# Optimization of Rapid Protein Digestion Using Pressure Cycling Technology.

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## 1.Introduction

Hydrostatic pressure has been previously shown to enhance enzymatic hydrolysis by trypsin [1,2], chymotrypsin and pepsin [3,4], as well as by Alcalase, Neutrase, Corolase 7089, Corolase PN-L, and papain [5,6]. In our preliminary experiments we have confirmed the positive effects of pressure and additional benefits of alternating hydrostatic pressure (pressure cycling) for several enzymes including proteinase K, PNGase F, Lys-C and lysozyme. However, mechanisms of pressure-induced acceleration remain speculative, while influence of various chemical agents on the enzymatic activity, substrate conformation and efficiency of the digestion process are not well understood, which leads to great variability of experimental results between groups which have attempted to employ PCT-enhanced digestion methods. In this work we explore stability and catalytic activity of trypsin and chymotrypsin under the influence of several chaotropes and organic solvents in combination with hydrostatic pressure at various temperatures. We have employed chromogenic substrates to measure enzyme activity independent of pressure-induced changes of substrate protein conformation. Additionally, using high performance LC-MS analysis, we have tested the effect of the factors outlined above on efficiency, selectivity, and throughput of proteolytic digestion. Analysis of the data obtained thus far leads to a set of guidelines for development of optimized and highly reproducible pressure-enhanced digestion methods.

## 2 Materials and Methods

### 2.1 Pressure Cycling Technology (PCT)

The Pressure Cycling Technology Sample Preparation System (PCT SPS) applies alternating hydrostatic pressure between ambient and ultra high levels to control molecular interactions [7]. The PCT SPS has been successfully used for cell and tissue lysis, extraction of proteins, lipids and nucleic acids [8]. Recently, PCT has been shown to accelerate enzymatic reactions such as proteolysis [1]. The PCT SPS is comprised of a small, semi-automated benchtop instrument (Barocycler NEP3229 or NEP2320) and single-use sample processing containers called FT500-ND PULSE Tubes (Pressure BioSciences, Inc., South Easton, MA). Used together, the PULSE Tubes transmit the pressure generated by the Barocycler to the sample, resulting in pressure enhanced proteolysis and accelerated genomic DNA isolation.

***Figure 1.*** *The PCT SPS platform*

### 2.2 PCT MicroTube sample containers

In order to address strong demand for the processing of smaller sample volumes and to enable higher sample throughput new disposable containers ("MicroTubes") have been developed. These containers have no moving parts and efficiently transmit hydrostatic pressure to the sample by flexible deformation of the polymer walls. The chemically inert fluoropolymer materials (FEP and PTFE) offer very low analyte binding, broad solvent compatibility and minimal extractability. MicroTubes maintain the seal and structural integrity during cryogenic storage in liquid nitrogen . Several different versions of the PCT MicroTube caps are available that will allow the use of the same size MicroTube to be used with multiple sample volumes (50, 100 and 150 µl) without causing irreversible mechanical deformation of the tubes under high hydrostatic pressure. These new containers are suitable for cell/ tissue lysis, PCT-fractionation, in-solution and in-gel protein digestion applications . The specialized cartridge system is designed for holding multiple (up to 48) MicroTubes in the cylindrical pressure chamber of the Barocycler instrument. The PCT MicroTube cartridge system keeps these containers sealed during rapid cycles of hydrostatic pressure even at temperatures exceeding boiling point of the solvent components. Additionally, several versions of the PCT MicroTube cap are intended to be used as gel spot picking tools (Figure 2) - this approach prevents any possibility of cross-contamination between gel spots and substantially simplifies the gel spot picking and transfer process.

