 50mM ammonium bicarbonate (ambic) using Amicon 3k MWCO filters (Millipore). Native (non-denatured, disulfide-intact) IgG was dissolved in 50mM ambic and used without further processing.

All reactions were carried out in 50mM ambic using 50 µl of reaction per MicroTube (unless indicated otherwise). In some cases the ambic was supplemented with urea, n-propanol or other reagents as indicated. Pressure cycling was performed in PCT MicroTubes at 20,000 psi using one minute cycling parameters (50 seconds at high pressure and 10 seconds at ambient pressure per cycle) unless indicated otherwise. Control reactions were incubated in MicroTubes at the same temperature but without pressure. All digests were performed at 50°C, except those containing urea which were incubated at 37°C to avoid protein carbamylation. Trypsin was used at 1.2 µg/ml for ubiquitin digestion and at 3.2 µg/ml for IgG digestion (in both cases this was equivalent to a ~1:30 enzyme-to-substrate ratio). SDS-PAGE was carried out using 8-16% Criterion gels (Bio-Rad). Proteins were visualized by staining with coomasie blue (Bloo Moose Staining Solution, Kerafast). Protein digestion was assayed by SDS-PAGE as the disappearance of the intact protein band(s).

Trypsin activity was measured using the chromogenic substrate, N_{α}-Benzoyl-D,L-argenine 4-nitroanilide hydrochloride (BAPNA). BAPNA assays were carried out in 50 mM ambic with or without 10% n-propanol, using 2 µg/ml trypsin and 500 µg/ml BAPNA (Sigma).

Results and Discussion

Pressure Cycling-Enhanced Trypsin Digestion

Pressure cycling-enhanced trypsin digestion was investigated using ubiquitin as the substrate. Ubiquitin is poorly digested by trypsin under normal conditions and therefore is a good model of a “difficult-to-digest” protein. Native human IgG was used to confirm that the results obtained using ubiquitin were likely to be generally applicable to other “difficult-to-digest” proteins.

Pressure cycling significantly accelerates trypsin digestion of ubiquitin, as can be seen in **Figure 1**. Standard overnight incubation results in very little digestion of ubiquitin as demonstrated by the minimal loss of the intact ubiquitin protein in samples treated with enzyme as compared to mock digests incubated overnight in the absence of trypsin. In contrast, digests performed with pressure cycling at 20,000 psi for 1 hour at 50°C exhibit significant reduction in the amount of intact ubiquitin. This loss of intact protein is confirmed to be due to proteolysis by trypsin, since the PCT control incubated without enzyme shows no loss of intact ubiquitin. Furthermore, the loss of ubiquitin in trypsin digests at 20,000 psi could be completely prevented by the addition of protease inhibitors (**Figure 2**), further confirming the specificity of the digestion reaction. IgG digestion by trypsin with pressure cycling is also significantly accelerated. A time-course performed with and without pressure shows that after just one hour, there is significantly less intact protein in pressure-treated IgG digests than in the unpressurized controls (**Figure 3**).

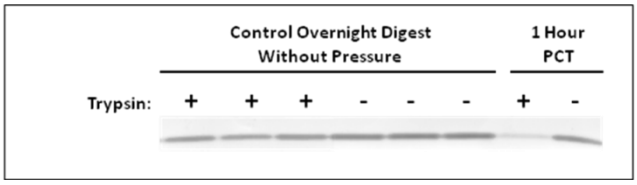


Figure 1. Comparison of Pressure-Enhanced and Standard Overnight Digestion of Ubiquitin Protein
Trypsin digestion of ubiquitin proceeds very slowly under standard digest conditions (overnight incubation at 37°C at ambient pressure). In contrast, when the digests are carried out with pressure cycling (20,000 psi at 50°C); most of the ubiquitin is digested within an hour.

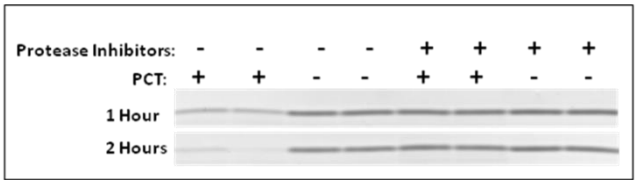


Figure 2. Trypsin Activity Under Pressure Remains Susceptible to Protease Inhibitors
Digests were carried out for 1 hour at 50°C in 50mM ambic with or without the addition of protease inhibitor cocktail. The results clearly show that even under pressure, trypsin activity is reduced or eliminated by the addition of the inhibitor cocktail. In the inhibitor-supplemented samples treated with pressure, the amount of intact ubiquitin is comparable to the controls incubated without pressure.

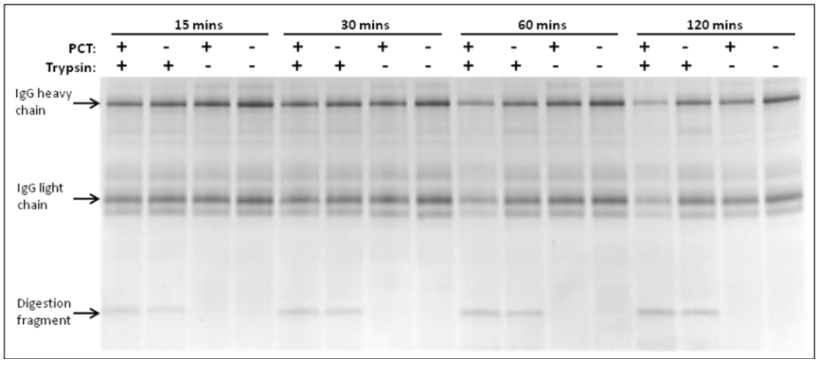


Figure 3. Trypsin Digestion of Native IgG Is Enhanced by Pressure Cycling Technology
Time-course (15, 30, 60 and 120 mins) showing trypsin digestion of IgG with or without pressure cycling (PCT) at 20,000 psi. Improved digestion in the PCT-treated samples is apparent within 1 hour and by 2 hours the pressure-treated sample exhibits significantly more digestion than the sample incubated at atmospheric pressure. All samples were incubated at 50°C for the indicated time. Mock reactions were performed without trypsin to control for non-specific protein loss.

To determine the optimal pressure level for PCT-accelerated trypsin digestion, the effect of pressure cycling on the trypsin enzyme itself was examined. Since trypsin 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. To assay the effect of pressure on trypsin itself, rather than on the substrate protein, these tests were carried out using BAPNA, a small chromogenic trypsin substrate. Cleavage of the BAPNA by trypsin releases a yellow digestion product, the accumulation of which is assayed by measuring the absorbance at 405nm. Trypsin activity can then be estimated based on the rate at which the absorbance increases. As shown in **Figure 4**, increasing the pressure from 20,000 psi to 35,000 psi leads a significant reduction in trypsin activity within the first 30 minutes of pressure cycling. However, the trypsin treated at 35,000 psi does retain some of its activity as indicated by the continued increase in absorbance at 405nm between 30 and 60 minutes. In contrast, after 30 minutes at 45,000 psi, trypsin activity is almost completely destroyed, as indicated by the plateau in absorbance at 405nm between 30 and 60 minutes in the samples treated at that pressure.