**PCT MicroTubes Will Be Commercially Available June 30, 2009**

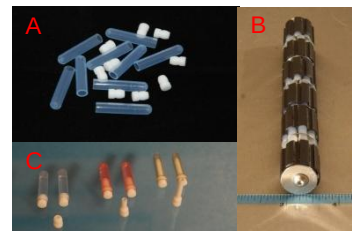


***Figure 2.*** *PCT MicroTubes. A) Standard PCT MicroTubes. B) PCT MicroTube cartridge system. C) PCT MicroTubes with gel-picking caps available in 50 µl, 100 µl and 150 µl sizes.*

### 2.3 Enzyme Activity Assays

Trypsin activity was measured using a chromogenic substrate, $N_{\alpha}$-Benzoyl-D,L-argenine 4-nitroanilide hydrochloride (BAPNA). Trypsin digests were performed in 50 mM ammonium bicarbonate using 2 µg/ml trypsin (Promega) and 500 µg/ml BAPNA (Sigma). PCT MicroTubes (Pressure BioSciences, Inc) containing 0.15 ml of reaction mixture were subjected to pressure cycling at 37˚C or 55˚C. Pressure cycling was performed at the indicated pressure. Each 1 minute pressure cycle consisted of 55 seconds at high pressure and 5 seconds at atmospheric pressure. To determine the effect of various denaturants on the stability and activity of trypsin under pressure enzyme activity was assayed in the presence of the denaturant and compared to control samples incubated at the same temperature and pressure without denaturant. For all samples, results were read at 405 nm.

Chymotrypsin activity was measured using a chromogenic substrate, N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma). Digests were performed in 100 mM ammonium bicarbonate using 0.6 µg/ml α-chymotrypsin from bovine pancreas (Sigma) and 125 µg/ml substrate. PCT MicroTubes (Pressure BioSciences, Inc) containing 0.15 ml of reaction mixture were subjected to pressure cycling at 53-55˚C for 20 cycles essentially as described above.

### 2.3 Digestion of Standard Proteins

A 1 pmol/µL mixture of standard proteins ("Protein Mix") with varying molecular weights, isoelectric points, and number of amino acid residues [Table 1] was used to examine various digestion protocols. Each protocol used 5 pmol of protein and was tested in triplicate. Sample digestion was done either in an incubated shaker or in a Barocycler NEP2320 (Pressure BioSciences, MA). Each sample was analyzed twice using NanoLC-2D HPLC system (Eksigent, CA) and LTQ Orbitrap (ThermoFisher, CA). MS data were analyzed with the SEQUEST-Sorcerer algorithm on the Sorcerer IDA2 (SageN Research, CA). Each bar presented on Figure 5 represents a mean of three individual technical replicates, analyzed twice by LC-MS/MS.

| Protein Description | SwissProt accession # | MW (Da) | PI | AAs |
|---|---|---|---|---|
| Ubiquitin human | P62988 | 9382 | 7.30 | 82 |
| Myoglobin equine | P68082 | 16941 | 7.36 | 154 |
| Cytochrome C bovine | P62894 | 11565 | 9.52 | 104 |
| ß –Casein bovine | P02666 | 23568 | 5.13 | 209 |
| Bovine serum albumin | P02769 | 66390 | 5.60 | 583 |
| α1- Casein | P02662 | 22960 | 4.91 | 199 |
| α2- Casein | P02663 | 24333 | 8.34 | 207 |
| κ -Casein | P02668 | 18963 | 5.93 | 169 |

***Table 1.*** *Standard protein mixture.*

## 3. Results and Discussion

### 3.1 Enzymatic activity

Effects of pressure cycling on α-chymotrypsin activity **(Figure 3A)**: Chymotrypsin activity was assayed at atmospheric pressure, and at 10,000, 20,000, 30,000 and 40,000 psi for 20 minutes at 55˚C. Results are expressed relative to a control performed at atmospheric pressure. The data indicate that α-chymotrypsin enzyme activity is greatly enhanced under pressure, and that this positive effect increases as pressure is increased to 40,000 psi, which is consistent with previously published data [9].

Effect of urea, methanol and guanidine on α-chymotrypsin activity **(Figure 3B):** α-Chymotrypsin activity at 55˚C was assayed after pressure cycling at 35,000 psi for 20 minutes in the presence of three common denaturants. The presence of 0.5 M urea and 0.5 M GuHCl had no significant effect on enzyme activity under these conditions. The addition of 10% methanol caused a slight decrease in enzyme activity. The data indicate that under pressure α-chymotrypsin activity is compatible with the presence of 0.5 M urea and 0.5 M GuHCl, but that the presence of methanol may be deleterious to the reaction.