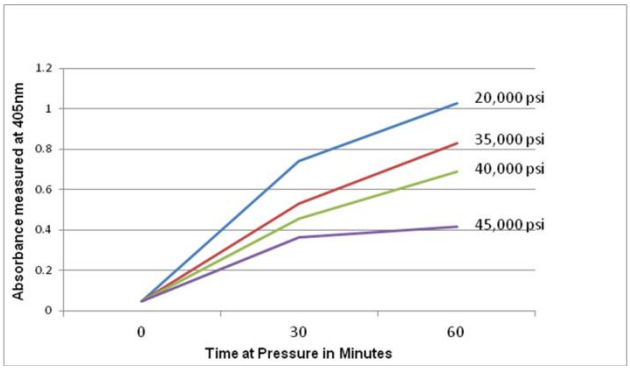


Figure 4. Effect of pressure level on trypsin activity
Trypsin was PCT-treated for 30 or 60 minutes in the presence of BAPNA. All reactions were carried out at 50°C at the indicated pressure (1 minute cycles) in 50mM ambic supplemented with 10% n-propanol. Each reading is an average of 3 replicates.

The pressure profile (i.e., the time at high and low pressure per cycle) for PCT-accelerated trypsin digestion was also investigated to determine whether cycling the pressure more rapidly was beneficial to the digest, or whether faster cycling would lead to more rapid inactivation of trypsin. Very rapid pressurization/depressurization cycles are expected to result in more rapid enzyme inactivation resulting in poorer digestion efficiency, but this might be offset in some cases if the increased denaturation of the substrate leads to better access of the trypsin to its target sites. **Figures 5 and 6** show the effect of static high pressure, slow pressure cycling (1 minute cycles) and fast pressure cycling (10 second cycles) on trypsin digestion of ubiquitin and native IgG. These results demonstrate that while more rapid cycling is clearly beneficial for the ubiquitin digest, the effect of rapid cycling on the IgG digest is much less pronounced. Thus, while pressure cycling accelerates the digest reactions, the exact cycling parameters may need to be optimized for different targets to obtain the maximum benefit while minimizing the negative impact on the enzyme.

Figure 5. Effect of Pressure Cycling Rate on Trypsin Digestion of Ubiquitin
Pressure cycling was carried out for 1 hour in 50 µl reactions at 50°C using either no pressure (control), static pressure at 20,000psi, slow pressure cycling (50 sec at 20,000 psi/10 sec at ambient per cycle) or fast pressure cycling (5 sec at 20,000psi/5 sec at ambient per cycle). As is clearly demonstrated, the rapid pressure cycling (10 second cycles) significantly improves digestion of the ubiquitin protein compared to the slow pressure cycling (1 minute cycles). Static high pressure has minimal effect on the digest and does not appear to be significantly different from the unpressurized controls.

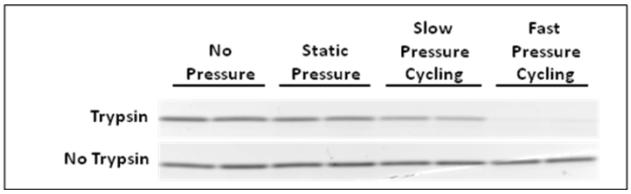
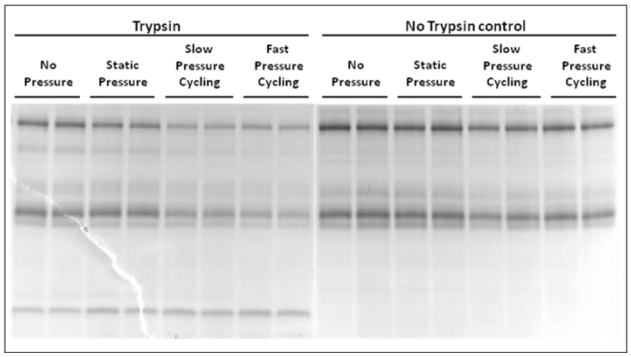


Figure 6. Effect of Pressure Cycling Rate on Trypsin Digestion of Native IgG
Pressure cycling was carried out for 1 hour in 50 µl reactions at 50°C using either no pressure (control), static pressure at 20,000 psi, slow pressure cycling (50 sec at 20,000 psi/10 sec at ambient per cycle) or fast pressure cycling (5 sec at 20,000 psi/5 sec at ambient per cycle). In contrast to the ubiquitin digest in **Figure 5**, the rapid and slow pressure cycling conditions appear to result in comparable levels of IgG digestion. However, static high pressure is clearly less effective and, similar to the ubiquitin digests in **Figure 5**, results in little improvement over the unpressurized controls.



Chemistry: Effect of Denaturing Additives

The addition of denaturants, such as urea or methanol, to trypsin digestion reactions is a common practice used to increase substrate protein denaturation and improve trypsin access to its target sites. However, these commonly used reagents may also be detrimental to trypsin activity at high pressure since their action is often greatly enhanced by pressure. It may therefore be possible (and often necessary) to reduce the concentration of these reagents, or to omit them entirely, when performing pressure-enhanced trypsin digestion.

The compatibility with pressure cycling of several common digest additives was assayed and examples of some of the results are shown in **Figure 7**. The BAPNA trypsin activity assay was used to examine the effect of urea, at different concentrations, on trypsin activity during pressure cycling at 20,000psi. **Figure 7A** clearly shows that the addition of urea does reduce trypsin activity, and that this effect is proportional to the concentration of urea in the sample. The effect of urea on pressure-enhanced trypsin digestion of ubiquitin is shown in the top panel of **Figure 7B**. The presence of 0.5M urea appears to be compatible with pressure cycling-enhanced trypsin digestion; however, the digests performed in 2M and 4M urea show significantly less digestion and demonstrate that higher concentrations of urea should be avoided.

The effect of several other reagents is examined in the lower panels of **Figure 7B**. Addition of methanol (MeOH) to the reactions results in poorer digestion than in the ambic control. At both 5% and 10%, the methanol reduced, but did not completely eliminate, the benefit of pressure cycling on the efficiency of the digest reaction. GuHCl also had a negative impact on pressure-enhanced trypsin digestion. As seen with methanol, 50mM guanidine reduced, but did not completely eliminate, the benefit of pressure cycling. At 100mM, the negative effect of guanidine was even more pronounced. Acetonitrile (ACN) at 10% or 20% had a very slight negative effect on the pressure-enhanced digestion. 10% n-propanol (prop) and 10% trifluoroethanol (TFE) appear to be quite compatible with PCT-enhanced digestion; however 20% TFE leads to significant inhibition of the reaction. The combination of 5% TFE+5% n-propanol also appeared to inhibit the reaction. Results obtained from multiple experiments using both ubiquitin and native IgG digestion are summarized in **Table 1**.