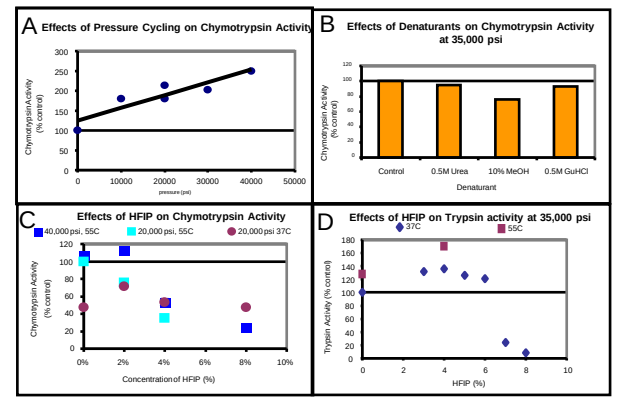


***Figure 3.*** *Enzymatic activity as a function of pressure, temperature and chemical composition. Part 1.*

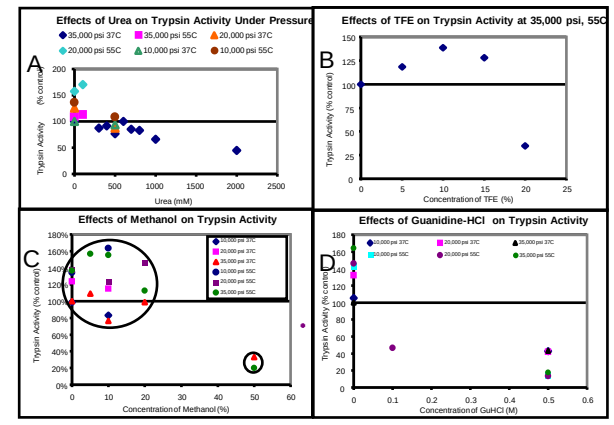


***Figure 4.*** *Enzymatic activity as a function of pressure, temperature and chemical composition. Part 2.*

Effects of hexafluoroisopropanol (HFIP) on α-chymotrypsin activity at 20,000 and 40,000 psi **(Figure 3C)**: α-Chymotrypsin activity at 37˚C and 55˚C was assayed after pressure cycling at 20,000 or 40,000 psi for 20 minutes in the presence of 2, 4 and 8% HFIP. Results are expressed relative to control (20,000 psi, 55˚C, no HFIP). The overall trend indicates that at 55˚C, the presence of HFIP negatively impacts chymotrypsin activity. Under less stringent conditions, however (20,000 psi, 37˚C), the effect of HFIP appears to be minimal. The data also suggest that there might be a slight increase in enzyme activity at very low concentrations (2%) of HFIP. However, this possible positive effect is not as pronounced as that observed with HFIP and trypsin.

Effects of hexafluoroisopropanol (HFIP) on trypsin activity at 35,000 psi **(Figure 3D)**: Trypsin activity at 37˚C and 55˚C was assayed after pressure cycling at 35,000 psi for 20 minutes in the presence of 3-8% HFIP. Results are expressed relative to control (35,000 psi, 37˚C, no HFIP). The data indicate that at concentrations below 6%, the presence of HFIP leads to an increase of trypsin activity of ~20% above the control level. However, at higher HFIP concentrations, there is a precipitous drop in trypsin activity, similar to what is seen with TFE. Therefore, for pressure-assisted proteolytic digestion with trypsin, HFIP concentrations should be kept below 6%.

Effects of urea on trypsin activity under pressure **(Figure 4A)**: Trypsin activity at 37˚C and 55˚C was assayed after pressure cycling at 10,000, 20,000 or 35,000 psi for 20 minutes in the presence of 0-2M urea. Results are expressed relative to control (35,000 psi, 37˚C, no urea). The results indicate that at very low concentrations (100 mM), the presence of urea has little or no effect on trypsin activity under pressure. At slightly higher concentrations (300-800 mM), the presence of urea in the reaction under pressure has a slight negative effect on enzyme activity. At higer concentrations (1-2 M), urea exhibits a significant inhibitory effect on the activity of trypsin. These findings indicate that at high hydrostatic pressure, high concentrations of urea are deleterious to trypsin activity and should be avoided. On the other hand, urea concentrations below 0.5M appear to be compatible with enzyme activity. Therefore, for pressure-assisted proteolytic digestion with trypsin, urea concentrations below 0.5M can be used.