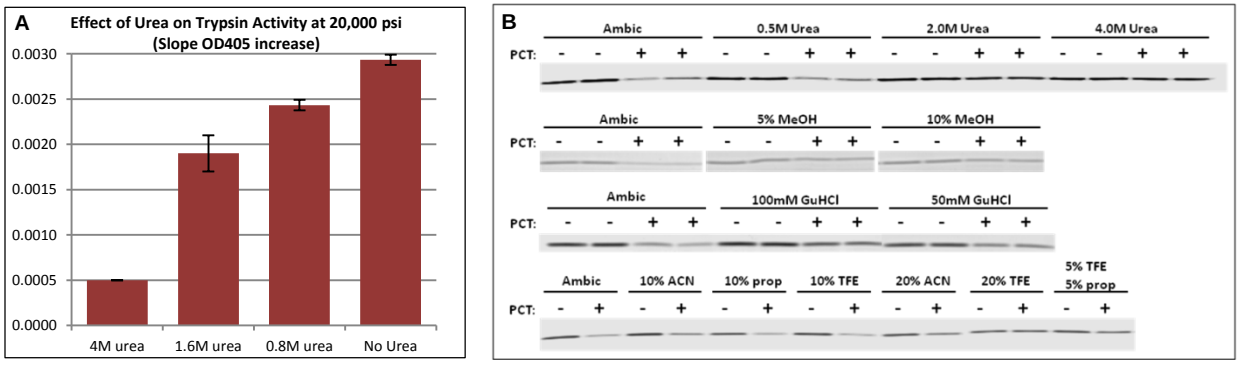


Figure 7. Effect of Denaturants on Trypsin Activity and Pressure Cycling-Enhanced Digestion of Ubiquitin.
Panel A. Trypsin activity assayed at 20 kpsi with synthetic substrate with different concentrations of urea (n=3 ± std dev). **Panel B**. Digest reactions carried out with or without PCT. Digests shown in the top panel were incubated at 37°C for 90 minutes to avoid carbamylation due to the presence of urea. All other digests were performed at 50°C for 1 hour. PCT was at 20,000 psi using slow pressure cycles (50 sec at high pressure/10 sec at ambient per cycle) for 60 cycles (90 cycles for digests at 37°C).

Table1. Compatibility of Various Commonly Used Denaturants with Pressure-Enhanced Trypsin Digestion

Denaturant	Compatibility	Notes
Urea	Compatible at or below ~0.8M final concentration.	Higher concentrations will inhibit reaction.
Methanol	Should be avoided.	If used, should be kept below 5%.
Guanidine-HCl	Should be avoided.	If used, should be kept below 50mM.
Acetonitrile	Compatible at 10% final concentration.	Higher concentrations will inhibit reaction.
n-Propanol	Compatible at 10-15% final concentration.	Higher concentrations may inhibit reaction.
Trifluoroethanol	Compatible at 10% final concentration.	Higher concentrations will inhibit reaction.
CHAPS	Should be avoided.	Inhibits reaction even at 0.2% final concentration.
SDS	Compatible at up to 0.025% final concentration.	Up to 0.05% can be used in some applications, but higher concentrations will inhibit reaction.

The effect of some denaturants on trypsin activity at pressure was also examined using the BAPNA colorimetric assay. These assays confirm that methanol, GuHCl and urea can have a detrimental effect on trypsin activity at high pressure [11], but that 10% n-propanol appears to protect trypsin from the denaturing effects of pressure (**Figure 8**). Although the BAPNA assay result shown in **Figure 8** suggests that 10% n-propanol should have a profound effect on

digest efficiency, the reactions performed using real protein substrates (ubiquitin and IgG) show only moderate additional benefit when 10% n-propanol is added to the pressure-enhanced reaction. This discrepancy in results highlights the need for protocol optimization using real proteins rather than synthetic substrates, as these are more representative of real-world sample types.

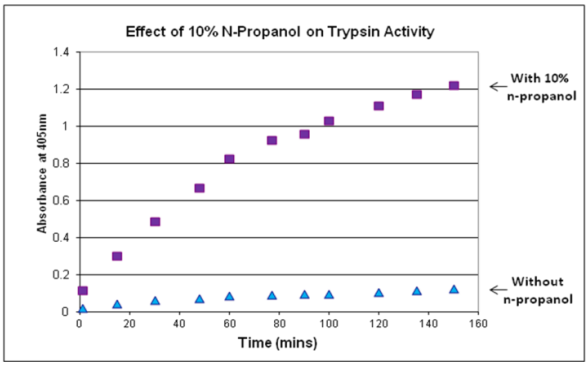


Figure 8. N-Propanol Appears to Protect Trypsin from Pressure-Induced Denaturation
Example of results obtained when trypsin was exposed to pressure cycling in ambic alone or in ambic supplemented with 10% n-propanol. Trypsin was pre-treated at 50°C with pressure cycling at 20,000 psi to test for denaturation and/or loss of enzyme activity. BAPNA was added to the pre-treated trypsin solution and enzyme activity was monitored at 37°C, over a 1.5 hour time-course. The result demonstrates that while trypsin that has been pressure cycled in ambic alone has lost much of its activity, the addition of 10% n-propanol protects the trypsin and allows the enzyme to retain much more of its function.

Effect of Sample Volume on Digest Efficiency: Proper Filling of PCT MicroTubes for Optimal Digestion with Pressure Cycling

The extent to which pressure cycling accelerates the trypsin reaction can be affected by the volume of sample in the PCT MicroTube. Digestion is most effective when the sample volume is low. This effect is more pronounced in the case of ubiquitin digestion (**Figure 9A**) and much less pronounced, but still evident, in the IgG digestion samples (**Figure 9B**). While it is not clear why sample volume should have this effect on the outcome of the digest reaction, several possible mechanisms were examined, including the effect of the ratio of sample volume to MicroTube inner surface, the ratio of sample volume to air volume, the length of the MicroCap and the total protein amount in the reaction. The results strongly suggest that the amount of air in the MicroTube has an impact on the digestion, and that a ratio of approximately 1:1 (sample volume-to-air volume) is necessary for optimal digestion under pressure. It is possible that this effect may simply be due to better mixing of the digest as the less-full MicroTubes are compressed and released at each pressure cycle. Alternatively, since high pressure compresses the sample and causes the air in the MicroTube to dissolve into the liquid, it is possible that the process of gas dissolution and out-gassing at each pressure cycle leads to improved digestion, perhaps by increasing mass transfer within the sample. While a mechanistic investigation of this phenomenon is outside the scope of this application note, and may be addressed at a later date, some options for increasing digest efficiency in larger volume samples were examined empirically. Model protein digests containing ubiquitin and myoglobin suggest that when sample volume is ~150µl (i.e. the maximum full volume of a PCT MicroTube), the reaction can be accelerated by increasing the pressure level during pressure cycling. Digestion reactions performed in full MicroTubes at 45,000psi appear to progress significantly more quickly than control reactions at either ambient pressure, or at 20,000 psi. However, since the effect of PCT at 45,000 psi on trypsin specificity has not been established, it is possible that miscleaved, semi-tryptic or other non-specific peptides may be increased in digests performed at pressures above 20,000psi.

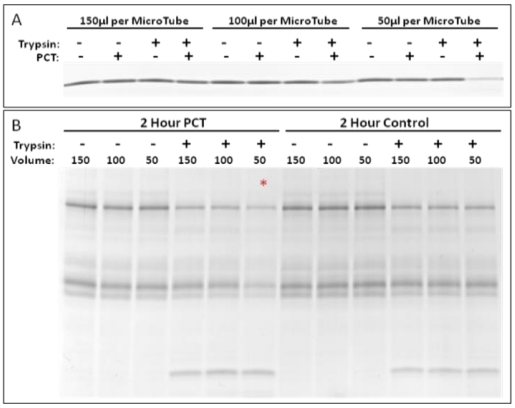


Figure 9. Effect of Sample Volume on Pressure-Enhanced Trypsin Digestion
A single digest reaction in 50mM ambic (containing either ubiquitin or native IgG) was prepared and the indicated volumes were dispensed into PCT MicroTubes. The 50µl samples were capped with 50 ul MicroCaps, the 100 µl samples were capped with medium MicroCaps and the 150 µl samples were capped with 150 ul MicroCaps. PCT was carried out at 20,000 psi using 1 minute pressure cycles. **Panel A**: Ubiquitin (± trypsin) digests were incubated at 50°C for 1 hour with or without pressure cycling. The pronounced effect of volume on this reaction is clearly illustrated on the gel. In the 150 µl samples, no significant digestion of intact protein is observed after 1 hour of pressure cycling. In the 100 µl reactions, the pressure-treated sample exhibits a very small reduction in the amount of intact protein, but in the 50 µl sample, the pressure-treated sample contains only a small amount of intact ubiquitin. The controls processed at ambient pressure show little detectable digestion in any of the samples. **B**: Native human IgG (± trypsin) digests were incubated at 50°C for 2 hours with or without pressure cycling. The effect of volume on the IgG digest is less pronounced than for ubiquitin, but a slight increase in digestion efficiency is observed in the PCT-treated 50 µl sample (*) compared to the 100 µl and 150 µl volumes.