Effects of trifluoroethanol (TFE) on trypsin activity at 35,000 psi **(Figure 4B)**: Trypsin activity at 55˚C was assayed after pressure cycling at 35,000 psi for 20 minutes in the presence of 5-20% TFE. Results are expressed relative to control (35,000 psi, 55˚C, no TFE). The data indicate that at concentrations ≤15%, TFE does not interfere with trypsin activity, and in fact appears to enhance trypsin function by 20-40% above control values. However, when the TFE concentration is increased to 20%, there is a precipitous drop in trypsin activity suggesting inactivation or denaturation of the enzyme. Therefore, for pressure-assisted proteolytic digestion with trypsin, TFE concentrations in the range of 0-15% are compatible with, and may even enhance, trypsin activity. However, concentrations ≥15% should be avoided.

Effects of methanol on trypsin activity under pressure **(Figure 4C)**: Trypsin activity at 37˚C and 55˚C was assayed after pressure cycling at 10,000, 20,000 or 35,000 psi for 20 minutes in the presence of up to 50% methanol**.** Results are expressed relative to control (35,000 psi, 37˚C, no methanol). At low concentrations (≤20%), methanol had minimal impact on trypsin activity at the various pressures and temperatures tested. However, at higher concentration (50%), the addition of methanol resulted in significant inhibition of trypsin activity under pressure. These findings indicate that at high hydrostatic pressure, concentrations of methanol at or below 20% appear to be compatible with enzyme activity. Therefore, for pressure-assisted proteolytic digestion with trypsin, methanol concentrations in the range of 0-20% can be used, however, higher concentrations should be avoided.

Effects of guanidine on trypsin activity during pressure cycling **(Figure 4D)**: Trypsin activity at 37˚C and 55˚C was assayed after pressure cycling at 10,000, 20,000 or 35,000 psi for 20 minutes in the presence of up to 0.5M guanidine HCl. Results are expressed relative to control (35,000 psi, 37˚C, no GuHCl). The data indicate that at all pressures and temperatures tested, the presence of guanidine results in significant inhibition of trypsin activity. Therefore, for pressure-assisted proteolytic digestion with trypsin, addition of guanidine to the reaction should be avoided.

### 3.2 Optimization of Protein Digestion. Peptide Identification.

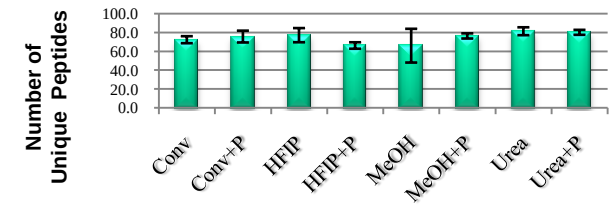


***Figure 5: Number of identified unique peptides.*** *Digestion in 2M Urea, 25% Methanol and 7% HFIP have been compared to the overnight digestion in 50mM ammonium bicarbonate (Conv); +P denotes PCT treatment for 30 minutes at 35,000 psi. Lower peptide counts for urea and HFIP digests under pressure are consistent with suboptimal enzyme activity. Decrease in concentration of these reagents is expected to further improve digestion results.*

## 4 Conclusions

Pressure cycling has been shown to significantly improve proteolysis with a number of enzymes. High hydrostatic pressure acts synergistically with chaotropes and organic solvents to boost the effects of these chemicals on protein denaturation and digestion These effects not only improve digestion efficiency and save significant time, but also allow the use of much lower concentrations of denaturants, which can simplify and improve downstream applications. While such effects are clearly beneficial, care must be taken in development of PCT-enhanced digestion methods to avoid impairment of enzymatic activity due to denaturation of the enzyme itself. The following points illustrate some of our recent findings:

1. Chymotrypsin activity can be significantly enhanced by performing digestion using cycled pressure.
2. Under cycled pressure, 0.5 M Guanidine-HCl inhibits trypsin but not chymotrypsin activities.
3. Under cycled pressure, 4% HFIP enhances trypsin but inhibits chymotrypsin activities.
4. Pressure levels above 20,000 psi exhibit negative effect on trypsin activity.
5. Under cycled pressure, trifluoroethanol below 15% enhances trypsin but inhibits chymotrypsin activities (data not shown).
6. Under cycled pressure, both methanol and urea can be included in trypsin digests at low concentration, but become rapidly inhibitory at higher concentrations.

## 4. References

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