Conclusions

Disruptive treatments, such as High Intensity Focused Ultrasound (HIFU) [12], microwave radiation [13] and high hydrostatic pressure [14, 15]; have been reported to accelerate digestion by trypsin and other enzymes. The increase in apparent activity is more likely to be due to pressure-induced denaturation of the substrate protein rather than by pressure-effects on the activity of the enzyme itself. By helping to maintain substrates in an unfolded state, PCT effectively makes them "better" substrates for the enzyme. Here we examined and optimized the pressure cycling parameters and pressure cycling-compatible chemistries that lead to efficient and reproducible trypsin digestion of two model hard-to-digest target proteins: bovine ubiquitin and native human IgG. These suggested conditions (Table 2) 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.

Table 2. Suggested Starting Parameters for PCT-enhanced Trypsin Digestion Protocol Optimization

Pressure	20,000 psi
Cycle profile	50 sec at high pressure/ 10 sec at ambient, per cycle
Temperature	50°C (35-37°C for urea-containing samples)
Buffer	Ammonium bicarbonate
Denaturant	10% n-propanol (15% may be beneficial under certain circumstances)
Volume	45-50 µl per MicroTube 50 ul MicroCap
Time	1-2 hours

References

1. ***Application of Pressurized Solvents for Ultrafast Trypsin Hydrolysis in Proteomics: Proteomics on the Fly***
López-Ferrer, D., *et al*., J Proteome Res. (2008) 7(8):3276-81
http://pubs.acs.org/doi/abs/10.1021/pr7008077

2. ***High Pressure Trypsin Digestion of Proteins for Proteomic Analysis***
Croley, T.R., Poster ASMS, May 2008
http://www.pressurebiosciences.com/downloads/3rdparty-2008-06-0/Virginia%20State%20Labs.Trypsin.pdf

3. ***Proteomics under Pressure: Development of Essential Sample Preparation Techniques in Proteomics Using Ultrahigh Hydrostatic Pressure***
Freeman and Ivanov 2011 J. Prot. Res. 10:5536-5546
http://pubs.acs.org/doi/abs/10.1021/pr200805u

4. ***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

5. ***Label-free mass spectrometry-based relative quantification of proteins separated by one-dimensional gel electrophoresis***
Getie-Kebtie, M,, A. Lazarev, M. Eichelberger, and M. Alterman 2011.
Anal. Biochem. 409:202-212
http://www.sciencedirect.com/science/article/pii/S0003269710006792

6. ***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

7. ***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

8. ***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

9. **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

10. ***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

11. ***Proteomics under pressure: Rapid extraction and digestion in a single tube***
Lazarev et al. ASMS poster 2009
http://www.pressurebiosciences.com/downloads/publications/2010-11/ASMS09%20posterTPE129.pdf

12. ***Pressurized Pepsin Digestion in Proteomics***
López-Ferrer D., *et al*., J. Proteome Res. 2005 Sep-Oct;4(5):1569-74
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3033671/

13. ***Rapid cyclopeptide analysis by microwave enhanced Akabori reaction***
Pramanik B.N., *et al.,* Protein Science. 2002, 11(11):2676-2687
http://144.206.159.178/ft/1010/86272/1459022.pdf

14. ***Application of high hydrostatic pressure for increasing activity and stability of enzymes***
Mozhaev V., *et al.,* Biotechnology and Bioengineering, 1996, 52:320-331
http://onlinelibrary.wiley.com/doi/10.1002/%28SICI%291097-0290%2819961020%2952:2%3C320::AID-BIT12%3E3.0.CO;2-N/abstract

15. **Influence of high hydrostatic pressure on the proteolysis of *b*-lactoglobulin A by trypsin**
Chicon R., *et al.,* Journal of Dairy Research, 2006; 73:121-128
http://www.aseanfood.info/Articles/11023877.pdf

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